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Review Article

Pathophysiological Roles of Cytokines in the Brain During Perinatal Asphyxia

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Abstract

The essential pathophysiology of perinatal asphyxia (PA) may be attributed to ischemia-reperfusion injuries.The resultant circulation failures contribute to cardio-respiratory dysfunctions at birth. The damage affects tissues and organs, leading to irreparable sequelae such as persistent cerebral palsy. In addition, ischemia-reperfusion injuries due to PA may cause aberrant immunological responses in various organs, such as excessive inflammation. These inflammatory responses appear to involve mainly the activation of microvascular endothelial cells and leukocytes that produce and release various cytokines. These cytokines modulate inflammation and tissue damage in PA, and inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α may be dramatically induced in PA. In contrast, anti-inflammatory cytokines such as IL-10 are also induced in an attempt to reduce the excessive inflammation caused by PA. Therefore, to understand better the pathophysiology of PA, it is essential to unravel the various roles of cytokines. In this review, we mainly focus on the cytokine-associated pathophysiology in the brain linked with PA.

ABBREVIATIONS

PA: Perinatal Asphyxia; **IL:** Interleukin; **HIE:** Hypoxic-Ischemic Encephalopathy; **PMNs:** Polymorpho Nuclear Leukocytes; **IFN:** Interferon; **TNF:** Tumor Necrosis Factor; **ROS:** Reactive Oxygen Species; **FIRS:** Fetal Inflammatory Response Syndrome; **CBF:** Cerebral Blood Flow; **CNS:** Central Nervous System; **CSF:** Cerebrospinal Fluid; **MMPs:** Matrix Metalloproteinases; **BBB:** Blood-Brain Barrier; **NO:** Nitric Oxide; **HMGB-1:** High-Mobility Group Box-1; **MAS:** Meconium Aspiration Syndrome; **SIRS:** Systemic Inflammatory Response Syndrome; **MCP-1:** Monocyte Chemoattractant Protein; **G-CSF:** Granulocyte Colony-Stimulating Factor; **VEGF:** Vascular Endothelial Growth Factor; **EPO:** Erythropoietin; **NMDA:** N-Methyl-D-Aspartate; **HIF:** Hypoxia-Inducible Factor; **ACD:** Anticonvulsant Drug

INTRODUCTION

The prevalence of moderate or severe hypoxic-ischemic encephalopathy (HIE) is approximately1–6 per 1000 births, with rates of mortality and sequelae of approximately 15–20% and 25%, respectively [1]. Perinatal asphyxia (PA) is a main cause of HIE and its pathophysiology appears to be mainly caused by ischemia-reperfusion injuries [2]. In general, most asphyxiated neonates have good outcomes. However, severe PA may cause irreversible damage in many organs, leading to poor outcomes, such as epilepsy, global developmental delay, cerebral palsy, or death [3].

From the pathophysiological point of view, PA is mainly caused by a reduction in organ blood flow and oxygen delivery. The pathophysiology of PA is closely associated with an excessive inflammatory response. Inflammatory cells, including lymphocytes and polymorphonuclear leukocytes (PMNs), are observed in tissues that have suffered a hypoxic insult [3-5]. The pathophysiological findings strongly suggest that such damage is associated with inflammation caused by ischemia-reperfusion injuries [3]. Indeed, asphyxiated neonates often have an episode of fever due to inflammation. Thus, inflammatory responses play a central role in hypoxia-ischemia injuries [3,5,6].

Cytokines play pivotal roles in immunologic regulation, including the proliferation and differentiation of most types of leukocytes [7]. Many cytokines, such as interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, are also involved in inflammatory responses *in vivo* [7,8]. These cytokines can activate inflammatory cells such as PMNs and monocytes/macrophages [7,8]. These cytokine-activated cells may release toxic substances, such as reactive

oxygen species (ROS) and toxic granules including proteolytic enzymes and myeloperoxidase, injuring cells and tissues [9]. Thus, various cytokines may be associated with the pathophysiology of ischemia-reperfusion injuries [10].

Previous studies reported relationships between PA and systemic inflammatory response [11,12]. Fetal distress may cause fetal inflammatory response syndrome (FIRS) [11]. FIRS are characterized bysystemic inflammation and an increase in the levels of various inflammatory mediators, such as IL-6 [11]. The presence of an excessive amount of inflammatory mediators induces multiple organ failure [11,12]. In addition, it is also known that neonatal asphyxia is associated with cytokinemia, which is called systemic inflammatory response syndrome (SIRS) [13-15]. Thus, many studies in neonates reported an association between PA and inflammatory mediators, such as proinflammatory and anti-inflammatory cytokines [13-15]. It may be important to control excessive inflammatory mediators in PA to reduce the damage caused.It is known that the pathophysiology of PA is closely associated with many inflammatory mediators, such as cytokines, complement proteins, and adhesion factors. However, to our knowledge, the temporal alteration of such inflammatory mediators and the relationships between cytokines and therapy, such as hypothermia, have not been elucidated in considerable detail. We focus this review on the pathophysiological roles of cytokines in the brain and the effects of PA therapeutics on cytokines.

The essential physiological changes in PA are ischemia-reperfusion injuries

Physiological phases in PA: The main cause of PA is an interruption of placental blood flow, which impairs oxygen supply and blood flow to many organs. In particular, impaired cerebral blood flow (CBF) causes neonatal brain injury. Gunn et al. [16] showed the phases of cerebral injury. The reduction in blood flow leads in turn to the primary energy failure phase, the reperfusion period, the latent phase, and, finally, the secondary phase of energy failure [16]. The energy failure of the secondary phase may be critical for the induction of irreversible injuries and has a close relationship with neurological outcomes in PA [7]. Therefore, it may be essential to initiate effective treatment as early as possible, such as hypothermia, oxygen free radical inhibitors and scavengers, and erythropoietin (EPO) [17,18].

The primary phase is directly affected by ischemic and/or hypoxic insults, which lead to primary energy failure due to the rapid depletion of adenosine triphosphate in anaerobic metabolism [17]. A lack of energy induces cell death via necrosis. Primary energy failure triggers several damaging reactions, including an excessive accumulation of calcium in the cytoplasm, increase in excito toxic amino acids in the neonatal brain, and oxidative stress [1,4]. These persistent reactions may result in the secondary phase of energy failure. However, the primary phase is first followed by a reperfusion period and a latent phase. In the latent phase, excessive calcium influx and pro- or anti-apoptotic proteins initiate a cascade that culminates in delayed cell death via apoptosis [19,20]. In addition, the restoration of blood flow and oxygenation drastically increases ROS generation, which damages tissues and organs. ROS attack the polyunsaturated fatty acid component of the cellular membrane, resulting in membrane fragmentation and cell death [21,22]. During secondary energy failure, the cardiac and respiratory condition is stable [23]. However, secondary energy failure accompanied by more severe injury is followed by progressive secondary deterioration, including seizures, cytotoxic edema, and excitotoxin accumulation [24]. Thus, the severity of secondary energy failure is strongly associated with long-term developmental outcomes at 1 and 4 years of age [25-27]. The mechanism of secondary energy failure is mainly mitochondrial dysfunction; mitochondria play a key role in determining the fate of neurons following hypoxia-ischemia.

Necrosis and apoptosis of neural cells in PA due to ischemia-reperfusion injuries

The mechanism of neuronal cell death following ischemia-reperfusion includes two major pathways: necrosis and apoptosis [28]. The degree of the initial insult determines the mode of cell death [29]. Necrosis is characterized by a passive process of cell swelling, disrupted cytoplasmic organelles, loss of membrane integrity, and eventual lysis of neuronal cells and activation of an inflammatory process [28]. In contrast, apoptosis is an active process distinguished from necrosis by the presence of cell shrinkage, nuclear pyknosis, chromatin condensation, and genomic fragmentation, which occur in the absence of an inflammatory response [28]. However, apoptosis is often triggered by inflammatory mediators. Thus, both necrosis and apoptosis may be associated with ischemia-induced inflammation. In particular, apoptosis appears to be more important than necrosis after injury and plays a prominent role in hypoxic-ischemic injury in the neonatal brain [30]. Therefore, the goal of therapy is almostalways associated with inhibiting cell death via apoptosis.

Immunological responses and the roles of cytokines in the ischemia-reperfusion injuries of PA

Excessive production of cytokines in the brain due to ischemia-reperfusion injuries: Ischemia-reperfusion injures tissues and organs. Cerebral ischemia also induces an inflammatory response in both the parenchyma and systemic circulation [31]. Microglia play an important central role in hypoxia-ischemia injuries in the brain. Microglial activation is the initial step in the inflammatory responses of the central nervous system (CNS) to various stimuli, including stroke [32]. This initial step is followed by the infiltration of circulating inflammatory cells, including monocytes, neutrophils, and T-cells [33]. In particular, neutrophils play a central role in ischemia-reperfusion. During ischemia, such as that seen in PA, neutrophils are the first to reach the site of inflammation [34] and can exacerbate brain injury through several mechanisms, e.g., generation of ROS, decreased microvascular flow resulting from capillary plugging by neutrophils, enhanced release of cytotoxic agents into the vasculature and brain parenchyma, and matrix metalloproteinase-9 (MMP-9) secretion [6,35,36]. Cytokines are producedat high levelsin the brain, and activated leukocytesmigrate into the injured brain [37]. Activated microglia contribute to phagocytosis, the production of proinflammatory and anti-inflammatory cytokines, antigen presentation, and the release of MMPs, as well as macrophage activation [31].

MMPs disrupt the blood-brain barrier (BBB) [38].Plasma MMP-9 levels in asphyxiated neonates are higher than in non-asphyxiated neonates [39]. Moreover, plasma MMP-9 levels in asphyxiated neonates are significantly associated with the severity of PA [39]. In reperfusion injury, MMPs participate in the biphasic opening of the BBB [39]. In the reversible initial phase, the induction of hypoxia-induced factor (HIF)-1α by hypoxia triggers the production of MMP-2 [40]. In the late phase after the insult (24–48 h), hypoxia-induced proinflammatory cytokines (e.g., TNF-α and IL-1β) lead to the production of MMP-3 and MMP-9. These activated MMPs degrade the basal lamina and tight junctions of endothelial cells [41]. As a result, opening of the BBB leads to vasogenic edema and circulating leukocytes can easily infiltrate the brain, further exacerbating theinflammation and brain damage [31]. Amoeboid microglia in the immature brain respond vigorously to hypoxia, accumulate in injured tissues, and produce excessive amounts of proinflammatory cytokines (e.g., TNF-α and IL-1β) along with glutamate, nitric oxide (NO), and ROS, which collectively cause oligodendrocyte death, axonal degeneration, and disruption of the immature BBB [6,42-44]. In addition, within minutes of an insult, astrocytes may be activated by inflammatory mediators and ROS [45]. Activated astrocytes can also produce proinflammatory cytokines [45,46]. Thus, many inflammatory cells, including microglia, amoeboid microglia, astrocytes, and neutrophils, may play important roles in the overproduction of cytokines in asphyxiated neonates with neuronal inflammation immediately after birth.

Is IL-6 proinflammatory or anti-inflammatory?

IL-6, a representative proinflammatory cytokine that acts as an exacerbation factor, is strongly associated with various inflammatory diseases, such as rheumatoid arthritis [47]. Chiesa et al. [14] demonstrated that IL-6 levels in umbilical cord blood increase in term neonates with asphyxia. Other studies have shown elevated levels of IL-6 in serum and cerebrospinal fluid (CSF) [15,48,49]. Thus, IL-6 mainly acts as an inflammatory mediator of brain damage and plays a central role in inflammatory responses. This cytokine orchestrates an inflammatory response between blood cells and other cells. In the brain parenchyma, IL-6 activates gliosis and leukocytes [50].

However, a recent study suggested that IL-6 plays a double role in cerebral ischemia, as an inflammatory mediator during the acute phase and as a neurotrophic mediator between the subacute and prolonged phases [51]. During the acute phase, IL-6 is involved in the induction of acute reactions and in controlling the level of acute inflammatory responses by decreasing the levels of proinflammatory cytokines and increasing the levels of anti-inflammatory molecules. During the prolonged phase, IL-6 is involved not only in eliciting an acute phase reaction but also in the development of specific cellular and humoral immune responses [52]. Many *in vivo* and *in vitro* studies have shown that IL-6 exerts a neuroprotective effect in several types of brain injury via leukemia inhibitory factor and ciliary neurotrophic factor [53-56]. IL-6 improves the survival of CNS neurons, reducing excitatory neuronal damage to N-methyl-D-aspartate (NMDA)-mediated injury and protecting neurons against apoptosis [54,57,58]. In human perinatal asphyxiated neonates with hypothermia, Jenkins et al. [59] reported that the levels of IL-6 were significant higher and showed a biphasic pattern.In an animal study, serial injections of recombinant IL-6 prevented learning disabilities and delayed neuronal loss [60]. Moreover, an anti-mouse IL-6 receptor monoclonal antibody increased the number of apoptotic cells [61]. Thus, IL-6 can potentially exert beneficial or detrimental effects depending on the pathologic context.

Relationships between cytokines and high-mobility group box 1 (HMGB-1)

HMGB-1 was originally described as a nuclear DNA-binding protein [62,63]. This molecule principally acts as a structural stabilizer of DNA [63]. Some types of leukocytes, such as macrophages, express the HMGB-1 receptor on their cell surface, and extracellular HMGB-1 induces the production of other proinflammatory cytokines, such as IL-1β and TNF-α, from inflammatory cells [64,65]. Thus, it is suggested that HMGB-1 is a physiologically multivalent molecule.

We demonstrated that serum HMGB-1 levels are significantly increased in asphyxiated neonates compared with non-asphyxiated neonates [66]. In addition, the plasma concentration of HMGB-1 in asphyxiated neonates can be decreased by head cooling therapy [67]. Recent studies indicate that HMGB-1 plays a critical role in several inflammatory diseases, including septic shock, rheumatoid arthritis, and acute lung inflammation [64,65]. The extracellular release of HMGB-1 is a common response to both cell and tissue injury and microbial invasion [68]. HMGB-1 induces endothelial cell activation by upregulating adhesion molecules and secreting proinflammatory cytokines and chemokines [69]. Moreover, HMGB-1 may be linked with the pathophysiology of hepatic ischemia-reperfusion injuries [70]. Thus, HMGB-1 may respond to other ischemia-reperfusion injuries, such as asphyxia.

Cytokine levels in the serum and CSF in PA

PA induces the excess production of proinflammatory cytokines, which contribute to the development of SIRS. Our previous reports suggested that there were differences in cytokine concentrations in serum among normal neonates, asphyxiated neonates, and adults [13]. Immediately after birth, the concentrations of IL-6, IL-8, and IL-10 were higher in the serum of asphyxiated neonates than in normal neonates [13]. In addition, the serum levels of IL-6, IL-8, and IL-10 were also higher in severely asphyxiated neonates (dead or poor outcome cases) than in asphyxiated neonates without poor outcomes, although the number of cases with poor outcomes in this study was small. The above results suggest that some cytokines are induced excessively by asphyxia. In addition, we demonstrated cytokinemia in neonates with meconium aspiration syndrome (MAS). The serum levels of most types of proinflammatory cytokines were higher inneonates with MAS than in those without MAS [71]. MAS is first triggered by fetal hypoxia or ischemia. Both fetal hypoxia-ischemia- and meconium-induced inflammation may increase drastically the serum levels of cytokines and lead to SIRS.

Others studies have measured the serum or CSF levels of cytokines in asphyxiated neonates in the early postnatal period (Tables 1 and 2). IL-6, IL-8, and TNF-α levels are significantly

increased in the CSF in the early postnatal period [15,72-76]. Indeed, microglia, astrocytes, and neutrophils are activated and release large amounts of proinflammatory cytokines within minutes of ischemia. Increased levels of proinflammatory cytokines in the CSF of asphyxiated neonates may be caused by activated inflammatory cells. As in the CSF, many studies reported the presence of proinflammatory (IL-1β, IL-6, IL-8, and TNF-α) and anti-inflammatory (IL-10) cytokines in the serum of asphyxiated neonates (Table 1). Cerebral ischemia induces an inflammatory response in both the parenchyma and systemic circulation. Therefore, these cytokines may also be increased in the serum of asphyxiated neonates in the early postnatal period.

Temporal alterations in serum cytokines in asphyxiated neonates have been investigated in six studies (Table 2). Jenkins et al. [59] reported that the levels of IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1 were significant higher and showed a biphasic pattern in the hypothermia group compared with the normothermia group [59]. This report may agree

Table 1: Cytokine levels in serum and CSF in asphyxia.

References	Cytokines	Sampling time	Subject	Results
Serum				
Okazaki et al.,2006, Japan [11]	8 cytokines: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, IFN-γ	<4 hours	Normal, Asphyxia	IL-6, IL-8, and IL-10 levels in the sera of asphyxiated neonates were higher than in normal neonates. IFN-γ in asphyxiated neonates was lower than in normal neonates. IL-6, IL-8, and IL-10 levels in asphyxiated neonates with either a poor outcome or death were higher than in those without poor outcomes. Subjects: normal (n=10), asphyxia (n=17).
Shang et al.,2014, China [72]	2 cytokines: IL-6, TNF-α	unknown	HIE mild, moderate, severe	Significant upregulation of levels of IL-6 and TNF-α in neonates with HIE compared with healthy neonates. The increase in the levels of these inflammatory mediators correlated with the severity of the disease and also had a positive correlation with the prognosis of the disease.Subjects: HIE mild (n=31), moderate (n=26), severe (n=17).
CSF				
Sävman et al.,1998, Sweden [34]	10 cytokines: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, GM-CSF, TNF-α, IFN-γ	<72 hours	Normal, HIE mild moderate severe	IL-6 was higher in asphyxia than in control neonates. There was also a significant relationship between IL-6 and the degree of HIE, and between IL-6 and outcome. IL-8 was higher in asphyxia than in control neonates and there was an association between IL-8 and the degree of HIE. IL-10, TNF-α, GM-CSF, and IL-1β did not differ between groups.Subjects: normal (n=7), HIE mild (n=4), moderate (n=8), severe (n=8).
Martin-Ancel et al.,1997, Spain [73]	IL-6	At 12 and 72 hours	No HIE HIE mild moderate severe	IL-6 (8 to 90 hours of life) levels were higher in neonates with severe HIE than in those with no to moderate HIE. IL-6 was significantly higher in neonates with signs of brain damage in cranial magnetic resonance imaging. IL-6 was higher in neonates with adverse outcomes than in neonates with favorable outcomes. Subjects: No HIE (n=3), HIEmild (n=5), moderate (n=6), severe (n=6).
Serum (plasma) and CSF				
Aly et al.,2006, Egypt [50]	3 cytokines: IL-1β, IL-6, TNF-α	<24 hours	No HIE HIE mild moderate severe	IL-1β, IL-6, and TNF-α levels in both serum and CSF were all significantly increased in HIE when compared with control. IL-1β in the CSF correlated with the severity of HIE more than IL-6 or TNF-α. IL-1β exhibited the highest CSF/serum ratio among the three studied cytokines. Abnormal neurological findings and/or abnormal DDST II at 6 and 12 months were best predicted by IL-1β in the CSF. Subjects: No HIE (n=13), HIEmild (n=5), moderate (n=7), severe (n=12)
Silveira et al.,2003, Brazil [48]	2 cytokines: IL-6, TNF-α	<48 hours	Control Asphyxia	Plasma IL-6 in asphyxia neonates was significantly higher than in control neonates. Plasma TNF-α was similar in asphyxia and control neonates. IL-6 and TNF-α CSF/plasma ratios in asphyxia were higher than in controls. CSF IL-6 and TNF-a levels in asphyxia neonates were significantly higher than in control neonates. The median CSF IL-6 was significantly higher in sepsis than in control neonates. Subjects: control (n=20), asphyxia (n=19).
Tekgul et al.,2004, Turkey [51]	IL-6	<24 hours	HIE mild moderate tosevere	IL-6 levels in both CSF and serum were significantly correlated with the degree of encephalopathy, as well as the outcome. IL-6 in cerebrospinal fluid had the highest predictive value among the biochemical markers. Subjects: HIEmild (n=12), moderate tosevere (n=9).
Oygür et al.,1998, Turkey [49]	2 cytokines: IL-1β, TNF-α	1-12 hours	At the age of 12 months, Group 1: normal Group 2: Abnormal Group 3: death	Plasma IL-1β and TNF-α were not significantly different between groups 1 and 2. CSF IL-1β and TNF-α levels in group 2 were significantly higher than those in group 1. IL-1β, but not TNF-α, in group 2 was even higher than in group 1, although non-survivors were excluded from group 2. Patients whose CSF samples were taken within 6 hours of the hypoxic insult had higher IL-1β and TNF-α than the patients whose samples were taken after 6 hours.Subjects: Group 1(n=11): normal development, Group 2(n=14): abnormal neurological development, and/or abnormal developmentat the age of 12 months, Group 3(n=5):death.

Abbreviations: IL: Interleukin; **IFN:** Interferon; **TNF:** Tumor Necrosis Factor; **HIE:** Hypoxic-Ischemic Encephalopathy; **GM-CSF:** Granulocyte Macrophage Colony Stimulating Factor; **CSF:** Cerebrospinal Fluid; **DDST:** Denver Developmental Screening Test.

Table 2: Temporal alteration of serum cytokines in asphyxia.

References	Cytokines	Sampling time	Subject	Results
Okazaki et al., 2012, Japan [53]	2 cytokines: VEGF, G-CSF	0, 6, 12, 18, 24 (hours)	Non-asphyxia Mild asphyxia Severe asphyxia with head cooling	G-CSF in sera markedly increased and was sustained in severely asphyxiated neonates treated with head cooling, whereas VEGF decreased and remained low. Subjects: non-asphyxia (n=4), mild asphyxia (n=5), severe asphyxia (n=5)with head cooling.
Chalak et al., 2014, USA [74]	9 cytokines: IL-1, -6, -8, VEGF, TNF-α, IFN-γ, RANTES, UCH-L1, GFAP	0, 6-24, 48-72, 78-96 (hours)	Mild (n=7) without hypothermia Moderate n=17 Severe n=3 with hypothermia	At birth, GFAP and UCH-L1 increased with the severity of HIE. Serial GFAP remained elevated in neonates with moderate to severe HIE. At 6-24 hours, IL-6, IL-8, and VEGF were greater in moderate to severe vs. mild HIE. The serial values were unaffected by hypothermia-rewarming. At 6-24 hours, elevated GFAP, IL-1, IL-6, IL-8, TNF-α, IFN-γ, and VEGF were associated with abnormal neurological outcomes. Subjects: mild (n=7) without hypothermia, moderate (n=17) Severe (n=3) with hypothermia
Jenkins et al., 2012, USA [42]	12 cytokines: IL-1β, -2, -6, -8, -10, -12, -13, TNF-α, MIP-1α, MCP-1, IFN-γ, IP-10	0, 12, 24, 36, 48, 60, 72 (hours)	Hypothermia Normothermia	MCP-1, IL-6, IL-8, and IL-10 were significantly higher in the hypothermia group. Association of death or severely abnormal neurodevelopment at 12 months of age: Elevated IL-6 and MCP-1 within 9 hours after birth Low MIP-1a at 60 to 70 hours of age IL-6, IL-8, and MCP-1 showed a biphasic pattern in the hypothermia group, with early and delayed peaks. In hypothermia neonates with better outcomes, uniform downmodulation of IL-6, IL-8, and IL-10 from their peak levels at 24 hours to their nadir at 36 hours was observed. Subjects: Systemic hypothermia of 33.0°C for 48 hours in HIE (n=28),normothermia (n=22).
Chiesa et al., 2003, Italy [33]	IL-6	0, 24, 48 (hours)	Normal Asphyxia no HIE mild HIE moderate HIE severe HIE	IL-6 in HIE was 376-fold as high as the values in normal infants and 5.5-fold as high as those in the no HIE group. There was also a significant relationship between IL-6 and the degree of HIE and between IL-6 and neurodevelopmental outcome at 2 years of age. Regardless of outcome, in the asphyxiated infants, the IL-6 values were significantly lower at both 24 and 48 hours of life than at birth, with a significant decline from 24 to 48 hours of life. Subjects: normal, asphyxia no HIE (n=21), mild HIE (n=11), moderate HIE (n=9), severe HIE (n=9).
Róka et al., 2013, Hungary [75]	12 cytokines: IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, MCP-1, EGF, VEGF, IFN-γ, TNF-α	6, 12, 24 (hours)	TOBY trial hypothermia 33-34°C normothermia	IL-6 (at 6 hours) and IL-4 (at all time points) were significantly lower in asphyxiated neonates treated with hypothermia than in normothermic neonates. VEGF was higher in the hypothermia than normothermia group at 6 and 12 postnatal hours. IL-10 levels decreased significantly between 6 and 24 hours of age in both groups. However, no difference in IL-10 levels was observed between the study groups. The duration of hypothermia before 6 hours of age correlated with lower levels of IL-6, IFN-γ, and TNF-α measured at 6 hours of age and IL-10 levels at 12 hours of age. Subjects: TOBY trial HIE with hypothermia [33-34°C] (n=10) or normothermia (n=8).
Liu et al., 2010, China [76]	3 cytokines: IL-1β, IL-8, TNF-α	1, 3, 7 (days)	Control HIE mild moderate severe	IL-1β, IL-8, and TNF-α levels in umbilical and peripheral blood were significantly higher in HIE patients than control groups. IL-1β in umbilical blood exhibited the best positive correlation with HIE grades compared with IL-8 and TNF-α. Abnormal neurological outcomes at 6 and 12 months of age were best predicted by umbilical levels of IL-1β. Subjects: control (n=40), HIEmild (n=12), moderate (n=18), severe (n=22).

Abbreviations: VEGF: Vascular Endothelial Growth Factor; **G-CSF:** Granulocyte Colony-Stimulating Factor; **RANTES:** Regulated on Activation, Normal T cell Expressed and Secreted; **UCH-L1:** Ubiquitin Carboxyl-terminal Hydrolase L1; **GFAP:** Glial Fibrillary Acidic Protein; **MIP:** Macrophage Inflammatory Proteins; **MCP:** Monocyte Chemoattractant Protein; **IFN:** Interferon; **IP:** Interferon gamma-induced Protein 10; **EGF:** Epidermal Growth Factor; **HIE:** Hypoxic-Ischemic Encephalopathy

with previous *in vivo* and *in vitro* studies showing that IL-6 can potentially exert beneficial or detrimental effects depending on the pathologic context [51]. We showed that the serum levels of granulocyte colony-stimulating factor (G-CSF) aremarkedly increased and sustainedin severely asphyxiated neonates treated with head cooling, while vascular endothelial growth factor (VEGF) levels decreased and remained low [77]. VEGF is thought to act as a growth facilitator of endothelial cells and an enhancer of vascular permeability. The increased permeability caused by VEGF may lead to brain edema [78]. Thus, high levels of VEGF in severely asphyxiated neonates may exacerbate brain edema. In relation to brain function, G-CSF may mediate anti-apoptosis pathways in neural cells [79]. Thus, the high levels of G-CSF in severely asphyxiated neonates may prevent excessive neural death due to hypoxia [80].

PA therapeutics that control the overproduction of cytokines

Hypothermia: The latent phase is the best time prior to the secondary phase to initiate appropriate therapy for PA because latent phase therapiesprevent the evolution of secondary energy failure or the initiation of anti-apoptosis pathways [17]. Head or whole-body cooling is applicable as a treatment strategy for severe brain damage. In asphyxia, these cooling therapies may lead to several reactions, including the inhibition of apoptosis, reduction of cerebral metabolism, prevention of BBB disruption,

and reduction of brain edema [81]. As previously mentioned, perinatal asphyxia is associated with the overproduction of inflammatory and anti-inflammatory cytokines (Tables 1 and 2). Previous studies showed that hypothermia at least partially blocks several damaging reactions, such as the production of IL-10, TNF-α, and NO by activated microglia, the activation of NF-κB, the mRNA expression of the anti-inflammatory cytokine IL-10 and the proinflammatory cytokines INF-γ, TNF-α, IL-2, IL-1β, and MIP-2 in the brain, and the induction of proinflammatory cytokines in human peripheral blood mononuclear cells [82-85]. *In vitro*, hypothermia suppresses the proliferation of microglia, migration of leukocytes, and induction of proinflammatory cytokines. Only a few studies have reported changes in various cytokines in the serum of human neonates (Table 2). The serum levels of some cytokines are higher in neonates treated with hypothermia than in controls, such as IL-1, IL-6, IL-8, IL-10, and MCP-1. Thus, it is very important to determine the influence of hypothermia treatment on temporal alterations in inflammatory mediators.

Neuroprotective treatments other than hypothermia

Hypothermia is the only properlyvalidated treatmentfor PA at the present time. However, it is not always effective for all asphyxiated neonates, particular in severely asphyxiated neonates. Unfortunately, PA therapeutics other than hypothermia have not demonstrated sufficient evidence of their effectiveness. Here, we mainly describe the effects of PA therapeutics, other than hypothermia, on cytokine levels.

Anticonvulsants

In asphyxiated neonates, seizure is one of the most common clinical symptoms. Recently, amplitude electroencephalography has been used to monitor seizures. During reperfusion, the rapid increase in CBF causes clinical seizures. Seizures are typically treated with anticonvulsants, such as phenobarbital, midazolam, carbamazepine, and phenytoin;however, some anticonvulsants may influence the immune system.

Interestingly, seizures are associated with altered levels of immune mediators, such as cytokines. After a seizure, the serum levels of IL-1RA can increase in humans [86]. In the CSF, IL-6 levels increased during seizures [87]. Furthermore, it was shown recently that some anticonvulsant drugs (ACDs) may also influence the immune system [88]. The most commonly used ACDs during the neonatal period are phenobarbital, midazolam, and phenytoin,which influence cytokine production. During PA, phenobarbital is the most commonly used anticonvulsant for the treatment of seizures during PA. High levels of phenobarbital suppress the production of IL-2 from phytohemagglutin in-stimulated mononuclear leukocytes *in vitro* [89]. Midazolam inhibits the production of cytokines, such as IL-6, in a time-dependent manner [90,91]. Phenytoin induces IL-1 activity and increasesthe production of IL-1 [92]. IL-1 and IL-6 are known as proinflammatory cytokines during the acute phase. During seizures, IL-1 inhibits glutamate reuptake by astrocytes and increases glutamate release by these cells [88]. On the other hand, other ACDs may also induce the expression of some cytokines. Carbamazepine significantly increases the levels of IL-1α, IL-1β, IL-6, and MCP-1 [94]. In addition, valproic acid also increases the levels of some cytokines [94]. Thus, several anticonvulsants affect the cytokine profile of asphyxiated neonates; therefore, anticonvulsants should be used carefully in such patients. However, most of these studies were *in vitro* experiments. In the future, *in vivo* studies are required.

Anti-inflammatory or antioxidant compounds

The neonatal brain is highly vulnerable to oxidative stress due to a high concentration of unsaturated fatty acids, high rate of oxygen consumption, low concentration of antioxidants, and low availability of redox-active iron [1]. Therefore, it is important to reduce oxidative stress, including ROS bursts, and increase HIF-1α andthe accumulation of H_2O_2 in the immature brain.

Edaravone, a hydroxyl radical scavenger, decreases inflammatory reactions. In a rat model of traumatic brain injury, edaravone treatment led to a significant decrease of proinflammatory cytokines (e.g., TNF-α, IL-6, and IL-1β) and a decrease of an anti-inflammatory cytokine (IL-10) [95]. In an animal sepsis model, edaravone reduced proinflammatory cytokines and prevented the increase ofBBB permeability [96]. The administration of edaravone to neonatal rats reduced NMDA-mediated cytochrome c release and apoptosis [97]. Moreover, in patients with acute brain infarction, edaravone suppressed the serum concentration of MMP-9, which inducesthedisruption of the BBB [98]. Thus, edaravone may be effective treatment to reduce cytokine levels in asphyxiated neonates.

The effectiveness of various antioxidant reagents as PA therapeutics has been assessed. Superoxide dismutase and catalase are antioxidant enzymes that can easily pass through the BBB and inhibit the action of oxygen free radicals. However, in a neonatal animal study, the neuroprotective action of these enzymes was only shown when they were administered prior to the hypoxic-ischemic insult [99]. Xanthine oxidase inhibitors, such as allopurinol and oxypurinol, reduced hypoxic-ischemic brain damagein an immature rat study [96]. Allopurinol decreased the production of TNF-α, downregulated the expression of intercellular adhesion molecule-1, and blocked the induction of MCP-1 and production of IL-6 [100,101]. In a recent clinical study, the administration of allopurinol to asphyxiated neonates reduced the blood concentration of oxygen free radicals [102].

High levels of free iron promote the formation of ROS, such as via the Fenton reaction. Therefore, in animal models of hypoxia-ischemia, iron-chelating agents prevent the formation of free radicals from iron, reduce the severity of brain injury, and improve cerebral metabolism when given during reperfusion [22, 103]. Indeed, hypoxia may induce apoptosis in oligodendrocytes by iron accumulation in neonatal rat periventricular white matter through the production of TNF-α and IL-1β and reactive oxygen/nitrogen species [104]. Iron-chelating agents may decrease iron accumulation and reduce these reactions.

Melatonin is of current interest as an antioxidanthormone. It is known to be highly protective against the oxidation of membrane lipids, cytosolic proteins, and nuclear and mitochondrial DNA [105]. Melatonin exertsanti-inflammatory effects principally though antioxidation. In neonates with respiratory distress syndrome, the administration of melatonin significantly decreased the serum concentrations of proinflammatory

cytokines, including IL-6, IL-8, and TNF-α [106,107]. Furthermore, combination therapy with melatonin and hypothermia in a newborn piglet model with perinatal hypoxia was shown to be safe and provided significant neuroprotection compared with hypothermia alone [105]. Thus, melatonin is very effective in neonatal disease. In addition, severe adverse effects of melatonin have not been reported in humans. In the future, melatonin may be used as an antioxidant.

EPO

EPO, a 30.4-kDa glycoprotein, is a cytokine with an emerging role in neuroprotection. The administration of EPO decreased the size of myocardial infarction in adult humans [108]. EPO may stabilize the BBB, reduce the invasion of inflammatory cells, and result in controlled inflammation [109]. NF-κB, one of the most important regulators of proinflammatory gene expression, leads to the synthesis of cytokines, including IL-1β, IL-6, IL-8, and TNF-α [110]. EPO play a crucial role in neuroprotection via NF-κB [109]. The EPO gene is targeted by HIF-1 and has beneficial effects on hypoxic-ischemic brain injury [111]. EPO receptors are expressed in glial cells, neurons, and endothelial cells [112]. Hypoxia-ischemia has been recognized as an important factor in the induction of EPO expression. EPO is a potent neuroprotective cytokine in asphyxiated neonates [113] and appears to be protective in neurons and endothelial cells and to enhance neurogenesis and angiogenesis [114,115]. The administration of EPO immediately after neonatal hypoxic-ischemic injury or stroke improved long-term memory deficits and brain injury in rats [116]. The widespread use of EPO in premature newborns may potentially be beneficial in treating perinatal brain damage [117]. Moreover, in asphyxiated neonates, the repeated administration of high-dose EPO improved neonatal outcomes [118]. Thus, EPO is associated with neuroprotection via cytokines.

Conflict of Interest

We have no financial support and no conflict of interest directly relevant to the content of this article.

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