

Full Length Research Paper

Microbiological study of cases of early neonatal sepsis and evaluation of the role of C-reactive protein, interleukin-6 and interleukin-8 as diagnostic biomarkers of such cases

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Neonatal sepsis is responsible for significant morbidity and mortality. Its early diagnosis is very important but very difficult. This study aimed to evaluate the value of C reactive protein (CRP), interleukin 6 (IL-6) and interleukin 8 (IL-8) as early diagnostic biomarkers for early neonatal sepsis (ENOS). Forty neonates with prenatal risk for neonatal sepsis with their mother were taken in this study (group I). Ten healthy neonates and their healthy mothers were taken as the control group (group II) in Tanta University Hospital in the period of March to December, 2016. White blood count (WBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), interleukin-6 (IL-6) and interleukin-8 (IL-8) were evaluated in cord blood of neonates and in mother's sera. Also, cultures were done for neonates to confirm neonatal sepsis. The results of this study showed that the gestational age was shorter in the study group than the control group. *Staphylococcus aureus* represent 55% of the organisms isolated from cases of early neonatal sepsis. WBC, ESR, CRP, IL-6 and IL-8 were significantly higher in the study group than the control group. In the study group, 50% had positive cultures, while there were only 10% in the control group with P value of 0.001. The sensitivity, specificity, positive predictive value (PPV) and negative predictive values (NPV) for CRP were 88.6, 84.7, 86.9 and 90.4%, for IL-6, they were 92.4, 97.6, 90.4 and 86.6%, for IL-8, they were 90.8, 88.9, 92.4 and 91.7%, respectively. The best sensitivity and NPV was for IL-6. Cord blood CRP alone has little utility in ENOS diagnosis. IL-6 and IL-8 has great superiority than CRP when combined with other hematological markers. IL-6 was better diagnostic biomarker for ENOS than IL-8 and CRP.

Key words: Early neonatal sepsis, C-reactive protein (CRP), IL-6, IL-8.

INTRODUCTION

Neonatal sepsis is defined as generalized bacterial infection of neonates and is considered one of the major

causes of morbidity and mortality in the newborn, especially in developing countries. Surviving infants can

have significant neurologic sequelae as a consequence of central nervous system involvement, septic shock or hypoxemia (Betty and Inderpreet, 2005).

Early onset neonatal sepsis (EONS), referred to infection occurring during the first 72 h after birth, is generally caused by pathogens prevalent in the maternal genital tract, delivery room and/or operation theatre. Infection can be acquired by ascending colonization following the rupture of membranes, during fetal passage through an infected birth canal, or at the time of resuscitation procedures. EONS has an incidence of 1.5 to 3.5%/000 live births with mortality rates ranging from 5 to 10% in developed countries (Vergnano et al., 2005).

The prognosis and outcome of neonatal sepsis depend on early diagnosis and treatment with efficient antibiotic therapy. The accurate diagnosis of early neonatal sepsis remains a challenge for the neonatologist because early signs and symptoms are often minimal, variable, nonspecific and often difficult to differentiate from the physiologic changes that occur during transition to extra uterine life (Ng, 2004)

Blood cultures were considered the gold standard method of diagnosis of neonatal sepsis for years but it is time consuming and can give negative results because of antibiotic treatment of mothers during labour (Kumar et al., 2001). In addition to the blood culture, other tests were usually used for the diagnosis of neonatal sepsis including: white blood cell count (WBC) and erythrocyte sedimentation rate (ESR). Unfortunately, these tests do not have a high sensitivity and specificity in diagnosing neonatal sepsis. CRP has also been used for long time as a biomarker for infection as it is simple, fast and effective but its sensitivity and specificity became a matter of questions (Gonzalez et al., 2003)

Meanwhile, infants who are suspected to have neonatal sepsis have to start antibiotic treatment which could be unnecessary and even harmful with higher incidence of antibiotic resistance and longer hospitalization with increased health costs (Bindlish et al., 2015). Therefore, efforts have been made to improve diagnosis and to find the ideal rapid biomarker that provides high sensitivity and specificity for diagnosis of presence or absence of sepsis, severity of sepsis, type of microorganisms, and prognosis during treatment (Modi et al., 2009).

In recent years, the search for new diagnostic tests for ENOS has turned to cytokines as more early and effective indicators of acute infections. IL-6 and IL-8 are potent proinflammatory cytokine and are responsible for a strong inflammatory reaction, which if left uncontrolled, may lead to severe hypotension, multiple organ dysfunction and death (Ng et al., 2004).

The aim of our study was to evaluate the role of CRP, IL-6 and IL-8 as early diagnostic biomarkers for early

onset neonatal sepsis.

METHODOLOGY

The study is a prospective one performed in obstetric department and neonatal intensive care unit (NICU) with cooperation of Medical Microbiology and Immunology Departments at Tanta University Hospital, Egypt during the period of March 2016 to December 2016. The study was approved by Tanta Ethical Committee and informed consent was obtained from the parents.

Fifty (50) neonates with gestational age above 28 weeks and their mothers were enrolled in the study. They were divided into two groups:

Group I (study group): 40 maternal and cord blood samples were taken at the same time with gestational age above 28 weeks from mothers with prenatal risk factors for infection. Eligible neonates had to fulfill one or more of prenatal risk factors for infection such as premature rupture of membranes (PROM), chorioamnionitis and/or maternal high fever.

Group II (control group): 10 maternal and cord blood samples were taken with the same gestational age above 28 weeks from mothers with no risk factors.

Maternal samples were taken during the 1st and/or 2nd stage of labor and cord samples were taken just after the fetal delivery and during resuscitation of the newborn by the help of obstetricians. All mothers were delivered vaginally.

CRP level was studied immediately by the latex immunonephelometric method (BNA analyser, Behring-Werke AG, Marburg, Germany). CRP levels ≤ 6 mg/L were considered normal. 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 and IL-8 using a micro enzyme linked immunosorbent assay (ELISA) method. All of the laboratory analyses were carried out in the central laboratory of Tanta University Hospital.

Exclusion criteria

Stillbirth, delivery outside the hospital, inadequate blood sampling, parents refusal to participate, presence of any other systemic infections e.g. chest, renal, hepatic, instrumental deliveries, Caesarean section, and mother taking antibiotics are the exclusion criteria.

All mothers were evaluated by ultrasonography, and biophysical profiles by obstetricians. Gestational age was calculated by mother's last menstrual date, and/or ultrasonographic fetal measurements. PROM was defined as a prolonged rupture of membranes exceeding 24 h. Clinical findings of chorioamnionitis were evaluated, and then placental remains from mothers with clinical chorioamnionitis were evaluated for histological chorioamnionitis.

All neonates of group I were admitted to NICU and subsequent complications such as early neonatal sepsis, pneumonia, intraventricular hemorrhage (IVH), respiratory distress syndrome (RDS), necrotizing enterocolitis (NEC) and chronic lung disease were recorded. Diagnosis of early neonatal sepsis was based upon criteria according to Cernada et al. (2012). The Tollner scoring system was used to evaluate risk of early neonatal sepsis. Both mothers' and cord blood serum were tested for IL-6, IL-8, CRP, ESR and CBC.

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Table 1. Age of mothers and gestational age of both groups.

Parameter	Group I	Group II	P. value
Gestational age	30.2±5.63	36.21±7.52	0.024*
Age of mothers	28.63±8.63	31.14±6.15	0.528

Diagnosis of microbiological sepsis was based on clinical signs and confirmed with blood culture where blood samples were collected from neonates who were showing signs and symptoms of sepsis. Each sample was 0.5 ml of blood. Blood cultures bottles (Salix®) were incubated at 37°C for 24 h. Subcultures on blood agar and mannitol salt agar were done, and incubated at 37°C for another 24 h. Characteristic colonies were identified by Gram stain, and biochemical reactions according to standard bacteriological procedures (Campos et al., 2010). Clinical sepsis was defined as the presence of 3 or more of the following:

- 1) Temperature instability (rectal temperature >38 or <36°C);
- 2) Respiratory symptoms (respiratory distress, apnea or cyanosis);
- 3) Cardiovascular symptoms: hypotension (blood pressure <5th percentile for age), tachycardia (HR >180/min), bradycardia (HR < 100/min) or poor perfusion; (iv) neurological symptoms: clinical or electrical seizures, bulging fontanelle, hypotonia or lethargy;
- 4) Gastrointestinal symptoms: vomiting, poor feeding or feeding intolerance and/or abdominal distension, without identification of a bacterial pathogen from a sterile site.

Statistics

Statistical presentation and analysis of the present study was conducted, using the mean, standard deviation and Chi-square test by SPSS V.20. P<0.05 was considered as significant. Sensitivity, specificity, negative predictive value and positive predictive value of the studied biomarkers were calculated.

RESULTS

The results show that the gestational age among group I in patients with early neonatal sepsis was significantly lesser than the age of the control group (group II) (P-value:0.024). Regarding the age of the mother, there was no significant difference between the two groups (P-value:0.528) (Table 1).

Leukocyte count for both mothers and neonatal cord samples in group I was significantly higher than in the group II. Also, ESR 1st and 2nd hours for both mothers and neonatal cord samples in group I was significantly higher than in the group II (Table 2). There was significant difference between both groups as regard levels of CRP, IL 6, and IL 8 of mothers which were higher in groups I as compared to II (Table 3).

There was highly significant difference between both groups as regarded CRP, IL 6, and IL 8 of neonatal cord blood which were significantly higher in group I than group II (Table 4). Number of newborn entered neonatal intensive care unit with proven ENOS by blood culture was 20 patients (50%) in group I, and one patient (10%) in group II with p-value < 0.05. There were high

significant difference between groups I and II regarding number of patient diagnosed with ENOS and high IL-6, IL-8 and CRP (Table 5). The sensitivity of CRP mother blood was 59%, specificity 85%, PPV 90.6% and NPV 74.6%, while sensitivity of CRP neonatal cord blood was 88.6%, specificity 84.7%, PPV 86.9% and NPV 90.4% (Table 6).

The sensitivity of IL-6 mother sample was 95.1%, specificity 91.8%, PPV 86.4% and NPV 92.1%. While sensitivity of IL-6 neonatal cord blood was 92.4%, specificity 97.6%, PPV 90.4% and NPV 86.6 % (Table 6).

The sensitivity of IL-8 mother sample was 91.2%, specificity 86.3%, PPV 85.9% and NPV 87.3%, while sensitivity of IL-8 neonatal cord blood was 90.8%, specificity, 88.9%, PPV 92.4% and NPV 91.7% (Table 6). Table 7 shows the results of blood culture among samples of cord blood of cases with early neonatal sepsis where *Staphylococcus aureus* was the main organism causing early neonatal sepsis in this study as it was isolated from 11 cases (55%), followed by *Klebsiella pneumoniae* (22.5%), *Acinetobacter baumannii* and *Pseudomonas auruginosa* isolated from 4 cases (10%) and *E. coli* isolated from only one case (2.5%). The best sensitivity for early neonatal sepsis was achieved by IL 6 (Table 6).

DISCUSSION

Early diagnosis of neonatal sepsis and appropriate management can be lifesaving and significantly decrease morbidity and mortality in newborns. However, early diagnosis of sepsis is very difficult because symptoms are minimal and non-specific. So, the search for an ideal rapid diagnostic test, which has maximum sensitivity and maximum negative predictive value, is very important for prognosis and to decrease unnecessary exposure to antibiotics (Ng et al., 2004). So, the authors measured CRP and cytokines (IL- 6 and IL-8) in high risk neonates to identify a reliable test for diagnosis of ENOS.

Presence of risk factors for neonatal sepsis usually results in preterm labour that was confirmed in the results by lower gestational age in the study group than control group. This is higher in comparison with the results of other authors (Modi et al., 2009). In this study, leukocytic count and ESR for both mothers and neonates in group I were significantly higher than in the control group, indicating the presence of infection. This showed agreement with other studies (Malik et al., 2003).

CRP is synthesized in the liver in response to IL 6, IL β and TNF-CRP. CRP is synthesized within 6 and 8 h of exposure to infection, peaks at 24 to 48 h, and then diminishes when the inflammation subsides (Kocabaş et al., 2007). This study reported increase in levels of CRP for both mothers and neonates in groups I as compared to group II with sensitivity, specificity, PPV and NPV of 88.6 and 84.7, 86.9 and 90.4%, respectively in neonates. These results are similar to that of other investigators

Table 2. Leukocytic count and ESR in both groups.

Parameter	Group I		Group II		P value
	Maternal samples	Neonatal cord samples	Maternal samples	Neonatal cord samples	
WBC	14.380±4.098 c/µl	15.180±6.992 c/µl	11.759±2.994 c/µl	10.898±3.556 c/µl	0.004*
ESR 1 st h	105±30 ml/h	97±35 ml/h	50±15 ml/h	30±10 ml/h	0.002*
ESR 2 nd h	122±40 ml/h	99±40 ml/h	40±20 ml/h	35±12 ml/h	0.008*

*h: Hour c/µl: cell/microliter.

Table 3. CRP, IL 6, IL 8 in serum of mothers in both groups.

Parameter	Group I	Group II	P value
CRP	17.63±14.16mg/l	12.25±6.71 mg/l	0.019*
IL 6	52.36±11.6 pg/ml	18.32±3.62pg/ml	0.001*
IL 8	395.2±85.6 pg/ml	311.2±42.6 pg/ml	0.019*

Table 4. CRP, IL 6, IL 8 in neonatal cord samples of both groups.

Parameter	Group I	Group II	P value
CRP	16.32±4.626 mg/l	13.9±2.93mg/l	0.011*
IL 6	65.35±8.25 pg/ml	35.4±8.52 pg/ml	0.001*
IL 8	385.4±74.1 pg/ml	291.4±63.8 pg/ml	0.019*

Table 5. Number of neonate admitted to NICU with EONS and that with high biomarkers.

Number of newborn entered ICU for ENOS	Group I	Group II	P value
Proven EONS by culture	20/40 (50%)	1/10 (10%)	0.001*
High CRP	18	2	0.004*
High IL-6	16	4	0.001*
High IL-8	15	1	0.001*

High CRP>6 mg/l high IL-6>9pg/ml high IL-8>33 pg/ml.

Table 6. Diagnostic value of blood cytokines in early-onset neonatal sepsis in both mother and neonate.

Parameter	CRP		IL-6		IL-8	
	Mothers	Neonates	Mothers	Neonates	Mothers	Neonates
Sensitivity	59	88.6	95.1	92.4	91.2	90.8
Specificity	85	84.7	91.8	97.6	86.3	88.9
PPV	90.6	86.9	86.4	90.4	85.9	92.4
NPV	74.6	90.4	92.1	86.6	87.3	91.7

(Reyes et al., 2003).

In contrast to the results, Dollner et al. (2002) found that CRP levels were low and undetectable in nearly all of the neonates, both in infectious and control groups. This

can be explained by the fact that infection may be initiated relatively close to delivery, resulting in low levels of umbilical CRP concentration. Moreover, CRP responses could be undetectable several days after birth

Table 7. Results of blood culture among samples of cord blood in cases with early neonatal sepsis:

Isolated organism	No. of cases (%)
<i>Staphylococcus aureus</i>	11(55)
<i>Klebsiella pneumonia</i>	4 (22.5)
<i>Acinetobacter baumannii</i>	2 (10)
<i>Pseudomonas aeruginosa</i>	2(10)
<i>E.coli</i>	1(2.5)
Total	20(100)

in infected extremely premature neonates. Also, cord blood CRP may not increase in presence of umbilical vasculitis which often reflects severe chorioamnionitis with neonatal diseases, whereas many other inflammatory markers do.

It is known that CRP does not cross the placenta (Beceiro-Mosquera et al., 2009). Accordingly, the present study confirmed that serum concentrations of CRP and its production in the mother and fetus/newborn are independent of one another as lower levels of CRP were present in mothers' blood. However, the same stimulus may be operating concurrently in each. The major obstetric condition in which determination of maternal serum CRP concentrations might be clinically useful is chorioamnionitis (Baltimore, 2002).

Possible sources of heterogeneity of results in different studies had wide differences in postnatal age, single versus serial measurements, different cutoff level, different sample sizes and different measurement methods. Thus, the fact that there are no established CRP reference intervals in the neonatal period can explain the wide range of reported CRP sensitivities (47 to 100%) and specificities (6 to 97%) for detection of neonatal sepsis (Beceiro-Mosquera et al., 2009).

IL-6 is a pro-inflammatory cytokine that promotes the synthesis of CRP by the liver. IL-6 is produced by monocytes, endothelial cells and fibroblasts. IL-6 is released soon after the inflammatory stimulus takes place, peaks within 6 h post-insult, preceding the increase in CRP, and decreases 48 h thereafter. Several studies that have used IL-6 as a biomarker for established neonatal sepsis have reported contradictory results (Beceiro-Mosquera et al., 2009).

It was found that IL-6 levels in group I was significantly higher than group II in both neonatal cord blood and maternal blood. IL-6 levels in maternal blood was valuable in detecting early fetal infection with a sensitivity, specificity, NPV, PPV of 95.1, 91.8, 92.1 and 86.4%, respectively and was comparable to the results of other investigators (Tasci et al., 2006). Also, the finding of higher IL-6 concentrations in cord sera of babies compared with the corresponding maternal samples argues against the dependency of neonatal serum IL-6 concentrations on maternal IL-6 concentrations.

This study reports higher values of IL-6 in newborns with culture-proven sepsis compared with IL-8 and similar to studies. IL 6 was found to have the highest sensitivity and NPV as a predictor of EONS as compared to CRP and IL-8 like the result of other investigators (Martin et al., 2012).

The different results of these studies may be partially explained by timing at which sampling was performed in the course of the disease as serum IL-6 values inversely correlate with timing of sample collection (Cernada et al., 2012). The samples were obtained immediately after birth before the patients featured any clinical sign of sepsis. This could explain why we found low values of CRP and high ones of IL-6 as compared to other publications.

IL-8 is a pro-inflammatory cytokine predominantly produced by monocytes, macrophages and endothelial cells. It has a role in release, activation and chemotaxis of neutrophils and rises early in the course of neonatal bacterial infections. IL-8 is considered to be an accurate early and late marker for neonatal sepsis in many studies (Dima et al., 2012).

In this study, it was found that IL-8 was significantly higher in group I than group II. This agreed with the results of other investigators (Dollner et al., 2002; Fukuda et al., 2012) who reported that a significant initial elevation of IL-8 in cord blood collected immediately at birth was observed in infected preterm neonates. Unlike the results of Fukuda et al. (2012) who studied cord levels of IL-6 and IL-8 and showed no increase in preterm with high risk for infection as compared to a control healthy group.

The sensitivity, specificity, PPV, NPV for IL-8 in neonatal cord blood were 90.8, 88.9, 92.4 and 91.7% and this was in agreement with the results of Fan and Yu (2012) who reported the sensitivity of cord blood IL-8 91%, specificity 93%, PPV 91% and NPV 97%.

Many investigators reported that IL-6 and IL-8 were produced rapidly in ENOS and peaked on day zero and they fell into their normal baseline within 24 h (Kocabaş et al., 2007). This makes them very important early predictors of ENOS but in the same time, could not be used alone for diagnosis as we could not be sure at which stage of infection blood samples were taken for IL-6 and IL-8. In contrast, CRP peak concentration occurs later so it is highly specific to confirm infection and the presence of high CRP in the presence of normal IL-6 and IL-8 suggest infection was there for 24-48 h (Ng et al., 2003). However, low sensitivity and NPV values for CRP in this study made this test alone not sufficient in the early diagnosis of neonatal sepsis. However, the relatively low cost of the rapid automated CRP test makes it an important test to screen for rather than to diagnose early neonatal sepsis. Combination of CRP with IL-6 or IL-8 improves its diagnostic value.

In this study, 50% of neonates in group I entered NICU with sepsis that was confirmed by positive blood culture and 10% of neonates in group II proved to have sepsis

also. There was statistically significant difference between number of newborns in NICU with EONS in both groups. This shows agreement with Jan et al. (2013) who reported incidence of neonatal sepsis (54%) among high risk newborn.

The results showed that 50% of cases suspected to be neonatal sepsis were positive for blood culture where *S. aureus* was the main organism causing early neonatal sepsis in the study as it was isolated from 11 cases (55%), followed by *K. pneumoniae* (22.5%), *A. baumannii* and *P. aeruginosa* isolated from 4 cases (10%) and *E. coli* isolated from only one case (2.5%).

The result of Shrestha et al. (2013) showed that out of 120 neonates suspected of having neonatal sepsis, 30.8% (37/120) were blood culture positive (prevalence = 30.8%). The most common causative agents of neonatal sepsis was *S. aureus* (56.8%; 21/37) followed by *K. pneumoniae* (21.7%; 8/37) and *P. aeruginosa* (13.4%; 5/37) and others. In another study by Vrishali et al. (2015), *K. pneumoniae* was the predominant pathogen (35.4%) among the Gram-negative pathogens and *S. aureus* (22.9%) was the predominant Gram-positive.

In conclusion, IL-6 and IL-8 levels in cord blood and mothers sera were superior markers for ENOS than CRP and did not show a significant difference in sensitivity and specificity and IL-6 had a better marker as compared to CRP and IL-8 with the highest sensitivity and PPV. Also, it is concluded that the use of multiple markers as CRP, IL-6 and IL-8 improves the diagnostic accuracy of ENOS than using each marker alone.

CONFLICT OF INTERESTS

Authors have declared that no competing interests exist.

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