Review Article

Birth asphyxia-induced brain damage: the long road to optimal reduction and prevention!

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Abstract: Moderate hypothermia (HT) is the only established therapy to reduce birth asphyxia-induced brain injury in (near) term neonates, but it is conceivable that pharmacological add-on therapy during or after HT can further reduce brain damage. This paper summarizes neuroprotective interventions with drugs that have possible potential to do so. We finally discuss the potential role of autologous and allogenic mesenchymal stem cell therapy for the repair of birth asphyxia-related brain injury.

Keywords: Birth asphyxia; (near) term neonate; pharmacological neuroprotection

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Introduction

Neonatal encephalopathy due to perinatal hypoxia-ischemia (HIE) is to date one of the most important causes of an adverse neurodevelopmental outcome and even death: 2 up to 26 neonates per 1,000 live births have an adverse long-term outcome such as cerebral palsy, epilepsy, cognitive impairment and learning difficulties (1-3). Moreover, birth asphyxia is responsible for 25% of the neonatal mortality rate in the world (4).

Etiologically a curtailed perfusion and, consequently, insufficient oxygenation is the most important cause for the injury to the (fetal) brain, the more the neonatal brain has high metabolic demands (5) and high concentrations of fatty acids and free ions leading to excessive free radical formation and other toxic compounds such as peroxynitrite (6,7).

Hypoxia-ischemia around birth starts for 20% of the birth asphyxia cases before birth, for 30% during the delivery and for 35% before and during the birth process. Only 10% of Hypoxia-ischemia develops after birth (8). In the majority of perinatal asphyxia there is an acute hypoxic-ischemic insult and in such situations the deeper brain structures such as brain stem, thalamus and basal ganglia are in particular affected, whereas subacute and chronic hypoxia-ischemia, which happens in about 10% to15% during perinatal asphyxia, induce damage at the borders of the vascular beds of the anterior, middle and posterior cerebral arteries, the so-called watershed damage (9,10). Hypoxic-ischemic insults that lead to a global pattern of injury are relatively rare, probably because in most cases this will result in fetal or intrapartum death, and infants will not undergo MRI examinations (9). See also *Figure 1*.

The process of brain damage due to perinatal hypoxia-ischemia is two-fold. During the actual insult, almost always starting before birth, (fetal) hypoxia-ischemia induces an abnormal production of excitatory neurotransmitters and lowering of intra- and extra-cellular pH already leading to neural cell death and accumulation of potentially damaging compounds such as free (pro-) radicals. Metabolization of these compounds after recovery of perfusion and oxygenation after birth can set in motion destructive

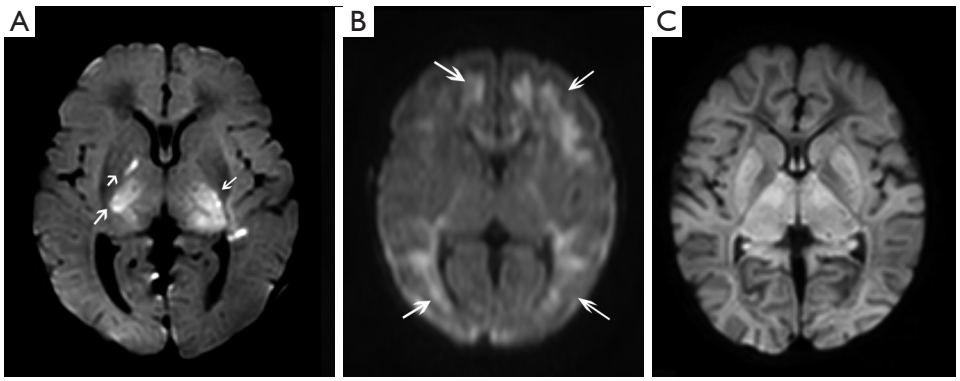

Figure 1 Axial diffusion weighted magnetic resonance images (DWI) showing 3 different patterns of brain damage i.e., acute, subacute, and severe global, respectively. (A) Basal ganglia/thalamus type of injury: DWI image on day 4 shows an increased signal in the basal ganglia and thalamus (see arrows) indicating brain damage after an acute perinatal hypoxic-ischemic insult. (B) Watershed type of injury: DWI image on day 5 shows increased signals in the "watershed" areas of the anterior, middle and posterior cerebral arteries indicating damage in these regions after subacute perinatal hypoxia-ischemia. (C) 'Near total' type of injury: DWI image on day 5 after very severe perinatal hypoxia-ischemia showing signal changes in the entire brain indicating severe global brain damage.

molecular pathways (see also below) leading to secondary energy failure after an initially recovered oxidative metabolism in the first hours of life (11). This delayed impairment of cerebral oxidative energy metabolism, mostly apparent after 6 to 8 hours after birth, can last up to 72 hours after birth and adds substantial brain damage due to necrosis and apoptosis neuronal cells (12). This postnatal process of cell death can even last for weeks (13).

Moderate hypothermia (HT) is the only neuroprotective therapy which has a proven positive impact on perinatal asphyxia-driven brain damage and will be briefly discussed. At present a large number of pharmaceuticals are under investigation as add-on therapy for HT. This paper summarizes the most promising of these drugs, expected to interrupt the devastating effects of destructive molecular pathways set in motion by perinatal hypoxia-ischemia. The most effective treatment of birth asphyxia is expected to be a combination of HT with consecutive (fetal) pharmacological therapies intervening in one (or more) of the destructive pathways (as described below). Finally it has to be expected that repair of the developing brain will play an increasingly important role in the coming years.

Neuroprotection and repair after perinatal hypoxia-ischemia

Moderate hypothermia

The only proven therapy is moderate hypothermia or HT down to a core temperature of 33.5 ℃ for 72 hours (14,15). Body temperature should be monitored rectally, transesophageally or nasopharyngeally (16,17). Clinical randomized studies showed that HT lowered adverse outcome at 18–24 month or mortality from about 55% to 60% back to 42% (17,18). Also long-term outcome up to 7 years of age showed that HT decreased cerebral palsy substantially as compared to non-cooled counterparts (19,20). HT should be installed in the first 6 hours after birth, in the so-called therapeutic window, when the energy metabolism is still apparently normal (11). Although the protective mechanism of HT is not yet fully explained and is linked to lowering of free radical formation and to anti-inflammatory and anti-apoptotic properties (21,22), its reducing effect on the hypoxic-ischemic-induced production of excitatory neurotransmitters seem to be important (23). Since neurotransmitter and free radical formation occurs during (fetal) hypoxia and intensifies upon reperfusion and reoxygenation early after birth (24,25), the earlier hypothermia can be installed the better its neuroprotective action may be! This is in fact also supported by earlier studies and passive or, better, active cooling (ice packs or servo-controlled) during transport to the NICU is therefore important (26-28). On the other hand, it has been suggested that delayed cooling after HIE, starting between 6 and 24 hours after birth, may have benefit as well as shown by Laptook *et al.* (29). HT is a relatively safe neuroprotective therapy although several (potential) complications are reported from which arrhythmia (mostly bradycardia) and

thrombocytopenia are the most common ones (18,30,31).

Two additional practical questions remain: will an increase of the length of cooling time and/or the depth of core temperature further improve neurodevelopmental outcome? This was investigated in a randomized clinical trial in the US where they compared duration of cooling of 120 hours with the standard cooling time of 72 hours; both cooling groups were either cooled to 32.0 ℃ or to the standard temperature of 33.5 ℃. The trial was discontinued because of futility or even possible harm, strongly suggesting that longer and/or deeper cooling was not beneficial indeed (32). Although both studies using selective head cooling or total body cooling showed significant improvements of outcome, whole body cooling may be superior (33,34).

Trials with HT and clinical practice nowadays limit the use of HT for term neonates who experienced birth asphyxia and developed a moderate to severe encephalopathy, mostly but not always defined using the Sarnat classification (35,36). A systemic review from 2018, including 20 studies, showed that infants with mild encephalopathy due to birth asphyxia had an adverse outcome in 25% of the infants, although the definition of "mild encephalopathy" used in these studies was not straight forward (36). However, Rao *et al.* reported recently that adverse outcome of infants with mild encephalopathy may be up to 43% (37). Moreover, van Handel et al investigated cognitive outcome at 9–10 years of age of a cohort of birth asphyxiated children with mild and moderate neonatal encephalopathy. They also found a substantial negative effect of mild neonatal encephalopathy on memory functioning in these children (38). This suggests that also neonates with a mild neonatal encephalopathy after birth asphyxia may benefit from HT. Another related issue is whether or not (moderate) preterm infants should be treated with HT: a retrospective study of 31 cooled preterm neonates born after a gestational age of 34–35 weeks compared to 31 term cooled neonates (37) showed that HT is feasible in this age group, but caution is warranted with respect to mortality and adverse effects (37,39,40).

At present, in our NICU, the inclusion criteria are a gestational age of 35.0 weeks or more, well documented perinatal asphyxia, followed by abnormal aEEG patterns (suppressed background patterns with a basline below 5 μV), or clinical criteria (Thompson score of 7 or more, or moderate to severe encephalopathy according to Sarnat). HT significantly decreased the composite outcome of mortality and adverse neurodevelopment in neonates with moderate (death/disability not cooled-cooled: 54% *vs.* 37%) and severe encephalopathy (death/disability not cooled-cooled: 86% *vs.* 70%) with a "number to treat" (NTT) of seven, meaning that one out of seven asphyxiated neonates treated with HT benefits from the intervention (18). However, with an overall composite adverse outcome (death; disabilities) of 40% to 50% negative consequences of birth asphyxia are still high (18,41) and it is conceivable that additional means of neuroprotection or recovery after birth asphyxia may further improve neurodevelopmental outcome. In this paper we will focus on pharmacological neuroprotection as add-on therapy for HT after severe perinatal hypoxia-ischemia (42).

Pharmacological add-on therapy for HT

To answer the question in what way pharmacological neuroprotection can further improve clinical outcome, a detailed knowledge of the time course of the already above mentioned destructive molecular pathways, set in motion after (severe) perinatal hypoxia-ischemia, is extremely important. When the time lines of (maximal) formation of (pro-) free radicals, cytokines and other toxic compounds are known optimal timing and optimal duration of antidotal treatment with specific drugs intervening in the formation of these destructive compounds can be determined.

Fetal therapy

In 90% of perinatal HI the starting point of birth asphyxia is fetal hypoxia leading to a failure of oxidative metabolism with consequent accumulation of hypoxanthine (8,43). Moreover, fetal hypoxia will trigger an excess formation of excitatory neurotransmitters, which activates the *N*-methyl-D-aspartate (NMDA) but also the voltage regulated receptors on the neuronal membrane (44). This results into a surge of extracellular calcium into the neurons and increase in intracellular Ca^{2+} leading to production of free radicals with damage of the membranes of neurons oligodendrocytes and astrocytes (24,44). This process is accompanied with a drop in intra and extra cellular pH. Liberation of metal-ions from the binding proteins leads to formation of so-called pro-radicals from which free or non protein-bound iron (Fe^{2+}) is an important one (45,46). See also *Figure 2*.

Trials with maternal magnesium therapy during fetal asphyxia intending to reduce the fetal excitatory transmitter production by blocking the NMDA-receptors were not successful (47,48). Attempts to reduce fetal formation

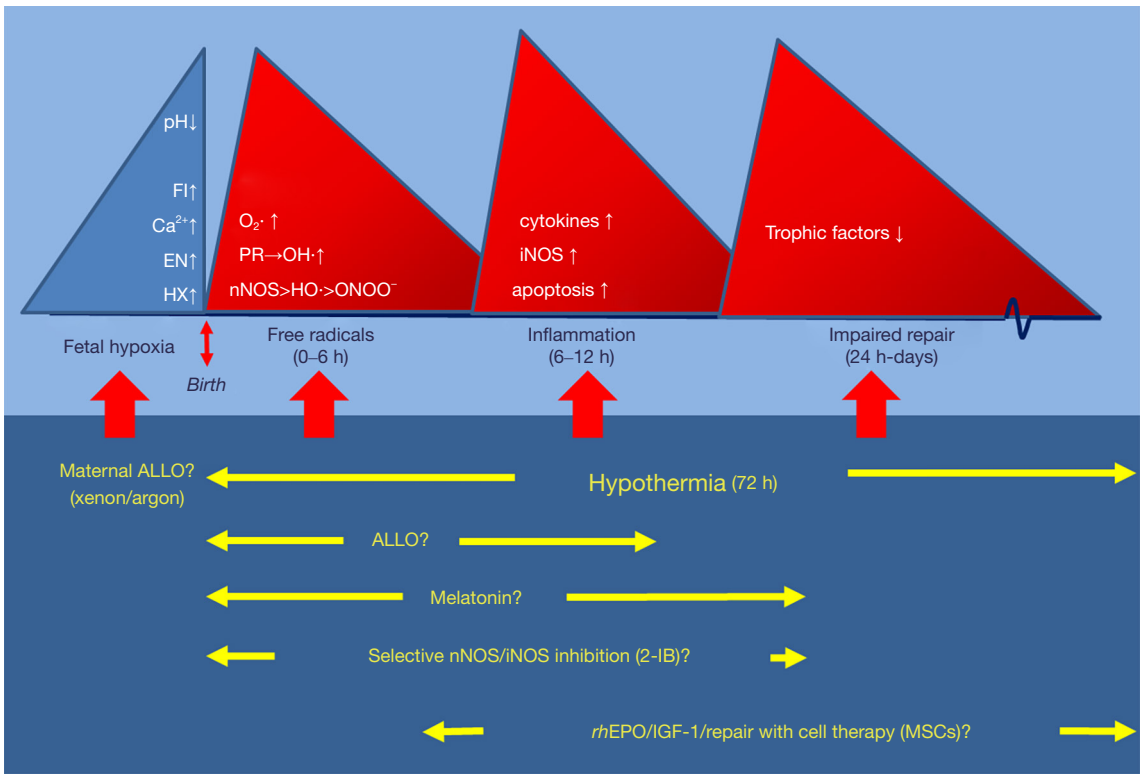

Figure 2 Time profiles of molecular destructive pathways set in motion by fetal hypoxia, perinatal hypoxic-ischemia and reperfusion and reoxygenation upon birth due to severe birth asphyxia. During fetal hypoxia (occurring in about 90% of cases of birth asphyxia) oxidative metabolism of the immature brain is curtailing the metabolization of xanthine to uric acid and thus leading to accumulation of hypoxanthine (HX), excessive formation of excitatory neurotransmitters (EN) leading to ionized calcium influx (Ca^{2+}) influx in the neurons with cell membrane damage, lowering interstitial pH and formation of pro-radical species (PR). Upon birth re-oxygenation facilitated metabolization of HX to uric acid by the xanthine-oxidase producing huge levels of the super-oxide free radical (O_2;); formation of neuronal and endothelial nitric oxide synthesis (e/nNOS) and (O_2;)-derived peroxynitrite ($ONOO^-$) and hydroxyl (OH.) production (maximal impact during first hours of life). From 6 hrs onward, inflammation is activated leading to (pro-) inflammatory cytokines, inducible NOS and excessive apoptotic cell death. These processes have eventually a negative effect on formation of trophic factors important for recovery of affected neurons.

of free radicals with maternal therapy with vitamin C, phenobarbital, N-Acetyl cysteine or melatonin, all drugs were supposed to pass the placenta, did not show or suggest positive or conclusive results (49-52). Allopurinol, which is predominantly a xanthine oxidase inhibitor but in higher concentrations also a direct free radical scavenger and free ion chelator (53,54) will exert its optimal action in the very early postnatal period given the excessive surge of (O_2·) and free radicals occurring as early as 30–60 minutes after reoxygenation and reperfusion (25,55). Uploading the fetus with therapeutical levels of allopurinol by treating the mother diagnosed with fetal hypoxia optimizes the free radical reducing effects of allopurinol since therapeutical levels of allopurinol and its active metabolite oxypurinol can optimally neutralize free radical formation which immediately accelerates upon birth because of a renewed availability of oxygen. Earlier studies showed that oral and intravenous maternal treatment led to therapeutical levels of allopurinol and oxypurinol in the fetus within 20 minutes after the maternal administration (56,57). A double blinded randomized clinical trial in term pregnant women diagnosed with fetal hypoxia just before delivery showed a gender specific short-term positive effect in girls, but also a considerable overtreatment of pregnant women who did not deliver an asphyxiated baby (58). Long-term developmental outcome of this cohort did not reveal differences between allopurinol- and placebo treated groups. However, in this study almost all fetuses experienced mild to moderate

hypoxia, not enough to eventually developing birth asphyxia (58,59).

Therapy in early neonatal period

After birth perfusion recovers normalizing oxygen availability. Since oxygen availability is very important for metabolization of the accumulated xanthine to uric acid by xanthine-oxidase during fetal hypoxia a huge production of O_2· occurs upon reoxygenation with a maximal production already 30 to 60 minutes after birth. O_2· Itself is destructive, but it also facilitates the metabolization of pro-radicals like free or non-protein bound iron, already produced fetally, generating the very aggressive hydroxyl free radical (OH·). It further reacts with the nitric oxide free radical (NO·) to form the very toxic peroxynitrite ($ONOO^-$) (5,6,25,42,55). So, very early after birth excessive amounts of free radicals and other toxic products may damage the brain and to prevent the damaging effects of these compounds it is important to install free radical scavenging therapy as early as possible after birth. Although a "therapeutic window" of 6 hours following birth is generally accepted to be the standard and is considered to be the time "slot" to start HT, reducing or preventing free radical and nitric oxide formation as early as possible and preferably within 30 minutes after birth can be crucial to safe the newborn brain after birth asphyxia. As already stated above allopurinol may be an ideal candidate here (60). We showed that maternal allopurinol therapy to prevent excessive production of the superoxide free radical (O_2;) is possible but led to a huge overtreatment of women diagnosed with fetal hypoxia (57-59). We therefore started a large European double blind randomized trial (ALBINO trial) where we aim to treat the neonates diagnosed with birth asphyxia within 45 minutes after birth with high dose allopurinol (or placebo) as add-on therapy of HT (ClinicalTrials.gov NCT03162653) (61). Clinical studies with allopurinol within the therapeutical window of 6 hours suggested a beneficial effect on short-term cerebral biomarkers and even on long-term developmental outcome, especially in moderately asphyxiated infants without serious adverse effects (62-64), but a Cochrane review in 2008 could not confirm that allopurinol was an effective neuroprotective therapy in the post hypoxic-ischemic neonatal brain since the clinical trials reported up to now were underpowered (65). The mostly relatively late treatment regimen in these clinical studies, up to 4 hours after the actual hypoxic-ischemic insult, may be an important cause for these ambiguous results.

As is the case with early allopurinol therapy, early inhibition of nitric oxide formation may be of value in relation with prevention of birth asphyxia-induced brain damage (66,67). Nitric oxide synthase (NOS) is an enzyme catalyzing production of NO· from L-arginine. Neuronal NOS (nNOS) and endothelial NOS (eNOS) are upregulated immediately after reperfusion/reoxygenation and inducible NOS (*i*NOS) from several hours onwards (68). eNOS is important in maintaining pulmonary blood flow, preventing pulmonary hypertension and maintaining adequate oxygenation. However, nNOS and iNOS are reported to have a damaging effect on the developing brain after perinatal asphyxia. Excessive NO· formation due to nNOS takes place especially in the early reperfusion/reoxygenation period shortly after birth, where it reacts with O_2· to produce the toxic peroxynitrite and nitrotyrosine, an end product of this process (7). iNOS upregulation occurs somewhat later after birth and reach its maximal production at an age between 6 and 12 hours and has also been shown to augment brain injury (69). Therefore selective inhibition of nNOS and iNOS can be neuroprotective. This was confirmed in a recent review of experimental studies on NOS inhibition (66). Our research lab investigated in several animal models 2-Iminobiotin (2-IB), a selective nNOS and iNOS inhibitor, and confirmed its neuroprotective actions after hypoxia-ischemia (67,70,71). Recently, pharmacokinetics of 2-IB were investigated by us in human infants with perinatal asphyxia requiring therapeutic hypothermia (72). Also here it may be very important to start treatment immediately after birth, especially in respect with the inhibition of the nNOS formation. A large blinded randomized clinical trial to show the effectiveness of 2-IB is in preparation.

Xenon, a noble gas and antagonist of the NMDA receptor, preventing or reducing the influx of calcium into neuronal cells, has also strong anti-apoptotic properties (73,74). Xenon inhalation may therefore be a potential neuroprotective strategy and has been investigated as an add-on therapy for asphyxiated neonatal species (75-77). It shows a rapid passage of the blood-brain barrier, and has no negative side effects are reported. Add-on therapy with HT enhanced neuroprotection which is demonstrated in several species as compared to neuroprotection by HT alone (78). Xenon use is costly, needs a closed loop system with adjustments in neonatal ventilators and cuffed tracheal tubes to stop loss of Xenon (79). The only clinical study using Xenon as add-on therapy for HT showed no adverse effects, but also no additional neuroprotective effect of Xenon (80). Ventilation with Argon may be an alternative:

Argon is another noble gas with reported neuroprotective actions in experimental studies in the neonatal animal, is much cheaper and does not need a closed loop system (81,82).

Magnesium has been shown to decrease hypoxic-ischemic excitotoxic activity by closing the NMDA glutamate channel by binding to the magnesium site and thus stabilizing the neuronal cell membrane (47). The neuroprotective action of Magnesium in term infants has been tested in several studies, but a meta-analysis showed no differences in mortality (83-85). At present there is no evidence that $MgSO_4$ as add-on therapy for hypothermia is neuroprotective after perinatal asphyxia (86,87).

Melatonin exerts a neuroprotective action after perinatal asphyxia by reduction of oxidative stress. It should have also anti-inflammatory, and anti-apoptotic activities (88). A neonatal piglet model of perinatal hypoxia-ischemia reported significant neuroprotection (89), and 2 pilot studies, from which one used melatonin as add-on therapy during HT, reported short-term neuroprotective effects as indicated by less free radical and NO· formation, and neurological improvement compared to hypothermia as single therapy respectively (90,91). Confirmation of its neuroprotective effects after birth asphyxia in the clinical situation is pending.

N-acetylcysteine, glutathione precursor, exerts a direct free radical scavenging activity (92). Although experimental studies revealed neuroprotective actions as add-on therapy for HT (93), adverse reactions were reported (78). Clinical studies in asphyxiated newborns are scarce and non-conclusive (94).

Some other drugs are mentioned in relation with neuroprotection after perinatal hypoxia-ischemia mostly because of their free radical scavenging effect such as Tetrahydrobiopterin and Topiramate, but their clinical significance is not yet well defined (78,95).

Finally, hyperbaric oxygen therapy should be mentioned in relation with its antioxidative actions: supranormal concentrations of oxygen aim to induce tissue super oxide dismutase expression and thus production and by that enhancing the antioxidative capacity of the body. Serious studies are needed to bring this antioxidative approach to the clinic (96).

As already stated above, a start as early as possible after birth of free radical scavenging therapy after perinatal asphyxia are expected to enhance the neuroprotective effects. *Figure 2* summarizes the timelines of production of free (pro) radicals and other toxic compounds and shows the preferred therapeutic actions of the most promising drugs as a function of postnatal age.

Delayed therapy (>6 hours of age); enhancement of anti-inflammation and neurotrophic factors

The transcription factor nuclear factor kappa B (NFB), which regulates expression of genes involved in inflammation and apoptosis, is upregulated after perinatal hypoxia-ischemia (97). This activation of NFB is partly due to and follows on the surge of free radicals upon and early after reperfusion and reoxygenation and manifests itself about 6 to 12 hours after birth. This potentially destructive pathway leads to an abundant formation of pro- and anti-inflammatory cytokines, starts production of inducible NOS (iNOS) and induces inappropriate apoptosis (see also *Figure 2*). At this particular postnatal time frame secondary energy failure of brain metabolism and subsequent brain damage becomes manifest. This brain damage is increasingly linked to pro-inflammation and excessive apoptosis due to lack of neurotropic repair activity (98).

Boosting anti-inflammatory capacity and repair by neurotropic factors seems essential. Several drugs and compounds are promising and most well investigated in this respect is probably Erythropoietin (EPO). EPO and EPO-derivatives (asialo-EPO and darbepoetin) have anti-inflammatory, anti-apoptotic and neurotropic properties as reported in experimental studies (99-101). EPO and its derivates exert its neuroprotective action in neurons, oligodendrocytes and astrocytes via hypoxia-mediated activation of the transcription factor hypoxia-inducible factor-1a (99). Recombinant Human EPO (rhEPO) has a safe pharmacological profile and is already used in preterm infants (102). Clinically a small trial suggested that therapy in asphyxiated newborns with rhEPO appeared safe and feasible (103). In China, a double-blinded randomized trial, before the HT era, in perinatally asphyxiated neonates showed that intravenously administered rhEPO improved developmental outcome at 18 months of age. Dosages used in this study where quite low (750 IU/kg) and there appeared a gender preference for girls who benefited most from this therapy (104). A pharmacokinetic study in 24 neonates with asphyxia undergoing HT and treated with rhEPO as add-on therapy for HT, showed that repeated high dose rhEPO (1,000 U/kg) were well tolerated (105). Randomized trials with moderate hypothermia and add-on therapy with high dose rhEPO was safe and feasible and suggested to reduce MRI-derived brain injury and improve 1-year motor function (106,107). A recent meta-analysis of

6 randomized trials with rhEPO or EPO-derivatives as add-on therapy for HT reported a lower risk for brain damage and cognitive outcome (108). Optimally powered studies are needed with appropriate (high) dosages of rhEPO and/or EPO-derivatives to ultimately confirm neuroprotective properties of this drug. A phase III double blind randomized study is currently underway (the HEAL Trial; ClinicalTrials.gov NCT02511263).

Melatonin has also anti-inflammatory properties, especially by preventing activation and translocation of NFB to the nucleus, in addition to its anti-oxidative and free radical scavenging actions (88). Although melatonin is a promising drug with respect to neuroprotection after perinatal hypoxia-ischemia, no clinical studies have been reported with respect to its anti-inflammatory action.

At present, two promising compounds, only investigated in the experimental setting at this point, should be mentioned: cannabinoids and azithromycin. Cannabinoids has anti-neurotoxic and anti-inflammatory properties and exert brain protection after neonatal hypoxia-ischemia as has been reported in the experimental setting, also as add-on therapy (98,109). Azithromycin is an antibiotic and a macrolide derivative of erythromycin (110). Azithromycin improved functional and neuropathology outcomes, probably based on its anti-inflammatory properties (111).

Research with respect to inhibition of NFB and pro-inflammatory cytokines or enhancement of anti-inflammatory cytokines or anti-apoptotic strategies are very interesting but still preliminary (112).

Downregulation of neurotropic-, maturational- and growth factors in the first days and even weeks after severe perinatal hypoxia-ischemia with an increased apoptotic activity has a negative influence on developmental outcome (99). It has been reported that erythropoietin has neurotropic and anti-apoptotic properties (113,114). EPO has a long lasting positive effect on neurogenesis but also on neovascularization, which is in particular true if given in supranormal dosages (1,000 to 5,000 IU/kg) improving developmental outcome. Sustained therapy may be important here, but need more research in relation with drug safety (115). HIF-1α-induced extra formation of transcriptional targets, enhanced formation of erythropoietin, endothelial growth factor and consequent brain repair may be a very important mechanistic issue here (100).

An intriguing recent field of research in respect with prevention and repair of immature brain damage is boosting and/or elevation of (endogenous) neurotropic factors such as basic fibroblast growth factor (BFGF), BDNF and especially IGF-1. Animal studies showed a reduction of injury of the (preterm) neonatal brain (116,117). Future research may demonstrate whether this therapy contributes to an improved long-term neurodevelopmental outcome after severe perinatal hypoxia-ischemia.

Repair of the brain after birth asphyxia-derived brain damage

Embryonic stem cells have the potential to differentiate into hematopoietic, neural or mesenchymal stem cells (118,119). Among the 3 lineages of the embryonic stem cell the mesenchymal stem cell (MSC) is the stem cell of choice at present for clinical studies and treatment options because of its neuro-regenerative and important immune-modulating actions and proven safety (120,121). Contrary to embryonic or neural stem cells, which are linked to the induction of tumorous formation, MSCs have a well investigated safety profile (121). As a detailed discussion of the potential benefits and hazards of several types of stem cells is beyond the scope of this review, we refer to a recent review paper we published about the therapeutic value of MSCs as compared to other progenitor cells (118).

Besides it safety profile the strength of MSCs in clinical care lies predominantly in the fact that MSCs can, in addition to differentiating into mesodermal cells (fat, bone and cartilage), induce proliferation of endogenous neural stem cells if entering a (previous) hypoxic environment (122,123): MSCs stimulate this formation of new brain cells by a paracrine action rather than by transformation themselves into neuronal tissue (118,124). See also *Figure 3*. A more detailed discussion of the beneficial actions of MSCs after neonatal brain injury are well-described in a recent review paper of our stem cell research group by Wagenaar *et al.* (118).

Optimal results by combining therapies at the right point of time

The ultimate answer towards a further reduction of brain damage after perinatal asphyxia by pharmacological neuroprotection is not yet available. However, if calcium influx can be reduced, free (pro-) radical production and toxic compounds in the fetal and early post-ictal phase can be reduced or even prevented. Subsequently, the inflammatory activation and loss of trophic factors can be restrained which requires besides HT multiple pharmacological interventions requiring optimal timing, dosing and duration for each drug dedicated to its specific intervention in the (destructive) molecular pathway.

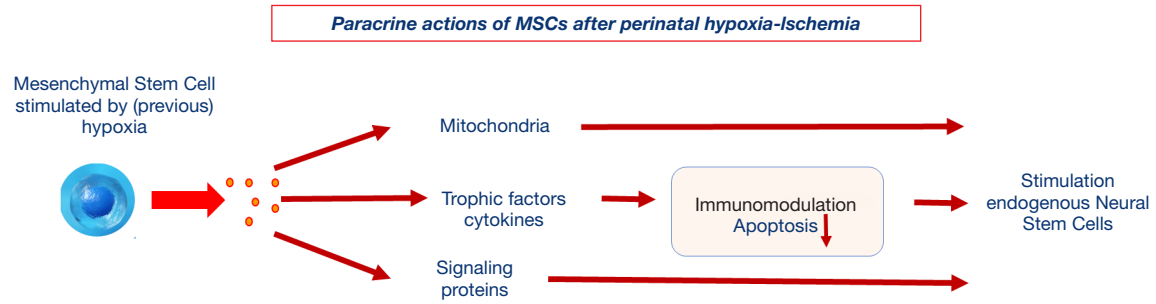

Figure 3 Production of exosomes set in motion by (post) hypoxia-ischemia-"activated" mesenchymal stem cells (MSCs): These vesicles are filled with growth- and maturational factors; mitochondria; cytokines creating the optimal inflammatory environment; and signaling proteins. This induces proliferation of the endogenous neural stem cells, situated in the paraventricular region, leading to formation of new neurons, astrocytes and oligodendrocytes.

The major challenge for pharmacologic therapy after perinatal hypoxia-ischemia is to figure out the optimal combination of pharmaceuticals as add-on therapy with HT. Unconventional study set-ups are necessary to prove the clinical value of such a pharmacologic approach! Additional issues such as gender specificity of the drugs used have to be resolved too. But if we are able to solve the complex questions as stated above and can "repair" remaining brain injury with cell therapy, the long-term outcome of neonates who experienced severe perinatal asphyxia can be improved substantially!

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None.

Footnote

Conflicts of Interest: Herewith the authors of this manuscript, Frank van Bel, MD and Floris Groenendaal, MD declare that Frank van Bel and Floris Groenendaal are, together with Cacha Peeters-Scholte, inventors of 2-iminobiotin as neuroprotective agent for neonates with cerebral hypoxia-ischemia. They have no financial or personal relationships with other people or organizations that could potentially and inappropriately influence their work and conclusions. They further declare to have no proprietary or commercial interest in any product mentioned or concept discussed in this article.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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