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Forum Review Article

Hypoxic-Ischemic Encephalopathy and Mitochondrial Dysfunction: Facts, Unknowns and Challenges

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Abstract

Significance: Hypoxic ischemic events due to intrapartum complications represent the second cause of neonatal mortality and initiates an acute brain disorder known as hypoxic ischemic encephalopathy (HIE). In HIE, the brain undergoes primary and secondary energy failure phases separated by a latent phase in which partial neuronal recovery is observed. A hypoxic ischemic event leads to oxygen restriction causing ATP depletion, neuronal oxidative stress and cell death. Mitochondrial dysfunction and enhanced oxidant formation in brain cells are characteristic phenomena associated with energy failure.

Recent advances: Mitochondrial sources of oxidants in neurons include complex I of the mitochondrial respiratory chain, as a key contributor to O2•- production via succinate by a reverse electron transport mechanism. The reaction of O2•- with nitric oxide (•NO) yields peroxynitrite, a mitochondrial and cellular toxin. Quantitation of the redox state of cytochrome c oxidase, through broadband near infrared spectroscopy, represents a promising monitoring approach to evaluate mitochondrial dysfunction in vivo in humans, in conjunction with the determination of cerebral oxygenation and their correlation with the severity of brain injury.

Critical issues: Being the energetic failure a key phenomenon in HIE connected with the severity of the encephalopathy, measurement of mitochondrial dysfunction in vivo provides an approach to assess evolution, prognosis and adequate therapies. Restoration of mitochondrial redox homeostasis constitutes a key therapeutic goal.

Future directions: While hypothermia is the only current accepted therapy in clinical management to preserve mitochondrial function, other mitochondrial-targeted and/or redox-based treatments are likely to synergize to ensure further efficacy.

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Introduction

Intrapartum events related with hypoxia ischemia, constitute the third cause of mortality in children under 5 years old, and the second cause of neonatal mortality (77, 86). The injury by hypoxia ischemia causes acute disordered brain function known as hypoxic ischemic encephalopathy (HIE). HIE represents a subset of neonatal encephalopathy, occurring in approximately 1.5 per 1000 live births and is the second most common preventable cause of childhood neuro-disability worldwide with deep psychosocial and economic resources consequences for families and the whole society (78).

Neurological dysfunction of neonatal HIE is manifested by clinical symptoms (Fig. 1) that begin immediately to the event of hypoxia ischemia which include difficulties in the initiation and maintenance of spontaneous breathing, lethargy or hyperexcitability, suckling impairment, and seizures (144). A variety of risk factors have been recognized, including maternal pyrexia, maternal hypotension, clotting of placental arteries, placental abruption, uterine rupture and inflammation (9, 87).

After a hypoxic ischemic intrapartum event occurs, a phase of primary energy failure begins in the brain characterized by a series of processes related to cellular energy failure, *i.e*., oxidative stress, neurochemical cascade and inflammation. These processes are responsible for brain injury, even after the hypoxic insult has ceased. Afterwards, the clinical signs progress and a latent period of partial recovery commence called the therapeutic window. Thereafter, a phase of secondary energy failure occurs, principally due to mitochondrial dysfunction which leads to cell death, clinical deterioration and seizures in neonates with moderate to severe injury (64).

It is known that mitochondrial failure caused by hypoxia determines neurological damage and long term neurodisability. In the neonatal intensive care unit (NICU), the patient with HIE is monitored using amplitude-integrated electroencephalogram (aEEG) during its first life hours to reveal neuronal electric activity. The current gold standard assessment of the HIE to unravel energetic failure is nuclear magnetic resonance spectroscopy, but in practical terms this technique is used towards the end of the first week of life when the “therapeutic window” has been dissipated once the secondary energy failure phase is established. Thus, the development and degree of mitochondrial and energetic dysfunction in the course of the first days of HIE remain largely unevidenced in the clinical setting; this opens opportunities for the

integration of direct spectroscopic methods to unravel mitochondrial redox activity as additional approaches to monitor disease progression and improve therapeutics in combination with clinical and neurophysiological data.

This review will focus on the basic biochemical and pathophysiological mechanisms of neonatal brain injury centered on mitochondrial dysfunction and associated alterations of redox homeostasis, their clinical correlation and possible mitochondrial-targeted therapeutic interventions.

Hypoxic ischemic damage of the developing brain

Mitochondria, oxygen and brain.

The brain depends on constant energy supply provided by glucose and oxygen but is incapable to store energy. The main function of the mammalian brain is the generation and transmission of electric impulses being these processes metabolically expensive, including the synthesis and fate of neurotransmitters. Although the adult human brain is 2% of the body weight, it represents about 20% of the body oxygen consumption to aerobically produce ATP (41). In the resting adult brain about the 50% of this ATP production, is spent on ionic movements and increases when neuronal activity is augmented. The key enzyme responsible for generating ionic gradients is the Na^+ and K^+ transporter across the plasma membrane, Na^+-K^+-ATPase. Other ion pumps of Ca^{2+}/Cl^- or Na^+/Ca^{2+} also consume energy to a lesser extent, and maintain the ion gradient across the plasma membrane, facilitating the generation of electric impulses (70). Another significant fraction of brain energy expenditure is directed to the synthesis, release and reuptake of neurotransmitters (42).

The ambient oxygen pressure is 150 mmHg; however, the oxygen concentration at tissues decreases and is close-fitting controlled by microcirculation adjustments to match oxygen supply and demand. Under physiological conditions, the intracellular pO_2 is *ca.* 10 a 40 mmHg (17.6 a 70.4 µM) (40). In air-saturated aqueous solutions at 37°C, O_2 is close to 200 µM; nevertheless, at the mitochondria micro-compartment where the terminal oxidase of the respiratory chain cytochrome *c* oxidase (complex IV) reduces oxygen to water, O_2 levels are in the order of 30 µM (17 mmHg) (55).

For the purposes of this review, we define hypoxia as a state where the O_2 concentration declines to a level below kinetic saturation of cytochrome *c* oxidase (considering Km ~ 1 µM) (30, 55). In the developing brain, the lack of O_2 availability results in an initial depletion of the

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so-called “high energy phosphates”, in particular ATP and phospho-creatine. After the hypoxic episode, ATP and phospho-creatine levels transiently return to the baseline, but a second longer depletion of cellular energy reserves follows; it is during this second phase that most brain damage occurs. These alterations in energy metabolism trigger a sequence of pathophysiological responses, which converge on a common level: mitochondria (61, 62, 66).

Mitochondrial oxidative stress induced by hypoxic ischemic encephalopathy.

Under basal physiological conditions, electrons can leak during electron transport chain at the mitochondrial inner membrane producing low fluxes of superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and peroxynitrite[1] (73, 111).

Neonatal HIE establishes a condition of cellular oxidative stress, as a disturbance between prooxidants and antioxidant in favor of the former, leading to a disruption of redox signaling and control and/or molecular damage (24, 122) initiated by NMDA glutamate receptors that through calcium influx to neurons activate the neuronal isoform of nitric oxide synthase[2] (nNOS) to synthetize the free radical nitric oxide ($^{\bullet}NO$). nNOS is the initial contributor for nitric oxide-dependent brain injury secondary to excess glutamate stimulation and calcium influx. Secondarily, endothelial NOS (eNOS) participates once the inflammatory process is established.

$^{\bullet}NO$ readily permeate membranes and can reach mitochondria to promote its site-specific diffusion-controlled reaction with mitochondrial-derived $O_2^{\bullet-}$ to yield peroxynitrite (Fig. 2) (48). Excess intracellular calcium also increases mitochondrial $O_2^{\bullet-}$ and H_2O_2 formation (90). Formation rates and steady-state concentrations of $O_2^{\bullet-}$, H_2O_2 y $ONOO^-$ under basal and pathological conditions have been reported elsewhere (49, 93, 146). In this scenario, high mitochondrial oxidant levels can overwhelm the mitochondrial antioxidant system detoxification capacity, that include peroxiredoxins 3 and 5 (Prx), manganese superoxide dismutases (MnSOD) and glutathione peroxidase (36, 121, 132, 133), and further promote the release of iron from aconitase and the generation of stronger oxidants such as hydroxyl radical ($^{\bullet}OH$) (92) (Fig. 3). This redox misbalance leads to mitochondrial oxidative damage that could

[1] Peroxynitrite refers to the sum of peroxynitrite anion (ONOO-) and peroxynitrous acid (ONOOH), pKa = 6.8

[2] The current nomenclature in this manuscript of nNOS, iNOS and eNOS corresponds with the most updated nomenclature of NOS1, NOS2 and NOS3, respectively.

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trigger apoptosis for example *via* activation of the mitochondrial transition pore in cardiac or hepatic tissue under chronic metabolic conditions (143) as during HIE (44, 83).

Complex I (NADH dehydrogenase) has been proposed as the main site of $O_2^{\bullet-}$ formation in mitochondria (21, 100). Furthermore, it is reported that in HIE brains, complex I contributes to elevated $O_2^{\bullet-}$ formation using succinate as preferred substrate oxidation supporting a reverse electron transfer mechanism (RET) (Fig. 2) (119). The role of succinate in hypoxia ischemia has been evidenced because is excessively accumulated on brain mitochondria after HIE (63). Moreover, in neonatal mice exposed to HIE, complex I is "deactivated" after ischemia but returns to the activated once the reperfusion ends with the concomitant production of $O_2^{\bullet-}$ (72). Another contributor to the increase of $O_2^{\bullet-}$ is coenzyme Q-cytochrome c reductase (complex III) induced by the low oxygen availability (20, 135).

Additionally, cytochrome *c* oxidase of the electron transport chain is particularly susceptible to $^{\bullet}NO$; indeed, $^{\bullet}NO$ has high affinity for complex IV (33, 130) reversibly competing with molecular oxygen for binding at the a_3 heme site leading to the transient inhibition of respiration, secondary accumulation of reduced electron transport carriers and subsequent formation of $O_2^{\bullet-}$ in the mitochondrial matrix (112) causing oxidative modification on proteins and/or lipids and forming H_2O_2 (spontaneously or enzymatically with MnSOD) (26, 28). Most of the $O_2^{\bullet-}$ formed inside mitochondria, is consumed by reaction with $^{\bullet}NO$ at diffusion-controlled rate to produce peroxynitrite (99, 113). Excess mitochondrial oxidants formation including peroxynitrite, causes oxidative modifications on target biomolecules such as mitochondrial proteins and lipids; notable examples are the nitration of MnSOD (35) the disruption of the iron-sulfur cluster of aconitase (29), thiol oxidation of permeability transition pore components (81), and cardiolipin oxidation (31) all of which contribute to alterations of mitochondrial redox and bioenergy homeostasis, potentially leading to energy collapse and apoptotic or necrotic cell death (26, 28, 108, 114). Figure 3 represents a schematic illustration of the oxidative modifications in intramitochondrial target biomolecules caused by peroxynitrite (136).

Hypoxic ischemic encephalopathy.

During acute injury some cells suffer necrotic cell death, the extent of which will depend on the severity and duration of hypoxia and ischemia, because an inadequate supply of blood glucose and oxygen to any brain region causes metabolic challenge to neurons and glia.

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The absence of oxygen and glucose in neurons originates a decrease in ATP production, ATP-dependent Na^+/K^+ pump failure and an increase in synaptic cleft glutamate that activates the α-amino-3-hydroxy-5-methyl-4-isoxazole receptor (AMPAR) and N-methyl-D-aspartate receptor (NMDAR) (50, 66). Additionally, glutamate induces intracellular calcium accumulation that generates mitochondrial swelling and endoplasmic reticulum damage, edema and cell lysis. Moreover, high levels of intracellular calcium activate nNOS, which in turn generates high levels of $^{\bullet}NO$ that reacts with mitochondrial-derived $O_2^{\bullet-}$ to produce $ONOO^-$, which causes inactivation of respiratory chain complexes, protein oxidation and nitration and peroxidation and nitrosylation of membrane lipids, among others (26, 48) (Fig. 2). Furthermore, Ca^{2+} triggers the activation of cytosolic phospholipases, which increase the release of eicosanoids that lead to inflammation (127).

Although a limited number of neurons may die during ischemia or asphyxia, many of the hypoxic ischemic-challenged neurons initially recover, at least partially, from the insult in the latent phase to develop progressive dysfunction and die many hours, or even days, later (Fig. 4). Magnetic resonance spectroscopy showed that many infants with evidence of moderate to severe asphyxia have initial transient recovery of cerebral oxidative metabolism after birth (latent phase), followed by secondary deterioration with cerebral energy failure from 6 to 15 h after birth (5). The apparent recovery of the cerebral oxidative metabolism involves an increased production of ATP and inhibition of the neurotoxic cascade. This period is known as a “therapeutic window” (1-6 hours, up to 24 hours) in which neuroprotective therapies are aimed to prevent to move forward to the next phase of the secondary energy failure. The duration of the latent phase is inversely proportional to the severity of the neuronal lesion (67).

The secondary energy failure phase is accompanied with a decrease in nucleotide triphosphate (NTP) and phosphocreatine and an increase in inorganic phosphate (140). Additionally, this phase is characterized by secondary cytotoxic edema, cytokine accumulation and mitochondrial dysfunction, being the latter a key step that trigger to programmed cell death, and long-term sequelae (62). The persisting effects years after injury are recognized as tertiary phase and are thought to be manifested with processes like gliosis, persistent inflammatory receptor activation and epigenetic changes (52, 61). The different characteristics

of each of the phases observed after HIE are summarized in Table 1 and the most relevant effects in the mitochondria on Fig. 4.

Inflammation and mitochondria.

Inflammation is a risk factor for injury to the developing brain (97). Indeed, hypoxia-ischemia triggers inflammatory processes that can continue for several weeks after the initial insult. For instance, induces rapid activation of microglia and mast cells in the lesion (95). During early reperfusion, neutrophils accumulate in postcapillary venules and myeloid cells, T cells and natural killer cells, infiltrate in the injured areas of the brain during the inflammation recovery phase (106). According to these findings, it was described that the blood–brain barrier undergo a transient opening at 6-24 h after hypoxia-ischemia, compromising both astrocyte and pericyte function (79).

Inhibition of the electron transport chain in neurons due to inflammation, increases susceptibility to hypoxia lesion in the immature brain. Microglia, the immune cells of the brain, ensemble the inflammatory response to various insults, and these cells can harmful effects in the brain that may be related to mitochondrial dysfunction in vicinal neurons due to the release of mediators such as $^{\bullet}$NO or peroxynitrite (48)(134).

Mitochondrial dynamics and turnover during HIE.

Recent studies suggest that mitochondrial dynamics are also affected in HIE (120). Mitochondrial function and morphology is regulated by a cycle of fission and fusion, coupled with mitophagy (mitochondrial recycling) and biogenesis that maintain mitochondrial integrity and health (149). Considering mitochondrial morphology after an HI event, there is an induction of mitochondrial fragmentation, in addition this effect had sex-specific effects with the degree of fission in men and women (34). The role of mitophagy in the development of brain lesions in newborns after HI is not fully known and it is not clear whether the induction of mitophagy would be beneficial or harmful (129). It is conceivable that in suffering neurons after the ischemic insult, and in particular during the secondary phase, re-establishing mitochondrial dynamics, successfully removing damaged mitochondria through mitophagy and, very importantly, replenishing with new mitochondria as a consequence of their biogenesis process are critical events for a successful functional and clinical recovery (Fig. 4) (148).

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Differential vulnerability of the brain regions induced by hypoxic ischemic encephalopathy

The magnitude of injury depends on the developmental level of the brain (mature and immature) and also on the severity of the insult. These two conditions establish the regional vulnerability, as well as are determinants of clinical signs and symptoms.

There are many differences between newborn and adult brain. For instance, in the adult brain, the vast majority of glucose-6-phosphate is metabolized aerobically. On the other hand, the developing brain of the newborn, has a predominantly anaerobic glucose metabolism, which is one of the reasons for the higher resistance to anoxia comparing with adult brain (15).

The relationship between maturity brain levels and regional vulnerability has been reported (138). It has been found that the most frequent lesions in the term newborn are on the basal ganglia (39%), focal ischemia (18%), subcortical hemorrhage (13%) and parasagittal cerebral injury (10%). On the other hand, in the preterm newborn 39% of the children had periventricular leukomalacia, 24% intraventricular hemorrhage and 18% persistent flares. A possible explanation of these regional differences may be the shifting location of intravascular "watershared" boundary zones as brain maturation occurs (138).

There is also greater tolerance to hypoxia at a lower gestational age. The immature brain has less oxygen consumption per 100 mg of tissue compared to the mature brain, greater dependence on non-oxidative metabolism for ATP formation, which makes it more tolerant to hypoxia (14, 38).

In a fetal sheep animal model, after carotid occlusion, it was observed that the more mature brain was associated with a greater increase in oxidized cytochrome *c* oxidase and greater suppression of cortical EEG activity, followed by markedly more rapid rise in cerebral impedance, indicating more rapid onset of neural depolarization and cell swelling. These results could indicate that more mature fetuses are more dependent on aerobic metabolism compared to more immature fetuses (37).

In summary, the extent of damage will depend on the maturity of the brain, the distribution of cerebral blood circulation and the differences in the metabolic requirements established between the white and gray matters.

Coupling neuronal activity with cerebral blood flow: neurovascular coupling

Neuronal function has great energy expenditure and is highly dependent on aerobic oxidative metabolism. To maintain the neuronal function, the brain has evolved a neurovascular

coupling mechanism to increase cerebral blood flow (CBF) to brain regions that are more active, this response is called functional hyperemia (107). Neuronal activation leads to the release glutamate that regulates the CBF by specific signaling in neurons and astrocytes.

In nNOS-containing neurons, the glutamate activated NMDAR causes a raise on the intracellular calcium levels, promoting the biosynthesis of $^{\bullet}$NO, which in turn diffuses to the adjacent microcirculation inducing a coupled increase in the CBF via the $^{\bullet}$NO-cGMP-dependent arteriolar vasodilation (23, 84, 88) Furthermore, elevated amounts of calcium, induce arachidonic acid (AA) production by phospholipase A2 (PLA2), which in turn is converted to prostaglandins (PG) by COX2 producing more vasodilation (23, 98, 104). In astrocytes, glutamate activate metabotropic glutamate receptors (mGluR) and intracellular calcium increases. Calcium activate PLA2 generating AA and therefore two types of metabolites: prostaglandin E2 (PEG2) and epoxyeicosatrienoic acids (EET) which dilate the vessels. A third metabolite, 20-hydroxyeicosatetraenoic acid (20-HETE) (by ω-hydroxylase) in smooth muscle, which contracts the vessels (56, 126).

Neurovascular coupling can be studied from different points of view, including spatiotemporal studies, which link changes in local blood flow to an artificially applied stimulus, and studies that investigate resting-state neurovascular coupling using spontaneous electrical activity of the brain. In spatiotemporal studies, functional magnetic resonance imaging (fMRI) and functional near-infrared spectroscopy (fNIRS) are the most frequently used methods. In the adult brain, increases in local neuronal activity result in increases in local blood flow to the activated region, neurovascular coupling (4, 65). This increase in the flow of oxygenated arterial blood exceeds the oxygen consumption by the tissue, which leads to localized increases in the oxygenation of hemoglobin. The functional magnetic resonance signal (fMRI) blood-oxygen-level-dependent (BOLD) reports these oxygenation changes, which represent decreases in the concentration of local deoxygenated hemoglobin (HHb) as "positive BOLD", which allows the image non-invasive neural activity (75). However, the results on functional images using fMRI in newborns differ from the results found in adults. These differences correspond to the immaturity of the neurovascular coupling, the differences in cerebral circulation and the differences in the metabolic characteristics of the immature brain. The newborn brain may have less oxygen demand than the adult brain, a concept backed by the high tolerance of the neonatal brain to hypoxia, as well as previous enzymatic expression

studies that have suggested that the postnatal early the brain mainly performs non-oxidative glucose metabolism (15, 19).

Neurological brain lesions due to hypoxia, typical of hypoxic ischemic encephalopathy (HIE) have been poorly evaluated with techniques that study neurovascular coupling. In a study conducted in infants with HIE and seizures in hypothermia, hemodynamic changes were correlated temporarily with seizures, using EEG and diffuse optical tomography (DOT) (124). DOT evaluates hemodynamic evidence in the form of changes in concentration of deoxygenated hemoglobin, and the simultaneous both techniques give information about neurovascular coupling. They observed distinct hemodynamic changes according to the type of seizure (124). The use of techniques that study neurovascular coupling in neonatal HIE, opens opportunities to measure the severity of neuronal damage and outline novel therapies.

Assessing brain mitochondrial function *in vivo*: broadband near infrared spectroscopy (NIRS)

Broadband near infrared spectroscopy (NIRS) is a technique that is increasingly used in the neonate, to monitor changes in cerebral oxygenation and in particular changes in oxygenated and deoxygenated hemoglobin (HbO_2 and HHb) concentration and recently changes in redox state in cytochrome *c* oxidase (CCO), which is directly linked to oxygen metabolism (Fig. 5). NIRS can cross the skull blade and penetrates deep into living tissue. This methodology can be used to monitor changes in the concentrations of absorbing compounds in mitochondria, such as the redox state of cytochrome c oxidase (*i.e*. at the CuA center) (10, 53).

As stated before, the first damaging event after a hypoxic ischemic injury is a burst of ROS production from mitochondria, specifically initiated by complex I (Fig. 2 and Table 1) (113, 125). In addition, •NO generation is augmented due to the activation of nNOS by a NMDAR-dependent mechanism. In this scenario, a state of mitochondrial oxidative stress is established because of the formation of RNS and ROS that are oxidative and nitrating agents leading to mitochondrial dysfunction (Fig. 3). Since CCO, the terminal oxidase of the electron transport chain, is inhibitable by •NO (26, 113) in its reduced state of Fe^{+2} through the formation of nitrosyl-heme CCO, its turnover becomes compromised and therefore the amount of oxidized CCO (Fe^{3+}) state and the capacity for oxygen consumption are decreased. Indeed, in patients with brain injury under hypoxic condition, the NIRS evaluation of the CCO redox state, reveals a marked decrease in the concentration of the oxidized form of CCO (oxCCO). Moreover, the timing and magnitude of the decrease of the oxCCO level depend on the preceding

oxygenation state, the depth and length of the desaturation event and the presence of a circulatory disorder (22).

In a recent study of neonatal HIE, during the hypoxic episode the response in terms of oxCCO levels was different according to the extent of brain injury (11). Specifically, the decrease in the oxidation state of the CCO in human brain tissue as a function of the drop of cerebral hemoglobin oxygenation, allows predicting the severity of the injury and the clinical outcome. The results show that oxidative metabolism during the brain injury of the newborn behaves differently depending on the severity of the injury; in unfavorable outcome cases of HIE, a greater oxygen dependence of mitochondrial metabolism is observed compared to favorable outcome cases.

Animal models for hypoxic ischemic encephalopathy studies

Translational medicine has the role to design unique models depending on the human disease to be studied, mimicking as closely as possible its evolution, clinical presentation and physiological behavior so as molecular, cellular or tissue dysfunction. Animal models must be as similar as possible to the human condition to extrapolate the outcomes in function and/or disease; other important characteristics are the availability to different research groups and the capacity to provide successive, reproducible and adequate biological samples. The animal models have largely help for understanding disease progression and to explore existing and new therapeutic strategies.

While the rat model is the most extensively used in HIE for practical reasons (115), it has become evident that data obtained in other larger animal models (*e.g*. pig and sheep) may better recapitulate what it is observed in the neonatal human brain (60). For example, we and others have used the pig model (17, 116) as a suitable alternative because of its size and anatomical and physiological similarities to humans allowing to monitor medically-relevant clinical and paraclinical parameters (147). The domestic pig (*Sus scrofa domesticus*) is an ungulate artiodactyla mammalian animal phylogenetically similar to primates and present in all types of environments (12). Neurologically, the pig brain is more developed at birth than that of the human, since neonate pigs can walk, vocalize, and establish social relationships. The piglet brain has also particularities, with higher rates of cerebral blood flow (CBF) and metabolism than the term human newborn. Instead, histologically, has a brain that closely resembles that of the term human infant but physiologically it is more mature. It is also more

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similar to humans than rodent brain in weight, volume, cortical surface area, myelination, composition and electrical activity (141). Brain development and maturation are also similar, meaning that neurogenesis in piglets and humans extend from late prenatal to early postnatal life, whereas in rodents this occurs almost entirely in the post-natal period. The cortical surface resembles the human gyrencephalic neocortex more closely than the lissencephalic rat cerebral cortex, thus, presenting some advantages for the understanding of neural and behavioral processes (12). Nowadays, we are actively developing methods for studying mitochondrial function in piglet brain biopsies, as previously characterized in bovine liver tissue (54), to assess the impact of HIE secondary to asphyxia.

Neuroprotection and neurorecovery: mitochondrial based therapies

Several therapeutic strategies to protect or restore mitochondrial function after a brain ischemic-reoxygenation insult are being developed and tested (Table 2). One currently approved therapy involves therapeutic hypothermia (8), a strategy that contributes to neutralize mitochondrial dysfunction (43). Hypothermia protocols have consistently involved starting treatment within the first 6 hours after the hypoxic event, with body cooling of 33.5 ± 0.5 ° C for the entire body, continuously for 48–72 hours (6–8, 51, 69). Regarding metabolism, hypothermia reduces brain metabolism by approximately 5% for each degree of temperature reduction (43, 76) and this reduction in oxygen metabolism in hypothermia suppresses the explosion of oxygen free radicals and lipoperoxidation in animal studies of HIE (Fig. 2) (74, 80).

Existing evidence shows that hypothermia significantly improves survival and decrease disability, including cerebral palsy and neurocognitive outcomes in childhood, in term infants with moderate to severe HIE (6–8). The development of therapeutic hypothermia is an example of how physiological understanding combined with robust models of large animals can support the development of effective clinical treatments. The use of highly translationally animal research should be at the forefront of our efforts to optimize hypothermia protocols, test possible combination therapies, and ensure the safety and efficacy of possible treatments before clinical trials in humans.

In an animal model of fetal sheep when therapeutic hypothermia started after a severe episode of hypoxic ischemia, cytotoxic edema was prevented, and the electroencephalogram (EEG) recovery improved. It was also observed a concomitant reduction in cortical infarction and a decrease in neuronal loss in all regions (58, 59). Comparably, in piglets exposed to

hypoxia and subjected to hypothermia immediately after the acute episode, it prevents secondary energy failure (128), reduces neuronal loss, and seizures (39). However, hypothermia treatment in fetal sheep is less effective if is initiated far away from the hypoxic ischemia episode or when the secondary failure begins (57).

Mitochondrial failure is a hallmark of the secondary phase (13). Clearly, maintaining mitochondrial function is crucial to avoid further neuronal death. Hypothermia suppresses mitochondrial oxidant-mediated damage in the hippocampus during reperfusion from hypoxic ischemia in rats (71). Moreover, prolonged cooling in the piglet and rodents preserves brain production of high-energy metabolites, and therefore strongly suggests that mitochondrial function was preserved by hypothermia (18, 128).

Taking in account that the $^{\bullet}NO$ formation by NOS is responsible for the generation of peroxynitrite-derived oxidants and neuronal damage, strategies using NOS inhibitors have been evaluated in animal model (rabbit, piglet and sheep) as potential neuroprotective strategy for neonatal HIE (2, 45). Among all the inhibitors evaluated the compound 2-iminobiotin (2-IB) showed to be successful in piglet and rat in a dose- dependent manner with or without therapeutic hypothermia, in fact, several phase II clinical studies are currently underway (Fig. 2 and 6) (2, 16, 46).

Another promising intervention is nicotinamide (vitamin B3) a precursor of nicotinamide adenine dinucleotide (NAD^+), a key coenzyme in complex I and Krebs cycle enzymes (85, 91). Nicotinamide at 250-500 mg/kg i.p. divided in two doses in newborn rat model of ischemic brain injury, reduced brain weight loss and improve behavior. On the other hand, this intervention reduce oxidative stress and caspase-3 activity (47).

On the other hand, being the mitochondria a principal site of oxidants formation after ischemia hypoxia insult, therapies that attenuate or prevent oxidant formation during the reoxygenation period can act in synergy with therapeutic hypothermia has been widely explored (Table 2) (101). For instance, the administration of a S-nitrosating compound, MitoSNO, a S-nitrosothiol linked to the lipophilic triphenylphosphonium cation (TPP^+), has been reported to attenuate the excessive $O_2^{\bullet-}$ and H_2O_2 production observed at the reperfusion immediately after an ischemic event. MitoSNO exert its protection by selective S-nitrosation of mitochondrial complex I at Cys 39 on the ND3 subunit, slowing down their reactivation at the beginning of reperfusion thus decreasing oxidative damage in mice after

ischemia-reperfusion injury in myocardial infarction (32). In neonatal mice subjected to cerebral hypoxia ischemia, MitoSNO was administrated at the onset of reperfusion, attenuating oxidative stress and improving neurological recovery (72).

To counteract the toxic effects of reactive oxidizing species a very powerful approach was implemented, namely the selective targeted antioxidants with a lipophilic cation conjugation such as tetra- and triphenylphosphonium by Murphy and collaborators as recently reviewed (101). The most utilized mitochondrial targeted redox active compound is mito-ubiquinone (MitoQ), although it has not been tested yet in HIE. Additionally, the mitochondrial-penetrating peptide, SkQR1 a conjugate of a plant plastoquinone and penetrating cation, also shows promising evidence of neuroprotection after neonatal hypoxic ischemic of a rat model (123). Other mitochondria-targeted neuroprotective compounds are peptides known as cationic arginine-rich peptides (CARPs) formed by 4 to 30 amino acids, positively charged due to the presence of cationic arginine residues, as well as cationic lysine and histidine residues (89).

Another group of mitochondrially-activated compounds are the metalloporphyrins, synthetic catalytic antioxidants that accumulate in mitochondria and use flavoenzymes like complex I and II (NADH dehydrogenase and succinate dehydrogenase, respectively) as electron sources to complete a catalytic cycle in the detoxification of superoxide anion and more importantly, $ONOO^-$ (131). Manganese-porphyrins are well known to protect mitochondria from peroxynitrite-associated damage in a variety of models of diseases (25, 27, 103, 137). Furthermore, there is a strong evidence that manganese-porphyrin can eliminate oxidants in animal models of Parkinson disease, permeabilizing the blood brain barrier (1); they also serve as radioprotectors in several cellular and animal models and more recently in normal brain, salivary glands (145) and during hippocampal neurogenesis (82). Although not tested yet, it would be interesting to evaluate whether manganese-porphyrin can afford mitochondrial and brain protection during HIE.

Last but not least, other therapeutic strategies directed to restore mitochondrial dynamics (*e.g*. melatonin, inhibits mitochondrial fission) or stimulate mitochondrial biogenesis (*e.g*. erythropoietin (EPO), PGC-1α stimulation) in HIE have been recently reviewed elsewhere (64).

In summary, while hypothermia is the only therapy accepted in clinical management of HIE and preserves mitochondrial function, it is conceivable that future combinations of it with

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other mitochondrial-targeted and/or redox-based treatments may ensure further efficacy and safety (see for example (2).

Conclusions and clinical implications in hypoxic ischemic encephalopathy

The deeper understanding on the energetic failure phases during HIE opens opportunities for more effective treatments. Mitochondria have a leading role in this process due to the large number of reports and evidences that associate mitochondrial dysfunction and disruption of the redox-bioenergy axis in both neonatal animal models and patients with HIE. In each of the phases of energetic failure in HIE, there is always some extent of mitochondrial injury which is implicated in neurological damage, so many efforts are focused to treat and revert mitochondrial dysfunction to attenuate brain lesions. Thus, revealing mitochondrial dysfunction *in vivo* and in real time in humans appears as a fundamental goal to optimize treatment and reducing sequelae. In this sense, promising technologies are emerging, as the case of the broadband near infrared spectroscopy (NIRS) that evaluates the redox state of cytochrome *c* oxidase as a parameter to assess mitochondrial respiratory function *in vivo*. Advances in NIRS are being used in infant and adult humans, to evaluate changes in cerebral oxygenation as the ratio HbO_2/HHb and their correlation with the severity of brain injury. This approach will likely help us to modulate the treatment and adjust it according to the phase and deepness of energetic failure of a specific patient.

Therapeutic hypothermia currently represents the only therapy accepted in clinical management that is known to preserve mitochondrial function; thus, it is likely that this therapy could be combined with other mitochondrial-targeted and/or redox-based interventions under development and testing to ensure synergism and, overall, further efficacy and safety for the treatment of neonatal HIE.

The fact that the progression of cerebral injury can be followed in real time by the measurement of cerebral metabolism would be highly helpful to optimize the treatment and monitor recovery during the crucial first days subsequent HIE. Following mitochondrial redox metabolism in vivo enables to obtain a clearer view of the pathophysiological condition of the patient and together with the clinical and aEEG data, optimize the treatment and prevent and/or eventually reverse the damage. In addition, a global clinical, neurophysiological and biochemical approach facilitates the research about neuroprotective therapies according to their mechanism of action.

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The authors declare that there are no competing financial or personal interests.

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Abbreviations used

20-HETE: 20-hydroeicositetraenoic

2-IB: 2-iminobiotin

AA: arachidonic acid

aEEG: amplitude integrated electroencephalogram

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazole receptor

CARPs: cationic arginine-rich peptides

CBF: cerebral blood flow

DOT: diffuse optical tomography

EET: epoxyeicosatrienoic acids

EPO: erythropoietin

fMRI: functional magnetic resonance imaging

HbO_2: oxygenated hemoglobin

HHb: deoxygenated hemoglobin

HIE: hypoxic ischemic encephalopathy

L-Arg: L-Arginine

mGluR: metabotropic glutamate receptor

mitoQ: mito-ubiquinone

MnSOD: manganese superoxide dismutase

MRS: magnetic resonance spectroscopy

NAA: N-acetylaspartate

NIRS: near infrared spectroscopy

NMDA: n-methyl-D-aspartate

NMDAR: n-methyl-D-aspartate receptor

nNOS: neuronal nitric oxide synthase

NTP: nucleotide triphosphate

oxCCO: oxidized cytochrome c oxidase

PG: prostaglandin

PLA2: phospholipase A2

Prx: peroxiredoxin

RET: reverse electron transfer

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Table 1. Key biochemical, pathophysiological and clinical features of HIE

	Primary Phase Acute HI	Latent phase 1 to 6-24 h	Secondary phase 6-24 to days	Tertiary phase Weeks to years	Ref.
Mitochondrial morphology	Mitochondrial swelling Loss of structurally intact mitochondria	Mitochondrial swelling	Loss of normal morphology		(96, 105)
Mitochondrial function	Energetic failure Inhibition of ETC by •NO Increase of ROS by RET Decrease of cytochrome c oxidase activity Succinate accumulation	Recovery of brain oxidative metabolism	Mitochondrial failure Deterioration oxidative metabolism. Loss of mobility linked to the loss of motor protein kinesin Abnormal shape probably due to malfunctioning fission or fusion	Normal oxidative metabolism	(68, 109, 118, 125)
ATP level	Depletion of ATP and	ATP recovery	Decrease of ATP and		(117 ,

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	phospho-creatine		phospho-creatine. Increase of inorganic phosphate		140, 142)
Biochemical and pathophysiological events	Increase of intracellular Ca^{2+} Increase of glutamate Increase of ROS Lipoperoxidation. Protein S-nitrosylation and nitration. DNA damage Prolonged activation of AMPK +Bcl2, acute activation +PGC1α	Cytotoxic edema Accumulation of excitatory amino acids Overshooted phosphocreatine *vs*. raised cerebral lactate and inorganic phosphate	Adequate oxygenation and circulation Increase of lactate Cytotoxic edema Bax-dependent mitochondrial permeabilization leads to apoptosis. Excitotoxicity, inflammation, and persistent uptake of intracellular Ca^{+2} Release of oxygen reactive species	Late cell death, astrogliosis, remodeling, and repair	(110)
Cell death mechanism	Necrosis	The apoptosis starts during	Apoptosis depending on		(105)

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		the latent phase Necroptosis	severity of insult, animal model, and brain region	
Clinical manifestations	Difficulties in spontaneous breathing Lethargy Apnea Hypotonia	Partially Recovery	Seizures Coma Lethargy or hyperexcitability Suction impairment	(64)

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Table 2. Selected examples of mitochondrial-based therapeutics for neuro-recovery in HIE.

Intervention	Mechanism of action	Experimental models/ trials	Observation	References
Hypothermia	Hypometabolism promoter	Animal model of neonatal HIE and human clinical trials.	Decreases the global cerebral metabolic rate for glucose and oxygen. Decreases utilization glucose, ATP, phosphocreatine Prevents secondary failure	(5–8, 43)
2-Iminobiotin	NOS inhibitor	Animal model of neonatal HIE and in vitro human neuronal cells. Human clinical trial.	Decreases oxidants	(2, 16, 46)
Nicotinamide	Precursor of NAD^+	Animal model of neonatal HIE.	Increases NAD^+ pool. Decreases oxidants and caspase-3 activity.	(47, 85)
SkQR1	Mitochondria-targeted antioxidant	Animal model of neonatal HIE.	Decreases oxidative stress.	(123)

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MitoSNO	Mitochondria-targeted nitrosating agent	Neonatal animal model of HIE	Decreases oxidative stress, improves neurological recovery, decreases neuronal mortality.	(72)
Erythropoietin (EPO)	Stimulator of mitochondrial biogenesis.	Human clinical trial of HIE	Increases mitochondrial metabolism	Reviewed in (64, 94)
Melatonin	Inhibitor of mitochondrial fission. Mitophagy regulator	Human clinical trial of HIE	Increases mitochondrial function	Reviewed in (64)

Figure legends

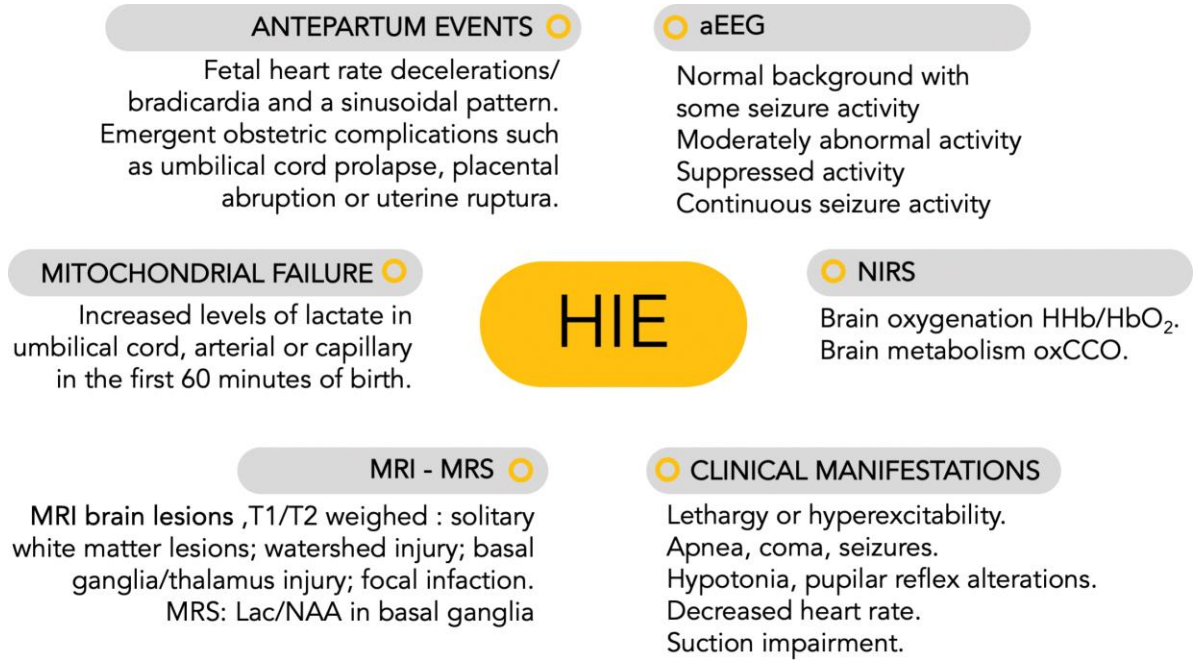

Figure 1: Main features of hypoxic-ischemic encephalopathy (HIE). This is basal criteria to define HIE in neonates that involves clinical parameters associated with an abnormal aEEG, increased metabolic acidosis in the umbilical cord, intrapartum risk factors and eventually, abnormalities in brain metabolism, oxygenation and imaging studies (3, 102, 139).

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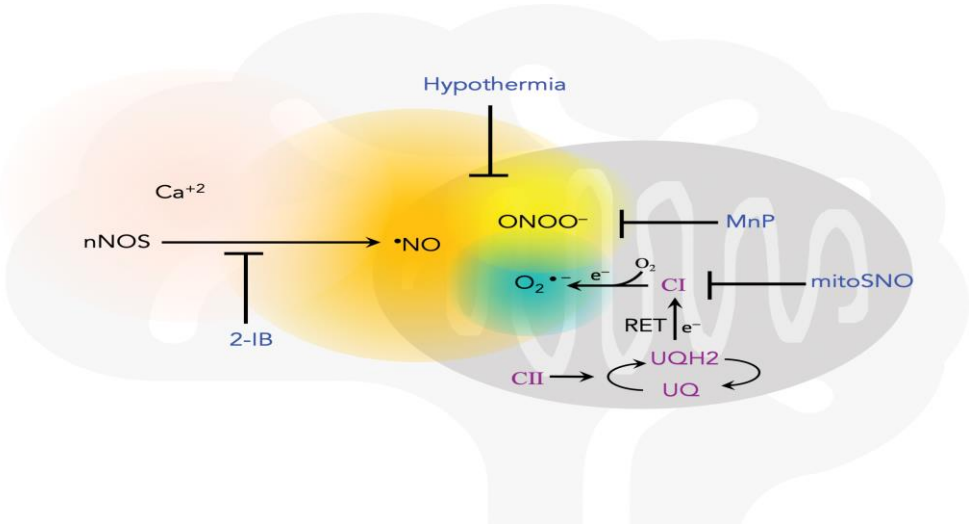

Figure 2: Mitochondrial oxidants in HIE. Elevation of intracellular calcium levels in nNOS-containing neurons induces cytosolic $^{\bullet}NO$ production that diffuses into mitochondria and may inhibit the mitochondrial electron chain by interactions with cytochrome c oxidase under low oxygen tensions. Succinate is accumulated in mitochondria because of the reverse activity of Complex II (32, 101), reducing UQ to UQH_2. UQH_2 in turn, transfer electrons to Complex I and forms $O_2^{\bullet-}$ by reverse electron transfer. In turn, peroxynitrite can be formed due to the reaction between $^{\bullet}NO$ and $O_2^{\bullet-}$ exerting toxic effect by reaction with key mitochondrial components and contribute to mitochondrial dysfunction, apoptotic signaling and neurological injury. Indicated mitochondrial and/or redox-based pharmacological interventions could attenuate damage or induce neuro-recovery (summarized in Table II).

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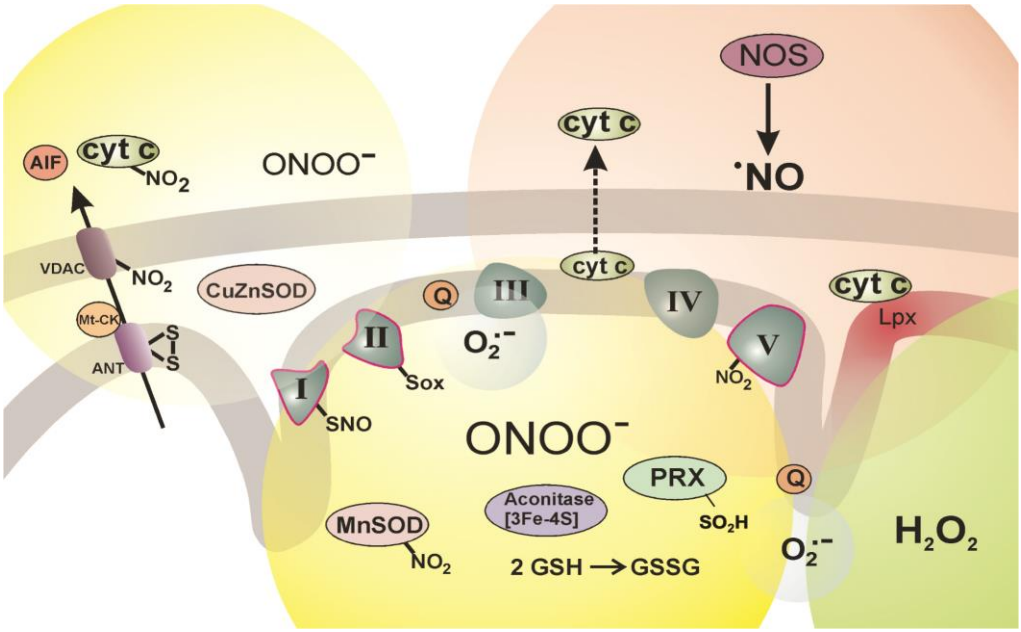

Figure 3: Peroxynitrite formation and targets in mitochondria. Oxidation and nitration reactions promoted by peroxynitrite in key mitochondrial proteins are indicated. Also, the peroxidation of cardiolipin as a relevant part of mitochondrial oxidative stress is indicated in red (as Lpx for lipid peroxidation) on the right side of the inner membrane. These biochemical processes constitute the basis for nitro-oxidative damage in the various mitochondrial compartments and may lead to disturbances in mitochondrial respiration and bioenergetics and/or the release of pro-apoptotic components to the cytosol. Adapted from Oxidative stress in mitochondria from Principles of Free Radical Biomedicine (136).

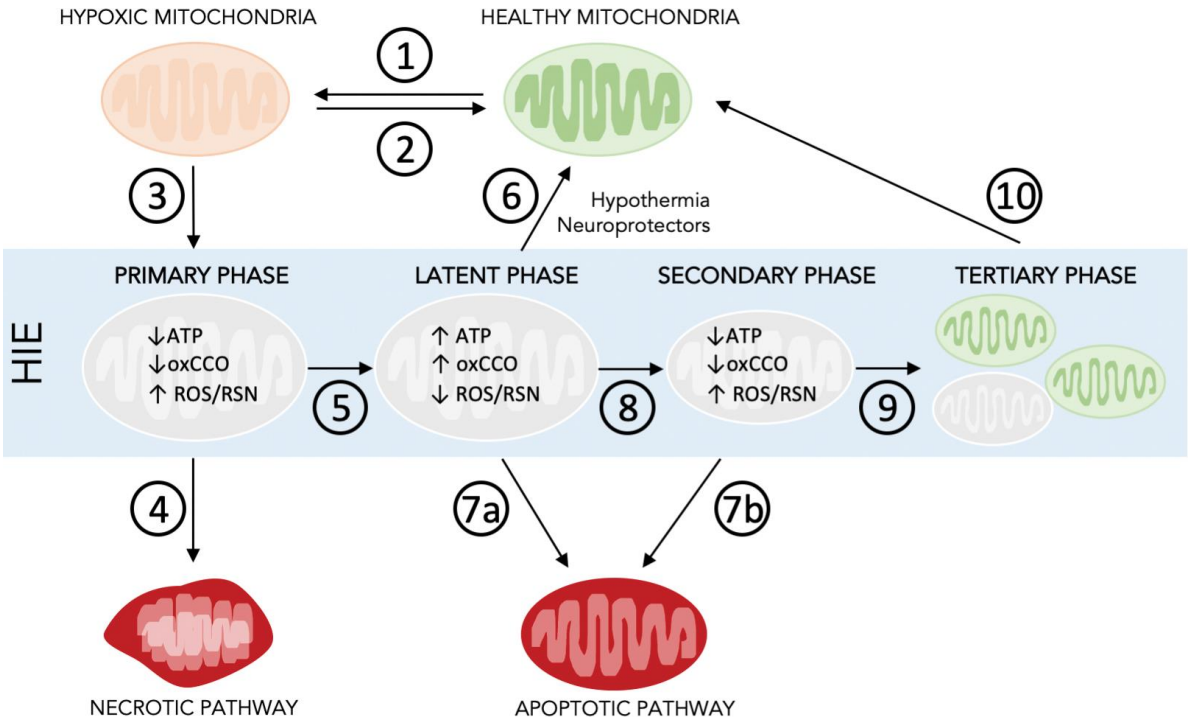

Figure 4: Possible fates of mitochondria during a hypoxic insult. Lowering oxygen levels can turn a healthy mitochondria into a hypoxic organelle (1) that can either fully recover reversibly depending on the severity and duration of the hypoxia (2). If hypoxic event triggers a HIE, neurons enters in the primary phase resulting in energetic failure, mitochondrial swelling and increased reactive oxygen and nitrogen species (3), which in turn can induced the cell to a necrotic pathway (4). The latent phase derives of the primary phase (5), a partial recovery of brain oxidative metabolism and clinical symptoms that can be fully recover mitochondria with neuroprotective pharmacology and hypothermia (6). However, if a secondary energy failure phase evolves mitochondrial function decline because of the deterioration of oxidative metabolism and malfunctioning of fission and/or fusion, inflammation (8). Both latent and secondary phase mitochondria can deviate to an apoptotic pathway (7a and 7b). Nevertheless, neurons can fully recover mitochondrial function and morphology, retrieving a normal oxidative metabolism and biogenesis (9) transforming into a heathy mitochondria (10).

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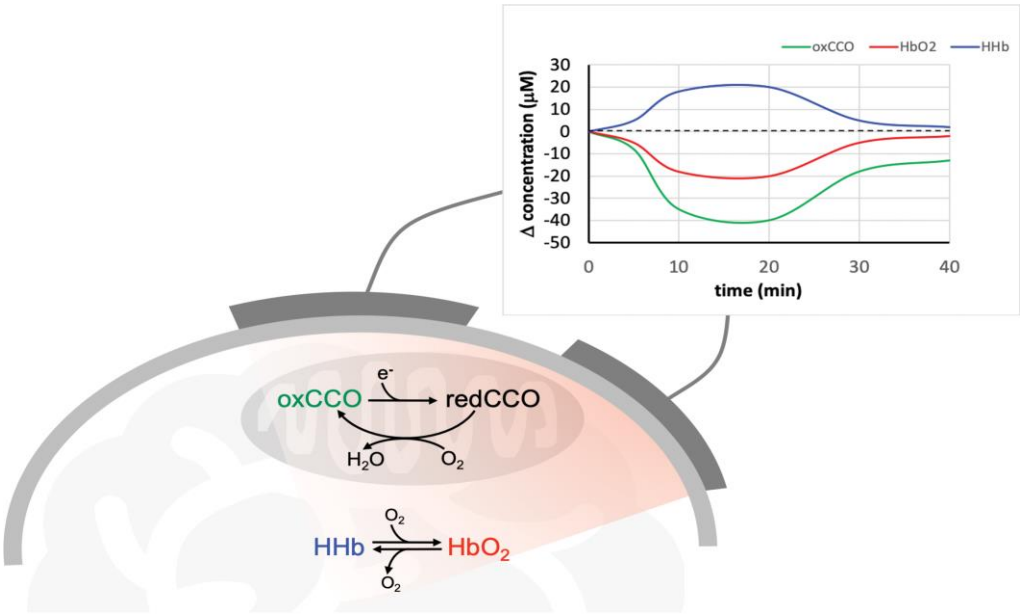

Figure 5: NIRS technique. This noninvasive methodology can monitor cerebral oxygenation levels in the regional tissue of neonatal patients. Two electrodes adhere to the head and measure near-infrared light absorbed in the underlying tissue (represented by the pink area). Oxyhemoglobin (HbO_2, red line) and deoxyhemoglobin (HHb, blue line) concentrations are measured. In the mitochondria, concentrations of the oxidized form of the cytochrome c oxidase (oxCCO, green line) is also measured. An illustration of the data obtained is presented in the graph.

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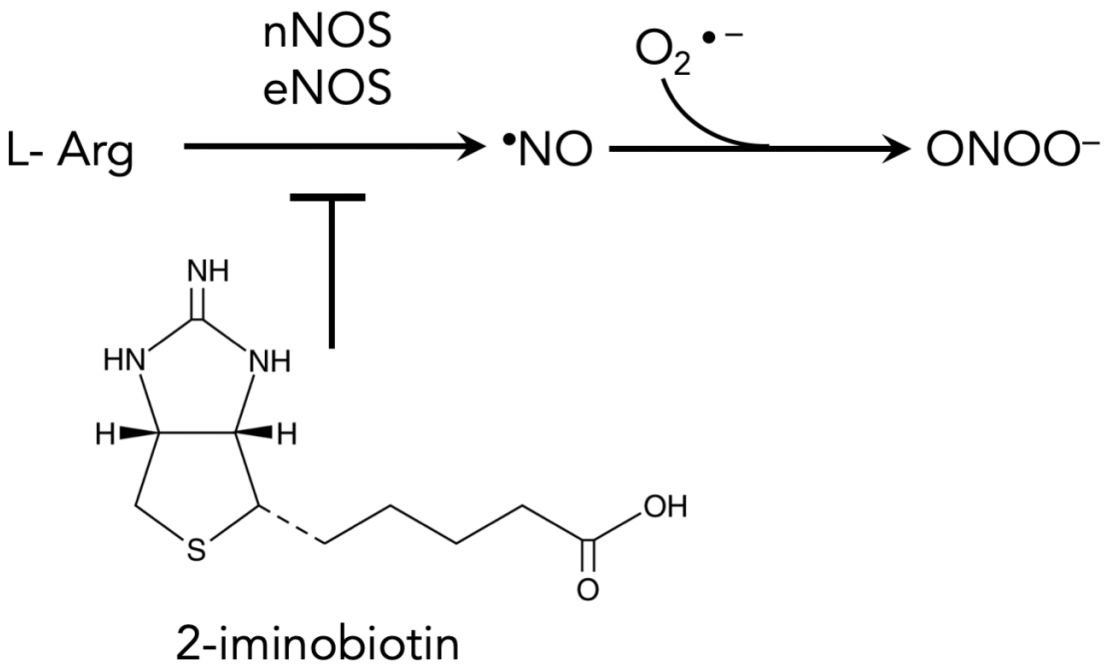

Figure 6: Inhibition of •NO formation with 2-iminobiotin (2-IB). 2-IB act as an inhibitor of nNOS and eNOS preventing •NO formation that can generate other toxic reactive species like $ONOO^-$.