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The Association of Cord Serum Cytokines with Neurodevelopmental Outcomes

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Abstract

OBJECTIVE—To test whether elevated umbilical cord serum inflammatory cytokine levels predicted subsequent cerebral palsy (CP) or neurodevelopmental delay (NDD).

STUDY DESIGN—Nested case-control analysis within a clinical trial of antenatal magnesium sulfate (MgSO4) before anticipated PTB for prevention of CP, with evaluation of surviving children at age 2. NDD was defined as a Bayley Psychomotor Developmental Index (PDI) and/or Mental Developmental Index (MDI) <70. Controls, defined as surviving children without CP and with Bayley PDI and MDI ≥85, were matched by race and gestational age. Cord serum was analyzed for IL-8, IL-1 β and TNF-α levels. Elevated cytokine levels were defined as ≥75th percentile in placebo-exposed controls. Analyses compared case/control cytokine levels, adjusting for MgSO4 exposure, gestational age, race/ethnicity and sociodemographic differences.

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RESULTS—Logistic regression analysis with 339 cases and 276 controls showed that elevated IL-8 and IL-1β were more common in cord blood serum from infants with subsequent low MDI compared with controls. After adjusting for additional confounders, the significant differences were no longer evident. Cytokine levels (IL-8, IL-1β, and TNF-α) were not elevated with CP or low PDI.

CONCLUSION—Cord serum IL-8, IL-1β, and TNF-α levels in preterm infants are not associated with subsequent CP or NDD.

Keywords

Cytokines; Cerebral Palsy; Neurodevelopmental Outcomes; Prematurity; Magnesium Sulfate

INTRODUCTION

Premature birth is a known risk factor for white matter injury and subsequent development of cerebral palsy (CP) and neurodevelopmental delay (NDD). CP is a disorder of posture or control of movement due to a non-progressive brain lesion[1]. In-utero infection has been hypothesized to increase the risk of CP and NDD, and numerous studies have associated positive clinical, histologic, or microbiological signs of infection with the development of CP or NDD[2–4]. It has been further hypothesized that even in the absence of overt infection, elevated cytokine levels may trigger lifelong neuronal damage. Pro-inflammatory cytokines, including IL-8, IL-1β, and TNF-α, have been implicated in the development of progressive neuronal damage during acute brain injury[5] leading to irreversible neurologic changes. IL-1β and TNF-α expression are increased in the first 48 hours following brain injury[6], and have been linked to hypoxic-ischemic encephalopathy (HIE) in perinatal animal models. Yoon et al have reported an association between elevated amniotic fluid cytokines and subsequent neonatal white matter lesions and cerebral palsy at age 3[7]. Elevated cerebrospinal fluid (CSF) and plasma cytokine levels have been reported in infants with HIE[8] and those who later developed poor neurological outcomes [9]. Additional studies have found elevated umbilical cord cytokine levels in infants with perinatal asphyxia or HIE[10–11]. However, umbilical cord cytokine levels have not generally predicted adverse long-term neurodevelopmental outcomes. Andrews and associates[12] found no correlation between cord levels of IL-6 and any neurodevelopmental markers in a cohort of 261 children delivered at < 32 weeks gestation and reevaluated at 6.8 +/− 0.7 years of age. Likewise, Nelson and colleagues[13] found, in a cohort of 34 children delivered at < 32 weeks gestation, no correlation between IL-1, IL-6, IL-8, or TNF-alpha levels in early neonatal blood samples and subsequent cerebral palsy diagnosed between ages 2 and 4.

Despite considerable effort and intervention strategies, the rate of cerebral palsy has not decreased substantially. Although the exact cause of CP is unknown, two mechanisms are most frequently cited: birth asphyxia and antenatal insult. Birth asphyxia is related to an acute hypoxic event at the time of delivery to a previously normal fetus, and is associated with subsequent HIE, a known risk factor for CP. Antenatal insult has been primarily ascribed to infectious causes or in-utero ischemic damage. A common pathway between these mechanisms is inflammation, which in turn is characterized by elevated levels of inflammatory cytokines. Both infection and ischemia are associated with increased levels of

cytokine release, and animal models have confirmed that elevated cytokines are capable of causing irreversible neuronal injury. Despite these findings, the direct cause of CP remains obscure, as not all infants with either of these risk factors develop CP, and some infants without these risk factors subsequently develop CP.

The goal of this study was to determine whether elevated levels of inflammatory cytokines IL-8, IL-1 β, or TNF-α in umbilical cord serum are associated with later development of CP or NDD. This information could provide a better understanding of the fetal inflammatory environment at the time of delivery and the role of inflammation in the pathogenesis of neuronal damage. Ultimately, we may be able to identify targets for in-utero therapy and prevent development of CP and NDD.

MATERIALS AND METHODS

This study was a planned secondary analysis of a double-blind, randomized controlled trial of magnesium sulfate (MgSO4) vs. placebo for the prevention of fetal or infant death or CP/NDD at age 2 conducted by the *Eunice Kennedy Shriver* National Institute of Health and Human Development Maternal-Fetal Medicine Units Network[14].

Inclusion criteria for the primary study included enrollment gestational age between 24 $^{0/7}$ weeks and 31 $^{6/7}$ weeks, singleton or twin gestation, advanced preterm labor, preterm premature rupture of membranes, or delivery planned within 24 hours. Exclusion criteria included delivery anticipated < 2 hrs, cervical dilation > 8 cm, fetal congenital anomalies or death, ruptured membranes < 22 weeks gestation, maternal hypertension or preeclampsia, maternal contraindication to MgSO4, or receipt of MgSO4 within the previous 12 hours.

Surviving infants underwent neurologic evaluation by an annually certified pediatrician or pediatric neurologist at 6, 12, and 24 months of age (corrected for prematurity). Infants with a normal neurologic examination at 1 year and who could walk 10 steps independently and had a bilateral pincher grasp were considered normal and did not undergo further physical examinations, although the scheduled neurodevelopmental examination was still performed.

The diagnosis of CP was made according to previously established criteria[1]. To assess severity, children diagnosed with CP were further classified by the Gross Motor Function Classification System (GMFCS)[15]. Scores of ≥ 2 were considered moderate or severe cerebral palsy.

Neurodevelopmental stages were assessed with use of the Bayley Scales of Infant Development II (BSID-II) [16]. Components include a Mental Development Index (MDI) and a Psychomotor Development Index (PDI). A score of 100 ±15 represents the standard ± one standard deviation. A score of <70 indicates significant impairment (> 2 SD below the mean).

All patient information was obtained from an existing de-identified database, and this study was considered exempt by the institutional review boards of both the University of Utah and Oregon Health & Sciences University.

Laboratory Assessments

Umbilical cord serum was collected at delivery and stored at −80° C. Duplicate cytokine assays were performed on each specimen by enzyme-linked immunosorbent assay kits (R&D Systems, Inc, Minneapolis, MN). Due to limitations in the amount of cord blood obtained, all 3 cytokine assays were not run on all subjects. The lower limits of detection are 0.438 pg/ml for IL-8, 0.008 for IL-1β, and 0.072 for TNF-α. Elevated levels were defined as the 75th percentile or above obtained in placebo-exposed controls. All samples were de-identified, thus blinding laboratory personnel from clinical outcome. We performed a nested case-control analysis. Subjects were matched by race/ethnicity and early preterm birth (<32 *vs* ≥32 weeks). Cases were children with CP or Bayley PDI and/or MDI <70. Controls were children without CP and with Bayley PDI and MDI ≥85.

Statistical Analyses

Cases and controls were frequency matched on race/ethnicity and gestational age at birth (<32 *vs* ≥32 weeks). Differences between cases and controls were tested using *t*-tests for continuous, normally distributed variables, Wilcoxon two-sample test for skewed distributions, and chi-square for frequencies. We used generalized estimating equations modeling outcomes (CP, MDI, PDI) as a logistic regression correcting for multiple maternal observations (i.e. twins) testing for the association between cases and controls and cytokine levels. We tested models for interaction between treatment group and cytokine levels. Models were adjusted for sociodemographic differences between cases and controls, MgSO4 treatment group, and matching criteria. Results are presented as odds ratios and 95% confidence intervals comparing those ≥ 75th percentile for each cytokine (vs < 75th percentile) for CP or NDD *vs* controls. Differences were considered statistically significant if the *P* value was less than 0.05. Analyses were performed using SAS v9.2 (SAS Institute, Cary, NC).

RESULTS

The primary study enrolled and randomized 2241 mothers carrying 2444 fetuses at risk for preterm delivery. Outcome data, including death or 2-year follow-up, was available for 2337 children (95.6%). One hundred fifteen children in the primary study met criteria for CP, and 501 children were diagnosed with NDD. Cord serum was available for 276 children who later developed CP or NDD (Table 1). Characteristics of cases and controls are presented in Table 2. There were no differences between cases and controls in the percentage of preterm premature ruptured membranes or spontaneous preterm birth with intact membranes. On average, cases weighed less at birth, had mothers who smoked and/or used drugs more frequently, and had less education than controls.

Descriptive statistics of cytokine levels are given in Table 3 for cases and controls. We found no significant interactions between cytokine levels and treatment group (MgSO4 vs. placebo) for any outcome in logistic regression models after adjusting for frequency matching on race/ethnicity and gestational age. In models adjusted for treatment group and frequency matching (race/ethnicity and gestational age), elevated IL-8 and IL-1β were more common in cord blood serum from infants with subsequent low MDI than controls, but there

were no differences for CP or low PDI (Table 4) even when adjusting for presence or absence of chorioamnionitis and time from steroid administration to delivery. IL-8 and TNF-α were not elevated with CP or NDD. After adjusting for confounders between the cases and controls (Table 4), IL-8 and IL-1β were no longer associated with MDI and, moreover, no cytokine levels were consistently elevated in infants with subsequent CP, low PDI, or NDD.

COMMENT

The proportion of patients with elevated umbilical cord serum levels of the inflammatory cytokines IL-8, IL-1β, and TNF-α in surviving children with either CP or NDD was not different from matched controls. Although treatment with MgSO4 in the parent trial decreased the incidence of CP in surviving children, treatment was not associated with altered cord serum inflammatory cytokine levels.

Perinatal inflammation and/or infection are commonly associated with spontaneous preterm birth, particularly early preterm birth[17–18] and are widely thought to increase the likelihood of subsequent neuropsychological dysfunction. These results support the findings of two earlier studies that also found no correlation of neonatal cytokine levels with either CP or NDD[13,19]. This is in contrast 2 studies of term infants that reported significant associations between infection and inflammation and subsequent CP[20–21] and suggests that mechanisms underlying subsequent development of CP may differ between affected individuals born remote from term and those born at term.

Matoba and colleagues[22] reported the cord blood serum values for 27 immune biomarkers from deliveries occurring from 23 weeks to term. They reported an increase in IL-8 and TNF-α values, but a decrease in IL-1β values, with preterm birth. Hansen-Pupp and colleagues[23] also reported an association between IL-8 and TNF-α cord serum levels in survivors of a cohort of very preterm infants who subsequently developed CP. While these findings differ somewhat from ours, it should be noted that in neither report were the etiology(ies) of their preterm birth population described, raising the possibility that some of their findings may be more representative of ongoing pregnancies delivered for reasons other than premature labor (fetal growth restriction, preeclampsia, etc).

A strength of this study includes its longitudinal design and careful, standardized follow-up assessments performed by trained and certified research staff. This is similar in design to the report of Andrews and associates[12] and distinguishes these two studies from other retrospective cohorts.

A potential weakness of this report is the absence of IL-6 assays, although the study of Andrews et al demonstrated no significant association between cord serum IL-6 levels and subsequent NDD, CP or low intelligence quotients[12]. In addition, systematic information on perinatal bacterial cultures and placental pathology was not available from the primary study. Of interest, however, Grether and associates were unable to demonstrate any association in a cohort of children born prior to 32 weeks gestation between clinical or placental pathology evidence of perinatal infection or inflammation and subsequent development of CP[19]. It is also possible that this secondary analysis was underpowered for the identification of more modest differences between cases and controls.

Likewise, we were unable to obtain follow up data in surviving children beyond age 2. Although follow-up to age 2 is generally adequate for the diagnosis of major motor disability, longer follow up would be necessary to exclude more subtle neuropsychological deficits. In addition, the sample size for this analysis was limited both by the relative infrequency of the primary outcomes in the parent trial as well as the availability and amount of cord serum.

Similar to the report of Andrews et al[12], our measurement of cord blood serum inflammatory markers also provides information on events immediately preceding delivery. While this more closely approximates the intrauterine milieu than do samples obtained at 2–3 days after birth[16], this report can still not provide information on the in-utero environment remote from delivery.

Our data suggest that cord serum markers of inflammation in preterm infants do not predict CP. Likewise, while administration of MgSO4 decreased the incidence of CP in survivors, this outcome does not appear to be mediated by any changes in these inflammatory markers.

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APPENDIX

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Table 1

Number of Subjects with Available Cord Serum

(Some Subjects Had More Than One Outcome)

Diagnosis	N with diagnosis	N (%) cord serum available
Cerebral palsy	102	52 (51.0%)
Neurodevelopmental delay		
PDI < 70	293	154 (52.6%)
MDI < 70	333	182 (54.7%)
PDI and MDI < 70	151	77 (51.0%)

Table 2

Characteristics* of Cases and Controls

Characteristic	Control (n=339)	Case (n=276)	*P* **
Mothers**	333	264	
MgSO4 treatment group	155 (46.6)	119 (45.1)	0.72
Maternal age (y)	26.1 ± 6.1	26.1 ± 6.6	0.98
Race/Ethnicity			0.69
African American	146 (43.8)	112 (42.4)	
Caucasian	117 (35.1)	88 (33.3)	
Hispanic	65 (19.5)	57 (21.6)	
Other	5 (1.5)	7 (2.7)	
Education (y)	12.2±2.7	11.5±2.6	<0.001
Smoked during pregnancy	73 (21.9)	82 (31.1)	0.01
Alcohol during pregnancy	20 (6.0)	27 (10.2)	0.06
Drug use during pregnancy	21 (6.3)	33 (12.5)	0.009
Steroids given	326 (97.9)	256 (97.0)	0.47
Time from steroid treatment to delivery (days)	5 (2 to 12)#	6 (2 to 15)#	0.09##
Chorioamnionitis	41 (12.3)	33 (12.5)	0.94
Neonates			
Gestational age at birth (wks)	30.2 ± 2.3	29.5 ± 3.0	0.001
Birthweight (g)	1505 ± 485	1363 ± 513	<0.001

* n (%) or Mean ± SD

** T-test for continuous variables and Chi-square for frequencies, except where indicated.

*** n reflects fewer mothers due to twin gestations

Median (25^{th} to 75^{th} percentile)

Wilcoxon rank sums test

Table 3

Cytokine Levels by Case-Control Status and Treatment Group

Cytokine levels (all in pg/mL)	Control (n=339)		Case (n=276)	
	n*	Median (25^{th},75^{th} Percentile)	n*	Median (25^{th},75^{th} Percentile)
IL-8	338	0.755 (<0.438, 199.72)	274	32.050 (<0.438, 287.46)
Placebo		1.290 (<0.438, 207.730)		37.040 (<0.438, 285.330)
MgSO4		<0.438 (<0.438, 165.98)		25.380 (<0.438, 287.460)
IL-1β	304	0.896 (0.120, 5.430)	251	1.77 (0.211, 6.13)
Placebo		1.192 (0.104, 5.586)		1.827 (0.246, 5.792)
MgSO4		0.813 (0.151, 4.479)		1.618 (0.193, 7.100)
TNF-α	247	2.527 (1.671, 3.956)	191	2.543 (1.718, 4.017)
Placebo		2.443 (1.656, 4.084)		2.556 (1.718, 4.017)
MgSO4		2.586 (1.692, 3.635)		2.543 (1.781, 3.928)

*Sample numbers vary based on amount of umbilical cord serum available for evaluation

Table 4

Odds Ratios for Cytokines (≥ 75th Percentile of Placebo Control Levels) by CP/NDD Outcomes

		Adjusted for Matching*			Full Adjustment**		
Outcome	**Cytokines*** **	**Odds Ratio**	**95% CI**	***P***	**Odds Ratio**	**95% CI**	***P***
CP	IL-8	1.30	0.67–2.52	0.44	0.94	0.47–1.86	0.85
	IL-1β	1.46	0.75–2.85	0.27	1.09	0.54–2.22	0.81
	TNF-α	1.05	0.50–2.22	0.90	1.45	0.61–3.43	0.40
PDI<70	IL-8	1.41	0.92–2.17	0.11	0.79	0.51–1.24	0.31
	IL-1β	1.29	0.82–2.04	0.28	0.99	0.60–1.61	0.95
	TNF-α	0.83	0.48–1.45	0.52	1.53	0.85–2.76	0.16
MDI<70	IL-8	1.51	1.01–2.26	0.05	0.78	0.51–1.19	0.25
	IL-1β	1.59	1.04–2.42	0.03	0.74	0.47–1.15	0.18
	TNF-α	0.92	0.55–1.53	0.75	1.39	0.81–2.38	0.23

* Adjusted for MgSO4 exposure, race/ethnicity, and gestational age at birth.

** Adjusted for MgSO4 exposure, gestational age at birth, race/ethnicity, birth weight, maternal drug use and education.

*** ≥ 75th percentile vs < 75th