Research Article

Assessment the Role of Phagocytic Neutrophil Cells among Different Wagner's Grades of Diabetic Foot Ulcers Infection

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Abstract

Foot ulcers complications in diabetes mellitus (DM) patients are one of the significant medical problems. This study aimed to assessment the role of phagocytic neutrophil cells and its relationship with the incidence of diabetic foot ulcers (DFU) infection in diabetic patients. A case-control analytical study was conducted on 60 DM, DFU patients and healthy control group. Blood samples were collected for phagocytic activity testing and swabs samples of DFU were collected and processed for culture and antibiotics susceptibility testing after the ulcers classified according to Wagner's grades system. Findings revealed that Gram-positive bacteria were the most prevalent in the DFU 57.1% with statistically significant relationship between the bacterial species and grades of Wagner's classification. Wagner's ulcers grade I and II were the most prevalence in DFU patients 30% for both. There was a weak negative correlation between phagocytic activity and grade ulcers classified. Amikacin and ciprofloxacin were the most effective antibiotics against bacterial isolates, whereas ampicillin, cefepime and cefadroxil were the most common resistance antibiotics of bacterial isolates. In conclusion, this is the first study carried out in Hadhramout, Yemen to assessment the phagocytic neutrophil cells and its role to fight the bacterial infection of diabetic foot ulcers. When the grade of ulcer increased, the bacterial resistance to antibiotics increased, and this was emphasis the correlation with prevalence of Gram-negative bacteria in the high grade of ulcers with high resistance of antibiotics. In contrast, the grade of ulcer increased, the efficiency of phagocytic neutrophil cells decreased.

Key Words: Neutrophil Cells, Bacterial Infection, Wagner's Grades, Diabetic Foot Ulcers, Phagocytic Activity

Introduction

Diabetes mellitus (DM) is one of the main global health problems. It's defined as a metabolic disorder resulting from a defect in insulin secretion, insulin action, or both, which leads to chronic hyperglycemia [1]. Several polymorphonuclear leukocyte cells (PMNLs) affected by hyperglycemia, neutrophil's functions impair occur in diabetic subjects including impaired migration, phagocytosis, intracellular killing and chemotaxis, which may be due to decreased PMNLs membrane fluidity [2]. There is clinical evidence pointing to the higher prevalence of infectious diseases among individuals with DM [3]. Foot ulcers remain one of the most distressing complications of a diabetic patient and it's one of the most significant and devastating complications of diabetes [4]. Wagner's ulcer grades classification is one of the most widely used and universally accepted grading systems for diabetic foot ulcers (DFU), consisting of six simplistic wound grades used to assess ulcer depth as following: grade-0; high risk foot and no ulceration, grade-1; superficial ulceration, grade-2; deep ulcer (cellulitis), grade-3; osteomyelitis with ulceration or abscess, grade-4; gangrenous patches and grade-5; gangrene of entire foot [5].

Phagocytic cells activity is affected by blood sugar levels, and infection of lesions grades of DFU also affected by phagocytic neutrophil cells activity. This study aimed to assessment the phagocytic neutrophil cells and its role to fight the bacterial infection of diabetic foot ulcers classified according to Wagner's grades system, as well as to determine the antibiogram patterns of bacterial species isolated from DFU.

Materials and Methods
Study Design
A case-control analytical study was conducted on diabetes mellitus and diabetic foot ulcer patients.

Research Setting

Some hospitals in Mukalla city, Hadhramout, Yemen were selected to achieve the research.

Participants

A total of 60 volunteers were selected randomly and classified into 20 DFU patients, 20 DM patients and 20 healthy control group.

Preparation of Samples

About 10ml of venipuncture blood samples were collected from all participants in ethylene diamine tetraacetic acid (EDTA) for phagocytic neutrophil activity test.

A total of 20 wound swabs were collected from DFU after cleaned with sterile saline to remove accumulated drainage and transient skin flora, then the swabs delivered to the microbiology laboratory for culture [6].

Classification of Diabetic Ulcers

The ulcers of diabetic foot patients were classified according to Wagner's grades classification system as an assessment of five grades at the time of study period.

Phagocytic Neutrophil Cells Activity Test

Phagocytic cells activity test was performed according to the methods described by Shodja et al. [7] with minor modifications. In briefly, normal saline suspension of pathogenic strain Staphylococcus aureus (S. aureus) colonies was prepared and the visible turbidity was adjusted to 0.5 McFarland turbidity standard yielding an approximately 1.5×108 CFU/ml. About 1ml of EDTA whole blood mixed with 1ml of saline bacterial suspension, then incubated for one hour at 37°C. One drop of incubated mixture was placed on a slide of microscope to make a thin smear. The smear was lifted to dry for 3 minutes, and fixed with ethanol 96%, then placed in hematoxylene stain for 10 minutes. After water washing, the smear placed in eosin stain for 30 seconds, then washed by water and examined under light microscope with 100x oil immersion. The total number of S. aureus ingested within 100 neutrophil cells were counted and divided by 100 to give the percentage of phagocytic cells activity. The results were interpreted as the following: less than 40% (low phagocytosis activity), 40%-70% (intermediate phagocytosis activity) and more than 70% (high phagocytosis activity).

Isolation and Identification of Pathogenic Bacteria

Samples of ulcers swab were inoculated into blood agar and Mac-Conkey agar media (Oxoid/England). The culture plates were incubated under aerobic conditions at 37°C for 24 hours. Identification of bacterial species was made based on bacteriological standard methods [8].

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was done using Kirby-Bauer disc diffusion method on Mueller Hinton agar (Oxoid/England) for testing ampicillin, cefepime, cefadroxil, ciprofloxacin, cefixime, cefaclor, trimethoprim, amoxicillin/clavulanic acid, amikacin and vancomycin. Resistant to methicillin was determined by the disc diffusion method to identify methicillin resistance S. aureus (MRSA) isolates. All susceptibility testing methods done according to the standard guidelines of the Clinical Laboratory Standards Institute (CLSI) [9].

Statistical Analysis

Data analyzed using the software of Statistical Package for Social Sciences (SPSS) version 25 (IBM Corp., Chicago Illinois, USA). Descriptive statistics for study variables were obtained and compared using least significant difference test (LSD) and one away ANOVA test. The association between different groups of the explanatory variables was measured and compared using Pearson Chi-square (χ2) test. The relationship between the variables examined by the Pearson correlation (r) test. The level of significance was set at P-value less than 0.05.

Results

Diabetic Foot Ulcers Classification

As an assessment of DFU grades by Wagner's classification system, the results showed that grade I and II accounts 6(30%), grade III 5(25%), grade IV 1(5%) and grade V 2(10%).

Distribution of Bacterial Species Isolated from DFU

Twenty-one bacterial species were isolated from DFU according to the conventional methods for identification of bacteria as presented in Table (1). Gram-positive bacteria showed an increased prevalence 57.1% in diabetic ulcers. The most common bacterial isolates recovered from diabetic ulcers were Staphylococci species 40.8%, with high rate of S. aureus and S. epidermidis 14.0% for each, S. saprophyticus 10.0% and MRSA 5.0%. Streptococci species were the second most commonly isolated pathogens in our study 14.0%. P. mirabilis and M. morganii accounted 9.0% of predominant Gram-negative bacteria in DFU in this study. The prevalence of P. aeruginosa, Klebsiella species, Esch. Coli, C. freundii and Serratia species isolates accounted 5.0% for each.

Table 1: Bacterial Species Isolated from Diabetic Ulcers Samples

Bacterial groups	Bacterial species	No.	%
Gram-positive bacteria	Staphylococcus aureus	3	14.0
	MRSA	1	5.0
	Staphylococcus epidermidis	3	14.0
	Staphylococcus saprophyticus	2	10.0
	Streptococcus species	3	14.0
Gram-negative bacteria	Proteus mirabilis	2	9.0
	Morganella morganii	2	9.0
	Serratia species	1	5.0
	Pseudomonas aeruginosa	1	5.0
	Escherichia coli	1	5.0
	Citrobacter freundii	1	5.0
	Klebsiella species	1	5.0
Total		21	100.0

Bacterial Species of DFU Associated with Wagner's Grades System

As shown in table (2), the diabetic patients have superficial wounds of Wagner's grades classification I and II were mostly infected with Gram-positive bacteria. Staphylococci and Streptococci species were the predominant pathogens, whereas Gram-negative bacteria were more frequently isolated from deep wounds (grades III and above) with Statistically significant of P-value 0.057.

Table 2: Distribution of Bacterial Isolated according to Wagner's Grades of Ulcers

Bacterial groups	Wagner's ulcer grades					P-value
	Grade I	Grade II	Grade III	Grade IV	Grade V	
Gram-positive bacteria	9.6%	33.5%	9.6%	4.8%	-	0.057*
Gram-negative bacteria	14.4%	-	14.4%	4.8%	9.6%	
*Statistically significant at P-value 0.05						

Antibiotics Resistance Patterns of Bacterial Isolates

The results of the antibiotics sensitivity assay showed the presence of resistant patterns of the isolated bacteria to antibiotics. The highest percentage of bacteria resistance was to ampicillin 95.2, followed by cefixime 90.5, cefadroxil 52.4%, cefepime and cefaclor 47.6% as given in Table (3).

Staphylococci species were resistant for ampicillin 100% and highly sensitive to ciprofloxacin, amikacin, trimethoprim and cefaclor. MRSA showed high resistant to cefixime, ampicillin, cefadroxil and trimethoprim 100% and highly sensitive for vancomycin, amikacin, ciprofloxacin, amoxicillin/clavulanic acid and cefepime 100%. Streptococci species were resistant for cefepime, cefixime, ampicillin, cefadroxil and cefaclor 100%, and sensitive for ciprofloxacin and amikacin 100%. Gram-negative bacteria showed high resistance to ampicillin and cefixime 88.8% followed by cefadroxil, cefaclor, cefepime 77.8% and trimethoprim 55.6%. Most of the isolates were susceptible to amikacin 77.8% and ciprofloxacin 55.6%.

Table 3: Antibiotics Susceptibility Patterns of Bacterial Isolates

Antibiotic class	Antibiotic agent	Susceptible No. (%)	Intermediate No. (%)	Resistant No. (%)
Cephalosporins	Cefepime	10 (47.6)	1 (4.8)	10 (47.6)
	Cefadroxil	9 (42.9)	1 (4.8)	11 (52.4)
	Cefixime	2 (9.5)	0 (0.0)	19 (90.5)
	Cefaclor	11 (52.4)	0 (0.0)	10 (47.6)
Penicillins	Amoxicillin/clavulanic acid	12 (61.9)	1 (4.8)	8 (33.3)
	Ampicillin	0 (0.0)	1 (4.8)	20 (95.2)
Fluoroquinolones	Ciprofloxacin	17 (81.0)	0 (0.0)	4 (19.0)
Sulfonamides	Trimethoprim	11 (52.4)	4 (19.0)	6 (28.6)
Aminoglycosides	Amikacin	19 (90.4)	1 (4.8)	1 (4.8)
Glycopeptides	Vancomycin	10 (83.3)	0 (0.0)	2 (16.7)

Antibiotics Resistance Patterns Associated with Wagner's Ulcers Grades

Our study revealed that a low significant positive correlation between the antibiotics resistant and increasing of Wagner's grades classification. When the resistance of bacterial isolates increased the grades of Wagner's classification increased too according to Pearson correlation test (P-value = 0.05; r = 0.369).

Phagocytic Neutrophil Cells Activity Test

According to phagocytic cells activity test, DM and DFU patients had no highly efficient phagocytic neutrophil cells activity, 1 and 0 respectively as given in table (4).

Table 4: Phagocytic Neutrophil Cells Activity among Studied Groups

Group study	Phagocytic neutrophil cells activity			
	Low	Medium	High	Total
Control	0	10	10	20
DM	2	17	1	20
DFU	6	14	0	20
Total	8	41	11	60

As shown in table (5), the mean of phagocytosis was completely different between the three groups studied (control, DM and DFU patients) using LSD test with high significant difference statistics.

Table 5: The Mean of Phagocytic Neutrophil Cells Activity among Studied Groups

Group study	Mean %		
	Control (71.35%)	DM (52.70%)	DFU (43.05%)
Control	-	0.000*	0.000*
DM	0.000*	-	0.001*
DFU	0.000*	0.001*	-
*Statistically significant at P-value < 0.05			

In this study we correlated the degree of wounds with phagocytic efficiency to understand the development of the ulcer grade and role of neutrophil phagocytic cells. Statistically, there was low insignificant correlation between these two variables. Also, the study results showed a weak inverse correlation between the ratio of phagocytic cells activity and the degree of wound in Wagner's classification (P-value 0.164; r = -0.323), as given in table (6).

Table 6: Correlation between Phagocytic Neutrophil Cells Activity and Wagner's Grades of Ulcer

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

Discussion

In the present study, the results of the assessment DFU grades by Wagner's classification system showed high prevalence of grades I and II followed by grade III. These results were compatible with other studies conducted in different countries [10-12], but disagree with a previous study reported that Wagner's grade II and III ulcers were the most common prevalence [13]. Grade IV was the lowest prevalence in this study, and this contrast with other studies revealed that the grade IV was the most common prevalence in DFU [14-15]. Identifying the risk factors of DFU might help to develop better prevention strategies in diabetic patients.

In this study, Gram-positive bacteria showed an increased prevalence 57.1% in diabetic ulcers with similar results of different studies 77% [16], 35% [17] and 51.1% [5]. The most common bacterial isolates from diabetic ulcers in this study were Staphylococci species 40.8% compatible to other findings showed that Staphylococci species accounted 43.37% [18], whereas Gram-negative bacteria were the most frequently pathogenic agents reported in other studies 51% [19], 56.1% [20], 61% [21], 67% [22] and 51.2% [23].

Interestingly, our study revealed high rate of S. aureus 14.3%, S. epidermidis 14.3%, S. saprophyticus 9.5% and MRSA 4.8%. These results were compatible with the findings of other studies showed that S. aureus 19.4%, S. epidermidis 18.4% and other Staphylococci species 3.6% [24]. Other study showed the most commonly isolated S. aureus 30%, followed by S. saprophyticus 19% and S. epidermidis 10% [25]. Another study showed that S. aureus 25%, S. saprophyticus 15% and S. epidermidis 7% were the most isolated from DFU [11].

Streptococci species were the second most commonly isolated pathogens in our study 14.3%, this is nearly with other studies isolated Streptococci species 17% [26], 16% [27] and 15.6% [28]. Poor control of blood glucose was associated with a high relative abundant colonization by Staphylococci species and Streptococci species [29]. So, the most cases of DFU who have mixed infection of Staphylococci species and Streptococci species were poor glycemic control patients in our study.

P. mirabilis and M. morganii accounted the predominant Gram-negative bacterial isolated from DFU in this study. High prevalence of Proteus species was reported in different studies [19,27,30], while M. morganii showed low prevalence isolated 3.16% [31]. P. aeruginosa accounted 4.8% with resistance to the most antibiotics used in this study. Similar results found that Pseudomonas species were the most common isolated from DFU [28-30,32]. P. aurginosa may cause an invasive form, sometimes in diabetics which fails to respond to topical antibiotic therapy [33]. Additionally, significant delay in wound healing cause of P. aeruginosa biofilms inhibit neutrophil movement [34]. So, the coordinated control of the production of virulence and antibiotic resistance factors and the ability to adapt to various environmental changes is a likely and important reason that P. aeruginosa is a successful and common pathogen [35].

In this study, the prevalence of Klebsiella species and Esch. coli agreed with other study results 4% [11], and disagreed with other study recorded a high rate of Klebsiella species 12.6% and Esch. coli 17.9% isolated from DFU [31]. Also, the prevalence of C. freundii and Serratia species isolates is comparable with other results 5% [17], 3.85% [5], 4.3% [36] for C. freundii and 2.3% for Serratia species [37]. Different geographical area could have contributed to different types of bacterial isolated [38]. The results of numerous studies conducted on the bacterial profiles of DFU showed a varied and often contradictory findings [39]. The reason for this difference in findings could be attributed to the difference in the causative agents, geographic variations or the severity of the infections [27].

In the current study, the diabetic patients have superficial wounds of Wagner's grades classification I and II were mostly infected with Gram-positive bacteria. Staphylococci and Streptococci species were the predominant pathogens, whereas Gram-negative bacteria were more frequently isolated from deep wounds (grades III and above). These findings are in agreement with various studies concluded that an increasing of Wagner's grades, the proportion of bacterial infections were increased [2, 21, 30, 40]. However, the percentage of S. aureus decreased with increased Wagner's grades similar findings reported in other study [19]. MRSA is an increasing problem in industrialized and developing countries. It is commonly believed to be an important cause of poor outcome, increased duration of hospital stays, increased cost and mortality [14]. In this study, MRSA were associated with Wagner's grade III ulcer classification, and other study showed that Wagner's ulcers grade III was commonly infected with MRSA [14].

This study showed the presence of antibiotics resistant patterns of bacterial isolated. The highest percentage of bacteria resistance was to ampicillin, cefixime, cefadroxil, cefepime and cefaclor, and these finding were similar to another previous studies reported high resistant patterns in a rate of 92.4% [37], 94.9% [41-42] and 50% [38]. The increased resistance may be attributed to the fact that ampicillin has been widely abused and frequently implicated in self-medication[40]. Cefepime was active against 47.6% of isolates, and this result agreed with other reports 41% [36], 61.9% [41], 42.4% [27] and 46.7% [42].

Our results revealed that amikacin was the most effective antibiotic against 90.5% of the bacterial isolates. This result was similar to other results showed 75.4% [43], 96.7% [37], 82.9% [44] and 90%

[21], while its less activity showed in other studies 54.7% [43], 54.5% [45], 62.7% [46]. Also, ciprofloxacin showed greater susceptibility to the isolates 81%, and this result was approved with several studies reported sensitivity of ciprofloxacin was 63.7% [47], 61.8% [42], 78.8% [28] and 77.3% [48], while other studies showed high resistant bacteria to ciprofloxacin 51.2% [49] and 71.8% [45].

In this study, vancomycin showed 83.3% sensitivity of Gram-positive bacterial isolates. This similar to other studies that showed 90.1% [47], 95% [31] and 98.2% [19], while other study revealed low sensitivity of vancomycin 44.5% [37]. However, other studies showed 100% of Gram-positive bacteria susceptible to vancomycin [5, 24, 29, 30, 44, 50].

In our study, amoxicillin/clavulanic acid was active against bacterial isolates 61.9%, and this result was in accordance with the studies showed amoxicillin/clavulanic acid sensitivity were 67.4% [22], 49% [47] and 57.2% [49]. In contrast, other studies showed high resistance amoxicillin/clavulanic acid 79% [37] and 81.8% [45]. Cefexime showed low sensitivity 9.5% for the isolated bacteria in this study. Similarly, low rate reported in other study 3.2% [51]. While the sensitivity for Gram-negative bacteria to cefexime was 70.7% [5], and for Gram-positive bacteria was 77.3% [28]. The sensitivity of cefaclor was 47.6% of bacterial isolates in this study, and this was compatible with the results of other study showed the second generation of cephalosporin was 50% [37]. Other studies showed that cefaclor was active 58.4% against Gram-positive bacteria [45], and 92.8% against Gram-negative bacteria [38].

Trimethoprim had sensitivity 52.4% of bacterial isolates in this study. It's similar with the results of other studies 50% [37] and 70.3% [38], while the sensitivity to co-trimoxazole was low in other studies 16% [31] and 14.3% [48]. Cefadroxil was active 52.4% against bacterial isolates. Other study indicated that first generation of cephalosporin was sensitive 41.7% [36] and 16.1% [27]. Other studies showed the sensitivity of cefadroxil to Gram-negative bacteria was 44.4% [42] and 11% [19].

Our study revealed that a low significant positive correlation between the antibiotics resistant and increasing of Wagner's grades classification. When the resistance of bacterial isolates increased the grades of Wagner's classification increased too according to Pearson correlation test (P-value = 0.05; r = 0.369). Such relation has previously reported that an increasing of Wagner's grades, the resistance rates to some antibiotics increased [52].

This study indicated a significant reduction in phagocytic activity in DM and DFU patients compared with control group. In addition, our baseline results confirm and extend previous reports concerning the impairment of phagocytosis that occurs in neutrophil cells from diabetic patients than non-diabetic patients [53]. Other studies showed a significant reduction in phagocytic cells activity in Type-1 DM and Type-2 DM [54-55]. This difference may be due to that hyperglycemia impairs granulocyte functions including adherence, chemotaxis, phagocytosis and bactericidal activity [56]. So, hyperglycemia reduced response of neutrophil function and disorders of humoral immunity as one of the long-term effects of elevated mean blood glucose (MBG) or HbA1c, also the impaired micro-vascular circulation in patients with diabetic foot limits the access of phagocytes favoring development of infection [3, 57, 23].

One of the strengths our study results find the relationship between the manner of immune response involving phagocytic cells activity and the degree of wound. In this study, we correlated the degree of wounds with phagocytic efficiency to understand the development of the ulcer grade and role of neutrophil phagocytic cells. Statistically, there was low insignificant correlation between these two variables. Our study results showed a weak inverse correlation between the ratio of phagocytic cells activity and the degree of wound in Wagner's classification (P-value 0.164; r = -0.323). However, in our knowledge, there have been no reports in the literature regarding the correlation between phagocytic cells activity and the degree of wound in diabetic foot patients to support our results to know the reason of this relation we need further deep studies to be clarified.

Conclusions
This study confirmed when the grade of ulcer increased, the bacterial resistance to antibiotics increased, and this was emphasis the correlation with prevalent of Gram-negative bacteria in the high grade of ulcers with high resistance of antibiotics. In contrast, the grade of ulcer increased, the efficiency of neutrophil phagocytic cells decreased. This study discover the correlation between diabetic foot ulcers and bacterial infection that can be beneficial for the surgeons to study antibiotics resistance in diabetic foot ulcer patients. The study will help the researchers to uncover the relationship between the manner of immune response involving the role of neutrophil phagocytic cells activity and the development degree of foot ulcer. Further immunological and bacteriological investigations needed to control the increasing of antibiotics resistance in diabetic foot ulcers patients.

Abbreviations
DM: Diabetes mellitus; DFU: Diabetic foot ulcers; PMNLs: Polymorphonuclear leukocyte cells; EDTA: Ethylene diamine tetraacetic acid; CLSI: Clinical Laboratory Standards Institute; SPSS: Statistical Package for Social Sciences; LSD: Least significant difference test; MBG: Mean blood glucose; MRSA: Methicillin resistance Staphylococcus aureus.

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Author's Contribution

Maryam Baras: conceptualization the idea, methodology, data analysis and writing the first draft preparation. Eidha Bin-Hameed: reviewing the manuscript formats, paper structure, final reviewing and editing.

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Availability of Data and Materials

All the data are available from corresponding author if required.

Ethics Approval and Consent to Participate

The study was approved by the board of Faculty of Science, Hadhramout University, Yemen. Ethical approval was also obtained from the hospitals in Mukalla City, Hadhramout.

Consent for Publication

Not applicable.

Conflict of Interest

It is declared that they have not conflict of interest.

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