

Evaluation of Immature Neutrophil Ratio and Calprotectin Level for the Diagnosis of Neonatal Sepsis

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ABSTRACT

Background: Sepsis is defined as a systematic inflammatory reaction against infection. Despite the recent advances in medical and pediatric sciences, sepsis remains a significant life-threatening health issue. The main problem associated with this disorder is the nonspecific and non-discriminative symptoms of noninfectious diseases. The present study aimed to introduce diagnostic parameters of high sensitivity and specificity for neonatal sepsis.

Methods: This case-control study was conducted in Mashhad, Iran during 2013-2015. Subjects included 40 septic neonates with confirmed diagnosis by a subspecialist of pediatrics and 40 neonates without clinical and laboratory findings of sepsis as the control group. Blood samples were collected from all the infants. In addition, differential white blood cell count of peripheral blood smear was performed, and immature/total neutrophil ratio (I/T) was calculated for all the PBS slides. Plasma calprotectin levels were also determined using the sandwich ELISA method.

Results: Mean plasma concentration of calprotectin was 33190 ± 23760 and 18980 ± 13410 ng/ml in the septic and control groups, respectively. Moreover, mean I/T was 0.61 ± 0.22 and 0.51 ± 0.26 in the septic and control groups, respectively. The obtained results indicated that calprotectin levels and I/T were significantly higher in the septic group compared to the control group ($P < 0.05$).

Conclusion: According to the results, nucleated red blood cell count and calprotectin levels were the most specific parameters for the definite diagnosis of neonatal sepsis, while neutrophils had the highest sensitivity in this regard.

Keywords: Calprotectin, ELISA, Immature neutrophils, Neonatal sepsis, NRBC

Introduction

Sepsis is a systematic inflammatory disease, which is associated with metabolic and hemodynamic disorders (1, 2). This inflammatory process is categorized based on the time of occurrence. Sepsis occurring within the first 28 days of birth is known as neonatal sepsis (1). In terms of the onset, neonatal early-onset sepsis (EOS) refers to the types of sepsis occurring within the first 72 hours of birth and late-onset sepsis (LOS) refers to the types occurring after the first 72 hours of birth (3, 4). Despite the recent advances in medical and pediatric sciences, neonatal sepsis remains a significant life-threatening health issue in newborns (4-9). According to the World Health Organization (WHO), four million neonatal deaths

are reported annually, more than 35% of which are due to infectious factors (7), affecting 1.5-2.7% of all neonates (3).

In Mashhad city (Iran), infection has been reported to be the fourth main cause of neonatal mortality, and septic infections account for 80% of these deaths. In general, 5-10% of all the neonatal deaths in this city are attributed to sepsis (10).

Sepsis has been associated with nonspecific indications (4, 6, 7, 9, 11), which are indiscernible from non-septic conditions, such as respiratory distress syndrome and maladaptation (6). Delayed treatment of sepsis due to the late diagnosis of the disease is known to increase the risk of mortality

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(12). In this regard, nonspecific indications consist of a set of influential factors that may delay the diagnosis and treatment of the disease (1, 3, 5, 13). Blood culture is considered the 'gold standard' diagnostic method for neonatal sepsis (1, 4, 6, 7, 14) despite certain limitations, such as low sensitivity and high rate of false negative results (1, 6), which are due to the low blood volume and drug administration (1, 4).

Diagnosis of infections involves the application of cytokines, chemokines, cell surface markers, acute-phase reactants, genomic and proteomic markers, nucleic acid, and molecular and metabolic markers (13). Sepsis screening tests should be able to detect all the certain and uncertain septic cases; in other words, they should be of high sensitivity (1). In general, the current standard parameters in this regard lack sufficient accuracy (3). Despite the few positive microbial cultures that can confirm neonatal sepsis (2 per 1,000 cases), a significant number of neonates (approximately 7-13%) are treated for possible sepsis infection (13).

Calprotectin is an inflammatory marker (7). It has been implicated that calprotectin and immature granulocytes/total mature neutrophil (I/T) ratio are proper markers for sepsis evaluation (14), as they are not affected by the type or site of the infection.

The present study aimed to assess the variations of complete blood cell count (CBC), calprotectin plasma level, and I/T, as well as some biochemical indices of septic patients, in comparison with a control group in order to introduce a sensitive and specific parameter for the diagnosis of neonatal sepsis.

Methods

This cross-sectional study was conducted in Ghaem Medical Center, affiliated to Mashhad University of Medical Sciences in Mashhad, Iran during 2013-2015. Subjects included 40 septic neonates and 40 neonates with no clinical or laboratory indications of sepsis as the control group; it is notable that the control subjects had icterus. Samples were collected from the infants within the first 72 hours of birth. All the infants in this study were referred to Ghaem Medical Center. The study was performed under the supervision of the Ethics Committee of Mashhad University of Medical Sciences.

Neonates presenting with the clinical indications of sepsis were enrolled in the study after obtaining parental consent by a physician. Confirmed clinical indications of sepsis included

lethargy, apnea, respiratory distress, restlessness, seizures, need for mechanical ventilation, abdominal distension, hypotension, and food intolerance. Confirmed laboratory indications included leukocytosis (white blood cell [WBC] $>2\times 10^4$ /dL), leucopenia (WBC $<5\times 10^3$ /dL), and thrombocytopenia (platelet $\leq 1.5\times 10^5$ /dL). In addition, infants with the suspicion of sepsis were enrolled in the study after the evaluation of the clinical indications and different laboratory findings by a pediatrician.

The control group consisted of the neonates with icterus, who required laboratory tests for determining the cause of jaundice. Blood, urine, and cerebrospinal fluid (CSF) cultures and CBC were obtained for all the septic patients. In total, 2 ml of blood was transferred to ethylenediaminetetraacetic acid (EDTA) tubes, and 2 ml of blood was transferred to the tubes without anticoagulants. CBC was carried out using the Sysmex KX-21N Automated Counter (Japan). Peripheral blood smears were prepared from the EDTA blood and stained with Giemsa. All the prepared slides were evaluated to control the CBC results and examine cell morphology. Additionally, differential WBC count, including the immature neutrophil count in 100 WBC, was reported. Absolute neutrophil and immature granulocyte counts were calculated to measure the I/T, which was obtained by dividing the immature granulocytes count by the total mature neutrophil count.

Immature granulocytes are the myeloid cells without perfect function. The most evident morphologic finding concerns the nucleus form that is not segmented completely. In this study, plasma calprotectin levels were measured by the sandwich ELISA method using the Biovendor ELISA kit (No: RD191217100R). To do so, sera of the studied cases were collected in accordance with the instructions of the manufacturer. Samples were centrifuged in 3,000 grams for five minutes, and the sera were separated and stored at the temperature of -20 degrees centigrade. Moreover, the ELISA test was performed on all the septic and non-septic neonates in accordance with the instructions of the manufacturer.

Data analysis was performed in SPSS version 16.0 (SPSS Inc., Chicago, Illinois, USA). Parametric and nonparametric tests were used to evaluate the collected data based on the normal and non-normal distribution. Nonparametric tests (e.g., Mann-Whitney U test) were applied for the data with abnormal distribution, and t-test was used for the normally distributed data. In addition, correlations between the parameters were

assessed using the logistic regression analysis.

Results

Among 40 septic neonates who were all in their first 72 hours of life, 22 cases (55%) were female, and 18 (45%) were male. In the non-septic group, 25 neonates (62.5%) were female, and 15 (37.5%) were male. Results of Chi-square demonstrated no statistically significant difference between the septic and non-septic groups sexing terms of gender distribution ($P=0.65$).

In this study, only two positive blood cultures (5.00%) were observed among the evaluated septic neonates, and no positive urine and CSF cultures were reported. Minimum and maximum values of calprotectin in septic neonates were 4,000 and 71,900 ng/mL, while these values were determined at 4,200 and 45,500 ng/mL in the control group, respectively. In addition, mean calprotectin was 27660 ± 20600 ng/mL in the male infants and 24970 ± 20360 ng/mL in the female infants. No significant difference was observed in the mean calprotectin level between the male and female neonates ($P=0.65$).

Minimum and maximum I/T were 0.21 and 1 in the septic group and 0.27 and 0.97 in the control group, respectively. According to the findings, I/T value was significantly higher in the septic neonates compared to the control group ($P=0.04$).

Hematological and biochemical laboratory findings of the septic and control groups are presented in Table 1. Platelet count, immature granulocyte and nucleated red blood cell counts,

I/T index, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and hematocrit (HCT) were observed to increase significantly in the septic patients ($P<0.05$). Moreover, leukopenia was reported in 3.57%, leukocytosis was detected in 17.85%, thrombocytosis was observed in 3.57%, and thrombocytopenia was reported in 60.71% of the septic neonates.

According to the results, mean platelet distribution width (PDW), hemoglobin (HB), and HCT was higher in the female neonates compared to the male neonates, and statistical analysis showed significant differences between the hematological parameters (PDW, HB, and HCT) and gender of the neonates in the septic group.

Calprotectin level was observed to have a direct correlation with WBC ($r=0.38$; $P=0.00$), MCV ($r=0.25$; $P=0.02$), and monocytes ($r=-0.26$; $P=0.02$). In addition, I/T had direct correlation with lymphocytes ($r=-0.25$; $P=0.22$), immature neutrophils ($r=0.43$; $P<0.01$), and erythrocyte sedimentation rate ($r=0.38$; $P=0.01$).

Test assessment features, including sensitivity, specificity, negative predictive values, and positive predictive values of the evaluated parameters were calculated in SPSS version 16.0 (Table 2).

The measured cutoff points were 38,300 ng/mL for calprotectin, 0.50 for I/T, 30.00% for immature granulocytes count, 14.50% for neutrophil count, and 14.00% for nucleated red blood cell count.

Among the five evaluated parameters (Table 2), calprotectin had the highest specificity and

Table 1. Hematological and Biochemical Laboratory Findings of Septic Patients and Controls

Variable	Septic Patients (N=40) SD±Mean	Controls (N=40) SD±Mean	P-value
White Blood Cell ($\times 10^3$ /dl)	10.42±14.28	3.85±11.58	0.78
Red Blood Cell ($\times 10^3$ /dl)	0.76±4.41	0.67±4.39	0.91
Hemoglobin (g/dl)	3.41±16.45	2.85±15.45	0.15
Hematocrit	8.00±47.95	7.48±44.06	0.02
MCV (fl)	11.82±109.44	6.65±100.15	0.00
MCH (Pg)	4.32±37.32	2.60±35.03	0.00
MCHC (g/dl)	1.97±34.13	9.40±36.88	0.09
Platelet ($\times 10^3$ /dl)	142.85±207.80	88.75±142.85	0.00
Neutrophil (100%)	14.16±24.87	11.65±22.32	0.39
Lymphocyte (100%)	17.91±35.05	18.48±44.45	0.02
Monocyte (100%)	2.47±2.77	4.39±5.85	0.00
Eosinophil (100%)	1.46±1.17	3.23±2.77	0.02
Immature granulocyte (100%)	16.63±36.20	15.16±24.35	0.00
MPV (fl)	2.55±9.92	1.59±10.81	0.22
PDW	5.95±13.87	5.37±17.90	0.00
NRBC (100%)	38.11±23.05	21.95±9.22	0.01
ESR	3.03±3.50	0.51±1.40	0.10
I/T	0.22±0.61	0.26±0.51	0.004
Urea (mg/dl)	24.75±39.75	6.33±16.85	0.004
Creatinine (mg/dl)	0.27±0.83	0.21±0.55	0.003
Calprotectin (ng/ml)	23760± 33190	13140±18980	0.002

Table 2. Sensitivity, Specificity, and Negative and Positive Predictive Values of Evaluated Parameters (%)

Parameter	Sensitivity	Specificity	PPV	NPV
Calprotectin	42.50	90.00	81.00	61.00
I/T	62.50	62.50	62.50	62.50
IMG*	80.00	67.50	71.10	77.10
Neutrophil	82.50	37.50	56.90	68.20
NRBC	35.00	90.00	77.80	58.10

*IMG: immature granulocyte

positive predictive value. On the other hand, the highest sensitivity and negative predictive value were observed in the peripheral blood neutrophil count and immature granulocyte count, respectively.

Discussion

Neonatal sepsis is a common inflammatory syndrome, which threatens the life of millions of neonates annually. Currently, rapid accurate diagnostic methods are not available for sepsis although blood culture has been approved as a proper diagnostic approach in this regard. Therefore, the results of blood culture, which are reported after seven days, could determine the presence of infection; however, it surpasses the golden time of treatment.

The present study aimed to introduce a highly sensitive and specific marker for the diagnosis of neonatal sepsis. According to our findings, mean plasma calprotectin (33190 ± 23760 ng/mL) was significantly higher in the septic neonates. In a study conducted in Egypt, Maaboud et al. (2012) investigated the association between the calprotectin level and blood culture results, reporting that calprotectin level was significantly higher in the positive cultures (5800 ± 610 ng/mL) compared to the negative cultures (3200 ± 1300 ng/mL) ($P=0.002$) (15). Furthermore, according to the results of Mann-Whitney U test, calprotectin values were higher in the septic neonates ($P=0.024$). In a similar study performed in Indonesia, Riskawa et al. (2012) reported the mean concentrations of plasma calprotectin to be 144,490 and 10,310 ng/mL in 10 preterm septic neonates and non-septic neonates, respectively. Findings of the two mentioned studies are consistent with the results of the current research (16). It is notable that the larger sample size was one of the advantages of the present study. It seems that the broad range of the reported values for plasma calprotectin is due to the differences in the applied kits. However, in the mentioned studies, the inflammatory marker was reported to be higher in the septic infants compared to the non-septic

subjects.

In another research, Decembrino et al. reported the specificity and sensitivity of calprotectin to be 69.7% and 62.5%, respectively (17), while the specificity and sensitivity of this parameter were respectively 42.50% and 90.00% in the present study. Despite the considerable differences between the patient and control groups, all the mentioned studies confirmed the higher concentration of calprotectin in the septic neonates compared to the control cases due to the infection.

As mentioned earlier, blood culture is considered the 'gold standard' for the evaluation of sepsis although it is not an ideal method due to the time-consuming process of providing the results. Recently, I/T, absolute neutrophil count, and WBC have been proposed as the three main incremental prognostic indices in this regard (13). Statistical analysis has revealed that all the three mentioned parameters are higher in septic neonates, while only the increment in I/T has been considered significant ($P=0.045$). In the current research, mean I/T was 0.61 ± 0.22 and 0.51 ± 0.26 in the septic and non-septic infants, respectively ($P=0.04$). In a study by Arif, the determined cutoff values were 0.5, 0.91, and 0.042 as the mean I/T in septic and control subjects ($P=0.025$) (1). Similar to the aforementioned studies, mean I/T was found to have a significant association with the occurrence of sepsis.

In the study by Maaboud et al., hemoglobin concentration in the septic neonates (10.1 ± 3.1 g/dl) was lower compared to the control group (13.8 ± 3.3 g/dl) with no significance in this regard ($P=0.125$) (15). In the mentioned study, the researchers examined LOS neonates aged 37-42 weeks, and the age of the infants may justify the differences in the reported mean values.

Nucleated red blood cells (NRBC) are the immature erythrocytes in the peripheral blood, which increase due to erythropoietin secretion (18). Different inflammations cause cytokine secretion, which is followed by the release of

NRBCs into the peripheral blood (19-21). In their research, Duley et al. evaluated 68 preterm neonates who were born to mothers with chorioamnionitis. According to the findings, NRBC count is an accessible, simple test with rapid response capability for the assessment of neonatal inflammations (22). In the present study, the observed increase in the NRBC was not significant compared to the immature granulocyte increment ($P=0.125$). Evaluation of the two mentioned parameters in larger populations could help with the identification of markers with higher accuracy in this regard.

The main limitation of the current research was the age of the neonates, as all the subjects were examined within the first 72 hours of birth.

Conclusion

According to the results, breastfeeding of infants whose mothers undergo physiologic delivery with no drug administration during labor was significantly stronger compared to the neonates whose mothers were administered with oxytocin during labor. According to the results, calprotectin, which is measurable by available ELISA techniques, could be used along with immature granulocyte count as a valuable diagnostic parameter for neonatal sepsis; the most important advantage of this marker is its rapid response capability. Therefore, it is recommended that gynecologists and midwives enhance the breastfeeding of neonates after birth by training women during pregnancy and preparing them for a safe childbirth without drug use.

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Conflicts of interests

None declared.

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