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REVIEW ARTICLE

Free radicals and neonatal encephalopathy: mechanisms of injury, biomarkers, and antioxidant treatment perspectives

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Neonatal encephalopathy (NE), most commonly a result of the disruption of cerebral oxygen delivery, is the leading cause of neurologic disability in term neonates. Given the key role of free radicals in brain injury development following hypoxia–ischemia–reperfusion, several oxidative biomarkers have been explored in preclinical and clinical models of NE. Among these, antioxidant enzyme activity, uric acid excretion, nitric oxide, malondialdehyde, and non-protein-bound iron have shown promising results as possible predictors of NE severity and outcome. Owing to high costs and technical complexity, however, their routine use in clinical practice is still limited. Several strategies aimed at reducing free radical production or upregulating physiological scavengers have been proposed for NE. Room-air resuscitation has proved to reduce oxidative stress following perinatal asphyxia and is now universally adopted. A number of medications endowed with antioxidant properties, such as melatonin, erythropoietin, allopurinol, or *N*-acetylcysteine, have also shown potential neuroprotective effects in perinatal asphyxia; nevertheless, further evidence is needed before these antioxidant approaches could be implemented as standard care.

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ROLE OF FREE RADICALS IN THE PATHOGENESIS OF NEONATAL ENCEPHALOPATHY

Neonatal encephalopathy (NE) most commonly results from an acute or subacute disruption of cerebral blood flow and oxygen delivery to the brain during the perinatal period. The incidence of NE ranges from 1 to 8 per 1000 live births in high-income countries to as high as 26 per 1000 live births in low-income countries.[1] Despite the advent of therapeutic hypothermia, this condition is still a major cause of death and neurodevelopmental disability in term neonates worldwide.[2]

Specific antepartum risk factors (e.g., maternal pyrexia, prolonged rupture of membranes, persistent occipito-posterior position) or a well-recognized intrapartum event responsible for an acute decrease of placental perfusion (e.g., placental abruption, prolapse of the umbilical cord, uterine rupture, shoulder dystocia) can be often identified.[3,4] The development of hypoxic–ischemic brain damage is more likely in the presence of umbilical cord arterial pH < 6.8, base excess <-20 mEq/L, Apgar score of ≤3 at 10 min, and other negative prognostic factors, such as absent fetal heart rate variability prior to birth, seizures in the first day of life, and multi-organ injury.[5] The characteristics of the asphyxiating insult (i.e., intermittent, persistent, chronic), together with the infant's gestational age, prior metabolic and cardiovascular status, and individual sensitivity to oxidative stress further contribute to the severity of NE.[5] The Sarnat staging system, based on the combination of specific clinical signs (e.g., abnormalities of consciousness, tone, reflexes, and/or electrical brain activity), classifies NE into three stages of increasing severity: the higher the stage, the lower the probability of survival without major neurological sequelae.[6]

The main phases of NE, with the related mechanisms of injury and the therapeutic strategies currently proposed in research settings, are summarized in Fig. 1. The event sequence leading to NE includes oxygen deprivation, energy depletion, and reoxygenation. As illustrated in Fig. 2, these events contribute to the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which harmfully interact with nearby proteins, nucleic acids, or membrane lipids, altering their function and converting them into free radicals.[7] ROS are finely regulated by specific antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), and glutathione peroxidase (GP); the imbalance between ROS production and clearance leads to oxidative stress (OS) in the newborn.[8,9]

The metabolic consequence of acute cerebral hypoperfusion is the inhibition of oxidative phosphorylation in the electron transport chain (ETC) of the mitochondria, resulting in anaerobic metabolism. Glucose utilization in anaerobic glycolysis is highly inefficient and contributes to a rapid depletion of cerebral glucose, the primary energy source for neural cells.[10] The resulting decrease in adenosine triphosphate (ATP) leads to the inactivation of ATP-dependent ion pumps, with intracellular accumulation of sodium and water, progressive cell swelling, and cellular necrosis. This event cascade culminates into glutamate excitotoxicity, which not only activates apoptotic pathways via *N*-methyl-D-aspartate (NMDA) and glutamate receptors, but also upregulates nitric oxide synthase (NOS) to induce a compensatory increase in cerebral blood flow.[11] The ensuing NO surge, however, triggers the production of potent RNS that actively contribute to brain damage.[12]

Following acute hypoxia–ischemia in term neonates, the deep gray nuclei appear most vulnerable. The enhanced susceptibility of this area is due to the presence of NOS-expressing (NOS+)

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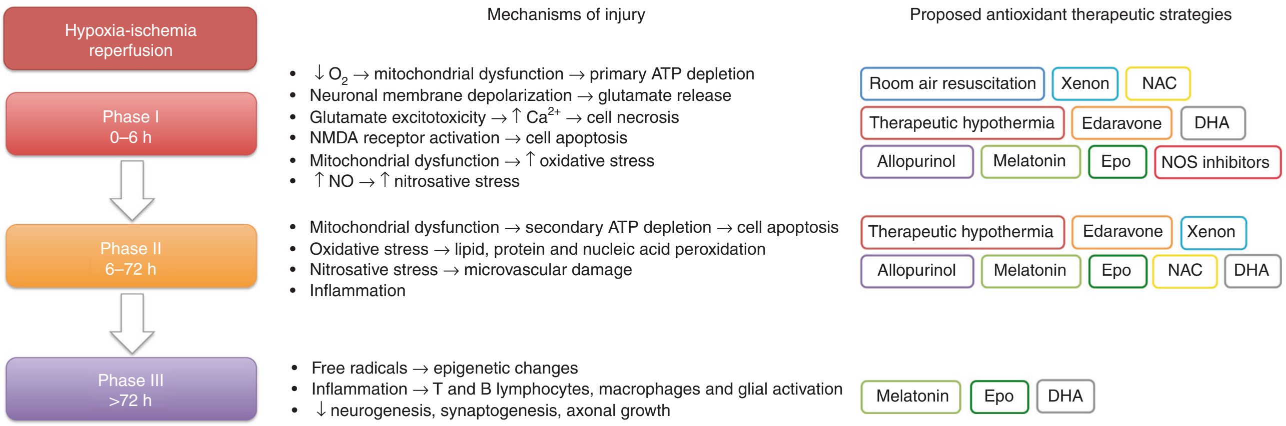

Fig. 1 Summary of the phases of neonatal encephalopathy with related mechanisms of injury and proposed antioxidant strategies

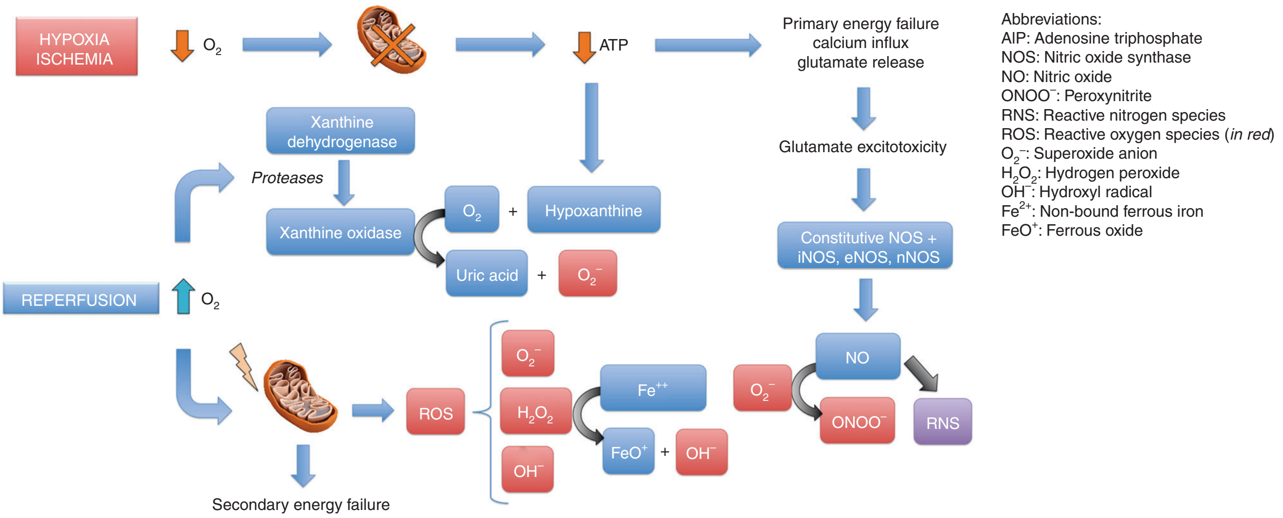

Fig. 2 Mechanisms of free radical production following hypoxia–ischemia and reperfusion

striatal neurons that are paradoxically resistant to hypoxic–ischemic injury but, by producing high amounts of RNS, exert a harmful bystander effect on nearby neural and glial cells.[13–16]

Following the acute insult, there is a restoration of cerebral perfusion, which, while essential for survival, paradoxically contributes to the so-called reperfusion injury, mediated by excessive free radical production.[17] This results not only in an oxidative burst but also in a progressive disruption of the mitochondrial ETC, leading to secondary ATP depletion and subsequent apoptotic brain damage.[18]

The progressive accumulation of hypoxanthine that follows ATP depletion and the reoxygenation-driven conversion of xanthine dehydrogenase (XD) to xanthine oxidase (XO), a superoxide-producing enzyme which, using O_2 as a cofactor, produces $\bullet O_2-$ and uric acid from xanthine or hypoxanthine, are primary sources of ROS after reperfusion.[19] Non-protein-bound iron (NPBI), released from hemoglobin, enhances hydroxyl radical formation via the Fenton reaction, thus raising ROS levels exponentially.[20] ROS and RNS further worsen the mitochondrial damage begun by primary ATP depletion and glutamate excitotoxicity, contributing to the progressive disruption of oxidative phosphorylation that ensues in secondary energy failure and neuronal apoptosis.[19] These latter events, however, are preceded by a phase of latency, which may last up to 6 h following reperfusion and represents a crucial therapeutic window.[4]

Understanding the role of free radicals in NE has led to the discovery of several biomarkers of oxidation or nitrosylation, with possible diagnostic and prognostic implications. Also, there is a potential of developing novel neuroprotective approaches based on antioxidant therapies. This review aims to provide an overview of the role of free radical biomarkers and antioxidant therapies in NE. Literature search methods are provided in Supplementary Table S1.

FREE RADICAL BIOMARKERS IN NE

The diagnosis of NE mainly relies on clinical, neurophysiological, and neuroimaging abnormalities. However, both the neurological status and cerebral electrical activity can be significantly altered by sedatives, anticonvulsants, and therapeutic hypothermia (TH).[21] Moreover, diagnostic changes on magnetic resonance imaging (MRI) may take several days to become apparent. The validation of molecular biomarkers of hypoxia–ischemia and ensuing free radical burden may help identifying neonates at higher risk of moderate-to-severe NE, who could qualify for early neuroprotective treatments. The following section focuses on current biomarkers for oxidative and nitrosative stress, which are summarized in Table 1.[22]

Table 1. Diagnostic and prognostic implications of the main biomarkers for oxidative and nitrosative stress in neonatal hypoxic–ischemic encephalopathy

Biomarkers	Role in oxidative/nitrosative stress	Changes in severe perinatal asphyxia/ hypoxic ischemic encephalopathy (HIE)	Predictive value on HIE severity or outcome	Suggested reference values
Superoxide dismutase (SOD) Glutathione peroxidase (GP) Catalase (CAT)	Antioxidant enzymes	↑ cord blood SOD, CAT and GP[25] ↑ plasma SOD and CAT at 0–24 h[26–28] ↑ CSF SOD at 0–72 h[29]	Cord blood SOD and CAT: positive correlation with Sarnat stage[25] Plasma SOD (24 h): positive correlation with Sarnat Stage and lipid peroxidation markers[27]	Not available
Urinary uric acid-to-urinary creatinine (uUA/uCr) ratio	Marker of xanthine oxidase activity	↑ uUA/uCr ratio at 0–72 h[30–35]	uUA/uCR ratio (0–72 h): positive correlation with Sarnat stage[31] Values ≥2.6 mg/mg (0–24 h) predictive of mortality[31]	Values >2.3 mg/mg (0–72 h) diagnostic of HIE[35]
Nitric oxide (NO)	Substrate for nitrosative species production	↑ plasma NO and nitrates/ nitrites ratio at 0–24 h[27, 36–38] ↑ CSF NO at 0–24 h[38]	Plasma and CSF NO (0–24 h): positive correlation with Sarnat stage[27, 37, 38] Increased plasma NO associated with brain injury development[37]	Not available
Non-protein-bound iron (NPBI)	Substrate for Fenton reaction-mediated ROS production	↑ plasma NPBI at 0–72 h in severe HIE[39–41] If therapeutic hypothermia (TH): higher NPBI before, but not during or after TH[42]	Undetectable NPBI (0–8 h): associated with normal neurological outcome at 12 month[39] Increased plasma and CSF NPBI: higher risk of mortality or neurological impairment[40, 41]	Normal cord values: <6.91 µmol/L[22]
Malondialdehyde (MDA) Urinary MDA/urinary creatinine ratio (uMDA/uCr)	Lipid peroxidation markers	↑ cord MDA in severely asphyxiated infants[31, 34, 44] ↑ serum MDA at 0–72 h[26–28, 36, 41, 45] ↑ CSF MDA at 0–72 h[25, 41] ↑ uMDA/uCr ratio at 0–48 h[31, 34]	Serum,[29, 47] CSF MDA,[23] and uMDA/uCr ratio[31, 34]: positive correlation with Sarnat stage Increased serum MDA (0–72 h): higher risk of mortality,[41, 45] seizures and neurological sequelae[40, 41, 45] CSF MDA (0–72 h): positive correlation with mortality risk[41] uMDA/uCr ratio >3.495 µg/mg (0–72 h) predictive of mortality[31]	Not available
4-Hydroxynonenal (4-HNE)	Lipid peroxidation marker	↑ cord 4-HNE in term and preterm infants[46]	Not evaluated	Not available
Isoprostanes	Lipid peroxidation markers	↑ cord isoprostanes in asphyxiated term neonates[49]	Cord 8-iso-15(R)-PGF2α: positive correlation with severity of perinatal asphyxia[49]	Normal cord values: <124.47 pg/mL[22]
Protein carbonyls (PC) Advanced oxidation protein products (AOPP)	Protein oxidation markers	↑ serum PC at birth and 48 h[45] ↑ cord AOPP in hypoxic preterm infants[50] If TH: higher plasma AOPP NPBI before, but not during or after treatment[28, 42]	Higher serum PC associated with seizures[45] Plasma AOPP (4–6 h): positive correlation with HIE severity[42] Plasma AOPP (0–5 days): positive correlation with MRI scores of brain damage[42]	Normal AOPP cord values: <80.39 µmol/dL[26]
8-hydroxydeoxy-guanosine (8-OHdG)	DNA peroxidation marker	↑ urinary and CSF levels in HIE infants[54]	Not evaluated	Not available

Gas chromatography coupled to mass spectrometry (MS) has long been the gold-standard technique for a qualitative and quantitative estimation of oxidative compounds on different specimens.[16] Recently, the development of high- or ultra-performance liquid chromatography (LC-MS/MS), which allows a simultaneous measurement of different compounds on smaller sample volumes, has contributed to ease the assessment of oxidative status in the neonatal population.[23] Nevertheless, high costs and need for trained personnel are major limitations to the routine clinical application of these methods, which are therefore often confined to research facilities.[24]

Antioxidant enzymes

SOD, GP, and CAT are the first-line antioxidant defense against OS. Following a hypoxic–ischemic hit, their activity acutely rises to counteract the ensuing ROS production and its harmful effects. Significantly higher SOD, CAT, and GP levels have been reported in cord blood samples of asphyxiated newborns compared to controls[25]; at 24 h, however, only CAT and SOD, but not GP, maintained higher plasma concentrations in infants with NE compared to controls.[26–28] A positive correlation between CAT and SOD cord levels and Sarnat stages was also observed,[25] suggesting that the upregulation of these enzymes may mirror NE severity. Given their acute increase, these data suggest that the evaluation of antioxidant enzymes in cord blood may add useful information on both timing and severity of perinatal asphyxia within the decision-making window for TH. Nevertheless, it should be noted that they are based on small cohorts, thus needing further confirmation in larger clinical trials.

Very little is known about antioxidant enzymatic activities in other biological fluids. One study has reported a significantly enhanced SOD activity in cerebrospinal fluid (CSF) between 0 and 72 h in 30 term neonates with NE, whereas GP and CAT activity increased only in the severe NE subgroup.[29] However, there was a wide time interval during which lumbar puncture was performed (anytime within 72 h of life) making it difficult to interpret.

Most of these cited studies were performed before the routine introduction of TH, whose potential modulatory effect on antioxidant enzymatic activities requires further evaluation.

Uric acid

The reoxygenation-driven conversion of XD to XO, which produces $\bullet O_2-$ and uric acid, plays a key role in oxidative brain damage. Urinary excretion of uric acid has thus been proposed as an economical and non-invasive marker for XO-related ROS production in asphyxiated newborns.

Several studies have consistently reported an increased urinary uric acid-to-urinary creatinine ratio within 48–72 h of life in term and preterm asphyxiated newborns,[30–35] and a positive correlation between this ratio and Sarnat stage has also been reported.[31] A cut-off of 2.3 mg/mg over the first 72 h has been proposed as diagnostic of perinatal asphyxia in a single study on 40 asphyxiated infants in a low/middle resource setting.[35] In this study, however, the diagnosis of perinatal asphyxia was mainly based on Apgar score and cord pH at birth; moreover, no postnatal clinical data of the infants enrolled are available, other than none of them were undergoing TH at the time of urine collection. Values ≥2.6 mg/mg may predict impeding death in asphyxiated term infants with good sensitivity and specificity,[31] but again, this data was based on a small study of only 20 infants and performed before the introduction of TH. Larger studies are therefore needed on patients undergoing TH.

Nitric oxide

Ischemia-induced upregulation of NOS enhances NO and RNS production. A raised concentration of NO in blood and CSF, as well as a higher plasma nitrate/nitrite ratio (which serves as a proxy for NO levels), have been detected in neonates with NE within the first 24 h,[27,36–38] with higher NO concentration related to higher Sarnat stage.[26,37,38] Furthermore, plasma NO was increased in asphyxiated infants with early evidence of brain damage compared to those with normal neuroimaging.[37] However, these studies were carried out before the introduction of TH. Future studies, though, could also help to evaluate the role of this biomarker in selecting those infants who might benefit of specific antioxidant treatments such as XO inhibitors, which hinder the formation of peroxynitrite from XO-derived superoxide and NO and the subsequent activation of downstream pathways that lead to cerebral endothelial and tissue injury.[38]

Non-protein-bound iron

Following hypoxia–reoxygenation, hemoglobin-released NPBI interacts with $\bullet O_2-$ and H_2O_2 and forms highly reactive •OH. Studies conducted in the pre-cooling era reported increased concentrations of plasma NPBI with increasing NE severity,[39] although low or undetectable NPBI levels were also seen in moderately and severely asphyxiated infants. However, low or undetectable concentration values were significantly associated with normal neurological outcome at 1 year, irrespective of NE stage; these latter data, however, were obtained from a small number of infants and need to be confirmed on larger samples.[39] Higher NPBI levels in plasma and CSF have been described within the first 72 h in neonates with moderate-to-severe NE who died or developed neurological sequelae.[40,41] More recently, increased plasma NPBI levels at 4–6 h, but not at 24–72 h and 5 days of life, were detected in 80 infants with severe NE who underwent TH, compared to those with mild–moderate NE,[42] suggesting not only a possible prognostic value of NPBI in the earliest phases after the perinatal insult but also a possible influence of TH on this biomarker or, more generally, on OS levels. Nevertheless, diagnostic NPBI levels for NE still have to be defined.

Lipid peroxidation markers

The extent of lipid peroxidation following hypoxia–ischemia can overwhelm the adaptive upregulation of antioxidant systems, leading to harmful effects on membrane phospholipids, functional loss, and programmed cell death. Once this process is initiated, it generates a wide variety of lipid peroxidation products that, given the rich lipid composition of the brain, might reflect the extent of cerebral oxidative damage.

Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). MDA, a biomarker for n-3 and n-6 fatty acid peroxidation, has been widely investigated, as it can be easily determined by thiobarbituric acid assay, a widely available spectrophotometric technique.[43] The current literature is consistent in reporting increased MDA levels in cord blood of severely asphyxiated neonates[31,34,44] and in the serum of infants with NE[26–28,36,41,45] compared to controls. As for prognostic implications, serum MDA at 24 h positively correlates with NE severity,[27,28] with highest values in infants who died[41,45] or developed persistent neurological abnormalities.[40,41,45]

Being water soluble, MDA is detected in urine, and its excretion rate has also been tested in the context of NE. The ratio between urinary MDA and urinary creatinine (uMDA/uCr) was significantly increased over the first 48 h of life in asphyxiated neonates compared to controls, and positively correlated with Sarnat stage.[31,34] A higher uMDA/uCr ratio was also observed in asphyxiated neonates who died compared to survivors, and values >3.49 µg/mg on day 1 have been proposed to predict mortality following perinatal asphyxia in a small number of asphyxiated infants.[31] Since most of these studies did not include infants who underwent TH, it is unclear whether this treatment can influence MDA levels.

A possible limitation of serum and urinary MDA is the lack of specificity for the cerebral tissue, as it may also reflect the extent of lipid peroxidation following extensive multiorgan damage.

MDA concentration in CSF should provide a better estimate of oxidative brain damage in asphyxiated infants. Significantly higher CSF levels of MDA have been reported at 24–48 h in asphyxiated term infants compared to controls, with a positive correlation with Sarnat stages,[25] and also in infants with NE who expired or developed neurological deficits compared to those with normal neurological status at hospital discharge.[41] However, further data are needed to validate CSF MDA as a reliable marker for oxidative brain damage in NE.

As for 4-HNE, a metabolite of n-6 fatty acid peroxidation, Schmidt et al. reported significantly risen cord blood levels following a perinatal hypoxic insult.[46] To date, however, no additional data are available to add knowledge on this biomarker in the context of NE. This could be due the unstable nature of 4-HNE that contributes to the technical complexity of its determination, which benefits from the use of advanced liquid chromatography methods.[47]

Prostaglandin-like peroxidation products (PPPs). Isoprostanes, neuroprostanes, and neurofurans are prostaglandin-like compounds derived from free radical-catalyzed peroxidation of arachidonic acid (AA) and docosahexaenoic acid (DHA).

F2-isoprostanes seem to best reflect OS extent after hypoxia–reperfusion[48] and, thanks to ultra-performance LC-MS/MS, can be determined even in the neonatal population. Higher cord blood levels of 8-iso-15(R)-PGF2α and total isoprostanes were detected in acidotic and depressed infants compared to healthy neonates; 8-iso-15(R)-PGF2α positively correlated with the severity of asphyxia.[49] However, serial evaluation of serum F2-isoprostanes over the first 5 days showed no difference between neonates who developed severe compared to mild-to-moderate NE nor any correlation with brain damage at neuroimaging.[42] Given this scarce and inconclusive evidence, further studies are necessary to define whether PPPs may reflect polyunsaturated fatty acid oxidation after perinatal asphyxia.

Protein oxidation markers

Protein carbonyls (PC) and advanced oxidation protein products (AOPP) result from protein carbonylation, nitration, crosslinking, or loss of thiol groups by free radicals. Increased serum PC have been observed at birth and 48 h in asphyxiated term neonates, with higher levels in those who developed seizures; however, no difference was observed in relation to Sarnat stage or developmental outcome at 9 months.[45] Buonocore et al. previously reported increased cord levels of AOPP in hypoxic compared to normoxic preterm infants and a positive correlation between this marker and plasma hydroperoxides.[50]

Recently, higher plasma AOPP at 4–6 h of life have been described in term infants with severe NE, who required TH, compared to mild/moderate NE.[42] Later measurements (i.e., 24–72 h and 5 days) showed no difference between cooled asphyxiated infants and controls. This is consistent with previous findings by Mutlu et al., who compared AOPP levels between 30 cooled NE infants and 30 healthy term controls at 6–24 h and 5 days.[28] Given this lack of difference in AOPP levels during and after TH, it suggests a possible role of cooling in modulating protein oxidation, which warrants further investigation. A significant independent association between MRI scores suggestive of brain injury and blood levels of AOPP over the first 5 days of life in neonates with NE has also been reported, with a stronger correlation in male infants, suggesting a possible role for AOPP as a biomarker of brain oxidative damage.[42]

DNA peroxidation markers

Oxidative stress can lead to harmful peroxidative changes to nucleic acids. The oxidized DNA nucleoside 8-hydroxydeoxyguanosine (8-OHdG), resulting from DNA peroxidation, has been proposed as a biomarker for oxidative DNA damage in the neonatal population[51,52] and has also been used to evaluate the efficacy of TH in reducing OS-related DNA damage.[53] Nevertheless, to the best of our knowledge, current clinical evidence on its diagnostic role in NE is limited to a small pilot study aimed at evaluating 8-OHdG concentration in urine and CSF samples of children with brain damage, including a small subgroup with NE.[54] Despite significantly higher CSF and urinary 8-OHdG levels were observed in this population compared to control subjects, the study sample is undersized to draw any conclusion, and data on the timing of specimen collection are not available. Moreover, this biomarker is also increased in other causes of brain injury (e.g., status epilepticus, central nervous system infections), thus suggesting a lack of specificity for NE.

ANTIOXIDANT STRATEGIES FOR NEUROPROTECTION IN NE

The role of free radicals in the development of brain injury following hypoxia–ischemia–reperfusion has provided the rationale to explore antioxidant therapeutic approaches for NE, aimed either at upregulating the physiological scavenging systems or hindering ROS and RNS production at different levels. While the use of room air for the resuscitation of depressed term infants has become part of standard neonatal care, the use of other antioxidant molecules is mainly limited to research settings, with only variable preclinical and/or clinical supportive evidence (see Table 2).

The main neuroprotective strategies currently adopted or proposed to dampen oxidative brain damage after perinatal asphyxia are analyzed and discussed below.

Use of room air and novel inhaled antioxidant strategies for neonatal resuscitation

The beneficial effects of the use of 21% oxygen for the resuscitation of asphyxiated neonates on clinical outcomes and mortality was first reported two decades ago[55] and largely confirmed over the following years, thus becoming the standard of care for term infants' delivery room management since 2010.[56] In parallel, the relation between OS and the oxygen amount provided during resuscitation after perinatal asphyxia has been investigated in animal and human studies, producing a growing body of evidence toward a reduction of the oxidative burden following room-air resuscitation.[57–60]

Based on this evidence, novel inhaled antioxidant strategies have been recently proposed for perinatal resuscitation. The use of inhaled hydrogen with room air has shown an encouraging attenuation of cerebral oxidative biomarkers in swine models of NE[61,62]; clinical evidence, however, is lacking. Owing to its ability to cross the blood–brain barrier (BBB), inhaled xenon has also attracted broad interest as a potential neuroprotective agent for NE, although its mechanism of action is mainly ascribable to NMDA receptor antagonism rather than to antioxidant properties. Current evidence from randomized controlled trials, however, are limited to Azzopardi et al., who did not demonstrate any added effect to TH in infants with NE,[63,64] despite the acknowledged possible limitations (e.g., timing of the treatment start and duration of dose).

Recently, owing to its cheaper costs compared to xenon, inhaled argon has also been proposed for neuroprotection following perinatal asphyxia. Encouraging preliminary evidence of reduced white matter lactate and *N*-acetyl aspartate (NAA) at 24 and 48 h, as well as of reduced apoptotic burden and faster recovery of amplitude-integrated electroencephalography, has been observed in asphyxiated piglets undergone TH and 45–50% argon inhalation 2 h after the insult, compared to a TH-only group.[65] Nevertheless, the safety of argon needs to be further assessed before translational clinical trials can commence.

Therapeutic hypothermia

The efficacy of TH in decreasing brain injury[66] and reducing major neurocognitive sequelae[67] in neonates with moderate-to-severe

Table 2. Current availability of preclinical and clinical evidence on antioxidant molecules proposed for neuroprotection in neonatal hypoxic–ischemic encephalopathy (HIE)

Molecule	Antioxidant mechanism	Availability of neuroprotective evidence on HIE			
		Neonatal administration		Maternal intrapartum administration	
		Preclinical	Clinical	Preclinical	Clinical
Erythropoietin	Controversial	Available[80–86]	Available[87–92]	Absent	Absent
Melatonin	Free radical scavenger Enhanced mitochondrial chain efficiency Increased antioxidant enzymatic activities	Available[95–100, 102]	Available[103,104] Ongoing trials	Limited[105]	Absent
Allopurinol	Xanthine oxidase (XO) inhibition Scavenger of hydroxyl free radicals and transition metals	Available[106–108]	Available[38, 109–111]	Available[112]	Available[113–115] Ongoing trials
Nitric oxide synthase (NOS) inhibitors	NOS activity inhibition	Available[14, 118–129]	Ongoing trials	Absent	Absent
N-acetylcysteine	ROS scavenger GSH replenishment NOS downregulation	Available[132–137]	Limited[136]	Absent	Absent
Docosahexaenoic acid	ROS scavenger	Available[141–145]	Absent	Absent	Absent
Edaravone	ROS scavenger	Available[147–149]	Absent	Absent	Absent

NE has been largely established and is currently the standard of care for term and late preterm infants with NE.[4] The beneficial effects of TH mainly result from the downregulation of cerebral energy metabolism, which dampens the apoptotic burden of secondary cerebral energy failure.[68] However, other suggested mechanisms through which TH exerts neuroprotection include inhibiting neuronal cell death, limiting excitotoxicity, modulating glial cell activation,[69] and activating cold-inducible RNAs.[70] Moreover, a direct reduction of oxygen-based free radicals following an ischemic insult and subsequent reperfusion has been reported in both in vitro[71] and in vivo animal studies.[72]

In animal models, TH has proved to effectively decrease striatal and cortical NO-mediated production of cyclic GMP[73] and lipid peroxidation products in white matter,[74] suggesting a protective effect against not only oxidative but also nitrosative stress. In clinical studies, decreased serum levels of MDA and PC and increased SOD, GP, and glutathione S-transferase activities have been documented in adults undergoing hypothermia after cardiac arrest,[75] whereas no significant difference in serum hydroperoxides was observed in hypothermic compared with normothermic asphyxiated term infants over the first 3 days of life.[76] The small amount of preliminary data would suggest that TH has a beneficial effect on free radical production.[28,42] Moreover, a combined role of TH and other neuroprotective strategies with known antioxidant properties (e.g., erythropoietin, melatonin, allopurinol, *N*-acetylcysteine (NAC), DHA) in reducing hypoxic–ischemic brain damage has also been reported, suggesting a synergistic mechanism of action.

Erythropoietin (Epo)

By binding to its receptors (Epo-R), which are largely expressed in the central nervous system, Epo inhibits apoptotic pathways, reduces proinflammatory cytokines, and dampens glutamate excitotoxicity,[77] with promising therapeutic implications in NE.[78] Under hypoxic conditions, Epo-R expression and Epo secretion are significantly upregulated but, while the former increases promptly, the latter rises more slowly[79]; hence, the potential therapeutic role of exogenous Epo is greatest during this period.

Preclinical research has provided robust evidence in support of the neuroprotective effects of Epo following perinatal hypoxia, reporting reduced neuronal apoptosis and inflammation, less damage to white and gray matter, lower mortality rates, and long-term improvements in motor and cognitive functions.[80–84] An in vitro suppression of ROS production in microglia and reduced lipid peroxidation products have also been reported in fetal murine brain following Epo administration,[85,86] suggesting possible antioxidant properties.

Consistent evidence has been obtained from controlled clinical trials on asphyxiated infants treated with Epo, either alongside TH[87] or alone.[88–92] Epo administration has been associated with decreased serum SOD and GP levels,[91] fewer seizures,[88,90] less abnormalities at neuroimaging,[87,90] better psychomotor outcomes up to 24 months of age,[89–91] and, when compared to supportive care, reduced mortality.[91,92] Current clinical evidence, however, is derived from small studies and, as such, it is burdened by pronounced differences in terms of settings (e.g., high or medium–low income), Epo dose ranges or timing of administration, outcome evaluation, etc. Hence, further data on a larger scale are needed to confirm these positive preliminary results. To this regard, two multicenter randomized double-blind controlled trials are currently recruiting (NCT02811263 and NCT03079167).

Melatonin

Melatonin has broad antioxidant, anti-inflammatory, and antiapoptotic properties. As an antioxidant, it acts as a ROS scavenger and also enhances ETC efficiency and the enzymatic activity of SOD, GP, and glutathione reductase[93]; moreover, by reducing NOS expression, it contributes to decrease peroxynitrite formation.[94] Owing to its lipophilic nature, this molecule can easily cross the placental interface and the BBB, with useful therapeutic implications.

Antioxidant benefits in NE are largely supported by preclinical evidence reporting lower brain levels of ROS, lipid and protein peroxidation products, free iron and NO, increased GSH in periventricular white matter, reduced neuronal apoptosis, and improved long-term developmental outcomes.[95–99] Alongside TH, melatonin significantly enhanced hypothermic neuroprotection in asphyxiated newborn piglets by reducing the area under the p-MRS (marginal rate of substitution) curve for lactate/NAA and lactate/total creatinine ratios in deep gray matter, improving cerebral energy metabolism and decreasing the apoptotic burden in basal ganglia and internal capsule.[100] In order to enhance its solubility in aqueous vehicles, however, melatonin administered in the above studies was diluted in ethanol, which, at low doses, has been associated with in vitro protective effects against ischemia/

reperfusion-mediated brain injury, and could have thus acted as a possible confounder.[101] This issue has been recently addressed by Robertson et al., who tested the neuroprotective effects of an ethanol-free melatonin formulation, administered at 5 and 15 mg/kg, compared to TH in a piglet model of NE.[102] Reduced cell death in the sensorimotor cortex was observed with 15 mg/kg melatonin but not with lower concentrations; moreover, no between-group difference in lactate/NAA was found at p-MRS evaluation.

In one small clinical study, lower serum levels of MDA, nitrosative biomarkers, and reduced mortality rates have been reported in a small cohort of asphyxiated neonates given melatonin.[103] In another trial, the combination of melatonin and TH resulted in decreased serum NO, fewer seizures, reduced white matter injury at neuroimaging, and improved survival without neurodevelopmental abnormalities at 6 months[104]; however, the different rates of severe NE in the two study groups (TH only vs. TH plus melatonin), together with the limited study sample, may have biased these observed results. A targeted trial aimed at assessing the appropriate melatonin dosage to achieve neuroprotective effects in infants with NE undergoing TH (NCT02621944) is currently recruiting. Based on the current literature, neonatal melatonin administration holds promising potential for translation to standard practice in NE, although further studies are warranted for its validation.

The ability of melatonin to cross the placenta has led to the evaluation of maternal intrapartum administration to minimize the harm of intrauterine fetal asphyxia. A reduction of lipid peroxidation products has been reported in the cerebral tissue of late gestation lambs,[105] but equivalent clinical studies are not available yet. Perinatal asphyxia, however, often results from a sudden and unpredictable event, thus possibly limiting the viability for research validation and subsequent clinical implementation of this approach.

Allopurinol

The neuroprotective potential of allopurinol results from its antioxidant properties, such as XO inhibition and free radical scavenging, combined with its ability to cross the BBB and the placenta.

Significantly reduced acute cerebral edema and extent of brain injury have been reported in the treated arm of a murine model of NE,[106,107] with possible gender-related effects.[108]

When first translated into clinical research, allopurinol administration had been associated with significantly lower serum levels of NPBI and uric acid, no rise in serum MDA, and more stable cerebral blood flow and electrical activity in treated compared to untreated asphyxiated infants.[109] Nonetheless, no effect on mortality and morbidity in severe neonatal asphyxia was documented.[110] Decreased NO levels in serum, but not in CSF, were also reported at 72–96 h of life following allopurinol treatment, commenced within 2 h from the perinatal asphyxiating insult.[38] Importantly, no adverse effects on blood cell count, skin, and liver enzymes have been reported.[38,109,110]

Longer-term effects of post-asphyxia allopurinol treatment on neurodevelopment have also been investigated. While Gunes et al.[38] observed improved outcomes at ≥12 months of age, the 5-year follow-up of Van Bel and Benders' trials demonstrated protective effects only for the moderate NE subgroup[111]; these studies, however, were performed before the routine introduction of TH.

The efficacy of antepartum allopurinol administration in the presence of fetal hypoxia has been examined in animal[111,112] and human[113–115] studies. While reduced cord blood levels of NPBI and neuronal damage biomarkers were preliminary reported in neonates born from treated mothers,[113] results from a multicenter randomized placebo-controlled trial (ALLO-trial) failed to demonstrate any decrease in cord blood biomarkers of neuronal damage and lipid peroxidation[114] or improved developmental and behavioral outcomes at 5 years in infants from the treated arm.[115] However, it should be noted that, as reported by the authors, none of the infants included in the ALLO-trial had developed NE, and no significant differences in Apgar scores, cord pH, and base excess were noted between the treated and control arms.[114] While these results would suggest that further clinical studies are warranted, there remains an issue regarding the feasibility of maternal intrapartum treatment in relation to the unpredictability of perinatal asphyxia, if the aim is to prevent NE.

Available clinical evidence on the neuroprotective effects of antenatal or postnatal allopurinol in NE is still inconclusive.[116] Data from an ongoing study (ALBINO, NCT03162653; http://www.albino-study.eu) evaluating the efficacy and safety of allopurinol administration immediately after birth to near term infants with NE in addition to TH may help to clarify the validity of postnatal therapy in the upcoming years.

NOS inhibitors

NO production is mediated by different NOS isoforms: neuronal (nNOS), endothelial, and inducible (iNOS). These isoforms, expressed in neurons, astrocytes, and endothelial cells, are significantly upregulated under hypoxic conditions; as such, NOS inhibition has been proposed as a possible neuroprotective strategy for NE.[117]

In murine models of NE, pre-hypoxic administration of iNOS, nNOS, and constitutive NOS inhibitors, such as aminoguanidine, 7-nitroindazole, and N^G-nitro-L-arginine, effectively reduced NO levels during hypoxia and reoxygenation.[118–126] Similar results, however, were not obtained after post-insult administration[120–122]; given the unpredictability of perinatal asphyxia, this represents a significant limitation.

The selective nNOS and iNOS inhibitor 2-iminobiotin (2-IB), administered in a repeated dosing regimen before and after the hypoxic insult, has shown neuroprotective effects in asphyxiated female mice.[14,123] Based on this preclinical evidence, 2-IB efficacy in human NE is currently being tested in two clinical trials (EudraCT2015-003063-12, NTR5221), whereas another one (NCT01626924) was prematurely terminated because TH, which was an exclusion criterion for the trial enrolment, had soon become the standard of care. The mechanisms of action of 2-IB, nevertheless, seem to ensue from the blockage of cytochrome C/caspase-3 apoptotic cascade rather than from NOS inhibition.[123–125]

Eventually, a novel class of computer-designed nNOS inhibitors has been tested in animal models of NE, proving better than 7-nitroindazole in nNOS downregulation.[126] These molecules have also shown protective effects against nitrosative stress on striatal neurons and improved motor and neurobehavioral outcomes,[127–129] paving the way for its potential clinical translation.

N-acetylcysteine

NAC is a cysteine precursor endowed with antioxidant effects, such as ROS scavenging and GSH replenishment in deficient cells.[130] These properties, together with its lipophilic nature and low-toxicity profile,[131] have made NAC an interesting candidate for neuroprotection in NE.

Post-resuscitation NAC administration in asphyxiated piglets has been associated with decreased inflammatory markers in the prefrontal cortex and cerebellum,[132,133] significantly attenuated H_2O_2 surge in the cortex, and decreased cortical levels of lipid hydroperoxide and oxidized glutathione.[134,135] Similar findings, together with reduced brain nitrotyrosine, were reported also in a rodent model of lipopolysaccharide-sensitized hypoxia–ischemia.[136] Moreover, when combined with TH, NAC effectively reduced brain volume loss, increased myelin expression, and improved functional outcomes after hypoxic–ischemic brain injury in neonatal rats.[137]

Preliminary clinical evidence from a small cohort of asphyxiated newborns who had previously undergone TH has shown a significant GSH surge in basal ganglia on MR spectroscopy within 30 min from intravenous NAC administration.[138] Nevertheless, further studies are needed to evaluate NAC efficacy and safety in NE.

Encouraging neuroprotective effects after maternal intrapartum NAC administration have been observed in newborns exposed to chorioamnionitis.[139] Trials on asphyxiated neonates, however, are not available yet.

Docosahexaenoic acid

Based on its free radical scavenging ability, and following evidence of decreased glutamate excitotoxicity, reduced NO, and increased antioxidant enzymatic activities in DHA-enriched neuronal cultures,[140] a possible neuroprotective role for DHA has been hypothesized. In a swine model of NE, post-insult DHA significantly reduced cortical and hippocampal lipid peroxidation markers,[141] increased hippocampal GSH levels, and showed an added effect to TH in decreasing urine F4-neuroprostanes[142] and cortical lactate/NAA ratio at MR spectroscopy.[143] In rodent studies, pre-insult DHA reduced brain volume loss and improved functional outcome after hypoxia–ischemia,[144] whereas post-insult administration achieved similar results only in combination with TH.[145] Clinical trials on DHA administration in NE, however, are lacking; hence, targeted studies are warranted to evaluate whether the above preclinical evidence can be translated to clinical settings.

Edaravone

Edaravone (3-methyl-1-phenyl-pyrazolin-5-one) is a free radical scavenger whose multiple antioxidant effects have been tested in different experimental settings, including animal models of NE.[146] In a murine study, pre- and post-insult edaravone administration effectively decreased the burden of apoptosis and necrosis and dampened down mitochondrial injury,[147] whereas its administration before, during, and after hypoxia led to a dose-dependent inhibition of lipid peroxidation in the neonatal rat brain.[148] Eventually, post-insult edaravone reduced both the number of apoptotic neurons and the expression of 8-OHdG, a marker of DNA peroxidation, within 48 h after the hypoxic–ischemic insult.[149] To date, however, clinical evidence on the neuroprotective effects of edaravone is limited to a small cohort of pediatric patients with cerebral infarction, who showed improved neurological outcome without significant adverse effects,[150] while data on its efficacy and safety in the neonatal population are still lacking.

CONCLUSION

Free radicals play a major role in the development of brain injury following hypoxia–ischemia–reperfusion in asphyxiated neonates. Over the past decades, a number of oxidative and nitrosative biomarkers from different biological fluids have been proposed to assess the burden of free radical damage following perinatal asphyxia. Among them, antioxidant enzymes, uric acid excretion rate, NO, MDA, and NPBI have been investigated more extensively in experimental and clinical research, showing also a possible predictive value for NE severity and outcome, whereas other biomarkers (e.g., F2-isoprostanes, AOPP) have come into the spotlight more recently and require larger evaluation. Nevertheless, small and highly heterogeneous study samples together with the lack of commercially available reference standards often hinder the clinical validation of these biomarkers and call for larger, multicenter trials, aimed also at establishing normal values and evaluating the influence of TH. Moreover, it is an open matter of debate whether plasma or urine metabolites truly report brain oxidation extent or rather reflect the multiorgan oxidative damage that often accompanies severe NE. While CSF specimens are more specific, they are harder to collect, particularly in sick or unstable infants, and only few biomarkers have been currently assessed in this biological fluid. Eventually, by enhancing OS, several conditions different from perinatal asphyxia, either antenatal (e.g., maternal pre-eclampsia,[151] exposure to maternal tobacco,[152] etc.) or postnatal (e.g., sepsis,[153] respiratory distress,[154] etc.) may alter the oxidant status of newborn infants and therefore should be taken into account when clinical trials evaluating oxidative or nitrosative biomarkers are designed.

Different antioxidant strategies have been explored as neuroprotective candidates for NE. While room-air resuscitation of asphyxiated term neonates is universally recommended and TH has become the standard of care for NE, a number of molecules endowed with antioxidant properties are currently under investigation. Melatonin and Epo have shown mild beneficial effects in clinical studies but require further large-scale validation before being introduced in routine neonatal care, whereas evidence on allopurinol efficacy is still inconclusive. Encouraging neuroprotective effects of NOS inhibitors, NAC, and DHA have been shown in preclinical trials, thus calling for clinical translation. In addition to the limitations already discussed for free radical biomarkers, such as undersized and heterogeneous samples, often from the pre-cooling era, current literature evaluating antioxidant treatments in clinical settings is further weakened by important differences in the dosages adopted and in the outcomes examined. Large, prospective multicenter randomized controlled clinical trials, assessing both the efficacy and safety of the above antioxidant treatments, are required before they enter routine clinical use.

AUTHOR CONTRIBUTIONS

L.C. and G.F. designed the review. S.M., T.A., and A.A. performed the literature search and revision. S.M. and T.A. wrote the first draft of the manuscript. All the authors critically reviewed the manuscript and approved the final version submitted for publication.

ADDITIONAL INFORMATION

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