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Amniotic Fluid Infection, Cytokine Levels, and Mortality and Adverse Pulmonary, Intestinal, and Neurologic Outcomes in Infants at 32 Weeks' Gestation or Less

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To what extent the risks of neonatal morbidities are directly related to premature birth or to biological mechanisms of preterm birth remains uncertain. We aimed to examine the effect of exposure to amniotic fluid (AF) infection and elevated cytokine levels on the mortality and pulmonary, intestinal, and neurologic outcomes of preterm infants, and whether these associations persist after adjustment for gestational age at birth. This retrospective cohort study included 152 premature singleton infants who were born at ≤ 32 weeks. AF obtained by amniocentesis was cultured; and interleukin-6 (IL-6) and IL-8 levels in AF were determined. The primary outcome was adverse perinatal outcome defined as the presence of one or more of the followings: stillbirth, neonatal death, bronchopulmonary dysplasia, necrotizing enterocolitis, intraventricular hemorrhage, and periventricular leukomalacia. Logistic regression analysis was adjusted for gestational age at birth and other potential confounders. In bivariate analyses, elevated AF IL-6 and IL-8 levels were significantly associated with adverse perinatal outcome. These results were not changed after adjusting for potential confounders, such as low Apgar scores, mechanical ventilation, and surfactant application. However, the independent effect of elevated cytokine levels in AF disappeared when additionally adjusted for low gestational age at birth; consequently, low gestational age remained strongly associated with the risk of adverse perinatal outcome. In conclusion, elevated levels of pro-inflammatory cytokines in AF are associated with increased risk of adverse perinatal outcomes, but this risk is not independent of low gestational age at birth. Culture-proven AF infection is not associated with this risk.

Keywords: Amniotic Fluid Infection; Cytokines; Gestational Age; Perinatal Outcome; Preterm Birth

INTRODUCTION

Early preterm birth (defined as delivery before 32 weeks) is a major cause of infant morbidity and mortality, and the determinant for these is known to be gestational age at birth (1,2). Among very preterm infants staying in the intensive care unit, the occurrence of bronchopulmonary dysplasia (BPD), necrotizing enterocolitis (NEC), severe intraventricular hemorrhage (IVH), and periventricular leukomalacia (PVL) is a major concern because these morbidities are at increased risk for later death or neurosensory impairments (3-5). However, to what extent the risks of these morbidities are directly related to premature birth or to biological mechanisms of preterm birth remains uncertain.

Evidence has shown that intra-amniotic infection and/or inflammation, defined as elevated pro-inflammatory cytokine levels in amniotic fluid (AF), is strongly associated with preterm birth in women with preterm labor or preterm premature rupture of membranes (PPROM) (6-8). Thus, a large proportion of very preterm infants born from these women are already exposed to AF inflammation in utero. In fact, several studies have shown a systemic inflammatory response, with elevated AF cortisol levels, in the fetus exposed to infected AF (9-11). In particular, it has been reported that AF inflammation may also contribute to the pathogenesis of adverse short- and long-term neonatal pulmonary and neurologic sequelae, especially in very preterm infants (12-15). However, most of these studies have been relatively small (13,14,16), and recruited patients with non-infection-mediated preterm birth (e.g., preeclampsia) (14,16) or premature infants with advanced gestational age as a study cohort (12,14-17), adjusted for gestational age at enrolment but not at birth (17), and did not consider whether AF inflammation can modify the effect of gestational age on infant morbidities (12-16). As a result, studies that examined the risk of AF inflammation for infant morbidities may have concluded that AF inflammation poses a high risk for these morbidities. The purposes of

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this study were to examine the effect of exposure to AF infection and elevated pro-inflammatory cytokine levels on the mortality and pulmonary, intestinal, and neurologic outcomes of preterm infants, and to determine whether these associations persist after adjustments are made for gestational age at birth.

MATERIALS AND METHODS

In this retrospective cohort study, we included all consecutive women who underwent amniocentesis and their infants who were admitted to a neonatal intensive care unit (NICU) at the Seoul National University Bundang Hospital (Seongnam, Korea) between August 2004 and August 2013. The inclusion criteria were: 1) singleton gestation; 2) preterm labor or PPROM; and 3) delivery with a gestational age between 23+0 and 32+0 weeks. Exclusion criteria included major congenital anomalies, twin and higher order multiple births, and transfer to another hospital after amniocentesis. Gestational age was calculated from the first day of the last menstrual period, and confirmed by first or second trimester ultrasound. The primary outcome measure was adverse perinatal outcome defined as the presence of one or more of the following: mortality (stillbirth or neonatal death), BPD, NEC, IVH, and PVL. Additionally, we investigated the associations of 4 individual morbidity variables with AF infection and elevated cytokine levels in infants who survived for at least 30 days after birth.

AF was obtained aseptically by transabdominal amniocentesis, and was cultured for aerobic and anaerobic bacteria and genital mycoplasma according to previously described methods (18). The remaining AF was centrifuged at 1,500 g at 4°C for 10 minutes, and the supernatant was aliquoted and immediately stored at –70°C until assayed. Interleukin-6 (IL-6) and IL-8 in stored AF were measured by an enzyme-linked immunosorbent assay human DuoSet Kit (R & D Systems, Minneapolis, MN, USA). The range of the IL-6 and IL-8 standard curves was 7.8–600 and 31.2–2,000 pg/mL, respectively. All samples were measured in duplicate. The intra- and inter-assay coefficients of variation for 2 different proteins were $< 10\%$ and $< 15\%$, respectively. Culture proven AF infection was defined as an infection with positive cultures of AF, regardless of the inflammatory state of the AF.

Our primary explanatory variables for outcome parameters were AF culture and AF IL-6 and IL-8. The other explanatory variables investigated were maternal and infantile demographic characteristics (maternal age, parity, gestational age at birth, birth weight, and gender), cause of preterm delivery, antenatal use of medications, mode of delivery, clinical diagnosis of chorioamnionitis, Apgar scores at 1 and 5 minutes, mechanical ventilation, and surfactant application.

Diagnostic criteria and management of preterm labor and PPROM have been described in detail elsewhere (18,19). The following clinical definitions have also been described in detail elsewhere: clinical chorioamnionitis, BPD, NEC, IVH, and PVL (6,19-23). The severity of NEC was graded according to the modified Bell's staging criteria; and stage IIa or higher was defined as the presence of NEC (21). The severity of IVH was graded according to the criteria of Papile et al. (22); and grade II–IV was defined as the presence of IVH.

Descriptive statistics were calculated to characterize all variables in the study cohort. Bivariate analysis of the association of adverse perinatal outcome with risk factors was conducted using Student's t-test, the Mann-Whitney U test, Fisher's exact test, or χ^2 test, as appropriate. The normality for continuous variables in groups was determined using the Shapiro-Wilk test. Odds ratio (OR) with 95% confidence interval (CI) was calculated for categorical variables, whereas continuous variables were summarized by the mean and standard deviation (SD) or by the median and range if not normally distributed. Multivariate logistic regression was then performed to determine the independent relationships of adverse perinatal outcome with AF infection, as well as with elevated cytokine levels, after adjusting for baseline variables showing a significant correlation or tendency towards an association with this outcome in bivariate analysis ($P < 0.1$). We checked for multicollinearity among the variables by using a χ^2-test, the Pearson's or Spearman's rank correlation test and variance inflation factor (VIF). Variables with a high correlation were summarized in the analysis; gestational age at birth alone was included instead of both gestational age and birth weight, and Apgar score at 5 minutes alone was included instead of Apgar score at both 1 minute and 5 minutes. Although the AF IL-6 and IL-8 levels were highly correlated, as main explanatory variables of interest, they were analyzed in separate models. Potential interactions between independent variables were evaluated. The receiver operating characteristic (ROC) curve was used to identify the best cut-off values for independent risk factors in predicting adverse perinatal outcome. The optimal cut-off values were obtained from the Youden index maximum ([sensitivity + specificity] – 1). All statistical analyses were performed using SPSS for Windows version 20.0 (IBM Corp., Armonk, NY, USA). *P* values of < 0.05 were considered statistically significant.

Ethics statement

The study was approved by the Institutional Review Board at Seoul National University Bundang Hospital (IRB No. B-1105/128-102). All women provided written informed consent for the amniocentesis procedure and use of AF samples.

RESULTS

During the study period, 152 women with preterm labor and intact membranes (n = 81) or PPROM (n = 71) and their babies

met eligibility criteria and were included in the final analysis. Infants who were transferred to another hospital prior to evaluation (n = 1) were excluded. The mean gestational age at birth was 29.1 weeks (SD 2.2 weeks; range 23.4–32.0 weeks) and the mean birth weight was 1,283 g (SD, 376 g; range, 500–2,275 g). The overall perinatal mortality was 5.2% with 1 stillbirth and 7 neonatal deaths occurring in the first 30 days of life. Seventy-four of 152 infants (48.7%) had an adverse perinatal outcome. Among 144 infants who survived for at least 30 days after birth, any stage BPD, NEC (≥ stage II), IVH (≥ grade II) or PVL developed in 56 (39%), 13 (9%), 10 (7%), and 5 (4%), respectively. Microorganisms were isolated from 61 (42.4%) of 144 AF specimens. The microorganisms isolated from the amniotic cavity included *Ureaplasma urealyticum* (n = 55), *Mycoplasma hominis* (n = 43), *Escherichia coli* (n = 1), *Streptococcus* spp. (n = 4), *Staphylococcus aureus* (n = 4), *Candida albicans* (n = 1), *Bacillus* spp. (n = 1), gram-positive bacteria (n = 5), and gram-negative bacteria (n = 2). Polymicrobial invasion was present in 43 of 66 cases (65.1%).

The levels of AF IL-6 and IL-8 were significantly correlated with each other ($r = 0.799$; $P < 0.001$). Gestational age at birth was also significantly correlated with AF IL-6 ($r = -0.270$; $P = 0.001$), AF IL-8 levels ($r = -0.306$; $P < 0.001$), or gestational age at amniocentesis ($r = 0.726$; $P < 0.001$), whereas gestational age at the time of amniocentesis was correlated with neither AF IL-6 ($r = -0.091$; $P = 0.263$) nor AF IL-8 levels ($r = -0.057$; $P = 0.484$).

Based on the bivariate analyses (Tables 1 and 2), elevated AF IL-6 and IL-8 levels were significantly associated with adverse perinatal outcome, as well as with BPD and IVH. Interactions between gestational age at birth and AF IL-6 and IL-8 levels were not found for the presence of adverse perinatal outcome, as well as of BPD and IVH (Fig. 1). The mortality, NEC and PVL were not associated with elevated IL-6 and IL-8 levels in AF. Intra-amniotic infection was not associated with adverse perinatal outcome, mortality and the 4 individual morbidity variables.

In bivariate analyses (Tables 1 and 2), the demographic and perinatal variables significantly associated with adverse perinatal outcome were gestational age at birth and amniocentesis, birth weight, low Apgar scores (< 7) at 1 and 5 minutes, mechanical ventilation, and surfactant application. The results for BPD and NEC were largely the same as those for adverse perinatal outcome. For IVH, an association was limited to gestational age at birth, birth weight, and surfactant application, and the asso-

Table 1. Demographic and perinatal characteristics in relation to the occurrence of composite adverse perinatal outcome

Characteristics	Composite adverse perinatal outcome (n = 74)	No composite adverse perinatal outcome (n = 78)	*P* value
Maternal age, yr	31 (21–40)	32 (22–41)	0.481
Nulliparity	35 (47.3)	35 (44.9)	0.764
No. of patients with PPROM	31 (41.9)	40 (51.3)	0.246
Gestational age at amniocentesis, wk	26.4 (18.0–31.3)	29.3 (19.3–32.0)	< 0.001
Antenatal corticosteroids	67 (90.5)	75 (96.2)	0.163
Antenatal antibiotics	57 (77.0)	63 (80.8)	0.572
Antenatal tocolytics	62 (47.7)	68 (87.2)	0.552
Cesarean delivery	33 (44.6)	43 (55.1)	0.194
Positive AF cultures	33 (44.6)	33 (42.3)	0.776
AF IL-6, ng/mL	8.7 (0.078–128.600)	4.2 (0.078–91.200)	0.037
AF IL-8, ng/mL	5.1 (0.031–101.300)	1.2 (0.031–87.600)	0.002
Clinical chorioamnionitis	7 (9.5)	8 (10.3)	0.869
Birth weight, g	1,060 (500–1,660)	1,520 (680–2,275)	< 0.001
Amniocentesis-to-delivery intervals, day	8.2 ± 14.1	9.2 ± 14.2	0.675
Gestational age at birth, wk	27.9 (23.4–31.6)	30.3 (24.5–32.0)	< 0.001
Male gender	41 (55.4)	40 (51.3)	0.611
Apgar score < 7 at 1 min	64 (84.5)	51 (65.3)	0.002
Apgar score < 7 at 5 min	40 (54.1)	22 (28.2)	0.001
Mechanical ventilation	54 (73.0)	33 (42.3)	< 0.001
Surfactant application	43 (58.1)	15 (19.2)	< 0.001
BPD all stages*	56 (38.9)	-	-
Mild BPD	25 (17.4)		
Moderate BPD	19 (13.2)		
Severe BPD	12 (8.3)		
NEC, ≥ stage II*	13 (9.0)	-	-
IVH, ≥ grade II*	10 (6.9)	-	-
PVL*	5 (3.5)	-	-

Data are presented as number (percentage), median (range) or mean ± SD.
AF = amniotic fluid, BPD = bronchopulmonary dysplasia, IL = interleukin, PPROM = preterm premature rupture of membranes, NEC = necrotizing enterocolitis, IVH = intraventricular hemorrhage, PVL = periventricular leukomalacia, SD = standard deviation.
*Based on 144 subjects who survived for at least 30 days after birth.

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ciation for PVL was further limited to amniocentesis-to-delivery interval.

The associations of adverse perinatal outcome, mortality and individual morbidity variables with AF infection as well as with elevated cytokine levels after adjusting for baseline parameters were compared using multivariate logistic regression analyses (Table 3). Because AF IL-6 and IL-8 levels were highly correlated, 2 separate multivariate analyses were carried out, whereby each excluded one of these terms. For adverse perinatal outcome, BPD and IVH, elevated AF IL-6 and IL-8 levels that were significantly associated in bivariate analyses remained significant risk factors for the aforementioned 3 outcomes, when we adjusted for low Apgar scores at 5 minutes, antenatal corticosteroids, mechanical ventilation, and surfactant application (AF IL-6 [ng/mL] for adverse perinatal outcome: adjusted OR [aOR], 1.022; 95% CI, 1.005–1.040; $P = 0.010$; AF IL-8 [ng/mL] for adverse perinatal outcome: aOR, 1.025; 95% CI, 1.005–1.046; $P = 0.016$, data on BPD and IVH not shown). However, the independent effect of elevated IL-6 and IL-8 levels in AF disappeared when additionally adjusted for low gestational age at birth; as a result, low gestational age at birth remained, in regression analyses, strongly associated with the risk of an adverse perinatal outcome and BPD (Table 3). The range of VIF in our models was from 1.012 to 1.821 which indicated absence of multicollinearity between our explanatory variables. The area under the curve (AUC) value for gestational age at birth predicting adverse perinatal outcome was 0.879 (95% CI, 0.822–0.936), and a cutoff value of < 29.0 weeks was identified as the optimal threshold, with a sensitivity of 81.1% and specificity of 82.1%.

Table 2. Bivariate analysis of AF cultures and cytokines and other risk factors for primary outcome variables

Outcome variables and potential risk factors	OR (95% CI) or median in infants with vs. without the indicated outcome variable	*P* value
Mortality		
Gestational age at amniocentesis, wk	23.3 vs. 28.3	0.001
Gestational age at birth, wk	24.7 vs. 29.1	< 0.001
Birth weight, g	755 vs. 1,305	< 0.001
Apgar score < 7 at 5 min	6.519 (1.334–31.841)	0.016
Positive AF culture	0.441 (0.101–1.916)	0.275
AF IL-6, ng/mL	6.816 vs. 6.555	0.610
AF IL-8, ng/mL	25.593 vs. 2.384	0.075
BPD*		
Gestational age at amniocentesis, wk	26.4 vs. 29.3	< 0.001
Gestational age at birth, wk	27.6 vs. 30.2	< 0.001
Birth weight, g	1,038 vs. 1,510	< 0.001
Apgar score < 7 at 1 min	2.437 (1.049–5.659)	0.035
Apgar score < 7 at 5 min	2.707 (1.345–5.448)	0.005
Mechanical ventilation	4.825 (2.245–10.369)	< 0.001
Surfactant application	7.000 (3.297–14.864)	< 0.001
Positive AF culture	1.165 (0.592–2.292)	0.658
AF IL-6, ng/mL	10.985 vs. 3.968	0.005
AF IL-8, ng/mL	6.131 vs. 1.158	< 0.001
NEC, ≥ stage II*		
Gestational age at birth, wk	28.2 vs. 29.3	0.005
Amniocentesis-to-delivery intervals, day	1.3 vs. 3.7	0.048
Birth weight, g	1,015 vs. 1,345	0.005
Mechanical ventilation	4.648 (0.991–21.797)	0.041
Surfactant application	4.300 (1.255–14.737)	0.018
Nulliparity	0.202 (0.043–0.949)	0.038
Antenatal tocolytics	0.291 (0.080–1.062)	0.072
Positive AF culture	0.378 (0.099–1.436)	0.238
AF IL-6, ng/mL	4.795 vs. 6.596	0.875
AF IL-8, ng/mL	5.364 vs. 2.020	0.875
IVH, ≥ grade II*		
Gestational age at birth, wk	26.5 vs. 29.3	0.002
Birth weight, g	1,008 vs. 1,353	0.005
Surfactant application	4.319 (1.067–17.485)	0.041
Antenatal corticosteroids	0.188 (0.033–1.082)	0.097
Positive AF culture	2.155 (0.581–7.994)	0.251
AF IL-6, ng/mL	15.416 vs. 4.998	0.019
AF IL-8, ng/mL	5.958 vs. 1.930	0.024
PVL*		
Amniocentesis-to-delivery intervals, day	18.0 vs. 3.1	0.038
Positive AF culture	0.904 (0.146–5.582)	> 0.990
AF IL-6, ng/mL	3.770 vs. 6.596	0.659
AF IL-8, ng/mL	0.675 vs. 2.420	0.308

Median in infants with or without the indicated outcome variable is given for all numerical variables, OR is given for all categorical variables. Only predictors with $P < 0.1$ are presented, except for the primary explanatory variables (i.e., AF culture and AF IL-6 and IL-8).

AF = amniotic fluid, CI = confidence interval, IL = interleukin, OR = odds ratio, NEC = necrotizing enterocolitis, IVH = intraventricular hemorrhage, PVL = periventricular leukomalacia.

*Based on 144 subjects who survived for at least 30 days after birth.

DISCUSSION

The principal findings of this study are as follows: 1) in bivariate analysis, elevated AF IL-6 and IL-8 levels were significantly associated with the risk of adverse perinatal outcome, but this relationship disappeared after adjustment for the gestational age at birth; and 2) culture-proven AF infection was not associated with the development of adverse perinatal outcomes. These observations are consistent with a recent study by Combs et al. (17) showing that AF IL-6 level was stronger than microbial invasion of the amniotic cavity as a predictor of composite perinatal morbidity and death, but IL-6 level was no longer predictive after adjustment for gestational age at delivery. Collectively, these findings suggest that the role of prenatal exposure to pro-inflammatory cytokines in the development of adverse neonatal outcomes may be overestimated, and underscored the importance of gestational age at preterm delivery to the risk of adverse neonatal outcomes in infants born at ≤ 32 weeks.

In the bivariate analyses, the risk of adverse perinatal outcome for infants exposed to elevated inflammatory cytokine levels in AF was increased, compared to those not exposed in utero. However, when low gestational age at birth was included in the multivariate model, the risk of elevated pro-inflammatory cytokine levels in AF disappeared, indicating the increased risk of elevated levels for adverse perinatal outcome may be mainly due to the large proportion of infants exposed to elevated levels who

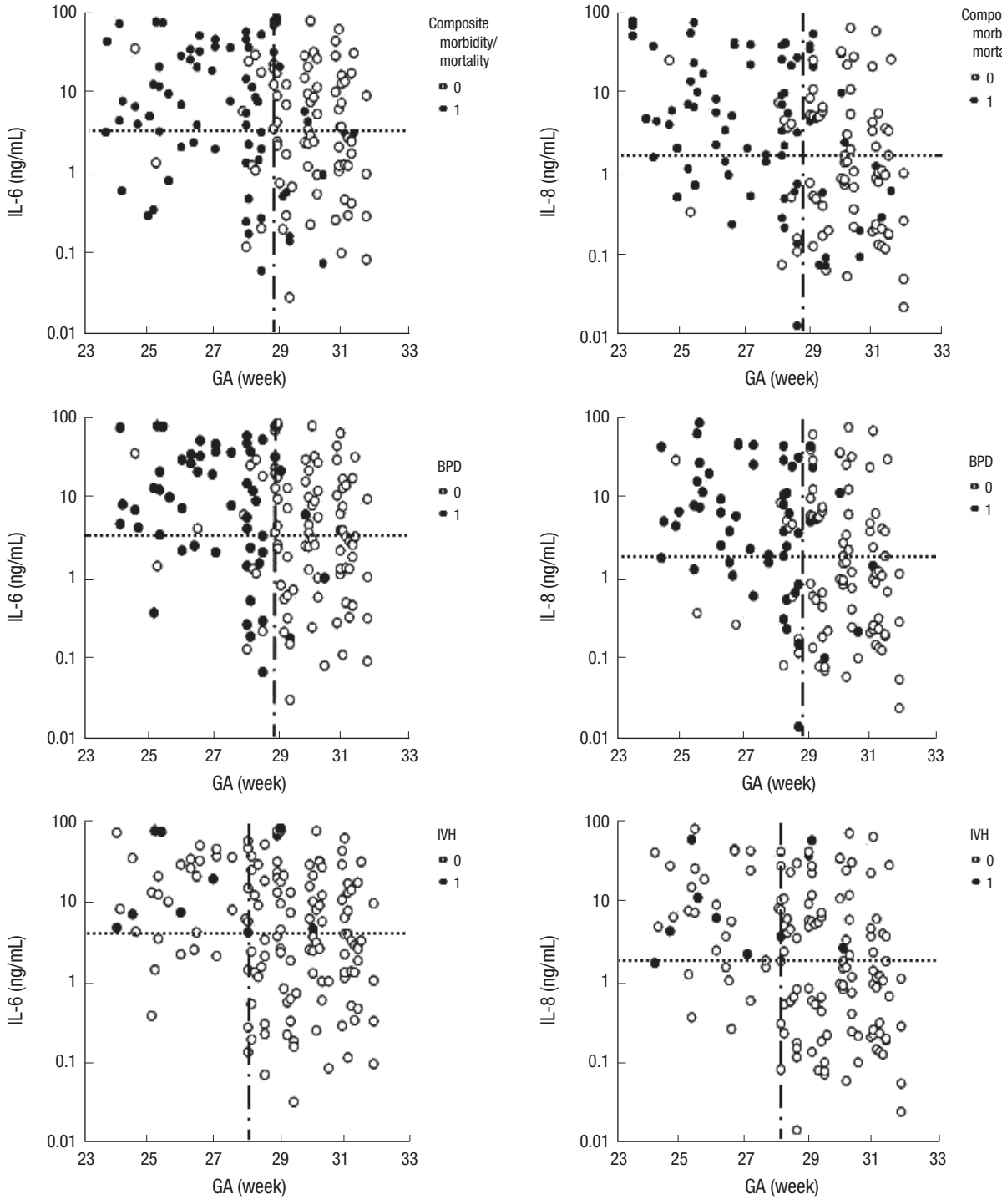

Fig. 1. The presence of composite adverse perinatal outcome, BPD, and IVH according to AF IL-6 and IL-8 levels and GA at birth. (.....): cut-offs for IL-6 and IL-8 levels; (.—.—): cut-off for GA at birth.
AF = amniotic fluid, BPD = bronchopulmonary dysplasia, GA = gestational age, IVH = intraventricular hemorrhage, IL = interleukin.

were born at lower gestational age (Fig. 1), given the tight inverse correlation between AF IL-6 and IL-8 levels and gestational age at birth. These findings suggest that delaying delivery in women with preterm labor or PPROM might reduce adverse outcomes in infants, especially in very preterm infants born at less than 29 weeks' gestation, despite prolonged exposure of the fetus to AF infection/inflammation in utero. Several case reports should be noted, in which antibiotic therapy eradicated microorganisms in AF in some patients with AF infection/inflammation, with subsequent continuation of pregnancy near or at term (24-26).

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Table 3. Regression analysis of risk factors for primary outcome variables

Outcome variables	Predictors*	OR (95% CI)	*P* value	*P* value for interaction term†
Adverse perinatal outcome	GA at birth, wk	0.421 (0.299–0.567)	< 0.001	-
	Apgar score < 7 at 5 min	0.694 (0.249–1.935)	0.485	-
	Mechanical ventilation	1.171 (0.409–3.358)	0.769	-
	Surfactant application	3.731 (1.160–12.006)	0.027	-
	AF IL-6, ng/mL‡	1.015 (0.996–1.034)	0.128	0.348
	AF IL-8, ng/mL‡	1.018 (0.994–1.043)	0.141	0.369
BPD§	GA at birth, wk	0.345 (0.233–0.510)	< 0.001	-
	Apgar score < 7 at 5 min	0.467 (0.138–1.581)	0.221	-
	Mechanical ventilation	1.421 (0.428–4.716)	0.566	-
	Surfactant application	4.885 (1.361–17.530)	0.015	-
	AF IL-6, ng/mL‡	1.014 (0.995–1.034)	0.154	0.601
	AF IL-8, ng/mL‡	1.015 (0.988–1.043)	0.282	0.704
NEC, ≥ stage II§	GA at birth, wk	0.764 (0.560–1.044)	0.091	-
	Amniocentesis-to-delivery intervals, day	0.997 (0.993–1.001)	0.153	-
	Nulliparity	0.176 (0.034–0.913)	0.039	-
	Tocolytics	0.369 (0.080–1.698)	0.200	-
	Mechanical ventilation	1.896 (0.257–14.009)	0.531	-
	Surfactant application	1.843 (0.364–9.339)	0.460	-
IVH, ≥ grade II§	GA at birth, wk	0.726 (0.492–1.072)	0.107	-
	Antenatal corticosteroids	0.405 (0.044–3.756)	0.426	-
	Surfactant application	1.745 (0.328–9.297)	0.514	-
	AF IL-6, ng/mL‖	1.019 (0.998–1.041)	0.070	0.151
	AF IL-8, ng/mL‖	1.016 (0.988–1.044)	0.272	0.140

AF = amniotic fluid, BPD = bronchopulmonary dysplasia, CI = confidence interval, GA = gestational age, IL = interleukin, IVH = intraventricular hemorrhage, NEC = necrotizing enterocolitis, OR = odds ratio.

*AF IL-6 and IL-8 levels were highly correlated with each other ($r = 0.804$, $P < 0.001$), and thus 2 separate regression models were used in multivariate analyses, in which each excluded one of these terms; †Interaction term between gestational age at birth and AF IL-6 or AF IL-8 levels; ‡Adjusted for gestational age at birth, low Apgar scores (< 7) at 5 minutes, mechanical ventilation, and surfactant application; §Based on 144 subjects who survived for at least 30 days after birth; ‖Adjusted for gestational age at birth, antenatal corticosteroids, and surfactant application.

Further studies are needed to confirm whether or not prolongation of pregnancy would be beneficial for the subgroup of women with both AF infection/inflammation and threatened birth of an infant of extremely low gestational age (e.g., 22–28 weeks of gestation), if antibiotic therapy can be targeted appropriately.

Previous studies have reported an association between elevated AF pro-inflammatory cytokines and the development of BPD, independent of gestational age at birth (13,16). However, these studies have the limitation of a small sample size (13,16) and the inclusion of a heterogeneous group of patients with regard to disease entity (16), cases with advanced gestational age as a study cohort (16), and the use of diagnostic criteria that differ from new criteria (20) for diagnosis and severity of BPD proposed by the National Institutes of Health in 2001 (13,16). In contrast to the results of these previous studies, we found that elevated AF IL-6 and IL-8 levels were significantly associated with the subsequent development of BPD in the bivariate analyses, but the association disappeared after adjustment for the gestational age at birth; traditional risk factors for adverse neonatal outcomes, such as gestational age at birth, rather than AF inflammation, remained significantly independently associated with BPD. Similarly, our observation of a lack of association between AF inflammation and grade II–IV IVH in gestational age-adjusted analysis, was different from the finding of only one study in which this association was investigated (15). This discrepancy may be related to which factor was adjusted for in the analyses (birth weight vs. gestational age at birth), which cytokines were used to define AF inflammation (tumor necrosis factor-α vs. IL-6 and IL-8), and different gestational age at enrolment (≤ 34 weeks vs. ≤ 32 weeks). In terms of association of AF inflammation with NEC, similar explanations can be applied to the discrepancy between our study and that of Hitti et al. (15).

The finding that positive AF cultures were not associated with adverse perinatal outcomes is consistent with previous studies (13,14,16). This observation is not surprising given 1) a recent report indicating that microbial colonization without inflammatory response appears relatively benign (17); and 2) the limitation of the standard microbiological culture technique used in the current study, which depends on many factors, including inoculum size, whether samples were obtained from an infected site, and the properties of the strains. With regard to microbial footprints in AF, a recent study in which culture and/or polymerase chain reaction (PCR) technique were used to detect *Ureaplasma* spp. showed that the presence of *Ureaplasma* spp. at the time of preterm cesarean delivery was strongly associated with BPD and IVH in preterm infants, even after adjustment for multiple risk factors (27).

A recent study suggested a synergistic detrimental effect of gestational age at birth and histologic and clinical chorioamni-

onitis as surrogate markers for prenatal inflammation on adverse neonatal outcomes (28). However, we did not find an interaction between gestational age at birth and elevated AF pro-inflammatory cytokines in association with adverse perinatal outcomes. The reason for this discrepancy may be related to the time difference at which the measured markers were taken to assess whether adverse neonatal outcomes were present. The measured markers in a previous study, which were late findings of antenatal inflammation, were assessed in samples taken at or near the time of delivery, and thus directly reflected adverse neonatal outcomes, whereas the measured markers in the current study, being early findings of antenatal inflammation, were assessed in samples taken remote from delivery. In fact, we found that gestational age at amniocentesis modified the association with elevated AF IL-8 levels on adverse perinatal outcome (data not shown).

The current study has several limitations. First, we did not use molecular techniques, such as PCR, to diagnose cases with actual AF infection that were falsely negative by standard microbiological technique for AF culture. Second, there were a relatively small number of patients in this study, which resulted in a low prevalence of certain individual adverse perinatal outcomes, such as PVL and mortality, with diminished statistical power for comparison with other groups. Third, the results of AF culture and white blood cell (WBC) counts were routinely reported to caregivers, which might affect the timing of delivery and initiation of antibiotic and tocolytic therapy. However, this bias is unlikely to change our main findings, because AF culture results take several days to become available, the results of AF IL-6 and IL-8 were unavailable to the caregivers for clinical use, and caregivers were not likely to deliver a pregnant woman solely based on the results of AF culture and WBC counts.

In conclusion, elevated levels of pro-inflammatory cytokines in AF are associated with increased risk of adverse perinatal outcomes, but this risk is not independent of low gestational age at birth. Low gestational age at birth is a major contributor to the risk of adverse perinatal outcomes. Culture-proven AF infection is not associated with the development of adverse perinatal outcomes.

DISCLOSURE

The authors have no potential conflicts of interest to disclose.

AUTHOR CONTRIBUTION

Conceptualization: Park KH. Data curation: Jung EY, Park KH, Han BR, Cho SH, Yoo HN, Lee J. Investigation: Jung EY, Park KH. Writing - original draft: Jung EY, Park KH. Supervision: Park KH, Han BR.

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