Neuroprotective strategies following perinatal hypoxia-ischemia: Taking aim at NOS

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ABSTRACT

Perinatal asphyxia is characterized by oxygen deprivation and lack of perfusion in the perinatal period, leading to hypoxic-ischemic encephalopathy and sequelae such as cerebral palsy, mental retardation, cerebral visual impairment, epilepsy and learning disabilities.

On cellular level PA is associated with a decrease in oxygen and glucose leading to ATP depletion and a compromised mitochondrial function. Upon reoxygenation and reperfusion, the renewed availability of oxygen gives rise to not only restoration of cell function, but also to the activation of multiple detrimental biochemical pathways, leading to secondary energy failure and ultimately, cell death. The formation of reactive oxygen species, nitric oxide and peroxynitrite plays a central role in the development of subsequent neurological damage.

In this review we give insight into the pathophysiology of perinatal asphyxia, discuss its clinical relevance and summarize current neuroprotective strategies related to therapeutic hypothermia, ischemic postconditioning and pharmacological interventions. The review will also focus on the possible neuroprotective actions and molecular mechanisms of the selective neuronal and inducible nitric oxide synthase inhibitor 2-iminobiotin that may represent a novel therapeutic agent for the treatment of hypoxic-ischemic encephalopathy, both in combination with therapeutic hypothermia in middle- and high-income countries, as well as stand-alone treatment in low-income countries.

1. Perinatal asphyxia

1.1. Background

The world has made substantial progress in reducing child mortality in the past several decades, and the total number of under-five deaths dropped to 5.6 million in 2016. However, neonatal deaths still account for 47% of all under-five deaths, increasing from 41% in 2000 [1]. The top 3 global causes of neonatal death include prematurity, infection and perinatal asphyxia (PA) [2]. The term "asphyxia" is derived from ancient Greek (ασφυξια), literally meaning "pulseless". PA describes a clinical condition that is characterized by perinatal hypoxia, hypercarbia, as well as combined respiratory and metabolic acidosis. PA leads to global cerebral hypoxia-ischemia (HI) around the time of birth and is one of the main causes of death accounting for about 23% of the neonatal deaths each year [2,3]. Despite major improvements in perinatal care, the incidence of PA remains constant at about 2–9 per 1000 live births in the Western world [4,5] rising up to 40 per 1000 live births in low-income countries (LIC) [6]. Short term consequences of PA are hypoxic-ischemic encephalopathy (HIE) and neonatal seizures.

Long term sequelae include cerebral palsy, mental retardation, cerebral visual impairment, epilepsy, learning disabilities and behavioral problems [7,8]. Even patients that have experienced a rather mild HIE have an abnormal long term outcome in at least 25% of cases [9,10]. As perinatal conditions, including PA, are among the leading diseases for global disease burden, intervention strategies have to be investigated that can reduce the amount of brain damage following PA in order to improve long-term neurodevelopmental outcome both in the Western world as well as in LIC.

1.2. Pathophysiology

1.2.1. Destructive pathways of perinatal asphyxia

During the period of PA the reduction in cerebral blood flow and oxygen delivery results in a switch to anaerobic metabolism with a depletion of high-energy phosphate, an accumulation of lactic acid and the inability to maintain cellular homeostasis. The energy deficit and reduction of cellular ATP lead to dysfunction of the transmembrane ion pumps with accumulation of intracellular Na^+ and Ca^{2+}. The resulting membrane depolarization activates and opens voltage gated Ca^{2+}

channels and the intracellular increase in Ca^{2+} furthermore triggers the release of excitatory amino acids, such as glutamate, from presynaptic neurons. In a next step, due to the overproduction and release of excitatory neurotransmitters, N-methyl D-aspartate-regulated (NMDA) cation channels are activated. As a consequence, even more extracellular Ca^{2+} enters the affected brain cells (e.g. astrocytes, microglial cells and neurons) resulting in an increased production of reactive oxygen species (ROS), a drop in inter- and intracellular pH, release of proradicals (e.g. free iron) from their respective binding proteins, hypoxanthine accumulation and excessive nitric oxide ($NO^{\cdot}$) synthesis by neuronal nitric oxide synthase (nNOS) that binds to and is regulated by the NMDA channel via the post-synaptic density protein-95 [11]. ROS and $NO^{\cdot}$ are also generated by mitochondria [12–14] and may under pathological conditions negatively influence integrity and function of the organelle itself [15,16]. The electron transport chain in the inner mitochondrial membrane represents the most important source of intracellular ROS production as it contains several enzymes that covert

molecular oxygen to superoxide ($O_2^{\cdot-}$) or hydrogen peroxide (H_2O_2) [14]. Under normal conditions, the activity of the electron transport chain generates a relatively small flux of ROS, but its production can be greatly magnified during PA and reperfusion-reoxygenation [14,17,18]. PA and reperfusion-reoxygenation mediated impairment of the electron transport chain (i.e. damage of complexes I-III) leads to electron leakage, $O_2^{\cdot-}$ generation, increased ROS production, and ROS leakage [14,19]. The overproduction of ROS via the electron transport chain and other sources (see below) finally results in increased mitochondrial permeability, collapse of the mitochondrial membrane potential, mitochondrial dysfunction and ROS release, which in turn can trigger ROS-induced ROS generation in neighboring mitochondria initiating a destructive vicious circle [16]. The depletion of high-energy phosphate and the resulting energy deficit, which are both related to the lack of oxygen and mitochondrial dysfunction, also attenuate the re-uptake of glutamate in the synaptic cleft, producing a vicious circle of excitotoxicity and cell death (Fig. 1, dark blue gearwheel) [20–25].
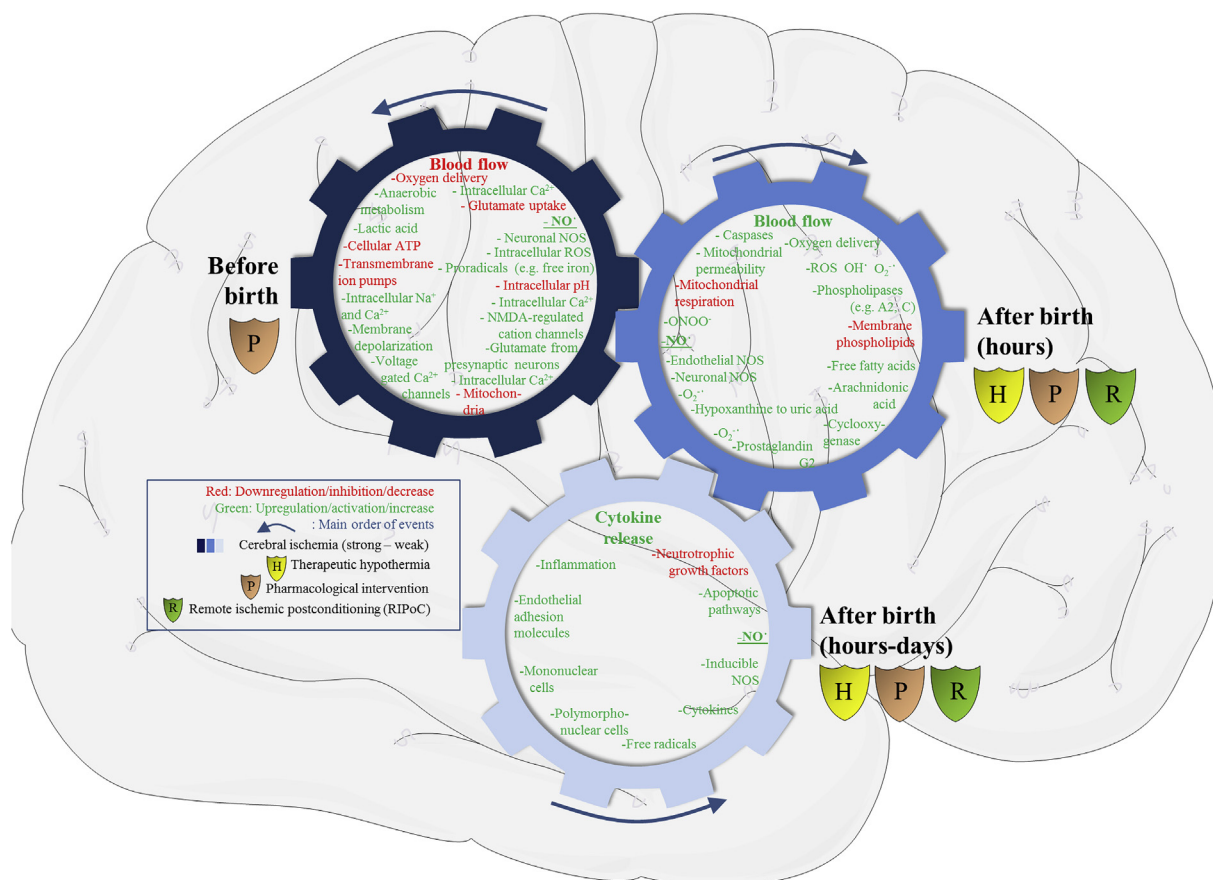

Fig. 1. Chain of main physiological, cellular and molecular events that are responsible for hypoxia-ischemia-mediated cerebral damage. The respective steps are interconnected but do not necessarily appear in sequence. Instead mechanisms may run in parallel, interfere with each other and include vicious circles that lead to an amplification of the overall cell damage. Note that the formation of **$NO^{\cdot}$** occurs before birth as well as in the early and late phases after birth and may therefore be targeted by different interventions (therapeutic hypothermia, remote ischemic postconditioning, pharmacological interventions).

Upon reperfusion and reoxygenation the renewed availability of oxygenated blood to the previously ischemic tissue causes formation of additional ROS during the latent phase that lasts for up to 6 h. Non-protein bound iron is a potent pro-radical that strongly contributes to the formation of the toxic hydroxyl ($\cdot$OH) radical via the $O_2^{\cdot-}$-driven Haber-Weiss reaction [26,27]. Furthermore, the Ca^{2+} induced production of free radicals and phospholipases A2 and C is involved in the breakdown of neuronal membrane phospholipids, releasing free fatty acids and especially arachidonic acid. Arachidonic acid stimulates the enzyme cyclooxygenase and catalyzes the formation of the prostaglandin intermediate PGG2 that in turn generates the $O_2^{\cdot-}$ anion free radical [27,28].

In addition, the conversion of hypoxanthine, which is formed in large amounts during PA, to uric acid by xanthine oxidase upon reperfusion-reoxygenation, gives rise to the further formation of large amounts of $O_2^{\cdot-}$ [29,30]. Concurrently, the levels of the neuronal and inducible isoforms of NOS increase considerably initiating formation of $NO\cdot$. $NO\cdot$ can react with $O_2^{\cdot-}$ to yield the non-radical peroxynitrite ($ONOO^-$) that may decompose to form more of the potent $\cdot$OH. $NO\cdot$, as well as $ONOO^-$, inhibit the mitochondrial respiration and increase mitochondrial permeability leading to irreversible caspase and non-caspase dependent neuronal cell death (Fig. 1, medium blue gearwheel) [31–36].

In addition to the above described radical and $NO\cdot$ driven mechanisms, both damaged neurons and activated endothelium produce various cytokines that lead to the activation of an inflammatory response and promote the biochemical cascade leading to secondary energy failure as well as delayed neuronal death. Important key players seem to be the activated in microglia, intercellular adhesion molecules, proteases and several cyto- and chemokines [37–40]. Through activation of endothelial adhesion molecules, polymorphonuclear cells, but also mononuclear cells are recruited to the brain. They further enhance inflammation by the excretion of free radicals and cytokines. The latter aggravate pre-apoptotic pathways and delayed brain damage by an induction of inducible NOS (iNOS) and additional production of $NO\cdot$ [11,41,42]. Besides increased NOS activity, delayed brain damage is also related to an attenuated formation of neurotrophic growth factors (e.g. IGF, NGF, GDNF, BDNF) that seem to inhibit apoptosis and improve cell growth and differentiation in the developing brain (Fig. 1, light blue gearwheel) [43–45].

1.2.2. Therapeutic window

Although significant neuronal cell death already occurs during PA,

reperfusion and reoxygenation during the latent phase induce additional biochemical cascades that result in secondary energy failure and substantial delayed neuronal cell damage (1.2.1. and Fig. 1). The delay in apoptotic and necrotic events may proceed up to days or weeks after birth [46–48] and is associated with a significant reduction of the phosphocreatine/inorganic orthophosphate ratio [49,50]. The latency period between the hypoxia-ischemia insult and the secondary energy failure [51,52] creates a temporary therapeutic window during which pharmacological interventions and remote ischemic postconditioning (RIPoC) can be applied in order to reduce neurological brain damage [53] (Fig. 2).

The temporary characteristics of the therapeutic window are dependent on several factors such as the depth and duration of the hypoxic-ischemic insult. In addition, the genetic background, phase of the brain development and activity of repair processes further influence the clinical outcome after PA and have to be considered in the course of potential therapeutic interventions [54–56].

Moreover, sexual dimorphism has been described in cell death pathways, in the response to oxidative stress as well as in neuroprotection. Recent studies suggest that females have greater memory deficits. In females cell death is dependent mainly on caspase activation, while males are more susceptible to oxidative stress. Therefore, treatments acting on distinct cell death pathways afford sex-dependent neuroprotection [57,58]. This means that potentially different neuroprotectants are necessary for males and females, and that in future efficacy trials gender has to be analysed in post-hoc analyses.

1.3. Global differences in perinatal asphyxia

Nearly one million babies die yearly from PA [3], of which the main burden (96%) falls in LIC [59]. The incidence of stillbirth is not included in this calculation, with an estimated rate of 46.6% of the stillbirths being caused by antenatal PA [60]. In a recent multi-country prospective cohort study performed in south Asia and sub-Saharan Africa, it was shown that 40–45% of pregnancy-related deaths, still-births, and neonatal deaths occurred during labour, delivery, and the 24 h postpartum period [61]. The presence of a low institutional delivery rate, a poor regionalization of care, lack of adequate transport facilities and ill-equipped neonatal intensive care facilities are part of the chain that cause the high incidence of PA and subsequent HIE in LIC [62].

A solution for the reduction in global morbidity and mortality caused by PA should be found in improving prenatal health care, better
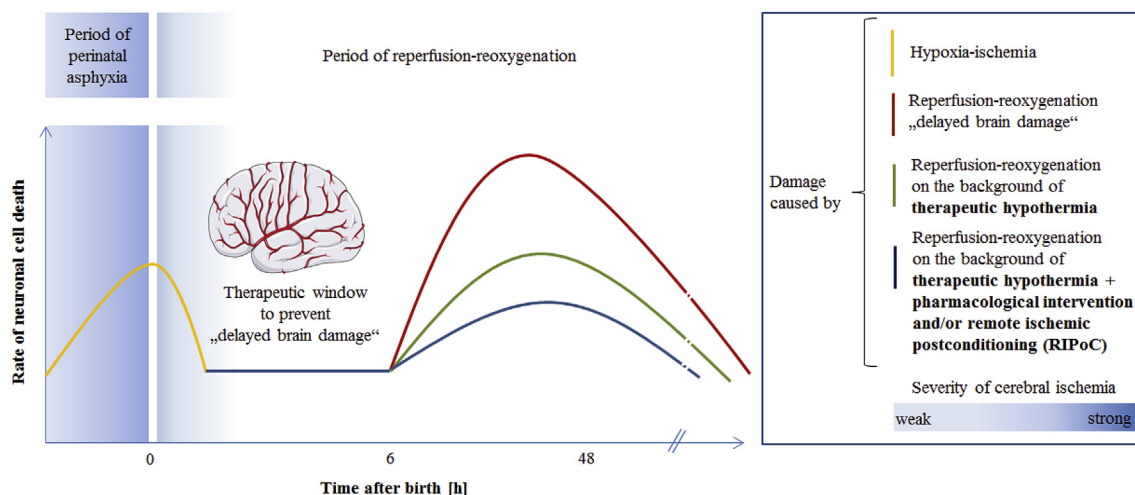

Fig. 2. Schematic representation of the rate of neuronal cell death taking place during the periods of perinatal asphyxia and reperfusion-reoxygenation. By administration of therapeutic hypothermia (TH) the amount of secondary brain cell damage can be reduced. Pharmacological interventions and/or remote ischemic postconditioning in the therapeutic window on the background of TH may further decrease the secondary cerebral cell damage in the reperfusion-reoxygenation phase. Severity of ischemia before and after birth is indicated by blue shaded areas in the diagram (light blue = weak ischemia; dark blue = strong ischemia).

education of midwives and gynecologists in delivery processes, pediatricians in the recognition and treatment of an asphyxiated neonate, and providing a neuroprotective rescue treatment when HIE is present.

Therapeutic hypothermia (TH) has become the standard of care for neonatal encephalopathy in middle- and high-income countries [63]. Because of the commonly associated multi-organ failure and severe encephalopathy, treatment mainly takes place at neonatal intensive care units and the neonate has to be transported from home or a level II hospital to a level III neonatal intensive care unit. In LIC this is a challenge, since there is a lack of good public transport and communications systems, and many patients cannot afford private transport. Also, the costs of equipment for controlled TH are high, and can often not be afforded in LIC.

Although TH has an effect size of 15% in the Western World [63], a meta-analysis on TH in low- and middle-income countries using low-cost cooling techniques, did not show a significant reduction in neonatal mortality [64]. It was suggested that adequately randomised controlled trials are required before cooling can be offered in LIC [65,66].

Alternative rescue treatment strategies include the administration of neuroprotective drugs or RIPoC within the therapeutic window. In LIC, therapy of PA needs to be cheap and easy to administer. The treatment should be stable and not be affected by high environmental temperature, and have a broad therapeutic range with minimal side effects. Additionally, the patient has to be able to reach the hospital in time to be treated efficiently [67].

2. Neuroprotective strategies

In recent years, different neuroprotective strategies have been described using numerous in-vitro and animal models, as well as clinical trials. The main focus of the following section will be on the results of clinical trials. Preclinical studies will however be mentioned if their outcome has the potential to directly or indirectly influence future clinical approaches. For an overview of all planned, recruiting and completed clinical trials, see Supplemental Table 1.

2.1. Therapeutic hypothermia

More than 60 years ago Burnard and Cross detected a reduced rectal temperature in babies suffering from asphyxia at birth and speculated that the lower temperature may have survival value [68]. Nowadays, TH is the only established treatment option that can significantly reduce brain damage after HIE. Several clinical studies showed that the combined adverse outcome of death and disability, such as hearing loss, cerebral palsy and other neuromotor disorders are reduced from 60% to 45% by the use of moderate TH, which has become the standard treatment of PA mediated brain injury in high-income countries [69–74]. During TH the brain is either cooled by selective head cooling or whole body cooling for 72 h at 33.5° Celsius. The specific mechanisms of hypothermic neuroprotection remain unclear, in part because TH suppresses a broad range of potential injurious factors [75]. Basically, all of the destructive mechanisms that are discussed in 1.2.1. and depicted in Figs. 1 and 2 are temperature-dependent and may therefore be influenced by TH. It is commonly accepted that TH regulates multiple aspects of brain physiology during and after ischemia. Especially pathways leading to excitotoxicity, apoptosis, inflammation and free radical production, as well as blood flow, metabolism and integrity of the blood–brain barrier are affected by TH [76]. In-vitro as well as in-vivo studies suggest that TH decreases enzymatic reactions that result in cell damage or death, including caspase-3 activation [77,78]. Moreover, expression of the pro-inflammatory cytokines TNF-alpha, IL-1beta and IL-18 is attenuated by TH, while the release of anti-inflammatory IL-10 is enhanced [54,79,80]. In addition, TH inhibits the activation of NMDA receptors, restraining intracellular Ca^{2+} levels [81–83]. Other mechanisms by which TH may reduce ischemia-reperfusion mediated neurotoxicity are suppression of free radical generation, protection of the fluidity of lipoprotein membranes, reduction of the oxygen demand in low-flow regions, reduction of intracellular acidosis as well as inhibition of the biosynthesis, release and uptake of excitatory neurotransmitters [83–86]. Based on the complexity of TH influenced mechanisms it is likely that no single factor can fully explain the neuroprotective effects provided by TH.
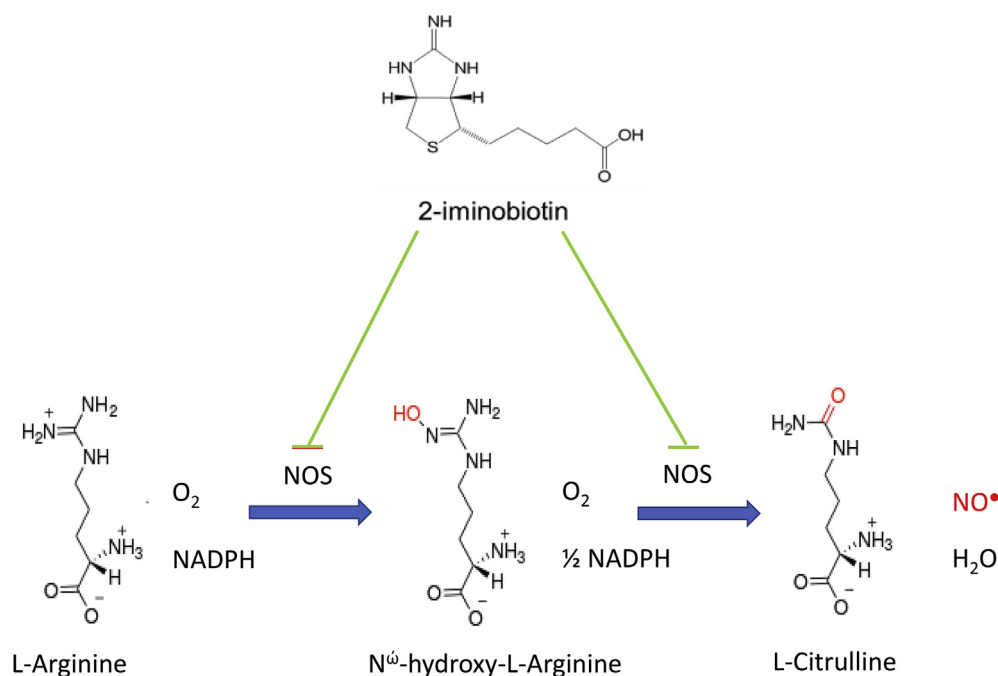

Fig. 3. Conversion of L-Arginine to L-Citrulline via nitric oxide synthase (NOS) and inhibition by 2-iminobiotin.

2.2. Inhibition of NOS

PA is associated with increased levels of NO˙ that is involved in several of the destructive pathways responsible for neuronal cell damage (for details see 1.2.1 and Figs. 1 and 3). Therefore, inhibition of NO˙ production by e.g. blocking the different isoforms of NOS alone or in combination with TH, may be a promising therapeutic option for the reduction of PA mediated neurotoxicity. For a summary of preclinical studies that investigated the potential of NOS inhibition after PA, see Favie et al. [87]. At this moment 2-IB is the only drug that has been tested in clinical trials. For an overview, see chapter 3.

2.3. Allopurinol

Allopurinol is a competitive inhibitor of the enzyme xanthine oxidase that catalyzes the oxidation of both hypoxanthine and xanthine. During reperfusion the renewed availability of oxygen starts the process of O_2^- and free radical formation, giving rise to lipid peroxidation and premature cell death. Allopurinol was evaluated in a Phase 2b trial after severe asphyxia without TH, but did not improve short term outcome when given within 4 h after birth [88]. This was also confirmed in a Cochrane review in 2012 [89]. However, a follow-up of two earlier performed randomized controlled trials with 4–8 years old children suggested a neuroprotective effect of neonatal allopurinol treatment in moderately asphyxiated infants [90]. Allopurinol is currently being investigated as a potential drug for additional neuroprotection on top of normothermia or TH in the treatment of moderate to severe HIE in the ALBINO trial (NCT03162653).

2.4. Erythropoietin/darbepoetin

Erythropoietin (EPO) is a glycoprotein known for its role in erythropoiesis, it also has a modulatory effect in the central nervous system. After PA, EPO exerts both anti-apoptotic and anti-inflammatory effects, as well as effects on neurogenesis, oligodendrogenesis, and angiogenesis in the long term [91]. In a Phase 2 placebo-controlled trial in neonates with HIE multiple doses of EPO (1000 U/kg) on top of TH resulted in less MRI brain injury and an improved short-term motor outcome [92]. At this moment three Phase 3 trials evaluating the long-term neurodevelopmental effect of EPO in combination with and following TH are recruiting patients (NCT2811263; NCT3079167 and NCT3163589). Darbepoetin alpha is a recombinant human EPO-derived molecule that has an extended circulating half-life but comparable biological activity to EPO. A Phase 2a clinical trial was conducted to look at safety and pharmacokinetics of darbepoetin (NCT01471015), which proved to be safe [93]. Currently a Phase 2b clinical trial is investigating the efficacy of darbepoetin on top of TH (NCT03071861).

2.5. Growth factors/cell therapy

Growth factors from umbilical cord blood [94], mesenchymal stem cells [95] or mixtures of growth factors have emerged as novel therapeutic agents with promising results in experimental studies of HIE. Cerebrolysin®, a mixture of growth factors purified from pig brains, was tested in a Phase 2a trial in 40 neonates with a history of HIE (NCT1059461). Afterwards, a randomized controlled trial was conducted in which neonates were twice-weekly injected with cerebrolysin® for 5 weeks. After 3 months infants' communication, especially symbolic behavior, was improved in the cerebrolysin® group [96]. Also infusion of fresh autologous umbilical cord blood cells, given in up to 4 doses adjusted for volume and red blood cell content, was safe and feasible for use in infants with HIE treated with cooling [97]. Another Phase 1–2 safety study (NCT02551003) and a Phase 2b efficacy study (NCT02612155) are currently recruiting patients. A Phase 1 study investigating the safety of one and two intravenous infusions of human umbilical cord tissue-derived mesenchymal stromal cells, administered

in the first 48 postnatal hours and at two months postnatal age respectively, is recruiting patients (NCT03635450).

2.6. Remote ischemic postconditioning

RIPoC, in which transient episodes of ischemia (e.g. by inflation and deflation of a blood pressure cuff) are applied after a prolonged HI/reperfusion injury, may have the potential to improve patient outcome and survival [98,99]. RIPoC has gained major attention during the last years, however, the triggers, mediators, and effectors responsible for the protective effects are mainly unknown [98,100]. Numerous animal studies and also several clinical trials proposed organ protective effects of RIPoC against ischemia/reperfusion injury in the heart, kidney, liver and also brain and suggested RIPoC as an effective treatment against ischemia/reperfusion injury [98,101–106]. Despite the lack of human studies investigating the efficacy of RIPoC in PA, several preclinical experiments have demonstrated that RIPoC is effective at reducing injury in cerebral ischemia models [107–109]. Combining RIPoC with TH or other interventions to treat PA could represent an attractive therapeutic approach, which has to be explored in further preclinical and most importantly clinical studies.

2.7. Melatonin

Melatonin is involved in regulating daily body rhythms and has been shown to be neuroprotective in different animal models of PA [110–112]. It reduces infarct volume, minimizes lipid and protein peroxidation, blocks apoptotic pathways, inhibits free radical production, and decreases inflammation [113]. Although melatonin has a good safety profile itself, the i.v. formula contains ethanol that is not acceptable for administration to newborns. Two small Phase 2 clinical trials showed promising results of oral administered melatonin after HIE, one without hypothermia [114] and one on top of hypothermia [115]. Currently, a Phase 2 dose-escalation study is ongoing with an oral solution of melatonin on top of TH (NCT02621944) and a 4-armed Phase 2 study with melatonin with normo- and TH is planned (NCT3806816).

2.8. Miscellaneous

Sildenafil, a phosphodiesterase-type 5 inhibitor and known for its boosting of NO-cGMP signaling, has been shown to be neuroprotective in animal studies of neonatal HIE [116]. In a Phase 2 study an oral blend of sildenafil is currently being investigated for safety and efficacy (NCT2812433).

Dexmedetomidine, an α2-adrenergic agonist, is investigated in a Phase 2 trial when given as maintenance infusion to asphyxiated neonates during 78 h of TH and rewarming (NCT02529202).

Topiramate, an anticonvulsant, was investigated for safety and efficacy in neonates with PA in combination with TH in a multi-center randomized-controlled Phase 2 study [117]. Topiramate was orally administered in a dose of 10 mg/kg once a day for the first three days of life. No statistically or clinically significant differences were observed for safety and outcome (combined frequency of mortality and neurodevelopmental outcome at 18–24 months), although a reduction in the prevalence of epilepsy was observed in the topiramate-treated infants.

Phenobarbital has frequently been studied for its ability to reduce neonatal seizures after HIE. Some studies investigated the neuroprotective capacities of phenobarbital on outcome after HIE [118]. In 2016, a meta-analysis was published on the prophylactic administration of phenobarbital for the prevention of morbidity and mortality after PA. It was concluded that prophylactic barbiturate therapy for late preterm and term infants in the immediate period following PA cannot be recommended for routine clinical practice [119].

Xenon, a noble gas with anti-NMDA properties, showed to be additive neuroprotective in several animal models of encephalopathy

treated with TH [120,121]. However, a pivotal trial in 92 patients investigating the administration of xenon within a delayed timeframe was apparently safe, but did not enhance the neuroprotective effect of TH after PA [122]. The results of the CoolXenon3 study in asphyxiated patients, receiving xenon gas at 50% concentration for 18 h, are awaited (NCT 02071394).

Magnesium sulphate has been reported to have anti-excitotoxic and anti-oxidative properties by blocking the NMDA receptor and downregulation of proinflammatory cytokines [123]. In two small Phase 2 trials $MgSO_4$ showed an increased risk on hypotension in high dosage [124] and on aEEG pattern [125], but was reported safe in 3 other Phase 2 trials on short term outcome [126–128]. One 4-armed phase III (MagCOOL) trial was conducted to determine the long-term outcome after combination treatment of $MgSO_4$ with normothermia and TH (NCT1646619), but no results are posted yet.

3. Neuroprotection by 2-iminobiotin

2-Iminobiotin (2-IB) is a vitamin H or B7 analogue, which contains guanidine and free carboxyl groups that allow 2-IB in a similar way as L-arginine to bind within the active site of NOS. 2-IB has been shown to inhibit iNOS and nNOS activity in a concentration-dependent way [129] resulting in a reduced production of NO˙ (Fig. 3).

3.1. Safety and efficacy of 2-iminobiotin in preclinical studies

In-vitro studies from our group suggested that 2-IB is able to protect human neuronal cells from hypoxia-induced cell damage [130] and that these effects are also evident if 2-IB is superimposed on TH [131]. Using a piglet model of bilateral carotid occlusion with inhalational hypoxia, we moreover showed a 90% improvement in cerebral energy state, 90% reduction in vasogenic edema, and 60% to 80% reduction in apoptosis-related neuronal cell damage after 2-IB administration. In addition, a significant reduction in tyrosine nitration in the cerebral cortex was observed in 2-IB-treated piglets, indicating decreased formation of reactive nitrogen species [132]. Clinical parameters, such as electro-cortical brain activity and the regional oxygen saturation and redox state of cytochrome aa3, were significantly preserved in 2-IB treated piglets compared to vehicle-treated animals [133,134]. These findings coincided with a preservation of the IGF-1 production suggesting an endogenous protection against perinatal HI after treatment with 2-IB [135]. Later on, a dose escalation study was performed in a piglet model of inhalational hypoxia with low blood pressure showing that 2-IB, administered i.v. every 4 h in doses of 0.1–1.0 mg/kg/day, is safe and effective [136]. The amount of nitrated tyrosine in different cerebral areas was found to be significantly reduced after 2-IB treatment. Based on survival with a normal aEEG, an exposure comparable to a dose of 0.2 mg/kg 2-IB in the piglet was likely to be the most appropriate dose for use in future clinical trials in neonates with perinatal hypoxia-ischemia. An AUC_{0-4h} of 365 ng h/mL was defined as the target AUC_{0-4h} for use in these trials.

In a p7 hypoxia ischemia rat model it was shown that nitrotyrosine formation preceded caspase-3 activation and that both the nitrotyrosin formation and caspase-3 activation were significantly reduced at 3 h post HI by i.p. treatment with 2-IB [137]. 2-IB administration in p12 day old HI rats reduced both short-term neuronal injury and long-term neuronal damage. However, no changes in nitrotyrosine levels were found at 24 h post HI [138]. In p3 and p7 rats a significant reduction of (apoptotic) cell death was confirmed at 6 weeks after HI but in female rats only, suggesting that, at least in rats, a sexual dimorphic effect on neuroprotection is present [139,140].

3.2. Safety and pharmacokinetics of 2-IB in clinical studies

3.2.1. Phase 1 clinical study

The primary objective of the Phase 1 clinical study was to investigate the safety and tolerability of single and multiple doses of 2-IB i.v. infusions in a range of a single infusion of 0.6 mg/kg to repeated infusions of in total 72 mg/kg/day in healthy male subjects and to determine their corresponding pharmacokinetics profiles. The study was defined as a randomised, double-blind, placebo-controlled, dose escalation study with 2 groups of 9 healthy male subjects receiving single or pulsed i.v. infusions of 2-IB or placebo in 3 periods. Captisol, a cyclodextrin, was used to enhance the solubility of 2-IB. Treatment with single and multiple doses of 2-IB with and without captisol by i.v. infusion of up to 6 doses of 6 mg/kg (36 mg/kg/day) was safe and well tolerated by all subjects. In a dose of 72 mg/kg/day, 2-IB was well tolerated by 4 out of 6 subjects. Two out of 6 subjects were withdrawn due to non-serious adverse events (nausea and dizziness). There were no clinically relevant differences in clinical laboratory, vital signs, ECG, or physical examinations between placebo and all treatments. It was concluded that 2-IB was a safe drug with the highest tolerable dose of 36 mg/kg/day in healthy male adults [141].

3.2.2. Phase 2 clinical studies

A first-in-man study was investigating the safety and pharmacokinetics of 2-IB in (near) term neonates with moderate to severe HIE treated with standard care (without TH) in 2 sites in Turkey (NCT01626924). The 2-IB dose of this study was defined at 0.2 mg/kg/dose every 4 h to a maximum of 6 doses, comparable to the dose used in piglets [136]. The first dose was administered within 6 h after birth. Five of 6 patients experienced severe PA. All 6 included patients received at least one dose of study medication. 2-IB was well tolerated, especially no signs of hypotension occurred and no relevant changes in hematology or biochemistry were noted, other than those that were related to the condition of PA. Two patients died because of HI-related brain injury. In all patients no (severe) adverse events that could be attributed to 2-IB were reported. A target AUC_{0-4h} of 365 ng h/mL was chosen in this clinical study. Based on pharmacokinetics results in these 6 neonates, it was advised to lower the 2-IB dose to 0.16 mg/kg/dose in a next Phase 2 clinical study (Tjabbes H, medical officer of Neurophyxia B.V., personal communication).

Aim of a second study was to investigate safety and pharmacokinetics of 2-IB in low-resource settings (www.clinicaltrials.eu; EudraCT 015-003063-12). Near term neonates, born in Kinshasa, Democratic Republic of Congo, with a Thompson score ≥7 were included and received 6 infusions of 0.16 mg/kg 2-IB starting within 6 h after birth, with 4 hourly intervals. Safety and pharmacokinetics were assessed with a target AUC_{0-4h} of 365 ng h/ml. Seven patients were included in the study. No significant changes in heart rate, oxygenation, blood pressure, and aEEG were observed around 2-IB administration. No adverse effects that could be attributed to the use of 2-IB were detected [142].

In a third study, short term safety and pharmacokinetics of 2-B were assessed in (near) term neonates with moderate to severe HIE treated with TH (www.clinicaltrials.eu; EudraCT 2014-004265-25). Two cohorts of 6 patients were included: Cohort A was treated with 4 dosages of 0.16 mg/kg 2-IB every 6 h for 24 h; cohort B with 8 dosages of 0.08 mg/kg every 6 h for 48 h. Treatment was started within 12 h after birth. A target AUC_{0-48h} was set at 4800 (range 3400–6000) ng.h/ml for maximal effectiveness. No statistically significant or clinically relevant change in vital parameters was found after 2-IB administration. There were no (severe) adverse events related to the study drug. No significant change in heart rate, oxygenation, blood pressure, cerebral saturation, and aEEG was observed after administration of 2-IB. It was concluded that the present dosing regimen resulted in AUC_{48h} within the target AUC. Eleven out of 12 patients survived in these 2 cohorts of patients with moderate to severe PA, treated with 2-IB on top of therapeutic TH. This is better than expected based on historical cohorts in the same center where 31.8% mortality was reported [143]. It was concluded that a phase 2b/3 study is urgently needed to evaluate the efficacy of 2-IB in combination with therapeutic TH [144,145].

4. Conclusions

PA is one of the main causes of neonatal death and represents a major problem in the Western world, even more so in LIC. TH has become the standard therapy for PA in developed countries and is able to partially reduce PA associated death and disability. In the last years several pharmacological compounds have been suggested for the therapy of PA. Within these pharmaceuticals, NOS inhibitors might play an important role, as destructive NO˙ driven mechanisms are involved in all phases of PA. Recently the biotin analogue 2-IB that represents a selective inhibitor of neuronal and inducible NOS, has gained major attention as 2-IB is able to reduce neuronal cell damage in-vitro as well as in-vivo. Clinical trials for 2-IB are now ongoing and will reveal weather NOS inhibition can be employed as one of the therapeutic treatments of PA in combination with TH in developed countries and might be an alternative to TH in LIC.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2019.02.025.

5. References

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