

We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900

Open access books available

186,000

International authors and editors

200M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



Neuroprotection in Perinatal Hypoxic-Ischemic Encephalopathy — Pharmacologic Combination Therapy

M^a Carmen Carrascosa-Romero and
Carlos de Cabo-de la Vega

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/57459>

1. Introduction

Hypoxic ischemic encephalopathy (HIE) currently constitutes one of the non-excluding causes of child cerebral palsy (CP) and, together with prematurity, is potentially preventable. For this reason there is an increasing interest in prevention policies as well as in research on neuroprotection therapies that minimize cerebral lesion and concomitant disabilities.

In the last few decades there has been an explosion of studies employing either animal models of global or focal hypoxia or cell cultures investigating the preventing effect of many chemicals on neuronal lesion. Recent clinical research has shown that certain pharmaceuticals have neuroprotective effects, suggesting that their use could be generalized for clinical practice in a near future. However, the use of some of these chemicals, such as nicardipine (calcium blocker) or magnesium (blocking NMDA-receptors), has been investigated in clinical trials showing no beneficial effects while causing severe hemodynamic adverse events. Therefore there is no generally accepted standard of care in the brain-oriented pharmacologic therapy for full-term neonates sustaining cerebral hypoxia-ischemia (H-I). In fact, neuroprotective treatment for HIE in the clinical practice is limited to the application of hypothermia in the newborn which is accepted now as a meaningful therapy, since no pharmaceutical has shown any benefit when administered by itself yet.

Future advances in the understanding of preconditioning may lead to the administration of neuroprotective agents earlier before childbirth. Although most of these neuroprotective strategies have not yet entered clinical practice, there is a significant hope that further developments will allow to incorporate them besides hypothermic neuroprotection. More specifi-

cally, maternal administration of allopurinol (xanthine oxidase inhibitor/ anti-oxidant) has been proposed as prebirth treatment when there is suspicion of an adverse event eliciting perinatal asphyxia.

Since it is conceivable that hypothermia postpones secondary energy failure, application of hypothermia immediately after the hypoxic event could prolong the window for pharmacotherapeutic intervention; furthermore, there is accumulating preclinical evidence that adjunctive therapies can enhance hypothermic neuroprotection. The question that still remains is whether a combination of therapeutic agents would be more efficient in reducing brain damage due to hypoxia-ischemia than applying just one pharmaceutical. The hypothesis is that combinations of therapies intervening at different levels in the cascade might lead to more prominent reduction of brain injury.

In this chapter we review the mechanisms of action of chemicals that have shown potential neuroprotection effect, with special regard to those already approved for use in the newborn and show no side effects. Finally, we propose a model of off-label combined neuroprotective therapy using a staggered design according to the severity of the asphyxia /encephalopathy.

2. Neonatal encephalopathy

The incidence of birth asphyxia is 9.4/1000 live term births whereas the incidence of neonatal encephalopathy secondary to intrapartum hypoxia-ischemia (H-I) is very low, estimated between 0.27 per 1000 (Palsdottir et al, 2007) and 1.5 per 1000 live full-term births; and about 15% to 20% of affected newborns die in the postnatal period, and an additional 25% of the survivors exhibiting permanent neuropsychological deficits (Kurinczuk et al, 2010). Birth asphyxia often appears to be a secondary symptom of an otherwise sick baby; thus, it is not the primary cause of CP in the majority of cases. One study of children with CP found that in only about 8% (15/183) of all the children with spastic CP was intrapartum asphyxia the possible cause of their brain damage; and the contribution of intrapartum events and obstetric mismanagement to overall CP rates is probably less than was previously thought (Blair & Stanley, 1998). In fact, due to this type of research, the term birth asphyxia has been replaced with the term neonatal encephalopathy because this later term does not imply a causal relationship (Fehlings et al, 2007).

Perinatal asphyxia can be defined as the injury caused to the fetus or newborn as a result of both reduced oxygen (O₂) supply to the brain and the sustained reduction in blood flow due to an inadequate cerebral perfusion. The term “asphyxia” is not synonymous with **HIE** despite been closely related: “asphyxia” is the *cause* whereas **HIE** is the *effect*. Besides, asphyxia does not always produce brain damage. **HIE** is defined as the neurological syndrome occurring in the newborn after a hypoxia / ischemia episode that affects consciousness in different degrees, with decrease of spontaneous movements, tone and reflexes, as well as the appearance of convulsions in the most severe cases. Hypoxic-ischemic events may cause multisystemic failure with impairment of pulmonary, cardiovascular, digestive, renal, hematological and metabolic functions, constituting the post-asphyctic syndrome. However, it is important to

emphasize that the clinical features of hypoxic ischemic encephalopathy are nonspecific, and a diagnosis of perinatal asphyxia should be made with caution and only after careful consideration of all data collected in the clinical history.

The essential criteria required to define an acute intrapartum hypoxic event as sufficient to cause CP were established by both the American College of Obstetricians and Gynecologists and the American Academy of Pediatrics, and the International Cerebral Palsy Task (ACOG, 2003), are listed as follows:

Essential criteria (must meet all four)

- Evidence of a metabolic acidosis in fetal umbilical cord arterial blood obtained at delivery (pH < 7 and base deficit ≥12 mmol/L).
- Early onset of severe or moderate neonatal encephalopathy in infants born at 34 or more weeks of gestation.
- Cerebral palsy of the spastic quadriplegic or dyskinetic type.
- Exclusion of other identifiable etiologies such as trauma, coagulation disorders, infectious conditions, or genetic disorders.

Criteria that collectively suggest an intrapartum timing (within close proximity to labor and delivery, eg, 0-48 hours) but are nonspecific to asphyxial insults

- A sentinel (signal) hypoxic event occurring immediately before or during labor
- A sudden and sustained fetal bradycardia or the absence of fetal heart rate variability in the presence of persistent, late, or variable decelerations, usually after a hypoxic sentinel event when the pattern was previously normal
- Apgar scores of 0-3 beyond 5 minutes
- Onset of multisystem involvement within 72 hours of birth
- Early imaging study showing evidence of acute nonfocal cerebral abnormality

Neonatal encephalopathy is a clinically defined syndrome of disturbed neurological function in the infant at or near term during the first week after birth, manifested by difficulty with initiating and maintaining respiration, depression of tone and reflexes, altered level of consciousness, and often seizures. Sarnat and Sarnat (Sarnat & Sarnat, 1976) were the first to define this syndrome as neonatal encephalopathy following foetal distress (TABLE 1). The clinical features and severity of encephalopathy have been well defined. They distinguished three stages of encephalopathy: stage 1, or mild encephalopathy associated with hyperalertness, sympathetic overdrive, and a normal EEG; stage 2, or moderate encephalopathy marked by obtundation, hypotonia, multifocal seizures, and an EEG showing periodic or continuous delta activity; and stage 3, or severe encephalopathy in which infants were stuporous and flaccid with an isoelectric or periodic EEG. Infants who did not enter stage 3 and who had signs of stage 2 for fewer than 5 days were normal on follow-up, but persistence of stage 2 for a week or failure of the EEG to normalise predicted later neurological impairment or death.

	State 1 Mild	Stage 2 Moderate	Stage 3 Severe
Level of Consciousness	Hyperalert	Lethargic or obtunded	Stuporous
Neuromuscular Control			
Muscle tone	Normal	Mild hypotonia	Flaccid
Posture	Mild distal flexion	Strong distal flexion	Intermittent decerebration
Stretch reflexes	Overactive	Overactive	Decreased or absent
Segmental myoclonus	Present	Present	Absent
Complex Reflexes			
Suck	Weak	Weak or absent	Absent
Moro	Strong; low threshold	Weak; incomplete; high threshold	Absent
Oculovestibular	Normal	Overactive	Weak or absent
Tonic neck	Slight	Strong	Absent
Autonomic Function	Generalized sympathetic	Generalized parasympathetic	Both systems depressed
Pupils	Mydriasis	Miosis	Variable; often unequal; poor light reflex
Heart Rate	Tachycardia	Bradycardia	Variable
Bronchial and Salivary Secretions	Sparse	Profuse	Variable
Gastrointestinal Motility	Normal or decreased	Increased; diarrhea	Variable
Seizures	None	Common; focal or multifocal	Uncommon (excluding decerebration)
Electroencephalogram Findings	Normal (awake)	Early: low-voltage continuous delta and theta Later: periodic pattern (awake) Seizures: focal 1-to 1-Hz spike-and-wave	Early: periodic pattern with isopotential phases Later: totally isopotential
Duration	<24 h	2-14 h	Hours to weeks

Table 1. Sarnat and Sarnat's [236] Clinical Stages of Perinatal Hypoxic Ischemic Brain Injury

The diagnosis HIE has often not been proven and has been assumed from a variety of clinical markers that do not accurately reflect hypoxia and ischaemia of either acute or chronic origin. Over 75% of cases of neonatal encephalopathy have no clinical signs of intrapartum hypoxia. Dammann et al (Dammann et al, 2011), under the title "Neonatal Encephalopathy or Hypoxic-

Ischemic Encephalopathy? Appropriate Terminology Matters” propose that by simply calling “neonatal encephalopathy” what is now called “HIE,” we might not only help reduce the number of unjustified convictions of obstetricians, midwives, nurses, and hospitals but also increase the amount of much needed research in perinatal brain injury not necessarily related to the hypoxia-ischemia paradigm.

Although in many newborns who have cerebral dysfunction, the brain injury may have occurred before the onset of labor and delivery, there exists a relatively small but clearly identifiable group of term newborns who sustain significant intrapartum H-I cerebral insult and who subsequently develop an acute encephalopathy during the first week of life. The Sarnat evaluation of staging of encephalopathy by clinical examination correlates well with subsequent neurodevelopmental impairment in infancy and childhood (Robertson, 2003; Badawi et al, 2005; Ambalavanan et al, 2006). The presence of abnormal neurologic examination results in the first few days of life highly predicts a brain insult in the perinatal period. Neonates with mild encephalopathy usually do not have an increased risk of motor or cognitive deficits. Neonates with severe encephalopathy have a high risk of death (up to 85%) and an increased risk of CP and intellectual disability among survivors. Neonates with moderate encephalopathy have significant motor deficits, fine motor disability, memory impairment, visual or visuomotor dysfunction, increased hyperactivity and delayed school readiness (Shankaran et al, 1991. Robertson, 2003; Marlow et al, 2005; Gonzalez & Miller, 2006. De Vries & Jongmans, 2010).

3. Neuropathologic aspects of hypoxic–ischemic brain damage

3.1. Gestational/perinatal age

H-I insults during critical cellular and tissue differentiation processes have a serious impact on brain maturation. Thus, the gestational/perinatal age of the infant is one of the main variables in determining the neuropathological picture of H-I brain injury. For this reason alone, the developmental stage at which a H-I insult occurs is of great importance. However, the mechanism(s) underlying the neuronal damage and death triggered by H-I in the developing brain leading to various forms of neurological disabilities remains inadequately understood. Due to selective ischemic vulnerability, the damage affects the gray matter in term newborns and white matter (WM) in preterm newborns with the typical neuropathological aspects of laminar cortical necrosis in the former and periventricular leukomalacia (PVL) in the latter (Volpe, 2003).

Reactive oxygen species (ROS) play a pivotal role in the development of PVL. The major type of injury involves cerebral white matter and the principal cellular target is the developing oligodendrocyte. The specific phase of the oligodendroglial lineage affected has been defined from the study of both human brain and experimental models. This premyelinating cell (pre-OL) is vulnerable because of a series of maturation-dependent events. The pathogenesis of pre-OL injury involves two upstream mechanisms, H-I and systemic infection/inflammation, both of which are common occurrences in premature infants. This differential susceptibility to

injury can also be related to the development of interneuronal connections and excitatory glutamate receptors that create the possibility of excessive neurotransmitter release and receptor overstimulation. Moreover, most forms of H-I in neonates cause injury to selected areas of the brain rather than the entire brain (Johnston, 2001; Johnston et al, 2001). Most importantly, elucidation of these factors has led to delineation of a series of potential therapeutic interventions, which in experimental models show marked protective properties. The critical next step, i.e., clinical trials in the living infant, is now on the horizon (Volpe et al, 2011).

3.2. Developing human cortex

The subplate zone (SPZ) is a transient cytoarchitectonic compartment of the fetal telencephalic wall, situated between the fetal WM (i.e. intermediate zone) and the cortical plate, and is the crucial laminar compartment for the development of the human cerebral cortex. The subplate contains numerous neurons of various morphological types and molecular phenotypes, including differentiated projection (glutamatergic) neurons and local (GABA and peptidergic) interneurons. The developing human cortex goes through three major early stages of functional development: (1) between 13 and 15 postconceptional weeks (PCW): initial-transient fetal circuitry, centered at the SPZ, which is endogeneously (spontaneously) driven; (2) 15 and 30 PCW: perinatal dual circuitry (co-existence of endogeneously driven subplate-centered transient circuitry with developing cortical plate-centered permanent circuitry), that slowly disappears towards the end of gestation and during the early postnatal period; and (3) postnatally established permanent (externally driven) cortical circuitry, centered at the cortical plate (that is, developing cortical layers I-VI). While the SPZ disappears during the perinatal and early postnatal period, numerous subplate neurons survive and remain embedded in the superficial (gyral) WM of adolescent and adult brain as so-called *interstitial neurons* (Judaš et al, 2010). The growth of the axonal pathways preterm explains their vulnerability and plasticity. In neonates the vulnerability is related to the intracortical circuitry. The neuronal elements in transient fetal zones form a developmental potential for plasticity after perinatal cerebral lesions (Kostovic & Judas, 2006).

3.3. Gender differences

This new information about gender differences in neuronal death pathways in experimental models is probably directly relevant to gender differences reported in the response of infants and children to brain injuries. Quantitative imaging showed that male premature infants are more vulnerable than girls to white matter injury from intraventricular hemorrhage (IVH), but girls are more vulnerable to gray matter injury. It follows directly from this information that an infant's gender could influence the efficacy of neuroprotective agents and the cell types most at risk. A striking example of this effect was reported from the prospective "Indomethacin Intraventricular Hemorrhage Prevention Trial" (Ment et al, 2004). In this study, indomethacin eliminated parenchymal hemorrhage and improved verbal scores in boys at ages 3 to 8 years, but had no effect on girls. "It is becoming increasingly clear that gender differences are not simply a result of hormonal influence but are profound properties of individual cells" (Fatemi et al, 2009).

4. Physiopathological and biochemical processes of H-I cerebral injury

The principal biochemical mechanisms of cellular death in hypoxemia, ischemia and asphyxia are very similar and derive from O₂ deprivation. Besides the main role of perinatal asphyxia, a key factor in the genesis of HIE is the loss of “cerebral blood flow (CBF) autoregulation”, a protective mechanism that maintains stable CBF velocity (CBFV) in normal infants, regardless of variations of systemic arterial pressure. At the cellular level, the reduction in CBF and oxygen delivery initiates a cascade of deleterious biochemical events. Clinical and experimental observations demonstrate that HIE is not a single “event” but is rather an “evolving process”, and reflect the evolution of a delayed cascade of molecular events triggered by the initial insult (Fatemi et al, 2009).

4.1. Physiopathological mechanisms of cerebral injury

H-I injuries develop in two phases: the ischemic phase, dominated by necrotic processes, and the reperfusion phase, dominated by apoptotic processes extending beyond ischemic areas. This second phase takes place two - six hours after H-I insult, such latency constituting a useful window in which therapeutic measures can be able to stop the evolution of cerebral damage (Hammerman & Kaplan, 2000; Inder & Volpe, 2000).

4.1.1. Acute injury: Primary energy failure (associated with anaerobic metabolism)

There is an initial immediate phase (FIGURE 1) characterized by alterations in glucose metabolism; the anaerobic metabolism is an energy-inefficient state, since anaerobic glycolysis produces lactate and only 2 ATP, whereas aerobic glycolysis produces 38 ATP. HI rapidly lowers the neonatal brain's stores of glucose and high energy phosphates (ATP and phosphocreatine), resulting in energy depletion and accumulation of lactate and inorganic phosphate; and a metabolic acidosis develops due to accumulation of lactic acid, with local and systemic consequences such as impaired vascular tone and cardiac contractility. In this phase, the crucial event triggering a cascade of chain reactions is represented by ATP depletion secondary to anaerobic glycolysis and metabolic acidosis induced by hypoxia. Reduced ATP availability determines the dysfunction of ATPase systems, in particular Na⁺, K⁺-ATPase and glial-ATPase. Na⁺, K⁺-ATPase dysfunction leads to membrane depolarization in neurons mainly causing intracellular calcium accumulation, and sodium and water accumulation with cytotoxic edema and/or cell lysis followed by inflammatory reaction with cytokines release. Disturbances of intracellular calcium homeostasis result in activation of calpains (*calcium-activated proteases*). At the same time, neuronal depolarization induces glutamate release which tends to accumulate in the intersynaptic and intercellular spaces because of the dysfunction of glial-ATPase (an astrocytic enzyme normally in charge of glutamate reuptake). Thus extracellular glutamate increases due to both enhanced release and lower recapture. Glutamate, a neuroexcitatory amino acid, stimulates specific NMDA and AMPA neuro-glial receptors and hence determines a massive intracellular entry of calcium that activates some endocellular enzymes including proteases, endonucleases and phospholipases. Proteases degrade neurofilaments causing cytoskeleton rupture and disintegration of the cellular body. Furthermore,

excessive amount of glutamate can cause excitotoxicity leading to cell death of neurons and glial cells. These events involve major mechanisms of fast neuronal death (edema, inflammation, necrosis) due to either direct or indirect neurotoxicity [mediated by free-radicals (FR) and nitric oxide (NO) generated during the first minutes / hours of anoxic insult]. In asphyxiated human neonates, the extent of depletion of high-energy phosphates, and the extent of accumulation of lactate, measured by magnetic resonance spectroscopy, correlate with the severity of eventual neurologic impairment (Hanrahan et al, 1999).

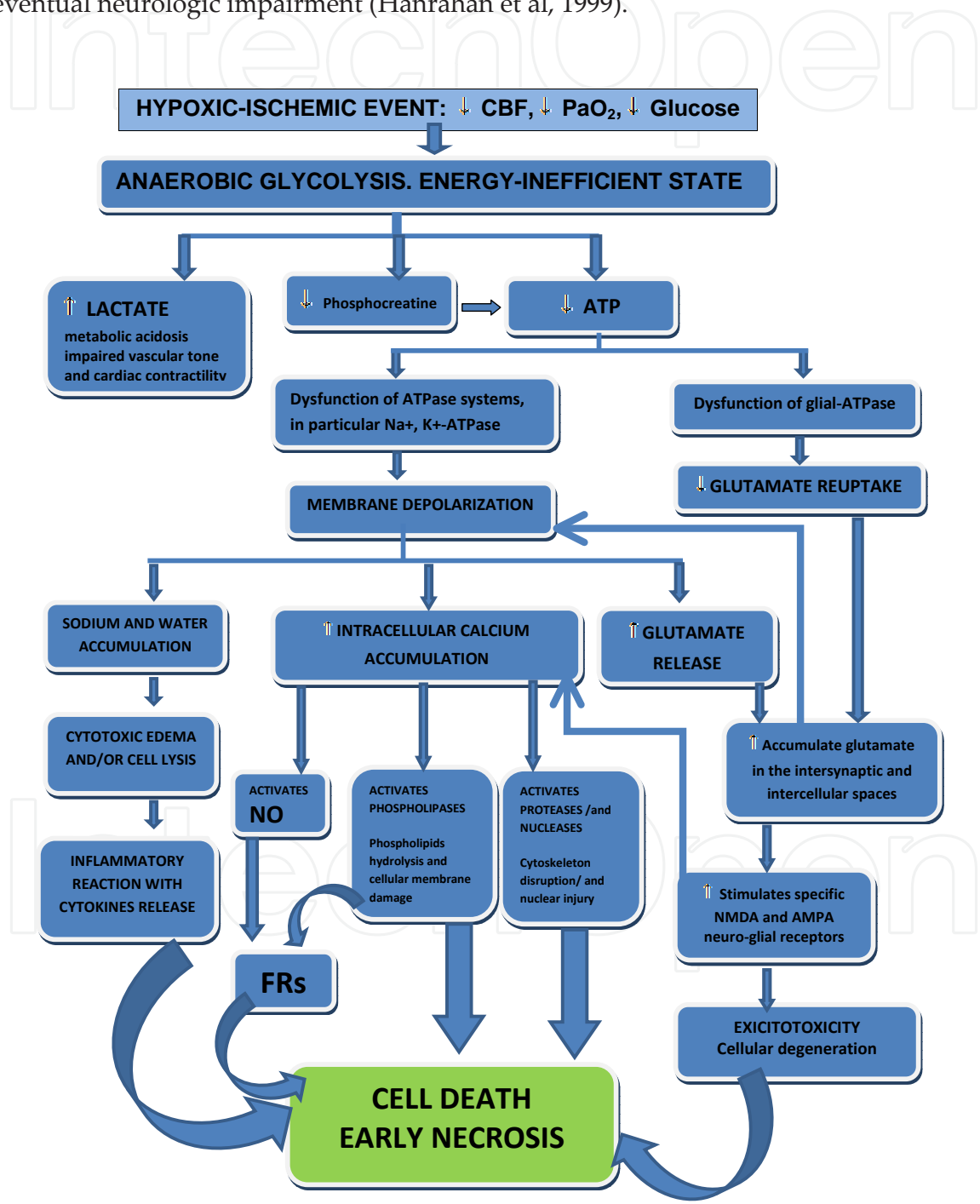


Figure 1. ACUTE INJURY: Primary energy failure (associated with anaerobic metabolism).

4.1.2. *Delayed brain damage: Secondary energy failure (associated with reperfusion)*

Secondly, a complex cascade of pathogenic mechanisms associated with reperfusion (or recovery of the blood flow) is triggered and this response is proportional to the severity of the first response. The secondary cerebral energy failure, also known as "delayed injury" (FIGURE 2), occurs from 6 to 48 hours after the primary event and may continue for days or weeks. Extended reactions from primary insults (eg, calcium influx, excitatory neurotoxicity, oxygen free radicals, or NO formation) secondary involve mitochondrial dysfunction. This mitochondrial impairment may lead directly to caspase-dependent and -independent apoptosis. This second phase also has an undoubtable prognostic value since it is associated with delayed neuronal death (apoptosis) during hours or days after the initiation of injury, and represents a window for therapeutic intervention. The exact mechanisms of secondary energy failure remain unclear but appear to be related to oxidative stress, excitotoxicity, and inflammation. In this phase, recovery of ischemia increases O₂ availability and hence activates xanthine-oxidase (via metabolizing the hypoxanthine formed during the initial period of HIE to uric acid) and cyclooxygenase enzymes and generates reactive oxygen species (ROS) responsible for oxidative cellular damage. Due to the increased intracellular calcium from the previous period, the reperfusion phase shows a sustained activation of phospholipase A₂ that hydrolyze phospholipids and can damage cellular membrane and induce the consequent release of free fatty acids (FFA), especially arachidonic acid (AA). Reperfusion also activates cyclooxygenase that catalyses the formation of prostaglandins and generate -among others- superoxide free radicals. The production of vasodilator prostaglandins that give rise to reperfusion of ischemia and build-up of platelet-activating factor (PLF) in brain tissues. Collectively, these processes lead to a surge of the superoxide free radical, which plays a central role in further production of free radicals and other toxic compounds (Lorek et al, 1994).

4.2. Biochemical mechanisms of injury

Key players in the pathophysiology of neonatal cerebral injury are accumulation of cytosolic calcium, oxidative stress, excitotoxicity, and inflammation leading to apoptotic and necrotic neuronal death (Grow & Barks, 2002; Hossain, 2005).

4.2.1. *Oxidative stress: Formation of free radicals (FRs)*

Definitions of Oxidative stress: The imbalance between free radical (FR) generation and free radical scavenging that leads to cell injury.

All biological systems that consume oxygen generate FRs, which are molecules with one or more unpaired electrons in their outer orbit. They readily accept electrons from iron and other metals to form more reactive radicals, which attack other biomolecules, especially lipids, proteins, and nucleic acids, generating more radicals that damage the developing brain. In aerobic cells, oxygen free radicals (reactive oxygen species –ROS–) are produced within the cytoplasm and mitochondria. The three most common ROS are superoxide (O₂⁻), hydroxyl radical (OH⁻), and hydrogen peroxide (H₂O₂). Two important sources of ROS are the byproducts of xanthine (derived from the breakdown of ATP) and prostaglandin synthesis (derived

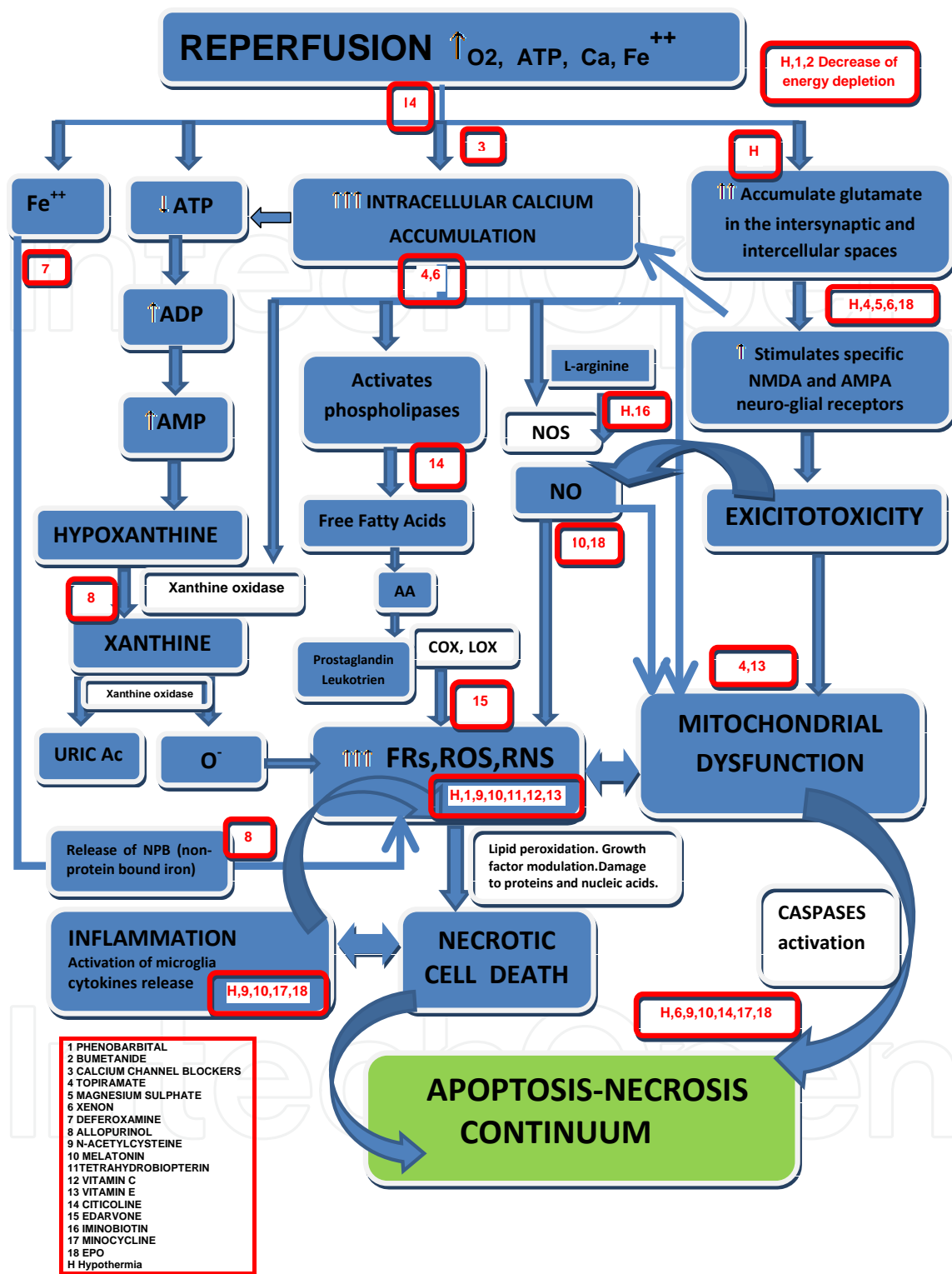


Figure 2. DELAYED INJURY: Secondary energy failure (associated with reperfusion). Neuroprotective interventions drugs according to predominants effects.

from the breakdown of free fatty acids). Low levels of ROS are indispensable in many biochemical processes, including intracellular messaging in the cell differentiation and cell

progression or the arrest of growth, apoptosis, immunity, and the defense against microorganisms. Interestingly, more recent data strongly suggest that low levels of NO and ROS are involved in normal events such as gene transduction control (Kroncke, 2003). In contrast, high doses and/or inadequate removal of ROS result in oxidative stress, which may cause severe metabolic malfunctions and damage to biological macromolecules. Increased production of ROS contributes to the pathogenesis of neonatal H-I brain injury. Acute restoration of blood flow after ischemia leads to the production of ROS, which are directly toxic to neurons and glia, and which may exacerbate leukocyte accumulation, microvascular thrombosis, and NO-mediated injury (Matés et al 1999).

Under normal physiologic conditions, low concentrations of O_2^- and H_2O_2 are produced as a byproduct of mitochondrial electron transport, a balance is maintained between the production of ROS and the capacity of the antioxidant enzyme system; however, if this balance breaks down, ROS can exert toxic effects. The oxygen FR are destroyed rapidly by the endogenous antioxidants: they are scavenged enzymatically by superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), and nonenzymatically by reaction with antioxidant molecules, such as alpha-tocopherol (vitamin E), and ascorbic acid (vitamin C), glutathione (GSH), b-carotene, and vitamin A (Grow & Barks, 2002). Treatment with the iron chelator deferoxamine, N-acetylcysteine (NAC), or with allopurinol (a xanthine oxidase inhibitor that also acts as a FR scavenger), attenuates H-I damage in the immature rat (Palmer et al, 1993; Palmer et al, 1994). A recently discovered antioxidant enzyme family, peroxiredoxin (Prdx), is also an important scavenger of FR: Prdx1 expression is induced at birth, whereas Prdx2 is constitutively expressed, and Prdx6 expression is consistent with the classical antioxidant enzymes (SOD, CAT, and GPX) (Shim & Kim, 2013).

It is recognized that during intra- and peripartum asphyxia the generation of ROS is increased exceeding the capabilities of the protective mechanisms and causing oxidative stress. FRs produced from xanthine by products and prostaglandin synthesis attack polyunsaturated fatty acids (PUFAs) of the plasma membrane, increasing membrane permeability, endothelial cell death compromises the blood-brain barrier (BBB), resulting in vasogenic edema and hemorrhage. The mitochondrial respiratory chain is also a major source of ROS, and mitochondrial dysfunction contributes to cellular necrosis as well. FRs are another example of the inability of the immature brain to handle reoxygenation. Although FR concentrations rise during hypoxia, a significant secondary elevation occurs during resuscitation.

The relationships between FR generation and perinatal brain damage are complex there are a number of potential mechanisms of FR generation (Mishra & Delivoria-Papadopoulos, 1999; Robertson & Finer, 1993):

1. Accumulation of intracellular Ca^{2+} and subsequent activation of:

- **Phospholipase a2** leading to increased generation of oxygen FRs from cyclooxygenase (COX) and lipoxygenase (LOX) pathways, and **phospholipase C** leading to IP3 formation resulting in release of Ca^{2+} from intracellular stores. Neonates also have high concentrations of PUFAs that break down to form more oxygen FR (Shalak & Perlman,

2004). ROS contribute to tissue injury by attacking the PUFAs component of the cellular membrane, resulting in membrane fragmentation and cell death.

- **Nitric oxide synthase (NOS)** leading to peroxynitrite formation and generation of FRs. NO is a weak free radical formed during the conversion of L-arginine to L-citrulline by NO synthase (NOS). NOS is strongly activated during H-I and reperfusion, producing a large amount of NO for extended periods. When combined with superoxide, NO generates a potent radical, peroxynitrite, which activates lipid peroxidation. In addition, NO enhances glutamate release (Palmer, 1995). The generation of ROS which interact with endothelial cell-derived NO leading to the formation of reactive nitrogen species (RNS). ROS and RNS target endothelial cells and neuronal cells, and both the oxidative stress and the nitrosative stress have been implicated in animal models of perinatal brain damage (Grow & Barks, 2002; McQuillen & Ferriero, 2004).
 - **Proteases** leading to conversion of **xanthine oxidase** resulting in increased FR generation.
2. **Reduction of electron transport chain components including ubiquinone** (a component that undergoes autooxidation to produce FRs) (Turrens et al, 1985).
 3. **Release of iron from ferritin under the condition of depleted cellular high energy compounds.** Physiologically, iron is maintained in a nontoxic ferric state and is bound to proteins (ferritin, transferrin). During H-I, free ferric iron is released from these proteins and reacts with peroxides to generate potent hydroxyl radicals. In addition, free ferric iron is reduced to a ferrous form, which further contributes to FR injury. Iron is a major mediator of cell damage. Differentiating OLs are particularly susceptible to FR damage because they are rich in iron, which is required for differentiation.

and

4. **Increased degradation of ATP during hypoxia and increased substrate for the xanthine oxidase reaction** Hypoxanthine accumulates as a product of ATP degradation and cannot be reconverted to ATP by the salvage pathway in anaerobic conditions. Xanthine dehydrogenase is converted to xanthine oxidase through the activation of a specific protease by calcium and this reaction produces ROS.

4.2.2. *Excitotoxicity (Excitatory neurotransmitter release)*

Definitions of Excitotoxicity: Excessive levels of extracellular neurotransmitters (NT's) causing excitatory overstimulation and neuronal damage.

The fundamental process responsible for H-I damage of neurons is called excitotoxicity, a term popularized in 1970s by John Olney that refers to cell death caused by excessive stimulation of extracellular excitatory amino acid (EAAs) receptors (Choi & Rothman, 1990). Both hypoxia and ischemia result in the failure of energy-dependent ion pumps and causes cellular depolarization, increasing the release of EAAs -glutamate and aspartate- into the extracellular space in the cerebral cortex and basal ganglia.

The amino acid glutamate is the major excitatory NTs in the central nervous system (CNS). During neurotransmission, glutamate is released from pre-synaptic neurons by means of depolarisation of the pre-synaptic neuronal endplate, and then diffuses across the synaptic cleft to activate post-synaptic glutamatergic receptors. Several studies indicate that glutamate receptor-mediated excitotoxicity is a key player in neuronal cell death and that it is more critically involved in the developing brain than in the adult brain (Johnston, 2001). The neuronal injury occurring with cerebral H-I has been attributed to overstimulation of the Nmethyl-D-aspartate (NMDA) and alfa-amino-3-hydroxy- 5-methyl-4-isoxazole-propionic acid (AMPA) subtypes of excitatory amino acid glutamate receptors. Acute energy deprivation leads to the excessive release of extracellular glutamate and uncontrolled activation of ionotropic glutamate receptors NMDA, AMPA, and kainate, impedes energy-dependent reuptake of glutamate and causes the rise in intracellular Ca^{2+} concentration. The massively increased levels of intracellular second messenger Ca^{2+} trigger activation of toxic intracellular pathways involving kinases, phosphatases, proteases, endonucleases, FRs, mitochondrial dysfunction, inflammation, DNA damage, and, ultimately, irreversible neuronal injury and death (Choi & Rothman, 1990; McDonald, 1998). Another important component of glutamate synaptic dysfunction caused by H-I is postsynaptic neuronal membrane depolarization with secondary opening of voltage-sensitive channels. Membrane depolarization due to high levels of synaptic glutamate produces maximum channel opening and flooding of calcium and sodium into neurons. Although high levels of glutamate can produce some degree of membrane depolarization under normal mitochondrial function, maximal excitotoxicity probably occurs when there is synergism between high synaptic glutamate levels due to disruption of glucose delivery and oxidative stress. The prominent role of the NMDA receptor-channel complex in perinatal H-I is related in part to its special transient role in brain development. The NMDA receptor subunits in the developing brain create populations of receptors that input more calcium, open more easily and block less frequently than mature forms, allowing them to serve these special developmental roles. However, this makes the immature brain more susceptible to excitotoxic injury if critical levels of energy failure are exceeded. For instance, neurotoxicity mediated by NMDA is more enhanced in the neonatal brain than the adult brain. Besides, NMDA receptors play an important role in activity-dependent neuronal plasticity during development. Therefore, development-dependent changes in the expression of NMDA receptor subunits and their composition are, at least in part, responsible for the fact that immature brains are far more excitable and epileptogenic than the adult brain.

A second important mechanism for the destruction of ion pumps is the lipid peroxidation of cell membranes, where enzyme systems, such as the Na^+/K^+ -ATPase, are located. This leads to water influx and cell swelling, causing cell death. EAAs also increase the local release of NO, which may exacerbate neuronal damage, although its mechanisms are unclear. It is quite possible that EAAs disrupt factors that normally control apoptosis, increasing the pace and extent of programmed cell death. The regional differences in injury severity may be explained by the fact that EAAs particularly affect the hippocampus, the developing oligodendroglia, and the subplate neurons along the borders of the periventricular region in the developing brain. This may be the basis for the disruption of long-term learning and memory faculties in infants with HIE (Barks & Silverstein, 1992).

4.2.3. *Inflammatory mediators (Microglial and astrocyte activation)*

Neuroinflammation, caused by activated microglia and astrocytes, plays a key role in the pathogenesis of CP. Maternal intrauterine infection and inflammation are risk factors for the development of PVL (characterized by focal necrosis around the ventricles, and diffuse microglial and astrocyte activation in the immature WM) and CP in the neonate (Haynes et al, 2003; Leviton et al, 2010). The microglia-immune cells in the brain-, play an important role in remodeling and growth during the fetal and postnatal periods, and they are proposed to be involved in the pathophysiological mechanism for the development of CP in humans. Activation of these cells can result in an exaggerated inflammatory response with formation of FR, excitotoxic metabolites, and pro-inflammatory cytokines, leading to brain injury (Dommergues et al, 2003; Li et al, 2008). In severe inflammation, astrocytes that normally participate in the protection of neurons and in preventing oxidative injury, are unable to maintain their neuroprotective role (Maragakis & Rothstein, 2006). H-I injury in the neonate is progressive, producing lesions of variable severity including focal necrotic cell death, diffuse WM injury, cystic or cavitory infarction and the resulting neuropathies linked to the activation of neuroinflammatory processes (inflammatory cytokines, chemokines, and matrix metalloproteinase (MMP) activity) that occur in response to the initial wave of cell death.

Cytokines may be classified as interleukins (IL), interferons, tumor necrosis factors, chemokines (proteins that stimulate leukocyte motility), and growth factors. Systemically, cytokines may be “proinflammatory” (eg, IL-1b, TNFa, IL-6, IL-8) or “antiinflammatory” (eg, IL-4, IL-10, TGFa). However, this subdivision may not apply to the CNS. Cytokines that are produced peripherally can send signals to the brain, but more importantly, they are produced locally within the CNS in response to acute insults, including H-I. Cytokines may act on neurons, astrocytes, microglia, and endothelium, and may influence CNS injury indirectly by way of systemic parameters, such as blood flow and temperature. Cytokines can also play trophic roles during development, and their effects may differ depending on cell type (Grow & Barks, 2002). In experimental models, there is strong evidence that increased IL-1b gene expression and bioactivity after HI contributes to the pathogenesis of H-I injury in the immature and adult brain, (Hagberg et al, 1996). Clinical data also suggest that inflammatory mediators play a role in the pathogenesis of H-I brain injury: In human infants, CSF IL-6 and IL-8 concentrations are increased after birth asphyxia in comparison with controls; the magnitude of the increases correlates with the severity of the encephalopathy (Savman et al, 1998).

Chemokines are cytokines that regulate leukocyte migration and activation. H-I induces increased expression of two potent monocyte chemokines (MCP-1 and MIP-1a). This chemokine response precedes, and may mediate, the recruitment and activation of monocytes and microglia. There is no direct evidence that these chemokines contribute to neonatal HI brain injury. A large body of evidence implicates neutrophils and leukocyte adhesion molecules in the pathogenesis of focal cerebral ischemia in the adult brain, but there is only limited information to substantiate a role for these mediators in the pathogenesis of neonatal cerebral H-I (Hudome et al, 1997).

Neural injury after H-I is exacerbated due to neuroinflammatory signaling from activated microglia and peripheral infiltration of macrophage, cell death via necrotic and apoptotic

mechanisms, and astrogliosis. Activation of microglia and macrophage infiltration is associated with the necessary phagocytosis of cellular debris, but it also results in a burden of increased production of neurotoxic substances, such as RNS and ROS, pro-inflammatory cytokines, and the NMDA agonist quinolinic acid. Thus, prolonged activation of these monocyte-derived cells for at least a week may elicit further deleterious changes in the brain (Nakajima & Kohsaka, 2004).

4.3. Mechanisms of neuronal cell death after hypoxia-ischemia

Excitotoxic cell death occurs through necrosis and/or apoptosis (also known as programmed cell death), the balance between modes of death may be influenced by the severity of the insult, cell phenotype and location, and maturational stage. One important characteristic feature of ischemia is selective neuronal necrosis (SNN), which is characterized predominantly by neuronal death whereas the astroglial cells are spared, at least initially. Necrosis predominates in more severely affected areas; apoptosis is seen more in penumbral areas of the injury. Several important features distinguish apoptotic from necrotic death (Northington et al, 2001):

1. Apoptosis is a physiologic process. Is the mechanism for refining cell connections and pathways during development by removing many neurons that will not be needed in adulthood, it is an essential part of normal brain development. This fact may underlie the observation that the propensity for experimental H-I to induce apoptosis peaks in the early, postnatal period.
2. Apoptosis is an active process, dependent on activation of a family of proteases called caspases that are modulated by other proteins (eg, those of the Bcl-2 family). The biochemical cascade of apoptosis can be blocked pharmacologically at several points.
3. Necrosis is characterized by cell swelling and eventual loss of cell membrane integrity followed by cell lysis. Resultant inflammation is a significant part of necrotic death. In contrast, apoptosis is an energy-dependent process and is characterized by a shrinking of the cytoplasm, condensation of the nucleus and eventual fragmentation of the cell body into smaller bodies. Markers of apoptosis such as cytoplasmic and nuclear condensation, as well as nuclear DNA fragmentation, appear in neurones, particularly in the infarct and penumbra of cerebral injury.
4. The time course of apoptotic death in H-I is slower than that of necrotic death. This feature may provide a more prolonged window of opportunity for therapeutic intervention than for necrotic death.

Early H-I-induced neuronal death occurs through necrosis (primary damage). Delayed neuronal death (secondary damage) occurs hours or days later through a series of complex and highly regulated biochemical and molecular events leading to apoptosis. Accumulating data suggest that apoptosis plays a prominent role in the evolution of H-I injury in the neonatal brain and may be more important than necrosis after injury. During neonatal brain injury, excitotoxicity, oxidative stress, and inflammation all contribute to accelerated cell death by means of either apoptosis or necrosis, depending on the region of the brain affected and the

severity of the insult (Blaschke, 1996). This apoptosis–necrosis morphological continuum of neuronal death after H-I is similar to that observed in neonatal rats after excitotoxic activation of NMDA and non-NMDA glutamate receptors, suggesting that H-I neuronal injury is triggered by the excitotoxic cascade (Portera-Cailliau, 1997; Martin, 1998; Bittigau, 1999).

Recent explosive progress toward dissecting the molecular basis of apoptosis revealed the existence of family acting proteases, known as caspases. These protein-splitting enzymes act in a cascade form when signals are transmitted from the various receptors. Caspases sequentially activate each other and several proapoptotic protein kinases and disable DNA repair mechanisms (e.g., by cleaving poly (ADP-ribose) polymerase, PARP). Apoptosis can be elicited through two pathways:

- Intrinsic pathway: in which translocation of cytochrome c from the mitochondria to the cytoplasm is an early step; when translocated cytochrome c combines with ATP, apoptosis protease activating factor-1 (Apaf-1) and procaspase-9, caspase-9 is cleaved to its active state.
- Extrinsic pathway initiated by cell membrane death receptor activation (e.g. TNF receptor). Death receptor-ligand interactions lead to caspase-8 activation.

Activation of either caspase-8 or -9 (initiator caspases) leads to cleavage and activation of caspase-3 (effector caspase). Caspase activation is modulated by the BCL-2 family of proteins, which includes proapoptotic (eg, Bax) and antiapoptotic (eg, Bcl-XL) members. ROS can induce apoptosis in neurons. This effect is mediated by mitochondrial cytochrome c release (Green, 2000). Current data implicate the caspase-9 pathway as predominant in acute neuronal injury. Ca^{2+} is an important inducing agent in the mitochondria-dependent apoptotic pathway. Increased free cytosolic Ca^{2+} may lead to uncoupling of mitochondrial oxidative phosphorylation, inducing the mitochondrial the release of cytochrome c. Once released from mitochondria, cytochrome c specifically activates caspase 3, which triggers a biochemical cascade involving activation of many other caspases and other substrates. Known substrates of activated caspase-3 include PARP (a nuclear enzyme that participates in DNA repair), and DNA-dependent kinase, endonucleases, and cytoskeletal proteins (eg, fodrins, actin, lamin). Caspase-3 is strongly activated in animal models of H-I, resulting in increased activity of PARP in neuronal nuclei, indicating activation of the DNA repair pathway (Mehmet, 1994). On the other hand, pancaspase inhibitors are strongly neuroprotective when given several hours after the insult (McDonald, 1997).

Severe cerebral ischemia also results in a major increase in intracellular Ca^{2+} , which is closely related to NO. Both play a fundamental role in signal transduction –controlling cell processes such as proliferation (Ashkenazi, 1998). NO has several important physiologic functions in the CNS. These include control of central and peripheral functions, modulation of synaptic plasticity, perception of pain and neuronal damage, and protection. High NO levels may be neurotoxic and induce apoptosis or necrosis. Changes in intracellular Ca^{2+} or NO may alternatively lead to blockade or activation of the cell cycle, and the decision as to whether the cell lives or dies is presumably a well-regulated phenomenon in which the duration and intensity of the Ca^{2+} and NO signals may play a fundamental role. Identification of enzymes

and substrates for these phosphorylation pathways would shed light on hypoxia-induced processes leading to cell recovery or death (Esplugues, 2002).

NMDA channel overactivity, calcium entry into neurons, production of NO, and mitochondrial dysfunction are intimately linked in a potentially lethal cycle. There is abundant evidence that excitotoxic injury to neuronal mitochondria is linked to activation of neuronal death, and mitochondria appear to play a central role in determining expression of injury as necrosis and/or apoptosis, determining whether neurons live or die following hypoxic-ischemic insult. The death receptor and mitochondrial apoptosis pathways converge at the caspases that cleave multiple cellular substrates, ending in DNA fragmentation and cell death. It is noteworthy that activation of apoptosis-executing caspases is much greater in the immature brain than in the adult brain (Hu, 2000).

From the above it follows, that one of the main difficulties for developing a neuroprotective pharmacotherapy is the existence of multiple cellular death mechanisms, occurring in different cells or even in the same cell, which may all need to be inhibited. In fact, the widely accepted apoptosis-necrosis dichotomy is being replaced by a more complex view involving a third type of cell death, named autophagic cell death, characterized by the presence of intense autophagy (Clarke, 2008). Whereas the roles of apoptosis and necrosis in such conditions have been studied intensively, the implication of autophagic cell death has only recently been considered. Autophagy is an essential pathway for the degradation and recycling of intracellular macromolecules. The most important autophagic mechanism, macroautophagy, consists in the sequestration of long-lived proteins and damaged organelles in multimembrane vesicles, named autophagosomes, which then fuse with lysosomes to degrade their contents. Whereas basal macroautophagy (called hereafter autophagy) plays a central physiological function in maintaining cellular homeostasis, induced autophagy may have both survival and deleterious roles. In neurons, autophagy has been demonstrated to be induced during development, starvation, neurodegeneration, and also after different excitotoxic stimuli. More recently, an involvement of enhanced autophagy in neuronal death following cerebral ischemia has been proposed (Koike, 2008).

4.4. Neurogenetic and gliogenetic processes after H-I injury

Recent studies in brains damaged by H-I insult have demonstrated that some cellular mechanisms can be activated in order to repair cerebral injuries. These mechanisms involve the neural stem/progenitor cells (NSPs) normally resident in the subventricular zone (SVZ) of the mammalian brain that can be stimulated by H-I to proliferate and differentiate into both neurons and OLs. These new cells are likely to play an important role in repairing neuronal and glial losses related to HIE. Perinatal H-I results in brain injury, whereas mild hypoxic episodes result in preconditioning, which can significantly reduce the vulnerability of the brain to subsequent severe H-I. "In vivo", hypoxic-preconditioning has been shown to enhance cell survival and differentiation of progenitor cells in the CNS, stimulating SVZ proliferation and neurogenesis. This phenomenon may be a positive adaptation for an efficient repair and plasticity in the event of a H-I insult (Ara, 2013). Therefore, the NSPs

in the SVZ could be a valuable target for therapeutic strategies to enhance recovery after cerebral H-I injury (Scafidi, 2008).

5. Therapeutic window

Brain injury following H-I insult is a complex process evolving over hours to days, which provides a unique window of opportunity for neuroprotective treatment interventions. The seminal concept emerging from both experimental and clinical studies is that brain cell death does not necessarily occur during H-I (the 'primary' phase of injury), but rather that the injurious event may precipitate a cascade of biochemical processes leading to delayed cell death, hours or even days afterwards (the 'secondary' phase). Experimental studies in piglets, immature rats, and fetal sheep have demonstrated the existence of both a primary phase of energy failure during H-I, a 'latent' phase during which oxidative metabolism normalizes, followed by secondary failure of oxidative metabolism. Consistent with these studies, although with exceptions (some newborn infants exposed to profound asphyxia show no initial recovery of oxidative metabolism after birth and typically have very severe brain injury and high mortality), in many cases infants show initial, transient recovery of cerebral oxidative metabolism followed by a secondary deterioration, with cerebral energy failure from 6 to 15 hours after birth. The severity of secondary energy failure correlates closely with the severity of neurodevelopmental outcome at 1 and 4 years of age. Critically, for understanding labor insults, experimental studies show that a single 'sub-threshold' insult that causes either minor or no neural injury can lead to a phase of increased vulnerability to further insults in a similar window of around 6 or more hours (Gunn, 2009). Advances in neuroimaging, brain monitoring techniques, and tissue biomarkers have improved the ability to diagnose, monitor, and care for newborn infants with neonatal encephalopathy, as well as to predict their outcome. The importance given to the role of oxidative stress in newborn morbidity with respect to the higher risk of FR damage in these babies is growing. However, challenges remain in early identification of infants at risk for neonatal encephalopathy, determination of timing and extent of H-I brain injury, as well as optimal management and treatment duration (Buonocore, 2012).

The literature review reinforces the notion that the spectrum of H-I encephalopathy outcomes represents a continuum, which has important implications for the prediction of outcome and the indications for intervention. Perlman & Shah (Perlman & Shah, 2011) summarize predictive clinical criteria at 3 time points: the first 6 hours of life, 6-72 hours of life, and at hospital discharge. They highlight the predictions at pivotal decision making times: at 0-6 hours (the earlier the better), to initiate or not to initiate neuroprotection, and at 6-72 hours, to identify, implement, and achieve the goal of withdrawing lifesustaining therapy.

The **"therapeutic window"** is this interval after reperfusion (*"reperfusion window": narrow therapeutic time-window within 6 hours of insult*), during which an intervention might be efficacious in reducing the severity of the ultimate brain damage. As we previously mentioned, the cascade of biochemical and histopathological events initiated by H-I can extend from days to weeks after the insult is triggered, which may provide a "therapeutic window of opportu-

nity", probably more extensive (ranging from 6 to 72 hours, called "*cytoprotection window*"), for intervening in the pathogenesis of the developing brain. But such interventions are currently limited by insufficient knowledge of the timing and duration of the so-called therapeutic window in newborns (Barks, 2008; Levene, 2010). Recent data suggests that interventions for perinatal HIE may be combined to enhance the protective and reparative processes, and thought must be given to the best time to administer these interventions. As we described before, injury evolves over long periods of time with different mechanistic phases, adding to the already slow process of neuronal necrosis and apoptosis that can extend for several hours to a day or more. Therefore, therapies will also need to be administered over long periods of time, with different combination of drugs aimed at these temporally evolving targets.

6. Neuroprotective therapies

Asphyxia thus constitutes one of the leading avoidable causes of morbi-mortality in the newborn, and, therefore neuroprotection strategies oriented to prevent neuronal lesion (or at least minimize its consequences) are currently one of the most important lines of research in perinatal medicine. Before the introduction of neuroprotective therapies, the management of neonates with HIE in Neonatal Intensive Care Unit (NICU), was limited to supportive intensive care, including resuscitation in the delivery room followed by stabilization of hemodynamic and pulmonary disturbances (hypotension, metabolic acidosis, and hypoventilation), correction of metabolic disturbances, (glucose, calcium, magnesium and electrolytes), monitoring for multiorgan dysfunction and treatment of seizures.

There has been significant research progress in HIE over the last 2 decades, and many new molecular mechanisms have been identified. Despite all these advances, therapeutic interventions are still limited. At present, magnesium sulfate in preterm- and hypothermia in term newborns are currently the only treatments recognized as effective in neonatal HIE, and they are the focus of completed or continuing clinical trials.

6.1. Hypothermia

Hypothermia has been shown to be neuroprotective at critical cellular and vascular sites of cerebral injury in fetal/neonatal models of HI by inhibiting many steps in the excitotoxic-oxidative cascade. The mechanism of action of hypothermia includes: 1) decrease in energy use, decrease in cerebral oxygen consumption and amelioration of secondary energy failure; 2) inhibition of the increase of lactic acid in the brain; 3) reduction/suppression of extracellular EAA –glutamate- accumulation; 4) inhibition of NOS activity and NO concentrations; 5) suppression of FR activity and lipid peroxidation; 6) decrease of inflammation: inhibits platelet activating factor (PAF); decreases the level of interleukin-1beta and the release of toxic cytokines themicroglial/glia cells and inhibits protease activation, 7) attenuation of secondary energy failure; 8) inhibition of mitochondrial failure; 9) inhibition of necrosis and decrease of caspase-3 activation and morphologic evidence of apoptosis. Hypothermia has been shown to

reduce cerebral metabolism, prevent edema and loss of membrane potential, decrease brain energy use, prolong the latent phase, reduce infarct size, decrease neuronal cell loss, and the extent of brain injury and epileptic activity, relieves the permeability of BBB and intracranial pressure, helps to retain sensory motor function, and preserves hippocampal structures (Shankaran, 2012).

Factors that influence the effects of hypothermia include the target body temperature, mode of hypothermia induction (selective head cooling vs total body cooling), duration of hypothermia, and rate of rewarming. Hypothermia after neonatal HIE, to a target temperature of 33–34°C for 72 hours has currently been shown to reduce death or disability at 18 months or increase the rate of survivors without disabilities. The safety and effectiveness of hypothermia as neuroprotection agent has been reported in many randomized controlled trials to date, enrolling infants born at greater than or equal to 36 weeks or greater than or equal to 35 weeks gestation, within the therapeutic window of 6 hours (Gluckman, 2005; Shankaran et al., 2005, Shankaran et al., 2008; Azzopardi, 2009; Edwards, 2010; Rutherford, 2010; Zhou, 2010; Simbruner, 2010; Jacobs, 2011; Tagin, 2012).

These trials have all included moderate/severe stage of encephalopathy (Sarnat & Sarnat, 1976) as eligibility criteria in random assignment studies at < 6 hours of age. Three trials included the additional criteria of abnormal amplitude-integrated electroencephalography (aEEG). (Gluckman, 2005; Azzopardi 2009; Simbruner 2010).

The Cool Cap trial (Gluckman, 2005) involved 234 term infants with moderate or severe encephalopathy and abnormal aEEG. Death or disability occurred in 66% conventional care and 55% cooled group (adjusted odds ratio [OR] [95% CI] 0.61 [0.34–1.09], $P = 0.10$). Predefined subgroup analysis suggested that head cooling had no effect in infants with the most severe aEEG changes but was beneficial in infants with less severe aEEG changes.

The National Institute of Child Health and Human Development (NICHD) Neonatal Research Network (NRN) randomized controlled trial (RCT) of whole-body hypothermia for neonates with HIE: 208 term infants with moderate or severe encephalopathy were randomly assigned to whole-body cooling to an esophageal temperature of 33.5°C for 72 hours or usual care. Death or severe disability occurred in 62% of the usual care group and 44% of the hypothermia group (relative risk [RR] [95% CI] 0.72 [0.54–0.95], $P = 0.01$) (Shankaran et al., 2005).

The Total Body Hypothermia for Neonatal Encephalopathy (TOBY) trial (Azzopardi, 2009) enrolled 325 infants. Death or severe disability occurred in 53% of the standard care group and 45% of the hypothermia group (RR 0.86 [0.68–1.07], $P = 0.17$). The rate of CP was lower, and improved mental and psychomotor indices were noted in the hypothermia group as compared with the usual care group (all $P < 0.05$). (Rutherford, 2010).

Hypothermia is currently the only treatment recognized as effective in neonatal HIE at present, and the only recommended therapeutic as a standard practice (Biban, 2011). Hypothermia is currently being recommended since 2010 by health care policy makers:

- The international Liaison Committee on Resuscitation (ILCOR). The most recent International Consensus Conference was held in Dallas in February 2010 and the published

conclusions and recommendations from this process form the basis of these 2010 ERC Guidelines (guideline of ILCOR CoSTR 2010) (Hazinski, 2010).

- American Heart Association Guidelines for Cardiopulmonary Resuscitation and Emergency Cardiovascular Care (Kattwinkel, 2010).
- The International Consensus on Cardiopulmonary Resuscitation and Emergency Cardiovascular Care Science with Treatment Recommendations (Perlman, 2010).
- The 2010 guidelines released by the European Resuscitation Council (ERC) (Nolana, 2010).
- and the American Academy of Pediatrics (AAP) (Shelov, 2011).

The guides recommend therapeutic hypothermia as a standard practice for term or near term infants with moderate to severe HIE, stating that during the postresuscitation period in greater than or equal to 36-week gestation neonates with evolving moderate or severe encephalopathy, hypothermia should be offered in the context of clearly defined protocols similar to published trials. Further trials to determine the appropriate techniques of cooling, including refinement of patient selection, duration of cooling and method of providing therapeutic hypothermia, will refine our understanding of this intervention (Cochrane Database; Jacobs, 2013).

6.2. Neuroprotective drugs

Although therapeutic hypothermia is a significant advance in the developed world and improves outcome, the rate of death or disability following hypothermia for neonatal HIE is unacceptably high and ranges from 31% to 55% in the published trials, therefore it is becoming evident that the association of moderate hypothermia with another neuroprotective interventions drugs may enhance the outcome.

Therefore, there still is an urgent need for other treatment options. Further, there are currently no clinically established interventions that can be given antenatally to ameliorate brain injury after fetal distress. One of the major limitations to progress is what may be called “the curse of choice” (Robertson, 2012). A large number of possible neuroprotective therapies have shown promise in pre-clinical studies (Painter, 1995; Kelen & Robertson, 2010). The lines of research currently include: 1) antenatal therapy for fetuses with a diagnosis of antenatal fetal distress at term; 2) and postnatal therapy of infants with moderate to severe neonatal encephalopathy. To date, there is no consensus on which drugs have a higher chance of success in preventing the continued neuronal loss for either antenatal or postnatal treatment in human neonates who have suffered from HIE (reviewed in Degos, 2008; Gonzalez & Ferriero, 2008; Kelen & Robertson, 2010; Rees et al, 2011; Buonocore et al, 2012; Robertson et al, 2012).

The majority of the drugs used as neuroprotective therapy in animal models does not penetrate in good condition the BBB. Thus, drug delivery across the BBB to target cells to treat diffuse brain injury is the hardest challenge. The development of new biocompatible dendrimers has become an important objective for the companies involved in Biotechnology. Among the potential applications of these *nanopolymer biopharmaceuticals* it would be improving the drug transport to increase both the bioavailability and the active fraction, as well as the controlling

the release of pharmaceuticals in order to custom and/or prolong the drug delivery over time. In this way, the use of nanoparticles opens a new door to cerebral HIE treatment.

Bellow, we review the drugs that have shown both higher neuroprotection and clinical safety and, therefore, could be applied to neonates. Since many of them have several mechanisms of action, they have been classified according to their predominant effect (see FIGURE 2).

6.2.1. Anticonvulsant drugs

The developing brain has both a higher incidence of seizures in human and animal models, and experiences seizures that can produce long-lasting consequences that are also stage-dependent (Ben-Ari, 2006). Perinatal H-I brain injury is one of the most important causes of epilepsy (Carrascosa et al, 1996), which occur in the majority (15–60%) of children with CP. Epilepsy is a disorder in which the balance between cerebral excitability and inhibition is tipped towards uncontrolled excitability. Selected neuronal circuits as well as certain populations of glial cells die from the excitotoxicity triggered by HI. The presence of seizure, practically occurring within the first hours, predicates a poor outcome of HIE.

The treatment of seizures is an essential component of the HIE management. Seizures are generally self-limited to the first days of life but may significantly compromise other body functions, such as maintenance of ventilation, oxygenation, and blood pressure. Additionally, seizures should be treated early and be well controlled, since even asymptomatic seizures (seen only on the EEG) may continue to injure the brain. The energy metabolism can be compromised by the hyperactive neurons, and both acute energy deprivation after HI insult and seizures are implicated in excitotoxicity. Thus, the therapeutic value of antiepileptic drugs (AEDs) may include not only the control of seizure activity but also the potential benefit for the compromised cellular energy metabolism (Aicardi, 2008).

Basic and clinical studies indicate that seizures in neonates have long-term neurodevelopmental and psychiatric consequences, highlighting the need for novel pharmacotherapeutics. The two most common classes of AEDs are GABAA receptor agonists and NMDA receptor antagonists. Currently, the first-line medical treatment for neonatal seizures is composed of drugs that increase GABA receptor channel chloride currents, like barbiturate and benzodiazepines (Calabresi et al, 2003).

- **Phenobarbital (FDA-approved)**

Phenobarbital (PB) increases GABA subtype A (GABAA)-receptor channel chloride currents. It is also important to acknowledge that PB has multiple potential modes of action in addition to anticonvulsant effects that could contribute to neuroprotection in this setting, including reduced cerebral metabolic demand, antioxidant effects and decreased cerebral edema.

PB controls seizures in less than half of newborns (Painter et al, 1999). This reduced efficacy of GABA-enhancing AEDs has been linked to neuronal chloride transport in the developing brain. In the adult nervous system, due to low intracellular levels of Cl⁻, GABA inhibits most neurons by activation of GABAARs, causing Cl⁻ influx, membrane hyperpolarization, and inhibition. In immature neurons, the Cl⁻-exporting activity of KCC2 is lower than in mature

neurons and in the context of NKCC1 expression, neuronal $[Cl^-]_i$ is higher and GABA_A reversal potential (E_{GABA}) is more depolarized. High levels of expression of the Na⁺-K⁺-2Cl⁻ (NKCC1) cotransporter in immature neurons cause the accumulation of intracellular chloride and, therefore, a depolarized Cl⁻ equilibrium potential. This results in the outward flux of Cl⁻ through GABA(A) channels, the opposite direction compared with mature neurons, in which GABA(A) receptor activation is inhibitory because Cl⁻ flows into the cell (**for review see De Cabo-de la Vega et al 2006**). This outward flow of Cl⁻ in neonatal neurons is excitatory and contributes to a greater seizure propensity and poor electroencephalographic response to GABAergic anticonvulsants such as PB and benzodiazepines (Khanna et al, 2013). It is also intriguing to consider that recent insights regarding the impact of maturational changes in neuronal chloride transporter expression on GABA receptor function may provide strategies that could improve the neuroprotective efficacy of PB in the neonate. Specifically, blocking the neonatal neuronal chloride transporter with bumetanide can augment the inhibitory activity of GABA agonists such as PB (Costa et al, 2006).

On the other hand, recent research have found that administration of PB is potentially harmful drug, associated with widespread apoptotic neurodegeneration throughout the brain when administered to immature rodents during the period of the brain growth spurt (Bittigau et al, 2003). Compounds that may cause neuronal apoptosis in the developing brain include antagonists of NMDA receptors (ketamine), agonists of GABA_A receptors (barbiturates and benzodiazepines), and sodium-channel blockers (phenytoin, valproate) (Olney et al, 2002). Nevertheless, PB remains the preferred drug for the treatment of seizures in neonates with HIE (Volpe, 2008; Slaughter et al 2013). It is still a controversial issue whether PB treatment should be administered before the seizure attacks. In a small randomized trial, treatment of infants with HIE with PB within 6 hours of birth resulted in a decrease in death or disability at three years of age (Hall et al, 1998). At the present time, anticonvulsant therapy to term infants in the immediate period following perinatal asphyxia cannot be recommended for routine clinical practice, other than in the treatment of prolonged or frequent clinical seizures (Evans et al, 2007).

In a neonatal rodent model the early post- H-I administration of PB may augment the neuroprotective efficacy of therapeutic hypothermia (Barks et al, 2012). Sarkar et al. (Sarkar et al, 2012) found that the PB treatment before cooling did not improve the composite outcome of neonatal death or the presence of an abnormal post-hypothermia brain MRI. Whether this combination treatment could also result in improved neuroprotective efficacy in conjunction with cooling is an interesting question for future research.

- **Bumetanide (FDA-approved)**

Low concentrations of the diuretic bumetanide have been shown to alter the ion gradient that underlies the excitatory effects of GABA. Blocking the NKCC1 transporter with bumetanide prevents outward Cl⁻ flux and causes a more negative GABA equilibrium potential in immature neurons. While several studies have reported anti-convulsant effects of bumetanide (Kahle et al, 2009), others have found no significant anti-convulsant effect. The alteration of Cl⁻ transport by bumetanide reduces electrographic seizures, and the combination of bumetanide

and PB is significantly more effective than PB alone on seizure occurrence, frequency, and duration (Dzhala et al, 2008). Currently there are two clinical trials evaluating bumetanide as a treatment for neonatal seizures. A phase I trial (NCT00830531) is actively enrolling patients in a randomized, double-blind, controlled dose-escalation study of bumetanide as an add-on therapy to treat refractory seizures caused by HIE. A second trial (NCT01434225) is being performed by a large, multi-center European group in an “open-label,” dose escalation fashion to assess the effect of bumetanide in addition to PB for the treatment of neonatal seizures caused by HIE. Data from these pilot studies will be utilized to guide the design of larger Phase III trials that will determine the efficacy of bumetanide in the treatment of neonatal seizures (Khanna et al, 2013).

6.2.2. *Anti-calcium drugs: Calcium channel blockers*

Calcium channel blockers such as **Nimodipine**, **Flunarizine** and **Nicardipine**.

Calcium antagonist pretreatment attenuates HI damage in immature animals models. However, there is no clinical evidence, that such agents are neuroprotective in asphyxiated newborn infants. This may be attributable to lack of selectivity of the available drugs for neuronal calcium channels, poor BBB penetration, or the inability of these agents to affect intracellular calcium stores. Besides, the use of calcium channel blockers in severely asphyxiated newborn infants has been associated with clinically important hypotension and fall in cerebral blood-flow velocity, and if there is no cerebral autoregulation, may cause further cerebral hypoperfusion (Levene et al, 1990).

6.2.3. *Antiexcitatory drugs: Glutamate antagonists*

Glutamate antagonists are the most studied neuroprotective agents.

A. Inhibition of glutamate release:

- **Adenosine A2A receptor antagonist including clonidine and dexmedetomidine (FDA-approved):** have been show to have neuroprotective potential in animal models of perinatal H-I. Adenosine acts as a NTs in the brain through the activation of four specific G-protein-coupled receptors (the A1, A2A, A2B, and A3 receptors). The A1 receptor has long been known to mediate neuroprotection, mostly by blockade of Ca²⁺ influx, which results in inhibition of glutamate release and reduction of its excitatory effects at a postsynaptic level. However, the development of selective A1 and A2 receptors agonists as anti-ischemic agents has been hampered by their major cardiovascular side effects (Abbracchio & Cattabeni, 1999).
- **Riluzole (FDA-approved):** a 2-aminobenzothiazole, is a drug inhibiting glutamate reléase, neuronal excitability, and interferes with the effects of proteins activated upon NMDA-receptor stimulation. After discovery of its neuroprotective effects in 1994, riluzole was approved by the FDA for amyotrophic lateral sclerosis (ALS). However, animal experimentation showed that modest neuronal losses in a H-I model evoked by excitotoxicity have a severe impact on locomotor network function, and that they cannot be satisfactorily blocked

by strong neurodepression with riluzole, suggesting the need for more effective pharmacological approaches (Sámano et al, 2012).

B. The blockers of glutamate receptors:

The blockers of glutamate receptors [non-nmda (particularly AMPA receptors) and NMDA receptors], have conflicting results. Antagonists of the NMDA receptors (NMDARs) for glutamate are potent neuroprotective agents in several animal models of perinatal brain lesions. Administration of pharmacologic antagonists of the NMDA, AMPA/kainate or metabotropic receptors attenuates H-I-induced neuronal injury. Administration of an AMPA/kainate antagonist (but not NMDA antagonists) attenuates H-I oligodendroglial injury (Follett et al, 2000). Conversely, increasing the expression of glutamate transporters has been shown to be neuroprotective in “in vitro” models of ischemic damage and an “in vivo” model of amyotrophic lateral sclerosis. Collectively, these data indicate a neuroprotective role of astrocytes by glutamate uptake via their glutamate transporters. Combination therapy with non-NMDA and NMDA antagonists could achieve a dual beneficial.

- **AMPA/kainate antagonist:**

Topiramate (FDA-approved)

Topiramate (TPM) is a novel anticonvulsant agent currently used in adults and children older than 2-yr-of-age, characterized by good absorption, high bioavailability, and good tolerability (Elterman et al, 1999). TPM has multiple mechanisms of action, including: blocking sodium channels, high voltage-activated calcium currents, enhancing GABA-induced influx of chloride, and inhibiting kainate/ AMPA glutamate receptors; but also blockade of carbonic anhydrase isoenzymes, and mitochondrial permeability transition pore). TPM blockade of AMPA and kainate receptors (glutamate receptors non-NMDA) shows little neurotoxicity, although their effects on other stages of brain development such as synaptogenesis have not been evaluated, prevention of excitotoxicity with TPM appears to be a particularly promising approach, since the agents shown to be effective experimentally are likely to be clinically safe, at least in developing animals (Glier et al, 2004). TPM protected pre-OL against excitotoxic or H-I death (Follett et al, 2004), a key event in the pathophysiology of WM lesions in preterm infants, and protected the PVWM against damage induced by an AMPA-kainate agonist in newborn mice (Sfaello et al, 2005). Subsequent studies that demonstrated that TPM treatment alone confers neuroprotection on H-I brain injury in neonatal rats, with more prolonged treatment (4 doses over 48h). It is more likely that greater neuroprotective efficacy is attributable to intrinsic AMPA-antagonist properties (Noh et al, 2006). TPM exert neuroprotective effects against PVL (Follett et al, 2004).

In clinical models no adverse effects attributable to TPM were detected (Filippi et al, 2010). Filippi et al. (Filippi et al, 2012) are doing a project (three-centre phase II pilot study entitled “Safety and Efficacy of Topiramate in Neonates With Hypoxic Ischemic Encephalopathy Treated With Hypothermia (NeoNATI)”) to evaluate whether the efficacy of moderate hypothermia can be increased by concomitant topiramate treatment, at 10 mg/kg once a day

for the first 3 days of life. Any favourable results from this research might open new perspectives about the reduction of cerebral damage in asphyxiated newborns.

- **Blockade of NMDA receptors:**

NMDA receptors play key roles in successive steps of brain development, including the proliferation, migration, survival, and differentiation of neurons (Lujan et al, 2005). Therefore, blocking NMDA receptors at specific neurodevelopmental stages might adversely affect brain development. Thus, in rats studied during the postnatal growth spurt, transient NMDA-receptor blockade with the potent noncompetitive antagonist **MK-801** led to massive cell death by apoptosis (Ikonomidou et al, 2002). These findings constitute a strong argument against the prolonged use of potent NMDA receptor antagonists during brain development.

Although glutamate receptor antagonists have shown excellent neuroprotective effects in animal studies (Lippman-Bell et al, 2013), these effects have not been validated in clinical studies. These agents all cause a similar spectrum of neuropsychological symptoms, and several have important cardiovascular effects. As a result, studies of several NMDA antagonists- **Selfotel** (CGS19755), **Gavestinel**, **Eliprodil** and **Aptiganel** (Cerestat, CNS 1102) - have been halted; **Dextrorphan** is not efficacious and may be harmful at higher doses (Labiche & Grotta, 2004; Jaffer et al, 2011). Other drugs that may interfere with glutamatergic neurotransmission include **Amantadine** and **Memantine**, two NMDA receptor antagonists devoid of the psychotomimetic and neurotoxic effects of phencyclidine (PCP) or dizocilpine (MK-801) when administered to adults. These drugs are neuroprotective in adults with conditions closely related to excitotoxicity. Unlike MK-801 and other NMDA blockers, **memantine** also appears to be relatively safe in the developing rat brain (Manning et al, 2010); their efficacy and safety in newborns need to be determined.

C. Other modulatory sites on the NMDA receptor complex:

- **Magnesium sulphate (FDA-approved)**

Magnesium is a nonspecific competitive blocker of calcium channel and plays many important roles in maintaining body homeostasis. Magnesium is involved in multiple physiological processes that may be relevant to cerebral ischaemia, including antagonism of glutamate release, NMDA receptor blockade, calcium channel antagonism, maintenance of CBF, cell membrane permeability, mitochondrial functions, the ionic membrane current in conducting cells. Prehypoxic treatment of magnesium sulfate ameliorates the severity of brain damage, but posthypoxic treatment deteriorates it. This deleterious effect may be attributable to hypotension caused by high-dose magnesium sulfate, which further worsens cerebral perfusion (Sameshima et al, 1999).

Use of antenatal magnesium sulphate (MgSO₄) for fetal neuroprotection of the preterm infant: MgSO₄ has been used for decades in pregnant women for different indications (to obtain tocolysis and to treat eclampsia), with no reported adverse effects in the neonates. MgSO₄ was neuroprotective in a model of neonatal WM damage. In a retrospective case control study, preterm infants exposed to antenatal magnesium sulfate were found to have a reduced risk of developing cystic PVL (FineSmith et al, 1997). It has been shown to be effective

for neuroprophalxis to decrease the risk of moderate to severe CP. The first multicenter controlled clinical trial, where mothers at risk of delivering before 30 weeks of gestation were given magnesium, was completed in 2003 by the Australasian Collaborative Trial of Magnesium Sulphate (ACTOMg SO₄) Collaborative Group (Crowther et al, 2003). Significant perinatal side effects occurred, and neurodevelopmental benefits were noted in survivors examined at 2-yr-of-age: substantial gross motor dysfunction (3.4% vs 6.6%; RR, 0.51; 95% CI, 0.29-0.91) and combined death or substantial gross motor dysfunction (17.0% vs 22.7%; RR, 0.75; 95% CI, 0.59-0.96) were significantly reduced in the magnesium group. No serious harmful effects were seen. Cochrane Systematic Review has confirmed this effect (Doyle et al, 2009), the review concludes that antenatal magnesium sulphate therapy given to women at risk of preterm birth substantially reduced the risk of CP in their children. Clinical practice guidelines on Magnesium Sulphate prior to preterm birth for neuroprotection have been developed in Australia (Clinical Practice Guidelines on Magnesium, 2011) and Canada (Magee et al, 2011) by the Societies of Obstetricians and Gynaecologists; and lately, by the American College of Obstetricians and Gynecologists and the Society for Maternal-Fetal Medicine (Committee opinion, 2013).

Postnatal therapy: In a randomised controlled trial performed by the “Intravenous Magnesium Efficacy in Stroke (IMAGES) Study” investigators (Muir et al, 2004), a mortality slightly higher in the magnesium-treated group than in the placebo group was shown, and did not reduce the chances of death or disability significantly. Magnesium also has a number of vascular effects, being that it inhibits NOS in a non-selective form, which in the case of eNOS would be harmful; therefore its use in postnatal therapy of infants with neonatal encephalopathy would be contraindicated.

- **Xenon (Not FDA Approved)**

Medical gases are pharmaceutical molecules which offer solutions to a wide array of medical needs. More specifically however, gases such as oxygen, helium, xenon, and hydrogen have recently come under increased exploration for their potential therapeutic use with various brain disease states including H-I, cerebral hemorrhages, and traumatic brain injuries. A colorless, heavy, odorless noble gas, xenon has been of particular interest to researchers because of its possible neuroprotective properties (Dingley et al, 2006). Since the discovery of xenon as an NMDA receptor antagonist, there has been growing interest in its potential use as a neuroprotectant. Some of the features of xenon that specifically interest scientists and researchers include its rapid introduction into the brain, favorable hemodynamic profile with little or no toxicity, as well as its inability to be metabolized (Liu et al, 2011). Xenon induces anesthesia and exerts its analgesic actions by inhibiting the NMDA receptor signaling pathway. Additional studies have demonstrated that xenon may act on the secondary messenger signaling pathway via increases in cyclic guanosine monophosphate. Xenon’s role in antiapoptotic mechanisms also demonstrates its neuroprotective qualities. Furthermore, Xenon plays an important role in the anti-inflammatory process. Xenon can influence mechanisms regulating the Ca²⁺ release channel on plasma membranes (inhibits plasma membrane calcium ATPase pump activity), resulting in an increase in neuronal Ca²⁺ concentration and an altered excitability in these cells.

Researchers have also explored the possibility of using Xenon in combination with other therapeutic strategies to evaluate its possible synergistic neuroprotective capabilities; Xenon may offer haemodynamic benefits in clinical neuroprotection studies (Chakkarapani et al, 2012).

6.2.4. Antioxidative drugs

The neonatal brain has a high rate of oxygen consumption and low concentration of antioxidants, making it susceptible to damage. In humans, mature OL carry increased antioxidant enzymes compared with the pre-OL present in the immature brain, which may partially explain the susceptibility of premature infants to WM damage (Haynes et al, 2005). In an effort to reduce oxidative damage to the neonate, a number of protective interventions have been used, including (1) FR reducers, (2) ROS scavengers: antioxidant enzymes, and FRs nonenzymatic scavengers, (3) lipid peroxidation inhibitors, and (4) NOS inhibitors. Antioxidant strategies have been used successfully to diminish ischemic cerebral tissue damage in animals, but the utility of a pharmacological agent as a clinically relevant therapeutic strategy may depend, in part, on its ability to cross the BBB, since although ischemic injury disrupts the integrity of the BBB, this disruption is by no means complete.

A. FR reducers:

- **Deferoxamine (DFO)** - FDA-approved-. The free iron induces the formation of ROS, and exogenous iron significantly exacerbates excitotoxic and aggravates cystic PLV in newborn mice (Dommergues et al, 1998). DFO is an iron chelator that decreases FR production by binding with iron and decreasing the production of OH \cdot . DFO is protective during exposure to H₂O₂ or excitotoxicity “in vitro”, and in animal models of H-I (Sarco et al, 2000).

- **Polyphenols**

Resveratrol (Res) -(FDA)-approved- could be a prophylactic factor in the prevention of ischemia/reperfusion (I/R) injury, they attenuates I/R injury in cardiomyocytes by preventing cell apoptosis, decreasing LDH release and increasing ATPase activity. NO, cGMP, PKC and K (ATP) may play an important role in the protective role of Res. Moreover, Res enhances the capacity of anti-oxygen FR and alleviates intracellular calcium overload in cardiomyocytes (Shen et al, 2012).

- **Allopurinol (FDA Approved)**

The xanthine-oxidase inhibitor allopurinol (ALLO) reduces FR formation, thereby limiting the amount of I/R damage. Hypoxanthine accumulates in the ischemic brain, and with reperfusion is oxidized to uric acid and superoxide. Elevated uric acid concentrations in the first postnatal day identify a subset of premature infants who are at high risk for having subsequent hemorrhagic or ischemic injury (Perlman et al, 1998). Furthermore, ALLO also has a non-protein bound iron (pro-radical) chelating and direct FR (hydroxyl) scavenging effect. Animal research in asphyxiated pigs demonstrated beneficial effects of postnatally administered ALLO on cerebral energy status and cytotoxic oedema (Peeters-Scholte et al, 2003). Treatment with ALLO reduces FR production following ischaemia and it reduces tissue injury in “in vitro”, and high doses (50-200 mg/kg) of ALLO effects cerebral protection in animal experiments and

also exerts benefits on reduction of cerebral edema and neuropathological damage after neonatal HIE (Palmer et al, 1993). In humans, the first work on the neuroprotective effect of allopurinol was carried out by Russell and Cooke (Russell & Cooke, 1995), in a randomized controlled trial of allopurinol prophylaxis in very preterm infants (between 24 and 32 weeks of gestation). In this trial of prophylactic ALLO for the prevention of PVL in preterm babies, no protective effect was apparent. A prospective randomized study in human neonates, examining the effects of ALLO in term asphyxiated neonates, showed an improvement of electrocortical brain activity and a reduction in FR formation after neonatal ALLO administration (Van Bel et al, 1998). A more recent paper by Gunes et al (Gunes et al, 2007) reports an improved neurological outcome after postnatal ALLO administration (40 mg/kg/day, 3 days, within 2 hours after birth) compared to a placebo in term asphyxiated neonates. Benders et al (Benders et al, 2006), however demonstrated that ALLO was not effective if administered 3 to 4 hours after the hypoxic incident to severely asphyxiated neonates. However, when the most severely asphyxiated children were excluded from the study, a beneficial effect of ALLO was seen on neurological development. Apparently, no advantage of neonatal treatment is seen anymore when the interval to the initiation of treatment is too long or when the brain damage is too severe. This has probably been the major disadvantage of late postneonatal treatment with ALLO on the NICU. ALLO administered at the NICU is likely to be given too late to provide adequate neuroprotection during the early period of reoxygenation in which the vast amount of FR is being produced. Apparently, when the asphyxia has been too severe, the inflicted brain damage can no longer be reversed. It is conceivable that earlier ALLO treatment, i.e. the use of ALLO during labour in case of suspected foetal hypoxia, provides the opportunity to start earlier with the treatment, thereby limiting the amount of I/R injury and improving neurological outcome. Animal and human studies suggest that administration of ALLO immediately prior to delivery in case of suspected foetal asphyxia might reduce HIE. In a study in the chronically instrumented foetal sheep, they were able to show evidence of cardio-and neuroprotection after antenatal ALLO administration to the pregnant ewe during repeated periods of ischaemia (Derkset al, 2006). Maternal administration of ALLO has been proposed as prebirth treatment when there is suspicion of an adverse event eliciting perinatal asphyxia. A prospective randomized placebo controlled pilot study, in which they administered ALLO to the pregnant woman when foetal asphyxia was imminent, showed an inverse correlation between the levels of ALLO and the amount of S100B, a biomarker for brain tissue damage, in cord blood (Torrance et al, 2009). A clinical trial of antenatal allopurinol is in progress (Kaandorp et al, 2010).

B. Antioxidant enzymes (Endogenous antioxidants):

SOD, GPX, and CAT, are considered the classical antioxidant enzymes. One therapeutic approach for the destruction of oxygen FRs generated during and after H-I is the administration of specific enzymes known to degrade highly reactive FR to a nonreactive component. SOD and CAT are enzymes poorly soluble and with short half life, so that should be conjugated to polyethylene glycol, which prolongs their circulatory half-life and facilitates penetration of the BBB. Notwithstanding that, the latency time to initiate therapeutic effects is unacceptably long, making them useful only as preventive. Because of their large molecular sizes, they are restricted

to the vascular space. In newborn animals, neuroprotection has only been shown when these agents have been administered several hours before the HI insult (Shimizu et al, 2003).

C. Free radical nonenzymatic scavengers:

- **N-Acetylcysteine (NAC) (FDA Approved)**

NAC is a precursor of glutathione and can therefore act as an anti-oxidant and is also a scavenger of ROS. NAC is used clinically for mucolysis and as an antidote for paracetamol intoxication in high doses. It also reduced oxidative stress, inflammation, and minimized H-I-induced brain injury in various acute models. In addition to reducing total tissue loss, NAC reduced WM injury, prevented endotoxin-induced degeneration of OL progenitors and hypomyelination in developing rat brain (Paintlia et al, 2004). The mechanism of NAC neuroprotection appears to be related to reduced oxidative stress, preservation of the scavengers GSH and Trx2, attenuated activation of apoptotic proteases (caspase-3, calpain), and reduced inflammation. The protective effect of NAC was much more pronounced than that produced by another FR scavenger, melatonin, when administered before and after lipopolysaccharide-sensitized H-I. NAC was also effective when administered directly after H-I (three days after) (Wang et al, 2007).

NAC is transported across the placenta and it is considered safe during pregnancy. Therefore, although transport across the BBB is believed to be poor, it has been assumed that NAC has potential therapeutic value in humans, performing its neuroprotective action at the level of the vascular bed (Schaper et al, 2002). NAC is associated with adverse reactions ranging from nausea to death (most of the latter due to incorrect dosing) which limits its use in humans. (Sandilands & Bateman, 2009; Knudsen et al, 2005).

NAC is the most effective therapy for acetaminophen (APAP) toxicity and is currently available for children for oral and intravenous (IV) administration; both routes are equally effective and safe (Green et al, 2013). Currently, there are three protocols that are used in acetaminophen ingestion:

72-hour oral-NAC: 140 mg/kg of oral NAC followed by 70 mg/kg every 4 hours for an additional 17 doses.

20- hours IV-NAC: consists of a continuous IV infusion - 300 mg/kg- of NAC, patients are given a loading dose of 150 mg/kg over 15 minutes, followed by 50 mg/kg over 4 hours and then 100 mg/kg over the next 16 hours.

48-hours IV-NAC: consisted of a loading dose of 140 mg/kg followed by 12 doses of 70 mg/kg every 4 hours.

The most frequently used protocol is the Simplified N-acetylcysteine dosing regimen -standard preparation of IV-NAC 30 g in 1 L of 5% dextrose in water, with a 150-mg/kg loading dose administered over 1 hour followed by an infusion of 14 mg/kg/h for 20 hours – this single intravenous bag protocol is effective and well tolerated, and there is infrequent interruption of therapy by dosing errors (Johnson et al, 2011). Both the IV and oral NAC have generally mild adverse drug reactions. Nausea and vomiting have been the most common reported

adverse events and were more common with oral treatment (9%). Anaphylactoid reactions were more common with IV administration (2%). The changing between administration routes, introducing deviations from “standard” treatment, may decrease these side effects (Bebarta et al, 2010). In a medication error in prescribing paracetamol for closing a patent ductus arteriosus in a preterm infant, NAC was indicated without showing adverse drug reactions (Brener et al, 2013).

There is no consensus on a neuroprotective dose, and a wide range of concentrations have been used in experimental studies. After neonatal I/R in piglets, NAC at doses of 150-mg/kg bolus and 20 mg/kg/h, IV for 24 hours, reduced cerebral oxidative stress with improved cerebral oxygen delivery and reduced caspase-3 and lipid hydroperoxide concentrations in cortex (Liu et al, 2010). With smaller dosages (30 mg/kg bolus then 20 mg/kg/h infusion) in newborn piglets with I/R, postresuscitation administration of NAC lowered cerebral lactate levels, reduced cerebral oxidative stress (significantly attenuated the increase in cortical H₂O₂, but not NO, concentration) and improved cerebral perfusion (Lee et al, 2008). Furthermore, the anti-inflammatory effect reduced lung edema and neutrophil influx into the lung and partly reversed surfactant dysfunction in the meconium aspiration syndrome model (Mokra et al, 2013). Combination therapy of NAC and systemic hypothermia induced immediately after neonatal H-I improves infarct volume, and reduced both white and grey matter damage after focal HI injury in neonatal rats (Jatana et al, 2006). By contrast, Olsson et al (Olsson et al, 1998), report that free NAC at 100 mg/kg showed some efficacy in attenuating inflammation and oxidative injury in the brain, but the improvement did not translate into myelination, neuronal counts or motor function in CP. The only randomized clinical trial in preterm newborns demonstrated that continuous infusion of NAC for 6 days after birth did not improve the incidence of chronic lung disease but did appear to reduce the incidence of PVL (Ahola et al, 2003).

Dendrimer-based N-acetyl-L-cysteine (D-NAC): Dendrimers are a nanopolymer biopharmaceutical emerging as potential intracellular drug delivery vehicles. The efficacy of the activity “in vitro” of anionic polyamidoamine (PAMAM-COOH) dendrimer-N-acetyl cysteine (DNAC) was significant, even at the lowest dose, and its activity compared to free NAC increase 16x higher dosage (Wang et al, 2009). The bioavailability “in vivo” of free NAC is poor (the terminal half-life was 5.58 h after IV administration and 6.25 h after oral administration. Oral bioavailability of total NAC was 9.1%). However, IV administration of a single 10 mg/kg dose of D-NAC resulted in a significant improvement in neuronal injury and motor function in CP kits. Moreover, the improvements seen with NAC-100 were similar to that seen with D-NAC at 1% of the dose improved uptake and efficacy of D-NAC when compared to free NAC in activated microglia, as shown previously “in vitro”, delivery of a higher drug-payload to the target cells (activated microglia and astrocytes) by the dendrimer “in vivo”, and decreased toxicity of the drug for neurons when conjugated with the dendrimer. The effectiveness of the D-NAC treatment, administered in the postnatal period for a prenatal insult, suggests a window of opportunity for treatment of CP in humans after birth (Kannan et al, 2012). The nanoparticles D-NAC open a new door to cerebral palsy treatment (Crunkhorn, 2012; Andón et al, 2012).

- **Melatonin (FDA Approved)**

Melatonin is an indoleamine that is formed in higher quantities in the adult. It is produced mainly by the pineal gland and is a naturally occurring hormone that binds to specific receptors and allows the entrainment of circadian rhythms in several biological functions. But it can also function as neuroprotective antioxidant (as a direct scavenger of ROS and NO), and has anti-apoptotic effects. Because of its lipophilic properties, melatonin easily crosses most biological cell membranes, including the placenta and the BBB. Several animal studies have shown neuroprotective benefits from melatonin treatment, both when given before and after birth. It has been found to provide long-lasting neuroprotection in experimental H-I and focal cerebral ischemic injury. Melatonin may exert some of its protection on developing WM in H-I sheep model via an anti-microglial effect (Welin et al, 2007). Newborns treated with melatonin were also found to have decreased proinflammatory cytokines (Gitto et al, 2004 and 2005).

Clinically, melatonin has been used safely in children with sleep abnormalities related to neurological disease (Jan & O'Donnell, 1996) and in septic newborns (Gitto et al, 2001) without serious adverse effects. Melatonin appears to have beneficial effects when given to asphyxiated newborns. It was shown to significantly reduce plasma levels of malondialdehyde and nitrate/nitrite, two robust indicators of oxidative stress (Fulia et al, 2005). In a small clinical trial, melatonin was given orally to newborn babies who had suffered birth asphyxia. In terms of mortality, 3 out of 10 asphyxiated babies died in the vehicle treated group, whereas there were no deaths in the post-asphyxia babies treated with melatonin. Importantly, this study did not report any adverse effects arising from the melatonin treatment, and clinical use of melatonin in the neonatal period has now been proposed (Gitto et al, 2009).

The optimal neuroprotective dose still needs to be determined, although Robertson et al (Robertson et al, 2013), in a piglet model of perinatal asphyxia, demonstrate that the therapeutic hypothermia plus IV melatonin (5 mg/kg/h) significantly reduced the H-I-induced. The safety and improved neuroprotection of a potential treatment with a combination of melatonin and cooling support the initiation of phase II clinical trials in infants with moderate and severe neonatal encephalopathy.

- **Tetrahydrobiopterin (FDA Approved)**

BH4 is an important co-factor for a number of enzymes, such as aromatic amino acid hydroxylases, which converts phenylalanine into tyrosine (phenylketonuria), tyrosine into L-dopa, and tryptophan into 5-hydroxytryptophan and NOS. BH4 may function as a FR, but it has also been reported that inhibition of biopterin synthesis reduces ischemic brain damage (Kidd et al, 2005). BH4 is a developmental factor determining the vulnerability of fetal brain to H-I. There is evidence that BH4 deficiency can exacerbate oxidative injury (Madsen et al, 2003) and that neonatal H-I can cause relative BH4 deficiency (Fabian et al, 2010). Fujioka H et al (Fujioka et al, 2008) found neuronal iNOS expression and increase of NO production in the acute phase of H-I in a newborn-piglet model, but brain biopterin did not increase despite plasma biopterin five-fold elevation. These findings suggest that the capacity of biopterin production in the CNS is inferior to that in other organs in the acute phase of H-I. The BBB is thought to prevent the transport of biopterin from the blood to the brain, therefore the initial shortage of biopterin in

a H-I brain may affect the severity of brain damage. Maternal treatment with BH4 increased fetal levels in basal ganglia and significantly ameliorated motor deficits and decreased stillbirths (Vasquez-Vivar et al, 2009).

- **Vitamin C (FDA Approved)**

Ascorbic acid (AA, vitamin C) is an important enzyme cofactor and water-soluble reducing agent that is highly concentrated in the adrenal gland and CNS. AA concentrations are lowest in plasma (0.01–0.1 mM), intermediate in cerebrospinal and extracellular fluid (0.05–0.5 mM) and highest in neuropil of the brain (1–3 mM). AA is a potent antioxidant, which scavenges various types of ROS, and its neuroprotective effect has not been established yet. Because vitamin C does not penetrate the BBB, therapeutic, nonenzymatic scavenging of FRs can be accomplished by AA only at very high physiological concentrations (Jackson et al, 1998). Some studies have shown that AA has a neuroprotective effect; however, the issue is still controversial. Since higher dose of AA may cause side effects such as oxaluria and kidney stone (Massey et al, 2005), erythrocyte damage leading to hemolytic anemia and hyperbilirubinemia in infant (Ballin et al, 1988), it might also have harmful effects on brain. AA can act as pro-oxidant and cause neurotoxicity “in vitro” by reducing transition metal ions under certain conditions (Buettner et al, 1996). Only one prospective, randomized double-blinded controlled clinical study in term infants with perinatal asphyxia has been performed, which found that the combination of AA with ibuprofen had no effect on outcome at 6 months of age (Aly et al, 2009).

Although the antioxidant vitamin C does not penetrate the BBB, its oxidized form, **dehydroascorbic acid (DHA)**, enters the brain by means of facilitated transport. AA can be oxidized to DHA in the stomach. Several studies demonstrated that AA and DHA have neuroprotective effects in adult animal models of HI. IV DHA would improve outcome after stroke because of its ability to cross the BBB and increase brain antioxidant levels. A dose of 250 mg/kg or 500 mg/kg DHA administered at 3 hours post-ischemia reduced infarct volume by 6- to 9-fold, to only 5% with the highest DHA dose ($P < .05$). Teratological and adverse effects have not been documented (Huang et al, 2001). AA may be given to the mothers if the safety of its use is established, since AA has been reported to cross the placental barrier (Rybakowski et al, 1995).

- **Vitamin E (FDA Approved)**

Vitamin E, a clinically safe agent, has been shown to be effective in cultured pre-OL models (Back et al, 1998). Clinical trials in very preterm infants have been confined to vitamin E for the prevention of IVH (Sinha et al, 1987). In rat pups, there was no benefit from postnatal treatment after H-I with Mito vitamin E, a mitochondrial antioxidant (Covey et al, 2006). However, in a study in asphyxiated rat pups, the combination of methylprednisolone with vitamin E therapy reduced H-I brain damage significantly (Daneyemez et al, 1999). Nakai et al. (Nakai et al, 2002) reported that the combined administration of AA and α -tocopherol (vitamin E) to pregnant rats before transient intrauterine ischemia was effective against secondary mitochondrial dysfunction in the neonatal rat brain.

D. Bioactive lipid mediators (Membrane “stabilizers”):

Considerable experimental evidence supports a pathogenetic role for lipid mediators in perinatal H-I brain injury (Grow et al, 2002). Bioactive lipid mediators that may play a role in cell signaling include arachidonic acid (AA), prostaglandins, leukotrienes, thromboxanes, and PAF. Membrane phospholipids are hydrolyzed by phospholipase A2 (PLA2) to release FFA, including AA, and lysophospholipid. Many studies have associated ROS-mediated damage with a disturbance of cell membrane integrity and subsequent increase in intracellular Ca^{2+} , which, in turn, activate a number of Ca^{2+} dependent enzymes including PKC and PLA, damaging membranes directly and initiating the production of lipid mediators, including AA and PAF. H_2O_2 has been shown to cause AA release in numerous cell systems, including cells in the CNS (Samanta et al, 1998). ROS produced during post I/R react with membrane phospholipids to form oxidized lipids, some of which have PAF-like activity. High concentrations of PAF or PAF-like oxidized lipids may contribute to neuronal injury by increasing intracellular calcium concentrations, by stimulating production and release of pro-inflammatory mediators from neurons or microglia, or by upregulating cyclo-oxygenase-2 (COX-2). Additionally, the involvements of different PLA2s, including cPLA2, iPLA2, and sPLA2, have been implicated in the oxidative-mediated AA release process (Martinez et al, 2001). XU et al (XU et al, 2003) showed that the response of astrocytes to oxidant compounds such as H_2O_2 , which stimulated signaling pathways leading to the activation of cPLA2 and iPLA2 (Ca^{2+} -independent) and the increase in AA release.

- **Citicoline (FDA Approved)**

Cytidine-5-diphosphocholine (CDP-choline as an endogenous compound that can also be administered exogenously as *citicoline*) is an endogenous nucleoside that is an essential intermediate in the synthesis of phosphatidylcholine, a major neuronal membrane lipid. Citicoline and its hydrolysis products (cytidine and choline) play important roles in the generation of phospholipids which are involved in membrane formation and repair, and it is known to have neuroprotective effects. The mechanisms that may explain the **neuroprotective actions** of citicoline include prevention of FFA release, stimulation of phosphatidylcholine synthesis, preservation of cardiolipin and sphingomyelin levels, increase of glutathione synthesis and glutathione reductase activity, restoration of Na^+/K^+ -ATPase activity, and antigitamatergic effects. The neuroprotective properties of CDP-choline seem to be related on glutamate-mediated cell death (Adibhatla et al, 2002; Hurtado et al, 2005). Citicoline might decrease the extracellular level of glutamate by inhibition of neuronal glutamate efflux and increased astrocytic glutamate uptake. It has been suggested that the neuroprotective effect of this compound is related to inhibition of the glutamate induced apoptotic pathway of cell injury (Mir et al, 2003). The protective effect of CDP-choline might also be associated with its actions on cell membrane stability because citicoline has distinctive membrane-modulating properties (Secades et al, 2006). Citicoline decreases phospholipase A2 stimulation and hydroxyl radical generation in cerebral ischemia (Adibhatla et al, 2003). Lately, it has been suggested a possible contribution of the gene *PNPLA4*, and codes for calcium-independent PLA2, as one of the mechanisms through which

the citicoline may act (Carrascosa-Romero et al, 2012); and regulative effect on the expression of intercellular adhesion molecule-1 (ICAM-1) mRNA in neonatal brain with H-I damage (Miao et al, 2005). The upregulation of the inflammatory genes and their products precedes leukocytes' adhesion to endothelial cells and their migration into the ischemic tissue, suggesting that these upregulated adhesion molecules on brain capillary endothelium play an important role in leukocyte migration into ischemic brain tissue (Wang et al, 1995). "in vitro", Matyja et al (Matyja et al, 2008) demonstrated that CDP-choline exerts neuroprotection in progressive motor neurons injury in a model of chronic excitotoxicity. It inhibited mainly neuronal apoptotic changes, whereas necrotic and autophagocytic abnormalities were not reduced. This confirms the suggestion that citicoline might protect neurons against the glutamate-induced apoptotic pathway probably via a negative effect on activation of the caspase cell death pathway (Mir et al, 2003); markedly reduced caspase-3 activation and Hsp70 expression 24 h after the insult, and dose-dependently attenuated brain damage in a rat model of birth asphyxia (Fiedorowicz et al, 2008). Diederich et al (Diederich et al, 2012) demonstrated that citicoline (100 mg/kg) for 10 consecutive days starting 24 hours after ischemia induction, have an extended therapeutic window.

In addition citicoline has convincingly been shown to also have **neuroregenerative effects** although the underlying mechanisms are unknown. As a first mechanism contributing to this more favorable neurological outcome, they could identify increased neurogenesis in the SVZ and migration of neural progenitors to the lesion with increased neurogenesis also within the peri-infarct area. A second component of the regeneration-enhancing effect of citicoline was a shift toward excitation in the perilesional cortex (Diederich et al, 2012).

Citicoline has been shown to have neuroprotective effects in a variety of CNS injury models, including focal and global cerebral ischemia (Trovarelli et al, 1981). In clinical trials, citicoline administered after acute ischemic stroke in adults, improved neurological outcome with mild adverse effects (Dávalos et al, 2002; Labiche & Grotta, 2004; Saver, 2008). A recent drug surveillance study on acute ischemic stroke in 4,191 patients (Cho and Kim, 2009) showed that oral citicoline (500-4000 mg/day) administered within less than 24 h after acute ischemic stroke improved neurological, functional and global outcomes without significant safety concerns. By contrast, in a randomised, placebo-controlled study (ICTUS trial), citicoline, (1000 mg every 12 h IV during the first 3 days and orally thereafter for a total of 6 weeks) in patients with moderate-to-severe acute ischaemic stroke, was not efficacious in the treatment of moderate-to-severe acute ischaemic stroke (Dávalos et al, 2012). These differences could be due to dosage, interaction with other drugs when they are administered simultaneously or to the fact that necrosis phenomena are more marked than those of apoptosis. However, the apoptosis in the newborn plays a prominent role in the development of H-I injury and may be more important than necrosis after injury. Additionally, the neurogenesis in the SVZ and migration of neural progenitors are more marked in the newborn than in adults. CDP-Choline administration to newborn infants (100 mg/kg/day, IV) was well tolerated without side effects (Valls et al, 1988; Wang et al, 1997). In the context of the well-known excellent safety profile of citicoline, these data suggest that success in the clinical evaluation of the efficacy of this drug in human neonatal asphyxia may be warranted.

- **Edaravone (Not FDA Approved)**

Edaravone, 3-methyl-1-phenyl-2-pyrazolin-5-one, (MCI-186) significantly decreased lipid peroxidation (thiobarbituric acid reactive substance levels) of the damaged brain hemisphere (Keda et al, 2002). Edaravone is a FR scavenger that improves the outcome after cerebral ischemia in humans and is used for treatment after acute stroke (Group EAIS, 2003) and traumatic brain injury (Itoh et al, 2009). Since edaravone has been approved in Japan for use in patients with cerebral infarction, it could be a promising candidate for the treatment of neonatal HIE.

E. nNOS inhibitors (Not FDA approved):

NO, a water-soluble, diffusible gas, has many physiological roles including regulation of gastrointestinal motility, vasorelaxation, and furthermore performs an important role in synaptic neurotransmission (intercellular messenger and signaling molecule), and is important for neuronal survival, differentiation, and precursor proliferation. NO can also contribute to tissue injury, and has been shown to play a dichotomous regulatory role in the brain; neuronal destruction and protection (Chen et al, 2004). NO prevents apoptosis of neuronal cells via two mechanisms; first, inhibition of caspase-3 activity through S-nitrosylation of cysteine residues in the protease, and second, cGMP-dependent (Lipton, 1995). NOS catalyzes the synthesis of NO from the conversion of arginine to citrulline. susceptibility to HI damage (Ferriero et al, 1996). Cerebral ischemia stimulates production of NO by neurons and microglia.

NO is generated by three distinct nitric oxide synthases: neuronal (nNOS), endothelial (eNOS), and inducible synthases (iNOS). Constitutively expressed nNOS and eNOS are activated by increased intracellular calcium, by way of the NMDA receptor stimulates; nNOS plays a physiologic role in excitatory neurotransmission; eNOS produces vascular smooth muscle relaxation; iNOS is upregulated by hypoxia, cytokines, or endotoxin in monocyte /macrophage/ microglia and is calcium independent; its induction can result in the production of large quantities of NO (Moncada et al, 1991). Selective inhibition of nNOS or iNOS has shown potential as a neuroprotective strategy, but nonspecific blockade of nNOS and eNOS is not protective (Marks et al, 1996). During I/R, nNOS plays a role in NO production, but iNOS only contributes to NO production during reperfusion.

Selective nNOS inhibitors:

The traditionally most employed nNOS inhibitor has been **7-nitroindazole (7-NI)** (Muramatsu et al, 2000), in adult stroke, protection by 7- NI has been inconclusive, this may reflect non-specific inhibition of eNOS, causing a decrease in CBF (Willmot et al, 2005). At present, the new inhibitors **HJ619** and **Jl-8** are at least few hundred fold more specific than 7-NI; the new compounds tested in a perinatal model of H-I, inhibited fetal brain NOS activity “in vivo”, reduced NO concentration, and dramatically ameliorated the number of deaths and CP in a rabbit model (Ji et al, 2009). These compounds are water-soluble and can be given IV. The starting dose is unknown. Adverse effects are also unknown (Robertson et al, 2012).

- **Iminobiotin (Not FDA approved)**

2-iminobiotin, a dual inhibitor with combined inhibition of nNOS and iNOS. Iminobiotin exhibits neuroprotective effects in rats following H-I (van den Tweel et al, 2005), showed protection only in female rat pups after H-I, but the protection was independent of the NO pathway (Nijboer et al, 2007). Orphan designation (EU/3/09/701) was granted by the European Commission to Neurophyxia for 2-iminobiotin for treatment of perinatal asphyxia on the basis of potential activity. At the time of submission of the application for orphan designation, no clinical trials with the designated product in patients with perinatal asphyxia had been started and 2-iminobiotin was not authorised anywhere in the EU for perinatal asphyxia.

6.2.5. *Anti-inflammatory drugs*

The CNS has its own resident immune system, in which glial cells (microglia, astrocytes, and OL) not only serve supportive and nutritive roles for neurons but also engage from time in several "inflammatory" processes that defend the CNS from pathogens and help it to recover from stress and injury. Cytokines and activated microglia/macrophages may extend neuronal injury and/or sensitize the developing brain to a second insult. Therefore, interference with their effects would be expected to reduce subsequent neurological deficits. However, cytokines such as IL-1 β or IL-6 were found to exert trophic effects on neurons, at least in cell cultures (Otten et al, 2000). Similarly, activated microglia/macrophages, in addition to exerting toxic effects, can display protective properties, such as scavenging of excess glutamate through increased expression of glutamate transporters (Vallat-Decouvelaere et al, 2003).

Accumulating evidence suggests that targeting delayed neuroinflammatory mechanisms may be a promising avenue for therapeutic intervention (Leonardo & Pennypacker, 2012). Anti-inflammatory interventions have shown promise in experimental models of combined gray and white matter injury: **cytokine antagonists (IL-1receptor antagonist)** (Hagberg et al, 1996), **platelet activating factor (PAF) antagonist** (Liu et al, 1996; Zhang et al, 1994) and **induced neutropenia** (Hudome et al, 1997). **Corticosteroids** theoretically may interrupt the inflammatory cascade that occurs during H-I; experimental and epidemiological studies support a protective role for antenatal steroids against PWMD (Whitelaw & Thoresen, 2000; O'Shea & Doyle, 2001), but this beneficial effect must be weighed against the adverse effects during a critical period of brain development of postnatal high-dose steroids used to prevent or to treat chronic lung disease in premature infants (Baud et al, 2004). Early synthetic glucocorticoid dexamethasone (DEX) exposure may lead the neonatal brain to be more vulnerable, exacerbated HI-induced injury on P7 by a glucocorticoid receptor-mediated mechanism. The aggravating effect of neonatal DEX treatment on HI-induced brain injury was correlated with decreased glutamate transporter-1 (GLT-1)-mediated glutamate reuptake.

- **Minocycline (FDA Approved)**

Minocycline is a semisynthetic second-generation tetracycline and a potential neuroprotective intervention following brain injury. However, despite the recognized beneficial effects of minocycline in a multitude of adult disease states, the clinical application of minocycline in neonates is contentious. Tetracyclines are broadspectrum antibiotics that have antiinflamma-

tory effects independent from their antimicrobial activity, but, as a class, are not usually administered to neonates. Minocycline inhibited microglial activation, reduces inflammation and protected neurons against ischemia in adult and developing rats in several studies (Yrjanheikki et al, 1999; Fan et al, 2006; Buller et al, 2009; Lechpammer et al, 2008). Minocycline treatment prevents the formation of activated caspase-3, a known effector of apoptosis, as well as the appearance of a calpain cleaved substrate, a marker of excitotoxic/necrotic cell death (Arvin et al, 2002). Although another study found that minocycline exacerbated H-I cortical injury in neonatal mice (Tsuji et al, 2004). Nevertheless, minocycline is not without clinical hazard, and further study of this agent and related analogs is needed (Volpe et al, 2011).

6.2.6. Anti-apoptotic drugs

One of the hottest topics in neurobiology is apoptosis, the highly orchestrated and possibly “controllable form of cell death” in which cells enter into a programmed suicide by chopping themselves into membrane-packaged bits. In stroke, neurons in the penumbra zone, deprived of oxygen and glucose tissue, gradually die because ischemic injury triggers their suicide programmes (Miller & Marx, 1998; Barinaga, 1998). Control of apoptosis involves a balance between expression of numerous apoptotic and anti-apoptotic proteins after injury, providing many potential approaches to modifying outcome (blockade of downstream effects).

- **Erythropoietin (EPO) (FDA Approved)**

EPO is a 165 amino acid glycoprotein produced mainly by peritubular cells in the adult kidneys and by hepatocytes in the fetus. EPO acts on the later stages of erythroid progenitor cells development, allowing maturation of erythroid precursors by inhibiting apoptosis and thus regulating red cell production. Recombinant human EPO (rhEPO) is currently effective and widely used to treat anemia of prematurity. Epo, the major haemopoietic growth factor, is now considered to have beneficial effects in various nervous system disorders based on the effects of prevention of metabolic compromise, neuronal and vascular degeneration, and inflammatory cell activation (Maiese et al, 2008). EPO is required for normal brain development in mammals (Juul, 2002; Yu et al, 2002). Exogenously administered EPO exhibits neuroprotective effects in numerous animal models, through the activation of anti-apoptotic, anti-oxidant and anti-inflammatory pathways as well as through the stimulation of angiogenic and neurogenic events. (for a review see Kumral A et al, 2011; Juul, 2012; Subirós et al, 2012). Moreover, EPO reduced the excitotoxic effect of glutamate and AMPA upon cortical neuron cultures (Sinor & Greenberg, 2000). EPO also prevented apoptosis induced by NMDA or by NO in neurons of cerebrocortical cultures (Digicaylioglu & Lipton, 2001).

Recombinant human EPO (rhEPO)- induced neurogenesis has been studied in “*in vivo*” and “*in vitro*” experiments, it has been shown that EPO regulates neurogenesis in the adult mouse brain (Shingo et al, 2001). Epo is not an appropriate antenatal therapy because it hardly crosses the human placenta (Widness et al, 1995). The capability of EPO to cross the BBB after systemic administration and its effective therapeutic window are advantages for H-I therapy. It requires small volumes, so it does not impose a fluid burden. The most commonly used treatment regimens used to stimulate erythropoiesis in neonates is 400 U/kg 3 times a week

given subcutaneously or 200 U/kg daily given IV. The optimal dose, number of doses, or dosing interval for Epo neuroprotection in humans have not yet been determined. Neonatal Epo treatment has been studied in randomized controlled trials of erythropoiesis, with few reported adverse effects, and the medication is thought to be safe at doses ranging as high as 2100 units/kg/week (Fauchere et al, 2008; Juul et al, 2008). Complications seen in adults (eg, hypertension, clotting, seizures, polycythemia, and death) (Ehrenreich *et al.* 2009) have not been observed in infants. Angiogenesis may be an important adverse effect in preterm infants at risk for retinopathy of prematurity. The first trial with EPO in full term neonates with moderate to severe H-I demonstrated that it reduced death and disability at 18 months from 44% (controls) to 25% (EPO-treated) with no adverse effect (Zhu et al, 2009). Human trials are just beginning, but they show promise (Elmahdy et al, 2010; Lakic et al, 2010).

Elmahdy et al (Elmahdy et al, 2010) in a prospective case-control study with 45 neonates, of which 15 were infants with mild/moderate HIE, received human recombinant EPO (2500 IU/kg, subcutaneously, daily for 5 days), demonstrated the feasibility of early administration of EPO to neonates with HIE for protection against encephalopathy.

- **EPO-Mimetic Peptides (Not FDA Approved)**

The possibility of developing Epo-mimetic that have specific subsets of Epo characteristics has been of great interest, because these molecules might circumvent unwanted clinical effects or provide improved permeability with the ability to cross the placenta or BBB. The tissue protective functions of Epo can be separated from its stimulatory action on hematopoiesis, and novel Epo derivatives and mimetics, such as **asialo-Epo** and **carbamylated-Epo**, have been developed. No studies have been done to assess safety or efficacy of these compounds as perinatal treatments (see Robertson et al, 2012).

Neuro-EPO is a variant with a low-sialic acid content and a short half-life. Drug transport from the nasal cavity directly to the brain has been shown to be feasible, even for challenging drugs such as small polar molecules, peptides and proteins, in animals and humans. Intranasally administered Neuro-EPO exhibits neuroprotective effects in gerbil models of brain ischemia. The use of the nasal route as a new delivery pathway to the brain is aimed to achieve quick delivery of neuroprotective concentrations to the nervous tissue using small drug doses (see Subirós, 2012).

- **Neurotrophic factors (Neurotrophins)**

Trophic factors are emerging as potential cytoprotective agents, although their role may be more important in the recovery phase (Labiche & Grotta, 2004). Neurotrophins are important cues for the migration and differentiation of neural stem cells (SCs). Neurotrophins are a family of growth factors that act through tyrosine kinase receptors and regulate the development and maintenance of brain cells by affecting growth, differentiation, maturation, maintenance and neuronal survival, as well as synaptogenesis and brain plasticity. They also exhibit neuroprotective activity in multiple neuronal populations after injury. The first neurotrophin discovered was neuronal growth factor (NGF). Further work identified other members of the family

such as Glial Derived Neurotrophic Factor (GDNF), Brain Derived Neurotrophic Factor (BDNF), and Neurotrophin-3 (NT-3).

The neurotrophins play a vital role in development, but also in the maintenance of the neuronal systems throughout life (Sizonenko et al, 2007). During the neonatal period, neurotrophins and their receptors are essential for brain development. After brain insult, neurotrophins levels increase, suggesting that they have an endogenous protective mechanism that limits neuronal cell death. Since discovery of the potent survival-promoting effects of neurotrophic factors (possibly through angiogenic mechanisms), they have been proposed as potential tools to be tested for the treatment of diseases of the CNS (e.g.: **basic fibroblast growth factor-bFGF**) (Binder & Scharfman, 2004). The protection of neurons, and perhaps most cells, from excitotoxicity and H-I injury may require extracellular ligand–receptor interactions and the activation of specific intracellular signaling cascades. In the CNS, these signals are provided, at least in part, by neurotrophic growth factors. Several neurotrophic factors (such as **platelet-derived growth factor, insulin-derived growth factor, and glial cell line-derived neurotrophic factor**) that have been reported to protect against excitotoxicity and H-I injury in immature animal models may act by inhibiting apoptosis (Nozaki et al, 1993; Hossain et al, 1998; Wang et al, 2013), these have not been studied in humans. Neurotrophins could guide migration and differentiation of stem cell transplants after brain injury, and once at the site of injury, enhance neuronal differentiation (Douglas-Escobar et al, 2012).

- **Neuropeptide-inhibitor**

Neuropeptides modulate neuronal activity and may therefore modulate glutamate-induced neuronal cell death. Neuropeptides are inactivated by enzymatic proteolysis, indicating that proteolysis inhibition may hold therapeutic potential. Among the peptidases identified, neural endopeptidase (NEP or neprilysin) is involved in the regulation and metabolism of a variety of biologically active peptides including tachykinins/ neurokinins (see Degos, 2008). Interestingly, the **NEP inhibitor Racecadotril** (Tiorfan®) is used in clinical practice to treat diarrhea, with a remarkably good safety profile. Racecadotril is rapidly and entirely metabolized to its active metabolite thiorphan. A recent study showed that systemic administration of thiorphan was neuroprotective against excitotoxic neuronal cell death in newborn mice (Schwartz, 2000). This neuroprotective effect was long-lasting and was still observed when thiorphan was administered 12 h after the insult, indicating a wide window for therapeutic intervention (Medja et al, 2006).

6.3. Neurorestorative therapies

There is increasing evidence from “in vitro” and “in vivo” preclinical studies that stem/progenitor cells may have multiple beneficial effects on the outcome after H-I injury. Stem cell (SCs) treatment can be administered by stimulating endogenous SCs (neurotrophic factors/growth factors such as EPO, insulin-like growth factor and brain-derived neurotrophic factor) or by transplanting exogenous SCs. **Stem/progenitor cells** (umbilical cord SCs, mesenchymal stromal cells, and bone marrow mesenchymal SCs) have been used for the experimental treatment of neonatal HIE models, and have shown great promise in animal studies in

decreasing neurological impairment. **Neural stem/progenitor cells** (NSCs) have also been transplanted in animal models of HIE, migrating long distances to ischemic brain areas and differentiating into neurons. SCs therapies are one of the promising options for the treatment of neonatal neurological diseases in the future. However, the mechanisms of action of the SCs, and the optimal type, dose, and method of administration remain surprisingly unclear, and some studies have found no benefit. Although cell-based interventions after completion of the majority of secondary phase cell-death appear to have potential to improve functional outcome for neonates after HI, further rigorous testing in translational animal models is required before randomized controlled trials should be considered. (Review articles: Pimentel-Coelho & Mendez-Otero, 2010; Bennet et al, 2012; Pabon et al, 2013). Additionally, their clinical use is strongly limited by the existence of the BBB that makes the human brain refractory to targeting of cell-sized agents delivered through the peripheral system. Intracerebral transplantation to bypass the BBB is a very invasive delivery method that cannot be proposed for human newborns.

- **Cord blood:** Umbilical cord blood cells (UCBCs), which are readily available at birth, have been shown to reduce sensorimotor and/or cognitive impairments in several models of brain damage, representing a promising option for the treatment of neurological diseases. The possible cell types and mechanisms involved in the therapeutic effect of UCBC transplantation, including neuroprotection, immunomodulation and stimulation of neural plasticity and regeneration have been recently reviewed by Pimentel-Coelho et al. (Pimentel-Coelho et al., 2012).
- **Mesenchymal stem cells (MSC):** The beneficial effect of MSC transplantation to treat neonatal brain injury might be explained by the great plasticity of the neonatal brain. The neonatal brain is still in a developmentally active phase, leading to a better efficiency of MSC transplantation than that observed in experiments using adult models of stroke. Enhanced neurogenesis and axonal remodeling likely underlie the improved functional outcome following MSC treatment after neonatal H-I brain injury. With respect to the mechanism of repair by MSCs, MSCs do not survive long term and replace damaged tissue themselves. MSC treatment after H-I reduced contralesional rewiring taking place after HI and increased the connection between the impaired forepaw and the ipsilesional motor cortex. These intrinsic adaptive properties of MSCs make them excellent candidates for a novel therapy to treat the devastating effects of HIE in the human neonate (van Velthoven et al, 2012). Intranasal MSC treatment may become a promising non-invasive therapeutic tool to effectively reduce neonatal encephalopathy (Donega et al, 2013).
- **Neural stem/progenitor cells (NSCs):** NSCs have also been transplanted in animal models of HIE, migrating long distances to ischemic brain areas and differentiating into neurons (Llado et al, 2004). The survival of transplanted NSCs was limited in these experiments for several potential reasons (Sato et al, 2008).
- **Adipose stromal cells (ASC):** In a rat middle cerebral artery occlusion model of ischemic brain injury, intracerebral transplantation of human ASC was followed by migration of these cells to areas of ischemic damage and by expression of neuronal specific markers in

conjunction with functional benefit. Therefore, the use of ASC could have potential to develop treatments to reverse or prevent the effects of H-I injury. (Kang et al, 2003)

7. Combined therapy

Following HIE, a complicated cascade of pathophysiologic processes is unleashed including excitotoxicity, oxidative stress, inflammation, and cell death via necrosis and apoptosis. These processes can lead to long-term neurologic injury. The post-injury time course can be divided into a latent (0–6 hours), secondary (6–72 hours) and tertiary phase (>72 hours) (Perlman, 2011). Studies in laboratory animals have shown that the immature brain responds differently to treatment than does the mature brain, which leads us to believe that an optimal treatment for a neonate would differ from that for a toddler and probably none of them would be the best option for an adult. Specific vulnerabilities that distinguish the response of the immature brain from that of the mature brain include:

Primary/latent phase (0–6 hours)

- Greater neuronal metabolism: The neonatal brain has a high rate of oxygen consumption. Such energetic costs seem also to exert a selective pressure towards metabolically efficient neural morphology, leading to metabolically efficient patterning of dendritic arborizations, neural codes and brain wiring patterns (Holliday, 1986).
- Antioxidant system insufficiency.
- The NMDA receptor subunits in the developing brain open more easily and block less frequently than mature forms, responsible for the fact that immature brains are far more excitable and epileptogenic than the adult brain.

Secondary phase (6–72 hours)

Increased susceptibility to excitotoxicity and FR injury, due to the production of large amounts of FR, high concentrations of PUFAs, and antioxidant system insufficiency (Grow & Barks, 2002).

Tertiary phase (>72 hours)

- Greater tendency to apoptotic death: activation of apoptosis-executing caspases is much greater in the immature brain than in the adult brain. During the tertiary phase, neurons and glial cells are lost due to chronic loss of trophic factors, loss of synaptic input from neighboring cells, and loss of or failure of recruitment of new progenitor neural stem cells and glial progenitor cells (heightened vulnerability of immature OL). Cell death involving a cell-autonomous active contribution of catabolic enzymes (or *apoptosis*) plays a prominent role in the evolution of H-I injury in the neonatal brain and it is at least as important for the loss of neurons as unregulated cell death (or *necrosis*) (Zhu et al, 2007). The prominence of autophagic neuronal death in the ischemic penumbra and the neuroprotective efficacy of postischemic autophagy inhibition indicate that autophagy should be a primary target in

the treatment of neonatal cerebral ischemia (Puyal et al, 2009). Therapy designed to ameliorate brain injury in adults may worsen outcomes in neonates, possibly by accentuating the apoptotic cell death cascade.

Neurogenetic and gliogenetic processes after H-I injury: neuroblast proliferation and migration from the neurogenetic niches take place after the lesion. Some cells differentiate into neurons (neuronal differentiation) and migrate to the injured area. Some other cells from the SPZ give rise to radial glia that contribute to neural progenitor expansion and support neuroblast migration. Thus, the neuronal elements in the transient fetal zones represent a potential for plasticity after perinatal cerebral lesions and neuronal migration could play a central role in brain repair (Kostovic & Judas M, 2006, Cayre et al, 2009, Distefano and Praticò, 2010). It is possible that the same chemical mediators that have deleterious effects during the initial stages of ischemia may also be involved in the ulterior process of neurorestoration. Therefore, the lack of effect found for some neuroprotective drugs could be due to either untimely or too prolonged period of administration, thus interfering with a given metabolic route at the time that is involved in the endogenous mechanisms of repair (Lo, 2008).

With the advent of hypothermia as therapy for term HIE, there is hope for repair and protection of the brain after a profound neonatal insult. However, hypothermia alone would not be sufficient to provide the required protection or stimulate the repair to ensure a normal neurodevelopment. Based on the theory of “secondary energy failure”, hypothermia may provide the possibility to “buy time”, in order to successfully use other (pharmacologic) interventions, by preserving energy metabolism elongating the therapeutic time window (Gunn & Gunn, 1997). At present, no individual neuroprotective agent has been proven safe and effective for the protection of neonates from neurological sequels after H-I insults as monotherapy. The persistent clinical failures might be due to many factors, including: heterogeneity in the causes of neural death in humans, associated toxicity at the doses required for drug-efficacy, the lack of adequate CNS penetration across the BBB and or the limited time window available to start the treatment (in *real-life* clinical practice, delays in the initiation of therapy are difficult to avoid).

Since there are many mechanisms involved in H-I process, it is reasonable to assume that the combination of several drugs or the use of molecules that combine two or more neuroprotective actions can exert synergistic effects by blocking diverse metabolic pathways. Traditionally, the above mentioned simultaneous drug co-administration has been discouraged, since it involves potential risks of interference, side effects and error in the sequence of administration. However, the use of hypothermia plus adjuvant therapies has been extensively reviewed (Cilio & Ferrriero, 2010; Robertson et al, 2012; Buonocore et al, 2012; Shankaran, 2012). According to the existing literature, the combined therapy of hypothermia and other neuroprotective strategies would be expected to increase the therapeutic time window, enhance neural repair and improve the neurological outcomes of HIE. Although both clinical observations and animal experimentation suggest that the cascade of damaging events in the developing brain may last for several days -thus extending the window of opportunity for intervention- the most successful outcome is likely to result from the earliest possible delivery of therapy (Rees et al, 2011). Few studies have examined possible interactions of medications with hypothermia and

whether combination therapies augment neuroprotection. Based on the preclinical studies, ongoing trials in neonates include: inhaled xenon and cooling (NCT01545271 and NCT00934700), safety of erythropoietin (NCT00719407), darbepoetin and hypothermia (NCT0147105), and topiramate plus hypothermia (NCT01241019).

Additional neuroprotective strategies to combat perinatal brain injury are urgently needed that may be used as possible synergies for therapy. Presumably the best outcome will be achieved by a multi-modal therapeutic approach such as a combination of hypothermia with anti-oxidants and glutamate receptor antagonists, using drugs with multiple effects (affecting multiple injury cascades and with neuroregenerative potential) without toxicity, no apparent interaction and previously used in children. Furthermore, the timing of the administration of medication may be critical to optimize the benefits and avoid neurotoxicity (e.g., early acute treatments targeted at amelioration of the neurotoxic cascade compared with subacute treatment that may promote regeneration and repair) (Kelen & Robertson, 2010).

8. Staggered design for a “off-label* combined therapy”

Given the urgency to find better therapies for HIE, and in the absence of ongoing clinical trials, we propose a model of “**off-label therapy**” based on hypothermia /antiepileptic drugs in combination with antioxidants, phospholipase A2 inhibitors, glutamate receptor antagonists and EPO using a staggered design in function of the intensity of the perinatal asphyxia and severity of the encephalopathy. However, we believe that a multicenter interventional randomized controlled pilot phase II clinical trial would be necessary.

Premises

- All drugs have been approved by the FDA.
- All the drugs have been currently available in infants without serious side effects.
- All drugs have demonstrated neuroprotection
- All drugs have and synergy with hypothermia, increasing the neuroprotective efficacy of therapeutic hypothermia in perinatal H-I brain injury – animals/clinical- models.
- All drugs have different mechanisms and multiple potential modes of action.

Timing of the administration of medications:

At the time of initiation of hypothermia, stage 1 drugs would also be administered for at least 72 hours. If moderate / grave encephalopathy (according to clinical and encephalographic evaluation) persists, administration of the drugs assigned to the next stages would successively proceed. It should be noted that the use of hypothermia delays the pathophysiological response to H-I (postpones secondary energy failure) and therefore “widens” the therapeutic window. This phenomenon allows staggering the application of the adjuvant drugs, thus providing the physician with the necessary time to assess the clinical situation of the patient and decide whether to proceed to the next therapeutic phase.

8.1. Primary/latent phase (0–6 hours)

- **Anticonvulsants for neonatal seizures: PHENOBARBITAL:** PB remains the preferred drug for the treatment of seizures in neonates with HIE. **Ways of action:** anticonvulsant effects by increase GABA subtype A (GABAA)-receptor channel chloride currents, reduced cerebral metabolic demand, antioxidant effects and decreased cerebral edema. **Doses:** loading dose 20 mg/kg IV, followed by 3- 5 mg/kg/day, every 12 hours
- **Antioxidative drugs: Free radical nonenzymatic scavengers (N-acetylcysteine and Vitamin E)**
- **N-acetylcysteine (NAC). Ways of action:** act as an anti-oxidant and is also a scavenger of oxygen FRs, reduce oxidative stress and inflammation; prevents endotoxin-induced degeneration of OL progenitors and hypomyelination; attenuated activation of apoptotic proteases.

Continuous IV infusion, by simplified N-acetylcysteine dosing regimen – Standard preparation of IV-

Prepare standard solution IV: 50ml NAC 20% + 200ml SG5% (5% dextrose in water) = Solution NAC 40mg/ml. Doses: 150mg/kg (3,75ml x kg) IV loading dose administered over 1 hour, followed by an infusion of 12 mg/kg/h (7.2ml x Kg) for 24 hours. Continue with infusion of 150 mg/kg/24 h (3.75 ml x Kg) for 24 hours, to complete 72 hours

Or 72-hour oral-NAC: 140 mg/kg of oral NAC followed by 70 mg/kg every 4 h. for an additional 17 doses.

- **Vitamin E. Ways of action:** protects OL during this special vulnerability maturation period from the oxidative stress-induced death caused by glutathione depletion. It also ameliorates secondary mitochondrial failure. **Doses:** 50 U.I. orally, followed by 1 UI/kg/24 hours. Equivalency: 1 U.I. Vitamin E = 1 mg tocopherol acetate.

8.2. Secondary phase (6–72 hours)

- **Antiexcitatory drugs: Topiramate. Ways of action:** blocking sodium channels, high voltage-activated calcium currents, enhancing GABA-induced influx of chloride, and inhibiting kainite/ AMPA glutamate receptors. It also blocks carbonic anhydrase isoenzymes and the mitochondrial permeability transition pore. **Doses:** 10 mg/kg/24 hours, divided into 2 doses, orally administered by orogastric tube.
- **Bioactive lipid mediators: Citicoline. Ways of action: Neuroprotective effects** - decrease of phospholipase A2 stimulation and hydroxyl radical generation; increase of glutathione synthesis and glutathione reductase activity, restoration of Na⁺/K⁺-ATPase activity, and antiglutamatergic effects. Roles in the generation of phospholipids involved in membrane formation and repair; prevention of fatty acid release, stimulation of phosphatidylcholine synthesis, preservation of cardiolipin and sphingomyelin levels. **Neuroregenerative effects** - related to inhibition of the glutamate induced apoptotic pathway, markedly reduces caspase-3 activation; increases neurogenesis in the SVZ and migration of neural progenitors

to the lesion area with increased neurogenesis also within the peri-infarct area. **Dosis:** 100 mg/kg/24 hours, every 12 h, intravenously during the first 3 days, and orally administered by orogastric tube thereafter for a total of 6 weeks.

8.3. Tertiary phase (>72 hours)

- **Anti-apoptotic drugs: Erythropoietin (EPO).** **Ways of action:** **Neuroprotective effects** - activation of anti-apoptotic, anti-oxidant and anti-inflammatory pathways as well as through the stimulation of angiogenic and neurogenic events; reduces the excitotoxic effect of glutamate and a glutamate receptor agonist (AMPA) on cortical neuron cultures. **Neuroregenerative effects** - prevents apoptosis induced by NMDA or by NO in neurons from cerebrocortical cultures and regulates neurogenesis. **Dosis:** Recombinant human EPO (rhEPO) given subcutaneously 400 U/kg daily for 5 days, thereafter 3 times a week.

9. Conclusion

Since hypoxic ischemic encephalopathy (HIE) is a potentially preventable cause of cerebral palsy (CP), much interest has been focused on prevention as well as research on neuroprotection therapies. Neuroprotective treatment for HIE in the clinical practice has been limited to the application of hypothermia in the newborn which is now accepted as a significant therapy, since so far no drug has shown any benefit when administered on its own. However, hypothermia alone may not provide complete protection or stimulate the repair that is necessary for a normal neurodevelopmental outcome. As we have described in this chapter, many mechanisms can be involved in the H-I process. It is therefore a reasonable assumption that the combination of several drugs involving two or more neuroprotective actions may exert synergistic effects by tackling several metabolic pathways at one time. We propose a model of **“off-label combined therapy”** based on hypothermia /antiepileptic drugs in combination with antioxidants, phospholipase A2 inhibitors, glutamate receptor antagonists or EPO using a staggered design in function of the intensity of the perinatal asphyxia and severity of the encephalopathy.

Note

*“Off- label” use is the use of already authorized pharmaceutical drugs for an unapproved indication or in an unapproved age group, unapproved dosage, or unapproved form of administration.

The term *“compassionate use”* (also known as *compassionate exemption* or *expanded access*) is used to define treatment options that allow the use of an unauthorised medicine. It may be applied to patients who cannot be treated satisfactorily by an authorised medicinal product or cannot enter a clinical trial. Although sometimes “off label use” has been considered a type of

“compassionate use”, those two concepts should not be mistaken since they have different legal requirements. For reference and discussion see:

- Randall S. Stafford. "Regulating Off-Label Drug Use — Rethinking the Role of the FDA". *N Engl J Med* 2008; 358: 1427–1429.
- Bombillar-Sáenz F.M. “The “Compassionate Exemption” In Spain: Not Asking For Compassion, Op. J., Vol. 2/2010, Paper n. 1, pp. 1 - 25, <http://lider-lab.sssup.it/opinio>, online publication July 2010.
- Guideline on compassionate use of medicinal products, pursuant to article 83 of regulation (EC) No 726/2004. Doc. Ref: EMEA/27170/2006.

Abbreviations

AA arachidonic acid	MBP myelin basic protein
AEDs antiepileptic drugs	NAC N- acetyl-L-cysteine
ALLO allourinol	NICU Neonatal Intensive Care Unit
AMPA α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid	NMDA N methyl-D-aspartate
BBB blood-brain barrier	NO nitric oxide
CBF cerebral blood flow	NOS Nitric oxide synthase
CBFV cerebral blood flow velocity	NT's neurotransmitters
CAT catalase	OL oligodendrocyte
CSF cerebrospinal fluid	PAF platelet activating factor
CNS central nervous system	PB Phenobarbital
CP cerebral palsy	PLA2 phospholipase A2
DC dendritic cell	Pre-OL premyelinating oligodendrocyte
DEX dexamethasone	PUFAs polyunsaturated fatty acids
EAA excitatory amino acid	PVL periventricular leukomalacia
EPO erythropoietin	PWMD periventricular white matter damage
FDA Food and Drug Administration (USA)	PCW postconceptional weeks
FFA free fatty acids	RNS reactive nitrogen species
FR free radical	ROS reactive oxygen species
GPX glutathione peroxidase	SCs Stem cell
H-I hypoxic–ischemic	SNN selective neuronal necrosis
HIE Hypoxic ischemic encephalopathy	SOD superoxide dismutase
IL interleukins	SPZ subplate zone
I/R ischemia/reperfusion	SVZ subventricular zone
IV intravenous	TLR toll-like receptor
IVH intraventricular hemorrhage	TPM Topiramate
LPS lipopolysaccharide	VLBW very low birth weight
	WM white matter

Author details

M^a Carmen Carrascosa-Romero¹ and Carlos de Cabo-de la Vega²

*Address all correspondence to: carlosd@sescam.jccm.es

1 Department of Neuropediatrics, Albacete General Hospital, Albacete, Spain

2 Neuropsychopharmacology Unit, Albacete General Hospital, Albacete, Spain

References

- [1] Abbracchio MP, Cattabeni F. Brain adenosine receptors as targets for therapeutic intervention in neurodegenerative diseases. *Ann N Y Acad Sci.* 1999; 890:79-92.
- [2] Adibhatla RM, Hatcher JF. Citicoline mechanisms and clinical efficacy in cerebral ischemia. *J Neurosci Res* 2002; 15: 133-139.
- [3] Adibhatla RM, Hatcher JF. Citicoline decreases phospholipase A2 stimulation and hydroxyl radical generation in transient cerebral ischemia. *J Neurosci Res* 2003; 73: 308-315.
- [4] Ahola T, Lapatto R, Raivio KO, Selander B, Stigson L, Jonsson B, et al. N-acetylcysteine does not prevent bronchopulmonary dysplasia in immature infants: a randomized controlled trial. *J. Pediatr.* 2003; 143:713-719.
- [5] Aicardi, J. Overview: neonatal seizures. In: Engel, JJ.; Pedley, TA., editors. *Epilepsy: a comprehensive textbook*. Philadelphia: Lippincott Williams & Wilkins; 2008. p. 2283-2285.
- [6] Aly H, Abd-Rabboh L, El-Dib M, Nawwar F, Hassan H, Aaref M, et al. Ascorbic acid combined with ibuprofen in hypoxic ischemic encephalopathy: a randomized controlled trial. *J Perinatol* 2009; 29:438-43.
- [7] Ambalavanan N, Carlo WA, Shankaran S, Bann CM, Emrich SL, Higgins RD, et al. Predicting outcomes of neonates diagnosed with hypoxemic-ischemic encephalopathy. *Pediatrics.* 2006; 118:2084-93.
- [8] American College of Obstetricians and Gynecologists and the American Academy of Pediatrics. Criteria required to define an acute intrapartum hypoxic event as sufficient to cause cerebral palsy. Neonatal encephalopathy and cerebral palsy: defining the pathogenesis and pathophysiology. ACOG 2003:73-80.
- [9] Andón FT, Pazinatto DB, Fadeel B. Perinatal nanomedicine: dendrimer-based therapy for neuroinflammatory disorders. *Nanomedicine (Lond).* 2012; 7(9):1294.

- [10] Ara J, De Montpelliér S. Hypoxic-preconditioning enhances the regenerative capacity of neural stem/progenitors in subventricular zone of newborn piglet brain. . *Stem Cell Res.* 2013; 11(2):669-86.
- [11] Arvin KL, Han BH, Du Y, Lin S-Z, Paul SM, Holtzman DM. Minocycline markedly protects the neonatal brain against hypoxic-ischemic injury. *Ann Neurol.* 2002; 52:54–61.
- [12] Azzopardi DV, Strohm B, Edwards AD, Dyet L, Halliday HL, Juszczak E, et al. TOBY Study Group. Moderate hypothermia to treat perinatal asphyxial encephalopathy. *N Engl J Med.* 2009; 361:1349–58.
- [13] Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. *Science* 1998; 281:1305 – 8.
- [14] Badawi N, Felix JF, Kurubczuk JJ, Dixon G, Watson L, Keogh JM, et al. Cerebral palsy following term newborn encephalopathy: a population-based study. *Dev Med Child Neurol.* 2005; 47:293–98.
- [15] Back SA, Gan X, Li Y, Rosenberg PR, Volpe JJ. Maturation-dependent vulnerability of oligodendrocytes to oxidative stress-induced death caused by glutathione depletion. *J Neurosci.* 1998; 18(16):6241–6253.
- [16] Ballin A, Brown EJ, Koren G, Zipursky A. Vitamin C-induced erythrocyte damage in premature infants. *J Pediatr* 1988; 113:114–20.
- [17] Barks JD. Current controversies in hypothermic neuroprotection. *Seminars in Fetal and Neonatal Medicine.* 2008; 13 (1): 30–34.
- [18] Barks JD, Liu YQ, Shangguan Y, Silverstein FS.: Phenobarbital augments hypothermic neuroprotection. *Pediatr Res.* 2010; 67(5):532-7.
- [19] Barks JD, Silverstein FS. Excitatory amino acids contribute to the pathogenesis of perinatal hypoxic–ischemic brain injury. *Brain Pathol* 1992; 2: 235–43.
- [20] Barinaga, M. (1998) Stroke-damaged neurons may commit cellular suicide. *Science* 281, 1302–1303.
- [21] Baud O. Antenatal corticosteroid therapy: benefits and risks. *Acta Paediatr Suppl* 2004; 93:6–10.
- [22] Ben-Ari Y.: Basic developmental rules and their implications for epilepsy in the immature brain. *Epileptic Disord.* 2006; 8(2):91-102.
- [23] Benders MJ, Bos AF, Rademaker CM, Rijken M, Torrance HL, Groenendaal F, et al. Early postnatal allopurinol does not improve short term outcome after severe birth asphyxia. *Arch Dis Child Fetal Neonatal Ed* 2006, 91:163-165.

- [24] Bebarta VS, Kao L, Froberg B, Clark RF, Lavonas E, Qi M, et al: A multicenter comparison of the safety of oral versus intravenous acetylcysteine for treatment of acetaminophen overdose. *Clin Toxicol (Phila)*. 2010; 48(5):424-30.
- [25] Bennet L, Tan S, Van den Heuvel L, Derrick M, Groenendaal F, van Bel F, et al. Cell therapy for neonatal hypoxia-ischemia and cerebral palsy. *Ann Neurol*. 2012; 71(5): 589-600.
- [26] Biban P, Filipovic-Grcic B, Biarent D, Manzoni P. International Liaison Committee on Resuscitation (ILCOR); European Resuscitation Council (ERC); American Heart Association (AHA); American Academy of Pediatrics (AAP). New cardiopulmonary resuscitation guidelines 2010: managing the newly born in delivery room. *Early Hum Dev*. 2011 Mar;87 Suppl 1:S9-11.
- [27] Binder DK, Scharfman HE. Brain-derived neurotrophic factor. *Growth Factors*. 2004; 22: 123–131.
- [28] Bittigau P, Siffringer M, Pohl D. Apoptotic neurodegeneration following trauma is markedly enhanced in the immature brain. *Ann Neurol* 1999; 45: 724–35.
- [29] Bittigau P, Siffringer M, Ikonomidou C. Antiepileptic drugs and apoptosis in the developing brain. *Ann NY Acad Sci* 2003; 993:103–114.
- [30] Blair E, Stanley FJ: Intrapartum asphyxia: A rare cause of cerebral palsy. *The Journal of Pediatrics*, Volume 112, Issue 4: 515-519.
- [31] Blaschke AJ, Staley K, Chun J. Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex. *Development* 1996; 122:1165–74.
- [32] Brener P, Ballardo M, Mariani G, Ceriani Cernadas JM. Medication error in an extremely low birth weight infant: paracetamol overdose. *Arch Argent Pediatr*. 2013; 111(1):53-5.
- [33] Buettner GR, Jurkiewicz BA. Catalytic metals, ascorbate and free radicals: combinations to avoid. *Radiat Res* 1996; 145:532–41.
- [34] Buller KM, Carty ML, Reinebrant HE, Wixey JA. Minocycline: a neuroprotective agent for hypoxic-ischemic brain injury in the neonate? *J Neurosci Res*. 2009; 87:599–608.
- [35] Buonocore G, Perrone S, Turrise G, Kramer BW, Balduini W. New pharmacological approaches in infants with hypoxic-ischemic encephalopathy. *Curr Pharm Des*. 2012; 18 (21):3086-100.
- [36] Calabresi P, Cupini LM, Centonze D, Pisani F, Bernardi G. Antiepileptic drugs as a possible neuroprotective strategy in brain ischemia. *Ann Neurol* 2003; 53: 693–703.
- [37] Carrascosa MC, Martínez Gutiérrez A, Onsurbe I, Vázquez MS, Catalan B, Tébar R.: "Neonatal convulsions in health care. Incidence, etiology and clinical aspects". *Rev Neurol* 1996; 24(134):1258-62.

- [38] Carrascosa-Romero MC, Suela J, Alfaro-Ponce B, Cepillo-Boluda AJ. Ictiosis ligada al cromosoma X asociada a epilepsia, hiperactividad, autismo y retraso mental, por microdelección Xp22.31. *Rev Neurol* 2012; 54: 241-8.
- [39] Cayre M, Canoll P, Goldman JE. Cell migration in the normal and pathological postnatal mammalian brain. *Prog Neurobiol*. 2009; 88(1):41-63.
- [40] Chakkarapani E, Thoresen M, Liu X, Walloe L, Dingley J. Xenon offers stable haemodynamics independent of induced hypothermia after hypoxia-ischaemia in newborn pigs. *Intensive Care Med*. 2012; 38(2):316-23.
- [41] Chen J, Tu Y, Moon C, Matarazzo V, Palmer A, Ronnett G. The localization of neuronal nitric oxide synthase may influence its role in neuronal precursor proliferation and synaptic maintenance. *Dev Biol* 2004; 269:165-82.
- [42] Cho H.J., Kim Y.J. Efficacy and safety of oral citicoline in acute ischemic stroke: drug surveillance study in 4,191 cases. *Methods Find Exp Clin Pharmacol*. 2009; 31: 171–176.
- [43] Choi DW, Rothman SW. The role of glutamate neurotoxicity in hypoxic–ischemic neuronal death. *Annu Rev Neurosci*. 1990;13:171–82.
- [44] Cilio M, Ferriero D. Synergistic neuroprotective therapies with hypothermia. *Semin Fetal Neonatal Med* 2010; 15(5):293-8.
- [45] Clarke PGH, Puyal J, Vaslin A, Ginet V, Truttmann A: Multiple types of programmed cell death and their relevance to perinatal brain damage. In perinatal brain damage: from pathogenesis to neuroprotection, chp 3. Edited by LA Ramenghi, P Evrard, and E. Mercuri. Mariani Foundation Paediatric Neurology Series 19, John Libbey Eurotext, Montrouge, France, 2008, pp. 23–35.
- [46] Clinical Practice Guidelines on Magnesium Sulphate Prior to Preterm Birth for Neuroprotection of the Fetus, Infant and Child. [Last cited on 15 Spe 2011]. Available from: <http://www.nhmrc.gov.au/guidelines/publications/cp128>.
- [47] Committee opinion no. 573: magnesium sulfate use in obstetrics. *Obstet Gynecol*. 2013 Sep; 122(3):727-8.
- [48] Costa C, Martella G, Picconi B, Prosperetti C, Pisani A, Di Filippo M, et al. Multiple mechanisms underlying the neuroprotective effects of antiepileptic drugs against in vitro ischemia. *Stroke* 2006; 37:1319–1326.
- [49] Covey M, Murphy M, Hobbs C, Smith R, Oorschot D. Effect of the mitochondrial antioxidant, Mito Vitamin E, on hypoxic-ischemic striatal injury in neonatal rats: a dose-response and stereological study. *Exp Neurol*. 2006 Jun; 199(2):513-9.
- [50] Crowther CA, Hiller JE, Doyle LW, Haslam RR. Effect of magnesium sulfate given for neuroprotection before preterm birth: a randomized controlled trial. *JAMA*. 2003; 290(20):2669-76.

- [51] Crunkhorn S. Neurological disorders: Nanoparticle opens door to cerebral palsy treatment. *Nat Rev Drug Discov*. 2012; 11(6):440-1.
- [52] Dammann O, Ferriero D, Gressens P: Neonatal Encephalopathy or Hypoxic-Ischemic Encephalopathy? Appropriate Terminology Matters. *Pediatric Research*. 2011; 70 (1): 1-2.
- [53] Daneyemez M, Kurt E, Cosar A, Yuce E, Ide T. Methylprednisolone and vitamin E therapy in perinatal hypoxic-ischemic brain damage in rats. *Neuroscience* 1999; 92: 693-7.
- [54] Dávalos A, Alvarez-Sabín J, Castillo J, Díez-Tejedor E, Ferro J, Martínez-Vila E, et al. International Citicoline Trial on acUte Stroke (ICTUS) trial investigators. Citicoline in the treatment of acute ischaemic stroke: an international, randomised, multicentre, placebo-controlled study (ICTUS trial). *Lancet*. 2012 Jul 28; 380(9839):349-57.
- [55] Davalos A, Castillo J, Alvarez-Sabin J, et al. Oral citicoline in acute ischemic stroke: an individual patient data pooling analysis of clinical trials. *Stroke* 2002; 33: 2850–57.
- [56] De Cabo-de la Vega C, Villanueva-Hernandez P, Prieto-Martin A. The neurochemistry of epilepsy, inhibitory neurotransmission and experimental models: new perspectives. *Rev Neurol*. 2006;42(3):159-68.
- [57] Degos V, Loron G, Mantz J, Gressens P: Neuroprotective Strategies for the Neonatal Brain. *Anesth Analg* 2008; 106:1670 –80.
- [58] Derks JB, Oudijk MA, Rosén KG, Thakor AS, Visser GHA, van Bel F, et al. Allopurinol maintains umbilical blood flow following repeated episodes of ischaemia-reperfusion in fetal sheep during late gestation [abstract]. *J Soc Gynecol Invest* 2006, 13:s35.
- [59] De Vries LS, Jongmans MJ. Long-term outcome after neonatal hypoxic-ischemic encephalopathy. *Arch Dis Child Fetal Neonatal Ed* 2010; 95:F220–4.
- [60] Diederich K, Frauenknecht K, Minnerup J, Schneider BK, Schmidt A, Altach E. Citicoline Enhances Neuroregenerative Processes After Experimental Stroke in Rats. *Stroke*. 2012; 43:1931-1940.
- [61] Digicaylioglu M., Lipton S.A. Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. *Nature*. 2001; 412: 641–647.
- [62] Dingley J, Tooley J, Porter H, Thoresen M. Xenon provides short-term neuroprotection in neonatal rats when administered after hypoxia-ischemia. *Stroke* 2006; 37:501–6.
- [63] Distefano G, Praticò AD. Actualities on molecular pathogenesis and repairing processes of cerebral damage in perinatal hypoxic-ischemic encephalopathy. *Ital J Pediatr*. 2010 Sep 16;36:63.

- [64] Dommergues MA, Gallego J, Evrard P, Gressens P. Iron supplementation aggravates periventricular cystic white matter lesions in newborn mice. *Eur J Paediatr Neurol* 1998; 2:313–8.
- [65] Dommergues MA, Plaisant F, Verney C, Gressens P. Early microglial activation following neonatal excitotoxic brain damage in mice: a potential target for neuroprotection. *Neuroscience*. 2003; 121:619–628.
- [66] Donega V, van Velthoven CT, Nijboer CH, van Bel F, Kas MJ, Kavelaars A, et al. Intranasal mesenchymal stem cell treatment for neonatal brain damage: long-term cognitive and sensorimotor improvement. *PLoS One*. 2013; 8(1):e512-53.
- [67] Douglas-Escobar M, Rossignol C, Steindler D, Zheng T, Weiss MD. Neurotrophin-induced migration and neuronal differentiation of multipotent astrocytic stem cells in vitro. *PLoS One*. 2012; 7(12):e51706.
- [68] Doyle LW, Crowther CA, Middleton P, Marret S, Rouse D. Magnesium sulphate for women at risk of preterm birth for neuroprotection of the fetus. *Cochrane Database Syst Rev*. 2009 Jan 21; (1):CD004661.
- [69] Dzhala VI, Brumback AC, Staley KJ. Bumetanide enhances phenobarbital efficacy in a neonatal seizure model. *Ann Neurol*. 2008; 63(2):222-35.,
- [70] Edwards AD, Brocklehurst P, Gunn AJ, Halliday H, Juszczak E, Levene M, et al. Neurological outcomes at 18 months of age after moderate hypothermia for perinatal hypoxic ischaemic encephalopathy: synthesis and meta-analysis of trial data. *BMJ* 2010; 340:c363.
- [71] Ehrenreich H, Weissenborn K, Prange H, Schneider D, Weimar C, Wartenberg K., et al. Recombinant human erythropoietin in the treatment of acute ischemic stroke. *Stroke*. 2009; 40: e647-e656.
- [72] Elmahdy H, El-Mashad AR, El-Bahrawy H, El-Gohary T, El-Barbary A, Aly H. Human recombinant erythropoietin in asphyxia neonatorum: pilot trial. *Pediatrics* 2010; 125:e1135-42.
- [73] Elterman RD, Glauser TA, Wyllie E, Reife R, Wu SC, Pledger G. A double-blind, randomized trial of topiramate as adjunctive therapy for partial-onset seizures in children. Topiramate YP Study Group. *Neurology* 1999; 52:1338–44.
- [74] Esplugues JV. NO as a signalling molecule in the nervous system. *Br J Pharmacol* 2002; 135: 1079 – 95.
- [75] Evans DJ, Levene MI, and Tsakmakis M. Anticonvulsants for preventing mortality and morbidity in full term newborns with perinatal asphyxia. *Cochrane Database of Systematic Reviews*, 2007; no. 3, Article ID CD001240,
- [76] Fabian RH, Perez-Polo J, Kent TA. Perivascular nitric oxide and superoxide in neonatal cerebral hypoxia-ischemia. *Am J Physiol Heart Circ Physiol* 2010; 295: h1809-14.

- [77] Fan LW, Lin S, Pang Y, Rhodes PG, Cai Z. Minocycline attenuates hypoxia-ischemia-induced neurological dysfunction and brain injury in the juvenile rat. *Eur J Neurosci* 2006; 24:341–50.
- [78] Fan LW, Pang Y, Lin SY, Tien LT, Ma TG, Rhodes PG, et al. Minocycline reduces lipopolysaccharide-induced neurological dysfunction and brain injury in the neonatal rat. *J Neurosci Res.* 2005; 82:71–82.
- [79] Fatemi A, Wilson MA, Johnston MV. Hypoxic-Ischemic Encephalopathy in the Term Infant. *Clin Perinatol.* 2009; 36 835–858.
- [80] Fauchere J, Dame C, Vonthein R, Koller B, Arri S, Wolf M, et al. An approach to using recombinant erythropoietin for neuroprotection in very preterm infants. *Pediatrics* 2008; 122:375–82.
- [81] Fehlings, D; Hunt C. & Rosenbaum P. (2007). Cerebral Palsy. In: A Comprehensive Guide to Intellectual & Developmental Disabilities. Brown, I. & Percy, M. 287– 295 ISBN-13: 978- 1-55766-700-7 Brookes Publishing Co. Baltimore.
- [82] Ferriero DM, Holtzman DM, Black SM, Sheldon RA. Neonatal mice lacking neuronal nitric oxide synthase are less vulnerable to hypoxic-ischemic injury. *Neurobiol Dis* 1996; 3:64– 71. 60.
- [83] Fiedorowicz M, Makarewicz D, Stańczak-Mrozek KI, Grieb P. CDP-choline (citicoline) attenuates brain damage in a rat model of birth asphyxia. *Acta Neurobiol Exp* 2008, 68: 389–397.
- [84] Filippi L, Fiorini P, Daniotti M, Catarzi S, Savelli S, Fonda C, et al. Safety and efficacy of topiramate in neonates with hypoxic ischemic encephalopathy treated with hypothermia (NeoNATI). *BMC Pediatr.* 2012; 5 (12):144.
- [85] Filippi L, Poggi C, la Marca G, Furlanetto S, Fiorini P, Cavallaro G, et al. Oral topiramate in neonates with hypoxic ischemic encephalopathy treated with hypothermia: a safety study. *J Pediatr.* 2010;157(3):361–6.
- [86] FineSmith RB, Roche K, Yellin PB, Walsh KK, Shen C, Zeglis M, et al. Effect of magnesium sulfate on the development of cystic periventricular leukomalacia in preterm infants. *Am J Perinatol.* 1997; 14(5):303–7.
- [87] Follett PL, Deng W, Dai W, Talos DM, Massillon LJ, Rosenberg PA, Volpe JJ, Jensen FE. Glutamate receptor-mediated oligodendrocyte toxicity in periventricular leukomalacia: a protective role for topiramate. *J Neurosci* 2004; 24: 4412–20.
- [88] Follett PL, Rosenberg PA, Volpe JJ, et al. NBQX attenuates excitotoxic injury in developing white matter. *J Neurosci* 2000; 20:9235–41.
- [89] Fulia F, Gitto E, Cuzzocrea S, Reiter R, Dugo L, Gitto P, et al. Increased levels of malondialdehyde and nitrite/nitrate in the blood of asphyxiated newborns: reduction by melatonin. *J Pineal Res* 2005; 31:343–9.

- [90] Fujioka H, Shintaku H, Nakanishi H, Kim TJ, Kusuda S, Yamano T. Biopterin in the acute phase of hypoxia-ischemia in a neonatal pig model. *Brain Dev.* 2008; 30(1):1-6
- [91] Glier C, Dzietko M, Bittigau P, Jarosz B, Korobowicz E, Ikonomidou C. Therapeutic doses of topiramate are not toxic to the developing rat brain. *Exp Neurol.* 2004; 187:403–9.
- [92] Gitto E, Karbownik M, Reiter R, Tan D, Cuzzocrea S, Chiurazzi P, et al. Effects of melatonin treatment in septic newborns. *Pediatr Res.* 2001; 50: 756-60.
- [93] Gitto E, Pellegrino S, Gitto P, Barberi I, Reiter RJ. Oxidative stress of the newborn in the pre- and postnatal period and the clinical utility of melatonin. *J. Pineal Res.* 2009; 46:128–139.).
- [94] Gitto E, Reiter RJ, Cordaro SP, La Rosa M, Chiurazzi P, Trimarchi G, et al. Oxidative and inflammatory parameters in respiratory distress syndrome of preterm newborns: beneficial effects of melatonin. *Am J Perinatol* 2004; 21(4):209–16.
- [95] Gitto E, Reiter RJ, Sabatino G, Buonocore G, Romeo C, Gitto P, et al. Correlation among cytokines, bronchopulmonary dysplasia and modality of ventilation in pre-term newborns: improvement with melatonin treatment. *J Pineal Res* 2005; 39(3):287–93.].
- [96] Gluckman PD, Wyatt J, Azzopardi DV, Ballard R, Edwards, Ferriero DM, et al. Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: Multicenter Randomised Trial. *Lancet.*2005; 365:663–70.
- [97] Gonzalez FF, Ferriero DM: Neuroprotection in the Newborn Infant. *Clin Perinatol.* 2009; 36 859–880.
- [98] Gonzalez FF, Miller SP. Does perinatal asphyxia impair cognitive function without cerebral palsy? *Arch Dis Child Fetal Neonatal Ed* 2006; 91:454–9.
- [99] Green JL, Heard KJ, Reynolds KM, Albert D. Oral and Intravenous Acetylcysteine for Treatment of Acetaminophen Toxicity: A Systematic Review and Meta-analysis. *West J Emerg Med.* 2013; 14(3):218-26.
- [100] Group, EAIS, Effect of a novel free radical scavenger, edaravone (MCI-186), on acute brain infarction. Randomized, placebo-controlled, double-blind study at multicenters. *Cerebrovasc Dis* 2003; 15: 222–229.
- [101] Grow J, Barks JD: Pathogenesis of hypoxic–ischemic cerebral injury in the term infant: current concepts. *Clin. Perinatol.* 2002; 29, 585–602.
- [102] Gunes T, Ozturk MA, Koklu E, Kose K, Gunes I. Effect of allopurinol supplementation on nitric oxide levels in asphyxiated newborns. *Pediatr Neurol* 2007; 36:17-24.
- [103] Gunn AJ, Bennet L. Fetal Hypoxia Insults and Patterns of Brain Injury: Insights from Animal Models. *Clin Perinatol.* 2009; 36 579–593.

- [104] Gunn, A.J, Gunn TR, de Haan HH, Williams CE, Gluckman PD. Dramatic neuronal rescue with prolonged selective head cooling after ischemia in fetal lambs. *J. Clin. Invest.* 1997; 99: 248-256.
- [105] Hall RT, Hall FK, and Daily DK. High dose phenobarbital therapy in term newborn with severe perinatal asphyxia: a randomized, prospective study with three-year follow-up *J. Pediatr.* 1998; 132 (2): 345–348.
- [106] Hagberg H, Gilland E, Bona E, Hanson LA, Hahn-Zoric M, Blennow M, et al. Enhanced expression of interleukin (IL)-1 and IL-6 messenger RNA and bioactive protein after hypoxia-ischemia in neonatal rats. *Pediatr Res.* 1996; 40:603–609.
- [107] Hammerman C, Kaplan M: Ischemia and reperfusion injury. The ultimate pathophysiologic paradox. *Clin Perinatol* 1998, 25:757-777.
- [108] Hanrahan JD, Cox IJ, Azzopardi D, Cowan FM, Sargentoni J, Bell JD, et al. Relation between proton magnetic resonance spectroscopy within 18 hours of birth asphyxia and neurodevelopment at 1 year of age. *Dev Med Child Neurol* 1999; 41:76 – 82.
- [109] Haynes RL, Baud O, Li J Kinney HC, Volpe JJ, Folkerth DR. Oxidative and nitrative injury in periventricular leukomalacia: a review. *Brain Pathol* 2005; 15:225–33.
- [110] Haynes RL, Folkerth RD, Keefe RJ, Sung I, Swzeda LI, Rosenberg PA, et al. Nitrosative and oxidative injury to premyelinating oligodendrocytes in periventricular leukomalacia. *J. Neuropathol. Exp. Neurol.* 2003; 62:441–450.
- [111] Hazinski MF, Nolan JP, Billi JE, Böttiger BW, Bossaert L, de Caen AR, et al. Part 1: Executive summary: 2010. International Consensus on Cardiopulmonary Resuscitation and Emergency Cardiovascular Care Science With Treatment Recommendations. *Circulation.* 2010; 122(16 Suppl 2):S250-75.
- [112] Holliday M. A. Body composition and energy needs during growth, in *Human Growth: A Comprehensive Treatise*, (1986). Vol. 2, 2nd Edn. eds Falkner F., Tanner J. M., editors. (New York, NY: Plenum); 101–117.
- [113] Hossain MA. Molecular mediators of hypoxic-ischemic injury and implications for epilepsy in the developing brain. *Epilepsy Behav.* 2005 Sep; 7(2):204-13.
- [114] Hossain MA, Fielding KE, Trescher WH, Ho T, Wilson MA, Lattera J. Human FGF-1 gene delivery protects against quinolinate- induced striatal and hippocampal injury in neonatal rats. *Eur J Neurosci* 1998; 10:2490–9.
- [115] Hu BR, Liu CL, Ouyang Y, Blomgren K, Siesjo BK. Involvement of caspase-3 in cell death after hypoxia–ischemia declines during brain maturation. *J Cereb Blood Flow Metab* 2000; 20: 1294–300.
- [116] Huang J, Agus DB, Winfree CJ, Kiss S, Mack WJ, McTaggart RA, Choudhri TF, Kim LJ, Mocco J, Pinsky DJ, Fox WD, Israel RJ, Boyd TA, Golde DW, Connolly ES Jr.: Dehydroascorbic acid, a blood–brain barrier transportable form of vitamin C, mediates

potent cerebroprotection in experimental stroke. *Proc Natl Acad Sci U S A*. 2001; 98(20):11720-4.

- [117] Hudome S, Palmer C, Roberts RL, Mauger D, Housman C, Towfighi J. The role of neutrophils in the production of hypoxic-ischemic brain injury in the neonatal rat. *Pediatr Res*. 1997; 41: 607–616.
- [118] Hurtado O, Moro MA, Cardenas A, Sanchez V, Fernandez-Tome P, Leza JC, et al. Neuroprotection afforded by prior citicoline administration in experimental brain ischemia: effects on glutamate transport. *Neurobiol Dis* 2005; 18: 336-345.
- [119] Ikonomidou C, Turski L. Why did NMDA receptor antagonists fail clinical trials for stroke and traumatic brain injury? *Lancet Neurol* 2002; 1:383–6.
- [120] Inder TE, Volpe JJ: Mechanisms of perinatal brain injury. *Semin Neonatol*. 2000, 5:3-16.
- [121] Itoh T, Satou T, Nishida S, Tsubaki M, Hashimoto S, Ito H. The novel free radical scavenger, edaravone, increases neural stem cell number around the area of damage following rat traumatic brain injury. *Neurotox Res*. 2009; 16(4):378-89.
- [122] Jackson T, Xu A, Vita J, Keaney JJ. Ascorbate prevents the interaction of superoxide and nitric oxide only at very high physiological concentrations. *Circ Res* 1998; 83:916-22.
- [123] Jacobs SE, Berg M, Hunt R, Tarnow-Mordi WO, Inder TE, Davis PG. Cooling for newborns with hypoxic ischaemic encephalopathy. *Cochrane Database Syst Rev*. 2013 Jan 31; 1:CD003311
- [124] Jacobs SE, Morley CJ, Inder TE, et al. Whole-body hypothermia for term and near-term newborns with hypoxic-ischemic encephalopathy: a randomized controlled trial. *Arch Pediatr Adolesc Med* 2011; 165:692–700.
- [125] Jaffer H, Morris VB, Stewart D, and Labhasetwar V: Advances in Stroke Therapy. *Drug Deliv Transl Res*. 2011; 1(6): 409–419.
- [126] Jan J, O'Donnell M. Use of melatonin in the treatment of paediatric sleep disorders. *J Pineal Res* 1996; 21: 193-9.
- [127] Jatana M, Singh I, Singh A, Jenkins D. Combination of systemic hypothermia and N-acetylcysteine attenuates hypoxic-ischemic brain injury in neonatal rats. *Pediatr Res* 2006; 59:684-9.
- [128] Ji H, Tan S, Igarashi J, Li H, Derrick M, Martasek P, et al. Selective neuronal nitric oxide synthase inhibitors and the prevention of cerebral palsy. *Ann Neurol* 2009; 65:209-17.
- [129] Johnson MT, McCammon CA, Mullins ME, Halcomb SE. Evaluation of a simplified N-acetylcysteine dosing regimen for the treatment of acetaminophen toxicity. *Ann Pharmacother*. 2011; 45(6):713-20.

- [130] Johnston MV. Excitotoxicity in neonatal hypoxia. *Ment Retard Dev Disabil Res Rev* 2001; 7:229–34.
- [131] Johnston MV, Trescher WH, Ishida A, Nakajima W. Neurobiology of hypoxic–ischemic injury in the developing brain. *Pediatr Res* 2001; 49:735–41.
- [132] Judaš M, Sedmak G, Pletikos M, Jovanov-Milošević N. Populations of subplate and interstitial neurons in fetal and adult human telencephalon. *J Anat.* 2010; 217(4): 381–99.
- [133] Juul S. Erythropoietin in the central nervous system, and its use to prevent hypoxic-ischemic brain damage. *Acta Paediatr Suppl.* 2002; 91: 36–42.
- [134] Juul S. Neuroprotective role of erythropoietin in neonates. *J Matern Fetal Neonatal Med.* 2012 Oct;25 Suppl 4:105–7.
- [135] Juul SE, McPherson, RJ, MR, Bauer LA, Ledbetter KJ, Gleason CA, Mayock DE. A phase I/II trial of high-dose erythropoietin in extremely low birth weight infants: pharmacokinetics and safety. *Pediatrics* 2008; 122:383–91.
- [136] Kaandorp JJ, BendersMJ, Rademaker CM, Torrance HL, Martijn A Oudijk MA et al: Antenatal allopurinol for reduction of birth asphyxia induced brain damage (ALLO-Trial); a randomized double blind placebo controlled multicenter study. *BMC Pregnancy and Childbirth* 2010, 10:8. <http://www.biomedcentral.com/1471-2393/10/8>.
- [137] Kahle KT, Barnett SM, Sassower KC, Staley KJ. Decreased seizure activity in a human neonate treated with bumetanide, an inhibitor of the NaC-KC- 2Cl- cotransporter NKCC1. *J. Child Neurol.* 2009; 24:572–576.
- [138] Kang SK, Lee DH, Bae YC, Kim HK, Baik SY, Jung JS. Improvement of neurological deficits by intracerebral transplantation of human adipose tissue-derived stromal cells after cerebral ischemia in rats. *Exp Neurol* 2003, 183: 355–366.
- [139] Kannan S, Dai H, Navath RS, Balakrishnan B, Jyoti A, Janisse J, et al. Dendrimer-based postnatal therapy for neuroinflammation and cerebral palsy in a rabbit model. *Sci Transl Med.* 2012; 4(130):130–46.
- [140] Kattwinkel J, Perlman JM, Aziz K, et al. Neonatal resuscitation: 2010 American Heart Association Guidelines for cardiopulmonary resuscitation and emergency cardiovascular care. *Pediatrics* 2010; 126(5):e1400–13.
- [141] Keda T, Xia YX, Kaneko M, Sameshima H, Ikenoue T. Effect of the free radical scavenger, 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186), on hypoxia-ischemia-induced brain injury in neonatal rats. *Neuroscience Letters.* 2002; 329(1):33–36.].
- [142] Kelen D, Robertson NJ. Experimental treatments for hypoxic ischaemic encephalopathy. *Early Hum Dev* 2010; 86:369–77.

- [143] Khanna A, Walcott BP, Kahle KT.: Limitations of Current GABA Agonists in Neonatal Seizures: Toward GABA Modulation Via the Targeting of Neuronal Cl(-) Transport. *Front Neurol*. 2013; 4:78.
- [144] Kidd GA, Hong H, Majid A, Kaufman DI, Chen AF. Inhibition of brain GTP cyclohydrolase I and tetrahydrobiopterin attenuates cerebral infarction via reducing inducible NO synthase and peroxynitrite in ischemic stroke. *Stroke* 2005; 36:2705–11.
- [145] Koike M, Shibata M, Tadakoshi M, Gotoh K, Komatsu M, Waguri S, et al. Inhibition of autophagy prevents hippocampal pyramidal neuron death after hypoxic-ischemic injury. *Am J Pathol* 2008, 172:454–469.
- [146] Kostovic I, Judas M. Prolonged coexistence of transient and permanent circuitry elements in the developing cerebral cortex of fetuses and preterm infants. *Dev Med Child Neurol*. 2006 May; 48(5):388-93.
- [147] Kroncke KD. Nitrosative stress and transcription. *Biol Chem* 2003; 384:1365–77.
- [148] Knudsen TT, Thorsen S, Jensen SA, Dalhoff K, Schmidt LE, Becker U, et al. Effect of intravenous N-acetylcysteine infusion on haemostatic parameters in healthy subjects. *Gut*. 2005; 54(4):515-21.
- [149] Kumral A, Tüzün F, Oner MG, Genç S, Duman N, Ozkan H. Erythropoietin in neonatal brain protection: the past, the present and the future. *Brain Dev*. 2011; 33(8): 632-43.
- [150] Kurinczuk JJ, White-Koning M, Badawi N. Epidemiology of neonatal encephalopathy and hypoxic-ischaemic encephalopathy. *Early Hum Dev*. 2010; 86:329–38.
- [151] Labiche LA & Grotta JC: Clinical Trials for Cytoprotection In Stroke. *NeuroRx*; 2004; 1 (1): 46-70.
- [152] Lakic N, Surlan K, Jerin A, Meglic B, Curk N, Bunc M. Importance of erythropoietin in brain protection after cardiac surgery: a pilot study. *Heart Surg Forum* 2010; 13:e185-9
- [153] Lechpammer M, Manning SM, Samonte F, Nelligan J, Sabo E, Talos DM, et al. Minocycline treatment following hypoxic-ischemic injury attenuates white matter injury in a rodent model of periventricular leukomalacia. *Neuropathol Appl Neurobiol*. 2008; 34: 379–393.
- [154] Lee TF, Tymafichuk CN, Bigam DL, Cheung PY. Effects of postresuscitation N-acetylcysteine on cerebral free radical production and perfusion during reoxygenation of hypoxic newborn piglets. *Pediatr Res*. 2008; 64(3):256-61.
- [155] Leonardo CC and Pennypacker KR: Neuroinflammation and MMPs: potential therapeutic targets in neonatal hypoxic-ischemic injury. *Journal of Neuroinflammation* 2009, 6-13.

- [156] Levene MI. Cool treatment for birth asphyxia, but what's next? *Archives of Disease in Childhood: Fetal and Neonatal Edition*, 2010; 95 (3): F154–F157.
- [157] Levene MI, Gibson NA, Fenton AC, Wyatt JS, Penrice J, Edwards AD, et al. The use of a calcium-channel blocker, nicardipine, for severely asphyxiated newborn infants. *Dev Med Child Neurol* 1990; 32:567–74.
- [158] Leviton A, Allred EN, Kuban KC, Hecht JL, Onderdonk AB, O'shea TM, Paneth N. Microbiologic and histologic characteristics of the extremely preterm infant's placenta predict white matter damage and later cerebral palsy. the ELGAN study. *Pediatr Res*. 2010; 67:95–101.
- [159] Li J, Ramenaden ER, Peng J, Koito H, Volpe JJ, Rosenberg PA. Tumor necrosis factor alpha mediates lipopolysaccharide-induced microglial toxicity to developing oligodendrocytes when astrocytes are present. *J. Neurosci*. 2008; 28:5321–5330.
- [160] Lippman-Bell JJ, Rakhade SN, Klein PM, Obeid M, Jackson MC, Joseph A, Jensen FE. AMPA Receptor antagonist NBQX attenuates later-life epileptic seizures and autistic-like social deficits following neonatal seizures. *Epilepsia* 2013. Article first published online: 1 OCT 2013. DOI: 10.1111/epi.12378.
- [161] Lipton ST. Neuronal protection and destruction by NO. *Cell Death Differ* 1995;6:943–51.
- [162] Liu J, Lee T, Chen C, Bagim D, Cheung P. N-acetylcysteine improves hemodynamics and reduces oxidative stress in the brains of newborn piglets with hypoxia-reoxygenation injury. *J Neurotrauma* 2010; 27: 1865-73.
- [163] Liu W, Khatibi N, Sridharan A, Zhang JH. Application of medical gases in the field of neurobiology. *Med Gas Res*. 2011; 1(1):13.
- [164] Liu X-H, Eun B-L, Silverstein S, Barks JD. The platelet-activating factor antagonist BN 52021 attenuates hypoxic-ischemic brain injury in the immature rat. *Pediatr Res*. 1996; 6: 797–803.
- [165] Lo EH. A new penumbra: transitioning from injury into repair after stroke. *Nat Med*. 2008; 14: 497-500.
- [166] Lorek A, Takei Y, Cady EB, Wyatt JS, Penrice J, Edwards AD, et al. Delayed ("secondary") cerebral energy failure after acute hypoxia-ischemia in the newborn piglet: Continuous 48-hour studies by phosphorus magnetic resonance spectroscopy. *Pediatr Res*. 1994; 36: 699-706.
- [167] Lujan R, Shigemoto R, Lopez-Bendito G. Glutamate and GABA receptor signalling in the developing brain. *Neuroscience* 2005; 130:567–80.
- [168] Llado J, Haenggeli C, Maragakis NJ, Snyder EY, Rothstein JD. Neural stem cells protect against glutamate-induced excitotoxicity and promote survival of injured motor

neurons through the secretion of neurotrophic factors. *Mol Cell Neurosci.* 2004; 27: 322–331].

- [169] Madsen JT, Jansen P, Hesslinger C, Meyer M, Zimmer J, Gramsbergen JB. Tetrahydrobiopterin precursor sepiapterin provides protection against neurotoxicity of 1-methyl-4-phenylpyridinium in nigral slice cultures. *J Neurochem* 2003; 85:214-23.
- [170] Magee L, Sawchuck D, Synnes A, von Dadelszen P. SOGC Clinical Practice Guideline. Magnesium sulphate for fetal neuroprotection. *J Obstet Gynaecol Can.* 2011; 33:516–29.
- [171] Maiese K, Chong ZZ, Li F, Y. C. Shang YC. Erythropoietin: elucidating new cellular targets that broaden therapeutic strategies. *Progress in Neurobiology.* 2008; 85 (2): 194–213.
- [172] Manning SM, Boll G, Fitzgerald E, Selip D, Volpe JJ, Jensen F. The clinically available NMDA receptor antagonist, memantine, exhibits relative safety in the developing rat brain. *J Neurosci.* 2010;
- [173] Maragakis NJ, Rothstein JD. Mechanisms of Disease: astrocytes in neurodegenerative disease. *Nat. Clin. Pract. Neurol.* 2006; 2:679–689
- [174] Marks KA, Mallard CE, Roberts I, et al. Nitric oxide synthase inhibition attenuates delayed vasodilation and increases injury after cerebral ischemia in fetal sheep. *Pediatr Res* 1996; 40(2):185–91.
- [175] Marlow N, Rose AS, Rands CE, et al. Neuropsychological and educational problems at school age associated with neonatal encephalopathy. *Arch Dis Child Fetal Neonatal Ed* 2005; 90: F380–7.
- [176] Martin LJ, Al-Abdulla NA, Brambrink AM, Kirsch JR, Sieber FE, Portera-Cailliau C. Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: a perspective on the contributions of apoptosis and necrosis. *Brain Res Bull.* 1998; 46:281–309.
- [177] Martinez, J.; Moreno, J. J. Role of Ca²⁺-independent phospholipase A2 on arachidonic acid release induced by reactive oxygen species. *Arch. Biochem. Biophys.* 392:257–262; 2001.
- [178] Massey LK, Liebman M, Kynast-Gales SA. Ascorbate increases human oxaluria and kidney stone risk. *J Nutr* 2005; 135:1673–7
- [179] Matés JM, Pérez-Gómez C, Núñez de Castro I.: Antioxidant enzymes and human diseases. *Clin Biochem.* 1999; 32 (8):595-603.
- [180] Matyja E, Taraszewska A, Nagańska E, Grieb P, Rafałowska J: CDP-choline protects motor neurons against apoptotic changes in a model of chronic glutamate excitotoxicity *in vitro*. *Folia Neuropathol* 2008; 46 (2): 139-148.

- [181] Medja F, Lelievre V, Fontaine RH, Lebas F, Leroux P, Ouimet T, et al. Thiorphan, a neutral endopeptidase inhibitor used for diarrhoea, is neuroprotective in newborn mice. *Brain* 2006; 129:3209–23.
- [182] Mehmet H, Yue X, Squier MV, Lorek A, Cady E, Penrice J, et al. Increased apoptosis in the cingulate sulcus of newborn piglets following transient hypoxia-ischaemia is related to the degree of high energy phosphate depletion during the insult. *Neurosci Lett* 1994; 181:121–5.
- [183] Ment LR, Vohr BR, Makuch RW, Westerveld M, Katz KH, Schneider KC, et al. Prevention of intraventricular hemorrhage by indomethacin in male preterm infants. *J Pediatr*; 2004 145(6):832–834.
- [184] McDonald JW, Silverstein FS, Johnston MV. Neurotoxicity of N-methyl-D-aspartate is markedly enhanced in developing rat central nervous system. *Brain Res* 1988; 459:200–3.
- [185] McDonald JW, Behrens MI, Chung C, Bhattacharyya T, Choi DW. Susceptibility to apoptosis is enhanced in immature cortical neurons. *Brain Res* 1997;759:228–32
- [186] McQuillen PS, Ferriero DM. Selective vulnerability in the developing central nervous system. *Pediatr Neurol* 2004; 30:227–35.
- [187] Miao H, Jiang L, Huang L. Effects of simvastatin on the expression of intercellular adhesion molecule-1 mRNA in neonatal brain with hypoxic-ischemic damage. *J Nanosci Nanotechnol*.2005; 5(8):1261-5.
- [188] Miller LJ, Marx J: Apoptosis-Special Section, Introduction. *Science* 1998; 281:1301.
- [189] Mir C, Clotet J, Aledo R, Durany N, Argemi J, Lozano R, et al. CDP-choline prevents glutamate-mediated cell death in cerebellar granule neurons. *J Mol Neurosci* 2003; 20: 53-60.
- [190] Mishra OP, Delivoria-Papadopoulos M. Cellular mechanisms of hypoxic injury in the developing brain. *Brain Res Bull* 1999; 48:233– 8.
- [191] Mokra D, Drgova A, Kopincova J, Pullmann R, Calkovska A: Anti-inflammatory treatment in dysfunction of pulmonary surfactant in meconium-induced acute lung injury. *Adv Exp Med Biol*. 2013; 756:189-96.
- [192] Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991; 43:109–42.
- [193] Muir KW, Lees KR, Ford I, Davis S; Intravenous Magnesium Efficacy in Stroke (IMAGES) Study Investigators. Magnesium for acute stroke (Intravenous Magnesium Efficacy in Stroke trial): randomised controlled trial. *Lancet*. 2004; 363(9407):439-45.
- [194] Muramatsu K, Sheldon RA, Black SM, Tauber M, Ferriero DM. Nitric oxide synthase activity and inhibition after neonatal hypoxia ischemia in the mouse brain. *Brain Res Dev Brain Res*.2000; 123(2):119–12.

- [195] Nakai A, Shibazaki Y, Taniuchi Y, Oya A, Asakura H, Koshino T, et al. Vitamins ameliorate secondary mitochondrial failure in neonatal rat brain. *Pediatr Neurol* 2002; 27:30–5.
- [196] Nakajima K, Kohsaka S: Microglia: Neuroprotective and neurotrophic cells in the central nervous system. *Curr Drug Targets Cardiovasc Haematol Disord* 2004; 4: 65–84.
- [197] Nijboer CH, Groenendaal F, Kavelaars A, Hagberg HH, van Bel F, Heijnen CJ. Gender-specific neuroprotection by 2-iminobiotin after hypoxia-ischemia in the neonatal rat via a nitric oxide independent pathway. *J Cereb Blood Flow Metab* 2007; 27:282–92.
- [198] Noh MR, Kim SK, Sun W, Park SK, Choi HC, Lim JH, et al. Neuroprotective effect of topiramate on hypoxic ischemic brain injury in neonatal rats. *Exp Neurol*. 2006;201:470–478
- [199] Nolana JP, Soarb J, Zidemanc DA, Biarentd D, Bossaerte L L, Deakin C: European Resuscitation Council Guidelines for Resuscitation 2010. Section 1. Executive summary. *Resuscitation*. 2010; 81:1219–1276
- [200] Northington FJ, Ferriero DM, Graham EM, Traystman RJ, Martin LJ. Early neurodegeneration after hypoxia-ischemia in neonatal rat Is necrosis while delayed neuronal death Is apoptosis. *Neurobiol Dis* 2001; 8: 207–19.
- [201] Nozaki K, Finklestein SP, Beal MF. Basic fibroblast growth factor protects against hypoxia-ischemia and NMDA neurotoxicity in neonatal rats. *J Cereb Blood Flow Metab*. 1993; 13:221–8.
- [202] Olney JW, Wozniak DF, Jevtovic-Todorovic V, Farber NB, Bittigau P, Ikonomidou C. Drug-induced apoptotic neurodegeneration in the developing brain. *Brain Pathol* 2002; 12:488–498.
- [203] Olsson B, Johansson M, Gabrielsson J, Bolme P. Pharmacokinetics and bioavailability of reduced and oxidized N-acetylcysteine. *Eur. J. Clin. Pharmacol.* 1988; 34:77–82.
- [204] O'Shea TM, Doyle LW. Perinatal glucocorticoid therapy and neurodevelopmental outcome: an epidemiologic perspective. *Semin Neonatol* 2001;6:293–307
- [205] Otten U, Marz P, Heese K, Hock C, Kunz D, Rose-John S. Cytokines and neurotrophins interact in normal and diseased states. *Ann N Y Acad Sci* 2000; 917:322–30.
- [206] Pabon MM, Borlongan CV Advances in the cell-based treatment of neonatal hypoxic-ischemic brain injury: *Future Neurol*. 2013; 8(2):193–203.
- [207] Painter MJ: Animal Models of Perinatal Asphyxia: Contributions, Contradictions, Clinical Relevance. *Seminars in Pediatric Neurology*, Vol 2 (1), 1995: pp 37–56.

- [208] Painter MJ, Scher MS, Stein AD, Armatti S, Wang Z, Gardiner JC, et al. Phenobarbital compared with phenytoin for the treatment of neonatal seizures. *N. Engl. J. Med.* 1999; 341, 485–489.
- [209] Paintlia MK, Paintlia AS, Barbosa E, Singh I, Singh AK. N-acetylcysteine prevents endotoxin-induced degeneration of oligodendrocyte progenitors and hypomyelination in developing rat brain. *J Neurosci Res* 2004; 78: 347-61.
- [210] Palmer C, Towfighi J, Roberts RL, et al. Allopurinol administered after inducing hypoxia-ischemia reduces brain injury in 7-day-old rats. *Pediatr Res* 1993; 33:405–11.
- [211] Palmer C. Hypoxic-ischemic encephalopathy. Therapeutic approaches against microvascular injury, and role of neutrophils, PAF, and free radicals. *Clin Perinatol.* 1995; 22: 481-517.
- [212] Palmer C, Roberts RL, Bero C. Deferoxamine posttreatment reduces ischemic brain injury in neonatal rats. *Stroke* 1994; 25: 1039–45.
- [213] Palsdottir K, Dagbjartsson A, Thorkelsson T, Hardardottir H: Birth asphyxia and hypoxic ischemic encephalopathy, incidence and obstetric risk factors. *Laeknabladid.* 2007; 93(9):595-601.
- [214] Peeters-Scholte C, Braun K, Koster J, Kops N, Blomgren K, Buonocore G, et al. Effects of allopurinol and deferoxamine on reperfusion injury of the brain in newborn piglets after neonatal hypoxia-ischemia. *Pediatr Res* 2003; 54:516-522.
- [215] Perlman JM, Risser R. Relationship of uric acid concentrations and severe intraventricular hemorrhage/leukomalacia in the premature infant. *J Pediatr* 1998; 132:436– 9.
- [216] Perlman JM, Wyllie J, Kattwinkel J, Atkins DL, Chameides L, Goldsmith JP, et al. Neonatal resuscitation: 2010 International Consensus on cardiopulmonary resuscitation and emergency cardiovascular care science with treatment recommendations. *Pediatrics* 2010; 126(5):e1319–44.
- [217] Perlman M, and Shah PS: Hypoxic-Ischemic Encephalopathy: Challenges in Outcome and Prediction. *J Pediatr* 2011; 158:e51-4.
- [218] Pimentel-Coelho PM, Mendez-Otero R. Cell therapy for neonatal hypoxic-ischemic encephalopathy. *Stem Cells Dev.* 2010; 19:299-310.
- [219] Pimentel-Coelho PM, Rosado-de-Castro PH, da Fonseca LM, Mendez-Otero R. Umbilical cord blood mononuclear cell transplantation for neonatal hypoxic-ischemic encephalopathy. *Pediatr Res* 2012; 71(4 Pt 2):464–73.
- [220] Portera-Cailliau C, Price DL, Martin LJ. Excitotoxic neuronal death in the immature brain is an apoptosis–necrosis morphological continuum. *J Comp Neurol* 1997; 378:70–87.
- [221] Puyal J, Vaslin A, Mottier V, Clarke PGH. Postischemic Treatment of Neonatal Cerebral Ischemia Should Target Autophagy. *Ann Neurol* 2009; 66: 378–389.

- [222] Rees S, Richard Harding R, Walker D. The biological basis of injury and neuroprotection in the fetal and neonatal brain. *Int J Dev Neurosci*. 2011; 29(6): 551–563.
- [223] Robertson CM. Long term follow-up of term infants with perinatal asphyxia. In: Stevenson DK, Benitz WE, Sunshine P, editors. *Fetal and neonatal brain injury*. 3rd edition. New York, NY: Cambridge University; 2003. p. 829–58.
- [224] Robertson CM, Finer NN. Long-term follow-up of term neonates with perinatal asphyxia. *Clin Perinatol* 1993; 20: 483 – 500.
- [225] Robertson NJ, Faulkner S, Fleiss B, Bainbridge A, Andorka C, Price D, et al: Melatonin augments hypothermic neuroprotection in a perinatal asphyxia model. *Brain*. 2013; 136(Pt 1):90-105.
- [226] Robertson NJ, Tan S, Groenendaal F, van Bel F, Juul SE, Bennet L, et al. Which neuroprotective agents are ready for bench to bedside translation in the newborn infant? *J Peds*. 2012; Vol. 160 (4): 544-552.e4.
- [227] Russell GA, Cooke RW. Randomised controlled trial of allopurinol prophylaxis in very preterm infants. *Arch Dis Child Fetal Neonatal Ed*. 1995 Jul; 73(1):F27-31.
- [228] Rutherford M, Ramenghi LA, Edwards AD, Brocklehurst P, Halliday H, Levene M, et al. Assessment of brain tissue injury after moderate hypothermia in neonates with hypoxic-ischaemic encephalopathy: a nested substudy of a randomised controlled trial. *Lancet Neurol* 2010; 9: 39–45.
- [229] Rybakowski C, Mohar B, Wohlers S, Leichtweiss H, Schreoder H. The transport of vitamin C in the isolated human near-term placenta. *Eur J Obstet Gynecol Reprod Biol* 1995; 62:107-14.
- [230] Sámano C, Nasrabady SE, Nistri A. A study of the potential neuroprotective effect of riluzole on locomotor networks of the neonatal rat spinal cord in vitro damaged by excitotoxicity. *Neuroscience*. 2012; 11(222):356-65.
- [231] Samanta S, Perkinton MS, Morgan M, Williams R.J. Hydrogen peroxide enhances signal-responsive arachidonic acid release from neurons: role of mitogen-activated protein kinase. *J. Neurochem*. 70:2082–2090; 1998.
- [232] Sameshima H, Ota A, Ikenoue T. Pretreatment with magnesium sulfate protects against hypoxic-ischemic brain injury but postasphyxial treatment worsens brain damage in seven-day-old rats. *American Journal of Obstetrics and Gynecology*. 1999; 180(3):725–730.
- [233] Sandilands EA, Bateman DN. Adverse reactions associated with acetylcysteine. *Clin Toxicol (Phila)*. 2009; 47(2):81-8.
- [234] Sarco DP, Becker J, Palmer C, Sheldon RA, Ferriero DM. The neuroprotective effect of deferoxamine in the hypoxic-ischemic immature mouse brain. *Neurosci Lett* 2000; 282(1–2): 113–6.

- [235] Sarkar S, Barks JD, Bapuraj JR, Bhagat I, Dechert RE, Schumacher RE, et al. Does phenobarbital improve the effectiveness of therapeutic hypothermia in infants with hypoxic-ischemic encephalopathy? *J Perinatol*. 2012; 32(1):15-20.
- [236] Sarnat HB, Sarnat MS: Neonatal encephalopathy following fetal distress. A clinical and electroencephalographic study. *Arch Neurol* 1976; 33: 696-705.
- [237] Saver JL. Citicoline: update on a promising and widely available agent for neuroprotection and neurorepair. *Rev Neurol Dis* 2008; 5: 167-77.
- [238] Sävman K, Blennow M, Gustafson K, Tarkowski E, Hagberg H. Cytokine response in cerebrospinal fluid after birth asphyxia. *Pediatr Res* 1998; 43:746- 51.
- [239] Sato Y, Nakanishi K, Hayakawa M, Kakizawa H, Saito A, Kuroda Y et al. Reduction of brain injury in neonatal hypoxic-ischemic rats by intracerebroventricular injection of neural stem/progenitor cells together with chondroitinase ABC. *Reprod Sci*. 2008; 15: 613-620.
- [240] Scafidi J, Gallo V: New concepts in perinatal hypoxia ischemia encephalopathy. *Curr Neurol Neurosci Rep* 2008, 8:130-138.
- [241] Schaper M, Gergely S, Lykkesfeldt J, Zbären J, Leib S, Těauber M, et al. Cerebral vasculature is the major target of oxidative protein alterations in bacterial meningitis. *J Neuropathol Exp Neurol* 2002; 61:605-13.
- [242] Schwartz JC. Racecadotril: a new approach to the treatment of diarrhoea. *Int J Anti-microb Agents* 2000; 14: 75-9.
- [243] Secades JJ, Lorenzo JL. Citicoline: pharmacological and clinical review, 2006 update. *Methods Find Exp Clin Pharmacol* 2006; 28 Suppl B: 1-56.
- [244] Shalak L, Perlman JM. Hypoxic-ischemic brain injury in the term infant-current concepts. *Early Hum Dev*. 2004; 80:125-141.
- [245] Shankaran S: Hypoxic-ischemic. Encephalopathy and Novel Strategies for Neuroprotection. *Clin Perinatol*. 2012, 39: 919-929.
- [246] Shankaran S, Barnes PD, Hintz SR, Laptook AR, Zaterka-Baxter KM, McDonald SA, et al. Brain injury following trial of hypothermia for neonatal hypoxic-ischaemic encephalopathy. *Arch Dis Child Fetal Neonatal Ed*. 2012; 97(6):F398-404
- [247] Shankaran S, Laptook AR, Ehrenkranz RA, Tyson JE, McDonald SA, Donovan EF, et al. Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. *N Engl J Med*. 2005; 353:1574-84.
- [248] Shankaran S, Pappas A, Laptook AR, McDonald SA, Ehrenkranz RA, Tyson JE et al. Outcomes of safety and effectiveness in a multicenter randomized, controlled trial of whole-body hypothermia for neonatal hypoxic-ischemic encephalopathy. *Pediatrics* 2008;122:e791-

- [249] Shankaran S, Woldt E, Koepke T, Bedard MP, Nandyal R. Acute neonatal morbidity and long-term central nervous system sequelae of perinatal asphyxia in term infants. *Early Hum Dev* 1991; 25:135–48.
- [250] Shelov, SP. American Academy of Pediatrics, American Heart Association - *Neonatal Resuscitation*. Editor American Academy of Pediatrics, 2011, sixth edition. ISBN: 1581105002, 9781581105001.
- [251] Shen M, Wu RX, Zhao L, Li J, Guo HT, Fan R, et al. Resveratrol attenuates ischemia/reperfusion injury in neonatal cardiomyocytes and its underlying mechanism. *PLoS One*. 2012; 7(12):e51223.
- [252] Shimizu K, Rajapakse N, Horiguchi T, Payne RM, Busija DW. Neuroprotection against hypoxia-ischemia in neonatal rat brain by novel superoxide dismutase mimetics. *Neurosci Lett*. 2003; 346: 41-44.
- [253] Shingo T, Sorokan ST, Shimazaki T, Weiss S. Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells. *J Neurosci*. 2001; 21: 9733–9743
- [254] Shim SY, Kim HS.: Oxidative stress and the antioxidant enzyme system in the developing brain. *Korean J Pediatr*. 2013; 56(3):107-11.
- [255] Simbruner G, Mittal RA, Rohlmann F, Muche R. neo. nEURO.network Trial Participants. Systemic hypothermia after neonatal encephalopathy: outcomes of neo.nEURO. network RCT. *Pediatrics*.2010;126:e771–8.
- [256] Sinha S, Davies J, Toner N, Bogle S, Chiswick M. Vitamin E supplementation reduces frequency of periventricular haemorrhage in very preterm babies. *Lancet* 1987; i: 466-71.
- [257] Sinor A.D., Greenberg D.A. Erythropoietin protects cultured cortical neurons, but not astroglia, from hypoxia and AMPA toxicity. *Neurosci Lett*. 2000; 90: 213–215.
- [258] Sizonenko SV, Bednarek N, Gressens P. Growth factors and plasticity. *Semin Fetal Neonatal Med*. 2007; 12: 241–249.
- [259] Sfaello I, Baud O, Arzimanoglou A, Gressens P. Topiramate prevents excitotoxic damage in the newborn rodent brain. *Neurobiol Dis* 2005; 20:837–48.
- [260] Slaughter LA, Anup D. Patel AD, and Slaughter JL, Pharmacological Treatment of Neonatal Seizures: A Systematic Review. *Journal of Child Neurology* 2013; 28(3) 351-364.
- [261] Subirós N, Del Barco DG, Coro-Antich RM Erythropoietin: still on the neuroprotection road. *Ther Adv Neurol Disord*. 2012; 5:161-73.
- [262] Tagin MA, Woolcott CG, Vincer MJ, Whyte RK, Stinson DA. Hypothermia for neonatal hypoxic ischemic encephalopathy: an updated systematic review and meta-analysis. *Arch Pediatr Adolesc Med* 2012; 166:558–66.

- [263] Torrance HL, Benders MJ, Derks JB, Rademaker CM, Bos AF, Berg Van Den P, et al. Maternal allopurinol during fetal hypoxia lowers cord blood levels of the brain injury marker S-100B. *Pediatrics* 2009; 124:350-357.
- [264] Trovarelli G, de Medio GE, Dorman RV, Piccinin GL, Horrocks LA, Porcellati G. Effect of cytidine diphosphate choline (CDP-choline) on ischemia-induced alterations of brain lipid in the gerbil. *Neurochem Res* 1981; 6: 821-833.
- [265] Tsuji M, Wilson MA, Lange MS, Johnston MV. Minocycline worsens hypoxic-ischemic brain injury in a neonatal mouse model. *Exp Neurol* 2004; 189:58–65.
- [266] Turrens JF, Alexandre A, Lehninger AL. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch Biochem Biophys* 1985; 237: 408 – 14.
- [267] Valls I Soler A, Sanjurjo P, Vázquez Cordero C. Controlled study of the administration of CDP-choline to preterm newborn infants with respiratory distress syndrome]. *An Esp Pediatr*. 1988 Jun;28(6):493-6.
- [268] Vallat-Decouvelaere AV, Chretien F, Gras G, Le Pavec G, Dormont D. Expression of excitatory amino acid transporter-1 in brain macrophages and microglia of HIV-infected patients. A neuroprotective role for activated microglia? *J Neuropathol Exp Neurol* 2003; 62:475–85.
- [269] Van Bel F, Shadid M, Moison RM, Dorrepaal CA, Fontijn J, Monteiro L, et al. Effect of allopurinol on postasphyxial free radical formation, cerebral hemodynamics, and electrical brain activity. *Pediatrics*.1998, 101:185-193.
- [270] van den Tweel ER, van Bel F, Kavelaars A, Peeters-Scholte CM, Haumann J, Nijboer CH, et al. Long-term neuroprotection with 2-iminobiotin, an inhibitor of neuronal and inducible nitric oxide synthase, after cerebral hypoxia-ischemia in neonatal rats. *J Cereb Blood Flow Metab*. 2005; 25(1):67-74.
- [271] van Velthoven CT, Kavelaars A, Heijnen CJ. Mesenchymal stem cells as a treatment for neonatal ischemic brain damage. *Pediatr Res*. 2012;71(4 Pt 2):474-81
- [272] Vasquez-Vivar J, Whitsett J, Derrick M, Ji X, Yu L, Tan S. Tetrahydrobiopterin in the prevention of hypertonia in hypoxic fetal brain. *Ann Neurol* 2009;66:323-31.
- [273] Volpe JJ, *Neurology of the Newborn*, 5th edition, 2008.
- [274] Volpe JJ. Cerebral white matter injury of the premature infant more common than you think. *Pediatrics* 2003; 112:176–80.
- [275] Volpe JJ, Kinney HC, Jensen FE, Rosenberg PA. The developing oligodendrocyte: key cellular target in brain injury in the premature infant. *Int J Dev Neurosci*. 2011 Jun; 29(4):423-40.

- [276] Wang B, Navath RS, Romero R, Kannan S, Kannan R. Anti-inflammatory and anti-oxidant activity of anionic dendrimer-N-acetyl cysteine conjugates in activated microglial cells. *Int J Pharm.* 2009 Jul 30;377(1-2):159-68.
- [277] Wang X, Feuerstein GZ. Induced expression of adhesion molecules following focal brain ischemia. *J Neurotrauma.* 1995; 12:825-32.
- [278] Wang X, Svedin P, Nie C, Lapatto R, Zhu C, Gustavsson M, et al. N-acetylcysteine reduces lipopolysaccharide-sensitized hypoxicischemic brain injury. *Ann Neurol* Mar; 2007 61(3):263–271.
- [279] Wang XL, Yu SL, Yu T, Li JH, Guo Ar, Liang HT. Treatment of neonatal hypoxic-ischaemic encephalopathy (HIE) with compound salvia miltiorrhizae and citicoline: a comparative study in China. *Singapore Paediatr J* 1997; 39: 120–123.
- [280] Wang Y, Cao M, Liu A, Di W, Zhao F, et al. Changes of inflammatory cytokines and neurotrophins emphasized their roles in hypoxic-ischemic brain damage. *Int J Neurosci.* 2013; 123(3):191-5].
- [281] Welin AK, Svedin P, Lapatto R, Sultan B, Hagberg H, Gressens P, et al. Melatonin reduces inflammation and cell death in white matter in the mid-gestation fetal sheep following umbilical cord occlusion. *Pediatr Res.* 2007; 61:153–158.
- [282] Whitelaw A, Thoresen M. Antenatal steroids and the developing brain. *Arch Dis Child Fetal Neonatal* Ed 2000; 83:F154–7.
- [283] Widness J, Schmidt R, Sawyer S. Erythropoietin transplacental passage: review of animal studies. *J Perinat Med* 1995; 23:61-70.
- [284] Willmot M, Gibson C, Gray L, Murphy S, Bath P. Nitric oxide synthase inhibitors in experimental ischemic stroke and their effects on infarct size and cerebral blood flow: a systematic review. *Free Radic BiolMed* 2005; 39:412-25.
- [285] Xu J, Yu S, Sun AY, Sun GY. Oxidant-mediated AA release from astrocytes involves cPLA2 AND iPLA2. *Free Radical Biology & Medicine.* 2003; 34 (12): 1531–1543.
- [286] Yrjanheikki J, Tikka T, Keinanen R, Goldsteins G, Chan PH, Koistinaho J. A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. *Proc Natl Acad Sci USA.* 1999; 96:13496–500.
- [287] Yu X., Shacka JJ, Eells JB, Suarez-Quian C, Przygodzki RM, Beleslin-Cokic B, et al. Erythropoietin receptor signalling is required for normal brain development. *Development.* 2002; 129: 505–516
- [288] Zhang RI, Chopp M, Li Y, Zalonga C, Jiang M, Jones M, et al. Anti-ICAM-1 antibody reduces ischemic cell damage after transient middle cerebral artery occlusion in the rat. *Neurology.* 1994; 44:1747–1751.
- [289] Zhou WH, Cheng GQ, Shao XM, Liu XZ, Shan RB, Zhuang DY, et al. Selective head cooling with mild systemic hypothermia after neonatal hypoxic-ischemic encephal-

opathy: a multicenter randomized controlled trial in China. *J Pediatr*. 2010; 157:367–72.

- [290] Zhu C, Kang W, Xu F, Cheng X, Zhang Z, Jia L, et al. Erythropoietin improved neurologic outcomes in newborns with hypoxic-ischemic encephalopathy. *Pediatrics* 2009; 124:e218-26.
- [291] Zhu C, Wang X, Huang Z, Qiu L, Xu F, Vahsen N, et al. Apoptosis-inducing factor is a major contributor to neuronal loss induced by neonatal cerebral hypoxia-ischemia. *Cell Death Differ*. 2007; 14(4):775-84.