Cord blood cytokine levels in neonates born to mothers with prolonged premature rupture of membranes and its relationship with morbidity and mortality

Mehmet Satar[1], Emin Turhan[1], Hacer Yapicioglu[1], Nejat Narli[1], Fatma Tuncay Ozgunen[2], Salih Çetiner[3]

[1] Department of Pediatrics, Division of Neonatology, Cukurova University Faculty of Medicine, Adana, Turkey
[2] Department of Obstetrics and Gynecology, Cukurova University Faculty of Medicine, Adana, Turkey
[3] Biochemistry Laboratories, Cukurova University Faculty of Medicine, Adana, Turkey

Correspondence : Dr Hacer Yapicioglu, Cukurova University Faculty of Medicine, Department of Pediatrics, Division of Neonatology, 01330 Adana/Turkey
<msatar@cu.edu.tr, yyhacer@hotmail.com>

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ABSTRACT. The purpose of this study was to determine cord blood cytokine levels and their relationship with morbidity and mortality in neonates with prolonged, premature rupture of membranes (PPROM). Forty two premature neonates of 29-35 weeks gestational age with PPROM exceeding 24 hours were considered as the PPROM group and simultaneously, 41 premature neonates without PPROM were considered as the control group. All the neonates were admitted to the Neonatology Unit for further evaluation of subsequent complications such as early neonatal sepsis, pneumonia, intraventicular haemorrhage (IVH), respiratory distress syndrome (RDS), necrotizing enterocolitis (NEC) and chronic lung disease (CLD). Cord blood and mothers' blood samples were obtained during delivery in both groups and tested for IL-6, IL-8 and TNF-α levels. Twenty one percent of patients with PPROM had histological chorioamnionitis. The risk for developing early neonatal sepsis increased significantly in neonates whose mothers had histological chorioamnionitis ($p < 0.05$). There was a statistically significant relationship between PPROM and risk of developing NEC ($p < 0.05$); no significant increase was seen as regards early neonatal sepsis, IVH, RDS, pneumonia, or BPD. The mean IL-8 levels in cord blood and mothers' serum were significantly higher in the PPROM group ($p < 0.001$, $p< 0.005$). In addition, IL-6 levels found in mothers' serum were significantly higher than those found in the control group ($p < 0.01$). However, levels in cord blood were similar ($p > 0.05$). TNF-α levels were similar in both groups ($p > 0.05$). Neonates who developed NEC had higher IL-8 levels in their cord blood when compared to those without NEC ($p < 0.05$). In conclusion, the presence of PPROM increases the risk of chorioamnionitis. In addition, PPROM increases the risk of NEC, and patients who developed NEC had significantly higher cord blood IL-8 values. We may conclude that patients with PPROM and higher IL-8 levels in cord blood might be considered as at possible risk of NEC.

Keywords: prolonged premature rupture of membranes, cytokine, chorioamnionitis

Prolonged premature rupture of membranes (PPROM) occurs in approximately 3% of all pregnancies and is responsible for about 1/3 of preterm births [1]. The most important complications related to PPROM are early neonatal sepsis, respiratory distress syndrome (RDS), necrotizing enterocolitis (NEC), intraventricular haemorrhage (IVH) and chronic lung disease (CLD). In particular, early neonatal infections are very frequently implicated in infant morbidity and mortality [2]. Subclinical or clinical amniotic fluid infections were evident in many pregnant women with PPROM and preterm delivery. Furthermore, pathogenic bacteria were found in amniotic fluid in 20% of the mothers of premature babies [3]. Inflammatory cytokines are produced by lymphocytes and activated macrophages in preterm deliveries. Inflammatory cytokines in amniotic fluid and mothers' serum have been investigated in many studies of PPROM and chorioamnionitis. However, amniosynthesis is an invasive procedure for mothers and their babies. Although cord blood is obtained easily for measurement of cytokine levels, cytokine levels in mothers and cord blood in cases of chorioamnionitis have shown variable results in many studies.

The purpose of this study was to determine cord blood cytokine levels and their relationship with neonatal morbidity and mortality and subsequent complications in neonates born to mothers with PPROM.

PATIENTS AND METHODS

In this study, 42 women at 29-35 weeks singleton gestations complicated with PPROM, and their babies, were taken as the study group (PPROM group). Forty-one

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women at similar gestational age without membrane rupture and their babies, were taken as the control group. Those women with a current or history of viral infection, or a urinary tract infection were excluded from the study. The Ethical Committee approved the study, and informed consent was obtained from the parents.

All mothers were evaluated with ultrasonography, and biophysical profiles by were prepared by obstetricians. Gestational age was calculated by mother's last menstrual date, or, if not known, by ultrasonographic measures and the New Ballard scoring systems [4]. Oligohydramniosis was determined by ultrasonographic examinations. PPROM was accepted as a prolonged rupture of membranes exceeding 24 hours. Clinical findings of chorioamnionitis were evaluated [5], and then placental remains from mothers with clinical chorioamnionitis were evaluated for histological chorioamnionitis.

All neonates with PPROM were admitted to the Newborn Intensive Care Unit and subsequent complications such as early neonatal sepsis, pneumonia, IVH, RDS, NEC, and chronic lung disease (CLD) were recorded. Diagnosis of early neonatal sepsis, pneumonia, IVH, RDS, NEC and CLD were based upon certain, defining criteria [6-8]. The Tollner scoring system was used to evaluate risk of early neonatal sepsis [9]. Cord blood and mothers' blood samples were obtained during delivery in both groups. The blood samples were then centrifuged and serum samples were stored at -70°C. Both mothers' and cord blood serum were tested for IL-6, IL-8 and TNF-α levels using a micro ELISA method (Pierce Endogen kit, Triturus, Grifols, Spain). The sensitivity of the tests for IL-6, IL-8 and TNF-α were < 1.0 pg/mL, < 2.0 pg/mL and < 1 pg/mL respectively. Specificity of the tests was 70-80% for IL-6, 72-78% for IL-8 and 75-89% for TNF-α. Internal quality control samples were studied in parallel with patients' serum samples. Titrations of the control and PPROM groups were performed under the same conditions, on the same day.

Statistical analysis was performed with SPSS 10.0 Software. The Mann-Whitney U, dependent t-test, one way ANOVA and linear regression analysis were used to evaluate data.

RESULTS

There were 42 babies in the PPROM group and 41 babies in the control group. There were no statistical differences between age of mothers, gestational age and number of pregnancies in both groups ($p > 0.05$). Mean birth weight was 1 742 ± 539 g and 1 847 ± 448 g; and mean gestational age was 31.6 ± 2.0 weeks and 32 ± 1.5 weeks in the PPROM group and the control group, respectively ($p > 0.05$). Thirty-eight mothers in the PPROM group and seven mothers in the control group were treated with antibiotics.

The leukocyte count for both mothers and newborns in the PPROM Group was significantly higher than in the control group (1 4380 ± 4 098/mm^3 in mothers and 15 180 ± 6 992/mm^3 in newborns in the PPROM group and 11 759 ± 2 994/mm^3 in mothers and 10 858 ± 3 556/mm^3 in babies in the control group), ($p < 0.05$).

The placenta from 31 mothers in the PPROM group and 25 mothers in the control group were evaluated histopathologically. Six mothers (19%) from the PPROM group were diagnosed with histological chorioamnionitis, but none of the patients in the control group. Clinical chorioamnionitis was diagnosed in nine (21.4%) from PPROM group but in none of the patients in the control group. Histopathological and/or clinical chorioamnionitis was significantly more frequent in the PPROM Group ($p < 0.05$).

Table 1
Cytokine levels in cord and mothers' blood in PPROM and the control group

	PPROM group Mean ± SD	Control group Mean ± SD	p
Mother (pg/mL)			
IL-6	55.2 ± 159.6	40.2 ± 91.3	< 0.01
IL-8	269.7 ± 445	37 ± 155	< 0.01
TNF-α	15 ± 65.2	4.8 ± 1.6	> 0.05
Cord (pg/mL)			
IL-6	135.9 ± 280.5	27.9 ± 44.9	> 0.05
IL-8	499.9 ± 597.1	22.3 ± 46.7	< 0.001
TNF-α	13.1 ± 38.6	5.1 ± 1.7	> 0.05

IL-8 levels in cord blood and mothers' serum were significantly higher in the PPROM group compared to the control group ($p < 0.001$, $p < 0.01$). In the PPROM group, although IL-6 levels in mothers' serum were significantly higher compared to those in the control group ($p < 0.01$), there were no statistically significant differences in IL-6 levels in cord blood between both groups ($p > 0.05$). TNF-α levels in mothers and babies were similar in both groups ($p > 0.05$). In *table 1* and *figure 1*, cytokine levels in cord and mothers' serum with PPROM and those in the control group are shown.

There was a statistically significant, positive correlation between mean mother and cord IL-6 levels in the PPROM group and the control group ($p < 0.05$). However, there was no correlation between TNF-α levels in cord and mothers' blood. In the control group, there was also a positive correlation between IL-8 levels in cord and mothers' serum ($p < 0.05$).

Although the mortality rate was similar in both groups, the number of days hospitalization in the PPROM group was significantly higher than in the control group (32 days *versus* 13 days) ($p < 0.05$). Cumulative relative risk for neonatal complications in PPROM babies was 3.2. No significant increase was seen as regards early neonatal sepsis, IVH, RDS, pneumonia and CLD in the newborns with PPROM when compared to the control group ($p > 0.05$). However, the odds ratio was 8 for NEC, 3 for

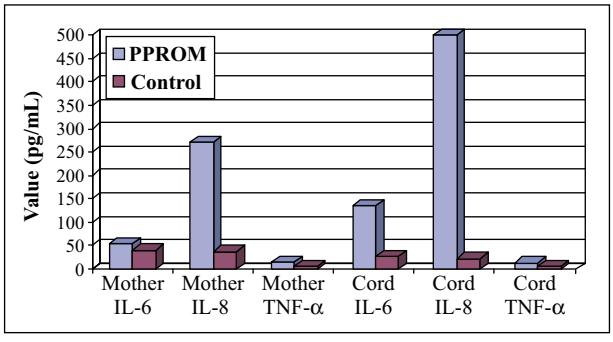

Figure 1
Cord and mothers' serum cytokine levels of PPROM and control group.

Table 2
Neonatal complications in both groups

	PPROM group (n = 42) Number (%)	Control group (n = 41) Number (%)	p
Early sepsis	9 (21.4)	3 (7.3)	> 0.05
RDS	6 (14.3)	5 (12.2)	> 0.05
Pneumonia	10 (23.8)	5 (12.2)	> 0.05
NEC	*8 (19)*	*1(2.4)*	*< 0.05*
IVH	11 (26.2)	6 (14.6)	> 0.05
CLD	4 (9.5)	2 (4.9)	> 0.05

early neonatal sepsis, 1.6 for pneumonia, 3 for IVH, and 2 for CLD. There was no significant increase for RDS. There was a statistically significant relationship between PPROM and NEC incidence ($p < 0.05$). Neonatal complications in the babies are shown in *table 2*.

Cord blood IL-8 levels were significantly higher in babies who developed NEC in both groups ($p < 0.05$). When the cut-off level for cord blood IL-8 was accepted as 12.1 pg/mL, sensitivity was 67%, specificity was 74%. There were no statistically significant differences in cord blood IL-6 and TNF-α and mothers' IL-6, IL-8 and TNF-α levels in patients with and without NEC ($p > 0.05$). In *table 3*, the mean cytokine levels in mothers and cord blood are shown for patients with NEC.

DISCUSSION

IL-6 and IL-8 are both well known cytokines secreted during inflammation. In preterm deliveries, von Minckwitz *et al*. [10] showed that IL-6 increased in mothers with preterm PROM compared to mothers without preterm PROM. Similarly, in preterm PROM with clinical or histological chorioamnionitis, Murtha *et al*. [11, 12] showed an elevation in maternal serum IL-6 levels. However, Bahar *et al*. [13] could not identify an increase in any of the cytokines IL-6, IL-8, or TNF- α in term or preterm labor; and choriamnionitis has been detected in only 42.1% of preterm PROM mothers with high IL-6 levels by Hadzidaki *et al*. [14]. Shobokshi *et al*. [15] also showed that maternal IL-6 levels did not increase in preterm PROM and increased in only half of the patients with histological and/or clinical chorioamnionitis. In the present study, we have detected increased maternal IL-6 levels in PPROM.

Lewis *et al*. [16] studied IL-6 levels in mothers with preterm PROM in order to detect potential infectious and/or neonatal complications of their babies. They found significantly elevated IL-6 levels in those mothers whose babies had these complications. Similarly Pfeiffer *et al*. [17] claimed that mothers' serum IL-6 levels may be of value in detecting fetal infections early; in their study, at a cut-off 11 pg/ml, IL-6 had a sensitivity of 81% and specificity of 76% for neonatal infections. Elevated IL-6 levels have been shown in preterm deliveries [10, 18, 19]. Tasci *et al*. [20] have studied cord blood IL-6 levels for predicting chorioamnionitis, funisitis and neonatal infection in term PROM, and reported that IL-6 levels were significantly higher in term PROM compared to healthy controls, and for predicting funisitis and positive newborn cord blood cultures, a cord blood IL-6 level of > 39 pg/mL had 100% sensitivity and 81% specificity. Fukuda *et al*. [21] studied cord levels of IL-6, IL-8 and TNF-α and showed no increase in preterm PROM compared to a control group. In the same study, amniotic fluid IL-6 levels had significantly increased in the preterm PROM group. Goepfert *et al*. [22] showed significantly increased cord blood IL-6 with preterm PROM or preterm labour. In that study, the increased levels of IL-6 were related to increased risk of periventricular leukomalasia, systemic inflammatory response syndrome and NEC in premature babies. Yoon *et al*. [23] found that cord IL-6 was significantly increased in premature babies compared to term babies, and in chorioamnionitis compared to healthy mothers. IL-6 has been shown to be released from placental endothelial cells during labour, but could be normal if there were a successful tocolysis [24, 25]. Similarly, Murtha *et al*. [11] showed that cord IL-6 levels increased in clinical and histological chorioamnionitis with preterm PROM, but were normal with preterm PROM lasting longer than 48 hours. In our study, although mothers' serum IL-6 levels were higher in the PPROM group, cord IL-6 levels were similar in both groups. Smulian *et al*. [5] showed that cord blood IL-6 was a better predictor of early neonatal sepsis than clinical signs of chorioamnionitis, in preterm PROM. Similarly Kashlan *et al*. [26] concluded that elevated cord blood IL-6 was an important sign of sepsis syndrome in histological chorioamnionitis. In some studies, elevated cord blood IL-6 was associated with early neonatal sepsis and pneumonia [22, 27]. However, in the studies by Santana *et al*. [28] and Berner *et al*. [29], cord blood IL-8 levels were found to be the most sensitive indicator of early neonatal sepsis diagnosis. While TNF-α has been found to be increased in early neonatal sepsis in some studies [30], no difference was found in others [28]. In our study, we could find no difference in maternal and cord cytokine TNF-α levels in PPROM.

There are a few studies involving maternal IL-8 levels in preterm PROM. Pfeiffer *et al*. [17] showed a slight increase in IL-8 levels in mothers with preterm PROM. Stallmach *et al*. [31] showed elevated IL-8 levels in cord and amniotic fluid, but only if there were chorioamnionitis, but there was no increase in maternal IL-8 levels. Uterine contractions and progressing labour have been linked with increased maternal IL-6 and IL-8 levels [32]. Von Minckwitz *et al*. [10] showed IL-6 and IL-8 significantly increased both in preterm labour and PPROM. However, Bahar *et al*. [12] did not detect an increase in maternal IL-6 and IL-8 levels in preterm labor. In our study, although cord blood IL-6 did not increase, maternal IL-6 levels increased in the PPROM group compared to the control

Table 3
Cytokine levels in cord and mothers' blood in patients with NEC

	NEC (+) 9 babies Mean ± SD	NEC (-) 74 babies Mean ± SD	p
Mother (pg/mL)			
IL-6	95.3 ± 221	42.0 ± 115	> 0.05
IL-8	252.5 ± 499	142.8 ± 333	> 0.05
TNF-α	4.6 ± 1.07	10.6 ± 49.2	> 0.05
Cord (pg/mL)			
IL-6	129.5 ± 231	76.9 ± 206	> 0.05
IL-8	*655.1 ± 765*	*216.2 ± 425*	*< 0.05*
TNF-α	5.4 ± 2.08	9.6 ± 29.2	> 0.05

group, and both cord and maternal serum IL-8 levels increased significantly in the PPROM group. We suggest that increase in IL-8 in cord blood might indicate fetal inflammation. We have not evaluated IL-6 and IL-8 in term labour, so we cannot comment about their effect in preterm labour.

Studies involving other cytokine, TNF-α, are mainly about its level in amniotic fluid [15, 33]. In a study by Von Minckwitz *et al*. [10], although IL-6 and IL-8 levels increased significantly, maternal TNF-α levels were not affected by preterm labour or preterm rupture of the membranes. Likewise, we could not find any increase in maternal TNF-α levels associated with with PPROM and chorioamnionitis.

There are only a few studies looking at cord blood TNF-α with PPROM. These studies mostly involve preterm PROM with chorioamnionitis [15, 26, 34]. Von Minckwitz *et al.* [10] could not find any significant increase in cord blood TNF-α levels in a preterm PROM group compared to a control group. While some studies showed cord blood TNF-α levels were not affected in preterm PROM with chorioamnionitis [26], some showed elevated levels [19, 35]. In term PROM, Zhang *et al*. [36] showed that maternal serum IL-6 and IL-8 levels and amniotic fluid IL-6, IL-8 and TNF-α levels were higher than the control patients. The levels of the cytokines were even higher in chorioamnionitis. In the present study, we could not show an increase in cord blood TNF-α with PPROM.

In the present study, we have found increased levels of cord IL-8 in babies with NEC. To our knowledge, although elevated levels of cord blood IL-6 have been reported in babies who later develop NEC [22, 37] there is no other study showing elevated cord IL-8 levels in NEC patients.

In conclusion, cord IL-8 levels increased significantly in PPROM and were found to be even higher in patients who later developed NEC. In addition, IL-8 levels and IL-6 levels in mothers' serum were both elevated in the PPROM group, but TNF-α levels did not differ. Therefore, we may conclude that elevated cord blood IL-8 levels might be a predictor of NEC in premature babies.

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