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Impaired polymorphonuclear neutrophil functions in diabetics

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Abstract

Background and objectives: Polymorphonuclear neutrophils (PMN) are the first line of host resistance against infections. Diabetics are prone to both bacterial and fungal infections. The present study evaluated the phagocytic and killing activity of PMN in diabetics.

Material and methods: Females aged 30 to 50 years with and without diabetes mellitus were enrolled. Functions of PMN were assessed by determining the phagocytic rate, phagocytic index and killing of *C. albicans* by PMN.

Results: A total of 36 diabetic patients and 15 age matched non-diabetic healthy individuals were enrolled. Phagocytosis and killing of *C. albicans* by PMN were significantly ($p<0.05$) lower in patients with diabetes mellitus compared to non-diabetic healthy individuals (86.5±14.6 vs. 94.5±4.2; 56.7±23.8 vs. 81.5±24.2).

Conclusion: Phagocytic and killing functions of PMN were significantly reduced in patients with diabetes mellitus.

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Introduction

Polymorphonuclear neutrophils (PMN) are the first line of host resistance against bacterial infection. The main mechanisms that allow microbial killing are migration of PMNs to the site of infection, phagocytosis and killing by both oxygen-dependent and oxygen-independent mechanisms. In addition, activated PMNs produce chemokines and cytokines which recruit and activate other immune cells [1]. Finally, activated PMNs undergo apoptosis, resulting in phagocytosis by macrophage [2].

Diabetes mellitus (DM) is a chronic metabolic disorder that is characterized by chronic hyperglycemia and causes long-term complications like retinopathy, neuropathy, nephropathy and increased susceptibility to infections. It is becoming one of the largest emerging threats to public health in the 21st century [3]. Several immune alterations have been described in diabetes especially changes in polymorphonuclear cells, monocytes and lymphocytes [4]. Several studies have shown alterations in neutrophil function, an effect that contributes to the high incidence of infections in diabetic patients [5]. Studies with neutrophils of diabetic patients reveal decreased bactericidal activity, impaired phagocytosis and decreased release of lysosomal enzymes and reduced production of reactive oxygen species [6]. This reduction in leukocyte phagocytosis and bactericidal activity is correlated with increase in blood glucose levels [7]. In poorly controlled diabetic patients abnormalities in granulocyte chemotaxis, phagocytosis and microbicidal activity have been described by several groups [8].

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Candida albicans is a part of the normal flora and is in the normal state kept under control by host defense mechanisms [9]. DM predisposes individuals to candidal infection. Several factors have influence on the balance between host and *C. albicans*, favoring the transition of *C. albicans* from commensal to pathogen and causing infection [10]. The main reason for this infection could be because of altered functions of the immune system in diabetic patients due to poor glycemic control [11]. Therefore, the present study evaluated the phagocytic functions of PMN in diabetics.

Materials and methods

The study protocol was approved by the Institutional Ethical Review Committee of Diabetic Association of Bangladesh. Informed written consent was obtained from all study participants prior to the enrollment in the study.

Study population and sample collection: Diabetic females aged 30 to 50 years with body mass index (BMI) 22-27, fasting plasma glucose 8-12mmol/L, on oral hypoglycemic agent (OHA) and free from diabetic complication(s) or systemic illness or pregnancy were enrolled. All diabetic cases were within normal limit of serum creatinine and C-reactive protein. Cases with hyperlipidemia and hypertension were excluded. Age matched healthy non-diabetic females were included as control. About 10 ml of venous blood was collected from each individual with aseptic precautions. Six milliliter of blood was taken in heparinized tube and 4 ml of blood was kept in a glass test tube for autologous serum and biochemical tests. AB serum was prepared from blood of AB positive healthy individual. The serum was separated and stored at -20^{0}C until used.

Assessment of PMN functions: Functions of PMN were assessed by determining the rate of uptake and killing of *C. albicans* by PMN as described earlier [12].

Yeast form of *C. albicans* was prepared by culturing *C. albicans* on Sabouraud dextrose agar media for 24 hours at 37^{0}C. Yeast cells were harvested and a suspension of 1x10^6/ml and 4x10^6/ml yeast cells were made in Hanks' balanced salt solution (HBSS, pH 7.4) for candidacidal and phagocytic assays respectively. Viability of yeast cells was checked by methylene blue dye-exclusion test.

PMNs were isolated from heparinized venous blood by Ficoll-Hypaque (MP Biomedicals) density gradient centrifugations. PMN purity was >95%, as determined by Giemsa staining and microscopy, while the cell viability was >98%, as determined by trypan blue exclusion test [13]. The PMNs were washed twice with HBSS and suspended in HBSS to a final concentration of 1x10^6 cell/ml.

For assessing PMN phagocytic function, a suspension of PMN and *C. albicans* was prepared at a ratio of 1:4 for PMN to *C. albicans*. Volumes of 100 µl of PMN suspension (1x10^5/100 µl), 100 µl *C. albicans* (4x10^5/100 µl), 100 µl autologous serum and 100 µl HBSS were made up to a final total volume of 400 µl in 1.5 ml microcentrifuge tube. A parallel assay in AB serum and appropriate controls without PMN were set up. The tubes were incubated at 37^{0}C for 2 hours with rotation. After 2 hours, the mixture was centrifuged at 3000g for 1 minute. Then 200 µl of supernatant was removed. The remaining mixture was shaken gently and smear was made on glass slide. The slide was fixed in absolute alcohol and stained with Leishman stain. At least 200 PMN cells were counted. The percentage of PMN with phagocytosed *C. albicans* was calculated by: {(Number of PMN with phagocytosed *C. albicans* ÷ Total PMN counted) × 100}. The phagocytic index per PMN was estimated by the formula: (Total number of intracellular *C. albicans* ÷ Total PMN with phagocytosed *C. albicans* counted).

For neutrophil candidacidal assay, 100 µl PMN (1x10^5/100 µl), 100 µl *C. albicans* (1x10^5/100 µl), 100 µl autologous serum and 100 µl HBSS were made up to a final total volume of 400 µl in a 1.5 ml microcentrifuge tube. A parallel assay in AB serum and appropriate controls without PMN were set up. The tubes were incubated at 37^{0}C for 2 hours with rotation. Then the mixture was centrifuged at 3000*g* for 1 minute and 200 µl of supernatant was removed. After mixing thoroughly, 50 µl of the mixture was taken in another microcentrifuge tube where 50 µl of 0.1% ice cold methylene blue solution was added. After 20 minutes, 1 drop of the mixture was taken on a glass slide and covered with a cover slip. The wet film was examined under

microscope and 200 yeast cells within PMN were counted. The percent of *C. albicans* stained blue (i.e. % kill) was scored.

***Optimization of neutrophil phagocytic assay and candidacidal assay**:* Uptake and killing of *C. albicans* by PMN were optimized in diabetics and healthy individuals. Ability of uptake and killing of *C. albicans* by PMN was observed at different time points namely 5, 30, 60, 90 and 120 minutes. Maximum uptake, phagocytic index and killing of *C. albicans* by PMN from both diabetic and healthy individuals were observed at 120 minutes.

Results

A total of 36 diabetic patients and 15 age matched non-diabetic healthy individuals were enrolled. Biochemical profile of study participants are shown in Table-1.

Table-2 shows the comparative rate of phagocytosis and killing of *C. albicans* by PMN from study population. There was significant difference (p=0.043) in the rate of phagocytosis of *C. albicans* by PMN between diabetic and non-diabetic healthy controls (86.5±14.6 vs. 94.5±4.2). Significantly (p=0.006) lower phagocytic index of PMN was observed in diabetic cases compared to non-diabetic controls (5.2±2.8 vs. 7.8±2.8). The candidacidal activity of PMN was significantly (p=0.001) higher in non-diabetic healthy controls than that of diabetic cases (81.5±24.2 vs. 56.7±23.8).

Table-1: *Biochemical profile of study population*

Study population	RBS mmol/L	Lipid Profile (mg/dL) Mean±SD				S.creatinine mg/dL Mean±SD	CRP mg/L Mean±SD
		Cholesterol	TG	LDL	HDL		
Diabetic (N=36)	7.1±1.8	152±46.9	139.2±7	78.1±42.1	46.4±9.1	0.8±0.1	6.4±1.4
Non-diabetic (N=15)	4.9±1.5	179.4±17.1	145.1±32.1	105.3±20.1	54.6±18.3	0.7±0.1	6±0

Note: RBS: random blood sugar, CRP: C reactive protein, HDL: high density lipoprotein, LDL: low density lipoprotein, TG: triglyceride

Table-2: *Comparison of rate of phagocytosis and killing of C. albicans by PMN from diabetic and non-diabetic healthy individuals*

Study population	PMN with phagocytosed *C. albicans* Mean % (±SD)	Killed *C. albicans* Mean % (±SD)	Phagocytic index Mean % (±SD)
Diabetic (n=36)	86.5±14.6	56.7±23.8	5.2±2.8
Non-diabetic (n=15)	94.5±4.2	81.5±24.2	7.8±2.8
p value*	0.043	0.001	0.006

*Note: *p value calculated by Student's t test*

Discussion

Diabetes is a major risk factor associated with candidiasis. One simple explanation for this is that PMN functions are altered in diabetic patients [9]. In our study, PMNs from diabetic cases exhibited reduced phagocytosis of *C. albicans*. A similar finding has been reported for patients with poor glycemic control who showed impaired PMN phagocytosis of virulent K1/K2 *Klebsiella pneumoniae* compared with patients with good glycemic control and healthy volunteers [14]. Also, PMN from diabetics displayed reduced uptake of *Burkholderia pseudomallei* compared to that of by PMNs from healthy controls [13].

Also, neutrophil candidacidal assay revealed reduced killing of *C. albicans* by PMNs from diabetic

patients. In the present study, killing of *C.* albicans was significantly reduced in diabetic than non-diabetic population. Previous studies have documented similar results; for example, PMN from DM subjects with poor glycemic control displayed lower killing rate of *B. pseudomallei* than PMN from healthy individuals [13]. Again, Mazade *et al*., found impairment of group B *Streptococcus* killing by neutrophils in diabetics [15].

For phagocytosis or killing of microbes, neutrophil requires energy. Metabolic changes are involved in the reduction of neutrophil function observed in DM [8]. Intracellular killing activity of PMN involves production H_2O_2, superoxide anion, molecular oxygen and nitric oxide [16]. The generation of these substances is dependent on activation of the pentose phosphate pathway of glucose utilization. Killing activity of PMN is thus closely connected with carbohydrate metabolism. PMN of diabetic persons may have decreased glucose consumption, disturbances of glycolytic processes, and decreased glycogen synthesis. Insulin improves carbohydrate metabolism in PMNs of diabetics [17].

Our results suggest that PMNs of diabetics are defective in resisting infection due to impaired phagocytic and killing functions.

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