Original article

Neutrophil functions in late preterm neonates with respiratory distress syndrome

Background: *Studies that have addressed the effects of respiratory distress syndrome (RDS) on neutrophil function suggested that neutrophil functions other than the generation of the respiratory burst are not impaired. Yet, results have been confusing and in some cases contradictory.* ***Objectives:*** *The aim of this cross-sectional controlled study is to assess neutrophil number and function in late preterm neonates with RDS.* ***Methods:*** *Thirty patients underwent clinical and laboratory evaluation including complete blood counts and tests of neutrophil functions (CD11b, CD62L and Dihydrorhodamine 123 by flowcytometry) in comparison to 15 healthy term controls. RDS was assessed clinically and radiologically (chest x-ray).* ***Results:*** *Fifty percent of patients (12 females and 18 males) had grade II respiratory distress followed by grade III then grade I. DHR, CD 11b and CD62L results were lower among the patients group (mean ± SD: 62.1± 12.23, 63.22 ± 11.41, 15.03 ± 8.7 respectively). There were no significant correlations between neutrophils count, DHR, CD11b and CD62L. Only CD11b was significantly lower with higher grades of RDS.* ***Conclusion:*** *Neonates with RDS show variable affection of neutrophil functions. Further studies are recommended to elucidate the exact mechanisms by which RDS can affect neutrophil functions and whether these effects are associated with increased incidence of infections.*

Keywords: Neutrophils, function, respiratory distress syndrome, late preterm, innate immunity, infections, adhesion molecules.

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INTRODUCTION

The innate immune system is the first line of host defense against invading microorganisms. In order to achieve this, neutrophils must be able both to circulate freely in the blood and to migrate as adherent cells into the extravascular tissues to phagocytose and kill invading microorganisms effectively[1].

Most studies of neutrophil function have been conducted under conditions of ambient oxygen, but inflamed sites where neutrophils operate may be extremely hypoxic[2]. However, neutrophils need to consume oxygen to maintain the NADPH oxidase-driven respiratory burst[3]. Previous studies that have addressed the effects of respiratory distress syndrome (RDS) on neutrophil functions suggested that neutrophil functions other than the generation of the respiratory burst are not impaired. Yet, results have been confusing and in some cases contradictory[2].

RDS is a common problem in preterm infants which is caused primarily by deficiency of pulmonary surfactant in an immature lung. This results in significant hypoxemia and constitutes a major cause of morbidity and mortality in preterm infants[4]. Approximately half of cases with respiratory distress syndrome are associated with infections[5]. Our cross-sectional controlled study aimed to investigate the effect of respiratory distress syndrome in late preterm infants on different aspects of neutrophil functions.

METHODS

Participants:

Patients group (n = 30): This group included late preterm neonates with RDS in 1st day of life. Late preterm was defined as neonates born at 35 or 36 weeks of gestation. The following conditions were excluded from enrollment in the study: Patients with causes of neonatal respiratory distress other than RDS like meconium aspiration or transient

tachypnea of newborn; patients with major congenital anomalies; neonatal sepsis and infants of diabetic mothers. A control group of apparently healthy term neonates (n=15) at 1st day of life at gestational age (37-38 weeks) was also included.

Methods:

Verbal consents were obtained from the parents or the care givers before enrollment of their neonates in the study.

All enrolled subjects were assessed by detailed history taking and thorough clinical examination for assessment of the gestational age using new Ballard score[6], mode of delivery, birth weight and signs of respiratory distress (e.g. tachypnea, working ala nasi, intercostal and subcostal retractions, grunting, cyanosis .., etc.).

Laboratory investigations:

Sampling: Three mL peripheral blood (PB) samples were obtained from all enrolled subjects on ethylenediamine tetra-acetic acid, dipotassium salt (K_2-EDTA) in vacutainer tubes (final concentration of 1.5 mg/mL) for:

a- Complete blood counts (CBC) on Coulter LH750 cell counter (Coulter, Electronics, Hialeah, FL, USA) with examination of PB smears stained with Leishman stain.

b- Flow cytometric assay of oxidative burst using DHR 123: Peripheral blood samples were processed on the same day of sample collection. The reagents in this test are the dihydrorhodamine123 (DHR123) and phorbol 12-myristate 13-acetate (PMA) (both supplied from Sigma-Aldrich, St Louis, MO, USA). In making stock solutions, these reagents are diluted to 2500 µg/ml and 100 µg/ml, respectively in dimethyl sulfoxide (DMSO). These stocks are then aliquoted and stored at -20°C until use. Three tubes are set up for each patient and control: (a) Blood only; (b) Blood + DHR123 (resting tube); (c) Blood + DHR123 + PMA (stimulated tube). To all tubes 100 µl of blood is diluted 1:10 with phosphate buffered saline with azide (PBA). 25 µl of thawed and diluted DHR123 is added to tubes 2 and 3. All tubes are incubated in a 37 ^{0}C water bath for 15 minutes. Following this incubation, 100 µl of the prepared PMA solution is added to tube 3 and all tubes are incubated an additional 15 minutes at 37^{0}C. This step allows the neutrophils to be stimulated to undergo the oxidative burst, thereby oxidizing the DHR123 to its resonance form (rhodamine) which is highly green fluorescent when exposed to the 488 nm laser. After washing and centrifugation, the samples are lysed with ammonium chloride-based erythrocyte lysing solution (Pharm Lyse, Becton Dickinson, Mountain view, CA, USA) for 10 minutes in the dark, followed by centrifugation and washing by phosphate buffered saline (PBS). Tubes were vortexed then analyzed using the 488nm laser and the FITC filter set up using Coulter Epics XL flow cytometer (Coulter, Electronics, Hialeah, FL, USA). Fluorescence is quantitated by mean peak channel fluorescence (MPC-FL).

Interpretation: Results were expressed as neutrophil oxidative index (NOI) which is the ratio of MPC-FL (PMA stimulated) over MPC-FL (unstimulated).

c- Flow cytometric immunophenotyping of CD11b (Integrin αM) and CD62L (L-selectin): Anti-human CD11b phycoerythrin (PE) labeled and CD62L fluorescein isothiocyanate (FITC) labeled monoclonal antibodies were used in this study (both were supplied by R & D systems, Minneapolis, MN, USA). Peripheral blood samples were processed on the same day of sample collection. They are counted using Coulter Cell Counter, and the total leucocytic count was adjusted to be around 5.0 x 10^9/L using phosphate buffered saline. 100 µL of adjusted sample were aliquoted in the control tube as well as each sample tube and then 20µL of each monoclonal antibody were added. The test tubes were incubated for 15 minutes at room temperature, protected from light. After incubation, 1-2 mL of ammonium chloride-based erythrocyte lysing solution was added to every tube. Tubes were vortexed then analyzed using Coulter Epics XL flow cytometer (Coulter, Electronics, Hialeah, FL, USA).

Radiological methods: Chest X ray postero-anterior view was done for all patients to assess the grade of RDS: Grade 1: slight reticular (slight granular) decrease in transparency of the lung; Grade 2: soft decrease in transparency with an aero-bronchogram, which overlaps the heart; Grade 3: like grade 2, but with gradual stronger decrease in transparency, as well as a blurry diaphragm and heart; Grade 4**:** white lung[4]**.**

Statistical Methods

The collected data were revised, coded, tabulated and introduced to a PC using Statistical package for Social Science (SPSS 15.0.1 for windows; SPSS Inc, Chicago). Data were presented and suitable analysis was done according to the type of data. Mean, standard deviation (± SD) and range were calculated for parametric numerical data and frequency and percentage for non-numerical data. Student T test was used to assess the difference between two study group means. ANOVA test was used to assess the difference between more than

two study group means. Chi-Square and Fisher's exact tests: were used to examine the relationship between two qualitative variables. A p value ≤ 0.05 was considered to be significant.

RESULTS

The 30 patients in the studied group included 11 patients who were born at the gestational age of 35 weeks and 19 patients at 36 weeks. All enrolled normal neonates were born term by 37-38 weeks of gestation. Mothers of patients who were born by cesarean section (C.S) have received antenatal steroids 24-48 hours before delivery. The patients group (n= 30) included 12 females and 18 males. Control group included 6 females and 9 males. Among enrolled patients, 22 patients (73.3%) were born by C.S. while 8 patients (26.7%) were born by NVD, results that were comparable to control group.

Radiological assessment of RDS revealed that 7 patients (23.3%) had grade I respiratory distress, 15 patients (50%) grade II respiratory distress and 8 patients (26.7%) grade III respiratory distress. None of the enrolled patients had grade IV respiratory distress.

On comparing the clinical parameters between patients and control groups, we found that patients group had significantly lower Apgar score at 1 minute ($t = -3.525$; $p< 0.001$) but comparable Apgar score at 5 min in comparison to controls ($t = 1.774$; $p=0.083$). Laboratory parameters of patients and control groups are depicted in tables 1-4.

Table 1. Laboratory parameters in patients and control groups

Parameter		Patients (n=30)	Controls (n=15)	T-test	
				t	*p*-value
WBCs (x10^9/L)	**Range**	10.5-17.6	12.3-28.3	-4.334	**<0.001***
	Mean±SD	13.74±1.92	17.44±3.85		
Monocytes (x10^9/L)	**Range**	0.3-0.8	0.1-0.8	1.146	0.258
	Mean±SD	0.51±0.16	0.44±0.24		
Lymphocytes (x10^9/L)	**Range**	2.1-4.1	2.0-4.0	2.856	**0.007***
	Mean±SD	3.15±0.47	2.70±0.56		
Eosinophil (x10^9/L)	**Range**	0.1-0.6	0.1-0.3	1.148	0.257
	Mean±SD	0.19±0.10	0.15±0.07		
Neutrophils (x10^9/L)	**Range**	7.1-13.1	10.0-24.0	-5.695	**<0.001***
	Mean±SD	9.86±1.63	14.15±3.44		
Segmented (x10^9/L)	**Range**	6.9-12.9	9.8-24.0	-5.628	<**0.001***
	Mean±SD	9.76±1.65	14.04±3.48		
Staff (x10^9/L)	**Range**	0.0-0.2	0.0-0.2	-0.112	0.911
	Mean±SD	0.10±0.09	0.11±0.10		

* Statistically significant
WBCs: White blood cells

Table 2. Comparison between studied groups in terms of DHR, CD11b and CD62L

Test		Patients (n=30)	Controls (n=15)	ANOVA	
				f	*p*-value
DHR (%)	Range	39.6-90.5	65.9-91.1	55.970	<0.001*
	Mean±SD	62.10±12.23	81.92±7.13		
CD 11 b (%)	Range	45.4-84.2	28.6-95.6	98.291	<0.001*
	Mean±SD	63.22±11.41	87.05±4.89		
CD 62 L (%)	Range	2.3-70.6	10.2-85.9	147.831	<0.001*
	Mean±SD	15.03±8.70	34.20±17.14		

* Significant
CD62 L: L selectin, DHR: Dihydrorhodamine 123 level after stimulation

Table 3. DHR, CD11b and CD62L among different grades of RDS

Parameter		Radiological grade of RDS				
		Grade I (n=7)	Grade II (n=15)	Grade III (n=8)	ANOVA	
					f	*p*-value
DHR (%)	Range	48.1-74.5	45.9-90.5	39.6-80.8	0.22	0.803
	Mean±SD	62.89 ± 10.2	63.09 ± 12.7	59.58 ± 14.2		
CD11b%	Range	59.6-84.2	48.9-79.4	45.4-80.1	3.48	**0.045***
	Mean±SD	71.74 ± 9.96	62.23± 10.5	57.63 ± 11.04		
CD62L%	Range	3.4-23.3	2.3-70.6	2.9-25.6	0.502	0.611
	Mean±SD	13.16± 6.58	17.94± 11.81	11.20± 6.83		

* Statistically significant
CD62 L: L selectin, DHR: Dihydrorhodamine 123 level after stimulation, RDS: Respiratory distress syndrome

Table 4. Correlations between neutrophil count and function in patients and control groups

Tests		Patients (n=30)			Controls (n=15)		
		ANC (10^9/L)	DHR %	CD 11b %	ANC (10^9/L)	DHR %	CD 11b %
DHR (%)	r	0.266			-0.716		
	p value	0.155			**0.003***		
CD 11 b (%)	r	0.311	0.025		-0.168	0.074	
	p value	0.094	0.895		0.549	0.794	
CD 62 L (%)	r	0.282	0.089	0.301	-0.034	0.003	-0.814
	p value	0.131	0.642	0.106	0.904	0.991	**<0.001***

ANC: absolute neutrophils count, CD62 L: L selectin, DHR: Dihydrorhodamine 123 flowcytometry test
*significant

DISCUSSION

Neutrophils must be able both to circulate freely in the blood and to migrate as adherent cells into the extravascular tissues to phagocytose and kill invading microorganisms effectively. Yet, several factors and disorders can affect neutrophil functions[1]. We aimed through this study to investigate the effect of RDS on different aspects of neutrophil functions in a group of late-preterm neonates. Late preterm age was suggested to avoid the effect of prematurity on our results.

On comparing laboratory parameters between patients and controls, we found that patients had significantly lower white blood cell counts, neutrophil counts and segmented neutrophil counts but higher absolute lymphocytic counts. Neutrophil infiltration and neutrophil mediated inflammation is well established in lungs of patients with RDS and neutrophil influx into the alveolar space correlates with lung injury as manifested by an increase in permeability of the alveolo-capillary membrane[7]. This may explain the relative neutropenia in patients in comparison to control group as the neutrophils might have accumulated at site of inflammation without sufficient compensation from the limited bone marrow stores. Age difference in our study is not suspected to have a major impact on the neutrophil count as neutrophils counts in majority of patients were within normal reference ranges of normal term infants below the 3rd day of life[8]. This is further supported in our study by finding that the less mature neutrophil form: band cells (staff) to be comparable among patients and control groups due to equivalent bone marrow stores. On the other hand, blood picture parameters and neutrophils counts were comparable among different grades of respiratory distress, despite lower neutrophil count measures in grade III RDS. Larger study population could have led to different results.

DHR, CD11b and CD62L were significantly lower in the patients' group in comparison to controls. DHR is a measure of neutrophil oxidative burst pathway and capability of superoxide production necessary for intracellular killing of catalase positive organisms[9]. Several studies have reported that hypoxia impairs neutrophils bacterial killing, and hypoxic incubation of neutrophils from healthy volunteers for 4 hours resulted in a 3- fold reduction in superoxide anion release as well as reduction in production of antimicrobial peptides[2,10]. Yet, in our study different grades of RDS were comparable in terms of their DHR values. This may indicate that RDS as a cause of stress and hypoxia even in mild degrees might affect the neutrophil capability for intracellular killing, but larger sample size is need for further elucidation of RDS effect on DHR results.

In our study, we noticed that CD11b expression on neutrophils was found to be significantly lower in patients with RDS in comparison to controls. Furthermore, CD11b was

found to be significantly affected by the grade of RDS being lower in higher grades. These findings do not represent the effect of RDS and its resultant hypoxia on CD11b as they disagree with what was reported by Jibiki et al., who noticed increased adhesion and CD11b expression on neutrophils exposed to hypoxia in vivo[11]. Also, Nupponen et al., had previously observed early transient postnatal neutrophil activation and increased CD11b expression in preterm infants in the first day of life with RDS that decreased to normal levels by the age of 10 days[12]. Furthermore, Zieba et al., reported that expression of CD11b receptors of neutrophils assessed in umbilical artery blood in preterm neonates does not show a statistical significant difference in comparison to term healthy neonates[13]. The mentioned studies exclude the effect of hypoxia, RDS, gestational age and postnatal age as a cause of lower CD11b in our patients. The only suggestion that may explain our results is what was reported by Ballabh et al., in 2004 who studied expression of adhesion molecules CD11b, CD18, and CD62L on neutrophils and monocytes in very low birth weight babies who developed RDS, and noticed their decreased expression with dexamethasone use after 2-3 days of steroids exposure[14]. Thus, antenatal steroids use in our patients could have led to such results; however it still doesn't explain the decreasing CD11b level with increasing severity of RDS.

L-selectin (CD62L), a marker of neutrophil activation and important mediator of neutrophil rolling and primary neutrophil adhesion to endothelial surfaces[15], was found to be significantly lower in patients in comparison to controls, but comparable among different grades of respiratory distress. Down regulation of CD62L on leucocytes was suggested as a possible mechanism of neutrophilia during inflammation. Dexamethasone use was found to be associated with even more decrease in the expression of CD62L within 24-72 hours of start of therapy. Different studies have noticed decreased serum levels of CD62 L in patients with stressful conditions like bronchopulmonary dysplasia[14] and those who were having cardiopulmonary by-pass surgeries[16]. Ballabh et al., suggested that the circulating neutrophil is not the same as the neutrophil migrating to the site of inflammation and neutrophils with reduced CD62L and CD18 expression would be preferentially retained in circulating blood, while neutrophils with normal or increased expression might be more likely both to migrate to be detained in the bronchi and lungs[14].

In our study, there were no significant correlations between neutrophil count and measures of neutrophil function in patients' group. On the other hand, in control group, there was a significant negative correlation between neutrophilic count and DHR and also between CD62L and CD11b. Siddiqi et al., 2001, found that leukocytes with a relatively high degree of adhesion molecule expression may display an average or decreased oxidative burst activity, and vice versa. They suggested that the divergence between oxidative burst activity and the responsiveness of cell adhesion expression in a particular individual may represent a "built in" protective mechanism against phagocyte-mediated tissue injury[17]. The same may apply for other neutrophil functions as well.

In conclusion, neonates with RDS shows variable affection of neutrophil functions which could be attributed to several factors including the disease itself with its resultant hypoxia, the gestational age, the postnatal age at time of assessment and the use of antenatal steroids. Special attention should be paid to those neonates and further longitudinal studies to assess the effect of the fore mentioned factors and their extent at different gestational ages and postnatal ages are required. Further studies on larger population size are required to evaluate the neonatal immune status born at different gestational ages to detect the critical level of prematurity that affects the immune system quantitatively and functionally. Additional studies are required for investigating the effect of hypoxia on different aspects of neutrophil functions to be linked with arterial blood gases results at postnatal ages after the first day of life. Neutrophil function tests on large study populations at different ages for setting of age related reference ranges and detection of levels of deficiency that can cause clinical manifestations are mandatory.

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