Research article
Phagocytic and oxidative burst activity of neutrophils in type 2 diabetic patients with foot ulcers

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ABSTRACT

Introduction and Aim: Diabetic foot ulcers are common complications seen in diabetic patients. Treatment of this disabling foot sore remains a challenge to health care professionals. This study aimed at evaluating whether the neutrophils from type 2 diabetic patients with foot ulcers present an impairment of phagocytic index and impairment in respiratory burst. We also aimed at understanding whether the impairment in neutrophil phagocytic activity can be alleviated with short course of standard treatment regime for foot ulcers.

Methodology: For this case-controlled study, 43 participants with type 2 diabetes (18 with foot ulcers and 25 without foot ulcers) were prospectively recruited along with 18 healthy volunteers. Phagocytic activity of neutrophils and respiratory burst of neutrophils was assessed along with ESR, percentage neutrophil counts before and after 2 weeks of standard treatment for foot ulcers.

Results: Neutrophils of type 2 diabetic patients (with and without foot ulcers) showed lower levels of phagocytic index and lower percentage of respiratory burst compared with non-diabetic subjects. Furthermore, on receiving treatment for foot ulcers, a significant improvement in neutrophil phagocytic indices were observed, along with improvement in wound ulcer score.

Conclusion: Phagocytic activity of the neutrophils is impaired in type 2 diabetics (with and without foot ulcers). Neutrophil phagocytic indices can be improved on glycemic control. Additionally, improvement in neutrophil phagocytic indices after short course treatment for foot ulcers can be useful markers to predict treatment efficacy and in prognosis of diabetic foot ulcers.

Keywords: Neutrophil phagocytic index; respiratory burst; type 2 DM patients; diabetes; foot ulcers; predictors for treatment efficacy of foot ulcers.

INTRODUCTION

Diabetic foot ulcers are common disabling sores on the feet which approximately 15% of people with diabetes mellitus experience (1, 2). Diabetic foot ulcers precede 84% of all diabetic-related lower-leg amputations, risk of which increases with age and duration of diabetes (2, 3).

Patients with type 2 diabetes mellitus (T2DM) are immunocompromised and have an increased incidence of infections (4). Phagocytic cells serve as cornerstones of innate immunity. Phagocytic activity of phagocytic cells (peripheral blood mononuclear cells-PBMCs) plays an important role in protecting the host from infectious diseases. Impaired PBMC functions are implicated in T2DM patients which predisposes them to risk of infections particularly foot ulcers (5, 6).

An essential mechanism involved in the elimination of pathogens by the innate immune system is the oxidative burst, or respiratory burst (increased superoxide radical and hydrogen peroxide produced to kill pathogens) in the phagocytes (6, 7). This involves a series of events that occur when these phagocytes are stimulated by a pathogen leading to increased oxygen uptake by macrophages and the PBMCs which are first-line defenders. This consequently leads to reactive oxygen species (ROS) production and oxidative stress. Oxidative stress conversely impairs immune functions (6–8). Phagocytic functions and attenuation of the pathogen can, therefore be impaired in DM patients (8, 9). A combination of increased oxidative stressors and impaired immune functions predisposes DM patients with micro and macro vascular complications susceptible to infections and thereby foot ulcers (9, 10). Effects of increased free radical production and oxidative stress environment in DM patients may be partially due to impaired antioxidant defenses and deranged carbohydrate metabolism (6, 9, 11).

Maintenance of oxidant and antioxidant homeostasis is important for optimum immune cell functions, maintenance of cell integrity, signal transduction, and gene expression. These events are compromised in DM patients due to the effect of hyperglycemia.
consequently putting them to risk of infections and in particular foot ulcers (11, 12). Several factors like genetic susceptibility to infection, poor blood supply, nerve damage, diabetes-associated alterations in metabolism along with defects in innate immune mechanisms are found to be associated with the pathogenesis of foot infections. Nevertheless, we still have no clear understanding of all the factors that make diabetic patients more vulnerable to frequent and protracted infections (13, 14).

Given the complexity of factors interacting with the mediators that contribute collectively to innate immune mechanisms, treatment for diabetic foot ulcers remains a challenge to health care professionals. This study addresses the association of impaired phagocytic capacity of neutrophils and deranged phagocytic respiratory burst in diabetic foot ulcers patients and focuses on whether these indices improve after standard therapy and whether the neutrophil indices serve to predict treatment efficacy of foot ulcers.

MATERIALS AND METHODS

Subjects

A total of 43 patients who underwent treatment for DM and Diabetic foot ulcers in a tertiary care hospital in Mangalore, Karnataka, India were considered for this cross-sectional prospective study. 18 of the recruited subjects were diabetic with foot ulcers (a total of 23 subjects were initially recruited and 5 of them underwent amputation and their results are not being discussed), 25 were diabetic patients without foot ulcers. All diabetic patients included were those patients who suffered from DM for the past 15-22 years. 18 healthy non-diabetic individuals were recruited as controls. (Table 1).

Ethical approval from the institutional ethical committee was obtained before commencing the study (ID: 03635).

Venous blood samples were collected in vacutainers from all participants after obtaining their informed consent. Blood samples from the participants with diabetic foot ulcers were collected twice, one sample before the commencement of treatment for foot ulcers and second sample after two weeks of standard treatment (antibiotics, insulin, surgical debridement, hyperbaric oxygen therapy).

The diabetic foot severity score was calculated by Umehese and Ogbemudia severity scoring. Indices for evaluation of neutrophil functions, the neutrophil phagocytic index and neutrophil respiratory burst was determined in the collected venous blood as briefly described below.

Determination of phagocytic index

The phagocytic activity of neutrophils was determined by a standard method. The phagocytic index, i.e. the number of microbial bodies absorbed by an average leukocyte, was evaluated in the isolated polymorphonuclear leukocytes (neutrophils).

Isolation of polymorphonuclear leucocytes from blood was done using commercially available polymorph- prep gradient following the manufacturer’s instructions.

The cells were resuspended in RPMI 1640 culture medium at a final concentration of 9 x 10⁶ cells/mL. Trypan blue dye exclusion staining was used to check cell viability.

About 5x10⁶ Staphylococcus aureus cells were centrifuged at 1200 rpm washed with phosphate buffer saline (PBS) and were treated with whole serum and kept at 37°C for 45 minutes with shaking for opsonisation. Following opsonisation, pellets got after centrifugation was washed in PBS and further reconstituted in PBS. Bacteria and neutrophils (1:9) were then processed for phagocytoses by incubating at 37°C for 30 minutes. Finally, smears were prepared, stained and observed under 100x objective for phagocytized bacteria. The number of bacteria engulfed by 200 neutrophils was counted. The ingestion bacteria was expressed as a phagocytic index which was a percentage of the total bacteria added in the reaction mixture (15).

Determination of respiratory burst in the neutrophils

Evaluation of superoxide production to evaluate the respiratory burst of the Neutrophils was assessed by the nitro blue tetrazolium (NBT) reduction test. Briefly, following stimulation of neutrophils and activation of the respiratory burst pathway, superoxide radicals reduce soluble NBT dye to insoluble formazan granules. The percentage of NBT-positive cells was determined for each sample by evaluating 1000 cells and the values were expressed as the percentage of phagocytes involved (16). All chemicals were obtained from Sigma, St. Louis, MO, USA.

ESR (erythrocyte sedimentation rate) FBS (Fasting blood glucose), Total Leucocyte count, percentage Neutrophil count was determined in the collected venous blood by routine standard protocols.

Statistical analysis

The prism software package from the Graph pad was used for the analysis of the data. All obtained data is presented as mean ± SD. data was compared for statistical significance using students t-test. Differences were accepted as significant at p < 0.05.

RESULTS

Description of study participants is given in table 1. Mean and SD values of different parameters that was assayed and the statistical significance of the parameters between the study groups as indicated by p values are given in tables 2 and 3.
The mean fasting blood glucose levels were significantly higher in group 2 and 3 in comparison with group 1. Phagocytic index and respiratory burst were significantly lower in group 2 and in group 3 when compared to group 1. No significant difference was observed in phagocytic index and respiratory burst between the diabetic groups (between group 2 and group 3). After a standard treatment regime for foot ulcers and glycemic control, a statistically significant lowering of FBS, ESR was achieved (Group 3b).

**Study Design:**

| Groups      | Description of study participants in table 1                                                                 |
|-------------|-------------------------------------------------------------------------------------------------------------|
| Group 1     | Non-diabetic individuals, age and sex-matched to that of group 2 and 3.                                       |
| Group 2     | Type 2 DM Patients of both sexes without foot ulcers and related complications.                             |
| Group 3     | Type 2 DM Patients of both sexes with foot ulcers and related complications.                               |

**Table 1:** Mean ± SD and statistical significance (p-value) of study parameters in different groups of participants.

| Parameter                  | Group 1       | Group 2       | Group 3       | P-value |
|----------------------------|---------------|---------------|---------------|---------|
| FBS (mg/dl)                | 92± 8         | 167±30        | 181± 42       | # p < 0.05 |
| Phagocytic Index           | 1.150 ± 0.61  | 0.33 ± 0.17   | 0.28 ± 0.13   | # p < 0.05 |
| Respiratory burst %        | 73 ± 12       | 36 ± 9.2      | 28 ± 8        | # p < 0.006 |

**Table 2:** Mean ± S.D and statistical difference after standard treatment for foot ulcers

| Groups | FBS (mg/dl) | ESR (mm/hr) | Total Leucocyte count (Cell/ cu mm) | Neutrophil count % | Wound severity score | Ulcer scoring (Area x Depth) |
|--------|------------|------------|------------------------------------|-------------------|----------------------|-------------------------------|
| Group 3a | 181.6± 41.51 | 55.78± 22.4 | 10601± 1147                        | 84.3 ± 16.5       | 17.28 ± 0.86         | 14.7± 6.194                   |
| Group 3b | 100.4± 12.43 # | 15.9 ± 5.88 # | 9203 ± 1245                       | 64.2 ± 6.212#     | 13.93± 1.06 #        | 9.503 ± 3.36 #               |

# significant decrease in values in patients treated for foot ulcers p values < 0.05

**Table 3:** Mean ± S.D and the statistical difference in the phagocytic index and Neutrophil respiratory burst index after standard treatment for foot ulcers

| Parameter                  | Group 3a       | Group 3b       | P-values Group 3a vs Group 3b |
|----------------------------|---------------|---------------|------------------------------|
| Phagocytic Index           | 0.28 ± 0.13   | 0.8 ± 0.2     | p<0.001                      |
| Respiratory burst %        | 28 ± 8.0      | 60 ± 14       | p<0.001                      |

**DISCUSSION**

The wound severity score was observed to be decreased in patients after they received a short course standard treatment regime, suggesting an improvement in foot ulcers in response to treatment. Along with the improvement in foot ulcers, neutrophil phagocytic index and neutrophil respiratory burst also improved significantly. This points towards an overall improvement in the functions of neutrophils, which are now more efficient to defend against the pathogens serving as more effective first-line defenders (14,17).

Results of our study indicate that the neutrophils of T2DM patients are less efficient in combating (lower levels of neutrophil function indices seen in group 2) invading pathogen and that impaired neutrophil functions can be reversed by glycemic control. Our results affirms few other previous studies, which

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reported that the phagocytic activity of peripheral blood mononuclear cells is impaired in type 2 DM subjects (14,18,19). However, there are no conclusive reports that address the responsiveness of neutrophils of DM patients to treatment targeting glycemic control (14,19,20).

Our findings reinforce the concept that hyperglycemia has an impact on immune deficits in DM patients which in turn imparts effect on the functions of the neutrophils. Therefore DM patients in whom hyperglycemia is not controlled for long periods can be predisposed to progressive deleterious effects on the immune dysfunctions (9,20). Immune dysfunctions in these patients impact neutrophil phagocytic functions and dysfunctional neutrophil functions increasing their risk for severe infections, microvascular and macrovascular complications (21-23). The cause for immune and phagocytic impairments in uncontrolled DM patients may be in part due to derangements of carbohydrate metabolism and oxidative imbalances. Derangement in carbohydrate metabolism increases utilization of NADPH for the sorbitol-aldose reductase pathway, making less NADPH available for the phagocytes and the antioxidant defenses, thereby effecting oxidative balance (24,25). Few studies have reported that the unstimulated polymorph neutrophils of DM patients exhibit increased respiratory burst under baseline conditions producing increased free radicals which is one of the contributors to oxidative stress in DM patients. Consequently, oxidative stress may in turn further harm the phagocytes, ultimately reducing the capacity for bactericidal functions (19,21).

As we observed, standard treatment regime for just 2 weeks significantly improved the neutrophil indices suggesting that neutrophil phagocytic index and neutrophil respiratory burst has a significant application as determinants of efficacy of treatment modalities for diabetic foot ulcers. We also observed that higher levels of ESR and percentage neutrophil count parallel to decreased neutrophil indices in foot ulcers patients. ESR, neutrophil counts decreased towards normal levels with simultaneously decreasing neutrophil function indices. Thus we hypothesis that ESR, Neutrophil count, phagocytic functions indices in combination can be very useful not only as predictors of foot ulcer healing process in response to treatment, but help understand the need for a change of treatment modalities if required.

CONCLUSION

In DM patients poor glycemic control can lead to defective neutrophil phagocytic function and neutrophils bactericidal capacity. These functions of the neutrophils improve when glycemic control is achieved. Impaired phagocytic and bactericidal functions in long-term uncontrolled DM patients may be a key player in pathogenesis of foot infections. Neutrophil phagocytic index and neutrophil respiratory burst improve in response to standard treatment of foot ulcers suggesting the usefulness of phagocytic index and neutrophil respiratory burst as markers for prognosis of foot ulcers and as predictors of effectiveness of treatment.

CONFLICT OF INTEREST

Authors declare no conflict of interest

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