Therapies for Neonatal Encephalopathy: Targeting the Latent, Secondary and Tertiary Phases of Evolving Brain Injury

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Abstract (150 words)

In term and near-term neonates with neonatal encephalopathy, therapeutic hypothermia protocols are well established. The current focus is on how to improve outcomes further and the challenge is to find safe and complementary therapies that confer additional protection, regeneration or repair in addition to cooling. Following hypoxia-ischemia, brain injury evolves over three main phases (latent, secondary and tertiary), each with a different brain energy, perfusion, neurochemical and inflammatory milieu. While therapeutic hypothermia has targeted the latent and secondary phase, we now need therapies that cover the continuum of brain injury that spans hours, days, weeks and months after the initial event. Most agents have several therapeutic actions but can be broadly classified under a predominant action (e.g., free radical scavenging, anti-apoptotic, anti-inflammatory, neuroregeneration, and vascular effects). Promising early/secondary phase therapies include Allopurinol, Azithromycin, Exendin-4, Magnesium, Melatonin, Noble gases and Sildenafil. Tertiary phase agents include Erythropoietin, Stem cells and others. We review a selection of promising therapeutic agents on the translational pipeline and suggest a framework for neuroprotection and neurorestoration that targets the evolving injury.

Key words
Neonatal encephalopathy, neuroprotection, neurorestoration, therapeutic hypothermia

1.0 Introduction

The incidence of NE varies across the world, affecting 1-3.5/1000 live births in high-resource settings [1, 2] and ~26 per 1000 in low-resource settings [3]. Therapeutic hypothermia (HT) is currently the only treatment for NE, and is routinely used in high-resource settings [4]. While HT is clearly beneficial with a number needed to treat to prevent death or disability of 7 [4], infants still experience unacceptably high rates of adverse outcomes. Recent UK single centre data show that, with the current practice of HT, mortality of NE has reduced from 25% to 9%, while disability dropped from 20% to ~16% with a reduction in the rate of cerebral palsy (CP) [5]. It is clear that not all children benefit from hypothermia treatment and some level of intellectual impairment, such as memory problems, remain high even in the absence of cerebral palsy, and become specifically apparent at school age [6]. Further attempts to refine HT suggest current cooling protocols are near optimal [7] and adjunct therapies to HT are needed to improve long-term outcomes [8]. Here we summarize the key phases of brain injury in NE and describe the main neuroprotective agents that have been studied pre-clinically and clinically as adjunct therapies with HT. Most have focused on the secondary phase, but the realization that brain injury processes continue into the tertiary phase is now shifting the focus into later therapies.

2.0 Primary, latent, secondary and tertiary brain injury epochs: can we target each epoch?

As described in detail in the present issue (Gunn et al), NE evolves over time; key phases of brain damage have been described, largely based on HI animal models [9] and supported by magnetic resonance spectroscopy (MRS) data in human term neonates with NE [10] **(Figure 1).**

2.1 Acute HI (Primary)

During an acute hypoxic ischemic insult, some cells undergo primary cell death; the magnitude of cell death will depend on the timing and severity of the insult (acute vs chronic insult) and sensitizing factors such as inflammation sensitization [11], background health and genetic factors. In the absence of oxygen and glucose, the neurons supply of high energy phosphates such as ATP falls below a critical threshold, and when the N+/K+ ATP-dependent pump begins to fail neuronal depolarization occurs. The synaptic cleft begins to flood with glutamate, which activates the N-methyl-D-aspartate (NMDA) receptor. Toxic cytoplasmic Ca^{2+} concentrations develop that trigger many downstream neurotoxic cascades, including activation of nitric oxide synthase and xanthine oxidase, which generates high levels of toxic reactive oxygen species (ROS) and cytosolic phospholipases, eicosanoid release and inflammatory cascade. While it is not feasible to start therapies before acute HI, it is clear that susceptibility in the developing CNS is affected by means of preconditioning (defined as a transient, sublethal stress which results in tolerance to later, otherwise lethal, cerebral ischemia)[12]. In addition, the pre-clinical and clinical literature has shown that the stage of brain maturity at the time of HI plays a central role in the response to injury.

2.2 Latent phase (Definition: *existing but not yet manifest*)

After the primary insult, upon resuscitation and recovery of oxygenation and perfusion, transient restoration of cellular glucose utilization, ATP levels, and phosphocreatine (PCr) occurs. This early latent phase has been termed the "therapeutic window" for cooling and is believed to span a period of 6-12 hours [13], although its duration depends on the insult severity [14]. In the fetal sheep, loss of protection was seen if initiation of HT was delayed to 8.5h after reperfusion [15] and the clinical benefit of delayed HT beyond 6h is attenuated compared to initiation of HT within 6h of birth[16]. It is thought that the neurotoxic cascade is inhibited during this phase, when there is endogenous inhibition of oxidative metabolism, and increased tissue oxygenation [17].

2.3 Secondary phase (secondary energy failure)

Thereafter, in neonates with moderate to severe primary injuries, a secondary decrease of high energy phosphates occurs that parallels a decrease in tissue oxidative metabolism and development of cell injury [10]. Although still a matter of intense investigation, mechanisms underlying this secondary

phase involve mitochondrial functional impairment [18], oxidative stress, apoptosis and necroptosis, and neuroinflammation. In animal models, this secondary phase starts between 6-12 hours following the HI insult and lasts for a few days. In neonates with NE during the secondary phase, MRS shows reduced cerebral PCr/Pi, NTP/total mobile phosphates [10], increased brain lactate [19] and reduced N-acetyl-aspartate (high Lac/NAA) [20], an alkaline intracellular pH (pHi) [21] and diffusion restriction or cytotoxic edema on diffusion-weighted imaging; the severity and location of changes on imaging accurately predict neurodevelopmental outcome at 2 years of age.

2.4 Tertiary phase
Even after secondary cell death has subsided, effects on the brain can persist including sensitization to inflammation or injury, increased seizure susceptibility, persistent inflammation and gliosis, impaired oligodendrocyte maturation and myelination, altered proliferation and synaptogenesis, and epigenetic alterations. These events have been combined under the term "tertiary phase" that could last between weeks to years after the initial perinatal insult [22].

The concept that the tertiary phase can be a target for neurorestoration is recent [22]; rehabilitation has been the only available approach, acting by promoting plasticity of the damaged brain. Other approaches targeting the tertiary epoch could potentially further improve long-term outcome by either promoting plasticity or modulating mechanisms that counteract plasticity.

Recent pre-clinical studies in adult ischemic stroke have shown that macrophages/microglia respond in a dynamic, changing way during the evolution of brain injury after HI, with harmful phenotypes promoting injury and healing phenotypes repairing and remodeling tissue [23, 24]; these are sometimes termed M1 and M2 phenotypes respectively, although this terminology is controversial [25]. Experimental evidence suggests that early after HI, local microglia and newly recruited macrophages adopt a 'healing' phenotype; then as the injury evolves, a pro-inflammatory harmful profile develops and this dominates the injured brain for days after HI, driving secondary brain injury via pro-inflammatory mediators and oxidative damage **(Figure 2).** Harmful, pro-inflammatory phenotypes are typically characterized by the release of neurotoxic mediators (tumour necrosis factor alpha (TNFα), interleukin 1 beta (IL-1 β), monocyte chemoattractant protein (MCP) 1, macrophage inflammatory protein (MIP)-1α and interleukin 6 (IL-6)) and ROS that exacerbate brain damage. Healing phenotypes, typically in less severely injured cells, secrete reparative cytokines, such as interleukin 4 (IL-4) and interleukin 10 (IL-10) [26]. Microglia and macrophages thus appear to undergo mixed polarization related to the spatio-temporal evolution of ischemic brain damage **(Figure 2).** Understanding these mechanisms and identifying cellular targets that promote repair in the tertiary phase, blocking the shift to harmful pro-inflammatory phenotypes, may be a powerful neuroprotective strategy [24].

3.0 Inflammation sensitization
A subgroup of neonates with NE are exposed to infection and inflammation during labor, prior to HI. This prior exposure (termed 'sensitization') increases the likelihood of death and disability for survivors [27, 28]. Pre-clinical studies confirm exacerbation of brain injury with the combination of inflammation induced by lipopolysaccharide *Escherichia coli (E.coli)* 4h before HI [11, 29, 30]. Given the significant risk of adverse outcomes with co-existing inflammation and birth asphyxia, it will be important to determine the impact of HT and other neuroprotective interventions in inflammation-sensitized models (reflecting both gram positive and negative organisms) [31, 32] as well as in models combining both HI and inflammation to guide future clinical trials (**Figure 2**).

4.0 Therapeutic hypothermia
Currently, therapeutic hypothermia (HT) is standard care for moderate to severe NE. The optimal protocol is core body temperature maintained at 33.5 ± 0.5°C for 72 hours, followed by slow

rewarming of 0.2-0.5°C per hour to normothermia, depending on local protocols [33]. While the effects of HT are significant, there is still an unacceptably high number of treated neonates with adverse outcomes, in particular cognitive and memory problems. There is evidence that HT should be started as early as possible within the latent phase to optimize protection [34]. Commencing HT within 6h of birth and continuing for 72h, spanning as much for the latent and secondary phase as possible is important so the phase of seizures and cytotoxicity is covered. Pre-clinical studies have shown that deeper cooling is detrimental such that there is a U shape protection response; cooling by 8°C lead to no protection compared to normothermia and cooling by 3.5-5°C provided significant protection compared to normothermia [35]. HT for 48h in the fetal sheep was less effective than 72h cooling in the same model [36], suggesting the importance of cooling duration and for clinicians to continue for the full 72h if possible. Slow rewarming in the fetal sheep model was seen to have no effect on oligodendrocyte survival, myelination or suppression of microgliosis compared to fast rewarming, but did modestly reduce astrocytosis [37] **(Figure 3).** Studies have thus shown that the current cooling protocols are near optimal and that deeper and longer cooling does not improve outcome [38, 39] **(Figure 3).**

Adjunct therapies with complementary or additive effects to HT are therefore urgently needed to improve outcomes in term neonates with moderate to severe NE. In the following sections, we give an update on the current status of pre-clinical and clinical studies in a selection of promising adjunct therapies. We focus on therapies given after birth (not to the mother with fetal distress). We suggest specific therapies might be most effective when targeted at particular phases of the neurotoxic cascade [8, 40]. The optimal response to most adjunct therapies is likely to occur when they are started as early as possible in the latent phase. In the sections below, we combine the latent and secondary phases as, during these phases, adjunct therapies are likely to be co-administered with HT. Following this we will discuss agents targeted to the tertiary phase **(Figure 1).**

5.0 A review of promising therapeutic agents
We describe here a selection of key therapies that have been tested pre-clinically (and a few clinically) to target key events of the Latent, Secondary and Tertiary phase of injury after a HI injury.

(i)   The latent and secondary phase (excitotoxicity, oxidative stress, inflammation, apoptosis).
We include the following therapies: Allopurinol, Azithromycin, Ascorbic acid and Ibuprofen, Exendin-4, Magnesium sulfate, Melatonin, Noble gases (Argon, Xenon), Sildenafil

(ii)   The tertiary phase (neurorestoration, regeneration, repair, neurogenesis, angiogenesis, immunomodulating).
We include the following therapies: Erythropoietin, Stem Cells. Other agents (e.g., Sildenafil) may also have some neurorestorative effects during this phase; further evidence is awaited.

(i)   ***THERAPIES SUITED FOR THE LATENT AND SECONDARY PHASE***

5.1 Allopurinol
Allopurinol and its metabolite oxypurinol, a xanthine oxidase inhibitor shows promise in reducing delayed cell death in experimental models of perinatal asphyxia [41].

Mechanisms of action:
Allopurinol chelates free ferric ion and cupric ion and scavenges hydroxyl radicals directly in high dosages, and additionally inhibits the formation of superoxides by inhibiting xanthine oxidase, which is activated by the hypoxic-ischemic event [41].

Pre-clinical evidence: In pre-clinical studies, allopurinol was shown to decrease brain injury in rodent models of neonatal brain injury [42]. On magnetic resonance spectroscopy in a piglet model of HI, a

preservation of the cerebral energy status was found in allopurinol treated animals [43]. Additionally, allopurinol treated sheep with fetal hypoxia had less cardiac oxidative stress, umbilical blood flow was preserved in the allopurinol-treated animals, while this was not the case in the control group. These findings suggest a cardioprotective effect of allopurinol in sheep [44].

Clinical evidence: In 2012, a Cochrane review and meta-analysis with three randomized/quasi-randomized controlled trials (N=114 infants) in neonates with NE found no clear difference in severe neurodevelopmental disability or death among survivors at 18 months or at 4 to 8 years after allopurinol versus placebo (RR 0.78, 95% CI 0.56 to 1.08) [45]. Long-term follow-up studies of 2 out of 3 studies included in the Cochrane review [46], suggested a positive effect of allopurinol in treated infants with moderate NE : 65% of controls had a severe adverse outcome, defined as death or severe neurodevelopmental disabilities, compared to 25% in the allopurinol-treated infants (p=0.047). Even with high allopurinol blood levels, no side effects were seen.
A recent study of 222 women in labor with suspected fetal hypoxia randomly assigned to receive allopurinol or placebo demonstrated that allopurinol administration does not lower biomarkers of neuronal damage in cord blood [47], except in the *ad hoc* analysis in girls. However, there was no improvement in long-term developmental and behavioural outcome at 5 years of age in the treated group [48].
The potential effect of adding early allopurinol to HT in neonates with moderate to severe NE on the incidence of death and severe neurodevelopmental impairment at 24 months of age (ALBINO) trial (NCT03162653) is currently recruiting (n=846 participants). In the ALBINO trial, an initial dose of 20 mg/kg is administrated in the first 30 minutes after birth in infants >36 weeks gestational age with perinatal acidosis and clinical signs of evolving NE. A second dose of 10 mg/kg is given 12h later in those infants who undergo HT. Outcomes will be reviewed at 2 years [49].

5.2 Azithromyin (AZI)
Azithromycin (9-deoxy-9a-aza-9a-methyl-9a homoerythromycin) (AZI) is a macrolide antibiotic used to treat infections. An important property of AZI is that it accumulates in white blood cells, particularly macrophages and neutrophils, [50] and crosses the placenta and blood-brain barrier where it has prolonged antibacterial, anti-inflammatory and immunomodulatory properties [51]. AZI accumulates in tissues, including brain, from which it is eliminated slowly. In P7 rats, blood AZI levels followed a 2-compartment model. AZI is administered by IV administration in humans and large animal models and intraperitoneally in P7 rats [52]. Data from a pre-clinical rodent model has shown that the likely target blood AZI levels for optimal neuroprotective effect is around 3000-5000 ng/ml and the brain AZI target range for optimal neuroprotective effect is around 700-1000 ng/g tissue [52]. The safety and efficacy of these levels need to be clarified in human newborns.

Mechanisms of action: AZI promotes a macrophage anti-inflammatory, healing phenotype expression [53, 54]; its neuroprotective properties are likely mediated by promotion of the microglial and/or monocyte anti-inflammatory and healing phenotype [53, 55]. The key aspects of macrophage biology are driven by the phenotype of macrophage arginine metabolism [53]. *Pro-inflammatory macrophages* express the enzyme nitric oxide synthase, which metabolizes arginine to nitric oxide (NO) and citrulline. NO can be metabolized to downstream oxygen free radicals. *Anti-inflammatory macrophages* are characterized by expression of the enzyme arginase, which hydrolyzes arginine to ornithine and urea. The arginase pathway limits arginine availability for NO synthesis; furthermore ornithine feeds into a downstream pathway important for cellular proliferation and tissue repair [56]. In preclinical studies it was observed that blocking macrophage/arginine metabolism resulted in loss of protection [53] **(Figure 2).**

Pre-clinical evidence: In an adult mouse model of middle cerebral artery occlusion, AZI reduced brain damage and neurological deficit [53]. Although all AZI doses improved function and reduced brain

damage, protection was dose-dependent (1.5-150mg/kg), with a therapeutic window of up to 4.5hours. In a P7 rat Rice Vannucci model, multiple treatment protocols were evaluated (doses ranging from 15 to 45 mg/kg; treatment onset 15 min to 4 h post-hypoxia, and comparison of 1 vs. 3 injections) [52]. Efficacy declined with increasing treatment delay. Three AZI injections, administered over 48h, improved performance on both function measures and reduced brain damage more than a single dose [52]. There is currently no data on AZI combined with HT and this needs to be assessed in pre-clinical models.

Clinical evidence: AZI has become the macrolide of choice with a better safety profile than erythromycin. In older adult patients, AZI is known to increase the risk of prolonged cardiac repolarization and QT interval on the electrocardiogram (ECG), which increase the possibility of cardiac dysrhythmias and torsades de pointes [57]. In neonates, previous studies have shown the association of a prolonged QT interval and sudden infant death syndrome [58]. HT is known to increase the QT during the cooling period with reversal following rewarming [59]. The safety of AZI with HT needs to be assessed in pre-clinical studies before planning carefully designed clinical trials of AZI in human neonates with NE to ensure safety and test efficacy.

5.3 Ascorbic acid and Ibuprofen
A combination of anti-inflammatory and anti-oxidant agents could hypothetically benefit infants with NE. Ibuprofen is a nonsteroidal anti-inflammatory COX (1 and 2) inhibitor, which gained popularity in preterm infants as an effective treatment for patent ductus arteriosus both in PO and IV formulation. [60]. Pharmacokinetic studies showed rapid increase in clearance rate with age and suggested increased dosing with maturation [61]. A study conducted in children demonstrated that IV ibuprofen readily penetrates CSF with a peak level attained after 30-40 minutes [62]. There is, however, no data about pharmacokinetics of oral administration in presence of NE and HT.

Mechanisms of action: Ascorbic acid is naturally concentrated in the brain [63]. The neuroprotective role of ascorbic acid is mainly due to its anti-oxidant effect and its ability of scavenging reactive oxygen species and reactive nitrogen species [63, 64].

Pre-clinical evidence: Ibuprofen demonstrates a potential anti-inflammatory neuroprotective effect via its ability to reduce the production of central inflammatory cytokines, decrease microglial activation, decrease expression of endothelial adhesion molecules, as well as increase in IL-1 receptor antagonist gene [65-68]. In neonatal rodent models of HI, subcutaneous ibuprofen injections starting 2 h after HI, and continued daily in the first week of life, was neuroprotective [66, 67]. In animal studies, higher intraventricular injection doses of ascorbic acid in a rat model of neonatal HI were associated with neuroprotection with reduced cell necrosis in the cortex, thalamus, caudate, putamen, and hippocampus CA1, and reduced apoptosis in cortex [69].

Clinical evidence: In a pilot randomized controlled study, with no HT, 30 neonates with NE randomized to receive combination of intravenous ascorbic acid 100 mg/kg/day for 3 days in addition to oral ibuprofen 10 mg/kg/day for one day followed by 5 mg/kg/day for 2 days, were compared to 30 neonates who received placebo. Although that regimen was not associated with significant complications, that study did not show significant difference in clinical outcome or developmental assessment at 6 months [70]. Thus, despite the hypothetical and preclinical promise, the current evidence does not support the use of ibuprofen and ascorbic acid for the treatment of NE.

5.4 Exendin-4
Exendin-4 is a small peptide drug approved by the Food and Drug Administration (FDA) in 2005 and European Medicines Agency (EMA) in 2006 for the treatment of type 2 diabetes mellitus [71]. It is an analogue of the human glucagon-like peptide-1 (GLP-1) and acts on GLP-1 receptors and plays a role

in regulating blood sugar levels by enhancing insulin release from the pancreas. Exendin-4 has a half-life of 60–90 min [72] and is administered twice daily subcutaneously as a therapy to lower blood glucose levels in children and adults. Exendin-4 can also be administered intravenously. In most rodent models it is administered intraperitoneally. It gains CNS access when administered systemically both in adult and neonatal animals [73]. There is, however, a lack of information on PK after intravenous administration in neonates with NE.

Mechanisms of action: Exendin-4 efficiently crosses the blood–brain barrier [74] and its cellular receptor (GLP1R) is found throughout the brain [75]. Exendin-4 has multiple actions including neurotrophic [76], neurogenic [77], immune-modulatory [78] and anti-apoptotic [79] effects, at least partly via activation of the PI3K/AKT pathway [80].

Pre-clinical evidence: Multiple studies in animal models of brain injury have shown that several GLP-1 receptor agonists have neuroprotective effects [81]. In neonatal mice, exendin-4 was well tolerated under control conditions and after HI. Blood glucose levels were not altered, there was no sign of adverse organ histopathology or inflammation. Despite reduced weight gain initially, weight was rapidly restored following end of treatment [73]. Exendin-4 is well tolerated in patients when given as treatment of type-2 diabetes but it appears as higher doses are needed in order to provide neuroprotection at least in rodents. However, according to the pharmacology and toxicity review of Exendin-4, doses of 450 times the clinical dose produces no hypoglycemia, no adverse effects or organ pathology (https://www.accessdata.fda.gov/drugsatfda_docs/nda/2009/021919s000pharmr.pdf). Recently, it was demonstrated that exendin-4, given directly at 2h after the insult (4 doses 0.5 ug/g), reduces tissue loss in postnatal day 7 and 10 mice after HI accompanied by attenuation of cell death as well as microglial and astroglial reactive responses [73]. In a neonatal rodent model of HI post-treatment with both HT and Exendin-4, brain injury was reduced. A moderate dose of exendin-4 enhanced neuroprotection provided by HT [73]. The neuroprotective efficacy of exendin-4 has not yet been assessed in large animal models of NE.

Clinical evidence: Exendin-4 improves clinical motor and cognitive functions in patients with Parkinson's disease and multiple clinical studies have shown that several GLP-1 receptor agonists have neuroprotective effects [81]. However, up to now, no randomized trial has tested the exendin-4 in human neonates with NE; more preclinical studies are needed.

5.5 Magnesium sulfate (MgSO4)
There has been significant interest in $MgSO_4$ for perinatal neuroprotection; in many western countries, $MgSO_4$ is standard treatment in pregnancies threatened by preterm labour at <32 weeks' gestation. Whether MgSO4 is a safe and effective adjunct therapy postnatally in combination with HT for term neonates with moderate to severe NE is still unclear.

Mechanisms of action: Magnesium ions reside within the glutamatergic NMDA receptor channel and competitively antagonize calcium ion entry. The excessive release of excitatory neurotransmitters such as glutamate is a key mechanism of injury in the hours following HI and is a target for MgSO4 neuroprotective intervention. Other effects of MgSO4 include a reduction in secondary inflammation and associated injury, stabilization of cell membranes, inhibition free radical production and improved cardiovascular stability. An important point is the preconditioning effect of MgSO4 in HI or excitotoxicity models in rodents. Preconditioning with MgSO4 was associated with improvement of mitochondrial resistance, attenuation of production of reactive oxygen species and pro-inflammatory cytokines [82].

Pre-clinical evidence: A recent pre-clinical piglet study comparing HT to a MgSO4 bolus and constant infusion with HT was well tolerated (no hypotension) and doubled serum magnesium (0.72 vs

1.52mmol/L), with a modest (16%) rise in CSF. With MgSO4 added to HT compared to HT alone, there was an overall reduced cell death and increased oligodendrocyte survival, but no improvement on aEEG recovery or MRS [83]. Maintaining a supra-systemic magnesium concentration in combination with HT had a small attenuation in the overall extent of cell death may not translate into a substantive improvement in outcome in clinical trials. However, such an incremental effect with a good safety profile may justify further pre-clinical studies of MgSO4 in combination with a cocktail of multiple complementary therapies in the future.

Clinical evidence: Hypotension is a common side effect of MgSO4. Severe hypotension is thus a significant concern with combination therapy. In a clinical study in neonates with NE, Rahman et al. reported a favorable safety profile of MgSO4 boluses with HT; however, hypotension was only broadly classified as either moderate (treated with volume therapy and/or one inotrope) or severe (on multiple inotropes) and they did not report cardiovascular parameters immediately following drug administration [84].

Tagin et al. [85] performed a systematic review and meta-analysis of 5 randomized controlled trials (n = 182 neonates) and reported that there was no difference in the composite outcome of moderate-to-severe neurodevelopmental disability or death at 18 months (RR 0.81, 95% CI 0.36 to 1.84), seizures (RR 0.84, 95% CI 0.59 to 1.19), mortality (RR 1.39, 95% CI 0.85 to 2.27), or hypotension (RR 1.28, 95% CI 0.69 to 2.38). They highlighted a significant reduction in the unfavorable short-term composite outcome (RR 0.48, 95% CI 0.30 to 0.77) between the MgSO4 and the control groups. However, the trend towards an increase in mortality in the magnesium group was a major clinical concern.

A recent systematic review of pre-clinical and clinical studies over the last 10 years [86] suggested further pre-clinical testing is needed before clinical trials. Some of the issues highlighted by this systematic review were: (i) none of the perinatal rodent studies that suggested benefit directly controlled body or brain temperature, (ii) most studies did not control for sex, nor study long-term histological and functional outcomes; (iii) pragmatic treatment regimens were not used; and (iv) many did not report controlling for potential study bias. More studies are needed.

5.6 Melatonin (MEL)

Mechanisms of action: MEL has diverse anti-oxidative mechanisms preventing free radical induced damage to the electron transport chain and mitochondrial DNA [87] as well as protection against excitotoxic damage [88]. MEL also exerts its effects through the downregulation of the pro-inflammatory transcription factor, nuclear factor-κB (NFκB), thereby reducing neuroinflammation. Mitochondrial integrity is preserved through the stabilization and protection from nitro-oxidative damage to membrane lipids and inhibiting pro-apoptotic proteins [89]. MEL stimulates neuronal differentiation and cell proliferation, which are critical in neuroplasticity.

Pre-clinical evidence: Preclinical studies have shown neuroprotective benefits of MEL combined with HT. In an HI piglet model, compared to HT alone, intravenously administered MEL at 30mg/kg, 10 mins after HI and repeated at 24h plus HT significantly improved cerebral energy metabolism (proton magnetic resonance spectroscopy studies), reduced cell death in deep brain and white matter structures, and decreased microglial activation in the cortex 48h after injury [90]. *In vitro* [91] and piglet studies [90] suggest the therapeutic level of MEL is ~15-30mg/L. The experience from previous pre-clinical piglet studies [90, 92, 93] suggest that MEL neuroprotection is time critical and dose dependent. Therapeutic melatonin levels are 15-30mg/L and for optimal effect on the high levels of oxygen free radicals (OFRs), these levels need to be achieved within the first 2-3h after birth.

Clinical evidence: Aly et al. [94] administered enteral MEL as an adjunct to HT in term NE with a total 50mg/kg dose over 5 days, commenced within 6h. Infants treated with MEL had reduced plasma levels

of NO and plasma superoxide dismutase after 5 days; there was also reduced white matter injury on MR, reduced seizure activity on EEG at 2 weeks and increased survival free of disability at 6 months compared with infants treated with HT. This study used the enteral route for MEL, dissolved in water [94]. Aly et al. demonstrated a modest doubling of plasma MEL levels from baseline to day 5 with 10mg/kg/day enteral melatonin (21±2.4 to 42.7±5.1 pg/ml (p<0.001), suggesting that the intravenous route of administration is more effective in elevating plasma concentrations. Interestingly the HT group (without enteral MEL) also showed an increase in plasma MEL from baseline to day 5 (20.6±2.5 to 32.1±3.5 pg/ml, p<0.001), confirming an endogenous MEL response previously seen in brain injury [95]. This feasibility study concluded that early administration of MEL to asphyxiated term neonates might improve brain injury, but larger powered clinical studies of HT with MEL reaching therapeutic MEL levels are urgently needed. Recently, the five trials performed so far in NE, involving 215 infants were systematically reviewed [96]. Limitations of these trials include their small size, inconsistent melatonin dosing regimens, lack of pharmacokinetic data, and a paucity of neurodevelopmental follow up.

5.7 Noble gases (Argon and Xenon)

Mechanisms of action: Noble gases have neuroprotective properties following different experimental brain injuries [97]. The most intensively studied noble gases following neonatal HI are Xenon and Argon.

Xenon, an approved and safe anesthetic drug [98], is a non-competitive antagonist of the NMDA subtype of the glutamate receptor. Other neuroprotective actions include activation of the two-pore K^+-channel, inhibition of the calcium/calmodulin dependent protein kinase II, activation of anti-apoptotic effectors Bcl-XL and Bcl-2 and induced expression of hypoxia-inducible factor 1 alpha and its downstream effectors Epo and vascular endothelial growth factor, which can interrupt the apoptotic pathway [99].

Argon, being a cost-efficient alternative to xenon, is not an anesthetic. Its predominant mode of action is thought to be mainly anti-inflammatory, being involved in ERK 1/2, PI3K/AKT/mTOR pathways [100].

Pre-clinical evidence: Several groups have demonstrated Xenon neuroprotection in in-vivo models [101, 102]. Combined Xenon and HT is effective, even when Xenon administration is delayed for some hours after HI [103]. Neuroprotective effects have been demonstrated by 50% xenon continued for 24 hours started 2 hours after HI with 24h HT in piglets [104]. In another model, when combined with HT, immediate or delayed 50% inhaled Xenon, enhances HT neuroprotection [105, 106].

In vitro, 70% Argon has been shown to be neuroprotective. In vivo animal studies of neonatal hypoxic-ischemic brain injury found that 70% [107] and 50% [108] inhaled argon, combined with HT were neuroprotective.

Clinical evidence: Two clinical trials of inhaled xenon as an adjunct to HT have been performed in the UK. The TOBY-Xe proof of concept randomized trial in 92 infants with moderate to severe NE found no evidence of short-term benefit based on the surrogate outcome measure 1H MRS Lac/NAA (Azzopardi et al., 2016), [109] confirmed subsequently at 2 years of age [110]. This was a logistically difficult study to perform with the mean age of starting xenon 10h after birth, which is likely to be beyond the therapeutic window for Xenon's action on the NMDA receptor release of glutamate. Data from the second trial, "Cool Xenon 3", using 50% of inhaled xenon, is not yet available.

A Cochrane review concluded that data is inadequate to show whether HT plus Xenon is safe or effective in NE [111]. Xenon's major disadvantage is that it is difficult to use in clinical practice due to its scarcity (0.0087 ppm in the air) and high costs, along with the need for closed-circuit delivery (including cuffed tubes) and recycling systems.

5.8 Sildenafil
Sildenafil is a highly potent selective inhibitor of phosphodiesterase type-5 (PDE5i), regulating the second messenger cyclic guanosine monophosphate (cGMP).

Mechanisms of action: The cGMP-mediated pathway is known to regulate neurogenesis, neuroinflammation, myelination, synaptic plasticity, cerebral blood flow, emotion and cognition in the adult brain [112]. Sildenafil appears to be both neuroprotective and neurorestorative in the adult brain through regulating neurons' survival and function, balancing neuroinflammation, alleviating white matter injury, and increasing angiogenesis, as well as improving learning ability and memory and an analgesic effect [113].

Pre-clinical evidence: In a rat model of NE, a dose of sildenafil given intraperitoneally immediately after HI at P7 improved functional outcome, reduced HI damage, and reduced apoptosis and inflammation [114]. Beneficial effects of sildenafil on functional outcome and HI damage were observed when sildenafil was started orally 12h after HI at P10 and continued for 7 days, with potential effect on neurogenesis and improvement of myelination [115], suggesting that this drug exerted a neurorestorative role in the tertiary phase in this model. There is currently no data on sildenafil combined with HT and this needs to be assessed in pre-clinical models.

Clinical evidence: Sildenafil is already used to treat neonates with persistent pulmonary hypertension. Clinical trials (NCT02812433 and NCT NCT04169191) are ongoing in neonates with NE treated with HT to determine PK and optimal safe dose.

(ii)   THERAPIES SUITED FOR THE TERTIARY PHASE

5.9 Erythropoietin (EPO)
EPO is a hemopoietic growth factor and pleiotropic cytokine [116]. Epo can be administered intravenously or subcutaneously. It requires small volumes, so it does not impose a fluid burden. The most commonly used treatment regimens used to stimulate erythropoiesis in neonates is 400 U/kg 3 times a week given subcutaneously, or 200 U/kg daily given intravenously.

Mechanisms of action [117]:
Anti-inflammatory: Prevents secondary, delayed rise in IL-1 beta and attenuates the infiltration of leukocytes.
Antiapoptotic/ Neurorestorative: Epo acts through several pathways to improve cell survival.
Via the Epo receptor dependent pathway, Epo activates Jak2 kinase leading to NFkB and Stat5 mobilization into the nucleus and gene expression of the antiapoptotic factors, bcl and bcl-xl [118]. Epo also reduces the expression of Fas/FasL which are part of the extrinsic apoptotic pathway [119].
Anti-oxidative: Increases glutathione peroxidase enzyme activity and decreases lipid peroxidation levels. Epo inhibits the transcription of iNOS mRNA responsible for NO production and oxidative stress [120].

Pre-clinical evidence: EPO has remarkable neuroprotective and reparative effects in the central nervous system [118]. Recombinant human erythropoietin (rEpo) is neuroprotective in immature animals. In neonatal rodent stroke models, multiple doses are more effective than a single dose, with 3 doses of 1000 U/kg given immediately after injury, 24 hours, and 7 days later showing equivalent benefit to 3 doses of 5000 U/kg given at 24-hour intervals for 3 days after injury [121, 122].

It is unclear whether the combination of high-dose rEpo therapy with HT can further improve outcomes. In a recent near-term fetal sheep study, HT and rEpo independently improved neuronal survival, with greater improvement with HT. There was no significant improvement in any outcome

after combined rEpo and HT compared with HT alone. It was concerning that the combination was associated with increased numbers of cortical caspase-3-positive cells compared with HT alone. These data suggest that the mechanisms of neuroprotection with HT and rEpo overlap and, thus, high-dose rEpo infusion does not appear to be an effective adjunct therapy for HT [123].

Clinical evidence: A recent systematic review and meta-analysis by Razak et al. [124] from six RCTs (EPO=5 and darbepoetin α=1) (n = 454 neonates) study concluded that EPO administration in neonates with NE reduces the risk of brain injury, cerebral palsy, and cognitive impairment. The results of the meta-analysis include:

i)   Trend towards lower death - EPO with or without HT: (five RCTs, 368 participants, relative risk (RR) 0.74, 95% confidence interval (CI) 0.47-1.19, Level of evidence: low)

ii) Trend towards lower death - EPO without HT: (four RCTs, 318 participants, RR 0.89, 95% CI 0.49-1.32, Level of evidence: low)

iii) Cerebral palsy risk reduced - EPO without HT, compared to placebo (two RCTs, 230 participants, RR 0.47, 95% CI 0.27-0.80, Level of evidence: moderate)

iv) Moderate to severe cognitive impairment risk reduced - EPO without HT compared to placebo (two RCTs, 226 participants, RR 0.49, 95% CI 0.28-0.85, Level of evidence: moderate)

v)   Brain injury risk reduced - EPO with or without HT (two RCTs, 148 participants, RR 0.70, 95% CI 0.53-0.92, Level of evidence: moderate).

Wu et al. showed that EPO treatment was associated with less brain (MRI) injury only in patients whose placentas exhibited no chronic histologic changes [125]. The High Dose Erythropoietin for Asphyxia and Encephalopathy (HEAL) Trial (NCT02811263) will evaluate whether high-dose Epo reduces the combined outcome of death or neurodevelopmental disability when given in conjunction with hypothermia to neonates with moderate/severe NE [126]. Recruitment of the HEAL trial is completed (n=501) and is pending determination of the primary outcome. The estimated study completion will be in September 2022. Preventing Adverse Outcomes of Neonatal Hypoxic Ischaemic Encephalopathy With Erythropoietin (PAEAN trial) (NCT03079167) is another ongoing study currently recruiting participants (n = 300); estimated study completion is December 2021.

5.10 Stem Cell Therapy
With development of reliable methodologies for collection, red blood cell and volume reduction, human umbilical cord blood mononuclear cells (hUCB cells) have been used for over 30 years for transplants, mostly for hematopoetic disorders, with FDA approval [127].

Mechanisms of action: hUCBs demonstrate a range of activities following injury, including increase in neurotrophic and angiogenic factors, decreased inflammation and microglial activation, as well as modification of T cell migration into injured areas of the brain [128-131]. Mesenchymal stromal cells (MSCs) work mainly via paracrine secretion of multiple cytokines and cargo-bearing exosomes, which affect the biology of adjacent and distant responder cells and tissue [132].

Pre-clinical evidence: In animal studies, hUCB cells obtained from cord blood and MSCs from multiple sources show promise as interventions in NE. In addition to decreasing markers of inflammation, administration of MSCs in a neonatal brain injury model was associated with increased differentiation towards neurons and oligodendrocytes and decreased proliferating inflammatory cells post injury. Additional work suggested repeat dosing, several days after injury, enhanced the cell differentiation and functional outcome benefits [133].

Virtually all of the animal model studies with hUCB cells have been done without concurrent HT. These studies are summarized in several reviews, and most report anatomic sparing of injured neural tissue,

less apoptosis and a range of improved neurologic outcomes [131]. There are limited dose-ranging studies for hUCB cells and for MSCs in the neonatal injury models. Tsuji *et al* catalogued many of the early hUCB studies, with anatomic and functional outcome benefits noted with doses ranging from $1.5 \times 10^4 - 1 \times 10^8$ cells in rat and mouse pup models of hypoxic-ischemic injury, with cells administered 0 - 7 days after injury [134]. Moderate to high doses performed better in one study, which included a range from $1 \times 10^6 - 1 \times 10^8$ cells given 24 h after injury [135]; there was no benefit for cell recipients with the lower dose alone. In the studies of administration of MSCs 3 and 10 days after HI, the early dose seems to primarily influence cell proliferation, with the second dose acting via different pathways involved with injury response and brain development [133], indicating possible additive effects from repeat doses. Caution is needed as some reports suggest the importance of type and timing of injury relative to brain development. In an excitotoxic brain injury model, modeling preterm brain injury, where hUCB cells were administered at P5, Dalous et al. reported increased microglial activation and increased white matter injury after intraperitoneal administration of hUCB cells in concert with intracerebral injection of ibotenate, a glutamate analogue, with more injury with higher doses [136]. Several pre-clinical MSC studies have included HT. The first report from Park et al., with cells administered prior to initiation of cooling, cell recipients had improved anatomic as well as functional outcome [137]. Following this, an important study by Herz et al. found less anatomic and functional benefit in the group of neonatal animals treated with cooling followed 3 days later with administration of MSCs derived from rodent bone marrow. Animals receiving cells alone, or only cooling, did better than those receiving both cooling and MSC's [138]. Ahn et al. subsequently reported that neonatal animals with hypoxic-ischemic injury who were cooled, and received human cord-blood derived MSCs immediately after cooling had improved anatomic outcome and functional outcomes, with a decrease in markers of apoptosis and inflammatory cytokines [139].

MSC treatment via nasal administration demonstrates a strong therapeutic potential for repair of neonatal ischemic brain damage using the mouse model [140-143], with significant improvement in motor and cognitive behavior and decreased gray and white matter damage at 5 weeks post-HI. MSCs promote repair by stimulating endogenous regenerative processes and do not engraft in the brain. Intracranial MSC treatment increases gene expression of several neurotrophic and anti-inflammatory factors in the brain. Intranasal MSC administration holds strong potential as a non-invasive therapeutic strategy to repair neonatal HI brain injury, and thus might be used as an add-on after HT. Safety studies have shown no induction of any malignancies or other pathological abnormalities in the brain over time.

In a recent neonatal piglet study, augmentation of brain protection was observed with 2 doses of 30 million huMSCs given intranasally at 24 and 48h after HI, with HT from 1-13h, compared to HT alone [144]. Evidence for protection included: (i) faster aEEG recovery after HI; (ii) improved brain energy metabolism on phosphorus-31 MRS compared to HT alone; (iii) reduced TUNEL positive cells and increased oligodendrocytes in the white matter. No benefit of intravenous huMSCs with HT was seen compared to HT alone. Fluorescent-labelled huMSCs, administered intranasally 1h after HI were detected in brain tissue 12h after administration, confirming "homing" of intranasal MSC to the brain. Further longer-term studies are needed.

Clinical evidence: Because of the perceived lower risk with fresh, autologous cells in neonates, the first phase I clinical trial was approved with a similar dose of autologous and red-blood cell-reduced hUBC cells to the dose used for transplants [145]. The study demonstrated feasibility and safety of collection, preparation and infusion of cells. In another phase I report from Japan, investigators used all available hUCB mononuclear cells in the first 72 postnatal hours, without safety concerns, with a range of total cumulative post-processing cells dosed of $1.4–10.9 \times 10^8$ cells, which were equally divided into 3 doses and administered intravenously [146]. A multicenter, double-blind, placebo-controlled trial was halted for problems with enrolment (not safety) concerns after enrolling over 30

infants with NE (NCT02612155). In a study of available autologous cord blood cells for children with cerebral palsy, those who received doses ≥2 × 10^7 /kg demonstrated significantly greater increases in GMFM-66 scores above those predicted by age and severity compared to children with lower doses [147]. Allogeneic MSCs are being tested in phase I and II (phase II: NCT01828957, results not yet published) trials in premature neonates with evolving lung disease in the first postnatal weeks, and in preterm neonates with intracranial hemorrhage [148, 149] and neonates with perinatal arterial ischemic stroke (NCT03356821, a safety-feasibility trial). Along with the accumulated safety evidence from these studies, multiple clinical trials for graft versus host disease, some with inclusion of children may not show clear efficacy, but they do continue to reinforce safety [150]. With these reports plus evidence of efficacy in the neonatal animal models, a phase I trial of allogeneic cord tissue derived MSCs has been completed in neonates with NE (NCT03635450), but results have not been reported.

In addition to work with hUCB cells and MSCs from various sources, other cells with specialized immunomodulatory activity, or cells classified as stem cells, are being tested in neonates. Human amnion epithelial cell (hAEC) therapy is in various stages of clinical trials in neonates, particularly preterm infants with lung disease. Multilineage-differentiating stress-enduring (MUSE) cells are non-tumorigenic endogenous pluripotent-like stem cells mobilized from the bone marrow to the peripheral blood. These cells distribute to the connective tissue of organs and have been demonstrated to improve anatomic and functional outcomes in animal models of hypoxic-ischemic injury and Phase I trials for neonates with NE are in progress in Japan with the estimated primary completion date in December 2021 (NCT04261335) [151].

While promising in most pre-clinical models, human trials are at a very early stage. Animal models suggest the importance of type and timing of injury as well as dose-response. So far in the human trials, short-term safety signals have been reassuring, but importantly, there is no evidence for long term efficacy, and much work remains.

6.0 Complementary therapeutic cocktails (Figure 1)
There is considerable evidence from pre-clinical studies that Stem cells and Epo have actions that specifically target brain injury in the tertiary phase, stimulating regeneration and repair [8]. Combination studies are needed where agents that target the early phase of injury (Melatonin, Magnesium, Azithromycin, Noble gases) are given within 1-2 hours at the initiation of HT and agents targeting regeneration and repair are given after rewarming and continued into the tertiary phase (Epo, Stem cells). For these studies, longer term pre-clinical survival models are needed.

Recently, a pre-clinical piglet study (72-hour duration) assessed neuroprotective effects of such complementary therapies – HT, melatonin and Epo. The following groups (i) HT & Melatonin, (ii) HT & Erythropoietin and (iii) combined HT, Melatonin & Erythropoietin were compared after HI [152]. MEL and Epo were safe when administered with HT and had complementary effects on brain protection. HT+MEL *double therapy* showed modest, but consistent beneficial effects across aEEG, Lac/NAA and TUNEL cell death immunohistochemistry. HT+Epo *double therapy* had most effect on oligodendrocyte survival; this concurs with Epo's effect on regeneration and indicates longer term studies are needed to assess its full potential. HT+MEL+Epo *triple therapy* did not augment brain protection further from that seen with *double therapies* and there was slower aEEG recovery with triple than with double therapy. The data suggests that staggering the administration of therapies with early MEL and later Epo (after HT) may be optimal; each therapy has complementary actions, which may be time critical during the neurotoxic cascade after HI. Future studies need to consider the timing as well as dose of therapies carefully.

Nonomura et al. [153] reported on the feasibility of combination therapy with 300 U/kg Epo every other day for two weeks, 250 mg/kg magnesium sulphate for three days, and HT in NE; efficacy data are awaited (ISRCTN33604417).

7.0 Conclusion

Neurodevelopmental outcomes in moderate to severe NE have improved with HT; overall there have been reductions in both the severity and incidence of CP. However, adverse outcomes, especially cognitive problems, persist despite HT. Optimizing and augmenting brain protection and supporting and stimulating neurorestoration will require therapies that cover the acute (latent), subacute (secondary) and chronic (tertiary) phases. Some therapies may be more suited to acute profound insults whereas others (for example, EPO) may be more effective in chronic partial insults affecting the white matter. Understanding the evolving cascade of injury and using therapies to their full potential in each phase is vital. Assessment of therapies across a range of animal models, with careful safety and pharmacokinetic studies as well as robust and translational outcome measures are needed. Clinical neuroprotection trials in neonates with NE need careful planning, with adequate power and pharmacokinetic information built into the protocols. Prior determination of optimal dose and timing of each agent will be important as we move into the era of therapeutic cocktails for optimizing outcomes. The use of MRS as a surrogate outcome measure will speed up the assessment of efficacy of potential therapies and thus accelerate clinical translation.

8.0 Practice Points

1. Assessment of therapies across a range of animal models, with careful safety and pharmacokinetic studies is vital before clinical translation.

2. Clinical neuroprotection trials in neonates with NE need careful planning, with adequate power and pharmacokinetic information built into the protocols.

3. Prior determination of optimal dose and timing of each agent will be important as we move into the era of therapeutic cocktails for optimizing outcomes.

4. The use of MRS as a surrogate outcome measure will speed up the assessment of efficacy of potential therapies and thus accelerate clinical translation.

9.0 Research Directions

1. Robust use of pharmacokinetics in pre-clinical and clinical studies to ensure therapeutic drug levels are reached with maximum exploitation of each therapy

2. Rational combinations of therapies in pre-clinical studies before clinical translation, with therapies that cover oxygen free radicals, excitotoxicity and acute inflammation in the acute phase and therapies that target regeneration repair for the tertiary phase after completion of HT.

3. Understanding the effects of long-term immunomodulation of microglia from damaging pro-inflammatory phenotypes to reparative anti-inflammatory phenotypes

4. Some therapies may be more suited to acute profound insults whereas others may be more effective in chronic partial insults affecting the white matter.

5. Ongoing trials:
    - The ALBINO trial (NCT03162653) is looking at the incidence of death and severe neurodevelopmental impairment at 24 months of age in neonates with moderate to severe NE where allopurinol was added to HT. Currently recruiting (n=846 participants).
    - Cool Xenon 3, outcome data awaited.
    - Clinical trials (NCT02812433 and NCT NCT04169191) are ongoing in neonates with NE treated with HT to determine PK and optimal safe dose of sildenafil.
    - The High Dose Erythropoietin for Asphyxia and Encephalopathy (HEAL) Trial **(**NCT02811263**)** will evaluate whether high-dose Epo reduces the combined outcome of death or neurodevelopmental disability when given in conjunction with hypothermia to neonates with moderate/severe NE [126]. HEAL trial recruitment is completed and pending determination of primary outcome. (n=501).
    - Preventing Adverse Outcomes of Neonatal Hypoxic Ischaemic Encephalopathy With Erythropoietin (PAEAN trial) (NCT03079167) is another ongoing study currently recruiting participants (n=300); estimated study completion is December 2021.
    - A phase I trial of allogeneic cord tissue derived MSCs has been completed in neonates with NE (NCT03635450), but results have not been reported.
    - Multilineage-differentiating stress-enduring (MUSE) cells are in Phase I trials for neonates with NE (NCT04261335) [151].
    - Combination therapy with 300 U/kg Epo every other day for two weeks, 250 mg/kg magnesium sulphate for three days, and HT in NE; efficacy data are awaited (ISRCTN33604417) [153].

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LEGENDS FOR FIGURES

FIGURE 1: Diagram illustrating the evolution of injury after cerebral HI. The primary phase (acute HI), latent phase, secondary energy failure phase and tertiary brain injury phase are shown (Adapted from Hassell KJ et al. Arch Dis Child Fetal Neonatal Ed 2015;0:F1–F11).
(A) Magnetic resonance spectra showing the biphasic pattern of NTP/EPP decline and lactate/NAA increase during primary and secondary phases following HI insult. Persisting lactic alkalosis is shown in tertiary phase.
(B) Amplitude-integrated EEG showing examples of normal trace at baseline, flat trace following HI, burst-suppression pattern in latent phase, emergence of seizures in secondary phase and normalisation with sleep–wake cycling in tertiary phase.
(C) Following HI, there is a period of hypoperfusion associated with hypometabolism during latent phase, followed by relative hyperperfusion in secondary phase, and somewhat normalization afterwards
(D) Cellular energetics and mitochondrial function are reflected in the biphasic response shown on MRS (A), with a period of recovery in latent phase followed by deterioration in secondary phase. There is partial recovery in tertiary phase.
(E) The most important pathogenic changes are shown for each phase, including generation of toxic free radical species, accumulation of EAAs, cytotoxic edema, seizures and inflammation. Cell necrosis occurs immediately following HI, while programmed cell death occurs in secondary phase; latent phase provides a therapeutic window. Persisting inflammation and epigenetic changes impede long-term repair.
(F) Damage is maximal in the secondary phase, but persists into the tertiary phase as dysregulated inflammation and gliosis evolve.
(G) In the future, neuroprotective and neurorestorative treatments are likely to involve a 'cocktail' of therapies targeting the different phases of injury. Acute therapies targeting OFR, inflammation and apoptosis need to be administered as early as possible in the latent phase and through the secondary phase to target and offset evolving damage. In the tertiary phase, therapies that target repair, regeneration, and inflammation are required.
HI, hypoxia-ischaemia; EAAs, excitatory amino acids; EPP, exchangeable phosphate pool; NAA, N-acetylaspartate; NO, nitric oxide; NTP, nucleoside triphosphate (mainly ATP); OFRs, oxygen free radical

FIGURE 2: A. Activation and polarization of microglia in resting condition (left) and during neuroinflammation (right). The morphology and the phenotype associated with different functional states of microglia are represented. In physiological conditions, patrolling microglia regulate central nervous system homeostasis. In neuroinflammation, microglia become amoeboid, losing their ramification. Depending on the local milieu and triggers, they acquire either a classical pro-inflammatory phenotype [producing TNFα, IL-1β, IL-2, IL6, IL-12, iNOS which metabolizes arginine to oxygen free radicals (OFRs)] or alternative anti-inflammatory phenotype [IL-10, chemokines (e.g., CCL_5) and arginase (which metabolizes arginine to ornithine, which is important for downstream tissue repair)]. The activation spectrum indicates the spectrum of polarization.
B. Early after HI, local microglia and newly recruited macrophages adopt a 'healing' phenotype; then as the injury evolves, a pro-inflammatory harmful profile develops and this dominates the injured brain for days after HI, driving secondary brain injury via pro-inflammatory mediators and oxidative damage. Immune modulating therapies (e.g., Azithromycin, Melatonin, Stem Cells) can change this polarization to favor the healing, anti-inflammatory phenotype

FIGURE 3: Therapeutic hypothermia protocols are near optimized, with 72-hour cooling starting as soon as possible in the latent phase with the target temperature (rectal or esophageal) being 33.5°C. The rate of rewarming appears to be less important than duration of cooling.