

Mechanisms Underlying Neonatal Hypoxia Ischemia

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Abstract: Neonatal hypoxic ischemic encephalopathy (HIE) is a common health problem and occurs in approximately 1-6 in 1000 term infants every year. After the initial hypoxic ischemic insult, brain injury evolves for days and possibly even weeks. The severity of the injury depends on the gestational age and duration of the insult. The exact underlying mechanisms of neonatal HIE remain undefined and many differences exist between adult and neonatal brain injury. In order to better understand the mechanisms underlying hypoxic ischemic injury and develop more effective therapies for HIE, different animal models of neonatal brain injury was developed. The proposed mechanisms include excitotoxicity, necrotic and apoptotic neurodegeneration, oxidative stress, inflammation and non-NMDA dependent pathways. New evidence suggests that non-NMDA-dependent pathways, such as the Na⁺/H⁺ exchangers and non-selective Ca²⁺-activated ATP-sensitive cation channels, may also be involved in HIE. Activation of these mechanisms after the hypoxic and ischemic insult contributes to pathogenesis simultaneously or sequentially. This review summarizes the different models of neonatal HIE, major cellular and molecular mechanisms, potential therapies that are currently investigated either in preclinical experimental animal models or in clinical trials in hypoxic ischemic brain injury in neonates.

Keywords: Neonatal hypoxia ischemia, apoptosis, NMDA, Na⁺/H⁺ exchanger isoform 1 (NHE-1), HOE 642 (cariporide).

1. INTRODUCTION

Neonatal hypoxic ischemic encephalopathy (HIE) occurs in approximately 1-6 in 1000 term infants every year [1]. Brain injury following the initial hypoxic ischemic event lasts for days and possibly even weeks. There are differences between adult and neonatal brain injury after hypoxia ischemia (HI). The immature brain may be more vulnerable to oxidative damage than the adult brain due to high concentration of unsaturated fatty acids and the availability of redox-active iron in the developing brain [2]. The underlying mechanisms of neonatal HIE remain undefined.

A considerable amount of research has focused on glutamate-mediated excitotoxicity, mainly through the N-methyl-D-Aspartate receptor (NMDAR). The NMDAR is over-expressed in early brain development [3]. In rodent models of neonatal HI, activation of the NMDAR was shown to be associated with excitotoxic brain injury [4]. However, direct inhibition of the NMDAR led to wide-spread apoptotic neurodegeneration in rodent brain under normoxic conditions [5-7]. Therefore, direct inhibition of the NMDAR for HI is problematic, considering its important roles in the developing brain. This article reviews the steps that have been taken to establish the animal model of HI in neonates, recent pre-clinical studies and clinical applications.

2. RESEARCH METHODS

2.1. Rodent Model of Neonatal HI

The model of neonatal HI was first developed by Vannucci and Rice in neonatal rats [8], modified from the Levine procedure for adult rats [9]. The Vannucci/Rice method involves a unilateral carotid artery ligation of postnatal day (P) 7 rats followed by exposure to 8% oxygen/balanced nitrogen at 37°C. The unilateral carotid artery ligation alone does not induce ischemic damage due to the intact circle of Willis. Exposure of neonatal rats to 8% oxygen after unilateral common carotid ligation results in hypoxemia and hypocapnia produced by hyperventilation [10]. Hypocapnia compensates for the metabolic acidosis which is due to lactic acid accumulation secondary to anaerobic glycolysis. Therefore, this model causes damage to ipsilateral hippocampus, striatum, thalamus and cortex [11].

The selection of the age of the neonatal rodent and duration of hypoxia vary among researchers. In rats, the accepted age has remained at P7, which correlates histologically to a 32 to 34 week human neonate [10]. In mice, the most frequently used age is P9, although P7 to P10 are commonly used [12-14]. Seven to 10 day old mouse brains are similar to a third-trimester neonatal brains, in terms of cellular proliferation, cortical organization, synapse number, neurotransmitter synthetic enzymes and electrophysiology [15].

The time course to induce HI related brain injury varies among different animal species and ages. Neonatal rats

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exposed to hypoxia for over three hours can induce brain injury before significant mortality occurs [10]. Severe forebrain injury was observed following 150 minutes (min) of HI in P7 rats [16]. Similarly, time course of HI varies from 15 min [7] to 2.5 hours (h) in P7 mice [17]. Thirty min of hypoxia can result in moderate injury and 1 h in severe injury in P7 mice [18].

In rabbits, a uterine model of ischemia has been developed to mimic acute placental insufficiency and fetal HI. Pregnant rabbits at 29 day of gestation are anesthetized. An embolectomy catheter is inserted in the left femoral artery to a distance of 10 cm with the balloon proximal to the uterine arteries and bifurcation of the aorta. The intraaortic balloon is inflated for 50 min to induce uterine ischemia, which causes premature fetal brain damage and neuronal death in hippocampus [19, 20].

Due to the nature of the immature brains, one of the limitations of the current HI model is the variability. To overcome this issue, scoring systems are developed to help analyze data. A commonly applied approach is to stain brain sections with a reliable cellular marker for cell damage, such as cresyl violet staining which shows damaged cellular nuclear morphology in each brain section. Eight regions in each brain section are then scored: the anterior, middle and posterior cortex, CA1, CA2, CA3 and dentate gyrus of the hippocampus and caudate, putamen, with the contralateral side serving as a control for uninjured tissue. A score of 0-3 is given to each region as follows: 0 = no detectable neuronal cell loss; 1 = small focal areas of neuronal cell loss; 2 = columnar damage in the cortex involving predominately layers II-IV or moderate cell loss in the hippocampus; and 3 = cystic infarction and gliosis. The score for each region is then added for a final score ranging from 0-24 [21]. Whole brains can also be scored based on appearance of the two hemispheres and presence of lesions [22, 23].

2.2. Detection Methods of Neuronal Degeneration

There are many different detection methods that are used to detect neuronal damage after HI. Cellular damage is often assessed by immunohistochemical staining in brain tissue sections (10-30 μm). Cresyl violet (Nissl) staining is used to study nuclear morphology in degenerating cells [16] and condensed chromatin in pyknotic neurons [24, 25]. Active Caspase-3 and TUNEL staining are combined indicators of apoptosis in striatal neurons at 6 h post HI and cortical neurons at 48 and 72 h post HI, respectively [25-27]. Fluoro-Jade (FJ) staining can be used to identify areas of neuronal degeneration in specific brain regions [14]. FJ-C is the most sensitive fluorescent marker of neuronal degeneration in terms of producing a high resolution with a great signal to background ratio. FJ-C has an higher affinity for the endogenous neurodegeneration molecules than its predecessors (FJ-A, FJ-B) [28].

Necrotic neurodegeneration is seen in the ipsilateral cortex and striatum at 3 h post HI [16]. Apoptotic cell death is detected in the thalamus at 24 h post HI [16]. At 48 h post HI, cells show both necrotic and apoptotic appearances in the ipsilateral cortex and striatum in rats [16]. In mice, damage is only detected in the ipsilateral hippocampus at 48 h post HI [18].

2.3. Brain Imaging Techniques

Recently, magnetic resonance imaging (MRI) and other imaging techniques have been adapted to detect brain injury following HI in neonates. This is important clinically as it allows for rapid detection of specific damage and determination of the appropriate treatments. In animal models, damage following HI can be assessed by MRI without sacrificing the animal, which allows the animal to be used for chronic studies. A recent study using diffusion tensor imaging (DTI) demonstrated neural network degeneration in P7 mice exposed to 45 min HI. At 24 h, rapid degeneration was seen in all regions of the ipsilateral hippocampus in three-dimensional T_2 and DTI images [14]. At P11 through P28, the ipsilateral fimbria fornix degenerated. By P15, there was injury and a loss of axons entering the ipsilateral septal nucleus [14]. Histological analysis of brain sections with FJ-C and cresyl violet staining revealed correlative damage with T_2 and DTI images [14].

Diffusion weighted MRI (DWI) of P7 rats found a hyperintense DWI signal and apparent diffusion coefficient (ADC) drop in the ipsilateral cortex at 24 h post HI. The increased DWI signal correlated with an increase in caspase-3 activity [29]. Surprisingly, unilaterally enlarged regions at the ipsilateral ventricle/white matter interface with an ADC increase also correlated to a modest increase in caspase-3 activity in the cortex even though the hemisphere had no apparent ADC drop [29]. Manganese-enhanced (ME) MRI has been used to depict delayed neuronal death in certain cell types. Manganese (Mn^{2+}) gives a positive contrast to T_1 -weighted images and has been shown to enter neurons and glial cells through voltage-gated Ca^{2+} channels [30]. 3D T_1 -weighted images and 2D T_2 -maps were acquired at 1, 3 and 7 days post HI in P7 rats [31]. Damage was detected only at 7 days post HI in parts of the ipsilateral cortex, hippocampus and thalamus. Immunohistochemistry staining revealed loss of microtubule associated protein 2 (MAP2), formation of activated microglia (cluster of differentiation protein CD68) and increased expression of glial fibrillary acidic protein (GFAP) and Caspase-3. T_1 -weighted images that showed increased ME areas in P14 correlated with delayed neuronal death and inflammation. Specifically, the ME-enhanced areas corresponded best to areas with accumulation of activated microglia. The Mn^{2+} may be accumulating in active microglia; thus ME MRI may be used to show active microglia following neonatal HI [31].

3. MECHANISMS OF CELL DEATH

3.1. Excitotoxicity

Current research shows the importance of glutamate-mediated excitotoxicity following neonatal HI. Glutamate is the main excitatory neurotransmitter in the central nervous system and acts through two broad classes of receptors: ion channel linked ionotropic receptors and metabotropic receptors (mGluR), which are coupled with G-proteins inducing intracellular messenger cascades [32,33]. Four main subtypes of glutamate-gated channels have been characterized pharmacologically and they have been named according to their preferred agonist, N-methyl-D-aspartate (NMDA), high affinity kainate (KA), α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and 2-amino-4-

phosphobutyrate (AP4) [32]. Following HI, excessive release of glutamate and/or impaired uptake by glia may result in overactivation of both types of receptors [1]. A prolonged hypoxia or HI results in depletion of cellular energy stores (ATP), depolarization of neurons and glia and release of excitatory amino acids into the extracellular space, particularly the glutamate. Excessive activation of NMDAR leads to an increase in Ca^{2+} influx which activates proteases and endonucleases, such as calpains, resulting in the breakage of cytoskeletal and nuclear structure and DNA damage. On the other hand, the influx of Na^+ ions *via* AMPAR causes cell swelling and depolarization. These are the major cell death mechanisms in response to excitotoxicity that eventually results in necrosis or apoptosis.

The ionotropic NMDA receptor has been studied extensively. Current research has focused on the subunits of the NMDA receptor. The NMDA subunits NR2A and NR2B showed increased tyrosine phosphorylation by Src and Fyn kinases following 15 min HI in P7 mice [7]. These kinases associate with NR2A and NR2B in the post synaptic densities for at least 6 h after HI. Inhibition of Src and Fyn with the potent inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo [3, 4-*d*] pyrimidine (PP2) resulted in decreased brain damage in ipsilateral cortex, hippocampus and striatum [7]. NR3A, a regulatory subunit of some NMDA receptors, was shown to be protective against NMDAR-mediated excitotoxicity in P7 mice following 2.5 h HI. Knockout of NR3A in P7 mice resulted in greater infarct size in the cortex at 7 days post HI [17].

The metabotropic glutamate receptor 1 (mGluR1) has also been studied for its involvement in cell death following neonatal HI. Stimulation of NMDA receptors triggers the activation of the Ca^{2+} -dependent protease calpain, which in turn can truncate the C-terminal domain of mGluR1 α , forming a cleavage product which can release Ca^{2+} from the endoplasmic reticulum [34]. Truncation of mGluR1 α also eliminates the link between mGluR1 and the neuroprotective PI₃K-Akt signaling pathways [35]. Inhibition of calpain-mediated mGluR1 cleavage by a TAT-mGluR1 peptide, which binds to the cleavage site, was shown to increase the number of neurons at 24 h after HI and maintain brain volume 6 days after HI [36]. There was also a greater reduction in hippocampal lesion and swelling of the hippocampal pyramidal neurons 24 h after HI with the TAT-mGluR1 peptide [36].

Neuronal pentraxins are neuronal proteins that have been shown to be involved in the excitatory synaptic remodeling. Neuronal Pentraxin 1 (NP1) has been shown to be upregulated following HI and associated with GluR1 AMPA receptors in the CA1 and CA3 pyramidal layer of the ipsilateral hippocampus. Blockage of AMPA-induced neuronal death following inhibition of NP1 expression implicates a regulatory interaction between NP1 and AMPA glutamate receptors. NP1 gene silencing alone was found to be neuroprotective against hypoxia-induced neuronal death. NP1 may be required to induce the AMPA excitotoxic cascade [37].

3.2. Necrosis and Apoptosis

Both necrotic and apoptotic cell death occurs following HI. Necrotic cells show early rupture of the plasma

membrane, swelling of intracellular organelles, shrinkage of cell volume and inflammation response activation resulting from the spilled cytosolic contents [38]. In contrast, apoptosis is an energy dependent programmed cell death mechanism occurring in development and neurodegeneration diseases [38]. Apoptosis in HI is morphologically different from developmental apoptosis [39]. Comparison of adult and neonatal animal models suggests that apoptosis may be more prevalent in the immature brains after HI [40].

Apoptotic events include both extrinsic and intrinsic pathways [41, 42]. The extrinsic pathway involves the Fas ligand and TNF- α receptor-mediated formation of the death-inducing signaling complex (DISC), which recruit scaffolding proteins, cleavage of pro-caspase 8 and activation of protease caspase 8 [43]. Caspase 8 then activates the other effector caspases (i.e. caspases 3, 7) which stimulate the enzymes responsible for chromatin condensation and packaging of cellular organelles into apoptotic bodies that prevent cytosolic leakage [38, 44]. The intrinsic (mitochondrial) apoptotic pathway involves trauma-induced cytochrome c release from the mitochondrial matrix to the cytosol where it interacts with Apaf-1 and pro-caspase 9 to form the apoptosome [44, 45]. The cleavage and formation of caspase-9 can then activate the downstream effector caspases in a manner similar to caspase-8 [42, 44, 45].

Studies found that the distinction between necrosis and apoptosis in P7 rats is dependent on time, brain region and neuronal network patterns. Early necrosis is seen in the ipsilateral forebrain at 3 h after HI. This initial wave of injury results in randomly distributed necrosis in the striatum and cortex [16]. At 24 h after HI, apoptosis is seen in ipsilateral ventral basal thalamus [16]. A second wave of injury in the ipsilateral cortex occurs at 48 h and is largely mediated by apoptosis [16]. Studies also show some cells experience a “cell death continuum” ranging from necrosis to apoptosis and sometimes a hybrid of the two is found that includes apoptotic factors as well as mitochondrial dysfunction indicative of necrosis. The ability to complete full apoptosis may be dependent on energy resources [27].

Cyclophilin D (CypD), a mitochondrial matrix protein that enhances inner mitochondrial membrane permeability through its interactions with the permeability transition pore, is critical for the development of injury in P60 mice following 30 min HI and knockout of CypD significantly reduces brain injury [23]. However, knockout of CypD worsened injury in P9 mice after 40 min HI [23]. Caspase-dependent and -independent cell death pathways were elevated more in P9 CypD knockout mouse than wild-type controls, whereas apoptotic activation was minimal in P60 mice [23]. Bcl-2-associated X protein (Bax), a well-known cell death signaling activator, was inhibited in both P9 and P60 mice. Bax inhibition by 5 μL of Bax-inhibiting protein injected intracerebroventricularly reduced caspase activation and brain injury in the P9 mice but was ineffective in P60 mice [23]. These findings suggest that CypD shifts from a prosurvival protein in the immature brain to a cell death mediator in the adult brain and that Bax-dependent cell death mechanisms prevail in the immature brain [23].

The 70 kDa heat shock protein (Hsp70) is up-regulated after HI and may be used as a marker for cellular damage.

Hsp70 functions as a molecular chaperone and has been shown to antagonize apoptosis by binding to Apaf-1 and preventing caspase-9 activation [46, 47]. Hsp70 overexpression sequesters apoptosis-inducing factor (AIF) and other pro-apoptotic proteins in P7 rats after 30 min HI [48]. Treatment with the nitric oxide synthase (NOS) inhibitor 2-iminobiotin resulted in neuroprotection in the hippocampus and cortex in P12 rats following 90 min HI, but less Hsp70 expression was seen in the ipsilateral side, possibly indicative of less brain damage [49].

Apoptosis can be inhibited at various places along the pathway. A variety of inhibitors from multiple pathways tested for reduction in apoptotic factors as a measure of delaying or attenuating cell death. Inhibition of neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) by 2-iminobiotin also reduces cytochrome C release [49]. The short peptides ADNF-9 and NAP from activity-derived neurotrophic factor (ADNF) reduced the number of apoptotic neurons in hippocampus [50]. Inhibition of the pro-death c-Jun N-terminal kinase (JNK) pathway with D-JNK inhibitor 1 (D-JNKI1, 0.3 mg/kg i.p.) significantly reduced early calpain release, late caspase-3 activation and thalamic autophagosome formation, indicating involvement of the JNK pathway in necrotic, apoptotic and autophagic types of cell death [51].

Glutathione peroxidase (GPx) was shown to be beneficial following HI by removing hydrogen peroxide (H_2O_2) [52]. H_2O_2 normally interferes with the Fas-associated death domain-like IL-1-converting enzyme inhibitory protein (FLIP), the dominant negative of caspase 8. Overexpression of GPx increased FLIP expression, thereby inhibiting cleavage of caspase 8 and promoting cell survival by interrupting the FAS death receptor pathway [52]. Neurotrophic factors were also found to be protective. Neurotrophic factor FGF-1 upregulation protects against HI by interfering with caspases and preserving anti-apoptotic protein X-linked inhibitor of apoptosis (XIAP) expression [53].

3.3. Oxidative Stress

Immature brains are particularly vulnerable to oxidative stress due to poor antioxidant capabilities, high concentration of free iron and high concentration of unsaturated fatty acids.

Following HI, energy failure leads to activation of NMDA receptors and calcium overload. This event triggers the direct activation of neuronal nitric oxide synthase (nNOS), generation of nitric oxide, increased formation of reactive oxygen species (ROS) and mitochondrial damage [54].

Oxidative stress response after HI differs in developing brains. H_2O_2 level, measured by the aminotriazole-mediated inhibition of catalase spectrophotometric method, was increased in P7 but not in P42 mouse cortex at 2, 12 and 24 h after HI. Also, less H_2O_2 was accumulated in P42 mouse hippocampus, compared with the P7 mouse hippocampus at 2 h post HI [2]. However, NADPH oxidase, which produces ROS in neutrophils and microglia/macrophages, does not contribute to brain damage in P9 mice [13], in contrast to its role in hippocampal damage of 8 to 10 week old mice following HI [55]. Sustained HI results in production of

reactive nitrogen and oxygen species and injury to the neurons in the premature fetal rabbit brains [20].

3.4. Inflammation

Inflammatory mediators also play an important role in the pathogenesis of HI in immature brains [56]. This inflammation could result from maternal infection which is a well known risk factor for the white matter disease in the newborns and leads to neuronal damage and cell loss [57]. Inflammation after HI has both beneficial and detrimental effects. Inflammatory cytokines may have a direct toxic effect *via* increased production of iNOS, cyclooxygenase and free radical release [58]. Expression of interleukin-1- β and tumor necrosis factor α (TNF- α) mRNA has been detected in the area of infarction within 1-4 h after HI [59]. Thus, inflammation may represent a common pathway of brain injury. The beneficial effects of inflammatory cytokines come from their neurotrophic effects. TNF- α knockout mice have increased neuronal cell damage with increased oxidative stress after ischemia, suggesting an important role for TNF- α in neuroprotection. Injury-induced microglial activation was also suppressed in TNF- α knockout [60].

3.5. Other Non-NMDA Dependent Pathways

NMDA-mediated excitotoxicity can lead to membrane depolarization, producing maximal channel opening and an influx of Ca^{2+} and Na^+ into neurons [61] and has also been shown to activate the Ca^{2+} -dependent protease calpain, which in turn can truncate the C-terminal domain of mGluR1 α , forming a cleavage product which can release Ca^{2+} from the endoplasmic reticulum [34]. However, non NMDA-dependent mechanisms can disrupt ionic homeostasis in neonatal brains after HI and contribute to brain damage. This includes Na^+/H^+ exchanger and non-selective Ca^{2+} -activated ATP-sensitive cation channels [62-65].

HI leads to a decrease in the intracellular pH (pH_i). To combat acidosis, the Na^+/H^+ exchanger (NHE) and H^+ pumps in the plasma membrane are activated to remove the excess H^+ ions. NHE isoform 1 is the most ubiquitously expressed isoform in the CNS [66, 67]. NHE-1 becomes overstimulated following ischemia-induced intracellular acidosis [68] and by extracellular regulatory kinase-mediated phosphorylation [66, 69]. NHE1-mediated ischemic and reperfusion damage largely result from an increase in intracellular Na^+ , which promotes Ca^{2+} influx by reverse mode operation of the Na^+/Ca^{2+} exchanger [70, 71]. In an adult mouse model of transient focal ischemia, both genetic knockdown (NHE-1 $^{-/-}$) adult mice and mice received pharmacological inhibition of NHE-1 by a potent, selective NHE-1 inhibitor HOE 642 had attenuated infarct size [64]. Recently, in a gerbil model of neonatal HI, NHE-1 protein levels were increased in the ipsilateral hippocampus following HI. Also, by using a non-specific NHE inhibitor, *N-methyl-isobutyl-amiloride* (MIA), pyramidal neurons in the CA1 region of the hippocampus were preserved [72]. MIA was also used in a P7 mouse model and reduced TUNEL-positive cells in the ipsilateral hippocampus [18]. Our recent study shows that the potent, selective NHE-1 inhibitor HOE 642 protected the hippocampal neurons in the CA1 pyramidal layer, reflected by less FJ-C positively stained cells in P9 mice following 55 min HI [62, 63]. In the

HOE 642-treated mice, HI-induced loss of MAP2 expression was reduced in the ipsilateral CA1 region following HI and an additional evidence of neuroprotection of the pyramidal neurons [62].

The non-selective $\text{NC}_{\text{Ca-ATP}}$ cation channel is involved in edema formation after neonatal HI. The $\text{NC}_{\text{Ca-ATP}}$ channel conducts monovalent cation currents and requires nanomolar concentrations of Ca^+ to open [73]. The channel is activated by depletion of intracellular ATP and regulated by the sulfonylurea receptor 1 (SUR 1). In adult stroke model, blockade of SUR 1 by glibenclamide reduced cerebral edema, infarct volume and mortality by 50% [74]. However, in a neonatal HI model of P10 rats, only moderately damaged (2 h HI) pups showed potential protection by glibenclamide, in contrast to the severely damaged (2.5 h HI) pups [65].

4. CURRENT THERAPIES

4.1. Therapies in Pre-Clinical Experimental Animal Models

A variety of potential therapies are being investigated in the animal models with some results being promising and some non-promising. It is hard to translate from animal models to human clinical trials due to possible differences in developmental age correlations and mechanisms. The following reviews potential therapies that have been studied in animal models with significant neuroprotection.

4.1.1. Anti-Excitotoxicity Therapy

Combination treatments of Memantine (a low affinity, non-competitive NMDAR blocker) and topiramate (an AMPA/KA receptor blocker and sodium channel blocker) reduced structural damage at 22 days post HI and improved performance on a foot-fault test at 21 days post HI in P7 rats [75]. Memantine when given alone after HI attenuated the acute white matter injury in immature brains and improved long-term histological outcome [76, 77]. It was also shown that preconditioning with the anesthetics, Xenon (also an NMDAR antagonist) and Sevoflurane (γ -aminobutyric acid type A receptor agonist) provide protection to the P7 rat assessed by reduced infarct size in ipsilateral hippocampus at 7 days post HI and improve performance on rotarod coordination test at 30 days post HI [78].

4.1.2. Antioxidation Therapy

In order to overcome the damage from oxidative stress, Edaravone, a free radical scavenger, was shown to reduce apoptotic neuronal number and 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in the cortex of P7 rats after 2 h HI. The drug was administered immediately after and at 24 h every day at 9 mg/kg i.p. [25]. Hydrogen gas (H_2) treatment following HI has been shown to be protective in brain by reducing oxidative stress and neutralizing free radicals [79]. Hydrogen saline administration (5 ml/kg) immediately and at 8 h post HI in P7 rats reduced caspase-3 activity, prevented activation of microglia, decreased the level of oxidative stress and reduced the infarct volume in the ipsilateral cortex and hippocampus [24]. At 5 weeks, rats showed improved scores in the Postural reflex test, reduced spontaneous activity and reduced escape latency in the Morris water maze [24].

Docosahexaenoic acid (DHA) is an essential long-chain fatty acid found in fish. Evidence suggests that maternal diets rich in fish are associated with reduced risk for cerebral palsy, one of the possible consequences of neonatal HI [80]. DHA may act through a variety of pathways to provide neuroprotection in neonatal HI. DHA pretreatment (4 hours prior to HI, i.p. 5 $\mu\text{L/g}$) was shown to improve forepaw placement in the vibrissae-stimulated forepaw placement test and lessen tissue loss in the ipsilateral hemisphere and specifically the hippocampus of P14 rats exposed to HI at P7 [81].

4.1.3. Anti-Apoptotic Therapy

Growth factors have been shown to be neuroprotective in rodent pups following HI. In P7 rats, adipose stromal stem cell-concentrated media (ASC-CM) was administered into the jugular vein either at 1 h prior or at 24 h post-HI and showed significant reduction in ipsilateral hippocampal and cortical volume loss [82]. The insulin-like growth factor (IGF-1) and brain-derived neurotrophic factor in the media may contribute to the protective effects of ASC-CM [82]. Intranasal IGF-1 administration one hour following HI in P7 rats attenuated cell death in the ipsilateral cortex, striatum, hippocampus; white matter area in the hippocampus at P9 and P22 with improved neurological outcomes in passive avoidance, pole and elevated plus-maze tests [83].

4.1.4. Stem Cell Therapy

Neural stem cells (NSC) have been investigated in both adult and neonatal ischemia models for neuroprotective effects. In neonatal HI models, intraventricular administration of NSC after HI showed migration of these cells to the area of injury and differentiating into neurons, astrocytes, oligodendrocytes and undifferentiated progenitors [84, 85]. Umbilical cord blood cells do not seem to protect against damage in neonatal HI in rat model, possibly because few cells were found to be localized in the brain [86].

4.1.5. Hypothermia Therapy

Preclinical studies using animal models shed some light on the underlying mechanisms of hypothermia treatment following neonatal HI. In rats, hypothermia preserved hippocampal neurons and resulted in an increase in glial cell line-derived neurotrophic factor (GDNF) protein expression and decreased TNF- α and IL-6 at both protein and mRNA levels in the hippocampus [87]. A reduction in glial fibrillary acidic protein (GFAP) mRNA and protein expression suggests hypothermia suppresses astrocyte activation and the inflammatory response in the hippocampus following HI [87].

4.2. Clinical Therapies

4.2.1. Barbiturates

Barbiturates in clinical trials after HI show variable success in neonates. When thiopental was used in 32 asphyxiated neonates for ~ 2 hours after birth, it did not appear to have a cerebral sparing effect [88]. Interestingly, Phenobarbital when given 20 mg/kg intravenously to neonates with HI within 6 hours of life, significantly decreased the incidence of seizures [89]. Three-year long term follow up of the newborns with HI after 40 mg/kg of Phenobarbital therapy revealed better neurological outcome

in the treated group [90]. Currently, the role of barbiturates in the severely injured population remains unclear [91].

4.2.2. Allopurinol

Allopurinol after HI has been studied as an antioxidant in animal and human neonates. At high concentrations, it decreases free iron by chelation, inhibits lipid peroxidation and decreases expression of heat shock factor [92]. Allopurinol reduces free radical production by inhibiting xanthine oxidase and scavenging hydroxyl radicals. Although promising in clinical trials, there may be short therapeutic window of administration of this medicine. Early postnatal administration of allopurinol in asphyxiated neonates did not decrease early reperfusion induced free radical increase [93]. However, neonates undergoing cardiac bypass surgery due to high-risk hypoplastic left heart syndrome, showed significant neurocardiac protection when administered before, during and after surgery [94].

4.2.3. Erythropoietin

Erythropoietin (EPO) is a glycoprotein and has various effects after HI in addition to its role in erythropoiesis. EPO has regulatory functions in inflammation, immune response, CNS repair, CNS development and angiogenesis [95-97]. EPO and EPO receptors were increased in animal models after HI in neurons, astrocytes, endothelial cells and microglia [98]. This makes EPO an important agent in CNS repair and neurogenesis after HI. Recently, in a randomized control trial of 167 term infants, EPO was administered less than 48 hours of life and continued every other day for 2 weeks (300 U/kg and 500 U/kg). EPO showed neuroprotection in neonates with moderate HI at 18 months of age. Neurological outcome was not different with two different doses [99].

4.2.4. Hypothermia

Hypothermia is the only standard treatment with many clinical trials in progress for HI. Two multi-centered trials (CoolCap and National Institute of Child Health and Human Development [NICHD] Body Cooling trials) are completed [100, 101]. Therapeutic hypothermia reduces the brain energy use rate and inhibits many of the processes involved in primary and secondary energy failure [102]. In both trials, infants around a gestational age of 39 weeks underwent hypothermia treatment for 72 h, with both trials initiating hypothermia between 3 and 6 h after birth. The NICHD Body Cooling trials used a cooling blanket to cool the infant to a temperature of 33.5°C measured by a probe in the lower third of the esophagus. The NICHD Body Cooling trials had a trend towards fewer deaths in the hypothermia group; a more prominent effect of hypothermia on primary outcome in infants with moderate compared to severe encephalopathy [101]. The CoolCap used a cap to cool the infant to a rectal temperature of 34 to 35°C. In the CoolCap trial, infants with intermediate amplitude-integrated electroencephalogram (aEEG) abnormalities show a more prominent effect of hypothermia on primary outcome than infants with severe aEEG abnormalities [100, 102]. Although the results appear promising, due to a lack of long term follow up, further studies are needed to determine the outcome.

5. SUMMARY

Many differences exist between adult and neonatal brain injury. The invention of a rodent model that closely mimics the damage seen in human neonates suffering from HIE has accelerated discoveries of underlying mechanisms and potential therapies. By using this model, the cell death pathways have been investigated. Immediate necrosis and delayed apoptosis develop based on time, brain region; cell type. Due to the second wave of damage presenting as apoptosis, there is a great chance that therapeutic interventions can be applied during this time window. As more mechanisms are uncovered, more potential therapies can be investigated. Excitotoxicity through the glutamate receptors has been shown to be important in the development of brain injury, although these receptors are also essential for normal brain development. Emerging research shows that non-NMDA-dependent pathways including the Na⁺/H⁺ exchangers may also contribute to cell death. Thus, combination of reducing the early ionic dysregulation and delayed apoptosis may provide synergistically effective therapies for HI.

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