Assessment of the Contribution of Cytokine Plasma Levels to Detect Retinopathy of Prematurity in Very Low Birth Weight Infants

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Purpose. To prospectively study the association of high cytokine plasma levels with later development of retinopathy of prematurity (ROP) in preterm infants with early-onset sepsis to assess a laboratory test to detect ROP.

Methods. A prospective cohort study was conducted of preterm infants with clinical early-onset sepsis whose birth weight (BW) was ≤1500 g and gestational age (GA) was ≤32 weeks. Plasma samples were assayed for cytokines IL-6, IL-8, IL-10, IL-1β, and TNF-α. ROP was diagnosed in screening assessments. For the univariate analysis of the known risk factors for ROP, all infants without ROP were designated as the No ROP group, patients with any stage of ROP formed the ROP group, and all treated patients formed the Severe ROP group. The best cutoff points for all cytokine levels were determined by ROC curves.

Results. Seventy-four patients were enrolled. Mean GA and BW were 29.6 ± 2.1 weeks and 1110.3 ± 232.5 g, respectively; 49 patients (66.2%) had no ROP and 25 (33.8%) had any stage of ROP (17 had stage 1 or 2 ROP and 8 had stage 3 ROP). IL-6 >357 pg/mL, IL-8 >216 pg/mL, and TNF-α >245 pg/mL were significantly associated with treatable ROP.

Conclusions. There is a relationship between high plasma levels of IL-6, IL-8, and TNF-α in the first days of life with the later development of ROP severe enough to treat in preterm infants with early-onset sepsis. Further epidemiologic studies are needed to explore other possible associations of high serum levels of cytokines with ROP in this population at high risk. (*Invest Ophthalmol Vis Sci.* 2011;52:1297–1301) DOI:10.1167/iovs.10-6279

The pathogenesis and the etiology of advanced retinopathy of prematurity (ROP) are not fully understood. In the past, many causative factors such as supplemental oxygen, excessive light exposure, and hypoxia have been suggested. Evidence for these as independent risk factors for the onset of ROP in recent years is not compelling.[1] A possible role of inflammation in ROP is generating a flurry of interest.

Systemic fungal infection and early-onset bacterial sepsis are described to be associated with the development of ROP in very low birth weight (VLBW) preterm infants.[2–5] Recently, Dammann et al.[6,7] have found some evidence in support of the contention that multiple "hits" of exposure to perinatal inflammation and infection might contribute to a gradual increase in the risk for ROP. Chen et al.[8] suggested that neonatal sepsis, oxygen exposure, and low gestational age (GA) are not only independently associated with ROP occurrence, they interact and have a multiplicative effect on development of ROP.

Some authors, on the other hand, have affirmed that the role of infection in ROP etiology remains unclear and that it is important to keep investigating inflammatory factors.[9,10]

Antenatal intrauterine infection and the fetal inflammatory response are actions mediated by cytokines. Cytokines include interleukin (IL)-6, IL-8, IL-10, IL-1β, and tumor necrosis factor-α (TNF-α). These are low-molecular-weight proteins produced and secreted by monocytes, macrophages, endothelial cells, and fibroblasts. They are intercellular signaling polypeptides produced by these activated cells.[11]

Cytokine levels in the vitreous of 19 patients with stage 4 ROP were recently analyzed by Sato et al.,[12] who demonstrated that vascular endothelial growth factor (VEGF) has likely the highest correlation with vascular activity in ROP eyes than all other cytokines studied. Sood et al.[13] studied cytokine levels in extremely low birth weight newborns on days 1, 3, 7, 14, and 21 and showed that some cytokines remained significantly different across ROP groups, suggesting that perinatal inflammation may be involved in the pathogenesis of ROP.

At present, the diagnosis of ROP is always, and exclusively, performed by ophthalmologists in charge of the screening sessions to detect and to treat ROP. This fact demands a high workforce load for ophthalmologists and many repeated ophthalmological examinations, causing stress and physical debilitation for the infants. No laboratory test is available to help in the early diagnosis of ROP.

Recently, plasma levels of sE-selectin were studied as a surrogate marker of ROP.[14] Other institutional and prospective studies are needed regarding the use of laboratory tests to help in the early identification of infants who may have treatable ROP. We chose to study VLBW infants with clinical early-onset sepsis and evaluate the plasma cytokine levels obtained in the first days of life to predict the late development of ROP. The initial hypothesis is that multiple "hits" of exposure to inflammation might contribute to increased risk for ROP. The evaluation of perinatal inflammation by cytokine levels as early as 72 hours after birth has not been investigated yet. Our aim was to examine a group of VLBW newborns that classically present high cytokine levels to determine whether there is a relationship between plasma levels of IL-6, IL-8, IL-10, IL-1β, and TNF-α

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in the first 72 hours of life and the later development of ROP in that high-risk population.

METHODS

Population and Setting

From July 2005 to October 2007, all preterm infants with BW ≤ 1500 g and GA ≤ 32 weeks at birth with diagnosis of clinical early-onset sepsis admitted to the neonatal intensive care unit (NICU) of the Hospital de Clinicas de Porto Alegre, Brazil, a teaching hospital, were included in a prospective cohort study.

According to published criteria, the diagnosis of clinical early-onset sepsis is based on the presence of three or more of the following conditions: maternal risk factors such as fever, premature rupture of membranes for >18 hours, and urinary tract infection; or newborn risk factors such as apnea, difficult breathing, cyanosis, tachycardia or bradycardia, perfusion deficit or shock; irritability, lethargy, hypotonia, and seizures; abdominal distention, vomiting, dietary intolerance, gastric residue, hepatomegaly, idiopathic jaundice, thermal instability, petechiae or purpura; and general poor appearance.[15,16]

Exclusion criteria were presence of major congenital malformation or malformation of the central nervous system, congenital infection such as syphilis, toxoplasmosis, rubella, cytomegalovirus, and herpes, and maternal HIV positivity. Preterm infants who died before of the first ophthalmologic examination were also excluded.

Outcomes and Variables

The following variables were prospectively analyzed: BW, GA, sex, score for neonatal acute physiology and perinatal extension II (SNAPPE II) at NICU admission, positive blood cultures <72 hours after birth (early-onset sepsis) and >72 hours after birth (late-onset sepsis), necrotizing enterocolitis, use of oxygen in mechanical ventilation or by nasal continuous positive airway pressure; occurrence of patent ductus arteriosus, respiratory distress syndrome, grades III and IV intraventricular hemorrhage, and mode of delivery. Gestational age was assessed by maternal dates and obstetric ultrasound performed at 12 weeks' pregnancy. VLBW with blood cultures positive for coagulase-negative staphylococci (CONS) and clinical sepsis met the following definitions to distinguish proven CONS infection from contaminants: two positive blood cultures drawn within 2 days of each other or one positive blood culture and elevated C-reactive protein (>10 mg/L) within 2 days of blood culture. For all other pathogens, proven sepsis was defined by the presence of the organism in the blood culture.

The main variables were the plasma levels of IL-6, IL-8, IL-10, IL-1β, and TNF-α. For the univariate analysis of the known risk factors for ROP, all infants without ROP were designated as the No ROP group, patients with any stage of ROP formed the ROP group, and all treated patients formed the Severe ROP group. For this study, severe ROP was defined as treatable ROP. Staging of ROP was recorded according to the International Classification of Retinopathy of Prematurity[17,18] and always corresponded to the highest stage of ROP found in any of the eyes during patient follow-up.

Ophthalmological Examination

All included patients underwent eye examination, which consisted of binocular indirect ophthalmoscopy using 28-D lens (Nikon, Melville, NY) after pupil dilation in both eyes with 0.5% tropicamide and 2.5% phenylephrine eye drops. An infant eyelid speculum (Alfonso Eye Speculum, Storz; Bausch & Lomb Inc., San Dimas, CA) was used. Scleral indentation was performed when necessary. All patients were examined in the NICU and, after hospital discharge, attended outpatient follow-up appointments until 45 weeks of maternal postmenstrual age. The initial ophthalmological examination was performed between the fourth and the sixth weeks of life. The screening sessions were performed according to the Brazilian guidelines to detect and treat ROP.[19,20] All eye examinations were performed by the same author (JBFF).

Cytokines Determinations

A blood sample was collected for sepsis work-up in the first 72 hours after birth, and an additional amount (200 μL) was obtained in EDTA tube for cytokine analysis. No blood samples or venous or arterial punctures were performed exclusively for the study. Blood samples were centrifuged immediately, and the plasma was frozen at −80°C until laboratory analysis. All tests were assayed in duplicate. The measurements of IL-6, IL-1β, IL-8, IL-10, and TNF-α were performed using commercially available kit (Human Cytokine Lincoplex; Linco Research, St. Charles, MO). This is a multiple assay kit for the simultaneous quantitative determination of these cytokines that has intra-assay and interassay precisions of 5% to 10% for cytokines. Readings were carried out with an automated laboratory analysis system (Luminex 100; Luminex, Austin, TX) with appropriate software.

Statistical Analysis and Ethical Considerations

We performed the statistical analyses using statistical software (SPSS 16.0 for Windows; SPSS Inc, Chicago, IL), and a significance level was established at $P < 0.05$. Univariate and logistic regression were performed for the risk factors for the No ROP, ROP, and Severe ROP groups. The results were expressed as mean ± SD or medians and interquartile range (p25–p75). Student's t, Mann-Whitney U, and χ^2 tests were applied. Differences among cytokine plasma levels of the No ROP, ROP, and Severe ROP groups were determined by Kruskal-Wallis test. All tests were 2-tailed.

Receiver operating characteristic (ROC) curves were performed for all cytokines studied. Because the possibility of the detection of ROP by any laboratory test requires more specificity than sensibility, once the final diagnosis of ROP was performed by binocular indirect ophthalmoscopy, we used a cutoff point for each cytokine. The best cutoff point for high specificity of each cytokine was, then, used to determine the association between the Severe ROP group and a new group formed by No ROP plus ROP 1 and ROP 2 patients. The χ^2 test was used for comparisons between these two groups.

The study protocol was approved by the Hospital's Research Ethics Committee (protocol 04-446), and it conformed to the provisions of the Declaration of Helsinki (as revised in Edinburgh, 2000). Informed consent forms were read and signed by parents or guardians before the study.

RESULTS

In our NICU, 180 VLBW infants were admitted during the study period and 106 had clinical early-onset sepsis. Thirty-two were excluded because they died before the initial ophthalmological examination and without obtaining the diagnosis of ROP. Then 74 VLBW infants (148 eyes examined) with clinical early-onset sepsis were enrolled in this study. The mean BW and GA for the entire cohort were 1110.3 ± 232.5 g and 29.6 ± 2.1 weeks, respectively.

Forty-nine preterm infants (66.2%) did not have ROP; 25 patients (33.8%) had any stage of ROP (17 patients [23%] had stage 1 or 2, and 8 patients [10.8%] had stage 3). The incidences of any stage of ROP and severe ROP in this high-risk cohort of patients were 33.8% and 10.8%, respectively.

After univariate analysis between No ROP and any stage of ROP patients (ROP group) only the variables BW ($P = 0.016$), SNAPPE II ($P = 0.039$), and use of oxygen therapy under mechanical ventilation ($P = 0.010$) were considered significant for any stage of ROP, whereas the significant variables for severe ROP were BW ($P = 0.014$), GA ($P = 0.010$), and SNAPPE-II ($P = 0.054$), as shown in Table 1. After logistic regression, the use of mechanical ventilation was significant as an independent risk factor for any stage of ROP (odds ratio

TABLE 1. Univariate Analysis of the Risk Factors for ROP

	No ROP Group (n = 49)	ROP Group (n = 25)	P ROP†	No ROP + ROP 1 + 2 (n = 66)	Severe ROP Group (n = 8)	P Severe ROP‡
BW, grams*	1,156 ± 228	1029 ± 219	**0.016**	1,133 ± 224	921 ± 229	**0.014**
GA, weeks*	29.9 ± 2.2	28.9 ± 2.0	0.064	29.8 ± 2.0	27.7 ± 2.0	**0.010**
Vaginal delivery, n (%)	11 (22.4)	8 (32)	0.543	16 (24.2)	3 (37.5)	0.415
Male, n (%)	29 (59.2)	16 (64)	0.881	40 (60.6)	5 (62.5)	1.000
SNAPPE II score	12 (0–19.5)	20 (6.5–36.5)	**0.039**	12 (0–22)	24 (10–39.7)	**0.054**
Oxygen therapy in nasal CPAP, n (%)	45 (91.8)	25 (100)	0.293	62 (93.9)	8 (100)	1.000
Oxygen therapy in mechanical ventilation, n (%)	27 (55.1)	22 (88)	**0.010**	42 (63.6)	7 (87.5)	0.253
RDS, n (%)	24 (49)	15 (60)	0.514	33 (50)	6 (75)	0.267
WMI, n (%)	10 (20.4)	8 (32)	0.416	14 (21.2)	4 (50)	0.092
NEC, n (%)	1 (2)	1 (4)	1.000	2 (3)	0 (0)	1.000
IVH, n (%)	43 (87.8)	20 (80)	0.492	57 (86.4)	6 (75)	0.339
Positive blood culture <72 hours, n (%)	4 (8.2)	1 (4)	0.657	4 (6.1)	1 (12.5)	0.445
Positive blood culture >72 hours, n (%)	10 (20.4)	8 (32)	0.416	15 (22.7)	3 (37.5)	0.393
PDA, n (%)	7 (14.3)	7 (28)	0.211	13 (19.7)	1 (12.5)	1.000

* Data are expressed as mean ± SD or median (p25–p75). WMI, white matter injury.

† Comparison between No ROP Group and any stage of ROP patients (ROP Group).

‡ Comparison between no ROP patients (49 patients) plus patients with stage 1 and stage 2 of ROP (66 patients) and Severe ROP Group (8 patients). Bold/underlined data are significant results.

[OR], 0.234; 95% confidence interval [CI], 0.059–0.932; P = 0.039), whereas GA (OR, 0.634; 95% CI, 0.413–0.974; P = 0.037) was significant for severe ROP.

The median collection time for cytokines was 18 hours after NICU admission, with no statistical difference between the ROP and No ROP groups. Median values of the cytokines plasma levels for the entire cohort were as follows: IL-6, 72 pg/mL; IL-8, 50 pg/mL; IL-10, 329 pg/mL; IL-1β, 1.3 pg/mL; TNF-α, 21.3 pg/mL.

Median IL-6, IL-8, and TNF-α plasma levels were higher in the Severe ROP than in the No ROP group, but the association was not significant among the groups. Median IL-10 and IL-1β plasma levels were very similar among both groups. The complete results for the median cytokines plasma levels between the both groups are displayed in Table 2.

In Table 3, we show the association between 66 VLBW without ROP or ROP stages 1 and 2 with 8 VLBW with severe ROP for each specificity best cutoff points of the cytokines.

DISCUSSION

In our study, IL-6 >357 pg/mL, IL-8 >216 pg/mL, and TNF-α >245 pg/mL enabled us to define the ROP requiring treatment. IL-10 >574 pg/mL and IL-1β >1.22 pg/mL were not associated with the ROP diagnosis in this high-risk population. Postnatal clinical indicators of illness severity, such as SNAPPE-II and low birth weight and GA, and oxygen therapy under mechanical ventilation were significant for any stage of ROP and severe ROP after univariate analysis.

Despite many recent advances in our understanding and management of ROP, the disorder is still an important cause of preventable childhood blindness, and it is not well understood why ROP progresses to a severe stage in some infants while in others, despite similar clinical conditions, it regresses spontaneously. A multiple "hits" of exposure to inflammation was suggested by Dammann et al.[6] Sood et al.[13,21,22] and other studies have demonstrated a role for genetic factors and polymorphisms. These conditions may contribute to higher ROP risk.

ROP is frequently associated with lower GA and BW and with oxygen therapy necessary for the survival of the preterm infant.[23] Our data are in accordance with previous studies, in which the most important risk factors for ROP were lower BW and GA at birth.[23,24] All groups were similar with respect to neonatal courses. We emphasize that we studied ROP occurrence and progression in a high-risk population with clinical early-onset sepsis because we wanted to determine whether high cytokine levels were still importantly associated with ROP, even in infants with increased ROP risk. Our studied patients had significantly high cytokine levels. We previously reported median IL-6 and TNF-α plasma levels of 38.64 pg/mL and 14.98 pg/mL, respectively, in the first 72 hours for control newborns.[16]

Scores for neonatal illness severity have been used to predict ROP.[25,26] SNAPPE II was previously evaluated in a prospective cohort of VLBW infants in whom we showed an association between higher scores at admission and later development of ROP, but after adjustments, as well as after the ROC curves results, this score did not enhance the assessment of ROP risk.[26] In the present study, we report SNAPPE II at admission with significance among No ROP, ROP, and Severe ROP groups after univariate analysis.

TABLE 2. Median Cytokines Plasma Levels and ROP

Cytokines (pg/ml)	No ROP Group (n = 49)	Severe ROP Group (n = 8)	P
IL-6	72 (29–262)	302 (37–2,175)	0.360
IL8	51 (11–191)	150 (45–585)	0.376
IL-10	320 (103–1,125)	395 (48–1,567)	0.906
IL-1β	1.3 (1.1–1.6)	1.2 (1.1–1.4)	1.000
TNF-α	21 (11–42)	137 (15–1,056)	0.064

Data are expressed as median (p25–p75).

TABLE 3. Cytokines Associated with the Later Development of Severe ROP

	No ROP plus ROP Stages 1 and 2 (n = 66)	Severe ROP (n = 8)	P*
IL-6 > 357 pg/ml	12 (18.2)	4 (50)	0.048
IL-8 > 216 pg/ml	13 (19.7)	4 (50)	0.076
TNF-α > 245 pg/ml	7 (16.6)	4 (50)	0.014
IL-10 > 574 pg/ml	23 (34.8)	4 (50)	0.452
IL-1β > 1.22 pg/ml	37 (56.1)	4 (50)	1.000

Data are expressed as n (%).

* Chi-square test.

In contrast to published data,[27] vaginal delivery was not correlated with the occurrence of ROP in our high-risk cohort of preterm infants because we had occurrences of maternal prenatal conditions, such as preeclampsia, that are known indications for cesarean section in preterm infants.

Neonatal sepsis, especially candidemia, has been reported as a factor associated with progression of any ROP stage to ROP requiring laser treatment. There were no patients with candidemia in this study. According to our institutional empiric therapy guideline based on risk factors for *Candida* infection, all patients with suspicion of candidemia had been treated with antifungal drug.[28]

Dammann et al.[6] investigated the role that sepsis plays in the occurrence of ROP and concluded that severe ROP was absent in infants more mature than 29 weeks' GA. However, when those patients had sepsis, there was an increased risk for any ROP stage and severe ROP. Both antenatal and neonatal exposure to inflammation seemed to contribute to increased ROP risk in preterm infants, which explains the "multiple hits hypothesis" for ROP.[6] Sepsis is frequently accompanied by hypotension, which may impair tissue perfusion and release angiogenesis factors secondary to hypoxic stress.[12]

Cytokines are secreted in response to several stimuli. High plasma levels of cytokines have been reported in newborn infants with early onset sepsis.[29] Given that proinflammatory cytokines have a half-life of only a few hours after exposure to inflammation, their concentrations should be performed as soon as possible to confirm neonatal sepsis.[16,29] Our patients' blood samples were collected within the first 72 hours after birth (median collection time, 18 hours), simultaneous with sepsis suspicion, and were obtained in the same sample for sepsis workup.

Because laboratory tests to detect ROP require more specificity, we used the best cutoff points of cytokines that enabled us to define the ROP treatable cases. Hence, IL-10 >574 pg/mL and IL-1β >1.22 pg/mL did not show any association with the late development of any stage or severe ROP among high-risk VLBW infants with clinical sepsis. On the other hand, IL-6 >357 pg/mL, IL-8 >216 pg/mL, and TNF-α >245 pg/mL were statically significant in the assessment of risk for the late development of ROP among infants with early-onset sepsis that already had high cytokine levels. Proinflammation at birth is associated with changes in the insulin growth factor (IGF) system, in which higher levels of IL-6 and IL-8 were associated with lower levels of IGF-I[30]; persistently lower IGF-I level is a known predictive factor for ROP.[31]

Our study did not show differences in the median cytokine plasma levels in VLBW preterm infants with early-onset clinical sepsis regarding later development of ROP, but this does not necessarily mean those difference do not exist. The small sample size and the low incidence of severe ROP in our NICU patients, as previously described, should be considered limitations to our study.[20,24,26,32] The study design did not permit an evaluation of sustained inflammation; cytokine plasma levels were obtained during sepsis workup and, in one instance, for ethical reasons. Despite the small number of patients in our study, they were all at high risk for ROP. We could not continue the study because in 2008 and 2009 none of our patients had severe ROP. Therefore, the sample size could not be enlarged.

When a cutoff point was used for each cytokine analyzed, we could find an association between cytokines released during inflammation or infection and a risk for progression to severe ROP requiring treatment. Further epidemiologic studies will be needed to explore other possible associations with ROP in this population.

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