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SEVERITY PREDICTION OF PERINATAL ASPHYXIA OF TERM NEWBORNS USING NUCLEATED RED BLOOD CELL COUNTS IN CORD BLOOD

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ABSTRACT
Background: Perinatal asphyxia is a major cause of neurologic morbidity and mortality. The purpose of this study was to investigate variations in nucleated red blood cell count per 100 white blood cells in cord blood of term newborns with perinatal asphyxia and its relationship to both the severity and short term prognosis of asphyxia.
Methods:A cross-sectional comparative study was undertaken between June 2016 and May 2017 in the Neonatal Intensive Care Unit of CSI holsworth memorial hospital and Cheluvamba Hospital, Mysuru. A total of 120 newborns completed the study, out of which 60 asphyxiated term newborns (case group) and 60 normal term neonates (control group) Immediately after birth, umbilical cord blood was collected and a thin blood smear slide was prepared which was stained with Leishman stain for NRBC count. The NRBC count of the case group and the control group were compared. The results were analyzed statistically.
Results: The mean (± SD) NRBC count in normal term newborns born of normal vaginal delivery was 5.3 ± 3.25 NRBC/100WBC (range 0-16). The mean NRBC count in asphyxiated babies was 23.65±12.9 NRBC/100WBC, ranging from 8 to 63. Hence, the NRBC count was significantly higher in the asphyxiated group compared to normal babies (p-value < 0.001).The NRBC count cut-off of >10/100WBC has sensitivity of 96.7% , specificity of 95%,positive predictive value of 95% and negative predicting value of 96.6% in predicting asphyxia defined as Apgar at 1 minute ≤ 6.
Conclusions: Nucleated red blood cell cord blood collected is a simple marker for assessment of severity and early outcomes in perinatal asphyxia. Early NRBC count in cord blood is a simple & cost effective strong marker for birth asphyxia.

Keywords: Cord blood, nucleated red blood cell, perinatal asphyxia

INTRODUCTION
Perinatal asphyxia is commonly defined using a combination of parameters including fetal distress, meconium stained liquor, low Apgar score, umbilical pH and clinical features of hypoxic ischemic encephalopathy.(1)Perinatal asphyxia is referred to a condition during the first and second stage of labour in which impaired gas exchange leads to fetal hypoxemia and hypercarbia.(2)Perinatal asphyxia is a major cause of acute mortality and chronic

neurologic disability amongst survivors and complication that occurs between 2-10% of deliveries. The WHO defines birth asphyxia as "failure to initiate and sustain breathing at birth"[3].National neonatal perinatal data base suggest that perinatal asphyxia contributes to 20% neonatal deaths in India. Perinatal asphyxia of moderate grade is defined as slow, gasping breathing or Apgar score of 4-6 at one minute of life. Severe asphyxia as no breathing or the Apgar score 0-3 at one minute of life.[4])Globally hypoxia of the new born (birth asphyxia) or the fetus (fresh still birth) is estimated to account for 23% of the 4 million neonatal death and 26% of the 3.2 million still birth each year. Majority of asphyxia insults occur in the ante partum or intrapartum periods asa result of placental insufficiency. Detection of nucleated red blood cell in the cord blood allows early prediction of severity of birth asphyxia – hypoxic ischemic insult as this parameter is related to neurodevelopment.[5]

Perinatal asphyxia results in hypoxic injury to various organs including kidneys, lungs and liver but the most serious effects are seen on the central nervous system.[4] Hypoxic ischemic encephalopathy (HIE) refers to the CNS dysfunction associated with perinatal asphyxia. The clinical features include altered consciousness, hypotonia, seizure activity, autonomic disturbances, and abnormalities of neonatal reflexes. HIE is of foremost concern in an asphyxiated neonate because of its potential to cause serious long-term neuromotor sequelae among survivors. A detailed classification of HIE (Stage I, Stage II and Stage III) in term neonates was proposed by Sarnat and Sarnat.[6] A simple and metabolic involvement may include hypocalcaemia, hyponatremia (as a result of syndromic inappropriate anti diuretic hormone or direct renal injury), and alterations in glucose metabolism. There may be haematological alterations (thrombocytopenia and disseminated intravascular coagulation).

The American Academy of Paediatrics has proposed the term perinatalasphyxia should be reserved to describe an infant who manifests with all of the following features:

(1) Umbilical cord arterial pH less than 7
(2) Apgar score of 0 to 3 for longer than 5 minutes
(3) Neonatal neurologic manifestations (seizures, coma, or hypotonia)
(4) Multisystem organ dysfunction (cardiovascular, gastrointestinal, hematologic, pulmonary, or renal system).[7]

Hypoxia or asphyxia should be labelled as a cause of disability and handicap only when the neonate demonstrates the four perinatal findings listed above and in whom other possible causes of neurologic damage have been excluded. In the absence of such evidence, subsequent neurologic deficiencies cannot be ascribed to perinatal asphyxia or hypoxia. Dr. Eastman of Hopkins called asphyxia "an infelicity of etymology" since the Greek derivation of asphyxia meant "without pulse."[8]

Several methods like scoring systems, markers (non protein bound iron, interleukin-6), EEG, cerebral function monitoring, imaging modalities (USG, CT,MRI) have been developed for early identification of neonates at high risk for brain injuries who may benefit from early neuroprotective interventions like induced mild hypothermia, antioxidant agents (allopurinol) and calcium channel blockers.[9,10,11] Further complications can be anticipated and corrective and preventive measures can be undertaken.

Most of the diagnostic and prognostic parameters used are available in few selected tertiary care hospitals, are expensive and require sophisticated equipments thus rendering them unreachable for most of the population. This problem is further compounded in country like India where there is a wide gap between the need and accessibility of health services. Therefore there is a need for simple tests to identify perinatal asphyxia.

There are few studies on exclusive term babies related to NRBC in perinatal asphyxia. Causes of high NRBC count include: fetal conditions such as prematurity, intrauterine growth retardation, congenital infection, cyanotic heart disease, and acute asphyxia. Maternal

conditions such as maternal diabetes, pre-eclampsia, ABO or Rh incompatibility, maternal smoking, and chorioamnionitis.[5,8]. In this study eliminating the above causes increased NRBC, can accurately can calculate NRBC due to perinatal asphyxia of term newborns. By looking NRBC at birth and along with clinical correlation, severity of perinatal asphyxia can be predicted. Hence this study can used for better management and prevention of neonatal mortality.

MATERIALS AND METHODS

A cross-sectional, comparative study was carried out at NICU of CSI Holdsworth Memorial hospital and Cheluvamba hospital , Mysuru, Karnataka,INDIA between the period of June 2016 to May 2017. The study was done after approval from ethical committee of our institute. After calculating the sample size by using the sensitivity of 87% as by Saracoglu F [12]and taking alpha as0.05 and precision as 10% of sensitivity total 120 term newborns including 60 cases (Asphyxiated) and 60 controls (non asphyxiated) were enrolled.

Inclusion criteria

Thecases were term neonates with perinatal asphyxia with 1-min Apgar of ≤6/10 asdefined by WHO and NNPD.Controls were term newborns delivered during the same period, fulfilling following criteria were enrolled as controls Apgar score ≥ 7 at 1 minute and absence of meconium stained amniotic fluid.

Exclusion criteria

Newborns with severe congenital malformations, chromosomal anomalies like Down syndrome, TORCH infections, septicemia, Rh incompatibility resulting in haemolysis, maternal diabetes mellitus, chorioamnionitis, intrauterine growth retardation, pre-eclampsia, pregnancy induced hypertension and eclampsia and caesarean section.

Sample collection

At birth 2ml of cord blood was collected in ethylene diamine tetra acetate (EDTA) tubes in both cases and control groups. Samples were stored in refrigerator if there was any delay in processing. Blood samples were used for making smears (for NRBCs) and complete blood count. Neonates with birth asphyxia were further admitted in NICU and investigated as per the routine NICU protocol for birth asphyxia. Samples were processed and analyzed by the same blinded pathologist. The ethylene diamine tetra acetate sample was processed by SYSMEX automated cell counter for obtaining total white cell count . The blood smears were stained by Leishman stain and manual differential count was done to count NRBCs. Number of nucleated red blood cells were counted per 100 leukocytes in peripheral smears and were reported as 'number of NRBC/100 WBC'.

Receiver operating curve (ROC) was used to derive cut-off value for NRBC count for predicting perinatal asphyxia as well as to predict adverse outcome in perinatal asphyxia. A p-value of <0.05 was considered statistically significant. The results are presented as means (± S.D.) unless otherwise indicated. All data were statistically analyzed using SPSS statistics software for Windows (Version 20).Descriptive statistics were applied, Sensitivity, Specificity, Positive predictive value and Negative predictive value were calculated.

RESULTS

In our study there were 60 babies in each study groups.

Table 1: There was no significant difference in the age of mother, parity, presentation, birth weight and sex of babies between the two study groups. Among cases 60% of asphyxiated babies had risk factors, 25% were associated with meconium stained amniotic fluid. Other risk factors associated were cord around the neck(18.3%), short stature(8.3%) and prolonged second stage of labour(5%). There was no statistically significant difference in the mode of delivery between the two study groups although

few number of babies with birth asphyxia were born of assisted (forceps) vaginal delivery. Babies born of emergency LSCS were excluded from both study groups.

Table 2: Among the cases, 56.7% of the newborns had severe asphyxia with one-minute Apgar score of 0-3 and the other 43.3% had moderate asphyxia with Apgar score of 4-6. Among controls all newborns had 1-minute Apgar score of more than 6. Out of 60 , 6 cases (10%) had severe asphyxia with 5-minute Apgar score of 3 or less and 41 cases (68.33%) had a apgar score between 4 to 6. Among controls, all babies had Apgar score of more than 6 at five minutes.Statistically significant between cases and controls with respect to apgar score at 1 minute and at 5 minute.

Table 3:In the present study, most controls (95%) had NRBC count between 0 to 9. Only 5 % of control had NRBCcount between 10 to 19. None of the controls had NRBC count more than 20. Most cases 58 (95%) had NRBC count of 10 or more. 29 cases (48.3%) had NRBCcount between 10 to 19 and 29 cases (48.3%) had NRBC count of 20 or more. Only two cases (3.4%) had NRBC count less than ten.

Table 4:In the present study, 100% neonates of HIE stage 3 and 61.5% of HIE stage 2 NRBC were 20 or more.90.5% neonates of HIE stage 1 and 38.5% of stage 2 NRBC were between 10 to 19.There was a positive correlation between NRBC count and HIE staging, which was also statistically significant.Nucleated RBC count increased with decreasing Apgar score at both 1 minute and 5 minute.

Table5: Nucleated RBC count was compared with Apgar score at 1 and 5 minutes in both cases and controls group. Among the cases, a statistically significant negative correlation exists between NRBC count and Apgar score at 1-minute as well as at 5-minutes. No such correlation was found among the controls.

Table 6:The mean NRBC count was 42.38 in stage 3 and 22.57 in stage 2 as compared to 13.38 in stage 1. Hence, NRBC count increased with increasing severity of HIE which was statistically significant.

Table 7:Correlation between HIE staging and NRBC count was significant at 0.01 level. As the severity of hypoxic ischemic encephalopathy increases, the NRBC count also increases.

The mean (± SD) NRBC count in normal term newborns born of normal vaginal delivery was 5.3 ± 3.25 NRBC/100WBC (range 0-16). The mean NRBC count in asphyxiated babies was 23.65±12.9 NRBC/100WBC, ranging from 8 to 63. Hence, the NRBC count was significantly higher in the asphyxiated group compared to normal babies (p-value < 0.001).The NRBC count cut-off of >10/100WBC has sensitivity of 96.7% , specificity of 95%,positive predictive value of 95% and negative predicting value of 96.6% in predicting asphyxia defined as Apgar at 1 minute ≤ 6.A statistically significant negative correlation was present between NRBC per 100 WBC count and Apgar scores at 1 minute and 5 minutes. A significant positive correlation was also established between NRBC count and hypoxic ischemic encephalopathy.

Table 8:There were total of 3 deaths in the study. All deaths occurred in the cases with NRBC count 20 and more 10.3% of total cases with NRBC count of 20 or more died. This group also had a significantly longer hospital stay compared to other groups which was statistically significant (p-value 0.001).

Table 1: Clinical variables in cases and controls

Parameters	Cases (n=60)	Control (n=60)	P value
Primi/ Multipara	40/20(66.6%/33.3%)	37/23(61.6%/38.3%)	0.654
Cephalic/ breech	57/3 (95%/5%)	58/2 (96.6%/3.3%)	0.648
Male/Female	30/30(50%/50%)	27/33(45%/55%)	0.583

Risk factors			
Nil	24 (40%)	60 (100%)	0.001
MSAF	15 (25%)	0	
Prolonged 2nd stage labour	3 (5%)	0	
Cord around neck	11 (18.3%)	0	
Short stature	5 (8.3%)	0	
MSAF+ prolonged 2nd stage	2 (3.3%)	0	

MASF- Meconium stained amniotic fluid

Table 2 :Distribution of study group according to Apgar at 1 min and 5 min

Apgar at 1 minute	**Cases-number (percentage)**	**Controls-number (percentage)**	**p-value**
< 3	34 (56.7%)	0	0.001
4-6	26 (43.3%)	0	
< 7	0	60 (100%)	
	60 (100%)	60 (100%)	
Apgar at 5 Minutes	**Cases-number (percentage)**	**Controls-number (percentage)**	**p-value**
< 3	6 (10%)	0	0.001
4-6	41 (68.33%)	0	
>7	13 (21.7%)	60 (100%)	
	60 (100%)	60 (100%)	

Table 3 : Distribution of Study Group According to NRBC Counts

NRBC	**Cases-number (percentage)**	**Controls-number (percentage)**	**P-value**
0-9	2 (3.4%)	57 (95%)	0.001
10-19	29 (48.3%)	3 (5%)	
> 20	29 (48.3%)	0	

Total	60	60	

Table 4:Distribution of Case Group According to NRBC and HIE Stage

		HIE stage			Total	P-Value
		1	2	3		
NRBC	0-9	2 (9.5%)	0	0	2 (3.3%)	0.001
	10-19	19 (90.5%)	10 (38.5%)	0	28 (48.3%)	
	> 20	0	16 (61.5)	13 (100%)	29 (48.3%)	
Total		21 (100%)	26 (100%)	13 (100%)	60 (100%)	

Table 5:NRBC Count and Apgar Score

APGAR 1 MINUTE		APGAR 5 MINUTE	
APGAR SCORE	NRBC COUNT	APGAR SCORE	NRBC COUNT
7-10	5.30 ± 3.25	7-10	13.84 ± 3.93
4-6	17.88 ± 10.65	4-6	23.80 ± 11.90
0-3	28.65 ± 12.91	0-3	43.50 ± 8.98
p-value	<0.001	p-value	<0.001

Table 5:Correlation between NRBC count and Apgar score at 1 minute and 5

	APGAR AT 1 MINUTE		APGAR AT 5 MINUTES	
	Case	Control	Case	Control
Pearson Correlation Correlation Coefficient	-0.529	0.211	-0.664	0.289
P value	0.000	0.106	0.000	0.025

Table 6:**NRBC count was compared with severity of HIE using one way ANOVA analysis**

HIE stage	Number of cases	NRBC Count (Mean ±SD)	Minimum	Maximum
Stage 1	21	13.38 ± 2.83	8.00	19.00
Stage 2	26	22.57 ± 5.50	14.00	35.00
Stage 3	13	42.38 ± 13.37	25.00	63.00
	Total 60	p value 0.001		

Table 7 : **Correlation between HIE and NRBC Count**

Correlation coefficient	0.805
P value	0.001

Table 8 :**Deaths in relation to NRBC count**

NRBC count (per100WBC)	Cases	Total hospital stayin days	Deaths
0-9	2 (3.40%)	6.23 ± 1.22	0
10-19	29(48.33%)	10.68 ± 2.19	0
20 and more	29 (48.33%)	14.71 ± 1.33	3 (10.3%)

		p-value 0.001	

NRBC count and perinatal asphyxia

As NRBC count correlated with various established markers of perinatal asphyxia like Apgar score at 1 minute and 5 minutes and development of HIE, Receiver operating characteristics (ROC) graph was plotted to determine the NRBCcount cut-off value for predicting birth asphyxia. Birth asphyxia was defined as Apgar score at 1 minute ≤ 6.

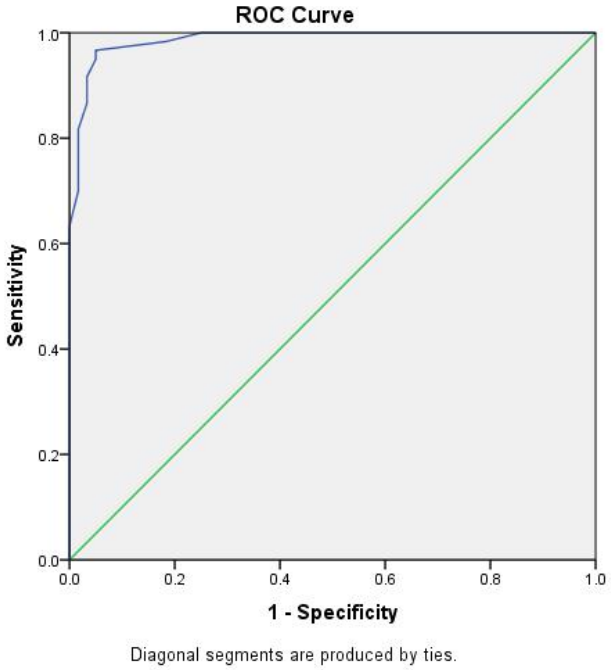

Figure 1: Receiver operating characteristics (ROC) graph to discriminate the sensitivity and specificity of NRBC/100 WBC count in the diagnosis of perinatal asphyxiated newborns.Area under curve 0.987

Discussion

NRBCs rarely circulate in older children, they are commonly seen in the blood of neonates. They are primarily produced in the fetal bone marrow in response to erythropoietin and are stored in the marrow as precursors to reticulocytes and mature erythrocytes. Many acute and chronic stimuli cause increases in the number of circulatingNRBCs from either increased erythropoietic activity or a sudden release from the marrow storage pools.[13]

Previously reported causes of a high NRBC count include: prematurity, ABO or Rh incapability, maternal diabetes, intrauterine growth retardation[14] , neonatal sepsis[15], congenital infection, cyanotic heart disease, pre-eclampsia[16], maternal smoking, and chorioamnionitis. However, neonates with these conditions were

excluded from the current study.In the present study, the NRBC count for normal neonates was found to be5.30±3.25NRBC/100WBC, which was consistent with previous reports.[12])NRBCcount/100 leukocytes increased during the first few hours of life in perinatal asphyxia as compared with healthy control subjects. The mean NRBC count in asphyxiated babies was 23.65±12.9 NRBC/100WBC, ranging from 8 to 63. Hence, the NRBCcount was significantly higher in the asphyxiated group compared to normal babies (p-value < 0.001).

The mechanism causing the rapid release of NRBC following perinatal asphyxia is not known, although increased erythropoietin results from hypoxia and probably has a major role in the process.[12]NRBCs are immature erythrocytes whose production is thought to be driven primarily by the interplay of hypoxia and erythropoietin (EPO) synthesis.[17] Previous studies suggest that EPO increases erythroid production and releases immature forms of erythrocytes into the peripheral circulation in response to hypoxia. It is possible that increased NRBC production in the immediate neonatal state primarily reflects hypoxic injury.[15]) Several studies have reported an increased NRBC in neonatal blood following perinatal asphyxia.[13,18,19]Boskabadi et al reported a cut-off value for NRBC as>70/mm^3 with sensitivity of 83.4% and specificity of 73.5% in predicting perinatal asphyxia.[18]Ferns et al observed significant difference between mean NRBC count of term healthy babies and babies with perinatal asphyxia having low Apgar score at five minutes (p < 0.001).[s20)]In the present study, a statistically significant negative correlation was demonstrated between NRBC per 100 WBC count and Apgar score at 5 minutes

The results of the present study give additional support to previous reports and also defines the cut-off value for NRBC as >10/100WBC with a sensitivity of 96.7%, specificity of 95%, positive predictive value of 95% and negative predicting value of 96.6% in predicting perinatal asphyxia.A statistically significant negative correlation was present between NRBC per 100 WBC count and Apgar scores at 1 minute and 5 minutes. A significant positive correlation was also established between NRBC count and hypoxic ischemic encephalopathy.Nucleated RBC count in cord blood is a low cost, simple and easily available test which can be done in any health care facility with minimal infrastructure.

Conclusion

In our country, a large number of deliveries occur in peripheral health facilities and at home by trained attendants. In such cases, accurate and reliable recording of well established markers of birth asphyxia like Apgar scores, intranatal cardiotocography recording for fetal distress, fetal scalp pH monitoring is often not available. Such babies pose diagnostic dilemmas for treating doctors in tertiary health care centres where these babies are referred. Early and accurate diagnosis of birth asphyxia is crucial in determining both short term and long-term prognosis. The present study establishes the role of NRBCcount in cord blood provide predictor of birth asphyxia. Hence, this simple and reliable test can be recommends in peripheral health centre.

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