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Brain injury with systemic inflammation in newborns with congenital heart disease undergoing heart surgery

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Abstract. The potential role of systemic inflammation on brain injury in newborns with congenital heart disease (CHD) was assessed by measuring levels of central nervous system (CNS)-derived proteins in serum prior to and following cardiac surgery. A total of 23 newborns (gestational age, 39±1 weeks) with a diagnosis of CHD that required cardiac surgery with cardiopulmonary bypass (CPB) were enrolled in the current study. Serum samples were collected immediately prior to surgery and 2, 24 and 48 h following CPB, and serum levels of phosphorylated neurofilament-heavy subunit (pNF-H), neuron-specific enolase (NSE) and S100B were analyzed. Systemic inflammation was assessed by measuring serum concentrations of complement C5a and complement sC5b9, and the following cytokines: Interleukin (IL)-1β, IL-6, IL-8, IL-10, IL12p70, interferon γ and tumor necrosis factor (TNF)-α. Analysis of cord blood from normal term deliveries (n=26) provided surrogate normative values for newborns. pNF-H and S100B were 2.4- to 2.8-fold higher (P<0.0001) in patient sera than in cord blood prior to surgery and remained elevated following CPB. Pre-surgical serum pNF-H and S100B levels directly correlated with interleukin (IL)-12p70 (ρ=0.442, P<0.05). pNF-H was inversely correlated with arterial pO_2 prior to surgery (ρ=-0.493, P=0.01) and directly correlated with arterial pCO_2 post-CPB (ρ=0.426, P<0.05), suggesting that tissue hypoxia and inflammation contribute to blood brain barrier (BBB) dysfunction and neuronal injury. Serum IL12p70, IL-6, IL-8, IL-10 and TNF-α levels were significantly higher in patients than in normal cord blood and levels of these cytokines increased following CPB (P<0.001). Activation of complement was observed in all patients prior to surgery, and serum C5a and sC5b9 remained elevated up to 48 h post-surgery. Furthermore, they were correlated (P<0.05) with low arterial pO_2, high pCO_2 and elevated arterial pressure in the postoperative period. Length of mechanical ventilation was associated directly with post-surgery serum IL-12p70 and IL-8 concentrations (P<0.05). Elevated serum concentrations of pNF-H and S100B in neonates with CHD suggest BBB dysfunction and CNS injury, with concurrent hypoxemia and an activated inflammatory response potentiating this effect.

Introduction

Approximately 8 in 1,000 live births are diagnosed with a congenital heart defect (1) and many require surgical intervention to ensure survival beyond the neonatal period. Recent advances in medical management, transcatheter intervention, surgical procedures and cardiopulmonary bypass, have significantly increased the survival rate of such children. Up to 50% of children with congenital heart disease (CHD) experience neurodevelopmental and behavioral problems including seizures, cognitive impairment, delays in speech, language, visual-motor and visual-spatial skills, attention deficit/hyperactivity disorder and learning disabilities (2-5).

The etiology of the neurobehavioral problems in children with CHD is complex, with contributions of innate factors including genotype that cannot be modified and acquired factors that potentially may be (6). These acquired factors are secondary to chronic and acute hypoxia, hypoperfusion,

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hematocrit, reperfusion injury, and surgery with cardio-pulmonary bypass and cross-clamp, and have a cumulative effect (7,8). The developing fetus with congenital heart defects may be exposed to hypoxia and abnormal cerebral perfusion *in utero* and these insults on the brain may continue during the neonatal period as a result of the failing heart (9,10). The mechanisms of brain injury are poorly understood but may involve multiple causative factors including maternal infection, inflammation, hypoxia and hypoperfusion, as observed in the development of periventricular leukomalacia (PVL), characterized by cerebral white matter damage (11-13). PVL has been documented by brain magnetic resonance imaging (MRI) in 16% of children with congenital heart disease and this incidence increases to 50% in children following cardiac surgery with cardiopulmonary bypass (CPB) (14). The duration of CPB, complexity of the anatomical defect and surgical repair, and the occurrence of cyanosis contribute to the postoperative systemic inflammatory response. This involves activated complements and lymphocytes, monocytes/macrophages, endothelial cells and myocytes expressing cytokines, such as tumor necrosis factor (TNF)-α and interleukins (ILs) (15-20). Postoperative systemic inflammatory response syndrome is a complication occurring in pediatric patients, particularly neonates undergoing corrective or palliative heart surgery and remains a challenge during the postoperative management of these patients (21-25). Inflammation during early brain development has been implicated in white matter damage and has been associated with cerebral palsy, autism and neuropsychological disorders (26-29). In experimental models, TNF-α induced myelin degeneration and oligodendrocyte apoptosis, and transgenic animals overexpressing TNF-α in the CNS exhibited demyelination and vacuolization (30,31). White matter injury is a complication of neonatal cardiac surgery and may occur in response to systemic inflammation; however, this association remains unclear and may be of relevance regarding patients undergoing highly complex cardiac surgeries (32,33).

The blood-brain and blood-cerebrospinal fluid (CSF) barriers restrict the passage of proteins into and out of the brain. Research on the blood-brain barrier (BBB) in experimental models has demonstrated that prolonged systemic inflammation in the early post-natal period leads to a transient increase in the permeability of blood vessels in the brain to serum proteins (34). Similar to that observed with ischemic or traumatic brain injury, it has been postulated that cardiopulmonary bypass with concomitant inflammation and activation of complements can lead to loss of integrity of the BBB and blood-CSF barrier (35-38).

Previous studies have determined that there is an association between the severity of the inflammatory response to heart surgery and an increased need for medical intervention or length of stay at hospitals (20,21,39-43). The current study hypothesized that newborns with CHD and in whom circulating pro-inflammatory mediators and complements were elevated, would have measureable concentrations of CNS-derived proteins, indicative of a compromised BBB and CNS injury. Serum markers of brain injury (43) include the high molecular weight (200 kDa) neurofilament protein (NF-H) that is abundant in neurons and concentrated in axons. The highly phosphorylated form of NF (pNF-H) is resistant to proteolysis and when released from damaged axons remains largely intact; thus, its presence in the blood is indicative of neuronal damage (44,45). Other biomarkers measured included a brain-specific isoform of enolase known as neuron specific enolase (NSE) (46) and S100, a 20 kDa protein belonging to the S100/calmodulin/troponin C superfamily of calcium-binding proteins that forms heterodimers (S100A1B) and homodimers (S100BB) in CNS glial cells and peripheral Schwann cells (47,48).

Patients and methods

Patient demographics and diagnostic classifications. The current prospective study conducted at the Cohen Children's Medical Center of New York at Northwell Health (Manhasset, NY, USA) was approved by the Northwell Health Institutional Review Board and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from parents on admission of patients to the hospital. A total of 23 patients with congenital heart disease requiring surgical repair with cardiopulmonary bypass were enrolled in the present study between January 2009 and August 2010. Full patient demographics are listed in Table I. The lesions consisted of hypoplastic left heart syndrome (n=8), transposition of the great arteries (n=5), co-arctation of the aorta (n=3), tricuspid atresia/pulmonary atresia (n=1), truncus arteriosus communis (n=1), aortopulmonary window (n=1), endocardial cushion defect (n=1), ventricular septal defect (n=1), anomalous right pulmonary artery (n=1) and total anomalous pulmonary venous connection (n=1). Two patients harbored known chromosomal abnormalities, including one with DiGeorge syndrome (22q11 deletion). Criteria for exclusion were body weight <1,600 grams, patent ductus arteriosus ligations, heart surgery without CPB, newborns of mothers with autoimmune disease and suspected congenital or postnatal infections.

Surgery, anesthesia and postoperative care. The conduction of surgical procedures including cardiopulmonary bypass and anesthesia followed our institutional standard practices. Sixteen of the 23 patients underwent CPB with deep hypothermic circulatory arrest with an average circulatory arrest time of 20±16 min. Eleven of these 16 patients were treated by ice cooling of the head. Twenty-one patients required blood pressure medication intra-operatively and all patients received methylprednisolone. Postoperative patient management in the pediatric intensive care unit (PICU) followed the standard institutional protocol. Decisions regarding the medical management of each patient were based on clinical assessment made by attending PICU physicians who were unaware of the study protocol and analysis.

Clinical data acquisition and sample collection. Patient clinical data were recorded, including pre-operative variables such as birth weight, gestational age, APGAR score at birth, head circumference, abnormal chromosomal findings, abnormal head ultrasound findings, need for respiratory support including intubation and fraction of inspired oxygen (FiO₂) requirement, pre-operative systemic saturation, arterial pO_2 and pCO_2, lowest pre-operative hematocrit, degree of acidosis, lactate levels, mean arterial pressures, urine output, presence of infection, presence of seizures, administration

of vaccines or Rhogam, need for blood transfusion, need of prostin infusion or inotropes, or balloon atrial septostomy. Intra-operative data were recorded, including weight and age at perfusion, length of cardiopulmonary bypass and aortic cross-clamp time, administration of intra-op steroids and degree of cooling. Postoperative variables were also measured and included presence of arrhythmia, medication including inotropes and diuretics, mean arterial blood pressure (MAP), degree of acidosis, blood lactate levels, hematocrit, pO_2 and pCO_2, FiO_2 requirement, duration of mechanical ventilation, duration of PICU care and hospitalization, systemic oxygen saturation at discharge and any neurological findings such as seizures, neurodevelopmental risk evaluation score or brain imaging results. Brain imaging protocols included brain ultrasound, computed tomography, electroencephalogram (EEG) or MRI during routine postoperative care of the patient.

Blood samples from patients were drawn from central intravenous catheters preoperatively and 2, 24 and 48 h following CPB. Blood was allowed to clot at room temperature for 4 h and subsequently centrifuged at 1,000 x g for 15 min at room temperature. Serum was stored at -70°C until analysis. Serum samples were also obtained from cord blood collected from 26 healthy term births that were donated to the Tissue Donation Program of Northwell Health but which were not used for stem cell collection due to insufficient cell counts. Data from these samples were used as normative newborn values.

Analyses of serum samples. Commercially available ELISA or EIA kits were used to measure serum concentrations of pNF-H (cat. no. RD191138300R; BioVendor LLC, Asheville, NC, USA), human NSE (cat. no. 0050; Alpha Diagnostic International, San Antonio, TX, USA), S-100B (cat. no. 708-85; Fujirebio Diagnostics, Inc., Göteborg, Sweden), C5a and sC5b-9 (cat. no. A021 & A020; Quidel Corp., San Diego, CA, USA). The Meso Scale Discovery multiplex assay kit (cat. no. K15008C-2; MSD, Rockville, MD, USA) was used for simultaneous measurement of cytokines IL-6, IL-8, IL-12p70, IL-1β, TNF-α, interferon (INF)-γ, and IL-10 in each serum sample.

Immunoblot analysis. The specificity of the anti-pNF-H antibody used in the ELISA was verified via immunoblot analysis comparing mouse brain tissue with patient plasma samples. Murine brain lysate was prepared as previously described (49). For immunoblot analysis, serum samples (12 μl) from 2 randomly selected patients were diluted in Laemmli buffer and separated by SDS-PAGE on 10% polyacrylamide gels, transferred to nitrocellulose membranes and incubated at 4°C overnight with monoclonal antibody against pNF-H (cat no. IMG-5018A-2; 1:1,000; Novus Biologicals, LLC, Littleton, CO, USA). Signal from horseradish peroxidase-conjugated secondary antibody (cat. no. 170-6516; 1:5,000; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was developed using chemiluminescence reagent (Western Lightning Electrochemiluminescence Pro; cat. no. NEL120001EA; Perkin-Elmer, Inc., Waltham, MA, USA) and detected by exposure to x-ray film. A sample of murine brain lysate (20 μg protein) was used to positively identify the antibody reactive pNF-H protein band.

Statistical analysis. Comparisons among variables were made using one-way analysis of variance or Kruskal-Wallis test, as appropriate, for continuous-type measures and the χ^2 or Fisher's exact test for categorical outcomes. Healthy controls were compared to patients using the Mann-Whitney test for continuous measures and the χ^2 or Fisher's exact test for categorical outcomes. Spearman correlations were calculated to determine the strength of correlation between serum biomarkers. A mixed models approach to repeated measures analysis of variance was conducted to determine if the patterns of change across time with respect to each of the biomarker levels differed between groups. Bonferroni-like adjusted pairwise comparisons (P<0.01) were used post-hoc to determine which time points differed from one another. Statistical analysis was performed using SAS V9.3 (SAS Institute Inc., Cary, NC, USA).

Results

Study patients and perioperative data. Table I lists patient demographics, mean (± standard deviation) and median values (min-max range) of specific pre-, intra- and postoperative physiological measures and outcome variables that were used for statistical analyses.

Role of cyanosis. A total of 70% (16/23) of patients were cyanotic prior to surgery, and they received surgery at a younger age compared with the seven acyanotic patients (5.9±3.2 vs. 11.4±6.4 days, respectively). The cyanotic patients had significantly longer bypass times (160±49 vs. 100±15 min; P=0.007) and total cross clamp times (75±38 vs. 40±17 min; P=0.012) indicative of greater complexity of surgical cardiac repair. When patients prior to surgery were grouped as cyanotic or acyanotic, no statistically significant differences in serum biomarkers either prior to or following surgery were measured. Therefore, data from all patients were combined for the statistical analyses presented herein.

Brain biomarkers prior to and following cardiopulmonary bypass surgery. The presence of pNF-H in the serum of newborns with CHD has not been previously reported. Therefore, the specificity of the detection antibody used in the ELISA assay was tested by immunoblot analysis of patient sera and mouse brain tissue known to express pNF-H. The antibody recognized both high- and medium-molecular weight NF proteins in mouse brain and a corresponding 200 kDa protein in two patient serum samples, thus confirming its specificity (Fig. 1). pNF-H concentration in the pre-surgical samples of patients was 2.4-fold higher (P<0.0001) than that measured in cord blood from healthy term births (Table II). The concentration of pNF-H in serum obtained 2 h after CPB was significantly reduced compared with pre-surgery (P<0.0001) but then accumulated during the subsequent period, reaching pre-surgical levels 48 h following CPB (Fig. 2). S100B, a protein expressed in glial cells, was also significantly higher in patient sera prior to surgery compared with normal cord blood (P<0.00001; Table II) and increased significantly 2 h following CPB (P<0.0001), further supporting potential CNS injury in these patients (Fig. 2). Both the rapid increase of S100B following CPB and subsequent rapid decrease in 24 h likely reflects its reported short

Table I. Patient demographics and peri-operative variables.

Variable	Mean ± SD	Median	Min-max
All patients (n=23)			
Male % (n)	48 (11)		
Gestational age, weeks	39±1.4	39.4	35.2-41.0
Birth weight, kg	3.235±0.410	3.345	2.381-3.750
Age at surgery, days	7.6±5	6.0	3-20
Weight at surgery, kg	3.2±0.4	3.2	2.5-3.8
Body mass index, kg/m^2	12.83±1.49	12.57	10.97-18.12
Pre-operative data			
SaO$_2$ (%)	87±11	90	60-99
Hb (g/dl)	12.5±2.5	12.3	8.6-18.0
PaO$_2$ (median value, mmHg)	48±29	40	25-154
PaCO$_2$ (highest value. mmHg)	50±22	45	30-142
HCO$_3^-$ (lowest value, mEq/l)	20.3±4.3	21.0	5-27
pH	7.27±0.16	7.31	6.71-7.42
Lactate (mg/dl)	5.3±4.3	3.7	1.4-21.0
MAP (mmHg)	48±4	48	40-56
Intra-operative data			
Bypass time, min	141±49	139	44-240
Cross-clamp time, min	71±32	72	21-14
Postoperative data (within 48 h)			
FiO$_2$ requirement	0.48±0.18	0.50	0.21-1.00
Hb (lowest value, g/dl)	11.3±1.7	11.0	8.3-15.3
PaO$_2$ (lowest value, mmHg)	74±37	64	29-160
PaCO$_2$ (median value, mmHg)	49±8	50	35-60
pH	7.36±0.09	7.37	7.07-7.50
Lactate (highest value, mg/dl)	8.0±6.6	6.0	1.9-25.0
SaO$_2$ at discharge %	90±10	96	66-100
MAP (mmHg)	52±5	50	45-66
Mechanical ventilation, days	10.0±15.9	5.0	1.0-69.0
Intensive care unit stay, days	13.3±14.5	9.0	4.0-69.0
Hospital stay, days	20.8±13.5	17.0	7.0-69.0

SD, standard deviation; Hb, hemoglobin; MAP, mean arterial blood pressure; FiO$_2$, fraction of inspired oxygen.
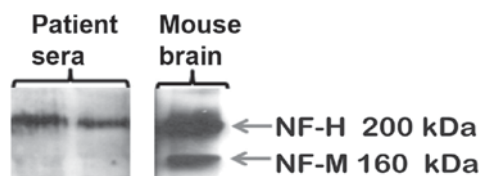

Figure 1. Immunoblot analysis of pNF-H in patient sera and mouse brain tissue. Molecular weight is indicated as kilodaltons (kDa). NF-H, neurofilament heavy chain subunit; NF-M, medium molecular weight subunit of neurofilament as identified in mouse brain.

half-life (~60 min). The amount of NSE in patient sera prior to surgery did not differ significantly from that in normal cord blood (Table II); however, it increased significantly 2 h post-CPB (P<0.0001) and then slowly decreased to pre-CPB levels over the subsequent 48 h, reflecting a longer reported serum half-life of ~30 h (Fig. 2).

Inflammation in neonates and response to surgery. Concentrations of cytokines, apart from INF-γ and IL-1β, were significantly lower in normal cord blood than in the sera of patients prior to surgery (P<0.05) (Table II). The responses of these inflammatory mediators to surgery are presented in Fig. 3. Serum levels of all ILs and INF-γ peaked immediately following CPB (P<0.01) and then decreased over the following 48 h (Fig. 3), although levels of IL-1β increased slightly but not significantly over 48 h post-CPB (data not shown). Serum TNF-α levels remained unchanged throughout the pre- and postoperative periods (Fig. 3). Complement activation, specifically the serum level of soluble C5b9, which potentially measures endothelial injury, was higher (P<0.0001) in patients prior to surgery than in normal cord blood (Table II). Serum C5a and sC5b9 concentrations increased progressively following surgery, with levels at 48 h being significantly higher than pre-surgical values (P<0.01; Fig. 4).

Table II. Serum concentrations of cytokines and brain-derived biomarkers.

Serum biomarker (concentration)	Normal cord blood Mean ± SD (n=20)	CHD patients pre-surgery Mean ± SD (n=23)
pNF-H (pg/ml)	136.8±53.9	328.7±155.7[d]
S100B (pg/ml)	126.3±113.6	358.0±210.2[d]
NSE (ng/ml)	20.27±9.93	15.50±15.17
C5A (ng/ml)	5.62±4.40	12.87±8.40[b]
sC5B9 (ng/ml)	79.0±48.1	652.2±491.1[d]
INF-γ (pg/ml)	0.18±0.13	0.40±0.39
IL-10 (pg/ml)	0.85±0.41	6.61±7.03[d]
IL-12p70 (pg/ml)	0.15±0.26	0.59±1.38[a]
IL-1β (pg/ml)	0.66±1.12	1.70±3.86
IL-6 (pg/ml)	1.86±1.08	11.01±17.99[c]
IL-8 (pg/ml)	6.61±1.94	36.49±48.62[d]
TNF-α (pg/ml)	4.43±1.12	14.02±8.01[d]

Values are mean ± SD of samples from cord blood of healthy term births and from patients with CHD obtained prior to heart surgery. n=number of patients. [a]$P<0.05$; [b]$P<0.01$; [c]$P<0.001$; [d]$P<0.00001$ vs. normal cord blood; TNF, tumor necrosis factor; IL, interleukin; INF, interferon; NSE, neuron specific enolase; CHD, congenital heart disease; SD, standard deviation.

Correlation between brain-derived biomarkers and inflammatory cytokines prior to surgery. The systemic inflammatory response to heart surgery with CPB in neonates has been well documented (15-21). However, immune function in neonates with CHD prior to heart surgery and its potential role on the clinical course of these patients is less clear. In the present study, Spearman correlation analysis demonstrated significant positive associations between the age of patients at surgery and pre-surgical serum levels of the cytokines ($P<0.05$; Table III). The age-dependent increases in serum IL-12p70, INF-γ TNF-α and IL-10 may be indicative of immune activation of T helper (Th) 1 immune response in these neonates. By contrast, the levels of the interleukins IL-1β, IL-6 and IL-8 were not correlated with patient age.

To support the hypothesis that inflammatory mediators serve a role in BBB permeability, it was observed that pre-surgical serum IL-12p70 concentrations correlated directly with levels of the brain proteins pNF-H and S100B (r=0.442, $P<0.05$; Fig. 5). Fig. 5 presents individual patient values for these biomarkers and a linear regression line drawn between serum pNF-H and IL-12p70.

It was examined whether pre-operative blood pressure and/or inotropic support of patients correlated with inflammatory biomarkers. A total of 15 patients treated with diuretics had significantly higher serum TNF-α, IL-10, IL12p70, IL1β and IL-6 levels compared with untreated patients ($P<0.05$; Table IV). Seven of these 15 patients also received dopamine and 8 patients (4 receiving both diuretics and dopamine) had elevated blood creatinine levels compared with the normal range for neonates (data not shown), suggesting that patients
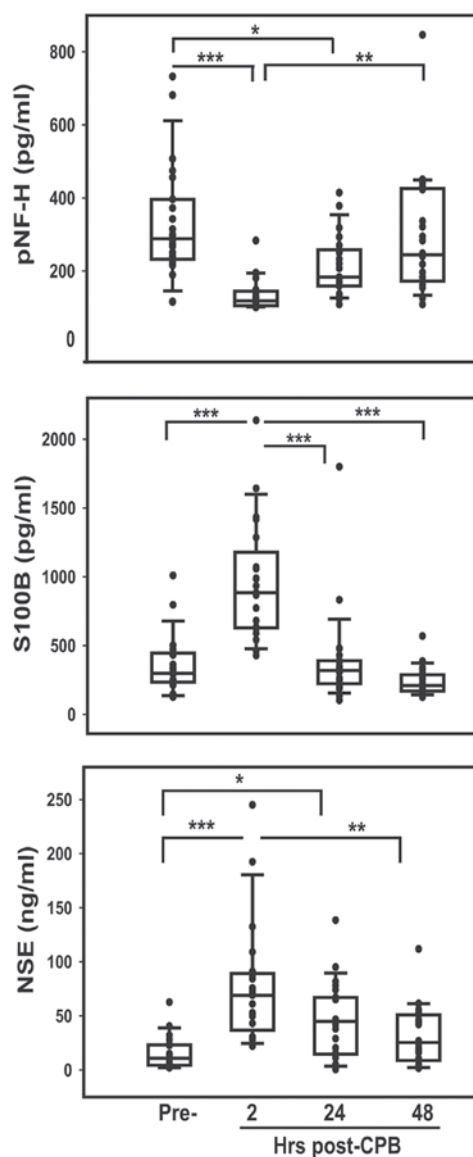

Figure 2. Concentration of CNS biomarkers in patient sera taken immediately prior to surgery (pre-) and at 2, 24 and 48 h post-CPB. Scatter plot represents individual patient data. Box plot: box boundaries indicates 25th and 75th percentile; line marks the median; whiskers indicate 10th and 90th percentiles. Statistical significance was determined by RM-ANOVA followed by Bonferroni post-hoc analysis for pairwise comparisons. *$P<0.01$; **$P<0.001$; ***$P<0.0001$. CPB, NSE, neuron-specific enolase; S100B, B subunit of calcium-binding protein S100; pNF-H, phosphorylated neurofilament heavy chain; repeated measures-ANOVA, analysis of variance; CPB, cardiac surgery with cardiopulmonary bypass.

who were hemodynamically unstable exhibited an augmented inflammatory response. Furthermore, 12 patients requiring ventilator support had significantly lower serum concentrations of the anti-inflammatory cytokine IL-10, compared with those who were not mechanically ventilated ($P<0.05$; Table IV). Of the other pre-operative variables recorded, arterial blood pO_2 correlated inversely with serum pNF-H, suggesting a potential causal relationship between hypoxemia and increased CNS injury (Table V).

Postoperative outcomes and serum biomarkers. Postoperative outcome variables demonstrating statistically significant correlations with serum brain biomarkers, cytokines and
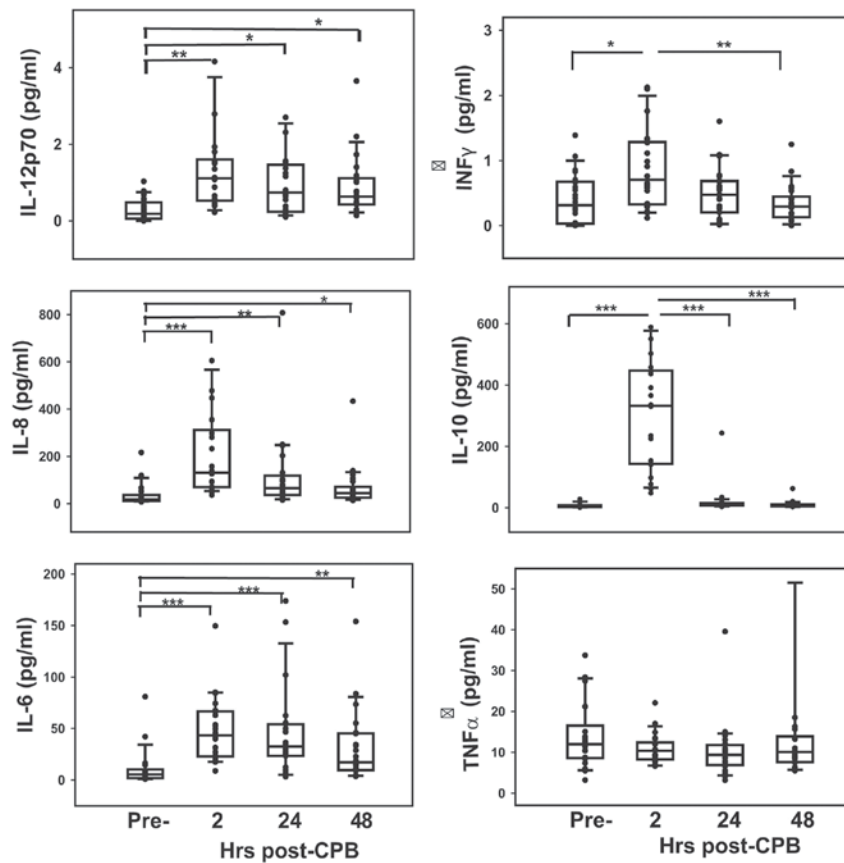
Figure 3. Concentration of cytokines in patient sera taken immediately prior to surgery (pre-) and 2, 24 and 48 h post-CPB. Scatter plot represents individual patient data. Box plot: box boundaries indicates 25th and 75th percentile; line marks the median; whiskers indicate 10th and 90th percentiles. Statistical significance was determined by RM-ANOVA followed by Bonferroni post-hoc analysis for pairwise comparisons. *P<0.01; **P<0.001; ***P<0.0001. CPB, cardiac surgery with cardiopulmonary bypass; RM-ANOVA, analysis of variance; IL, interleukin; INF, interferon; TNF, tumor necrosis factor.
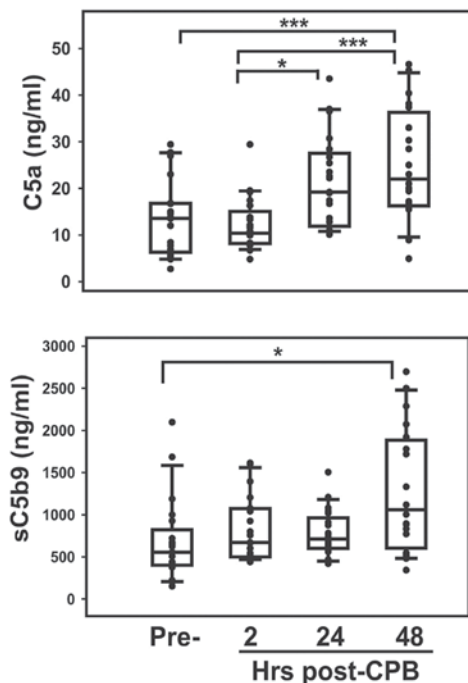

Figure 4. Concentration of complement in patient sera taken immediately prior to surgery and at 2, 24 and 48 h post-CPB. Scatter plot represents individual patient data. Box plot: box boundaries indicates 25th and 75th percentile; line marks the median; whiskers indicate 10th and 90th percentiles. Statistical significance was determined by RM-ANOVA followed by Bonferroni post-hoc analysis for pairwise comparisons. *P<0.01; ***P<0.0001.

complements are presented in Table V. Correlations were determined between physiological measurements recorded during the first or second 24 h period post-CPB and blood samples obtained at 24 or 48 h. MAP during the first 2 days post-CPB correlated positively with serum TNF-α and C5a. Measurements indicative of tissue hypoxia including arterial blood gases (pO_2, pCO_2) and lactate, and increased requirement for oxygen inhalation (FiO_2) correlated significantly with inflammatory responses and complement activation. Levels of the brain biomarkers pNF-H and NSE correlated directly with blood pCO_2 and lactate, respectively (both P<0.05) during the first 24 and 48 h post-CPB, suggesting that hypoxia may have contributed to BBB permeability and release of these brain proteins. Serum levels of S100B and NSE, as well as IL-8 and IL-10, were inversely correlated with blood oxygen saturation at hospital discharge (P<0.01, P<0.05, P<0.01 and P<0.01, respectively) suggesting that systemic inflammation following surgery was an important determinant of clinical course (Table V). The postoperative duration of mechanical ventilation (length of intubation) was directly correlated with serum levels of IL-12p70 and IL-8 measured 48 h post-CPB (both P<0.05; Table V).

Although only a small number of patients (six) without chromosomal abnormalities exhibited anomalies in brain imaging during their hospital course, they had significantly higher serum levels of IL-12p70 (1.21±0.58 vs. 0.77±0.86 pg/ml, P<0.05) and NSE (50.64±32.19 vs. 23.52±20.86 ng/ml, P<0.05)

Table III. Correlations between serum biomarkers and patient age.

	TNF-α	INF-γ	IL-10	IL-12p70
Age at blood draw/surgery	0.500[a]	0.638[c]	0.491[a]	0.656[c]
IL-12p70	0.544[b]	0.737[d]	0.576[b]	-
IL-8	0.660[c]	ns	ns	ns
sC5b9	0.450[a]	ns	ns	ns

Correlations between biomarker concentrations in patient sera obtained immediately before surgery and patient age at surgery at the time of blood draw. Numbers are Spearman correlations ρ-values; [a]$P<0.05$; [b]$P<0.01$; [c]$P<0.001$; [d]$P<0.0001$. ns, not statistically significant correlation; IL, interleukin; TNF tumor necrosis factor; INF, interferon.
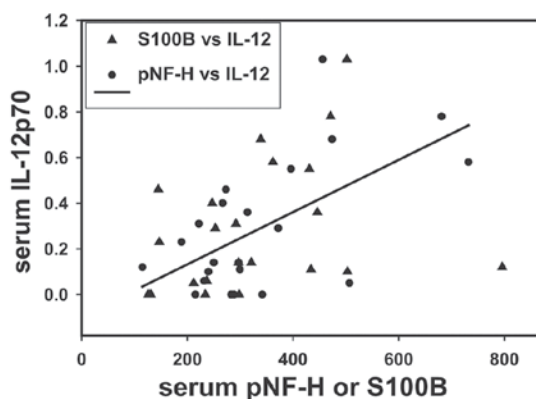

Figure 5. Spearman correlation analysis between brain biomarkers, pNF-H, S100B and cytokine IL-12p70 in patient sera obtained prior to surgery demonstrated similar correlation coefficients of ρ=0.442 and P<0.05. The straight line represents the best-fit linear regression between pNF-H and IL-12p70. Triangles or circles represent individual patient serum S100B or pNF-H values, respectively, plotted vs. serum IL-12p70. IL, interleukin; pNF-H, phosphorylated neurofilament-heavy subunit.

following surgery compared with the 15 patients without any suspicion of CNS pathology or clinical findings.

Discussion

In the present study, it was hypothesized that newborns with CHD and specifically those with tissue hypoxia, experience systemic inflammation as a consequence of the disease, which results in dysfunction of the BBB and subsequent injury to the CNS as measured by the presence of circulating CNS-derived proteins. A number of studies have linked elevated levels of circulating pro-inflammatory cytokines, including TNF-α and IL-6 that are derived from peripheral immune cells or sites of organ/tissue injury, to BBB failure (50,51). Evidence suggests that brain microvascular endothelial cell damage may occur due to cytokine-induced reactive oxidative species produced by NADPH oxidases or by altered function of endothelial junction proteins (52,53) and that hypoxia-induced cytokine production may be an initiating event (54). Brain biomarkers, including pNF-H, S100B and NSE were detectable in patient

serum prior to surgery and the concentrations of pNF-H and S100B were ~2.5-fold higher in patients than in normal cord blood. Both pNF-H and S100B levels were directly correlated with serum IL-12p70 and the correlation between pNF-H and IL-8 trended towards significance. These data support a potential causal link between pro-inflammatory mediators and BBB dysfunction with neuronal injury in these neonates. Furthermore, pre-operative arterial pO_2 correlated inversely with serum pNF-H, while the strongest correlation with pre-operative arterial pCO_2 was with TNF-α, suggesting that hypoxia and acidosis in neonatal patients with CHD may be an initiating event for the peripheral inflammatory response with a direct effect on CNS injury. This conclusion is further supported by results obtained from the 24-48 h postoperative period, during which serum pNF-H correlated significantly with arterial pCO_2, while NSE correlated directly with serum lactate. Furthermore, NSE and S100B were inversely associated with arterial oxygen saturation at discharge.

Modified ultrafiltration that allows for hemoconcentration and recovery of circuit blood following CPB, has been shown to remove certain inflammatory cytokines and components of the complement system from the circulation (55). Serum concentrations of IL-6, IL-10, TNF-α and C5b9 prior to and immediately following CPB in the present study are similar to those published in a study by Berdat et al (56) that demonstrated that various ultrafiltration methods were effective at removing such mediators to a varying extent. Despite ultrafiltration, levels of the inflammatory mediators NSE and S100B, increased significantly immediately following CPB, reflecting their release/secretion and accumulation. Serum concentration of pNF-H, a large 200 kDa protein, decreased following CPB compared to pre-surgical levels, suggesting that it had been effectively removed by ultrafiltration. One explanation for these changes could be either volume expansion or hemoconcentration as a result of the CPB procedure. However, the results of the current study suggest that the temporal differences in serum concentrations of pNF-H, NSE and S100B possibly depended on their individual half-lives. The short half-life of S100B (90 min) predicted its rapid rise and fall immediately following CPB (<24 h), whereas the relatively longer half-life of NSE (~30 h) was reflected in its slower decline with serum concentrations at 24 h remaining significantly higher than pre-surgery. By contrast, the stable hyperphosphorylated NF-H protein with a half-life >3 days, was slow to rise in serum and took 48 h to reach concentrations similar to pre-surgery. Previous studies have demonstrated that pNF is elevated in serum for weeks following spinal cord or mild brain injury and in children with febrile seizures (45,57). Although the kinetics of these molecules in serum remain largely unknown, these data support the hypothesis that the BBB remains permeable in the initial h following CPB but begins to close within the first postoperative day. Abu-Sultaneh et al (48) demonstrated a significant correlation between peak serum S100B following CPB with cerebral oxygen desaturation during surgery, as measured by near-infrared spectroscopy. Furthermore, it has been demonstrated that cerebral oxygen saturation is correlated with neurodevelopmental outcomes and brain MRI at one year of age (58). While brain biomarkers, including S100B and NSE, have been studied in children in response to cardiac surgery with CPB, to the best of our knowledge this study isthe

Table IV. Effects of pre-operative treatments on serum cytokine concentrations.

Treatment variables	n	Serum cytokines (pg/ml)				
		TNF-α	IL-10	IL-12p70	IL-1β	IL-6
Ventilator support						
(+)	12	ns	3.72±2.88[a]	ns	ns	ns
(-)	10	ns	10.15±8.93	ns	ns	ns
Diuretics						
(+)	15	16.83±8.14[a]	8.73±7.93[a]	0.39±0.31[a]	1.60± 2.73[a]	14.84±21.58[a]
(-)	8	8.41±3.60	2.99±2.65	0.11±0.10	0.30±0.22	4.09±4.82

Fisher's exact test for categorical outcomes was used to determine statistical differences between pre-operative treatment groups. [a]$P<0.05$; numbers are serum cytokine concentrations (pg/ml); ns, not statistically significant; TNF, tumor necrosis factor; IL, interleukin; n, number of patients/group; (+) treated, (-) not treated.

Table V. Correlations between pre- or postoperative physiologic measurements and serum biomarkers.

Variables	Serum brain biomarkers, complement, cytokines								
	pNF-H	S100B	NSE	C5a	sC5b9	TNF-α	IL-10	IL-12p70	IL-8
PRE-OP									
pO_2 (median value on ABG)	-0.493[a]	ns	ns	ns	ns	ns	ns	ns	ns
pCO_2 (highest value on ABG)	ns	ns	ns	ns	ns	+0.448[a]	ns	ns	ns
POSTOP									
MAP (median daily value)	ns	ns	ns	+0.551[b]	ns	+0.407[a]	ns	ns	ns
pO_2 (lowest daily value ABG)	ns	ns	ns	ns	-0.520[b]	ns	-0.437[a]	ns	-0.430[a]
pCO_2 (median daily value ABG)	+0.426[a]	ns	ns	+0.423[a]	ns	ns	+0.449[a]	ns	ns
Lactate (maximum daily value)	ns	ns	+0.631[a]	ns	-0.500[a]	ns	+0.553[b]	+0.727[c]	+0.475[a]
FiO_2 requirement	ns	ns	ns	ns	ns	+0.425[a]	+0.483[a]	ns	ns
O_2 saturation at discharge	ns	-0.584[b]	-0.489[a]	ns	+0.663[c]	ns	-0.611[b]	ns	-0.556[b]
Length of intubation	ns	ns	ns	ns	ns	ns	ns	+0.442[a]	+0.445[a]

Correlations between physiological parameters measured pre-operatively or during the first or second 24 h period post-CPB and serum biomarkers measured in serum collected at those time points. Numbers are Spearman correlation ρ-values; [a]$P<0.05$; [b]$P<0.01$; [c]$P<0.001$. ABG, arterial blood gas; MAP, mean arterial pressure; FiO_2, fraction of inspired oxygen; NSE, neuron specific enolase; IL, interleukin; TNF, tumor necrosis factor; ns, not statistically significant correlation.

first to measure pNF-H in the serum of newborns with CHD undergoing open-heart surgery with CPB.

Newborns with complex CHD have an increased risk of white matter injury, reduced brain growth and delayed brain development (6,59) and those requiring corrective surgeries with CPB during the neonatal period experience an inflammatory response (2,13,14,21,60,61). Although inflammation during the perinatal period has been determined to affect brain development in the pre-term infant (28,29), the significance of inflammation on the long-term neurocognitive development of children with CHD who have undergone surgical cardiac repair remains unclear and its significance is controversial (7,33). Upon examination of various pre-operative treatments and postoperative physiological outcome measurements, it was determined that patients receiving diuretics and/or dopamine had significantly higher serum concentrations of many cytokines, which cannot be explained solely by changes in intravascular/extracellular fluid volumes. In the postoperative period, levels of IL-10 and IL-8 correlated with arterial hypoxemia and increased with blood lactate levels and inhaled O_2 requirements. Other cytokines, including TNF-α and IL-12p70, similarly correlated with these post-surgical parameters but with varying strengths of association. Thus, taken together these data support an augmented systemic inflammatory response to tissue hypoxia and acidosis, with an increased need for mechanical ventilation with higher oxygen requirements in the postoperative period, suggesting that these neonates may be at higher risk of CNS injury.

In light of reports that activation of complement leads to loss of integrity of the BBB and blood-CSF barrier, the

serum content of C5a was measured to assess activation of the complement system and serum sC5b-9 to assess potential cellular damage mediated by complement. Activation of the complement system has been observed in intracranial inflammation following traumatic brain injury with upregulation of soluble terminal complement complex sC5b-9 in spinal cord fluid that correlated with degree of BBB dysfunction (36). C5a and C3a are known leukocyte chemotactic factors that can induce the release of lysosomal enzymes and oxygen-derived free radicals, and can stimulate cytokine production and release (62). In the present study, serum C5a and sC5b9 were significantly higher in patients who had red blood cell transfusion prior to surgery and sC5b9 correlated directly with serum levels of TNF-α and IL-1β. In the postoperative period C5a and sC5b9 correlated with various parameters indicative of arterial hypoxemia, elevated blood pressure and increased length of hospital stay. To the best of our knowledge, this is the first report demonstrating complement activation in this setting. Pagowska-Klimek *et al* (63) have recently reported that cardiac surgery with CPB activated the lectin pathway of complement activation and this response was significantly greater in pediatric patients presenting with post-bypass systemic inflammation. Further study is warranted in assessing the role of complement activation in brain injury in infants with CHD, particularly regarding novel therapeutic advances directed at the complement system to treat autoimmune diseases that may be repurposed for treatment of newborns with CHD (64-66).

It was observed that there was a significant correlation between patient age at surgery (between 3 and 20 days) and pre-surgical serum levels of TNF-α, IL-12p70, INF-γ and IL-10. The strongest correlation was between patient age at surgery and levels of IL-12 and INF-γ, which are Th1 cytokines. Newborns exhibit a bias toward a Th2 cell-dominated cytokine response, including the release of IL-4, IL-6 and IL-10; however, with increasing age and maturation of dendritic cells and their production of IL-12 in response to INF-γ, a shift to a Th1 cell response occurs (67,68). The predominance of Th2 cytokine production in the fetus and neonate serves to dampen innate immune responses, protecting the newborn from Th1-induced affects (69,70). The capacity of mononuclear cells to secrete a number of inflammatory cytokines increases with age from 2 months through one year to adulthood; however, monocytes from normal cord blood, which is essentially newborn blood, have increased capacity in response to pathogens to secrete TNF-α, IL-6, IL-10 and INF-γ (but not IL-12) at levels observed in adults (71,72). Similarly, the observation that serum concentrations of TNF-α, INF-γ and IL-12p70 increased in neonates as they lived longer with their disease prior to heart surgery suggests that the production of Th1 cytokines was in response to disease-induced effects rather than normal maturation of immunity towards Th1 cell polarization. Gestational age and weight have been reported to be important determinants of the neonate's immune response to infection (73). In the current study, mean gestational age was 39 weeks and no neonates were small for their gestational age, suggesting that these newborns had the capacity to elicit an age-appropriate innate immune response. Therefore, the current study concludes that term newborns with congenital heart disease may be able to elicit an age-appropriate immune response; however, this response to their disease process

may promote an excessive pro-inflammatory Th1 response potentially leading to end-organ damage, including increased permeability of the BBB and neuronal injury. New avenues of investigation, including studies examining the response of isolated peripheral blood mononuclear cells from neonates with CHD to various pathogenic and danger signals may advance understanding of innate immunity in this unique patient group and improve approaches to their medical care.

Limitations of the current study are the small sample size and absence of data from age-matched normal neonates aged between 3 and 30 days. Cord blood was analyzed from healthy term deliveries to provide surrogate normative newborn values. Although none of the study patients were small for gestational age or born pre-maturely, prospective collection of cord blood from these subjects would be preferable. A further limitation is the inclusion of congenital heart disease pathologies with varying degrees of severity and complexity. Despite these limitations, consistent immune responses were observed in this patient population, lending support to the significance of the results.

In summary, the results of the present study demonstrate that neonates with congenital heart disease requiring surgical repair/palliation during the first month of life have elevated serum levels of pNF-H, a CNS-derived axonal protein that suggests brain injury with loss of BBB integrity. Concentrations of complement and pro-inflammatory cytokines in the patients' serum were found to be higher than normal cord blood, and these biomarkers of inflammation were significantly correlated with pre- and post-operative measurements of tissue hypoxia/acidosis, and an increased need for mechanical ventilation with higher oxygen requirements, suggesting that these neonates may be at higher risk of CNS injury.

Acknowledgements

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