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ORIGINAL ARTICLE

Thiobarbituric Acid Reactive Substance Cord Blood as a Diagnostic Marker of Early-onset Neonatal Sepsis in Preterm Neonates

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ABSTRACT

Background: *Neonatal sepsis (NS) promotes unbalanced production of oxidant and anti-oxidant substances, causing excess of free oxygen radicals which may lead to tissue damage. NS carries high risk of morbidity and mortality, thus identification of biomarker to optimize early diagnosis and therapeutic interventions is highly desirable.* ***Objectives:*** *to detect cord blood thiobarbituric acid reactive substance (TBARS) in preterm neonates with maternal risk factor for sepsis as predictors of early onset neonatal sepsis (EOS).* ***Methodology:*** *cord TBARS was measured in 80 preterm neonates with antenatal risk factors for EOS, and classified into two groups: sepsis (n=25) and no-sepsis (n=55).* ***Results:*** *TBARS was significantly higher in sepsis than no-sepsis groups 10.50 (6.5 – 20.5) vs 3.00 (2.2 - 3.8) nmol/ml, (p=0.000). TBARS was significantly higher in culture proven sepsis than negative culture patients. TBARS was significantly higher in died neonates than survivors.* ***Conclusion:*** *cord TBARS in preterm neonates with maternal risk factor for sepsis can be used as diagnostic and prognostic biomarker for EOS.*

INTRODUCTION

Neonatal sepsis (NS) is a serious condition leading to morbidity and mortality in neonates, especially preterm infants, particularly in developing countries. Accurate and timely diagnosis of early-onset neonatal sepsis (EOS) occurring within the first 72 h after birth remains challenging to the clinician and the laboratory. Moreover, rapid diagnosis is very essential to decrease sepsis life threatening complications and to avoid unnecessary use of antibiotics. The blood culture is considered the gold standard for the diagnosis of NS, however, it takes at least 48–72 h and occasionally produce false negatives. Thus EOS diagnosis involves the use of combinations of clinical signs which usually are non-specific, with hematological and serological markers for identification and intervention in babies at risk[1,2,3,4].

Different studies have shown that any injurious tissue event; (infection, trauma, anoxia) is recognized by monocytes and macrophages, which secrete cytokines, subsequently they activate inflammatory cells that release large amounts of toxic oxidizing substances [5]. These reactive oxygen species (ROS) could attack all biomolecules including DNA, lipids, and proteins. These oxidized molecules could be measured in biological fluids, being protein carbonyls a marker of protein oxidation and thiobarbituric acid reactive species (TBARS) a marker of lipid oxidation, the most frequently oxidative stress biomarkers measured in humans [6].

In NS, both oxidative stress and antioxidant pathways are stimulated, however, redox unbalance favoring oxidative stress occurs. Total oxidant state (TOS) and total antioxidant state (TAS) are both raised in septic patients before treatment versus controls and oxidative stress index (OSI), and the percentage ratio of TOS/TAS, were also increased. Interestingly, TOS and TAS were also measured to monitor therapy and significantly dropped after treatment in septic patients in comparison to pretreatment levels [7].

The purpose of our study is to detect cord blood level of thiobarbituric acid reactive substance (TBARS) in preterm neonates with maternal risk factor for sepsis as a diagnostic and prognostic biomarker of early onset neonatal sepsis.

METHODOLOGY

Study Population

This is a prospective case control study done in Neonatal Intensive Care Units (NICU), Pediatrics Hospital, Ain Shams University, Cairo and Armed Forces Hospital, Alexandria, from June 2017 till April 2018. Informed consent was provided to the care giver of recruited neonates and the study was approved by the pediatrics department council of Ain Shams University Hospitals.

The study included 80 preterm (≤36 weeks gestation) infants having one or more maternal risk factors for EOS; evidence of chorioamnionitis, foul smelling liquor, antepartum/intrapartum maternal fever[8],

prolonged and/or premature rupture of membranes ≥ 18 hours, offensive vaginal discharge and/or maternal blood leucocytosis (>15,000 leucocytes/ml[9]. Neonates with major congenital birth defects, chromosomal anomalies or perinatal asphyxia were excluded from the study.

Detailed perinatal history was obtained for all neonates, followed by a thorough clinical examination. The gestational age was determined by maternal last menstrual period and further confirmed by using the modified Ballard score[10]. Birth Anthropometric parameters including birth weight, birth length and occipito-frontal circumference were measured. All neonates received routine neonatal care according to our NICU protocol. All studied neonates were followed up for 72 hours for detection of clinical and/or laboratory results suggestive of EOS.

After a maximum of 72 hours of age, the included neonates were distinct into 2 groups; neonates with EOS 25/80 (31.25%) based on the development of clinical evidence suggestive of sepsis within 72 hours of birth [11], Rodwell's hematological scores ≥3 [12] and/or Tollner's scores ≥10 [13], and 55/80 (68.75%) no-sepsis neonates in whom EOS was ruled-out on the basis of absence of any clinical or laboratory evidence suggestive of infection.

Laboratory analysis

Blood samples from the umbilical cord were obtained after the delivery of the neonate and before the delivery of the placenta for TBARS analysis. Blood samples were added in iron-free polyethylene tubes without addition of anticoagulant and centrifuged for 15 minutes for separation of the serum. They were stored at -70°C till assay. Repeated freeze-thaw cycles were avoided.

The level of TBARS in the samples was determined spectrophotometrically using TBARS assay kit supplied by R&D systems (KGEO13, Minneapolis, USA). The principle of the technique is based upon direct measurement of TBARS which are low-molecular-weight end products (mainly malondialdehyde) that are formed during the decomposition of lipid peroxidation products. In this technique, in the presence of heat and acid, malondialdehyde reacts with thiobarbituric acid to produce a colored end product that absorbs light at 530-540 nm. The intensity of the color at 532 nm corresponds to the level of lipid peroxidation in the sample. A standard curve was constructed from which the concentrations of the samples were determined.

For complete blood count (CBC), venous samples were obtained at birth on potassium-EDTA in sterile vacutainers, and right after collection, the tube was gently inverted several times and placed into a cold box at 4–8°C. The samples were transported and analyzed on the same day. CBC was performed using the automated hematology analyzer; Sysmex XT-1800i (Sysmex, Kobe, Japan). The XT-1800i performs an analysis of white blood cells with an optical detector based on the flow cytometry method. Red blood cells (RBCs) and platelet count analyses were done by the RBC detector using the Hydrodynamic Focusing method.

As for CRP, venous blood samples were obtained at birth in gel tubes without addition of anticoagulant and centrifuged for 15 minutes for separation of the serum and were measured using HEALES analyser (Shenzhen Huisong Technology Development Co., Ltd, China) by latex-enhanced turbidimetric assay. The CRP antigen (present in serum) unites with anti-human CRP polyclonal antibody combined with latex particles to form latex-antibody-antigen combo. Turbidity leading to light scattering by this combo is measured at wavelength 630 nm. The scattering intensity is proportional to the content of antigen-antibody immune complex. The detection limit for CRP is 3mg/L.

Samples for blood cultures were collected soon after birth using BD BACTEC PEDS PLUS/F culture vials (BENEX Limited, Shannon, County Clare, Ireland). Blood culture samples were collected into the culture bottles, transported immediately to the laboratory, and processed using the BD BACTEC FX40 systems (Becton–Dickinson Microbiology Systems, Sparks, Ireland). A single blood culture bottle was used per patient. The minimal volume inoculated was 2 mL (3 mL was an ideal volume) and blood culture bottles were incubated for a maximum of 5 days (unless they flag positive). The BD BACTEC FX40 system detects positive cultures based on CO_2 production. Blood culture bottles which flagged positive were cultured on standard media with the use of routine microbiological techniques. Analytical Profile Index (API) biochemical test kit (BioMerieux, France) were used to confirm suspected pathogens.

Sample Size Calculation:

It is estimated that a sample size of 33 preterm neonates with one or more risk factors for EOS would yield 11 neonates with EOS *(positive group)* and 22 neonates without EOS *(negative group)*. This sample size would have a power of 92% (type 2 error, .08) to detect a statistically significant difference between the area under the ROC curve (AUC) associated with TBARS and a null AUC of .5 using a two-sided z-test with a confidence level of 99% (type 1 error, .01). This computation assumes that the positive group to negative group ratio is 1:3, the rate of EOS in the study population is 33.3% (Cancelier et al.)[6].

Statistical Analysis

Data were collected, revised, coded and entered to the Statistical Package for Social Science (IBM SPSS) version 23. The quantitative data were presented as mean, standard deviations and ranges when their distribution found parametric. Also qualitative variables were presented as number and percentages. The comparison between groups regarding qualitative data was done by using Chi-square test. Skewed numerical variables were presented as median (interquartile range), and between group differences were compared using the Mann–Whitney test. The comparison between

two independent groups with quantitative data and parametric distribution were done by using Independent t-test. Spearman correlation coefficients were used to assess the correlation between two quantitative parameters in the same groups.

Also Receiver operating characteristic curve (ROC) were used to assess the best cut off point with sensitivity, specificity, positive and negative predictive value and area under curve (AUC).

The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, the p-value was considered significant as the following: P-value >0.05: Non significant, P-value < 0.05: Significant, P-value < 0.01: Highly significant.

RESULTS

There were no statistically significant differences between sepsis and no-sepsis groups as regards the gender and mode of delivery (all neonates were delivered by lower segment caesarian section), however, the sepsis group showed statistically lower gestational age, birth weight, length, occipitofrontal circumference and Apgar score than no-sepsis group. Also, PROM ≥18 hours and maternal pre-eclampsia were significantly higher in the sepsis group than no-sepsis one (table 1).

Table 1: Comparison between sepsis and no-sepsis groups as regards neonatal and maternal demographic characteristics:

Demographic data	Sepsis group (No. = 25)		No- sepsis group (No. = 55)		Test value	P-value
Gender (males)	15 (60.0%)		34 (61.8%)		0.024*	0.877
Gestational age (weeks)	32.00 ± 2.29		34.42 ± 1.20		-6.208•	0.000
Weight (kg)	1.32 ± 0.40		2.35 ± 0.51		-8.891•	0.000
Length (cm)	40.48 ± 3.11		43.09 ± 2.24		-4.263•	0.000
OFC (cm)	32.88 ± 0.93		34.31 ± 0.57		-8.446•	0.000
Apgar score at 5 minutes Median(IQR)	6 (6-8)		8 (8-9)		-3.99‡	0.001
Risk factors	**No.**	**%**	**No.**	**%**	**Test value***	**P-value**
PROM ≥ 18	25	100.00%	43	78.20%	6.417	0.011
Maternal fever	7	28.00%	17	30.90%	0.069	0.792
Pre eclampsia	3	12.00%	0	0.00%	6.857	0.009
DM	2	8.00%	7	12.70%	0.385	0.535
UTI	3	12.00%	5	9.10%	0.162	0.688
IUGR	1	4.00%	2	3.60%	0.006	0.937

*:Chi-square test; •: Independent t-test, ‡: Mann Whitney test
DM: diabetes mellitus, IGUR: intrauterine growth retardation, OFC: occipitofrontal circumference, PROM: premature rupture of membrane, UTI: urinary tract infection.

Table 2 shows initiative laboratory data for sepsis and no-sepsis groups.

Table 2: Comparison between sepsis and no-sepsis groups as regards initial CBC and TBARS

		Sepsis group No. = 25	No-sepsis group No. = 55	Test value	P-value
Hb g/dl	Mean±SD	15.97 ± 1.00	16.51 ± 1.07	-2.050•	0.044
	Range	13.5 – 17.6	14.5 – 17.8		
HT %	Mean±SD	37.70 ± 1.29	39.31 ± 0.84	-6.558•	0.000
	Range	36 – 40	38 – 40		
TLC (*10^3/ µL)	Mean±SD	16.33 ± 1.08	12.73 ± 1.52	10.313•	0.000
	Range	14.8 – 18.4	8.5 – 15.2		
Platelets 10^3/ µL	Mean±SD	309.78 ± 110.40	399.05 ± 22.06	-5.776•	0.000
	Range	140 – 423	343 – 440		
TBARS (nmol/ml)	Median(IQR)	10.50 (6.5 – 20.5)	3.00 (2.2 - 3.8)	-6.567‡	0.000
	Range	4.2 – 24	0.5 – 17		

•: Independent t-test ‡: Mann Whitney test
CRP: C reactive protein, Hb: hemoglobin, HT: hematocrite, TLC: total leucocytic count

In the sepsis group 13/25 (52%) had positive blood culture; *Escherichia coli* (24.0%), *Klebsiella pneumoniae* (12.0%), Staphylococci (8.0%), Group B streptococci (8.0%). CRP median (IQR) was 12 (9-24) mg/L, Tollner's score median (IQR) was 6 (5-7) and Rodwell's score median (IQR) was 4 (4-5). All of the no-sepsis group had negative blood culture.

No death was reported in the no-sepsis group while 11 (44%) neonates died in the sepsis group (p <0.001)

In table (3) TBARS was found to be negatively correlated to gestational age (r = -0.338, p = 0.002), Apgar score (r = -0.631, p = 0.000), birth weight (r = -0.543, p = 0.000), length (r = -0.343, p = 0.002) and occipitofrontal circumference (r = -0.494, p = 0.000). Also TBARS was found to be negatively correlated to platelet count (r = -0.285, p = 0.011), while positively correlated to Tollner score (r = 0.744, p = 0.000), Rodwells score (r = 0.756, p = 0.000), total leucocytic count (r = 0.450, p = 0.000), and CRP (r = 0.676, p = 0.000).

Table 3: Correlation of cord TBARS (nmol/ml) with different studied parameters in all patients

	Cord TBARS nmol/ml	
	All patients	
	r	p-value
Gestational age (weeks)	-0.338	0.002
Birth weight (kg)	-0.543	0.000
Length (cm)	-0.343	0.002
Occipitofrontal circumference	-0.494	0.000
APGAR score	-0.631	0.000
Tollner score	0.744	0.000
Rodwells score	0.756	0.000
TLC (*10^3)	0.450	0.000
Platelets	-0.285	0.011
CRP	0.676	0.000

CRP: C reactive protein, TLC: total leucocytic count

From table 4 there was significant relation between TBARS and blood culture results (median IQR); no growth 6.45 (5.25 – 7.85), *Group B Streptococci* 23 (22 – 24), *Staphylococci* 20.25 (16.5 – 24), *Escherichia coli* 20.25 (10.5 – 21), and *Klebsiella pneumonia* 18 (17.5 – 24), (p= 0.000).

TBARS was significantly higher in patients who died than who survived median (IQR) 21 (18 – 24) vs 7 (6 – 8.5) nmol/ml, respectively, (p = 0.000).

Table 4: Relation between TBARS and blood culture results

	Median (IQR)	Range	Test value	P-value
No growth	6.45 (5.25–7.85)	4.2–8.5	18.617	0.000
Escherichia coli	20.25 (10.5–21)	10.5–24		
Klebsiella pneumoniae	18 (17.5–24)	17.5–24		
Staphylococci	20.25 (16.5–24)	16.5–24		
Group B streptococci	23 (22–24)	22–24		

Receiver operating characteristics (ROC) curve analysis of cord blood TBARS for prediction of EOS showed a cut off value of 4 nmol/ml with 100% sensitivity and 90.91% specificity with AUC of 0.960. Another ROC curve was constructed to examine the diagnostic performance of TBARS for prediction of mortality caused by sepsis. The best cut off was 16 nmol/ml with a sensitivity of 100%, specificity of 98.55% and AUC of 0.999 (Figure 1 and 2).

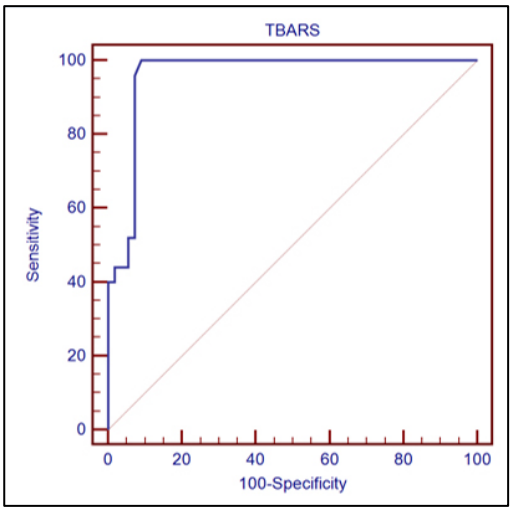

Fig. 1: Roc curve of predicting sepsis cases

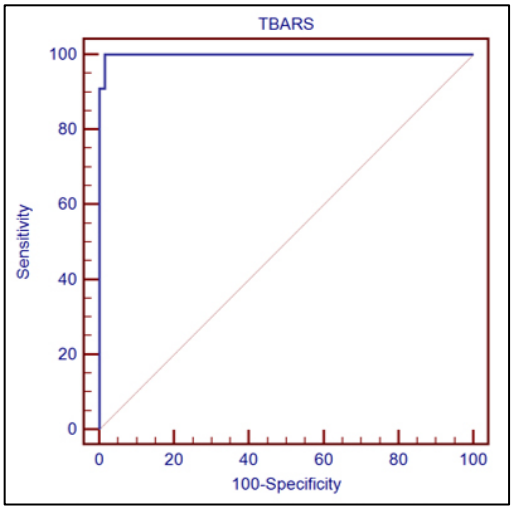

Fig. 2: Roc curve of predicting died cases

DISCUSSION

Maternofetal bacterial infection causing EOS has significant morbidity and mortality. Early diagnosis and timely initiation of appropriate antibiotics are vital to improve outcomes [14]. Oxidative stress is incriminated in the destructive pathways activated during NS, eventually leading to organ dysfunction and death [15]. In our study we found that neonates with EOS showed higher levels of oxidative stress markers in cord blood.

In the present study, the sepsis group showed significant lower gestational age, birth weight, length, occipitofrontal circumference and Apgar score than no-sepsis group, similarly Simonsen et al, [2] declared that infant factors associated with EOS include prematurity/low birth weight, congenital anomalies, instrumental or complicated delivery, and low APGAR scores (score of ≤6 at 5 min). Also, [16] El Nadaf et al, 2018, found that the neonates in the sepsis group showed significant lower gestational age and birth weight than control group.

Blood culture is the gold standard for the diagnosis of neonatal sepsis. However, positive results are low and is affected by inoculated blood volume, antenatal antibiotic use, level of bacteremia and laboratory capabilities. In developing countries, negative cultures are responsible for the majority of cases [17].

In our study, we documented that 13/25 (52%) had positive blood culture, and that *Escherichia coli* (24.0%), *Klebsiella pneumoniae* (12.0%), *Staphylococci* (8.0%), *Group B streptococci* (8.0%) were the causative organisms. Another study from Egypt documented culture proven sepsis in 39% of the examined neonates, and that *Staphylococcus aureus* and *Klebsiella pneumoniae* were the most encountered organisms in 27.3% each [18]. In one study in India, blood culture proven sepsis was encountered in 63% *Klebsiella pneumonia* (54%) followed by *Pseudomonas aeruginosa* (15.9%) and *Escherichia coli* (11.1%) were the most common isolates [19]. Very low birth weight (VLBW) infants are more likely to have sepsis from Gram-negative organisms with *Escherichia coli* rather than *group B streptococci* or other Gram-positive organisms [20].

Escherichia coli is a common pathogen in neonatal sepsis especially in preterm infants. In premature infants, a growing problem of an increased trend in the incidence of EOS caused by antibiotic-resistant *E coli* and other pathogens has been observed [21].

In the present analysis the sepsis group had lower hemoglobin, hematocrit and platelet count and higher leucocytic count and CRP than the no sepsis one. In their study, Cosar et al,[22] found that in the EOS group, culture-positivity rate was detected as 41.86 % and that in the EOS group significantly higher levels of WBC and CRP, while platelet counts were significantly lower than no sepsis group. While Hornik et al, [23] declared that low WBC and platelet counts are associated with increased odds of EOS, and that a high WBC count is not associated with increased odds of infection. In their study Abdel-Latif et al. [24], showed that platelets count was significantly lower among sepsis group compared with control group ($p<0.01$) however, there was no significant difference between both groups as regards total leucocytic count and hemoglobin.

Forty four % neonatal deaths were reported in the sepsis group, this was comparable to a study on VLBW in Taiwan with EOS related mortality reporting 40%, with *E. coli* causing the highest mortality rates [20].

Free radicals and ROS generated have been implicated in the pathogenesis of NS and its complications [5]. In our study, cord blood TBARS is significantly higher in the sepsis group than no sepsis group (approximately three times higher in the sepsis than no sepsis) this is similar to Cancelier et al.[6] who described that cord blood levels of IL-6, IL-10, TBARS, and protein carbonyl were higher in the sepsis group when compared with healthy control.

Lipid peroxidation substances; malondialdehyde (MDA) and 4-hydroxylalkenals were nearly 2-fold higher in septic neonates when compared with healthy infants [5] and as reported in other studies [3,25,26,27,28]. Discordant with this observation, intra-amniotic infection, histological chorioamnionitis, and funisitis did not significantly affect cord blood TBARS and products of oxidative stress in the neonates of 165 pregnancies complicated by premature rupture of membranes[29].

There was significant negative correlation between TBARS and gestational age, Apgar score, birth weight, length and occipitofrontal circumference. In their study, Cancelier et al,[6] declared that there was no significant correlation between Apgar scores and TBARS levels, while there was significant negative correlation between TBARS and birth weight.

In our study, there was significant negative correlation between TBARS and platelet count, while significant positive correlation between TBARS and Tollner score, Rodwells score, total leucocytic count, and CRP. However, Valerio et al,[30] did not find correlation between TBARS and CRP.

There were significant relation between TBARS and blood culture results. Cancelier et al,[6] declared that TBARS was significantly higher in culture proven sepsis than negative culture sepsis.

In our study, TBARS was significantly higher in patients who died than survivors, this was in accordance to other study [27], however, Valerio et al,[30] did not find correlation between TBARS and mortality from EOS, but they found that TBARS in preterm patients showed a mild to moderate correlation with clinical sepsis severity score. However, at present, no relationship has been confirmed between oxidative stress biomarkers and the occurrence of long-term adverse outcomes in septic neonates [15].

On performing ROC curve analysis to examine the performance of TBARS as a diagnostic marker for prediction of EOS in preterm. TBARS showed a cut-off value of 4 nmol/ml with 100% sensitivity and 90.91% specificity with AUC of 0.960. As a mortality predictor, the cut-off value of TBARS was 16 nmol/ml with a sensitivity of 100% and a specificity of 98.55%. In a study by Cancelier et al,[6] ROC curves from the studied markers in the sepsis group, IL-6 and TBARS had equivalent areas under the ROC curve (0.88).

Limitations of our study is that we included IUGR, Caesarian Section and neonates with maternal diabetes and pre eclampsia, all those factors can increase the oxidative stress markers.

CONCLUSION

Cord blood TBARS levels are higher in preterm infants with early onset neonatal sepsis and may be used as a diagnostic and prognostic marker.

Further studies need to be done to show whether TBARS can be used to detect neonatal sepsis severity and if it can be used to follow up the effect of adequate antibiotic therapy.

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Contributors' Statement:

Ismail R and Shaaban HA conceptualized and designed the study.

Shaaban MAA contributed to laboratory analysis

Emam AM selection of obstetric patients and diagnosis of maternal risk factors for early onset neonatal sepsis

All authors contributed to data interpretation and manuscript writing and have read and approved the final submission.

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