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REVIEW

Pathophysiology of Perinatal Hypoxic-Ischemic Encephalopathy – Biomarkers, Animal Models and Treatment Perspectives

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Summary

Hypoxic-ischemic encephalopathy (HIE) is one of the leading pediatric neurological conditions causing long-term disabilities and socio-economical burdens. Nearly 20-50 % of asphyxiated newborns with HIE die within the newborn period and another third will develop severe health consequences and permanent handicaps. HIE is the result of severe systemic oxygen deprivation and reduced cerebral blood flow, commonly occurring in full-term infants. Hypoxic-ischemic changes trigger several molecular and cellular processes leading to cell death and inflammation. Generated reactive oxygen species attack surrounding cellular components resulting in functional deficits and mitochondrial dysfunction. The aim of the present paper is to review present knowledge about the pathophysiology of perinatal hypoxic-ischemic encephalopathy, especially with respect to novel treatment strategies and biomarkers that might enhance early detection of this disorder and thus improve the general outcome of patients.

Key words

Hypoxic-ischemic encephalopathy • Excitotoxicity • Oxidative stress • Inflmmation • Biomarkers

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Introduction

Hypoxic-ischemic encephalopathy (HIE) is the brain injury caused by oxygen deprivation and is one of the leading pediatric neurological conditions in terms of long-term disabilities and socio-economical burdens. Statistics suggest the incidence of HIE is variable, reaching 1.5-4 per 1000 live term births; an estimated four million children per year (Mir and Chalak 2014, Vannucci 2000, Vannucci and Hagberg 2004). Nearly 20-50 % of asphyxiated newborns with HIE die within the newborn period and another third will develop severe health consequences and permanent handicaps (Vannucci and Hagberg 2004). Generally, it is believed the rate of HIE and its consequences are higher in developing countries (Kurinczuk *et al.* 2010, Ellis *et al.* 2000). In essence, the phenomenon occurs as a consequence of severe systemic hypoxemia and reduced cerebral blood flow (CBF), most commonly in full-term infants. Although the newborn possesses several potent physiological compensatory mechanisms, a lasting hypoxia (mainly due to asphyxia) leads to a spectrum of neurological deficits. These involve behavioral and motor disabilities, general developmental delay, seizures or even structural and irreversible brain damage (Alexander *et al*. 2014, Badawi *et al*. 2001, Gadian *et al*. 2000). HIE due to perinatal asphyxia is a leading cause of death or severe impairment among infants. The severity of consequences of HIE depends upon how long the hypoxia lasted, the condition of the new-born at the time of the

hypoxia and the severity of the damage. The broad symptomatic spectrum of hypoxia makes it very hard to rapidly detect in clinical practice. The same problem arises with reasonable interpretation of animal models outcomes, not to mention the possible cure of this phenomenon. The most common long term consequences of HIE include cerebral palsy, epilepsy and seizure disorders, severe learning and mental impairments, blindness or severe vision impairments, cognitive developmental problems and motor and behavioral problems (Alexander *et al*. 2014, Boksa *et al*. 1995, Lun *et al*. 1990, Ikeda *et al*. 2001). The effects of HIE have often been observed as an all-or-nothing phenomenon; patients have been believed to show either a completely normal development or become severely disabled.

Despite the increasing knowledge about the pathophysiology and risk factors of HIE along with the boom of molecular diagnostic methods, only a limited number of diagnostic methods and preventive strategies are available at present. Despite this, when HIE is suspected to endanger the newborn, appropriate methods should be immediately used to aid the diagnosis. The reasoning emerges from the idea that the treatment is only effective for a relatively short time frame following the onset of the cellular cascades causing brain damage. However, the situation is complicated by many processes that control physiological birth in both the mother's and infant's body, not to mention other relevant conditions such as the psychosocial aspects of a problematic delivery. Unfortunately, the classical molecular biomarkers of brain injury lack necessary selectivity and thus ubiquitous intra-partal brain compressions cause their clinical irrelevance in the acute period of HIE (Douglas-Escobar and Weiss 2012, Mir and Chalak 2014). Suspicion of HIE is thus in clinical practice mainly based on the whole clinical picture (including traumatic birth, presence of risk factors such as fetal stroke, signs of organ dysfunction and early seizures). The Sarnat grading system (Sarnat and Sarnat 1976) scores different clinical criteria allowing classification of the neonates into mild, moderate and severe categories which aid in the prediction of their prognosis (Finer *et al.* 1981, Douglas-Escobar and Weiss 2012). Once HIE is suspected, only amplitude integrated electroencephalography or neuroimaging techniques such MRI are able to confirm the diagnosis (Douglas-Escobar and Weiss 2012). Nevertheless such tests become irrelevant in unstable patients or during hypothermia treatment.

The aim of the present paper is to review present knowledge about the pathophysiology of perinatal hypoxic-ischemic encephalopathy especially with respect to novel treatment strategies and biomarkers, which might enhance early detection of this disorder and thus improve general outcome of patients. Such biomarkers could help to recognize and quantify the severity in everyday practice, identify those who require hypothermia therapy and could help others to avoid unnecessary treatment.

Pathophysiology of perinatal hypoxic-ischemic encephalopathy

HIE may occur at different stages of pregnancy. Predisposing factors in the prenatal period include toxemia and systemic disease of the mother, whereas perinatal factors include prolonged delivery, sepsis and shock (Douglas-Escobar and Weiss 2012).

Impairment of cerebral blood flow as a result of perinatal asphyxia triggers several processes on both a systemic and cellular level. Low levels of blood oxygen initially activate general compensatory mechanisms such as redistribution of blood flow to vital organs (Fig. 1). Prolonged asphyxia results in the exhaustion of these mechanisms, moreover the physical obstruction of cerebral vessels leading to ischemic damage of the corresponding brain areas. In similar fashion to adults, the immature brain reacts to hypoxic-ischemic conditions by releasing excitatory molecules and producing reactive oxygen species causing inflammation and blood brain barrier (BBB) impairment. However, important developmental norms have to be taken into an account when describing the pathophysiology of perinatal hypoxic-ischemic encephalopathy. The metabolic turnover (and its rate) of the immature brain is lower compared to an adult one and depends mainly on the level of development and synaptic maturation (Cremer 1982, Nehlig and Pereira de Vasconcelos 1993). Immature neuronal tissue shares the ability to utilize substrates other than glucose to cover its metabolic demands. This includes, among others, lactate and ketone bodies. Furthermore, their ability to transport the aforementioned particles in and out of cells is enhanced as compared to the adult brain. Two thirds of the energetic requirements during the initial postnatal days are covered by ketone bodies, with glucose playing a minor rule as a metabolic fuel (Nehlig and Pereira de Vasconcelos 1993). This is accompanied by lower

expression of transporters for glucose (Vannucci *et al.* 1994). The immature brain is therefore less demanding for metabolic fuel, which is especially problematic during the period of hypoxia (when anaerobic glycolysis prevails) as there is failure to deliver enough energetic substrates *via* blood brain barrier (Vannucci *et al.* 1994). Immature brain tissue can thus withstand a more significant period compared to an adult one, but is consequently more prone to excitotoxic damage if a critical point of energy deprivation is reached. Destabilization of energetic homeostasis within the brain and failure of adequate substrate or fuel delivery leads to a cascade of events that could end with cell death (Puyal *et al.* 2013). As the energy reserves fall during HIE, the release of excitatory amino acids (EAA) into the extracellular space is more pronounced. This is accompanied (and partially preceded) by depolarization of neuronal cells. The resultant picture is characterized by the energy-dependent reuptake and buffering of already falling extracellular EAA, with mainly glutamate reaching potentially toxic levels (Doble 1999, Johnston 2005).

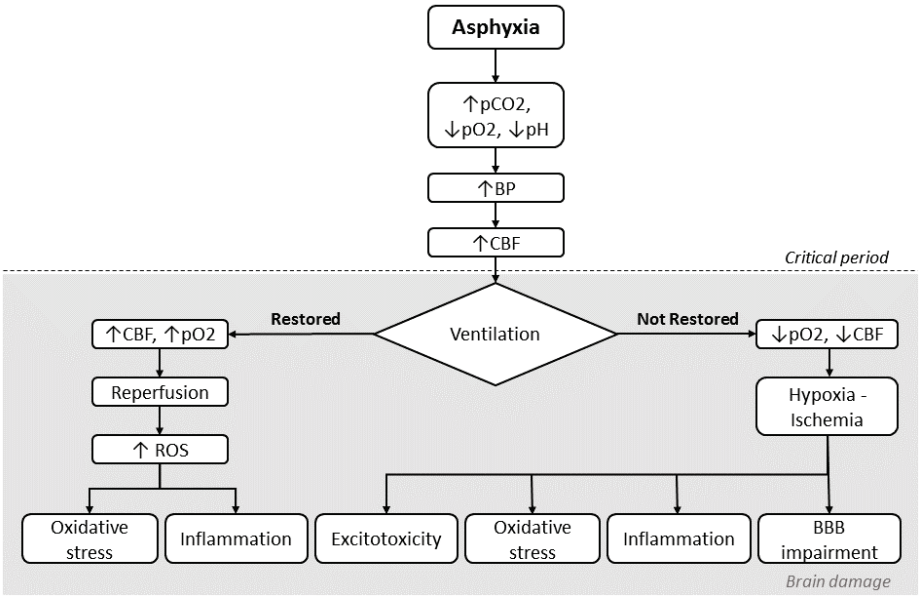

Fig. 1. The cellular sequence of events following the period of hypoxia and energy deprivation in HIE. pO2 – partial pressure of oxygen, Glc – glucose, ROS – reactive oxygen species, RNS – reactive nitrogen species, BBB – blood brain barrier.

Excitotoxicity

Under normal circumstances, excitatory amino acids such as glutamate play an important role in immature brain development. It contributes to synaptogenesis, neural plasticity and is a crucial trophic factor (Komuro and Rakic 1993). However, after the consumption of cellular energetic reserves during hypoxic-ischemic insults, the neuronal and glial cells tend to depolarize and release a high amount of glutamate and other excitatory amino acids into the synaptic cleft and extracellular space (Fig. 2). Glutamate reuptake mechanisms are not capable of balancing its release as the energy supply for such processes are compromised. Tonic activation of glutamate ionotropic receptors trigger sequentially damaging pathways leading finally to cell injury or death (Doble 1999). The concept of excitotoxicity, an injury caused by excessive excitation, was firstly described by Olney *et al.* (1974). He observed the phenomenon that the exaggeration of the excitatory properties of glutamate can lead to neurotoxicity. The major molecular pathways of excitotoxicity include intracellular calcium elevation and consequent activation of calcium dependent enzymes, loss of mitochondrial membrane potential and overproduction of reactive oxygen and nitrogen species. A significant part of excitotoxicity is attributed to the glutamatergic system and its receptors. As noted above, glutamate plays an important role in brain cognition, memory formation and generation of basic excitatory responses (Dong *et al.* 2009). The aforementioned effect of glutamate is mainly expressed *via* ionotropic glutamate receptors, permeable to various cations. According to its affinity to different exogenous ligands, ionotropic glutamate receptors are classified into three major subgroups: N-methyl-D-daspartic acid (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and kainic acid (KA) (Møllerud *et al.* 2017, Zhu and Gouaux 2017).

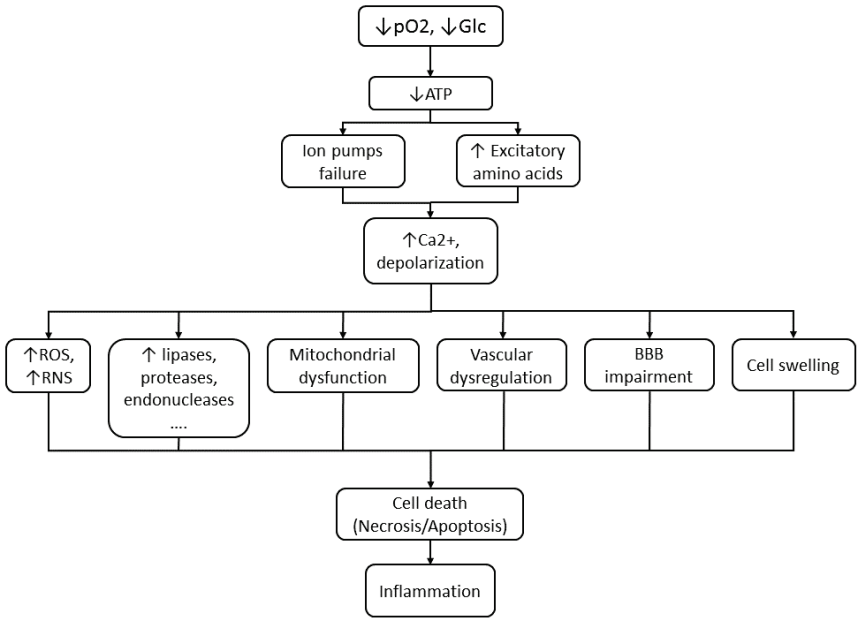

Fig. 2. Pathophysiological changes in asphyxia and their consequences. pCO2 – partial pressure of carbon dioxide, pO2 – partial pressure of oxygen, CBF – cerebral blood flow, ROS – reactive oxygen species, BBB – blood brain barrier.

NMDA receptors are heteromeric complexes of four various subunits surrounding a central ion channel permeable for calcium. Seven different subunits were identified (NR1, NR2A-D, NR3A and NR3B). NR1 subunit is able to bind glycine and is an obligatory constituent of any functional NMDA receptor. The ion channel of the NMDA receptor is under resting membrane potential which is maintained by magnesium or zinc cations which block and thus prevent the flux of other cations through the open ion channel. When the membrane is depolarized the magnesium and zinc leave ion pore allowing a flow of sodium and calcium ions. AMPA receptors are heteromeric glutamate receptors consisting of four subunits GLUR 1-4 (Hollmann and Heinemann 1994). If AMPA receptor lacks the GLUR2 subunit, it is permeable to calcium, sodium and potassium. The presence of the GLUR2 subunit makes the channel calcium impermeable (Hsu *et al.* 2010). Kainate receptors can form homomeric or heteromeric receptors by combination of GLuK 1-5 (previously named as GLUR5-7 and KA 1 and 2). GLuK 4-5 require the heteromeric combination with GLuK1-3 subunits. Kainate receptors are distributed both pre- and postsynaptically (Mollerud *et al.* 2016, Herb *et al.* 1992, Fletcher and Lodge 1996).

The unequal distribution of glutamate receptors within the developing CNS is the structural background for the region specific sensitivity to hypoxic-ischemic conditions. As the experiments further continued, the role of calcium in this process of neuronal tissue damage under hypoxic-ischemic conditions is evident and the importance of NMDA receptors was recognized. Such disruption is especially pronounced during the neonatal period as the hypersensitivity of NMDA receptors has been described in the period of early ontogenesis (McDonald *et al.* 1988, Bittigau *et al.* 1999, Johnston 2005). Over activation of ionotropic glutamate receptors, especially the NMDA receptor, by excess excitatory amino acid production under hypoxic-ischemic conditions sustain a plethora of cellular events. This involves intracellular calcium overload, triggering of downstream effects like mitochondria membrane depolarization along with generation and activation of reactive oxygen and nitrogen species and caspases (Doble 1999). A prolonged calcium influx through glutamate receptors channel is thus a crucial event in excitotoxicity. Another important part of excitotoxicity is its osmotic component. Huge depolarization of the postsynaptic membrane causes an influx of sodium and chloride into the cell followed by water. If uninterrupted and non-buffered, such process can lead to rupture and lysis of the cell (Doble 1999). The disbalance in neuromediator physiology, accompanied by the unique ontogeny of GABA and glutamate receptors and their formation and maturation of synaptic functional networks, often creates the convenient background for elicitation of acute symptomatic seizures, which frequently manifest after hypoxic-ischemic insult (Doble 1999, Ming *et al.* 2015).

Oxidative stress

Free radicals, highly reactive molecules with unpaired electrons, play important functions in cell signaling and physiological regulations including smooth

muscle tonus, regional cerebral blood flow regulation, inflammation and modulation of excitatory neurotransmission. However, under pathological circumstances such as hypoxia – ischemia or reperfusion – reoxygenation, they are believed to participate in the degenerative processes leading to the acute and chronic brain injury. In humans the most important source of free radicals is oxidative metabolism, especially in the mitochondria. In particular Complex I and III of the mitochondrial transport chain have shown to be a major source as well as a target of highly toxic superoxide anions under acute hypoxic or reoxygenation conditions. Besides mitochondrial superoxide production, NADPH and xanthine oxidases churn out significant amounts of superoxide. This production is usually tonic and has a latency of tens of minutes or hours after the insult. Irrespective of the source, a superoxide reacts with surrounding cellular components such as proteins, nucleic acids, lipids and other reactive species like nitric oxide whose production is enhanced by activation of both constitutive and inducible nitric oxide synthases. Neurons and glial cells dispose of effective scavenging enzymes such as superoxide dismutase which deactivate superoxide to form hydrogen peroxide which is then degraded by several catalytic systems to oxygen and water. Massive superoxide overproduction, however, causes elevated levels of both superoxide and hydrogen peroxide, which prove to be detrimental. The negative effects of reactive oxygen species can be subdivided into acute and chronic. Although molecular mechanisms in both effects are similar, an attack of surrounding covalent bindings, the difference in consequences is fundamental. Acutely, oxidative stress causes alterations to structural components of cells like receptors, transporters, respiratory complexes, which can lead to cellular death if a certain level of damage is reached. On the other hand, the chronic effects are caused by defective genetic information from oxidative alterations to nucleic acids and thus the resultant proteins have impaired function (Folbergrová *et al.* 2016, Otahal *et al.* 2014). Moreover, we have recently found that oxidative stress accompanying status epilepticus in immature rats significantly inhibits Complex I. Such inhibition surprisingly lasts for a long period after the initial insult when one would expect total replacement of defective complexes (Folbergrová *et al.* 2016, Folbergrová *et al.* 2012). Complex I is thus the major source of superoxide in acute hypoxic-ischemic conditions but also the main target for reactive species. Inhibited Complex I then produces more superoxide starting thus a vicious circle (Otáhal *et al.* 2014). From a chronic point of view, a modern concept of so-called "selective vulnerability" seems to be very interesting. It has been shown recently that in the brain, besides the glutathione peroxidase system, a thioredoxin/peroxiredoxin system plays a critical role in neuronal peroxide detoxification (Drechsel and Patel 2010). Using hippocampal slice cultures and auranofin, a thioredoxin/peroxiredoxin blocker, a significantly higher rate of peroxide formation in interneurons as compared to pyramidal cells has been revealed (Kudin *et al.* 2012). Recent research also suggests that especially parvalbumin (PV) expressing interneurons and interneurons in general may be crucial, especially in an ontogenetic sense, to the development of epilepsy and schizophrenia (Behrens and Sejnowski 2009, Folbergrova and Kunz 2012, Gulyas *et al.* 2006, Druga *et al.* 2010). Excessive oxidative stress caused by glutathione peroxidase system (GSH) deficit led to significant and selective decreases of PV-expression and GABAergic phenotypes resultantly decreasing the power of gamma rhythms in the hippocampus (Steullet *et al.* 2010). Significant reduction of GSH and imbalance of thiol-redox potential is a common finding in schizophrenic patients and is implicated in the pathophysiology of schizophrenia (Behrens and Sejnowski 2009). Similar findings have been shown in models of pharmacologically induced schizophrenia-like behavior by the application of NMDA receptor antagonists. This led to a loss of PV immunoreactive fast spiking interneurons and resulted in lasting psychosis-like behavioral changes (Behrens *et al.* 2007).

Inflammation

Hypoxic-ischemic condition in the immature brain can massively initiate an inflammatory response over a few hours. Activated microglial cells and astrocytes produce various immunoactive molecules such cytokines, growth factors and chemoattractants with subsequent infiltration of blood derived immunocompetent cells. Microglial phagocytosis contributes to the restoration of tissue homeostasis by clearing necrotic cells and cellular debris. Activated microglia produce matrix metalloproteinases that further disrupt the BBB. Interestingly, cytokines released by activated microglia can have either pro-inflammatory (IL-1, IL-6) or anti-inflammatory roles (IL-10) depending on their microglia phenotype (Lee *et al.* 2014). Microglial

cells with M1 phenotype (typically activated microglia) express pro-inflammatory mediators (IL-1β, IL-6, TNF-α), MHCII class on the cell membrane and present antigens to T cells. M2 microglia are regarded as "healing" that contribute to recovery after damage and secrete predominantly anti-inflammatory mediators (IL-10, IL-4, IL-13, TGF-β, IGF-1) (Lee *et al.* 2014). In early stages of hypoxic-ischemic insult the M2 phenotype is dominant, while M1 populations of microglia usually expressing the ED-1 marker of phagocytosis dramatically increase with time in both the core and peri-infarct regions. A growing body of evidence suggests that inflammatory mediators might alter neuronal functions and synaptic transmission in acute as well in chronic states. Indeed, it has been shown that pro-inflammatory cytokine IL-1β can induce rapid an increase in excitability by activating its receptor IL-1R which might result in acute seizures (Vezzani *et al.* 2013). Application of IL-1 receptor antagonists lead to rapid termination of experimentally induced seizures (Librizzi *et al.* 2012). This finding led to clinical testing of the commercially available IL-1 receptor antagonist anakinra. On the contrary, IL-10 released by microglia of the M2 phenotype possesses anticonvulsive properties (Godukhin *et al.* 2009). Additionally cytokines and growth factors released during neuroinflammation initiate complex transformation of glial cells. For instance, TGF-β induces changes in expression of astrocytic proteins, which result in astrocytic dysfunction. TGF-β mediated transformation of astrocytes leads to impaired potassium buffering (Kir4.1) and decreased glutamate withdrawal (glutamate transporters) (Vezzani 2013). Neuroinflammation processes induced by stroke thus might induce acute seizures as well mediate epileptogenesis.

Blood brain barrier alterations

Cerebral vessels (small arterioles and capillaries) are fully covered by astrocytic processes, which together with remaining structures of the mature vessel form the semipermeable BBB. Neurons, astrocytes, pericytes and vascular cells are all components of the neurovascular unit and work in concert to maintain the homeostasis of the brain microenvironment (Iadecola and Nedergaard 2007). Acute brain insults (such stroke) seriously affect components of BBB on both molecular and cellular levels and result in BBB dysfunction (Otahal *et al.* 2014). Increased BBB permeability leads to extravasation of plasma constituents and vasogenic brain edema (Stanimirovic and Friedman 2012). Localized and transient BBB breakdown have been shown to trigger both acute epileptiform activity and chronic seizures in experimental conditions (Friedman 2011, Maggio *et al.* 2013). The main mechanism is probably astrocytic transformation and inflammatory processes induced by albumin and TGF-β leading to changes in brain extracellular ionic environment (Ivens *et al.* 2007, Marchi *et al.* 2007, Otahal *et al.* 2014, Tomkins *et al.* 2007). Seizures are usually caused by hyperexcitability of brain tissue, for which serum albumin and TGF-β signaling play a critical role. Nadal *et al.* (1997) have shown that albumin induces calcium signaling and DNA synthesis in astrocytes in brain cultures and acute slices. Ivens *et al.* (2007) elucidated the causal role of albumin in neuronal hyperexcitabilty after experimental local BBB opening. They have shown that albumin is selectively transported into astrocytes *via* TGF-β receptors, where it triggers transcriptional and functional changes. Besides chronic epileptogenic transformation after the BBB disruption, an acute loss of BBB integrity during insult such as hypoxia-ischemia may directly cause seizures. Thrombin and other proteases have been identified as a trigger of seizures when they are in direct contact with brain tissue (Lee *et al.* 1997).

Biomarkers of perinatal brain damage

Guidelines from the American Academy of Pediatrics (AAP) and the American College of Obstetrics and Gynecology (ACOG) for HIE indicate that all of the following must be present for the diagnosis of perinatal asphyxia severe enough to result in acute neurologic injury: profound metabolic or mixed acidemia (pH<7) in an umbilical artery blood sample, if obtained; persistence of an Apgar score of 0-3 for longer than 5 min; neonatal neurologic sequelae (e.g. seizures, coma, hypotonia) and multiple organ involvement (e.g. kidney, lungs, liver, heart, intestines). Apgar score less than 7 after first 5 min after birth is usually the sign of elapsed hypoxia, although it usually does not correspond to the blood pH. Analysis of pH, blood gasses and base excess (BE) from newborn blood taken from the umbilical artery is the common method used to diagnose the elapsed hypoxia. The analysis allows us to determine prognoses of the newborn.

Nowadays, there are a number of routine ante- and intrapartal diagnostic methods that can detect signs of

hypoxia of the fetus. Methods that can be used in the antenatal diagnostics include cardiotocography, Doppler blood flow velocity in the middle cerebral and umbilical arteries. Cardiotocography, intrapartum fetal pulse oximetry and ST-analysis of the ECG are used for intrapartal diagnostics. The most common method how to diagnose the impending hypoxia of the fetus during labor is cardiotocography and the intermittent auscultation of the fetus at predetermined intervals (Salamalekis *et al.* 2002). A suspicious heart rate has low specificity and many false positive outcomes. It could result in unnecessary intervention during the labor, which has increased the caesarean section delivery rate (Salamalekis *et al.* 2002). Fetal pulse oximetry is able to detect the heart rate even during fetal arrhythmia. It can detect the saturation of oxygen in fetal blood in real time in combination with cardiotocography and could be useful during high-risk labor and to reduce rate of caesarean deliveries.

The tools and assessments mentioned above help physicians to judge a level of probability of serious organ damage, which special focus on brain damage. However, none of them are able to evaluate the extent and severity of real brain damage and they should be used for routine selection of patients at risk. In such patients, it is then necessary to extend diagnostic evaluations with more selective and sensitive methods to elucidate presence and extent of brain damage. In clinical practice ultrasound and/or MRI is largely used. These methods possess good sensitivity and selectivity, however they are time consuming and the patient has to be transported to the imaging unit as per usual. These factors limit their routine use. Moreover, in initial states when morphological changes are still in development, the real extent of the lesion may be misinterpreted. It is therefore easier to use peripheral biomarkers of brain damage whose level correlate with the extent of damage. Ideally, such biomarkers can serve also as reliable parameter for longitudinal control of treatment efficacy. Nowadays, several molecular biomarkers of brain damage have been described and tested. In some cases, they can be detected in peripheral blood and others are detectable only in CSF due to their limited ability to cross the BBB. Although the idea that the assessment of hypoxic-ischemic brain damage using routine, and fast peripheral blood analysis is very attractive, one should keep in mind that sensitivity and selectivity to hypoxic-ischemic damage is problematic. Present candidate biomarkers are molecular components (proteins) released from damaged brain cells, oxidatively altered molecules and general markers of neuroinflammation. The family of biomarkers of neuronal tissue injury consist of proteins specifically expressed in brain tissue and are released during their damage. For example, neuron specific enolase (NSE) is especially found of the neurons of the brain. Elevated serum NSE levels have been found in mild and severe HIE at 4 and 48 h (Celtik *et al.* 2004). However, Nagdyman *et al.* (2001) did not observe different levels between the groups. Myelin basic protein (MBP) is the major protein of myelin. The MBP levels in serum and CSF rapidly increase after the insults and correlates with white matter damage (Lv *et al.* 2015). Specific patterns of MBP release in HIE is not well documented and thus requires further investigation. Acidic calcium binding protein S-100β is a brain specific protein synthesized and released by astrocytes. The plasma levels of S-100β dramatically rise during the first hour after hypoxia-ischemia with reasonable specificity and sensitivity. S-100β is thus a promising molecule for routine use as biomarker for early diagnosis, management and prognosis of HIE (Lv *et al.* 2015, Gazzolo *et al.* 2015). Glial fibrillary acidic protein (GFAP) is a specific skeletal protein in astrocytes. During brain damage GFAP levels in serum and CSF increases. GFAP serum levels also seem to be a very sensitive marker of blood brain barrier opening. It has been shown that serum GFAP levels correspond to morphological damage assessed by MRI after hypoxia-ischemia suggesting that GFAP might be a reliable molecular biomarker of HIE (Lv *et al.* 2015, Ennen *et al.* 2011). Lactate dehydrogenase (LDH) is an important enzyme catalyzing oxidation of lactate to pyruvate. LDH is increased in HIE and when used in combination with more neuronal damage biomarkers such as NSE, the specificity is rather high (Lv *et al.* 2015). Oxidative stress can be revealed by detection of malondialdehyde (MDA) or glutathione peroxidase. Studies by Kumar *et al.* (2008) have shown significantly elevated serum levels of MDA in newborns with diagnosed HIE. MDA was more elevated in patients with seizures after HIE (Mondal *et al.* 2010) which prove low specificity of the MDA as biomarker to HIE. Neuronal damage caused by any insult, including neonatal hypoxia-ischemia, triggers inflammatory reactions. Biomarkers of neuroinflammation such as interleukins (IL-1 and 10), TNF-α, high-sensitivity C-reactive protein and adhesion molecules are extensively studied in neuronal damage and HIE. The induction of these molecules has longer delay and they have low specificity to HIE. For more

detailed information on individual molecular biomarkers we recommend an excellent recent review by Lv *et al.* (2015).

Potential treatment approaches for perinatal HIE

Nowadays, hypothermia is the only intervention against HIE clinically used on humans (Wu *et al.* 2015). Review of clinical trials has shown that hypothermia reduced the mortality rate without increasing the disability rate in asphyxiated newborns. This outcome was indicated by a decrease in the rate of major disability and an increase in the rate of survival with normal neurological function (Tagin *et al.* 2012). Besides hypothermia, also other treatments (e.g. erythropoietin, melatonin, allopurinol, xenon, growth factors, nitric oxide synthase inhibitors) have proven to have beneficial effects on animal models (Wu *et al.* 2015). Erythropoietin (EPO) possesses complex action. It modulates NMDA excitotoxicity, potently reducing free radical toxicity and inflammation. A clinical trial with EPO has been started with promising results. Melatonin and allopurinol, both anti-oxidative substances, have been extensively tested in preclinical experiments and clinical testing has begun (Muller and Marks 2014). There are also gender differences in responding to the therapy. Studies showed, that females appear to benefit more from neuroprotective interventions after HI brain injury, such as hypothermia, EPO and 2-IB (Nijboer *et al.* 2007a, Fan *et al.* 2013), whereas other studies showed more treatment benefits of EPO for males (Fang *et al.* 2012).

Long term consequences of HIE

Cerebral palsy is the most common long term consequence of HIE. The proportion of cerebral palsy associated with intrapartal hypoxia-ischemia is about 14.5 % (Graham *et al.* 2008). Standardized testing with the Griffiths Mental Development Scales showed that almost all children with moderate HIE who had been diagnosed with cerebral palsy at the age of 12 months were severely delayed in development. Children with moderate HIE without cerebral palsy at this age performed in the average range or above, although the range of scores varies widely across studies (Van Handel *et al.* 2007). Moderate HIE does not seem not to be related to post-neonatal epilepsy, regardless of its association with neonatal seizures (Pisani *et al.* 2009).

Neonatal seizures and their comorbidities could be the leading long-term consequence of perinatal hypoxia (Sun *et al.* 2016). We have shown that photothrombotic focal cerebral ischemic lesions induced in seven days old rats increased their susceptibility to pentetrazol seizures induced later in development (Brima *et al.* 2013b). The data suggests that processes triggered by perinatal ischemia significantly alters brain tissues and irrespective of what the mechanism is, it finally makes the brain more prone to seizures. Indeed, the severities of perinatal HIE and neonatal seizures in humans are a potential risk factor for the development of late-onset seizures. Moreover, therapeutic hypothermia may reduce the risk of the development of epilepsy in such cases (Inoue *et al.* 2014).

Animal models of HIE

The first models trying to describe the consequences of hypoxia and hypoxia itself were published in early thirties of the last century (Ferraro 1933). Injection of potassium cyanide produced toxic encephalomyelopathy and CNS demyelination. Hurst noticed that observed consequences of cyanide injection were not necessarily ascribed to its toxicity, but that the "bouts of anoxia" were the mechanism of white matter damage (Hurst 1940). Later on Levine published a model of surgical double ligation of right carotid, followed by anoxic anoxia (oxygen replaced by nitrogen or nitrous oxide gas in jar with single adult rat) (Levine 1960). Adjustment of this technique was later seen in the Rice-Vannucci model of ligation of the common carotid artery in immature rats (postnatal day 7) followed by normobaric hypoxia with 8 % of oxygen in a controlled atmosphere and at stable temperatures (Rice *at al.* 1981). This model took the developmental aspect in account. Nowadays it is widely accepted that the postnatal day 7 in rats represents the same level of CNS maturity after 32-34 gestational weeks in humans (Huang *et al.* 2016) and the maturity equivalent of in-term newborns equals approximately the rat's post-natal day 10-12 (Clancy *et al.* 2001). Mentioned developmental parallels are not absolute. Brain sub-compartments (white matter, glia) mature at different speeds in comparison to humans and the growth spurt of a newborn rat is enormous relative to humans (Rumajogee *et al.* 2016, Semple *et al.* 2013, Craig *et al.* 2003). The Rice-Vannucci model is best described in literature by the authors themselves who have published excellent reviews of it (Vannucci *et al.*

1999, Vannucci and Vannucci 2005). As the model developed over more than thirty-five years, its variability decreased and damage to the particular brain area is rather the same. Another HIE model is the ligation of both common carotids followed by normobaric hypoxia (oxygen at 8 %). Such closure could be either definitive or temporary and was demonstrated at different levels of the rat's postnatal development period (Uehara *et al.* 1999, Jelinski *et al.* 1999, Cai *et al.* 2001). Another possibility to mimic HIE conditions, with special focus on the inflammation process, is the injection of lipopolysaccharide (LPS). Such a model could be applied pre- or postnatal. If LPS is injected directly to a pregnant rat, it triggers the placental inflammatory cascade following into the lesions of white matter of fetuses, activation of microglia and apoptosis of neuronal tissue (Cai *et al.* 2000, Huang *et al.* 2016). If LPS was injected into very immature rats and then normobaric hypoxia treatment followed, there was severe white matter injury, pronounced microglia activation and blood brain barrier damage (Wang *et al.* 2010, Wang *et al.* 2016). We have elaborated the model of focal neocortical cerebral ischemia on postnatal-day 7 old rat pups using photothrombosis. Intravenous application of rose bengal with consequent illumination of desired cortical areas with green laser lead to reliable focal circular lesions of gray and white matter with minimal mortality. Animals with ischemic lesions demonstrate poor motor performance and developed higher susceptibility to seizures later in life (Brima *et al.* 2013a, Brima *et al.* 2013b). If considering the appropriate animal model, one must keep in mind there are several factors that could influence the results. One of the most significant is temperature. Hypothermia is nowadays accepted as the major treatment procedure and different temperatures of the animals throughout the experiment cause very different results (Vannucci and Vannucci 2005). Another limitation that has to be taken into account is the difference in sex. Even pre-pubertal animals have sex specific hypoxia responses and not all molecular or cellular mechanisms of brain damage are very probably shared between sexes (Hagberg *et al*. 2004, Nijboer *et al*. 2007b).

Conclusions

Although the knowledge about pathophysiology of hypoxic-ischemic encephalopathy is improving, only limited enhancements have been achieved in clinical practice. One of the major challenges is to detect this critical situation early during its development and to start immediate therapeutic interventions. Typical clinical manifestations together with elevated molecular biomarkers of brain damage may reduce the time to a precise diagnosis and commencement of treatment. Biomarkers can be also used, as their results can be obtained from minimally invasive procedures and at a low cost control of treatment efficacy. They could help to recognize the severity of HIE and could help others to avoid exposure to unnecessary treatment.

Conflict of Interest

There is no conflict of interest.

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