Original article

Impact of maternal gestational diabetes on neutrophil functions of full term neonates

Background: *Maternal gestational diabetes is associated with an inflammatory environment that may contribute to fetal and placental inflammatory profile changes. Few studies investigated the effect of maternal gestational diabetes on neonatal innate immunity.* ***Objectives:*** *Our objective was to study neutrophil number and function in neonates born to mothers with gestational diabetes.* ***Methods:*** *Neutrophil number (complete blood count) and functions [CD11b, CD62L and Dihydrorhodamine 123 (DHR) by flow cytometry] were assessed in the cord blood of 30 full term neonates born to gestational diabetic mothers on insulin during pregnancy and another 15 born to healthy mothers as controls.* ***Results:*** *The mean total leucocytic and absolute neutrophil count were significantly lower in neonates of diabetics than in normal neonates (13.55± 2.51 and 17.89± 3.66 p> 0.001; 9.01±1.59 and 14.18±3.44 p>0.001 respectively). Mean CD11b, CD62L and DHR were lower among neonates of diabetic mothers than normal neonates (82.48± 8.09 & 87.85± 4.87 p < 0.05; 8.63±4.41 and 24.98±10.47 p <0.001; 68.71± 10.24 and 79.57±8.64 p< 0.001 respectively). Unlike the control neonates, neonates of gestational diabetic mothers had positive correlation between the functional neutrophil parameters (r0.39 p<0.05).* ***Conclusion:*** *Gestational diabetes affects cord blood neutrophil count and functions leading to high susceptibility to infection.*

Keywords: Gestational, diabetes mellitus, neutrophils.

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INTRODUCTION

Neutrophils play a basic role in host defense against infection and any decrease in their functional capability adds to high susceptibility and seriousness of infection[1]. Abnormalities in granulocyte chemotaxis, phagocytosis and anti-microbial activity have been described in experimental studies on diabetic rats and mice and clinical investigations of diabetic patients1. During the neonatal period, response against infections mainly depends on the innate immunity and any impairment of innate immunity during the intrauterine life may decrease microbicidal activities[2]. Gestational diabetes mellitus is associated with down regulation of the placental inflammatory response affecting nutrient transporter expression and activity. These changes in placental function may be the cause of reduced cell-mediated immunity and lower chemotactic activity of cord blood neutrophils from infants of diabetic mothers[3]. Previous studies have addressed the deleterious effect of diabetes on patients' neutrophils functions, and neutrophil functional activity in neonates born to mothers with gestational diabetes[4]. In our study we focused on changes in the neutrophil chemotactic and phagocytic activities of neonates due to maternal gestational diabetes. Furthermore, we addressed only those who have been following up regularly and controlled on insulin to exclude any bias that may be caused by microvascular complications of uncontrolled diabetes.

METHODS

Patients' selection:

This was a cross-sectional controlled study performed over 6 months, on 45 full term neonates delivered at Ain Shams university neonatology units in 2014.

Study group (Neonates of gestational diabetes mothers (NGDMs): included 30 full term neonates (38-40 weeks) born to mothers with gestational diabetes mellitus recruited at delivery (NGDMs). All women who were followed up regularly during pregnancy at the Ain shams University obstetrics clinic and who had their blood glucose levels controlled on insulin therapy without other diabetes

comorbidities or complications were candidates for inclusion in the study. The mother was approached and informed consent was obtained. Eligible women had to be admitted at least 2 hours before delivery to allow time for enrollment

Control group: included 15 sex and age matched apparently healthy full-term neonates recruited on their 1st day of life.

Exclusion criteria:

- Any newborns with history suggestive of respiratory distress syndrome, neonatal sepsis or family history of immune deficiency.
- Newborns with major congenital anomalies or multiple gestations.
- Neonates born to mothers suffering from long standing diabetes mellitus or its microvascular complications and comorbidities.

Methods

All enrolled neonates were subjected to history taking and clinical examination (gestational age, birth weight, organomegaly and vital data).

Laboratory investigations:

Random blood sugar (RBS)) for assessment of any hypoglycemia at delivery where neonatal hypoglycemia was defined as a plasma glucose level of less than 30 mg/dL (1.65 mmol/L) in the first 24 hours of life and less than 45 mg/dL (2.5 mmol/L) thereafter[5].

Verbal consents and approval were obtained from the parents after explanation of the subject and procedure.

Sampling:

a) Two mL cord blood (CB) 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 CBC and flow cytometric assays.
b) Two mL PB samples were obtained on K_2-EDTA in vacutainer tubes from controls for flow cytometric assay.

1) Complete blood counts (CBC) on Coulter LH750 cell counter (Coulter, Electronics, Hialeah, FL, USA) with examination of cord blood (CB) smears stained with Leishman stain and differential white cell count with emphasis on absolute neutrophilic count.
2) Flow cytometric assay of oxidative burst using DHR 123:
   - 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). 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:
     1. Blood only.
     2. Blood + DHR123 (Resting tube).
     3. 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 degrees C water bath for 15 minutes. Following this incubation, 100ul of the prepared PMA solution is added to tube 3 and all tubes are incubated an additional 15 minutes at 37 degrees 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, Mountainview, CA) for 10 minutes in the dark, followed by centrifugation and washing by 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 are expressed as neutrophil oxidative index (NOI) which is the ratio of MPC-FL (PMA stimulated) over MPC-FL (unstimulated).
3) Flow cytometric immunophenotyping of CD11b (Integrin α M) and CD62L (L-selectin) on neutrophils:
   - 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).
   - CB 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 (PBS).

- 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 were added to every tube. Tubes were vortexed then analyzed using Coulter Epics XL flow cytometer (Coulter, Electronics, Hialeah, FL, USA).

Statistical methods:

The collected data were collected and analyzed using statistical package for social science (SPSS) version for windows (version 15.0.1). All data were expressed as mean values ± SD. Comparisons of parameters among groups were made using paired t test. Comparisons between two qualitative variables were performed using chi-square and fisher's exact tests. A p value ≤ 0.05 was considered significant

RESULTS

Neonates of gestational diabetes mothers (NGDMs) (n= 30) were compared to control neonates (n=15) by gestational age, sex, birth weight, length, mode of delivery and Apgar score (shown in Table 1). We did not report any case of hypoglycemia at time of sample withdrawal after birth in both groups. Comparative parameters of the CBC results between the two groups showed that NGDMs had significant lower mean total leucocytic and neutrophil counts (13.55±2.51 and 9.01±1.59 respectively) than those of controls (17.89±3.66 and 14.18±3.44 respectively) P<0.001. The tested parameters of the neutrophil functions (DHR, CD11b, and CD62L) were significantly reduced in the NGDMs than controls as in table 2. A positive correlation was found between DHR and CD11b in neonates of gestational diabetic mothers in contrast to the control group where those parameters were negatively correlated (table 3, 4 and figures 1and 2).

Table 1. Demographic data of the studied groups

		NGDMs (n=30)		Control (n=15)		Chi-square	
		N	%	N	%	X^2	P-value
Sex	Female	10	33.3	4	26.7	1.392	0.499
	Male	20	66.7	11	73.3		
Mode of delivery	Normal	7	23.3	11	73.3	10.417	<0.001*
	Cesarean	23	76.7	4	26.7		
Apgar score 1min.	Range	8-10		8-10		0.007	0.993
	Mean± SD	9.23±0.86		9.27±0.88			
Apgar score 5min.	Range	10-10		6-10		2.907	0.066
	Mean± SD	10.00±0.00		9.53±1.06			
Weight (Kg)	Range	2.6-4.5		2.4-3.6		0.141	0.869
	Mean± SD	3.26±0.37		3.20±0.32			
Length (cm)	Range	48-60		36-55		6.220	0.004*
	Mean± SD	51.53±2.43		46.93±6.23			

** Statistically significant*

Table 2. Comparison between the studied groups as regard CBC DHR, CD11b, CD62L.

	IDMS		Infant		Anova test		
	Mean	SD	Mean	SD	f	P-value	Sig.
DHR	68.71	10.24	79.57	8.64	38.497	<0.001*	HS
CD11b	82.48	8.09	87.85	4.87	33.588	<0.001*	HS
CD62L	8.63	4.41	24.98	10.47	861.035	<0.001	HS

DHR= dihydrorhodamine 123, CD62L = L selectin

Table 3. Correlation between DHR & CD11b and CD11b & CD62L in IDMS

IDMS	DHR		CD11b	
	r	P-value	r	P-value
CD 11b	0.390	0.033*		
CD 62L	0.096	0.612	-0.187	0.322

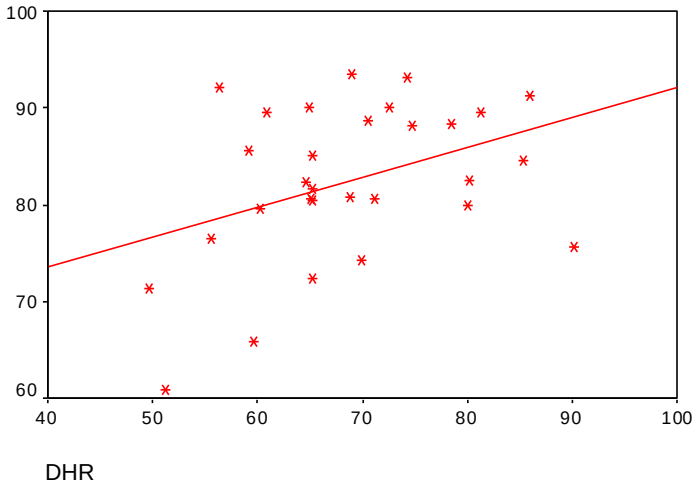

Figure 1. positive correlation between DHR and CD11b in NGDMs (r= 0.390 / P = 0.033)

Table 4. Correlation between DHR & CD11b and CD11b & CD62L in control.

control	DHR		CD 11b	
	r	P-value	r	P-value
CD 11b	-0.518	0.048		
CD 62L	-0.056	0.844	-0.059	0.834

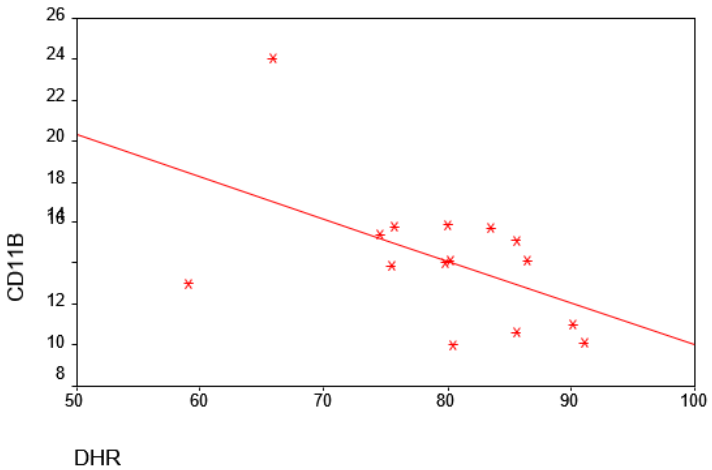

Figure 2. Negative correlation between DHR and CD11b in control infants (r=-0.518/ P = 0.048)

DISCUSSION

Neutrophils act as first-line-of-defense cells and the alteration of their functional activity contributes to high susceptibility and severity of infections[1]. During the neonatal period, the defense against infection is mainly dependent on innate immunity[6]. We assumed that gestational diabetes may affect the neutrophil function of the newborns affecting their innate immunity and defense mechanisms against infections.

Our data indicate that the maternal gestational diabetes is accompanied by impairment of cord blood neutrophil function represented in relative lower levels of DHR, CD11b, and CD62L in neonates of gestational diabetic mothers than their matched controls.

Normal pregnancy is associated with highly regulated inflammatory response that is vital to the process of placentation, from implantation through to labor at term[7]. Maternal gestational diabetes has been associated with altered inflammatory profiles in the mother, placenta and fetus leading to the co-morbidities observed in these pregnancies. Vascular, endothelial changes, oxidative stress and tissue damage do occur in response to inflammation. Intrauterine inflammatory response evokes remarkable changes in development of microvessels, hematogenous cells and other components[8]. During inflammatory response, leucocytes roll along the lining endothelium of post capillary venules and eventually become attached to the vascular wall before migrating into tissue[9]. This may explain the relative leucopenia and neutropenia in IDM in comparison to controls.

The finding that hemoglobin and hematocrit in neonates of gestational diabetes mothers is higher than those in controls as well as their positive correlation with the CD62 level and negative correlation with the CD11b is consistent with the effect of relative hypoxia and peroxidation effect of high placental level of glucose during pregnancy on the red blood cell lipid membrane together with the protein synthesis, microtubular, cell membrane depolarization and microtubular structure of neutrophils[10].

We have demonstrated that neonates of gestational diabetes mothers have lower cord blood expression of CD11b, CD62L (L selectin) and DHR and that they are positively correlated to each other. Adhesion molecules such as CD11b and CD62L are important for rolling and adhesion as well as useful markers of neutrophil activation[11]. It has been found that neonatal neutrophils have lower functional adherence to the activated endothelium compared with those of adults[12]. This has been proved in many studies by finding defective expression and storage of CD18/CD11b and lower functional expression of CD11b on neonatal neutrophils[13]. In addition, Koeing etal[14] correlated the defect in neutrophils' endothelial adherence to the diminished total cellular CD11b on neonatal neutrophils[14]. Moreover, maternal diabetes creates pro-inflammatory placental environment, thus potentially influences the immune regulatory process of the neonates. In this context, deficiencies in neutrophil functions have been described in diabetic host [8]. It has been proven by murine experimental models that diabetic mice are at higher risk of bacterial infections [15]. Furthermore, studies showed that failure of neutrophil migration to infection sites is associated with poor outcome in experimental sepsis[16].

L-selectin (CD62L), is a cell adhesion molecule found on lymphocytes and the preimplantation embryo. It is a marker of neutrophil activation and an important mediator of neutrophil rolling and adhesion to activated endothelium[17]. Our results demonstrated that neutrophils from neonates of gestational diabetic mothers had a significant lower CD62L compared with control neonates. In Patients with diabetic microangiopathy were shown to have a significant decrease in surface CD62L expression. Changes in adhesion molecule expression also could contribute to the impairment of rolling and adhesion of leukocytes to endothelial cells found in diabetic mothers that may affect also their neonates[18].

DHR is a measure of neutrophil oxidative burst pathway and capability of superoxide production necessary for intracellular killing of catalase positive organisms[19]. Our data showed significantly lower mean values of DHR in NGDMs compared to control neonates. Studies have reported that DHR is higher in adult than in infant especially NGDMs[20, 21]. As previously known, microbicidal response of human neutrophils lies on the generation of reactive oxygen species (ROS) and activation of NADPH dependent oxidase releasing enzymatic and antimicrobial protein contents in the granules. Such responses are triggered by numerous agonists promoting adhesion or by phagocytic targets and as both are impaired during neonatal period this contributes to newborn incapability to generate O2 in response to neutrophils stimulation and adds to increase susceptibility to infection[22].

We conclude that even controlled gestational diabetes without complications is still a risk factor for impaired neutrophil function in term neonates. This may be based on pathophysiology of the disease itself rather than the type of treatment or

blood glucose control during pregnancy. Further studies could address the effect of impaired glucose control during pregnancy on the neutrophil activity.

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