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Identification of a panel of cytokines in neonates with hypoxic ischemic encephalopathy treated with hypothermia

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ABSTRACT

Purpose: Inflammation is a crucial but understudied mechanism of neuronal injury after hypoxia-ischemia. The aim was to identify a panel of cytokines involved in brain injury in neonates with hypoxic ischemic encephalopathy (HIE).

Methods: Ten newborns with HIE undergoing to therapeutic hypothermia (TH, HIE Group) and 8 healthy newborns (CTRL Group) were enrolled. For the HIE group, 5 samples were collected: between 0 and 6 h of life (time 1), 12 h (time 2), 24 h (time 3), 48 h (time 4) and 96 h of life (time 5). For the CTRL group, one sample was collected. A panel of 48 inflammatory cytokines was determined in all samples. Data were analyzed using multivariate statistical analysis (Principal component analysis, PCA)

Results: 17 cytokines, among 48 analyzed, were found to be significantly different, initially, between the CTRL and HIE groups: 12 with reported pro-inflammatory effects and 5 with reported anti-inflammatory effects. In the HIE group cytokines showed a decreasing trend during the TH and at the end of treatment comparable to the CTRL group. IL-18 did demonstrate a slight increase at time 3 during HT but decreased steadily at sampling times, 4 and 5.

Conclusions: Our data demonstrates that many pathways of the inflammatory cascade are activated following hypoxic-ischemic injury. This information will increase our understanding of changes in cytokines over time in neonates with HIE undergoing TH.

1. Introduction

Hypoxic-ischemic encephalopathy (HIE) is a significant cause of morbidity and mortality in neonates. The incidence of HIE ranges from 1 to 8 per 1000 live births in developed countries and is as high as 26 per 1000 live births in underdeveloped countries [1,2]. Between 10 and 60% of babies who exhibit HIE will die during the newborn period [3,4]. Of the surviving neonates with HIE, up to 25% have permanent neurodevelopmental handicaps in the form of cerebral palsy, mental retardation, learning disabilities, or epilepsy [5–7]. Despite the use of therapeutic hypothermia (TH), the devastating neuro-behavioral outcomes from HIE continue to be a major individual, familial, and social issue. No one knows why hypothermia is effective in alleviating long-term neurodevelopmental impairments in some, but not all, treated neonates. As a consequence, neonatal brain injury represents one of the most urgent medical as well as socio-economic challenges.

Inflammation is a crucial but understudied mechanism of neuronal injury after hypoxia-ischemia. Inflammation is characterized by activation of microglia, migration of peripheral macrophages, release of cytotoxic and proinflammatory cytokines and chemokines, and phagocytosis of injured and uninjured neurons [8–11].

It has been suggested that blocking the inflammatory reaction promotes neuroprotection and could be used in the clinical treatment of hypoxic-ischemic brain injury [12–14]. Conversely, inflammatory signals play an equally important role in the restoration and reparative processes after neuronal injury [15]. Hypothermia blocks the pro-inflammatory pathway, reduces production of reactive oxygen species, and reduces metabolic rate [16–18]. However, present knowledge about the effect of TH on the inflammatory response triggered by hypoxic-ischemic insult in babies with HIE is limited. The aim of this

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Table 1
Characteristics of the 10 patients with HIE enrolled (GA = Gestational Age, BW = Birth Weight, pH = Initial pH, BD = Initial Base Deficit, SS = Initial Sarnat Score, ECS = Emergency C-section).

#	GA (wks)	BW (Kg)	Apgar scores (1, 5, 10 min)	pH	BD	Inborn	SS	Seizures	ECS	Intubation in DR
1	36	2.377	0, 5, 6	6.76	−29.3	no	II	no	yes	yes
2	38	2520	3, 6, 8	6.87	−17	no	II	yes	yes	yes
3	38	3.210	2, 5, 5	7.19	−14	yes	II	yes	no	no
4	35	2.790	1, 6, 7	6.91	−14.1	yes	II	no	yes	no
5	35	2.855	1, 2, 5	6.95	−20	no	III	yes	yes	yes
6	38	3.660	3, 5, 6	6.70	−26	no	I	no	yes	yes
7	39	3.660	0, 1, 3	6.90	−26	no	II	yes	yes	yes
8	39	3.271	1, 2, 5	6.90	−24	no	II	yes	yes	yes
9	40	3.430	2, 3, 5	6.99	−17	no	II	yes	no	yes
10	41	3.630	1, 3, 7	7.25	−14	yes	II	yes	no	no

study is to identify a panel of cytokines in neonates with HIE and to examine the changes in the cytokines over time during TH. We hypothesized that pro-inflammatory cytokines would be elevated in neonates with HIE when compared with control neonates.

2. Materials and methods

2.1. Study population

The University of Florida Institutional Review Board approved the study. Informed consent was obtained from parents of neonates who were eligible for TH and for entry into the Florida Neonatal Neurologic Network registry and biorepository. This study was a retrospective analysis of serum inflammatory cytokines from randomly selected specimens in the biorepository and registry information. For this study, 10 neonates with HIE and qualified for TH were enrolled. Eight healthy neonates, who did not have any perinatal events associated with HIE, were enrolled as a control population.

Entry criteria for hypothermia included a gestational age ≥35 weeks or greater, a birth weight ≥1.8 kg or greater, and less than or equal to 6 h of age. Enrolled neonates had evidence of encephalopathy defined by seizures or abnormalities on a modified Sarnat examination [19]. Evidence of hypoxic–ischemic injury was defined by (1) a pH of 7.0 or less and/or a base deficit of greater than 16, or (2) a pH between 7.01 and 7.15 and/or a base deficit between 10 and 15.9, or (3) no blood gas available and an acute perinatal event (cord prolapsed, heart rate decelerations, uterine rupture) [19].

2.2. Blood sample collection and processing

From each patient in the HIE group, 5 samples at 5 different times was collected as follows: between 0 and 6 h of life (time 1), 12 h (time 2), 24 h (time 3), 48 h (time 4) and 72 h of life (time 5). These timepoints respectively correspond to before, during, and after the TH. In the control group (CTRL) only one sample was collected from each patient's umbilical cord at the time of birth. Samples were collected from either the umbilical artery or vein.

Blood (1 ml) was collected and centrifuged at 1200 RCF (g) at room temperature for 15 min. Then, serum was transferred into 2 ml cryovials with red cap inserts (USA Scientific, Ocala, Florida, USA) and stored at −80 °C until processing.

2.3. Cytokine analysis

Cytokines were quantified with ultrasensitive ELISA technology according to manufacturer instructions (Kit Bio-Plex Pro™ Human Cytokine Standards Group I 21–Plex, Kit Bio-Plex Pro™ Human Cytokine Standards Group II 27–Plex, Bio-Rad, Hercules, CA, USA). The following were analyzed: Cytokines IL-1α, IL-2Rα, IL-3, IL-12p40, IL-16, IL-18, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17; Chemokines CTACK, GRO-α, LIF, monocyte chemotactic protein-3 (MCP-3), macrophage migration inhibitory factor (MIF), MIG, TRAIL, IP-10, MCP-1, monocyte inflammatory protein 1a (MIP-1α), MIP-1β, IFN-α2, IFN-γ; Growth factors hepatocyte growth factor (HGF), M-CSF, GM-CSF, β-NGF, SCF, SCGF-β, SDF-1α, TNF- β, Eotaxin, Basic FGF, G-CSF, M-CSF, PDGF-BB, RANTES, TNF-α, and vascular endothelial growth factor (VEGF).

2.4. Statistical analysis

All statistical analyses were performed using principal component analysis (PCA). In this case, data have been autoscaled. The mean was subtracted and each variable was divided by its standard deviation. A Welch Two Sample *t*-test was performed to assess the statistical significance of the difference of any selected cytokine. Data were analyzed using R (R Core Team, 2017 with the following libraries: ChemometricsWithR, ggplot2, dplyr, RColorBrewer, broom, tidyr. (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/).

3. Results

Clinical characteristics of the patients are shown in Table 1.

Out of 48 cytokines, 8 were discarded from the analysis because more than half of the samples were below or above the limits of detection. The discarded cytokines were RANTES (over upper limit of detection), IL-2, IL-13, MCP-3, IL-12p40, TNF-β, β-NGF, and IL-1α (all below lower limit of detection). Time 2 was also not included in the statistical analysis due to the missing samples of some patients.

3.1. PCA of control and HIE subjects

The data, composed of 43 samples with 48 cytokines analyzed on each sample, were analyzed by PCA. A clustering of the samples emerged. The controls (pink points) are grouped on the right top corner of the plot (Fig. 1). The samples from time 5 (96 h of age, 24 h after hypothermia) are also grouped on the right side of the same plot. Samples at times 3 and 4 (24 and 48 h of life, during hypothermia) are located in the center of the plot. Samples at time 1 (0–6 h of life, pre-hypothermia) are different from samples at time 3–4–5 and did not form a cluster.

At different times of treatment, the HIE group was identified in the score plot with a color intensity that differed from time 1 to time 5. Notably, the various colored time point markers moved from left to right. HIE samples at the end of the TH were positioned in the right portion of the graph, similar to the CTRL samples. This pattern indicates that the cytokine concentrations in HIE samples at time point 5 progressed in a manner similar to the control concentrations. The change in the cytokine profile of the treated patients appeared clear; the score plots show the differences in the cytokine profile of treated patients at the four-time points (Fig. 1).

Among the 48 cytokines analyzed, 17 cytokines at time 1 were