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REVIEW ARTICLE

Pathophysiology of hypoxic–ischemic encephalopathy: a review of the past and a view on the future

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Abstract

Hypoxic–ischemic encephalopathy, also referred as HIE, is a type of brain injury or damage that is caused by a lack of oxygen to the brain during neonatal period. The incidence is approximately 1.5 cases per 1000 live births in developed countries. In low and middle-income countries, the incidence is much higher (10−20 per 1000 live births). The treatment for neonatal HIE is hypothermia that is only partially effective (not more than 50% of the neonates treated achieve an improved outcome). HIE pathophysiology involves oxidative stress, mitochondrial energy production failure, glutaminergic excitotoxicity, and apoptosis. So, in the last years, many studies have focused on peptides that act somewhere in the pathway activated by severe anoxic injury leading to HIE. This review describes the pathophysiology of perinatal HIE and the mechanisms that could be the target of innovative HIE treatments.

Keywords Hypoxic–ischemic encephalopathy · Neonatal brain · Oxidative stress

Abbreviations

AIF	Apoptosis-inducing factor
BBB	Blood–brain barrier
BIP	Bax-inhibiting peptide
ETC	Electron transport chain
HI	Hypoxia–ischemia

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HIE	Hypoxic–ischemic encephalopathy
HNSC	Human neural stem cells
MMPs	Matrix metalloproteinases
MPTP	Mitochondrial permeability transition pore
NAD+	Nicotinamide adenine dinucleotide
NO	Nitric oxide
PARP	Poly(ADP-ribose) polymerase
PDHC	Pyruvate dehydrogenase complex
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha

Introduction

Hypoxic–ischemic encephalopathy (HIE) is a term used to describe the complex physiological, cellular, and molecular changes resulting from a severe anoxic brain injury during neonatal period. This can lead to premature mortality or a variety of life-long morbidities, including acute symptoms such as seizures, alteration of consciousness, weak breathing, poor muscle tone or metabolic derangement as well as chronic conditions such as cerebral palsy, epilepsy, intellectual disability, and behavioral disorders [1, 2].

HIE has an incidence of approximately 1.5 cases per 1000 live births in developed countries and 10−20 per 1000 live births in low and middle-income countries [3–5].

HIE is not a single event, rather an ongoing process causing neuronal cell deaths over hours to day after the initial injury. In fact, it is possible to recognize three distinct phases [6]. The first immediate phase (primary neuronal death) is determined by the primary energy failure during hypoxic–ischemic (HI) event, resulting in oxidative metabolism failure, cytotoxic edema, and accumulation of excitotoxins [7]. After restoration of cerebral circulation, the second phase begins (a latent phase lasting about 6 h). Between 6 and 15 h after the HI insult, occurs the secondary energy failure (delayed neuronal death) that is associated with encephalopathy and increased seizure activity. The mechanisms involved in this phase include excitotoxicity, apoptosis, and microglial activation [8].

Therapeutic hypothermia is the current standard of care for infants with HIE, cooling the whole body to a core temperature of 33.5 °C for 72 h starting within 6 h of birth [9]. According to scientific evidences, therapeutic hypothermia inhibits key steps in the excito-oxidative cascade [1]. However, it does not provide complete neuroprotection and it is only partially effective. Even after treatment, there is a high prevalence of neurologic morbidity and mortality, affecting 40–50% of neonates who underwent therapeutic hypothermia [10].

In the last years, many studies have focused on research of new treatments for HIE that, together with hypothermia, could enhance neuroprotection and lower adverse outcomes [11]. Since HIE pathophysiology involves oxidative stress, mitochondrial energy production failure, glutaminergic excitotoxicity, and apoptosis [12], these researches are focusing on peptides that act somewhere in the pathway activated by severe anoxic injury leading to HIE.

In this review, we conducted a literature search to better clarify the pathophysiology of perinatal HIE and the inflammatory mechanisms involved in it, those mechanisms that could be the target of innovative HIE treatments.

Materials and methods

A review of the literature was conducted to identify the most relevant studies reported in the English since 2017 on PubMed MEDLINE electronic database. Based on the abstracts, we made a selection of these studies, focusing on articles that concerned HIE pathophysiology and inflammatory mechanisms. The exclusion criteria were articles not in English and not relevant to the review and abstracts (Fig. 1).

The keywords used were “hypoxic-ischemic encephalopathy”, “Hypoxic-Ischemic Injury”, “developing brain”, “neonatal brain”, “neonatal”, “perinatal”, “pathophysiology”. Different combinations of the terms were used. Moreover, references in each article were searched to identify potentially missed studies.

Results and discussion

Pathophysiology

Fetal brain requires a constant supply of energy in form of ATP that obtains metabolizing lactate, ketone bodies, and glucose. Comparing to adult, fetal brain has a greater ability to tolerate hypoxia–ischemia (HI), due to its capacity to reserve energy when needed. However, in case of a critical depletion of ATP also, the fetal brain becomes susceptible to injury. This critical ATP depletion can be caused by prolonged or sudden and important HI, originating from various conditions (i.e. chronic maternal hypoxia, pre-eclampsia, umbilical cord knotting, umbilical cord prolapse, shoulder dystocia and placental abruption) leading to an impairment of oxygenated cerebral blood flow to the fetus causing systemic and cellular responses [13]. HI brain injury is an ongoing process composed by several different phases. When the primary critical energy failure occurs, an uncontrolled release of excitatory neurotransmitters begins, starting the ischemic cascade that damages neuronal cells (both at cytoplasmic and mitochondrial level), disrupts the brain–blood

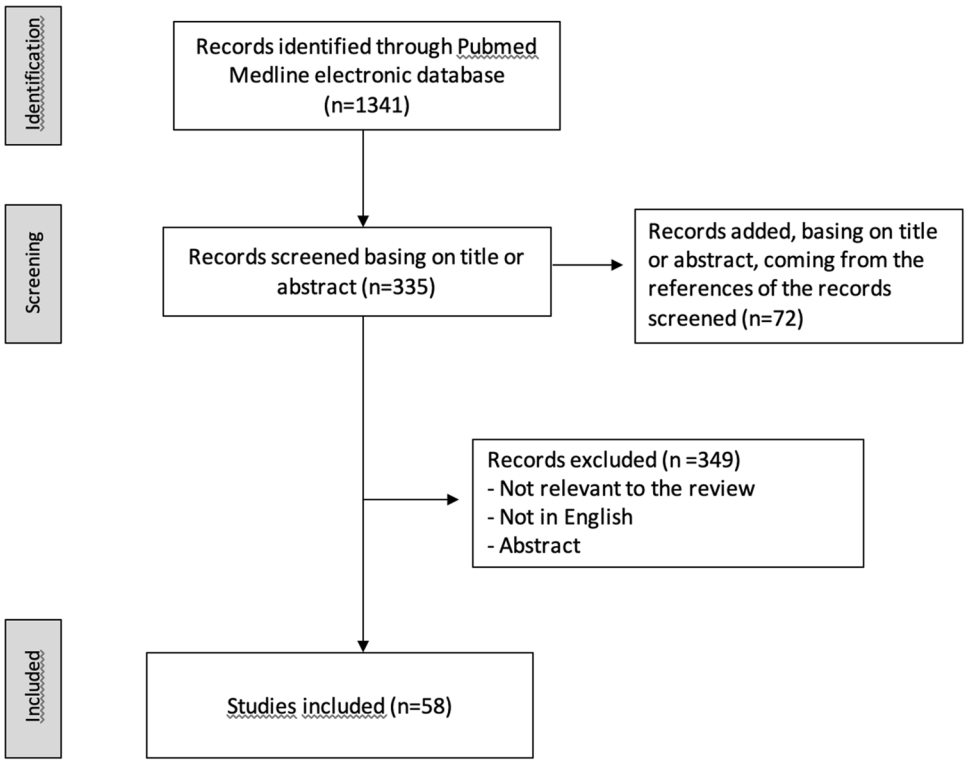

Fig. 1 The screen procedure for article inclusion for review

barrier (the amount of membrane peroxidation is directly related to the severity of ATP depletion) and activates an important inflammatory response. These result in failure of oxidative metabolism, cytotoxic edema, and accumulation of excitotoxins [5]. After the cerebral circulation restoration, a latent phase begins, lasting around 6 h, followed by (6–15 h after HI) a secondary energy failure that can last for days. Typical of this phase is seizures, renewed cytotoxic edema, release of excitotoxins, impaired cerebral oxidative energy metabolism, and finally, neuronal cell death [14].

HIE pathophysiology (Fig. 2) can be outlined in five main events, linked one to the others: oxidative stress, intracellular Ca^{2+} accumulation, mitochondrial dysfunction, excitotoxicity, and inflammation [13].

Oxidative stress

Fetal brain is an environment particularly susceptible to oxidative stress, since unsaturated fatty acids and metals catalyzing free radical reactions are heavily present, while antioxidant levels are low [15]. Oxidative stress and reactive oxygen species (ROS) produced by HI cause an important damage to lipids, proteins, and nucleic acids, leading to lipid and protein oxidation and DNA degeneration.

Normally ROS, mainly generated in mitochondria, are cleared by superoxide dismutase and glutathione peroxidase. During HI, ROS cannot be immediately eliminated by antioxidant enzymes because of interrupted metabolism, thus an excessive ROS accumulation occurs [16]. This, together with oxidative modification of lipids, proteins, and DNA leads to metabolic failure and mitochondrial dysfunction following newborn hypoxic–ischemic insult.

In HIE, ROS are generated from mitochondrial electron transport chain, NADPH oxidases, xanthine oxidase, arachidonic acid (12/15 lipoxygenase), and nitric oxide (NO) synthase.

NO synthase is activated by a mechanism enabled by hypoxia-induced energy depletion. This causes alterations of the NMDA receptor ion channel, inducing influx of Ca^{2+} into the cytoplasm. Increase of intracellular Ca^{2+} leads to the activation of NO synthase via a Ca^{2+}/calmodulin-dependent mechanism, generating NO free radicals.

NO produced during hypoxia has several adverse neuronal effect through different mechanisms. First, NO increases the expression of apoptotic proteins, activating CREB protein, through a NO-mediated pathway involving CaM kinase IV activity and CaM kinase IV-dependent phosphorylation of CREB protein, thus leading to increased expression of proapoptotic genes [15, 17]. Second, NO reaction with superoxide generates peroxynitrite, a toxic free radical known to alter cell membranes, to interfere with protein and receptor activity and to activate pre-apoptotic pathway [18]. In addition, NO causes lipid peroxidation, protein oxidation and nitration of nuclear membranes, DNA damage and increasing in intranuclear Ca^{2+} [15].

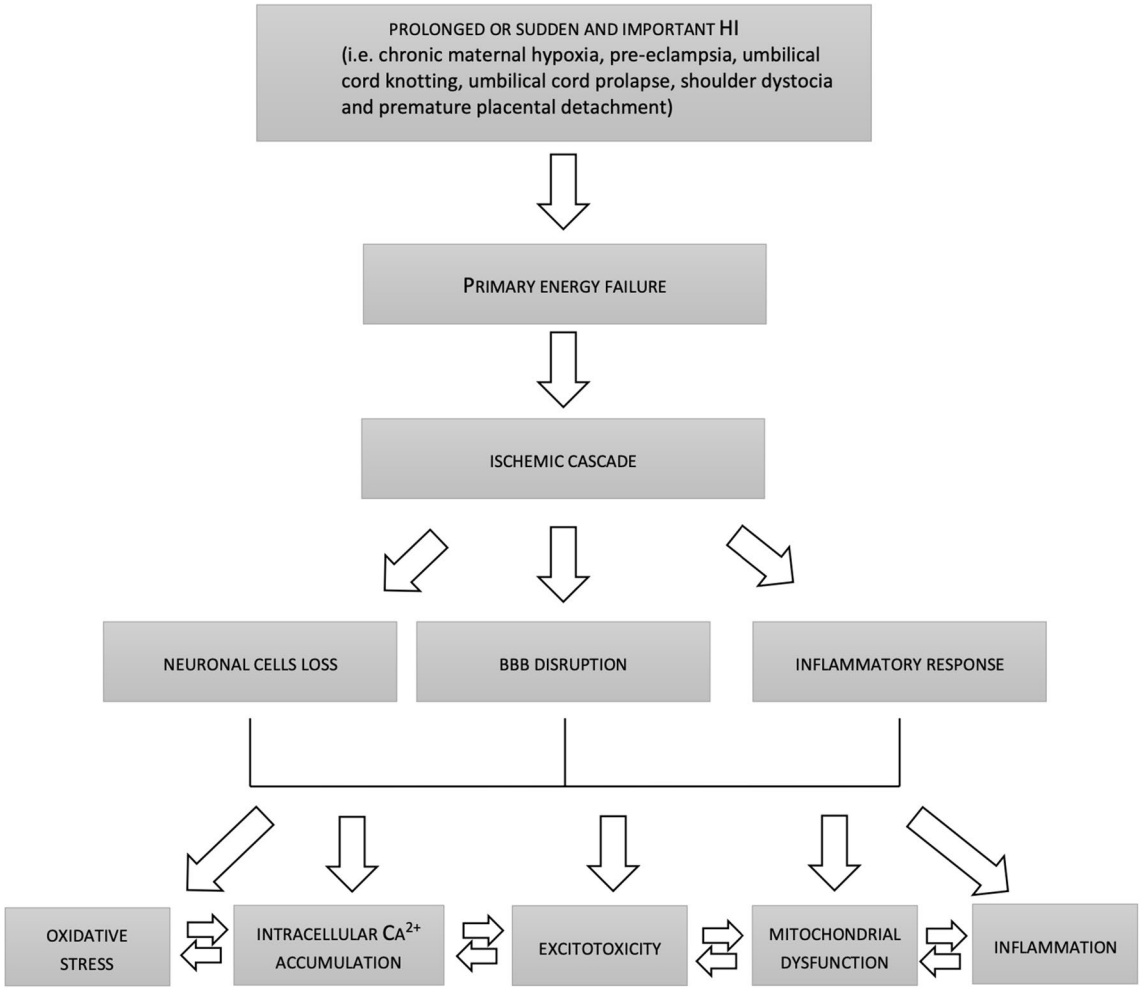

Fig. 2 Pathophysiology of HIE

Because of all these adverse neuronal effects, the inhibition of NO synthase has been a target in HIE studies on neuroprotection [6, 15, 19].

Moreover, a surplus of ROS limits glucose metabolism. In fact, excessive ROS accumulation inhibits pyruvate dehydrogenase complex (PDHC) activity. Because of this alteration in PDHC activity and the impairment of reducing power transportation from glycolysis-generated NADH to mitochondria, lactate dehydrogenase, normally activated by elevated Ca^{2+}, will use NADH to form massive amounts of lactate in cytosol. Acidosis is the obvious consequence of this lactate accumulation in neurons during HI [20].

Intracellular Ca^{2+} accumulation

Ca^{2+} extracellular concentration can reach up to 1–2 Mm, while intracellular Ca^{2+} is normally very low (about 100 nM) [21]. Several mechanisms contributes in maintaining the intracellular Ca^{2+} concentration (i.e. Ca^{2+} release from endoplasmic reticulum; Ca^{2+} release through Ca^{2+}–ATPase or Na^{+}–Ca^{2+} exchanger in cell membrane; Ca^{2+} transportation into mitochondria through an electrophoretic uniporter, entry of extracellular Ca^{2+} into cytosol through NMDA receptors or voltage-gated Ca^{2+} channels). NMDA receptor channel is probably one of the main pathway in intracellular Ca^{2+} accumulations, as shown by studies on NMDA antagonists, found to block Ca^{2+} entry in neurons and thus significantly attenuating neurodegeneration after HI [22].

In HI condition, the deprivation of oxygen and glucose caused by abnormal blood circulation triggers the overstimulation of glutamate release. Glutamate stimulates the opening of NMDA receptor channels, allowing Ca^{2+} to flow inside neurons [20] (Fig. 3).

High intracellular Ca^{2+} concentration has various effects: as seen before, activates NO synthase via a Ca^{2+}/calmodulin-dependent mechanism; moreover, it can induce mitochondrial dysfunction and mediate irreversible immature neuron death by the activation of several Ca^{2+}-dependent proteins.

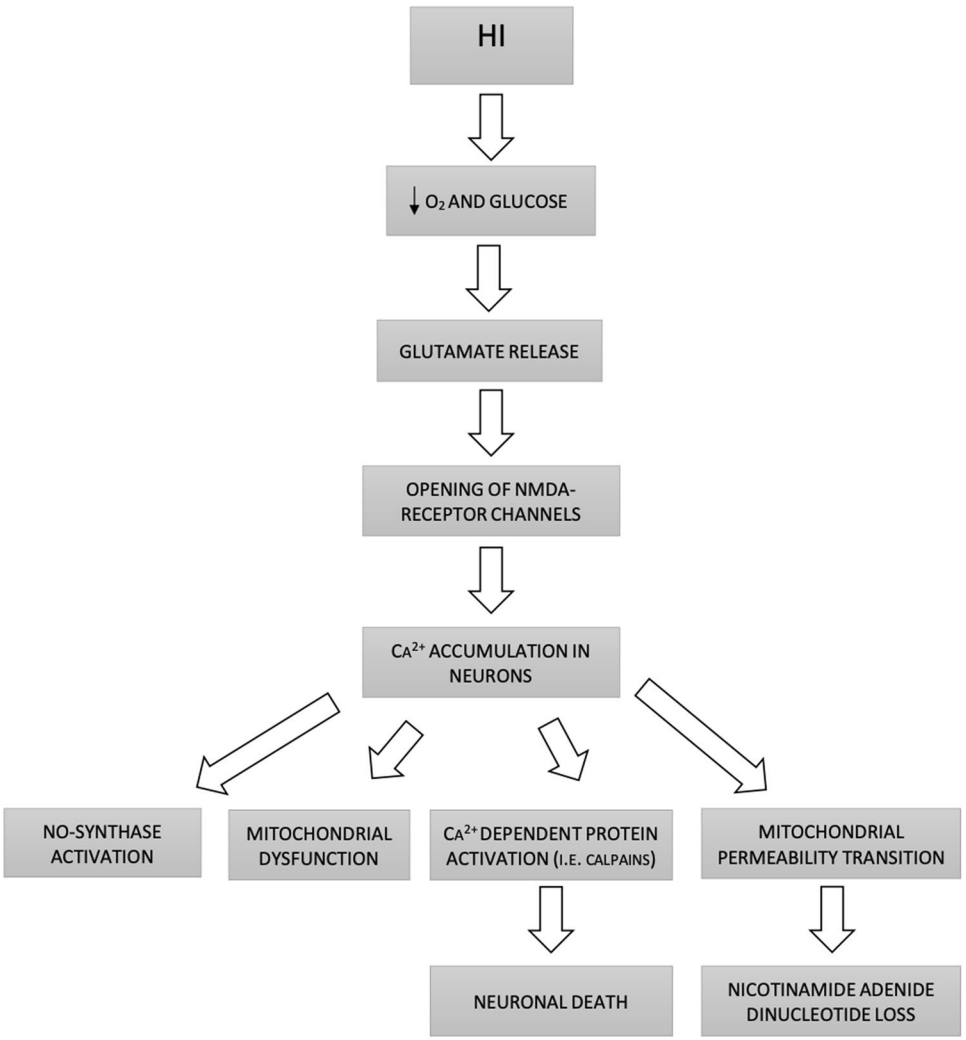

Fig. 3 Effect of $Ca2^{+}$ surge in neurons

Among these, there are proteins, as calpains, involved in cell remodeling, membrane destruction and neuron degeneration [23, 24]. Furthermore, increase in intramitocondrial Ca^{2+} concentration, together with ROS, contributes to mitochondrial permeability transition and, consequently, to nicotinamide adenine dinucleotide loss, important for ROS detoxification and cellular energy metabolic processes [20].

Mitochondrial dysfunction

Mitochondria carry out many important functions and their alteration assumes a central role in HI-induced neurodegenerations, thus determining the destiny of cells subjected to HI. Mitochondria produce energy indispensable for multiple cellular activities, a process that generates ROS, and are also the most important intracellular Ca^{2+} buffers. Their dysfunction, indeed, can lead to mitochondrial energy failure and, consequently, to intracellular Ca^{2+} accumulation. This is caused by the reduction of the energy needed to maintain membrane ion gradient and worsened by the opening, in HI condition, of NMDA receptors and voltage-gated Ca^{2+} channels [25, 26]. Moreover, the normal functioning of electron transport chain (ETC) is interrupted because of ROS excess present in HI, thus amplifying the production of mitochondria-free species [27]. Oxidative modification of proteins and lipids of mitochondrial inner membrane alters its permeability since membrane depolarization occurs. This alteration uncouples oxidative phosphorylation processes resulting in ATP production deficiency. This drastic energetic failure contributes to cell membrane depolarization and, consequently, to Ca^{2+} influx, instituting a sort of vicious cycle [20].

Therefore, mitochondrial dysfunction can cause a series of lethal problems, as oxidative stress, intracellular Ca^{2+} accumulation, and mitochondrial energy failure that lead to neuron apoptosis and, consequently, neurodegeneration.

Studies based on mitochondrial energy failure have focused on AMPK role [28]. AMPK is a sort of cell energy sensor and is activated when there is an imbalance in the AMP:ATP ratio, as happens in HI. Once activated, AMPK

acts to inhibit energy consumption (fatty acid/cholesterol synthesis) and promote energy production (e.g. glycolysis), trying to restore energy balance. AMPK activation is due to two kinases: LKB1 and CaMKKβ [29]. The latter is activated by intracellular Ca^{2+} excess. Moreover, another AMPK mediates apoptosis during excitotoxicity, through the expression of the proapoptotic protein Bim [30]. More specifically, excitotoxicity and ROS overload seen in HI cause calcium surge that not only activates CaMKKβ and, consequently, AMPK, but also, at the same time, challenges the mitochondrial respiratory chain. In this way, the imbalance in AMP:ATP ratio increases and AMPK activation is reinforced. This AMPK activation restores energy balance, explaining the return to basal level of ATP after the primary energy failure [28]. Later, the other mechanisms involved in HI, such as inflammation, damage mitochondrial function once again, causing the second energy failure and the rebound of AMPK over activation. These deleterious events could cause a final mitochondrial challenge, leading to mitochondria membrane permeabilization and cellular apoptosis.

Apoptosis too, as many other HI events, is linked to Ca^{2+} accumulation in cytoplasm together with ROS. As a matter of fact, accumulation of Ca^{2+} and ROS activates the mitochondrial permeability transition pore (MPTP) within the mitochondria inner membrane, inducing mitochondrial permeability transition (MPT). The role of MPTP in HI has been confirmed by studies on MPTP inhibitor cyclosporine A. It helps in protecting against HI neurodegeneration by binding a component of MPTP (cyclophilin D, CypD) thus interrupting its involvement in MPTP construction [31, 32]. Despite these studies, the exact role and entity of MPT involved in neonatal HIE is still unclear and the CypD-based treatment remains controversial.

MPTP releases in cytoplasm nicotinamide adenine dinucleotide (NAD+), a cofactor important for energy metabolic reactions and ROS detoxification. Mitochondrial distress will determine the formation of Bax/Bak megapores inside the mitochondria outer membrane, causing release of mitochondrial contents, including proapoptotic proteins such as cytochrome c (CytC), apoptosis-inducing factor (AIF), endonuclease (endo) G and Smac/Diablo. Each protein has different downstream targets, but they all contribute to cell death. CytC and Smac/Diablo triggers caspase activation, determining an irreversible apoptosis [33], while AIF, after interacting with cyclophilin A, translocates to the nucleus where it causes DNA fragmentation [34]. Caspases, ROS, and AIF induce DNA fragmentation that activates poly (ADP-ribose) polymerase (PARP), a DNA repairing enzyme. The DNA repairing process consumes NAD+. This NAD+ loss plus oxidative failure will worsen mitochondrial dysfunction, by depleting NAD+ needed to maintain mitochondrial energetics, and will extend the impairment of neonatal hypoxic–ischemic brain injury.

During neurodegeneration, two forms of neuron death could happen: necrosis or apoptosis.

Many studies, focusing on high expression of proapoptotic proteins, such as Caspase-3, Bax, and BCl, have elucidated that apoptosis is the more prevalent form in immature brain injury compared with adult models [20, 35].

Subsequently, largely due to advances in cell biology and to experimental animal studies, emphasis has been switched also to autophagy mediated by programmed cell death (PCD) mechanisms as important forms of degeneration in HIE. For instance, preclinical evidence has now shown that hypoxic–ischaemia exacerbates autophagic flux in neonatal rats and leads to learning and memory impairment. Moreover, recent clinical data showed an upregulation of MALAT1 after HIE which controls the HIF-1α axis and autophagic cell death [36–40].

Accordingly, several recent studies have focused on mitochondrial-related therapeutic targets, such as Bax-inhibiting peptide (BIP), PARP inhibition, MPT inhibition, NAD administration, with promising results for future applications [20]. In summary, the mitochondrial dysfunction is the key point of neurodegeneration in HIE.

Excitotoxicity

Excitotoxicity, a term first used in the 1970s, refers to cell death mediated by excessive stimulation of extracellular excitatory amino acid receptors [41]. These receptors mediate the glutamate physiologic excitatory effects. During HI, they are excessively stimulated by elevated glutamate levels and membrane depolarization, leading to their inappropriate opening and, consequently, to a lethal flow of Ca^{2+} inside neurons. In fact, following prolonged HI, cellular homeostasis is disrupted due to ATP depletion and to the incapacity to maintain ionic gradients. This causes neuronal depolarization and glutamate release into the synaptic cleft, with an overload of extracellular glutamate, due also to a reduced activity of the glial pumps that normally keep synaptic glutamate levels low [26]. This leads to excitotoxicity in neurons and other cells (glial progenitor cells) that express glutamate receptors [13], with their inappropriate opening. As depicted above, the consequent Ca^{2+} flow inside neurons and glial progenitor cells results in activation of calcium-dependent proteases, lipases, and deoxyribonucleases, ROS production, oxidative stress, cytotoxic edema, mitochondrial dysfunction and, eventually, stimulation of proapoptotic cellular pathways.

Inflammation

In the immature brain, within minutes after an HI injury, an innate immune response takes place. Microglia, neutrophils, lymphocytes, cytokines, selections, and immunoglobulins,

all have a role in the inflammatory process activated by hypoxia.

Microglia

Microglia provides immuno-surveillance to the brain. When HI happens, microglia activates and develops macrophage-like abilities, such as phagocytosis, antigen presentation, inflammatory and anti-inflammatory cytokines production and matrix metalloproteinases (MMPs) release, which lead to blood–brain barrier (BBB) breakdown [42]. Consequently, peripheral leukocytes have free access to the brain, and the normally immune-privileged brain environment is exposed to systemic responses, further exacerbating inflammation and brain damages [8].

The role of microglia in HIE is supported by different studies: some, focusing on postmortem examinations of neonatal brains, have found that patient died from HIE had a dense infiltrate of microglia in the hippocampal dentate gyrus, whereas those neonates who died of other acute causes (trauma or sepsis) had significantly fewer microglia [43]. Other studies, instead, have focused on cytokines released by activated microglia after HI, e.g. IL-18 and Caspase-1. Genetic deletion of IL-18 or of Caspase-1 attenuates brain injury [44]. However, it is important to highlight that no intervention targets microglia selectively. Indeed, pharmacological depletion of microglial prior to neonatal stroke aggravates rather than improves outcome, exacerbating the release of inflammatory cytokines. This suggests that at least a part of microglia has beneficial effects [45]. Indeed, for example, microglial phagocytosis of remains has been hypothesyzed to be critical for tissue recovery during the second delayed phase after HI. These contrasting findings can be explained with different microglial activation phenotypes. Some microglia participates in acute early pro-inflammatory responses and aggravates injury, whereas others might be involved in the late anti-inflammatory responses and protect against injury [44]. According to literature [8, 46], indeed, there are two microglial activation phenotypes. Classical activation (M1), responsible of innate immune response, that leads to the production of cytokines, chemokines, and reactive intermediates. Another activation phenotypes (M2) responsible of anti-inflammatory signaling (M2a) and clearance of ROS and nitrogen species (M2b). The M1 phenotypes can lead to increases neuronal death compared to M2, this is why nowadays there is a growing interest in controlling the classical activation pattern of microglia.

Astrocytes

As well as microglia, astrocytes are activated within minutes after injury by pro-inflammatory mediators, cytokines, and ROS secreted by injured cells. Astrocytes have opposite role in HI: from one side, they release glutathione and superoxide dismutase (SOD) [47] and enhanced extra-synaptic glutamate uptake [48]; from the other, they produce and release pro-inflammatory cytokines (IL-6, TNF-α, IL-1α, and β and interferon γ). This cytokines exacerbate HI injury directly inducing neuronal cells apoptosis, inhibiting neurogenesis, and increasing toxic NO levels [8]. Moreover, astrocytes release chemokines that attracts immune cells to the ischemic site, thus worsening the brain injury.

Neutrophils

Neutrophils can worsen brain injury determined by HI through multiple mechanisms, such as ROS production, release of cytotoxic agents, MMP-9 secretion, and decreased microvascular flow due to neutrophils accumulation inside vessels. Differently from adult brain, where neutrophil infiltration can be massive, neonates have a lower capacity to mount a neutrophil response after ischemia, as proven by the fact that neonatal neutrophils have reduced extravasation from blood vessels [8].

This finding can explain why treatment based on neutrophil inhibitory factor, neuroprotective in adult animals, is not so efficacious in neonatal rats [49].

Lymphocytes and mast cells

Differently from microglia and neutrophils, the role of lymphocytes and mast cells in HI is not yet totally clear. Lymphocyte infiltration of neonatal brain after HI seems to be, differently from what happens in adult, less profound or only briefly present, reflecting the immaturity of lymphoid progenitor cells. Studies suggest that a lymphocytic response is involved in the more chronic immunoinflammatory activation following HIE, since CD4 have been found in damaged areas 7 days after HIE, persisting there for 14–35 days [50]. However, it is still unknown whether this lymphocytic presence enhances injury or, conversely, neuron restoration.

Mast cells, on the other side, seem to promote inflammation acutely after damage and to contributes to excitotoxic injury early releasing TNF and exacerbating transforming growth factor β1 (TGFβ1) toxicity. However, the contributions of mast cells to the ongoing evolution of damage or reparation are still unclear.

Cytokines and chemokines

Activated microglia, astrocytes, and endothelial cells release chemokines and pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α), transforming growth factorβ (TGF-β), IL-1, IL-6, IL-8, and IL-10. Cytokines attract leukocytes and facilitate their stop in the brain stimulating

the production of adhesion molecules on leukocytes and endothelial cells. TNF-α and IL-1 β, often expressed simultaneously, are two of the best-characterized early response cytokines and are secreted by microglia, astrocytes, and neurons [8, 51]. These cytokines determine the accumulation of inflammatory cells in the injured brain attracting them. Neonates with HIE have higher level of TNF-α and IL-1 β in peripheral blood samples compared to control and IL-1 β correlates positively with HIE severity [52]. Studies on administration of IL-1 receptor antagonist in HIE models have demonstrated the neuroprotective potential of this treatment, reaffirming the damaging involvement of this cytokine in HIE [53, 54].

Chemokines also play a central role in cerebral injury in HIE controlling inflammatory cell traffic. They act through specific receptors belonging to the family of G-protein-coupled receptors [55]. Studies on HIE in rats have demonstrated the upregulation of α- and β-chemokines before the expression of lymphocytes receptor in the damaged area [50].

Neuroprotective agents for neonatal hypoxic–ischemic brain injury and future prospects with neural regeneration agents

Different potential neuroprotective treatments are being studied to prevent the cascade of injurious effects after hypoxia–ischemia. These promising neuroprotective agents tested on animal models and pilot clinical studies of neonatal H–I brain injury target different phases of injury: the early phase of excitotoxicity, oxidative stress and apoptosis as well as late-phase inflammatory reaction and neuronal and oligodendrocyte regeneration.

The present neuroprotective agents for early treatment of neonatal hypoxic–ischemic brain injury are classified into four categories according to currently known mechanisms: antiexcitotoxicity, antioxidation, anti-inflammation, and antiapoptosis [56, 57].

Xenon

Xenon has two therapeutic effects (antiexcitotoxic and antiapoptotic). Acute hypoxic–ischemic insult leads to NMDA receptor activation. Xenon inhibits NMDA signaling and thus may play a role in reducing the acute cell injury. Studies of birth asphyxia in the piglet model suggest a benefit in the association of treatment with hypothermia and xenon [58, 59]. But a proof of concept, open-label, randomised controlled trial (Total Body hypothermia plus Xenon-TOBY-Xe) [60] concluded that xenon is unlikely to enhance the neuroprotective effect of cooling after birth asphyxia. Additionally, considering that xenon is an expensive noble gas (it requires a specialized delivery system) [61], alternative therapies are currently being evaluated.

Argon

Argon is a less expensive noble gas with antiapoptotic effect that has demonstrated significant neuroprotection in animal models of HIE. An extensive piglet study of perinatal asphyxia showed that inhaled 45–50% argon augments hypothermic brain protection [62].

Melatonin and erythropoietin (EPO)

Melatonin and erythropoietin (EPO) have three therapeutic effects (anti-inflammatory, antioxidative, and antiapoptotic). In a randomized controlled pilot trial evaluating melatonin with cooling in term infants with HIE, compared to controls, combination of melatonin to therapeutic hypothermia was efficacious in reducing oxidative stress and improving survival with favorable neurodevelopmental outcomes at 6 months of age [63]. Melatonin seems to be safe and beneficial in protecting neonatal brains from perinatal HIE. However, larger randomized controlled trials in humans are required.

Erythropoietin and darbepoetin (a long acting erythropoietin analogue that offers the additional benefit of once weekly administration) have neuroprotective properties in animal models of hypoxic–ischemic brain injury and neonatal stroke [57]. High doses of erythropoietin, both in conjunction with hypothermia and as monotherapy, have shown promise in preliminary randomized trials for reducing brain injury and improving motor outcomes in infants with HIE [64]. A small randomized trial evaluating the use of darbepoietin as adjunctive therapy to hypothermia in the first 12 h of life and repeated at 1 week of life demonstrated a good safety profile of this medication [65]. However, confirmation from larger trials is needed.

Allopurinol

Allopurinol is a xanthine oxidase inhibitor with one therapeutic effect (antioxidative). It reduces the production of oxygen radicals as superoxide, which contributes to secondary energy failure and apoptosis in neurons and glial cells after reperfusion of hypoxic brain tissue.

Preclinical studies in animal models of HIE have shown neuroprotective effects with use alone and as a complement to TH. A review in 2012 did not reveal any statistically significant difference in the risk of death or a composite of death or severe neurodevelopmental disability.

Currently, the ALBINO trial, a European double-blinded randomized placebo-controlled parallel group multicenter trial (Phase III), is evaluating the effect of

postnatal allopurinol administered in addition to standard of care (including therapeutic hypothermia if indicated) on the incidence of death and severe neurodevelopmental impairment at 24 months of age in newborns with perinatal hypoxic–ischemic insult and signs of potentially evolving encephalopathy [66, 67].

Magnesium sulfate

Magnesium sulfate is an NMDA receptor antagonist with two therapeutic effects (antiexcitotoxic and antiapoptotic) that is widely used antenatally for neuroprotection in preterm deliveries. A prospective, longitudinal, placebo-controlled trial of $MgSO_4$ use in infants with severe asphyxia, without hypothermia therapy, demonstrated good short-term outcomes compared to standard supportive treatment [68]. A systematic review of preclinical evidence for $MgSO_4$ use in HIE demonstrated no benefit and little consensus in dose and timing of administration [69]. Another meta-analysis evaluating $MgSO_4$ in HIE concluded that there was improvement in short-term outcomes and no increase in side effects [70]. However, further large studies are needed to determine if there are long-term benefits of magnesium and to confirm its safety.

Stem cells

Different studies recently suggested that after an HI event, there is increase of regeneration pathways, opening up new research into therapies not only to attenuate brain damage but also to promote cell repair and regeneration in a developmentally disorganized brain long after the perinatal insult [71, 72]. Exogenous stem cell transplantation for neonatal HIE shows promising preliminary results. The delivery routes for stem cell transplantation include intracerebral, intrathecal, arterial, intravenous, intraperitoneal, and intranasal approaches.

Stem cell-based therapy has the potential to rescue and replace the ischemic tissue caused by HI and may facilitate endogenous brain repair [73]. The implantantion of neural precursor cells (derived from embryonic stem cells) into the deep motor cortex of HIE newborn rats can improve motor function, within 3 weeks post-implantation [74].

Intranasal delivery of human neural stem cells (HNSC) could improve neurobehavioral outcomes in neonatal HI rats, which is possibly related to the modulation of NF-κB signaling [75].

The noninvasive nasal cavity implantation provides a simple method to perform stem cell transplantation in the future. The clinical application of HNSC has been preliminarily attempted in infants with HIE. Luan et al. [76] reported that HNSC were transplanted into one 75-day old male infant with neurological disability that was caused by severe HIE; 28 days after transplantation, remarkable improvement occurred not only in his myotonia but also in his intelligence and movement, which became similar to those of the normal infants of the same age. Positron emission tomography (PET) showed significantly increased radioactivity at temporal and occipital lobes which suggested that the cellular metabolism had increased greatly.

In another study, HNSC were implanted in six cases of neonatal HIE; the second day after cell transplantation, all patients' sucking and swallowing reflexes appeared, convulsions stopped, and muscle tension was improved. All patients were evaluated at 12 months of age; four cases showed normal mental motor development, whereas two cases presented with cerebral palsy [77]. The above studies have provided very valuable experience for further research and clinical applications.

Cord blood mononuclear cells

Cord blood cell transplantation may be the most potent therapeutic candidate for neonatal HIE, with wide application prospects.

These cells show a strong potential for nerve regeneration and they are easier to obtain than other stem cells. Animal experiments have demonstrated that cord blood mononuclear cells can reduce the activity of microglia, inhibit neuronal cell death, and promote the recovery of sensorimotor reflex function [78]. Also, they can promote the differentiation of endogenous neural stem cells into mature neurons to aid in the recovery and development of damaged neurons [79] and the upregulation of neurotrophic factor in the brain after cell transplantation [80]. The optimal timing, dosage, and delivery of stem cell transplantation for neonatal HIE will require further study.

Conclusions

HIE is the result of severe anoxic brain injury during neonatal period that can lead to premature mortality or can result in life-long morbidity.

Nowadays, the only recognized treatment for neonatal HIE is hypothermia that, although positively influencing the neurological outcome of neonatal HIE [81], is only partially effective. In fact, no more than 50% of the neonates treated achieve an improved outcome [11, 82].

At the present, efforts are focusing on finding adjuvant therapies for HIE and understanding the pathophysiology of HIE is the first, indispensable, step to better clarify the mechanism underlying it.

Pathophysiology of HIE involves several different events, strictly linked one to the others. Mitochondrial dysfunction, excitotoxicity, calcium surge, reactive oxygen species

accumulation, and inflammation are the five main events caused by severe anoxic brain injury. Starting from them, a variety of complex pathways begins. Some gaps in our knowledge concerning the pathophysiology and the timing of important endogenous neuroprotective and neuroregenerative mechanisms still exist.

Although there have been significant steps in the basic sciences to create novel neuroprotective and intervention strategies to combat HIE, there is still much more research needed to be conducted to translate potential life-saving and brain damage-limiting therapies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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