Original article

Serum calprotectin as a diagnostic marker of late onset sepsis in full-term neonates

Background: *Calprotectin, a complex of two calcium-binding proteins that belong to the S100 protein family, is abundant in the cytosolic fraction of neutrophils. A high level of calprotectin reportedly exists in extracellular fluid during various inflammatory conditions, but its role in neonatal sepsis was investigated only in one study as a marker of sepsis in very low birth weight neonates.* ***Objective:*** *This study aimed to measure the serum calprotectin level by ELISA in full-term neonates with late onset neonatal sepsis, its correlations with other laboratory markers of sepsis as interleukin-6, C-reactive protein (CRP), total leucocytic count and platelet count and its relation to the outcome of cases.* ***Methods:*** *This study comprised 48 full-term neonates with gestational ages of 37 to 42 weeks with manifestations of late onset neonatal sepsis admitted to the neonatal intensive care unit, Minia University Hospital during the period from February, 2011 to December, 2011 and 40 healthy neonates, age and sex matched as a control group. Serum levels of calprotectin, IL6 and CRP were measured for all neonates recruited in this study.* ***Results:*** *Serum calprotectin levels were significantly higher in term neonates with late onset neonatal sepsis than controls (3.77±1.85 µg/ml and 0.70±0.33 µg/ml respectively, P-value = 0.000). Cases with positive blood cultures and poor outcomes had the highest levels of calprotectin (5.8±0.61 µg/ml and 6.1±0.42 µg/ml respectively). Significant positive correlations were found between calprotectin levels and IL6 (P-value =0.000, r=0.92), C-reactive protein (p=0.000,r=0.95) and total leucocytic count (P-value =0.000, r=0.72), and negative correlations were found between its level and platelet count (P-value =0.000, r=-0.87), gestational age (P-value =0.014, r=-0.35) and body weight (P-value=0.018, r=-0.34). No significant differences were observed between males and females as regards calprotectin levels (3.96±2.10 µg/ml vs 3.55±1.52 µg/ml, P-value=0.444).* ***Conclusions:*** *Serum calprotectin levels are significantly higher in full-term neonates with late onset neonatal sepsis. Its levels correlated well with other laboratory markers of sepsis and neonatal mortality. It is a sensitive diagnostic marker for late onset neonatal sepsis.*

Key words: Calprotectin, IL6, Full-term, Late-onset sepsis.

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INTRODUCTION

Severe infections represent the main cause of neonatal mortality accounting for 11 million neonatal deaths worldwide every year[1]. Neonatal septicemia is associated with hyperinflammatory host responses that subtend activation of immune system. A broad spectrum of inflammatory markers has been proposed for the diagnosis of neonatal sepsis. However, most of these markers are mediators of an acquired immune response, which is largely immature in the neonatal period[2]. On the contrary, innate immunity is fully developed in the first weeks of life, but the potential diagnostic role of components of innate immunity has not been investigated[3,4]. The neonatal immune response to many pathogens is largely immature[5]. At this age, infections are characterized by a high mortality and morbidity. An early diagnosis is crucial because the clinical course may be fulminating in neonates, in whom the onset is often inconspicuous, with minimal, subtle, and nonspecific signs[5-7]. Isolation of bacteria from central body fluid (usually blood) is the standard test for neonatal systemic infection; however, the result of culture is not available before 24–48 h and is negative in many instances, even in cases of a clear clinical picture of sepsis[6,7]. Thus, a reliable infection marker or a set of markers are required to promptly and accurately identify the infected cases so that treatment can be started without delay.

Calprotectin, a complex of two calcium-binding proteins that belong to the S100 protein family, is abundant in the cytosolic fraction of neutrophils[8]. A high level of calprotectin reportedly exists in extracellular fluid during various

inflammatory conditions, such as rheumatoid arthritis, cystic fibrosis and abscesses[9-12]. However, the exact biological role(s) of the factor is now under investigation. The findings suggest that calprotectin exerts a regulatory activity in inflammatory processes through its effect on the survival or growth states of cells participating in the inflammatory reaction. It is also possible that calprotectin, at a high concentration, might have a deleterious effect on fibroblasts and influence the recovery of inflammatory tissue. Therefore, the protein factor may be a new drug target to control inflammatory reactions. It is found that a few of the amaryllidaceae alkaloids effectively inhibited the growth-inhibitory and apoptosis-inducing activities of calprotectin[13,14].

Calprotectin is released by innate immunity cells immediately after host pathogen interaction and is detectable in body fluids by means of a simple ELISA technique. Calprotectin has been proposed for the diagnosis of many inflammatory conditions. However, its use in diagnosis of neonatal sepsis in term neonates remains unexplored.

This study aimed to measure the serum calprotectin levels in full-term neonates with late onset neonatal sepsis and its correlations with other laboratory markers of sepsis as IL6, CRP, total leucocytic count and platelet count and its relation to the outcome of cases.

METHODS

Patients

This is a case control study in which 48 full-term neonates (26 males 54.17% and 22 females 45.83%) were enrolled. Their gestational ages ranged from 37-42 weeks and were admitted to the neonatal intensive care unit, Minia University Hospital, Egypt, with a picture of late onset neonatal sepsis (in newborns aged older than 3 days to 30 days of life). All fulfilled the following inclusion criteria:1) at least 1 of the following symptoms: (feeding intolerance, lethargy, irritability, temperature instability, prolonged capillary refill or jaundice) and 2) signs of respiratory or circulatory dysfunction (tachypnea > 60 bpm, recurrent apnea >20 seconds, tachycardia >160 bpm, or bradycardia <100 bpm); and 3) a blood culture (or CSF) turning out positive within the first 30 days of life or at least two of the following criteria being met: (C-reactive protein (CRP) > 10 mg/ml within 48 h after onset of clinically suspected sepsis, pneumonia as diagnosed by X ray or by microscopic or cultural evidence in tracheal aspirate, thrombocytopenia or proportion of immature (bands and less mature forms) to total neutrophils of >0.2 documented in any complete blood count (CBC) within 48 h after clinically suspected sepsis. Blood cultures yielding either coagulase-negative staphylococci or staphylococcus aureus were considered to be contaminants if the infant was not fulfilling all the above-mentioned criteria.

Forty full-term neonates (24 males and 16 females) attending neonatal follow-up clinic of the same hospital were enrolled as a control group. Their gestational ages ranged from 37-42 weeks with a mean age of 39.40±1.26 weeks.

Exclusion criteria:

Infants with early onset sepsis (within 3 days of delivery) or born to mothers with chorioamnionitis or PROM, unstable ventilated infants with respiratory and/or circulatory failure, and infants with neonatal asphyxia or congenital malformations were excluded from this study.

Parental informed consent was obtained for every neonate before admission to the study. The protocol was approved by the local ethics committee of the faculty.

Methods

Blood samples of 2-3 mls were collected by venipuncture from all neonates recruited in the study for bacterial culture and for serum calprotectin and IL-6 determinations before starting the treatment. CRP concentrations were measured using nephelometric assay (Dade-Behring, France), any levels greater than 10 mg/L were defined as abnormal[15]. Complete blood count was done by coulter (Sysmex). For blood cultures: samples were cultured in blood culture bottles (Egyptian diagnostic media, Egypt), incubated at 37°C and examined each 24 hours for turbidity. Subcultures from the blood cultures flasks were made on sheep blood agar, incubated both aerobically and anaerobically at 37°C for 48-72 h and any growth was identified according to the standard microbiological protocol[16,17]. For IL-6 determination: Plasma was separated by centrifugation and then stored in aliquots at −70°C until analysis. We used an IL-6 enzyme-linked immunosorbant assay kit (Quantikine, R&D Systems, Minneapolis, MN, USA), the detection limit of the assay, as indicated by the manufacturer, was 10 pg/ml. All samples were run in duplicate[18,19]. Serum calprotectin was measured by a commercial ELISA assay (Calprest, Eurospital, Trieste, Italy).

A blood sample (0.5 mL) for serum calprotectin measurements was collected in blood

collection tubes containing (EDTA).The sample was centrifuged and the extracted serum was collected and frozen at −20°C for subsequent measurement. The serum was diluted 1:50, and 200 µL of each sample was added to the wells of a plate and incubated at room temperature for 1h. The plate was then washed 3 times with diluted washing solution, and 200 µL of purified rabbit anticalprotectin antibodies conjugated with alkaline phosphatase were added and incubated for 1h at room temperature. A second washing procedure was performed, 200 µL of enzyme substrate solution was added to each well, and optical density was read at 405 nm. Serum calprotectin concentration was calculated from the standards and expressed as µg/mL[20,21].

Statistical Analysis

Values are given as means ± SD, range or as the number of subjects and percentage. 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 Chi-square test was used to compare proportions. $P < 0.05$ was considered significant. Analyses were performed using the SPSS software package (SPSS 11 for Windows).

RESULTS

Both of the demographic and laboratory data of cases and controls were presented in table (1). No significant differences were present as regards mean gestational age, sex or birth weight. Significant differences between cases and controls were present as regards mean values of hemoglobin levels (0.03), platelet counts (p=0.001), total leucocytic counts (p=0.001) and CRP (p=0.001) levels. Calprotectin and IL6 levels were significantly higher in cases than controls (p=0.001). Blood cultures were positive in eight neonates (16.6%); three for coagulase-negative staphylococci (CoNS), two for group B streptococci, one for staphylococcus aureus, one for haemophilus influenzae and the last one for escherichia coli. Five cases (10.4%) died within one week after diagnosis of sepsis, three of them were blood culture positive (two of them were positive for CoNS and the third one was positive for group B-streptococci). Cases with positive blood cultures and/or poor outcomes had the highest levels of serum calprotectin and IL-6.Table (2).

Significant positive correlations were found between calprotectin levels and IL6 Fig. (1). Both correlated positively and significantly with C-reactive protein and WBCs, but correlated negatively with platelet counts, gestational ages and body weights. Table (3). No significant differences were noticed between males and females as regards calprotectin and IL-6 levels. At cut-off value of 1.4 µg/mL of calprotectin and 160 pg/mL of IL-6, the sensitivity, specificity, positive and negative predictive values were 91.3%, 94%, 97.7% and 87% for calprotectin versus 89%, 92.2%, 96.7% and 84.8% for IL-6. Table (4).

Table 1. Comparison between patients and controls as regards demographic and laboratory data.

Variable		Patients (n=48)	Controls (n=40)	p-value
Gestational age (weeks)	Range Mean± SD	37.00- 42.00 38.71±1.15	37.00-42.00 39.40±1.26	0.23
Weight (grams)	Range Mean± SD	2.30-4.40 3.29±0.47	2.60-4.10 3.43±0.51	0.20
Sex	Males Females	26(54.17%) 22(45.83%)	24(60%) 16(40%)	0.34
Hb (gm/dl)	Range Mean± SD	9.5-16 10.1±3.1	12-18 13.8±3.3	0.03*
Platelets ($\times10^3$/dl)	Range Mean± SD	10.00-220.00 72.71±46.65	160.0-500.0 273.00±84.56	0.001**
WBCs ($\times10^3$/dl)	Range Mean± SD	8.00-31.00 17.66±7.60	3.80-16.00 7.42±3.77	0.001**
CRP (mg/L)	Range Mean± SD	8.00-29.00 19.88±5.69	0.50-9.00 3.31±2.59	0.001**
Blood culture	Positive Negative	8(16.6%) 40(83.4%)	0(0%) 20(100%)	0.02*
IL-6 (pg/mL)	Range Mean± SD	10.00-250.00 131.46±75.48	10.00-15.00 9.48±4.38	0.001**
Calprotectin (µg/mL)	Range Mean± SD	1.50-6.50 3.77±1.85	0.50-1.40 0.70±0.33	0.001**
Outcome	Survival Death	43(89.58%) 5(10.42%)	40(100%) 0(0%)	0.04*

*= Significant
**= highly significant

Table 2. Comparison between calprotectin and IL6 levels in patients as regards blood culture and outcome

Parameters	Blood culture		Outcome	
	Positive (n= 8)	Negative (n= 40)	Survival (n=43)	Death (n= 5)
Calprotectin (µg/mL)				
Range	5-6.5	1-5.5	1-5.5	5.5-6.5
Mean± SD	5.8±0.61	3.2±1.3	3.5±1.7	6.10±0.42
p-value	0.002**		0.002**	
IL-6 (pg/ mL)				
Range	180-250	10-180	10-220	200-250
Mean± SD	218±26.24	115.30±42.66	120.47±71.74	226±20.74
p-value	0.002**		0.002**	

*= Significant **= highly significant

Table 3. Correlation between calprotectin and IL6 levels and other parameters in patients.

Parameters	Calprotectin (µg/mL)		IL-6 (pg/ml)	
	r	(p)	r	(p)
Age (weeks)	-0.35	0.014*	-0.55	0.026*
Weight (grams)	-0.34	0.018*	-0.40	0.005**
Platelets (×10³/dl)	-0.87	0.001**	-0.79	0.001**
WBCs (×10³/dl)	0.72	0.001**	0.66	0.02*
CRP (mg/L)	0.85	0.001**	0.89	0.001**
IL-6 (pg/ mL)	0.92	0.001**	--	--

*= Significant **= highly significant

Table 4. The sensitivity, specificity, positive and negative predictive values for calprotectin and IL-6.

Parameters	Calprotectin (µg/mL)*	IL-6 (pg/ml)*
Sensitivity	91.3%	89.0%
Specificity	94.0%	92.2%
Positive predictive value	97.7%	96.7%
Negative predictive value	87.0%	84.8%

**Cut-off value= 1.4 µg/mL of calprotectin & 160 pg/mL of IL-6*

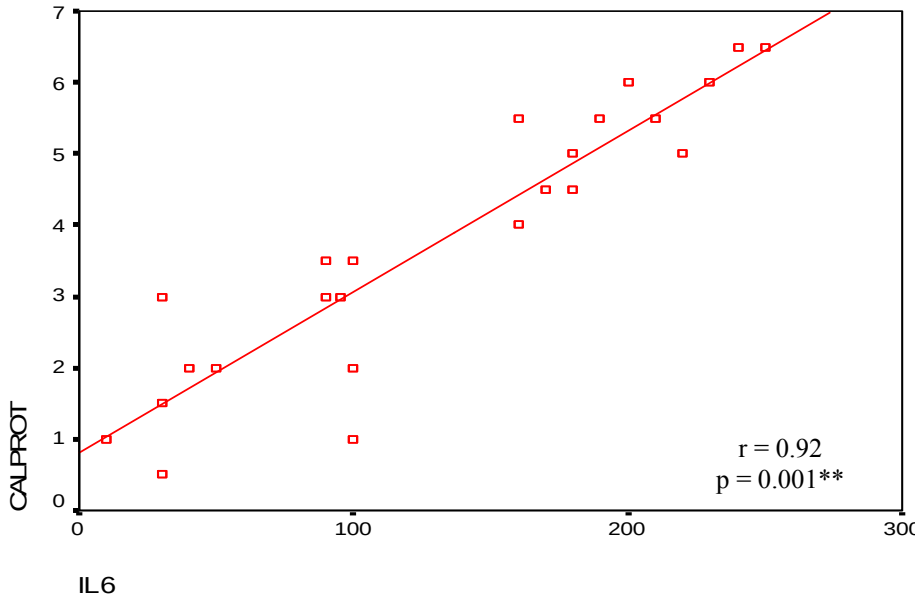

Figure 1. Correlation between calprotectin and IL6 in patients

DISCUSSION

In this study the plasma calprotectin levels were significantly higher in septic neonates than controls (3.77±1.85#0.70±0.33 µg/ml respectively, p-value =0.001). Terrin and coworkers[22] reported that calprotectin levels were increased in LBW neonates with early onset neonatal sepsis.

In the present study, only eight patients (16.6%) had positive blood cultures. Blood cultures are reported to be positive only in10-30% of cases suspected of neonatal sepsis[23]. Nosocomial sepsis caused by coagulase negative staphylococcal (CoNS) infections continues to be an important cause of morbidity in NICUs. Isaacs et al[24] reported that coagulase-negative staphylococci are the major causative microorganisms in neonatal nosocomial sepsis and several studies demonstrated that CoNS sepsis is caused by predominant molecular types which are widely distributed among both neonates and staff, suggesting cross-contamination[25,26]. These results supported our results as three cases of eight (37.5%) were positive for CoNS followed by group B streptococcal infection.

Patients with positive blood cultures, and/or those who died within one week after diagnosis of sepsis had significantly higher levels of calprotectin than cases that were negative for blood cultures or those who survived beyond the first week after diagnosis of sepsis. These findings may reflect the reliability of serum calprotectin level as a marker of the severity and outcome of cases with sepsis.

CRP, an acute-phase protein involved in coagulation, is a biomarker widely used in diagnosis of sepsis. Acting as an opsonin for gram-positive bacteria to aid in their phagocytosis, so a conventional CRP value 10 mg/L, in the presence of one (or more) clinical sign(s) compatible with infection, was considered as a criterion to make a diagnosis of clinical septicemia at any neonatal age in NICU babies[27,28]. Results of this study showed that both calprotectin and IL-6 levels correlated positively with CRP levels and total leucocytic count which are two laboratory markers of neonatal sepsis[27-29] pointing to their usefulness as additional markers of sepsis.

The results of this study revealed that the correlations between calprotectin and IL-6 levels were more significant than the correlations between calprotectin and CRP levels and this may be attributed to the concomitant early appearance of both calprotectin and IL-6 during the course of sepsis in contrast to the late peak of CRP levels during the course of sepsis[27,28].

Thrombocytopenia is one of the most common complications of neonatal sepsis[29] and is considered one of the hematological parameters of severity of neonatal sepsis[29,30]**.** The significant negative correlations of both calprotectin and IL-6 with platelet counts indicate that both calprotectin and IL-6 may be used as parameters of the severity of sepsis.

The significant negative correlations between calprotectin, IL-6 levels and both of ages and weights in this study may be attributed to the fact that sepsis is more severe in neonates with younger ages and lower weights resulting in higher levels of these inflammatory markers and this is in agreement with many previous studies[3,17,19] .

Several problems in this study need to be addressed in further studies; first, definition of sepsis was based on clinical definitions of infection. Second, the blood cultures yielding coagulase negative staphylococci may represent contaminations and lastly the small sized sample.

Serum calprotectin levels are significantly higher in full-term neonates with late onset neonatal sepsis than controls. Its levels correlated well with other laboratory markers of sepsis and neonatal mortality, and proved itself as both a sensitive and specific marker of neonatal sepsis. These findings may be helpful in planning strategies for diagnosis and hence the treatment of late onset neonatal sepsis in full terms.

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