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Plasma myeloperoxidase enzyme assay in cases of neonatal sepsis

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Abstract

Background: Myeloperoxidase (MPO) is a heme enzyme of azurophilic granules in neutrophils that has a strong oxidative activity. MPO has been demonstrated to be a major pathway for O_2-dependent microbicidal activity.

Methods: This study was conducted on 84 neonates. 41 with culture proven sepsis and 43 healthy neonates who served as control. Complete blood count, C-reactive protein quantitative assay and blood gases were done in cases group, MPO enzyme level measured by ELISA was done and compared in both groups.

Results: MPO enzyme level was statistically higher in case group compared to control group (P-value < 0.001). There was no statistically significant difference in whether the infected were pre-term or full term. MPO enzyme levels were statistically higher in cases with sepsis without septic shock compared to cases with septic shock (P-value < 0.001). MPO level showed positive correlations with total leukocyte count and absolute neutrophil count. MPO assay (using the cutoff value of 146.5 ng/ml) had a sensitivity of 80% and specificity of 83.9% with a positive predictive value of 92.9%, negative predictive value of 61.5% and accuracy of 82.9%.

Conclusion: MPO enzyme level increases significantly in neonates with sepsis. MPO assay is not affected by gestational age but can be affected by deterioration to septic shock. Early diagnosis of sepsis cannot rely on a single laboratory test and clinical decision remains to have the upper hand in diagnosis. (El Med J 2:2; 2014)

Keywords: Myeloperoxidase, Neonatal Sepsis, Septic Shock, Systemic Inflammatory Response Syndrome

Introduction

Sepsis neonatorum is the term used to describe any systemic bacterial infection documented by a positive blood culture in the first month of life. Bacterial sepsis in the neonate is a clinical syndrome characterized by systemic signs of infection accompanied by bacteremia [1]. Approximately 99% of the 4 million annual neonatal deaths occur in low and middle income countries, and 36% in others are attributed to serious infections; in high mortality settings this proportion may approach 50% [2].

Neonatal sepsis can be classified into two subtypes depending upon whether the onset of symptoms is before 72 hours of life: early onset neonatal sepsis (EONS) or late onset sepsis (LONS). These definitions have contributed greatly to diagnosis and treatment by identifying which microorganisms are likely to be responsible for sepsis during these periods and the expected outcomes of infection [3].

At the time of term birth, the immune system has not fully matured. The inexperienced adaptive immune system must still develop specificity and memory, which is completed only in the early childhood years. As such, normal term neonates rely heavily on their innate immune response but this too is immature. Immaturity of the immune system is more pronounced in infants born preterm [4].

Polymorphonuclear leukocytes (PMNL) are the first cell type in human beings activated in host immune defense against infection. These cells driven by chemotactic gradients migrate to inflammatory loci, where they recognize and phagocytose bacteria and other extrinsic microorganisms by release of hydrolytic enzymes and bactericidal proteins pre-stored in granules as well as newly generated reactive oxygen species (ROS) [5]. PMNLs isolated from premature human neonates have impaired phagocytosis, decreased capacity to generate oxygen radicals, and deficient intracellular bacterial killing [6].

Vascular leakage and recruitment of circulating PMNLs to the site of injury represent the early phase of the host defense mechanism and response to tissue injury or sepsis. Clinically, the increased number of PMNLs in blood is generally used to determine the development of inflammation/sepsis [7]. A more feasible and quantitative approach is the use of the biochemical assay of PMNLs associated MPO enzyme activity. This enzyme is highly enriched in the azurophilic granules of PMNLs recruited to injured tissue to mediate the acute phase of the inflammatory response [8]. Circulating lipopolysaccharides released from bacteria may activate both neutrophils and monocytes. The activated neutrophils release MPO a major granule enzyme in neutrophils which accounts for 5% of the total neutrophil protein [5]. MPO enzyme promotes oxidative stress in numerous inflammatory pathologies. It uses hydrogen peroxide to catalyze the production of strong oxidants including reactive oxygen species and free radicals that have bactericidal action [9].

The isolation of microorganisms from blood and/or CSF still remains the gold standard for definitive diagnosis. For years, investigators have sought a test or panel of tests able to identify septic neonates accurately and rapidly while awaiting culture results, in order to obtain an early diagnosis and develop a specific effective treatment for a successful outcome. This study aimed: 1) to determine the diagnostic utilities [sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV)] of myeloperoxidase (MPO) enzyme for detection of neonatal sepsis; 2) to define the optimal cutoff value for MPO level using the receiver operating characteristics (ROC) curve so that it may be used as a reference with which future studies can be compared.

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Methods

This cross-sectional study was carried out in neonatal intensive care units (NICU) of Cairo University Hospital (Kasr El Aini) and New Children's Teaching Hospitals (El-Monira) between January and August 2013, with the approval of the Ethical Committee of NICU and parents of the neonates. Data was confidentially preserved according to the revised Helsinki Declaration of Bioethics [10].

Patients

The study was carried on forty one newborns with confirmed neonatal infection. It included preterm (PT) and full term (FT) neonates of both gender with clinical symptoms and signs of sepsis within 1st month of life and positive blood culture. Exclusion criteria for enrolment into this study were congenital anomalies, chromosomal abnormalities, inborn errors of metabolism and neonates with surgical emergencies.

Forty three healthy stable newborns were enrolled in this study as a control group. They were born to healthy mothers with negative medical and obstetric history. All were free on clinical examination, their blood samples were taken within the first 28 days of life for MPO measurement. The collection of the blood specimens coincided with other routine blood sampling procedures, such as hematocrit, electrolytes, or glucose measurement.

Methods

All septic newborns were subjected to the following:

Comprehensive history taking including:

1. Antenatal history: maternal diseases (diabetes and hypertension), maternal infections (TORCH infections) and maternal medications during present pregnancy.
2. Obstetric history: includes mode of delivery and premature rupture of membrane (PROM) > 24 hours.
3. Natal history: gestational age, neonatal sex and birth weight.
4. Postnatal history: resuscitation data and Apgar score at 1 and 5 minutes, respiratory distress and cyanosis, onset of sepsis, severe sepsis (sepsis complicated by organ dysfunction), occurrence of septic shock (tachycardia with signs of decreased perfusion), duration of hospital stay and outcome (survival or mortality).

Clinical examination for neonates:

1. Assessment of gestational age (GA) through analysis of maternal dates and Ballard scores [11].
2. Assessment of general condition and reflexes (Moro / suckling).
3. Assessment of vital signs (respiratory rate, heart rate, blood pressure, temperature, capillary refilling time) at time of sampling.
4. Complete examination including cardiac, chest, abdominal, and neurological laying stress on tolerance to oral feeding, abdominal distension, residual gastric aspirate, oliguria, jaundice, cyanosis, convulsions, bleeding tendency and signs of septic shock (tachycardia, decreased peripheral pulses, altered alertness, cool extremities, reduced urinary output and lately hypotension).

Laboratory investigations:

1. Complete blood count with differential leukocytic count (Abott Cell - Dyn3700 - Abbott diagnostics - USA).
2. Analysis of plasma myeloperoxidase enzyme level by enzyme-linked immunosorbent assay (ELISA) (MPO ELIZA kit - Immundiagnostik AG - Germany): 1ml peripheral blood sample on EDTA vacutainer tube was collected. The sample was taken at the time of sepsis confirmation. Freshly collected EDTA blood was centrifuged to collect plasma. The assay utilizes the two-site "sandwich" technique with two selected polyclonal antibodies that bind to human MPO.
3. C-reactive protein (CRP) quantitative assay (NEPHSTAR CRP kit-Goldsite Diagnostics - China): normal range of CRP concentration of healthy infant is < 5 mg/L.
4. Measurement of venous blood gases: Metabolic acidosis is defined as an arterial blood pH < 7.35 (if venous sample pH < 7.32) with plasma bicarbonate < 22 mEq/L (if venous sample < 19 mEq/L).
5. Blood culture for aerobic and anaerobic organism and antibiotic sensitivity testing: 1 to 5 mL of blood was drawn from venipuncture using sterile needle and then blood was injected in the blood culture bottle for Bactec microbial detection system (Bactec 9050, Becton - Dickinson).

Statistical methods

Data analysis was performed using Statistical Package for Social Sciences (SPSS) version 17. Numerical data was summarized using median and ranges. Categorical data was summarized as percentages. Comparisons between two groups with respect to normally distributed numeric variables were done using the t-tests. Non-normally distributed variables were compared by Mann-Whitney test. Comparisons between septic shock cases, septic cases without shock and controls were performed by Kruskal–Wallis test followed by the post hoc Bonferroni test. To measure the strength of the association between MPO and other factors, Spearman's correlation coefficients were used. The receiver operator characteristic (ROC) curve was used to display the relationship between sensitivity and specificity [12]. All p-values were two-sided. P-values < 0.05 were considered significant.

Results

Among the total of 41 neonates in the case group, there were 23 (56.1%) males and 18 (43.9%) females. 14 (34.1%) neonates were delivered normally (NVD) and 27 (65.9%) neonates by cesarean section (CS). 8 (19.5%) neonates were delivered after history of PROM and 33 (80.5%) neonates with no history of PROM. Among the total of 43 neonates in the control group, there were 30 (69.8%) males and 13 (30.2%) females that represent. 20 (46.5%) neonates were delivered by NVD and 23 (53.5%) neonates by CS. 3 (7%) neonates were delivered after history of PROM and 40 (93%) neonates with no history of PROM that represent. Table 1 shows comparison between the case and control groups regarding GA, postnatal age, weight and Apgar score at 1 and 5 minutes and revealed that there was a significant difference between the two groups regarding these variables, p-value < 0.001. The following data were found in study group of neonates with sepsis: 1) PT neonates (61%) were more than FT ones (39%); 2) LOS is of higher prevalence (78%) than EOS (22%); 3) Regarding outcome of neonates; 73.2% were discharged while 26.8% died. Table 2 shows the laboratory data (CBC with differential count, blood gases and CRP) of the case group.

Table 1: Demographic data of the case and control groups

Item	Cases (n=41)				Controls (n=43)				P-value
	Mean	SD*	Minimum	Maximum	Mean	SD*	Minimum	Maximum	
GA® (weeks)	34.1	4.1	28.0	41.0	38.0	2.1	30.0	40.0	<0.001
Postnatal age (days)	15.0	7.7	3.0	28.0	9.1	4.3	3.0	21.0	<0.001
Weight (Kg)	2.1	1.0	0.9	5.0	3.2	0.6	1.3	4.2	<0.001
Apgar 1 min	3.7	1.9	1.0	7.0	6.4	1.4	3.0	8.0	<0.001
Apgar 5 min	6.8	1.8	2.0	9.0	8.8	0.5	7.0	9.0	<0.001

*SD=Standard Deviation; ®GA=Gestational Age

Table 2: Laboratory data of the case group

Variable		Mean	Standard Deviation	Median	Minimum	Maximum
Blood Count	Platelets (1000/ml)	132.6	91.4	-	17.0	413.0
	Total leukocyte count (1000/ml)	16.0	11.5	-	3.4	44.0
	Absolute neutrophil count (/mm^3)	-	-	6.12	1.51	31.68
	Absolute band cells count (1000/mm^3)	-	-	1.08	0.1	14.08
	Band (%)	13.0	8.4	-	1.0	34.0
	Absolute segmented cells count (1000/mm^3)	-	-	4.3	0.93	26.84
	Segmented cells (%)	44.5	18.1	-	16.0	82.0
	Immature to total neutrophil ratio (I/T ratio)	0.2	0.1	-	0.0	0.6
Blood Gases	pH	7.3	0.1	-	7.1	7.6
	pCO_2	46.4	12.1	-	16.0	80.0
	pO_2	45.7	23.1	-	20.0	124.0
	HCO_3	24.1	6.7	-	9.2	35.0
	Base excess	-	-	-0.6	-16.0	12.7
C-reactive Protein		-	-	24	6	192

Table 3 shows thrombocytopenia in 61% of cases, leukocytosis in 26.8% of cases, leukopenia in 7.3% of cases, shift to left in 63.4% of cases and metabolic acidosis in 29.3% of cases.

Table 3: Thrombocytopenia, total leukocyte count, shift to left and metabolic acidosis frequency in the case group

Variable		N	%
Thrombocytopenia (<150,000/µl)	No	16	39.0%
	Yes	25	61.0%
Total leukocyte count	Normal	27	65.9%
	Leukopenia*	3	7.3%
	Leukocytosis®	11	26.8%
Shift to the left (I/T ratio ≥ 0.2)	Yes	26	63.4%
	No	15	36.6%
Metabolic acidosis	Yes	12	29.3%
	No	29	70.7%

*Leukopenia <5000/mm^3; ®Leukocytosis >20,000/mm^3

Table 4 demonstrates the frequencies of organisms isolated from blood culture of neonates with sepsis. The highest frequency was for Klebsiella that was isolated from 17 neonates (41%).

Figure 1 shows a comparison between cases and control groups regarding MPO level. MPO level differs significantly in both groups. In the case group; the median was 238 ng/ml while in the control group; it was 67 ng/ml with p-value < 0.001.

Table 4: Blood culture organism frequency

	Frequency	Percentage
Klebsiella	17	41.5%
Pseudomonas	10	24.4%
Acenitobactear	3	7.3%
CoNS	3	7.3%
Candida	3	7.3%
Klebsiella and Pseudomonas	2	4.9%
MRSA*	1	2.4%
E.coli	1	2.4%
Klebsiella & MRSA	1	2.4%
Total	41	100%

*Methicillin-resistant Staphylococcus aureus

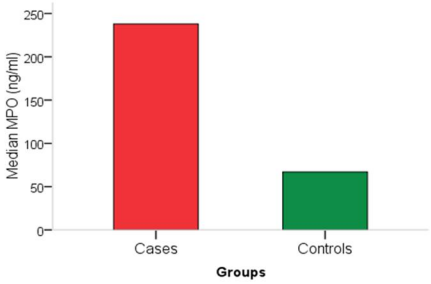

Figure 1: Comparison of MPO level in the case and control groups

A comparison regarding MPO level between PT neonates (median = 238 ng/ml) and FT neonates (median = 232 ng/ml) among case group was done and it was found that MPO level was not significantly different (p-value=0.979).

Figure (2) revealed that MPO level was lower in neonates with septic shock (median=100 ng/ml) than other cases in group of sepsis (median = 276 ng/ml) with statistically significant difference (p-value < 0.001).

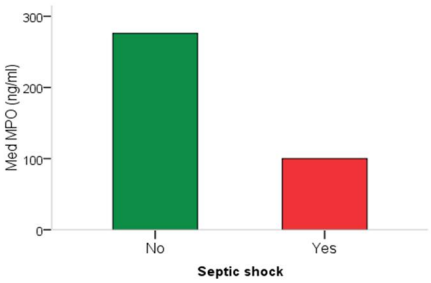

Figure 2: Comparison of MPO level in neonates with septic shock and other cases with sepsis

Figure 3 revealed no statistically significant difference in MPO level between neonates with septic shock (median = 100 ng/ml) and control group (median = 67 ng/ml) while both groups when compared to the group of other cases of sepsis (not in septic shock) (median = 276 ng/ml) show statistically significant difference (p-value < 0.001).

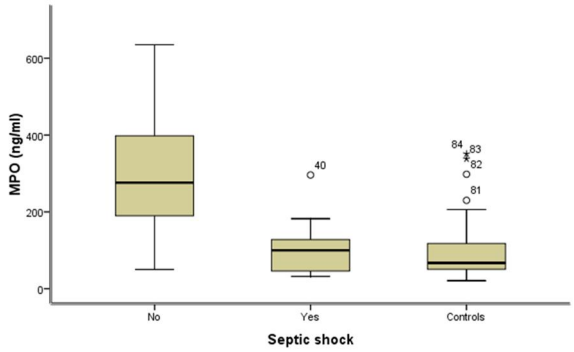

Figure 3: Comparison of MPO level in neonates with septic shock, other cases and control group

NB.: Samples (numbered according to their number in master sheet) whose results were plotted outside the ranges of septic shock group and control group in figure 3 is considered to be extreme values.

Table 5 revealed no statistically significant difference of MPO level between neonates with severe sepsis (median = 187, minimum = 32, maximum = 635) compared to other cases (median = 262, minimum = 50, maximum = 588) as p-value was not significant (0.629). Figure 4 revealed moderate positive correlation between MPO level and TLC with r-value (0.503) and p-value (0.001). A moderate positive correlation was found between MPO level and absolute neutrophil count with r-value (0.536) and p-value (<0.001) (figure 5). Table 6 and figure 6 revealed the diagnostic utilities of MPO assay (using the cutoff value of 146.5 ng/ml); it had a sensitivity of 80%, specificity of 83.9% with a positive predictive value of 92.9%, negative predictive value of 61.5% and accuracy of 82.9% as per the ROC curve.

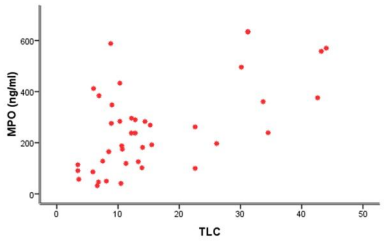

Figure 4: Correlation of MPO level with total leukocyte count

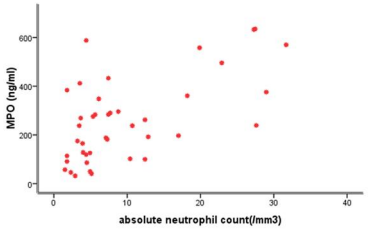

Figure 5: Correlation of MPO level with absolute neutrophil count

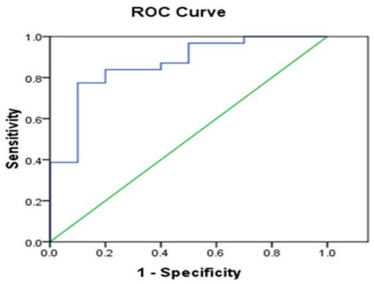

Figure 6: ROC curve for MPO as a predictor for diagnosis of sepsis

Table 5: Demographic data of the case and control groups

Item	Neonates in case group (n=41)						P-value
	Severe sepsis (n=22)			Other cases (n=19)			
	Median	Minimum	Maximum	Median	Minimum	Maximum	
MPO (ng/ml)	187	32	635	262	50	588	0.629

Table 6: Diagnostic utilities of MPO assay

Area	Standard Error	P-value	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
0.865	0.066	0.001	0.735	0.994

Discussion

The present cross-sectional study was done on neonates in whom infection was confirmed by positive blood culture and were then recruited into the study. CBC, CRP, venous blood gases and plasma MPO enzyme level were measured.

Regarding maternal risk factors of neonatal sepsis in our study; PROM accounted for 19.5% in cases diagnosed with sepsis. In the study by Leal et al, PROM was reported for 32.7% [13]. These differences may be attributed to large sample size (11,790 neonates) compared to our sample size. In agreement with the study of Fahmey, the highest frequency pathogen among culture-proven cases was for Klebsiella that was isolated from 41.5% neonates in our study and 42.8% in the comparative study [14].

Currently, we need a predictive marker in diagnosis of sepsis that follows two main strategies: 1) starting an antibacterial therapy as early as possible in a case of suspected sepsis; and 2) initiating therapy only in infected patients. Thus, a high sensitivity and negative predictive value of approximate 100%, and a good specificity and positive predictive value in excess of 85% are recommended. In addition, standardized cut-off values are crucial, making results comparable between laboratories [15].

Total leukocyte count (>20000 or <5000), differential leukocyte count and morphology, total neutrophil count, total non-segmented neutrophil count, neutrophil ratios and platelet count are the indices most commonly used. These hematological counts and ratios showed a limited accuracy with wide range of sensitivity (17–90%) and specificity (31–100%), due to the relatively long period necessary to become positive and the significant influence of non-specific factors. However, I/T ratio of >0.2 may reach a sensitivity of 90% and negative predictive value of 98% [16].

Our study revealed that MPO enzyme level was higher in cases of sepsis compared to controls. This comes in agreement with Kothari and his colleagues [5]. MPO enzyme activity was higher in cases of sepsis (mean, 2.4 ± 1.8) than control group (mean 0.32 ± 0.11) and the difference was statistically significant (p-value <0.01), while in SIRS group (mean 1.86 ± 1.2 in) the level of MPO was slightly lower than that of sepsis with no significant difference despite of statistically significant (p-value <0.01) when compared to control group, noting that MPO specific activity was expressed as nanomoles of H_2O_2 degraded per milligram protein per 10 minutes.

In accordance of Mühl et al study, MPO enzyme activity was higher in cases of sepsis than control group (cases of SIRS due to acute burn injury patients not infected) with the difference being significant [17]. In their study, daily samples were withdrawn from cases and control groups. Comparison between both groups revealed that the p-value < 0.05 on first and second days of intensive care unit admission and this value changed on third day to be < 0.01.

In the study by Yunanto et al, MPO enzyme activity was assessed not only in the blood but also in the saliva and was found to be higher in neonates with sepsis than healthy ones with statistically significant difference in both conditions (p-value<0.001) [18].

In agreement with Kothari et al, MPO enzyme level was expected to increase with the progression of sepsis and development of septic shock, but it was constantly observed to be on the lower side in patients with advanced stages of septic shock [5]. As MPO enzyme level reflects the neutrophil function, neutrophil count was found to be low in patients with septic shock that may be explained by pancytopenia in these patients due to bone marrow suppression in advanced stages of septic shock.

Spearman's correlation coefficients between MPO level and laboratory data showed moderate positive correlation of MPO level with TLC and absolute neutrophil count. These correlations are explained by the fact that MPO is a major granule enzyme in neutrophils, accounting for 5% of the total neutrophil protein as mentioned by Kothari and his colleagues [5].

In our study, MPO assay (using the cutoff value of 146.5 ng/ml) had a sensitivity of 80% and specificity of 83.9% with a PPV of 92.9%, NPV of 61.5% and accuracy of 82.9%. Compared to other markers of neonatal sepsis, Adib and his colleagues reported that CRP (using the cutoff value of 12 mg/l) had a sensitivity of 45% and specificity of 95% with a PPV of 30%, NPV of 30% while procalcitonin (PCT) (using the cutoff value of 1.1 ng/ml)was concluded to be a better marker than CRP in the diagnosis of neonatal sepsis due to 70% sensitivity, 80% specificity, 80% PPV and 75% NPV [19]. The increase in the serum concentration of CRP is rather slow during the first 14-48 h of infection and this may negatively affect the sensitivity of the test. In addition, increase in CRP concentration in non-infected clinical conditions such as meconium aspiration, prolong rupture of membranes are thought to affect the specificity of the test while PCT is detectable in the plasma as early as 2 h after the exposure to the bacterial products.

Advantages of using MPO as a diagnostic marker are: 1) The measurement can be "quantitative" and thus enables comparison of results among different centers; 2) The MPO level is not affected by gestational age; 3) It can be done in saliva rather than blood sample rendering it noninvasive utility in the diagnosis of neonatal sepsis [18].

As with any other study, there are certain points of weakness in our study: 1) It would have been better to do assay on serum samples to avoid factors that may cause MPO concentration shift in plasma samples such as the time between serum collection and analysis as well as repeated freeze-thaw cycles; 2) We didn't perform the diagnostic utilities of the hematologic parameters (CBC and CRP) in comparison with that of MPO for more confirmative data in control group; 3) Our study had a relatively small number of cases compared to others.

Conclusion

MPO can be used as a marker of neonatal sepsis with considerable diagnostic utility. MPO assay in addition to other markers of neonatal sepsis such as CRP may be of better value in early diagnosis of neonatal sepsis.Further studies on larger scale should be done to clarify the importance of MPO and assess diagnostic value of combination of MPO and other sepsis markers in achieving higher sensitivity, specificity, PPV, and NPV.

Authors' Contributions: Hanna Abul Ghar: conception and design of study, critical revision of the article and final approval of the version to be published. Eman Abdel Ghany: study design, data collection and interpretation of data, drafting of the article and final approval for publishing. Eslam Madbooly: data acquisition and an analysis and critical revision for important intellectual content. Rania Samy: laboratory work of the study, analysis and interpretation of data and final approval of the article.

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