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Cytokines (IL-1 β, IL-6, IL-18, TNF-α) in Blood and Cerebrospinal Fluid in Neonatal Hypoxia/Ischemia)

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Perinatal brain injury is an important clinical and socioeconomic entity. It is a syndrome of impaired brain function in the early days of life, and it is a consequence of inadequate brain oxygenation before, during or shortly after birth, with high mortality rates and early and late morbidity rates [1,2].

The most common consequence of this devastating pathophysiologic event is neonatal encephalopathy (NE), or what is commonly recognized in clinical practice as hypoxic-ischemic encephalopathy (HIE). It is estimated that about 30% of children with NE in well developed countries and 60% of children with NE in less developed countries have some evidence of intrapartum hypoxia-ischemia. A still high percentage (20-50%) of asphyxiated children with HIE die in the newborn period, with the other quarter developing more severe neurological impairment [3].

The inflammatory response involving multiple cytokines and chemokines has been confirmed in a series of research studies as in an animal-controlled so in a human model of hypoxic-ischemic brain injury [4-7]. Yet, despite the discovery of the phenomenon of these biomarkers, their individual occurrence, role, and consequences are still the subject of research. The great advancement in biomarker science has significantly changed the practical approach in the clinical care of children with PBI. However, daily clinical experience suggests that the causes and consequences of hypoxia-ischemia (H-I) in perinatal period are sometimes recognizable and sometimes unrecognizable pathological events. Human HIE is complex, context-specific, and delicate syndrome in each case [8].

New specific non-brain markers of cardiac health and global asphyxia have been discovered and now play an important role in interpreting stroke severity. The emergence and significance of new biomarkers, as well as the understanding of how so-called “old biomarkers” can play a double role, all of this, makes “Cytokine Story” just getting started [8].

The cytokine concept (back more than two decades) was a revolutionary theoretical explanation of the complex mechanisms in hypoxic-ischemic pathophysiology. With the rise of valid research, it has been found that the results of the cytokine role are often inconsistent and divergent and sometimes contradictory. Extrapolation of results from an animal model to human biology and pathology is not always a plausible pattern. It seems that even today, more than twenty years after the first results on cytokines, the role of individual cytokines is insufficiently defined and the cytokine concept remains open and intriguing. Difficulties in identifying and assessing the role of individual cytokines exist due to their pleiotropism and redundancy. Identifying and assessing their role in the body of a newborn with neonatal encephalopathy is an extremely delicate and serious task. During fetal and neonatal maturation, both the immune system’s properties and the anti-inflammatory response change [9,10].

The short half-life makes cytokines sometimes undetectable in clinical settings, although it is assumed that they have already “played their part” and “shaken the cytokine network”. In perinatal hypoxia/ischemia, the child responds with the “whole body” because it is a largely global hypoxia injury. Infant’s capacity to respond to hypoxia injury is limited by stroke strength, length, maturity of the infant’s immune system and genetic predisposition [11]. The underlying pathophysiological process in hypoxia/ischemia is the redistribution of blood

flow in favor of vital organs. It means that hemodynamic changes in response to changes in the partial pressures of pO2 and pCO2 in the body after ischemic lesions are basic and primary pathological defense response [12]. This system of defense and self-regulation also includes the blood-brain barrier (BBB), which has its own capacity [13,14]. Cerebral tissue injury is a consequence of exhausted adaptive circulatory mechanisms. The occurrence of biomarkers of this injury is a guide to understanding the severity of the injury as well as a possible guide to understanding the reversibility or irreversibility of the stroke. The occurrence of vasoactive amines (prostaglandins) (PGI2 and PGE2) in CSF and serum in a group of children with PBI would indicate a rapid adaptation of the circulatory system to H/I and a possible self-defense mechanism in the brain sparring process [15].

The main question of our study on cytokines in perinatal hypoxia was: Do cytokines appear at a noticeable concentration in the body of a hypoxic newborn in the acute phase of the disease (the first four days of life)? Second: Are cytokines appearing equally in both tested and comprehensible compartments (blood/CSF) or actually, where the primary sites of their production are in hypoxic-ischemic injury? Third: Is there a correlation between cytokine concentrations and the degree of hypoxic-ischemic encephalopathy (HIE) as an acute pathological condition (early brain injury)? Fourth: Is there a significant association between cytokine concentrations and pathological neurological outcome at 12 months of age (late brain injury) [16]? Neonates who fulfilled three of proposed criteria for perinatal hypoxia/asphyxia were included in prospective study (2006-2010) [17]. CSF and blood samples were taken from the participant at 3-96 hours of age and stored at -70°C at the time of the analyses. Cytokines were determined by the enzyme linked immunosorbent assay (ELISA) using the original kits of Quantikine R&D, USA, (IL-6, IL-1 beta, TNF-alpha), and Diaclone Research (France) for IL-18. Neonates with PNH were evaluated by Sarnat/Sarnat method as mild (I grade), moderate (II grade) and severe HIE (III grade) [18]. Neurological status was estimated at 12 months of age by the Amiel-Tison method (1. normal outcome; 2. mild motoric damage; 3. abnormal neurologic outcome (cerebral palsy or death) [19].

Our main research results have shown in the first place that the values of the selected cytokines differ depending on the source.

1. In the blood (serum) of children with PNH, unlike the control group, IL-6 and TNF alpha (p=0.04; p=0.02) were significantly elevated;

2. In CSF, IL-1 beta and IL-18 were undetectable (both in patients and in the control group). None of the CSFs showed significantly higher concentrations in children with PNH than the control group;

3. No significant correlation between cytokine concentrations and grade HIE was observed, although slightly higher IL-6 values were observed in children with IIIrd HIE but without statistical significance (p=0.07)

4. A significant correlation was found between elevated cytokine values and poor neurological outcome: serum IL-6 and TNF alpha with poor outcome, serum and CSF IL-6 as well as serum IL-18 with moderately poor outcome.

Cytokines, their source and role, spectrum of action, cell lines stimulated by their activity, short and long term effects - all of this are still the subject of research in human biology and pathology [20]. Newborn hypoxia is a very complex condition with numerous interfering influences, and prevention is almost impossible in the case of unpredictability and dramatic nature. This is especially true today in less developed countries where the incidence of asphyxia/hypoxia in infants is still high. There is still a visible discrepancy between the high degree of understanding of the "molecular mechanism of injury" or the so-called "Theoretical knowledge" and its extrapolation into practical pediatric everyday life [21].

By comparing cytokine values in patients with PNH and controls, only for serum IL-6 and TNF- alpha values we did obtain significant differences suggesting increased production of these cytokines in the first 96 hours after HI stroke. For the CSF values of any one of the investigated cytokines, we did not obtain any significant differences between the patients and the control group. A possible explanation for this finding may be that most of our patients were from the mild HIE group.

IL-6 is a cytokine that appears rapidly after HI stroke (15 minutes) and returns to normal values 20 hours after stroke [22]. In the newborn, hypoxic-ischemic stroke affects mainly the whole organism, and more severe systemic increase in cytokines causes multiorgan post-asphyxiated failure [23,24]. More significant reflection in serum than in the liquor of our patients (an average of 37.5 hours after injury) could be a reflection of this dynamic. Increased concentration of IL-6 is explained by its local production in injured tissue [25-28]. Several other studies also find elevated IL-6 levels in the serum of children with HIE [28-31]. Boskabadi et al. reviews the results of 13 selected searched papers and concluded that "IL-6 and IL-1beta values of serum samples are useful predictors for the outcomes of perinatal asphyxia and its intensity" [32]. There are only few studies on the neonatal hypoxia model that confirm higher concentrations of IL-6 in CSF [32]. This is understandable because of the delicacy of sampling in the vulnerable period. Ellis at al. in their study compare CSF and serum cytokine values

of premature newborns with cerebral white matter injury (WMI) and healthy controls. They found significantly elevated levels of IL-6, IL-10 and TNF alfa in the CSF of premature infants with WMI. But, no significant correlations were seen between CSF and plasma values for any paired cytokines for the whole investigated cohort [33].

For a better understanding of the origins of interleukins in pathological conditions, and also in normal biology, we need more evidence from both body compartments at the same time.

The IL-1 beta in our study was determined in the CSF of 11 children and in the serum of 8 children with PNH. Values were undetectable in CSF and barely detectable in serum (0.503 pg/mL on average). Savman et al. also found no significant values of IL-1 beta in the CSF of children with HIE [26], while Aly et al. found significantly higher values of this cytokine in the CSF of children with HIE [34]. Samples were taken within the first 24 hours of life. Elevated serum levels of children with perinatal hypoxia are also found by other researchers [25,28] but without measurement in CSF. The perceived inconsistency of methodologies is a major problem in assessing the role of cytokines. This led to the launch of the BAMRI (Birth Asphyxia-Magnetic Resonance Injury) project [28]. From the 1990s, studies have confirmed that IL-1beta and TNF-alpha have potent proinflammatory activity and the potential to modulate cellular growth [35]. The biological effect of this cytokine (IL-1 beta) is that it can affect the progression of injury, but also the regulation of ischemic injury healing. In the injured brain, mRNA expression for IL-1 beta peaks 4 hours after stroke and returns to normal 24 hours after HI stroke [35]. IL-1 beta and TNF alpha can stimulate microglia to synthesize other cytokines that attenuate injury. IL-1 beta may also have a direct effect on neural transmission. The cumulative impact of these pleiotropic cytokine effects depends on a wide variety of interacting factors in the early-stage of recovery [36].

In our study, TNF alpha was significantly higher in the serum of children with PNH (p=0.023). Silveira et al. finds also higher serum TNF values [27]. Oygur et al. find elevated values in the CSF of children with PNH [37]. Aly et al. find TNF both in serum and CSF of children with PNH [34]. The role of TNF-alpha in hypoxic-ischemic injury also has been demonstrated in animal [38-40] and in human models [26,27,37]. TNF-α and IL-1beta are the two major cytokines found within the parenchymal microglia at the lesion site. IL-1beta is important in enhancing the production of neurotropic factors by astrocytes, and TNF acts on microglia in an autocrine and paracrine manner. TNF alpha-induced microglial activity, can be detrimental to the brain by promoting oligodendrocyte apoptosis and preventing remyelination [38,39]. TNF can lead to early neuronal injury but later to better recovery. It depends on the type of TNF receptor stimuli (TNFr1 and TNFr2) [40]. During the perinatal period, high plasma adenosine levels can inhibit TNF alpha and modulate the innate and acquired response [41]. Sampling time is an important factor in analyzing this dual role of TNF-α (biphasic behavior!). All of this make the role of TNF still intriguing [26,37].

IL 18 is an important anti-inflammatory and immune-stimulating cytokine, rarely examined in neonatal pathology. In our study, increased concentrations of this cytokine were observed in the serum of newborns with PNH (patients: median 111.13 vs. 10.7pg/mL-control), but the difference was not statistically significant (12 children - small sample !?). In CSF of patients and also in controls was immeasurable. In their study, Minagawa et al. found elevated concentrations of this cytokine in the umbilical blood of infants who later developed white matter damage and cerebral palsy (CP) [42]. Elevated IL-18 levels are also found by Schmitz et al. in the CSF of premature infants who developed posthemorrhagic hydrocephalus and periventricular leukomalacia [43].

In our analysis, the concept of tissue sections and barriers as well as the pathology that impairs their homeostasis is imposed. This diversity requires further studies of paired samples (CSF/serum) in the future with consideration of the pathophysiological context.

Some authors examining blood-brain barier (BBB) and blood-cerebrospinal fluid barier (BCSFB)) come to the important realization that their function or dysfunction is likely to be specifically involved in the cytokine story [44,45]. They conclude that there are differences in the transport of individual cytokines through BBB and that this transport is dependent on both the type of cytokine and the region in the brain and the pathophysiological conditions [45,46]. The cardiocirculatory system is involved in these pathophysiological conditions, whether compensated or decompensated, which can significantly alter the image of the injury [47].

In general, we did not observe significantly higher concentrations of cytokines in relation to different stages of hypoxia, or their role as biomarkers of early hypoxic injury. But, our analysis confirmed that significantly higher serum levels of some of them (IL-6, IL-18, TNF alpha) better corresponded to an abnormal outcome at 12 months of age of the CSF values.

After perinatal encephalopathy, the injury process can quietly persist for months and years, so it is assumed that there is a third mechanism of injury (epigenetic?). The cytokine hypersensitivity system reflects this vulnerability. All of this opens up new points of view, and makes that view on “the longer therapeutic window” could be possible [48].

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