Estimating The Efficiency Of Phagocytic Neutrophil Cells And Studying Its Risk Factors Among Diabetic Foot Ulcers

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Abstract. There are several factors that affect the efficiency of phagocytic activity in diabetic patients. In this study, a significant difference of phagocytosis efficiency showed in diabetic patients and controls (non-diabetic). The means of phagocytic activity were 43.05%, 52.70% and 71.35% for diabetic foot ulcer, diabetes mellitus patients and controls respectively. Age groups, mean blood glucose levels and controlling fasting blood glucose were significant factors effects on phagocytic activity (P-value = 0.022, 0.013 and 0.045) respectively. There was reverse significant correlation between the mean blood glucose and phagocytic activity (P-value = 0.049; r = -0.314). Wagner grade ulcers classified I and II were the most prevalent 30% for both in diabetic foot ulcer patients. And we found that there is a weak negative correlation between the phagocytic activity efficiency and the grades of the classified ulcers (r = -0.323). In this study, we revealed that the efficiency of phagocytic activity was affected by many factors. Level of blood glucose control was the most important factor.

1. Introduction

Diabetes and its complications have a long-term effect on the immune response, with a direct impact on wound healing [1]. Polymorphonuclear neutrophil cells are the first line defence have breached the epithelial barriers and the most abundant leukocytes in human blood and are particularly adept at phagocytosing and killing the microbes [2], [3], [4]. Initially, neutrophil cells direct the innate immune response by engaging in complex interactions with natural killer cells, dendritic cells, macrophages and through cross-talk with most of the cellular effector mediators [5]. Phagocytosis was defined as recognition and ingestion of particles larger than 0.5 μm into a plasma membrane derived vesicle, known as phagosome. It is a critical step in the removal of bacteria during infection [6].

Diabetes is a metabolic disorder that certainly affects the immune system and altered immunological aspects may be responsible to manifest as diabetic complications [7]. Increasing the risk of infection are related to several immunological factors, the observed changes in neutrophil cells include impairment of adhesion to endothelium and migration to the inflammation site, chemotaxis process, bacterial killing activity, phagocytic activity and production of reactive oxygen species. Because of the exaggeration of the hyperglycemia and blood glucose level, many impairments are happened in the serum level of immunoglobulins, cell mediated immunity and complement function [8], [9]. Foot ulcers remain one of the most significant and devastating complications of diabetes and it's the most distressing complications of a diabetic patient [10]. Wagner classification is one of the most widely used and universally accepted grading systems for diabetic foot ulcer (DFU) patients. Wagner's classification is consisting of six simplistic wound grades which used to assess ulcer depth as follows: grade-0 (high risk foot and no ulceration), grade-I (superficial ulcer involving the full skin thickness not but underlying tissues), grade-II (deep ulcer - cellulitis), grade-III (Deep abscess,
osteomyelitis), grade-IV (localized gangrene) and grade-V (extensive gangrene of the whole foot) [11].

Based on that neutrophil's activity is affected by blood sugar levels in blood, we hypothesized that the infection of lesions grades in DFU is affected by phagocytic neutrophil cells activity. Therefore, the present study was aimed to assess the efficiency of phagocytic neutrophil cells and studying the risk factors affected the Wagner grades of the diabetic patients' foot ulcers.

2. Materials and Methods

2.1 Study design and participants
This is a cross-sectional study which was carried out in Mukalla city-Hadramout/Yemen. About 5ml of venipuncture blood samples which was collected in ethylene diamine tetra acetic acid (EDTA) obtained from a sample size of 60 participants included 20 diabetes mellitus patients, 20 hospitalized DFU patients and 20 healthy persons (control).

2.2 Inclusion and exclusion criteria
The patients with diabetes mellitus type-2 were included in the study, whereas patients excluded when were they are having diabetes mellitus type-1.

2.3 Samples preparation and processing.
Blood glucose level determination test was done according to the instructions of a kit manufactured by SPECTRUM Company (UAE) using Spectrophotometer (Stat Fax, Germany).

Phagocytosis activity estimation test was performed according to different methods with minor modifications. Briefly, normal saline suspension of pathogenic strain of *Staphylococcus aureus* (*S. aureus*) colonies was prepared and the visible turbidity was adjusted to 0.5 McFarland turbidity standard yielding an approximately 1.5×10^8 bacterial cells. About 1ml of EDTA whole blood mixed with 1ml of bacterial suspension, then incubated for one hour at 37°C. One drop of incubated mixture was placed on a slide of the microscope to make a thin smear. The smear was lifted to dry for 3 minutes, then fixed with ethanol 96%, and placed in hematoxyline stain for 10 minutes. After washing with water, the smear is placed in an eosin stain for 30 seconds, then washed by water and examined under the light microscope using 100x oil immersion. The total number of *S. aureus* ingested within 100 neutrophil cells were counted in several fields and divided by 100 to give the percentage of phagocytosis activity (bacterial killing by neutrophil cells). The results were interpreted as the following: less than 40% (low activity of phagocytosis), 40%-70% (intermediate activity of phagocytosis) and more than 70% (high activity of phagocytosis) [8], [9], [12], [13].

2.4 Ulcers classification
Ulcers of diabetic foot patients were classified into five grades according to Wagner's grades classification.

2.5 Data collection tool
The risk factors for diabetic patients and controls were collected and filled with participants interviewed using a standardized questionnaire included the variables of gender, age groups (years), level of the blood glucose and the control of it and smoking.

2.6 Statistical analysis
Data in this study were analyzed using SPSS program version 25. The graphs were presented by using the software program (Excel for Windows Microsoft) version 10. Descriptive statistics were presented as frequencies, percentages and means, then calculated and compared using least significant difference test (LSD), and analysis of variance (ANOVA) was performed between factors. Pearson Chi-square (χ2) test was used to compare and measure the association between different explanatory variables of the studied groups. The relationship between the variables examined by the Pearson correlation (r) test. The level of significance was set at (P-value ≤ 0.05).
3. Results and Discussion

According to the phagocytosis activity test of the studied groups, diabetic foot ulcer patients and diabetes mellitus patients had no highly efficient phagocytosis activity as given in (table 1). *S. aureus* phagocytized by neutrophil cells are shown in (Fig. 1).

**Table 1**: Phagocytosis activity among the studied groups.

| Group             | Phagocytic activity |
|-------------------|---------------------|
|                   | Low | Medium | High | Total |
| Control           | 0   | 10     | 10   | 20    |
| Diabetes mellitus | 2   | 17     | 1    | 20    |
| Diabetic foot ulcer| 6   | 14     | 0    | 20    |
| Total             | 8   | 41     | 11   | 60    |

**Figure 1**: *S. aureus* phagocytized by neutrophil cells.

The mean of phagocytosis activity was completely different between diabetes mellitus patients, diabetic foot ulcer's patients and healthy persons (control) using the least significant difference test (LSD) with high statistical difference, (table 2) and (fig. 2).

**Table 2**: Phagocytosis activity among the studied groups.

| Group                  | Control (71.35%) | Diabetes mellitus (52.70%) | Diabetic foot ulcer (43.05%) |
|------------------------|------------------|----------------------------|------------------------------|
| Control                | -                | 0.000*                     | 0.000*                       |
| Diabetes mellitus      | 0.000*           | -                          | 0.001*                       |
| Diabetic foot ulcer    | 0.000*           | 0.001*                     | -                            |

*P-value < 0.05 is considered statistically significant
These results of significant reduction in phagocytosis activity in both groups of diabetes mellitus and diabetic foot ulcer patients in compared to control group confirm the impairment of phagocytosis activity of neutrophil cells in diabetic patients than non-diabetic patients [14]. Other studies showed a significant reduction of phagocytosis activity in both type 1 and 2 diabetes mellitus [15], [16], and this difference may be due to that hyperglycemia impairs the functions of the granulocyte including: adherence, chemotaxis process, phagocytosis activity and bacterial killing activity [17]. So, one of the long-term effects of elevated mean blood glucose or glycated hemoglobin (HbA1c) that resulting from hyperglycemia is reduced the response of neutrophil function, also the impaired micro-vascular circulation in diabetic foot ulcer patients limits the access of phagocytic cells which leads to development of infection [18], [19], [20].

In this study, there was no significant relationship effect between gender and activity of phagocytosis, while a significant relationship showed between efficiency of phagocytosis activity and the age groups ($P$-value= 0.045). High phagocytosis activity was found in the age groups of <18 years and 18-25 years, whereas low phagocytosis activity showed in the participants of age group >55 years as showed in (table 3). Some studies revealed that phagocytosis activity in diabetic patients didn't affected by differences relating to gender and age groups [21], [22], [13].

Table 3: Effect of gender and age on phagocytic activity.

| Characteristics       | Group | Phagocytic activity | $\chi^2$ test value | $P$-value |
|-----------------------|-------|----------------------|----------------------|-----------|
|                       |       | Low  | Medium | High | Total |                      |                      |
| Gender                | Male  | 7    | 28     | 5    | 40    | 3.839                 | 0.147                |
|                       | Female| 1    | 13     | 6    | 20    |                       |                      |
|                       | Total | 8    | 41     | 11   | 60    | 3.839                 | 0.147                |
| Age group (years)     | < 18  | 0    | 1      | 3    | 4     |                       |                      |
|                       | 18-25 | 2    | 6      | 3    | 11    |                       |                      |
|                       | 26-35 | 0    | 11     | 2    | 13    |                       |                      |
|                       | 36-55 | 2    | 5      | 2    | 9     | 15.8                  | 0.045*               |
|                       | > 55  | 4    | 18     | 1    | 23    |                       |                      |
|                       | Total | 8    | 41     | 11   | 60    | 15.8                  | 0.045*               |

* $P$-value < 0.05 is considered statistically significant

The present study provides evidence that fasting blood glucose level has significant associated effects with phagocytosis activity ($P$-value= 0.022) as shown in (table 4). This is similar to results of different studies that showed fasting plasma glucose was independently associated with phagocytosis activity [21], [22], [8], [23], [13]. Other studies have linked the state of glycemic with immune function to surgery site infection (SSI) including postoperative hyperglycemia was the most important risk factor.
for SSI [24], [25], [26]. So, evaluate the perioperative glucose and insulin in patients with hyperglycemia are very important targets [17], [25], [27]. Thus, our findings that we revealed may have relevance for clinical outcomes about increasing the risk of infections.

**Table 4:** Effect of fasting blood glucose levels on phagocytic activity.

| Phagocytic activity | Fasting blood glucose levels (mg/dl) | F-value | P-value |
|---------------------|-------------------------------------|---------|---------|
| No.                 | Mean                                |         |         |
| Low                 | 8                                   | 230.9   |         |
| Medium              | 41                                  | 163.5   | 4.077   | 0.022* |
| High                | 11                                  | 124.7   |         |
| Total               | 60                                  | 165.4   |         |

*P-value < 0.05 is considered statistically significant

Among enrolled participants in our present study, 6 of them had low phagocytosis activity, 15 had moderate phagocytosis activity and only just one had high phagocytosis activity with statistically significant difference (P-value= 0.013), whereas high activity of phagocytosis found in controlled. However, there was no significant effect of smokers and non-smokers participants on the efficiency of phagocytosis activity, (table 5).

**Table 5:** Effect of blood glucose control and smoking on phagocytic activity.

| Characteristics          | Group          | Phagocytic activity | χ² test value | P-value |
|--------------------------|----------------|---------------------|---------------|---------|
| Blood glucose control    | Controlled     | Low 2, Medium 26, High 10 | 8.664         | 0.013*  |
|                          | Uncontrolled   | Low 6, Medium 15, High 1  |               |         |
|                          | Total          | Low 8, Medium 41, High 11 |               |         |
| Smoking                  | Non smokers    | Low 6, Medium 27, High 3 | 5.423         | 0.066   |
|                          | Total          | Low 8, Medium 41, High 6  |               |         |

*P-value < 0.05 is considered statistically significant

Herein, we revealed a significant reverse correlation between level of fasting blood glucose and phagocytosis activity. Wherever, the mean of blood sugar increased, the activity of phagocytosis was decreased as shown in (table 6) and (fig. 3). In contrast, other studies showed significant negative correlation between phagocytosis activity and fasting blood glucose [28], [22], [8], [29].

**Table 6:** Correlation between levels of fasting blood glucose and phagocytosis activity.

| Variables                  | No.  | P-value | Pearson correlation (r) |
|----------------------------|------|---------|-------------------------|
| Level of fasting blood glucose | 40   | 0.049*  | -0.314                  |
| Phagocytosis efficiency     | 40   |         |                         |
*P*-value < 0.05 is considered statistically significant

![Figure 3: Correlation between phagocytosis activity and fasting blood glucose level.](image)

In poorly controlled participants, there was an abnormality in granulocyte phagocytic cells and bactericidal activity have been described in the different groups with significant relationship between blood glucose control and phagocytosis activity in our study. Some studies revealed that the controlling of blood glucose plays an essential role in phagocytosis activity [28], [8], [22], [15], [20]. In this study, there was no significant relationship between smoking and decreased phagocytic efficiency, for this reason there are no previous studies regarding the relationship between the effect of smoking and phagocytosis activity in diabetic foot patients.

As an assessment of grades of the ulcers of diabetic foot patients as described by Wagner's classification at the time of study period, the results showed that grade I and II were seen in 6(30%) cases for both, grade III in 5(25%) cases, grade IV in 1(5%) case and grade V in 2(10%) cases, (fig. 4). These results were compatible with other studies where Wagner grade I and II were the most common prevalence in ulcers of diabetic patients [30], [31], [32], but disagree with a previous study reported that Wagner grade II and III ulcers were the most common prevalence [33]. Also, the grade IV was the lowest prevalence in this study, this contrast with other studies revealed that the grade IV was the most common prevalence in DFU [34], [35].

![Figure 4: Percentages of distribution diabetic foot ulcers according to Wagner grades.](image)

One of the point strengths of our study is to find the relationship between the manner of immune response involving phagocytosis activity and the degree of wound as shown in (table 7) and (fig. 5). We correlated the degree of wounds with phagocytosis activity efficiency to understand the development of the ulcer grade and the role of neutrophil phagocytic cells. Statistically, there was low insignificant correlation between these two variables. However, to our knowledge, there have been no reports in the literature regarding the correlation between phagocytosis activity and the degree of wound in diabetic foot patients, but to support our result and know the reasons for this relation we
need further deep studies to be clarified.

### Table 7: Correlation between phagocytosis activity and Wagner's grades of ulcer.

| Variables          | No. | P-value | Pearson correlation (r) |
|--------------------|-----|---------|-------------------------|
| Grades of ulcers   | 20  | 0.164   | -0.323                  |
| Phagocytosis       | 20  |         |                         |

![Figure 5: The correlation between phagocytic activity and Wagner's grades of ulcers.](image)

### 4. Conclusion
Neutrophil malfunction leads to increase the infection risk among diabetic patients, and to the difficulty in treating infections. Age, mean blood glucose and blood glucose control were the risk factors associated with efficiency of phagocytosis activity. Enhancing blood glucose control may enhance white blood cells function. Wagner's classification of grade I and II were the most prevalent in diabetic foot ulcers. The efficiency of the phagocytosis activity process is getting low with the increase of the wound degree. Hyperglycemic control should be adjusted to avoid developing the ulcers of diabetic foot patients and infections resulting from malfunction of neutrophil cells.

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