Original article

Neutrophil CD64 in early-onset neonatal sepsis

Background: *Neonatal sepsis is a life threatening disease with an incidence of 3.5 to 8 cases per 1,000 live births; and mortality rate 16 to 30%. Cytokines, produced by monocytes, macrophages, and endothelial cells in response to infectious stimuli are important proinflammatory mediators in the early phases of the sepsis syndrome. Elevated serum levels of interleukin-6 (IL-6) had been found in both neonatal and adult sepsis. However, for neonatal sepsis, little is known about a group of molecules playing a central role in the innate immune system. Among them is the neutrophil CD64 which is expressed on neutrophil surface in many inflammatory conditions.*

Objective*: To study the neutrophil CD64 expression in neonates with early onset sepsis and its relation to other laboratory markers as IL6, CRP, total leucocytic count and platelet count.*

Methods: *This study comprised 30 neonates with a gestational age of 28 to 40 weeks with a picture of early onset neonatal sepsis within 48 hours of life admitted to neonatal care unit, Suzan Mubarak Hospital, El-Minia University, Egypt during the period from February, 2008 to January, 2009 and 20 healthy neonates age and sex matched as a control group. Neutrophil surface expression of CD64 was quantified with flow cytometry. We measured plasma IL6, C-reactive protein, complete blood count and blood culture.*

Results: *Neutrophil CD64 expression was increased significantly in neonates with neonatal sepsis than controls (p=0.001). Cases with history of premature rupture of membranes (PROM) ≥48 hours, with positive blood culture or poor outcome had the highest levels of neutrophil CD64 expression (528±50.7, 558±58.4 and 560.9±43.9 relative fluorescence units (RFU) respectively). A significant positive correlation was found between CD64 levels and the levels of IL6 (r=0.71, p=0.001),C-reactive protein (r=0.74, p=0.001) and total leucocytic count (r=0.76 ,p=0.01) and negative correlation with gestational age (r=-0.92, p=0.001) and body weight (r=-0.92, p=0.006), but there was no correlation between it and platelet count (r=-0.32, p=0.08).*

Conclusion: *Neutrophil CD64 expression is increased in neonates with early-onset neonatal sepsis and correlated well with other laboratory markers of sepsis.*

Keywords: Neonatal sepsis, Cytokines, Neutrophil CD64, IL6, PROM.

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INTRODUCTION

Neonatal sepsis is a common life threatening disease with an incidence of 3.5 to 8 cases per 1,000 live births; and mortality rate 16 to 30%[1], causing about 1.6 million deaths annually in developing countries[2]. Early diagnosis and treatment of neonatal septicemia may help to decrease neonatal mortality. Recently, one of the new generation of tests to detect early systemic infections measures the up regulation of an Fc receptor (Fc gamma R1, or CD64) on neutrophils. The Fc receptors on white blood cells are very important for effective phagocytosis of bacteria and are upregulated during infections[3].

Our aim of the work was to estimate the percentage of neutrophils that express CD64 by flowcytometry in patients with early onset neonatal sepsis and to correlate it with other laboratory markers of neonatal sepsis.

METHODS

This study comprised 30 neonates, with a gestational age of 28 to 40 weeks, admitted to the neonatal unit, Suzan Mubarak Hospital, El-Minia University, Egypt, with a picture of early-onset neonatal sepsis, within 48 hours after birth. All patients either fulfilled the following inclusion criteria: 1) at least 1 risk factor for infection (rupture of membranes ≥24 hours, maternal body temperature of ≥38°C, chorioamnionitis, maternal colonization with group B streptococci, or preterm delivery); 2) signs of respiratory or circulatory dysfunction (tachypnea >60 bpm, recurrent apnea >20 seconds, tachycardia >160 bpm, or bradycardia <100 bpm); 3) at least 1 of the following symptoms: feeding intolerance, lethargy, irritability, temperature instability, or jaundice and (4) blood culture positive or at least two of the following criteria were met: C-reactive protein (CRP) > 10 mg/l within 48 h after onset of clinically suspected sepsis, pneumonia diagnosed by X ray, by microscopic or cultural evidence in tracheal aspirate, proportion of immature (bands and less mature forms) to total neutrophils of >0.2 documented in any complete blood count (CBC) within 48 hours after clinically suspected sepsis.

Infants who were more than 48 hours old at onset of suspected sepsis, infants who were unstable and ventilated with respiratory and/or circulatory failure, and infants with neonatal asphyxia (Apgar score <7 at 5 minutes) or those with congenital malformations were excluded from this study.

Complete blood count with differential count determined by automated cell counter sysmex k-800 (TAO Medical Incorporation, Japan, CRP using CRP-Turbi Latex quantitative turbidmetric test using kits supplied by SPINEACT,S.T., Spain , IL-6 using IL-6 ELISA TEST based on the principle of a solid phase enzyme linked immunoassay using Enzyme-linked immunosorbent assay kits (Quantikine, R&D systems, Minneapolis, MN) were used and neutrophil CD64 expression by (flowcytometry) was performed. Parental informed consent was obtained for every neonate before admission to the study. The protocol was approved by El-Minia Faculty of Medicine.

Blood samples: From each neonate with suspected infection, a blood sample of 1000 µL to 1500 µL was drawn by venipuncture for CD64 and IL-6 determinations, between 0 and 48 hours of life before the first doses of antimicrobials. Each blood sample was placed into a pyrogen-free tube containing citrate phosphate dextrose (Baxter Health Care Ltd, Norfolk, England; 0.14 mL/mL blood) and cooled immediately to 0°C in an ice-water bath to minimize neutrophil activation ex vivo.

Blood culture: After skin preparation with 70 % alcohol, between 1.0 and 2.0 ml of blood was obtained and inoculated into Bactec Peds Plus/F (Becton Dickinson and Company, Sparks, MD) culture vials under sterile conditions. All positive vials were Gram-stained and subcultured for organism identification on sheep's blood, chocolate, and MacConkey agars.

Determination of CD64 expression by flow cytometry: We used monoclonal anti-human CD64 (Fc gamma R I)-fluorescein (Quantikine, R&D systems, Minneapolis, MN). Whole blood was collected in evacuated tubes containing EDTA or heparin as an anticoagulant. Contaminating serum components were removed by washing the cells three times in an isotonic phosphate buffer (supplemented with 0.5% BSA) by centrifugation at 500×g for 5 minutes. Fifty microliters of packed cells were transferred to a 5 ml tube for staining with the monoclonal antibody.

Determination of IL-6: Plasma was separated by centrifugation and then stored in aliquots at −70°C until analysis. IL-6 enzyme-linked immunosorbent assay kit (Quantikine, R&D systems, Minneapolis, MN) the detection limit of the assay, as indicated by the manufacturer, was 10 pg/mL . All samples were run in duplicate.

CRP determination: Plasma CRP concentrations were measured immunoturbidimetrically (detection limit: 5 mg/L). Any levels greater than 10 mg/L were defined as abnormal.

Statistical analysis:

Values are given as means ± SD, range, or as the number of subjects and proportions. The Student *t* test was used for group comparisons of normally distributed variables, and the Mann-Whitney U test and Wilcoxon signed-rank test were used for comparisons of variables with skewed distribution. The χ^2 test was used to compare proportions. Correlation coefficients were used to describe associations between variables, and multiple regression analysis was used to detect any relationships between the variables. $P < 0.05$ was considered significant. Analyses were performed using the SPSS software package (SPSS V 8.0 for Windows).

RESULTS

Demographic data of the study group were presented in table (1). Patients and controls were comparable with respect to mean gestational age, sex or birth weight. PROM was significantly more common in patients group.

Significant differences were observed in some laboratory data. The platelet count was significantly lower *(p=0.001)* and WBCs *(p=0.01)* and CRP were significantly higher *(p=0.001)* in patients. Levels of both CD64 and IL6 were significantly higher in cases than controls *(p=0.001)*. Five patients were blood-culture positive: 2 for group *B streptococci*, 1 for *Streptococcus agalactiae*, 1 for *Escherichia coli*, and 1 for *Staphylococcus aureus*. Eleven cases (36.7%) died within the first week of this study; two of them were blood culture positive (one for *Streptococcus agalactiae* and the other for group *B streptococci)*. (Table 2)

Significant positive correlations were found between CD64 and CRP, IL6 and total leucocytic count of patients. Significant negative correlations were present between CD64 and platelet count, age and weight of patients. PROM for 48 hours or more was associated with significant higher levels of CD64 and the same was seen in patients who died within the first week of life compared to patients who survived beyond the first week of life *(p=0.04 &p=0.005)*. No statistical significant difference in CD64 values was found between in patients with positive blood culture and those with negative blood culture *(p=0.08)*. (Table 4)

Similarly significant positive correlations were found for IL6 level and total leucocytic count as well as CRP levels in patients group. A significant negative correlation was present between IL6 and age, weight and platelet count in patients. A significant higher level of IL6 was present in cases with positive blood culture *(p=0.03)*. No statistically significant difference was present in cases as regards PROM or outcome. (Table 5)

Table 1. Demographic data of patients and controls.

ITEM		**patients (n=30)**	**Controls (n=20)**	***p-value***
Gestational age *(weeks)*	Range Mean± SD	28-40 33.4±4.1	28-40 33.3±4.2	0.9
Sex	Male Female	19(63.3%) 11(36.7%)	12(60%) 8(40%)	0.5
Weight (*grams)*	Range Mean± SD	1700-3150 2481.6±503.8	1750-3150 2582.5±537.3	0.5
PROM	≥48 hours	23(76.6 %)	0(0%)	0.01*
	≥24hours	7(23.3 %)	3(15%)	
	≤24 hours	0	17(85%)	

* Significant, ** Highly significant, PROM: premature rupture of membranes

Table 2. Laboratory data of patients and controls.

ITEM		**Patients (n=30)**	**Controls (n=20)**	***p-value***
Hb *(gm/dl)*	Range Mean± SD	11-15 13.9±1.3	12-17 14.7±1.5	0.4
Platelets *(×10³/µl)*	Range Mean± SD	10-190 58.3±40.7	110-400 282±103.08	0.001**
WBCs *(×10³/µl)*	Range Mean± SD	3-32 17.8±8.6	4-12 7.3±2.7	0.01*
CD64 *(RFU)*	Range Mean± SD	450-610 528±50.7	110-190 144.5±26.8	0.001**
IL6 *(pg/ml)*	Range Mean± SD	8-280 142.9±68.09	0-15 5.4±4.4	0.001**
Outcome	Survived Died	19(63.3%) 11(36.7%)	20(100%) 0	0.01*

* Significant, ** Highly significant, RFU= Relative Fluorescence Units

Table 3. The relation of CD64 to other studied parameters of patients.

Parameters	CD64 *(RFU)*	
	r	P
Age *(weeks)*	-0.92	0.001**
Weight (*grams)*	-0.91	0.01*
Platelets *(×10³/µl)*	-0.70	0.008**
Total WBCs *(×10³/µl)*	0.76	0.01*
CRP *(mg/l)*	0.74	0.001**

* Significant, ** Highly significant

Table 4. The relation of IL6 to other studied parameters of patients.

Parameters	IL6 *(pg/ml)*	
	r	P
Age *(weeks)*	-0.68	0.001**
Weight (*grams)*	-0.71	0.001**
Total WBCs *(×10³/µl)*	0.65	0.001**
Platelets *(×10³/µl)*	-0.69	0.02*
CRP *(mg/l)*	0.63	0.001**

* Significant, ** Highly significant

Table 5. Relation of CD64 and IL6 to PROM, blood culture and outcome in neonates with sepsis.

		PROM		Blood culture		Outcome	
		≥ 48 hours	≤ 48hours	positive	negative	survived	died
CD64 (RFU)	**Range**	450-610	450-500	460-610	450-590	450-580	480-610
	Mean ± SD	528.6±50.7	465 ±48.1	558±58.4	522±48.1	508.9±45.07	560.9±43.9
	P-value	0.04*		0.08		0.005**	
IL6 (pg/ml)	**Range**	8-280	8-195	120-280	8-250	8-240	128.05±59.5
	Mean ± SD	142.9±68.9	125 ±59.3	202±59.3	125 ±59.3	10-280	168.6±76.9
	P-value	0.9		0.03*		0.1	

* Significant, ** Highly significant, PROM: premature rupture of membranes

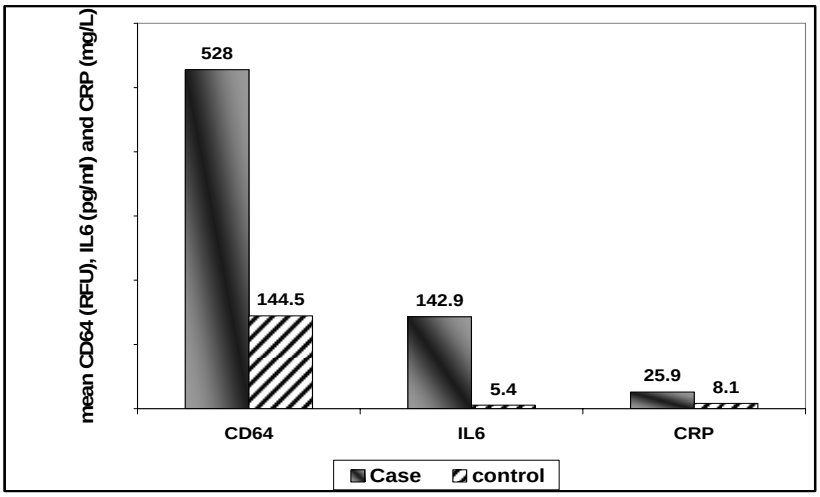
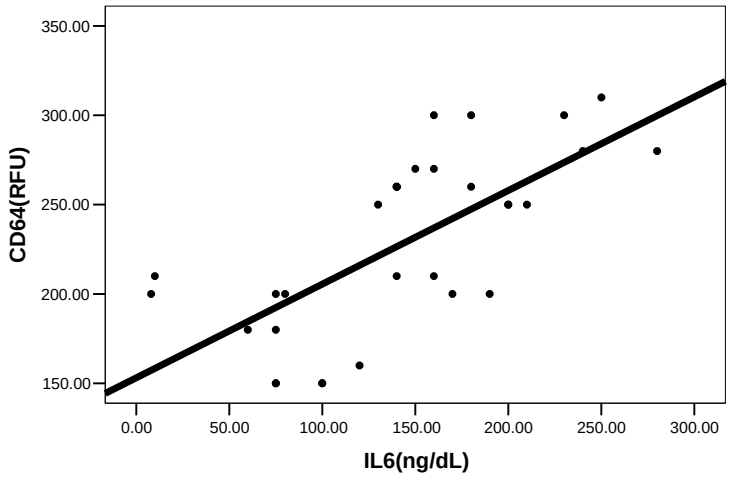


Figure 1. Comparison between patients and controls as regards CD64, IL6 and CRP levels.



r=0.70 **p=0.001****

Figure 2. Correlation between IL-6 and CD64 in the patients' group

DISCUSSION

There is a clear need for improved indicators of neonatal sepsis to increase the sensitivity and specificity of both diagnosis and therapeutic monitoring. One of the effects of inflammatory cytokines on the innate immune response is the rapid up-regulation of CD64 expression on the neutrophil membrane. During the last years, several clinical studies, have demonstrated that levels of proinflammatory cytokines, such as TNF-α, IL-1ß, IL-6, and IL-8 are elevated in cord blood of neonates with sepsis[4-8].

Davis and Bigelow[9], Nuutila and coworkers[10] and Rudensky and coworkers[11] reported that neutrophil CD64 expression is an improved diagnostic indicator of infection/sepsis. The results of our study are in line with this as we found significantly elevated levels of CD64 in septic neonates when compared to healthy controls. These findings are also in agreement with other studies[12-14] which reported the same results in neonates with early onset sepsis. Another study reported similar results in very low birth weight neonates with late onset neonatal sepsis[15]. Among the various cytokines, many studies16-18 besides ours reported the utility of IL-6 as an early marker of neonatal sepsis.

In this study cases with high risk factors for sepsis such as those with PROM ≥48 hours and/or positive blood culture, or those who died during the first week of life had significantly higher levels of CD64 than cases with PROM ≤48 hours, negative blood culture or those who survived beyond the first week. These findings may reflect the reliability of this test as a marker of the severity of cases with sepsis and are in concordance with other studies[19,20].

In this study, both CD64 and IL-6 correlated positively with CRP levels and total leucocytic count which are laboratory markers of neonatal sepsis pointing to their usefulness as additional markers of sepsis. This is in agreement with previous studies[19,26-28].

Thrombocytopenia is one of the most common complications of neonatal sepsis[21,22] and is considered one of the hematological parameters of severity of neonatal sepsis[23]**.** The finding in the present study of a significant negative correlation between both CD64 and IL-6 and platelet count indicates that both CD64 and IL-6 can be considered too as parameters of the severity of sepsis.

No significant difference was noticed between patients and controls with respect to the results of blood culture in this study. This may be due to the small sample size and that only five cases (18%) had positive blood cultures. Positive blood cultures are reported to be positive only in 13 to 20 % of cases suspected of neonatal sepsis[24,25]. Vineet et al[3] reported that CD64 index had the highest area under the curve of all hematological variables, with a higher sensitivity and specificity for blood culture positive cases.

The significant negative correlation between IL-6 level and both of age and weight in this study indicates that sepsis is more severe with younger age and lower weight resulting in higher levels of these inflammatory cytokines and this is in agreement with many studies[29,30].

It is concluded that neutrophil CD64 expression is increased in neonates with early-onset neonatal sepsis and correlated well with other laboratory markers of sepsis as well as outcome of cases.

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