Assessment the level of CD4 among patients with diabetic foot ulcers

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Abstract

In the population, the incidence of *S. aureus* isolates among diabetic foot ulcers (DFUs) has become a major issue. As a result, the goal of this study to investigation and distribution for immunological characterization among *S. aureus* from diabetic foot ulcer infection. There were 42 (70 %)) men and 18 (30 %)) females among the 60 patients (non-duplicate). The *S. aureus* isolates were identified using biochemical tests, and the Vitek-2 compact system verified that 37/60 (61.6 %) of the isolates were *S. aureus*. The immunological study included investigation the level of CD4 in patients human and experimental animals(rabbits) serum. The investigation of CD4 level explained a significantly lower in DM- foot ulcer compared with both the healthy control group and DM non - foot ulcer (P<0.05). The age group > 61 years within the DM-foot ulcer group patients showed significantly decreased in CD4 level compared to both DM-non foot ulcer and controls healthy with a significant difference.P <0.05. As for there is no effects of sex and comorbidity, type DM, and treatment on CD4 level in all study groups. The greatest percentage of infected was Grade2 (43.3%) then Grade3 (23.3%) with significant difference,P <0.05. Also, the study showed that the level of CD4 level in rabbits groups (after inducing the infection by *S. aureus* that isolate from diabetic foot ulcer patients) significantly decreased compared with both the healthy control rabbits and rabbits groups (before the infection) P <0.05 while we noticed that there was no significant difference between the rabbits groups healthy control and before infected (P>0.05).

Keywords: DFU, Bacteria, CD4, DM-foot ulcer, Rabbits, DM.

1. Introduction

Diabetic foot Ulcer (DFU) is defined, according to the World health Organization, as ulceration of the foot (distally from the ankle and including the ankle) associated with neuropathy and different grades of peripheral vascular disease and infection. It represents a serious long-term complication of diabetes mellitus leading to amputations, disability, and reduced quality of life [1].

The most common pathogen identified in diabetic foot infections (DFI) is *Staphylococcus aureus* (a Gram-positive extracellular Bacterium*). S. aureus* produces a range of virulence factors and toxins during an infection, which have been extensively investigated [2]. There are some components of host immunity, which create the first-line defense activated regardless of the type of pathogen. They include humoral and cellular innate immunity [3]. Because pro- and anti-inflammatory processes are important in the different phases of wound healing, it is conceivable that disturbances of the immune system interfere with tissue homeostasis and wound healing after the manifestation of ulcers and lead to the chronic, non healing wounds that are characteristic of diabetic foot syndrome [4].

In humans, CD4 marker (Cluster of Differentiation 19) is a co-receptor of the T cell receptor (TCR).it is a member of the cluster of differentiation family of proteins, mainly expressed on the surface of thymocytes and a specific subset of mature T-cells and assists the latter in communicating with antigen-presenting cells. The TCR complex and CD4 bind to distinct regions of the antigen-presenting MHC class II molecule. Foot ulcers appear to be preceded by the increase of T cells and a loss in TCR repertoire diversity [5, 6].

2. Material and Methods

Sample collection

The current study was included 60 non-duplicate patients age (30-80 years)suffering diabetic foot ulcer whose admitted to Marjan Teaching Hospital, Al-Sadiq Al-Turkish Hospital in Babylon Province as well as some chief clinical laboratories in Al-Najaf City-Iraq, during several months during 2021. For each diabetic patients with foot ulcer (DF), two samples were taken which included the blood samples for immunological study and swabbing wound for bacteriological study and used the laboratory animal (male New Zealand white rabbits) with (age, 2 years old; male, weight, 2.5-3.0 kg), the experimental animals under constant temperature (22±3)ºC and humidity (45%).A sterile swab from foot ulcer sites of patients then sent quickly to the Advance Microbiology Laboratory, Department of Biology, Faculty of Science, University of Kufa, Iraq.

The immunological study

Five ml of blood was collected from (84) samples of study groups: (32) diabetic with foot ulcer and (26) samples for each control groups:(diabetic without foot ulcer and healthy persons). Also from (30) samples of laboratory animals (male white New Zealand) included:(10) samples for each: infected rabbits with

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foot ulcers after stimulating infection and the control groups (the healthy group and the group before infection stimulus) and transferred into a gel tube for serum separation and kept frozen at -20 C for the immunological investigation to determine level both cluster of Differentiation 4 (CD4), in human and laboratory animal by the sandwich ELISA Kit according to (Bioassay Technology Laboratory, China).

Bacterial isolation and identification

All specimens have been cultured and lawn on blood agar and Mannitol Salt Agar, unto gained a single colony. Isolates of *S. aureus* had been identified according to the biochemical tests, coagulase, motility and oxidase assessments [7]. Vitek-2 system (bioMèrieux France)used to affirm the identification the usage of ID-GP cards. The rabbits injecting subcutaneously (S/C) with infection dose: (1×10^8 C.F.U./ml) of bacterial isolate *S. aureus* previously diagnosed in patients with a diabetic foot ulcer. After 3 days (72h.) (period of observation), it was noticed that the infection.

Statistical analysis

Normally distributed expressed as mean±SD (standard deviation) and using the chi-square test, independent t-test, Paired t-test (before and after treating samples) for comparison between two groups; therefore ANOVA for among two groups. Considered statistically significant at p-value <0.05 The statistical analysis was performed using SPSS v.25 (SPSS Inc., Chicago, IL, USA), and graphic with software Microsoft Excel 2019.

3. Results

The results of bacterial growth revealed that 44 (73.3%) of the gram positive bacteria isolates represented by 37(61.1%) of the isolates were identified as *Staphylococcus aureus* which give catalase and coagulase positive while others species of *staphylococcus* were 7(11.6%) isolates which represented by 5 (8.3%) *S. epidermides* and 2 (3.3%) of isolates return to *Streptococcus* spp., which give catalase positive and negative for coagulase test, while the bacterial growth of gram negative bacteria which revealed 16 (26.6. %) isolates were identified 7 (11.6%) of *Escherichia coli*, 5 (8.3%) isolates of *Pseudomonas aeruginosa,* and 3 (5%) and 1 (1.6%) isolates of *Klebsiella* spp., and *Protus* spp. Respectively.Table (1).

Table (1): Distribution of bacterial isolates with diabetic foot ulcer according the sex.

Total	Sex		Type of bacterial isolates
	female	Male	
			Gram positive bacteria
37(61.1%)	17	20	*Staphylococcus aureus*
5 (8.3%)	2	3	*S. epidermides*
2 (3.3%)	1	1	*Streptococcus spp.*
44(73.3%)			**Total**
			Gram negative bacteria
7 (11.6%)	2	5	*E.coli*
5 (8.3%)	2	3	*Pseudomonas aeruginosa*
3 (5%)	1	2	*Klebsiella pneumoniae*
1 (1.6%)	-	1	*Protus spp.*
16 (26.6. %)			**Total**

The Table(2), figure(1) appears significantly decrease in CD4 level in DM- foot ulcer (2.18±0.44 ng/ml) compare with both healthy group(3.16±0.72). (P<0.05). p=0.0001,and DM non - foot ulcer (2.42±0.37 ng/ml) (P<0.05). (P=0.03).

Table (2):Compression level (CD4) in all study groups (DM- foot ulcer, DM - non foot ulcer, healthy Control.

Study groups	Healthy control N=26	DM -Non foot ulcer N=26	DM -foot ulcer N=32	p-value of patients
CD4	3.16±0.72 (ng/ml) **A**	2.42±0.37 (ng/ml) **B**	2.18±0.44 (ng/ml) **C**	*0.03
***The different letters significant differences at p-value <0.05.**				

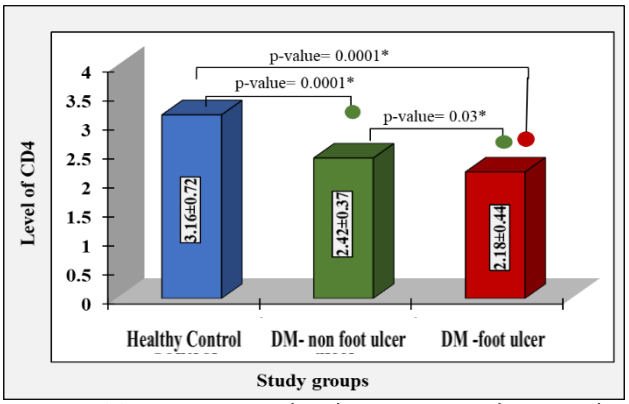
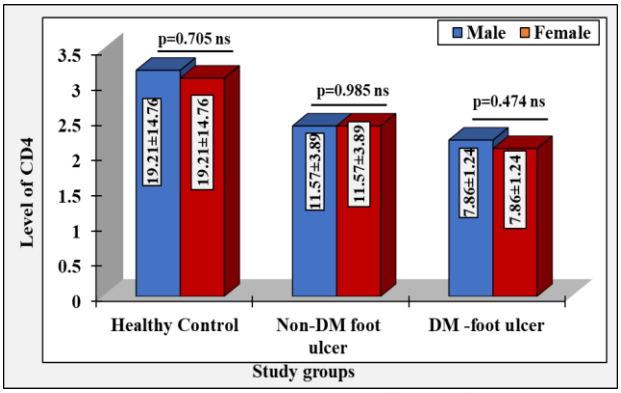


Figure (1): compression level CD4 among three study groups.



The Figure (2) shows there is no effects of sex on CD4 level in each of the study groups with regard to sex, the mean *value of CD4 for DM -foot ulcer (male, female) was (7.86±1.24,7.86± 1.24 ng/ml), DM-non foot ulcer (11.57±3.89, 11.57±.89 ng/ml) healthy controls (19.21±14.76,19.21±14.76 ng/ml) respectively.*

The figure (2): Effects of sex on CD4 level in DM -foot ulcer, DM -non foot ulcer and healthy controls.

No relevant correlation has been observed, according to Table (3) within group: comorbidity (Ve+Ve-,), type DM (DMT1, DMT2,), and treatment (Insulin,Oral) on CD4 level in all study groups. (P. Value >0.05).

Table (3): effect of Comorbidity, Type DM, and Treatment on CD4 level in DM - non foot ulcer and DM -foot ulcer.

Variables		Mean ±SD of CD4 level (ng/ml)	
		DM -non foot ulcer	DM -foot ulcer
Comorbidity	Ve+	2.36±0.32	2.27±0.42
	Ve-	2.44±0.40	2.04±0.45
p-value		0.855	0.587

Type DM	DMT1	2.49±0.25	2.20±0.37
	DMT2	2.38±0.44	2.17±0.47
p-value		0.482	0.885
Treatment	Insulin	2.24±0.41	2.09±0.48
	Oral	2.52±0.32	2.35±0.29
p-value		0.365	0.369

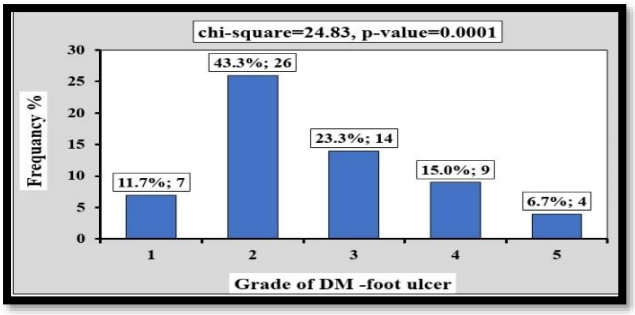


Figure (3): Grade of DM - foot ulcer patients

In the Figure (3), the highest percentage of infected patients appeared with Grade2 (43.3%), after that Grade3 (23.3%), Grade4 (15.0%), Grade1 (11.7%) and Grade5 (6.7%) with significant difference,P-value <0.05. P-value=0.0001.

The age group > 61 years within DM -foot ulcer group was more effective compared to the other study groups at the level CD4 as shown in Table (5)with significantly decrease P-value <0.05.

Table(5):Effects of CD4 level among age groups in studied groups:

Study groups Age groups	Healthy Control		Non-DM foot ulcer		DM -foot ulcer	
	n	mean±SD(ng /ml)	n	mean±SD(ng /ml)	n	mean±SD(ng /ml)
<45 year	7	3.08±0.53	6	2.46±0.23	3	2.32±0.58 A
46-60 year	10	3.34±0.57	8	2.43±0.52	14	2.10±0.42 B
> 61 years	9	3.04±0.99	12	2.40±0.35	15	1.78±0.46 C
p-value	0.641		0.930		0.010*	

*** Significant difference,P-value <0.05.The different letters significant differences at p-value <0.05.**

The comparison the level CD4 among the three study groups in the rabbit (healthy control, before infected,after stimulating infection) was significantly decreased (362.12±23.31 ng/ml) compared with both the healthy control groups and rabbits groups (before the infection) (383.23±21.87 ng/ml, 441.85±79.80 ng/ml with P <0.05, p=0.045, p=0.022 respectively), while we noticed that there was no significant difference between the rabbits groups healthy control and before infected (P>0.05) as shown in figure(4).

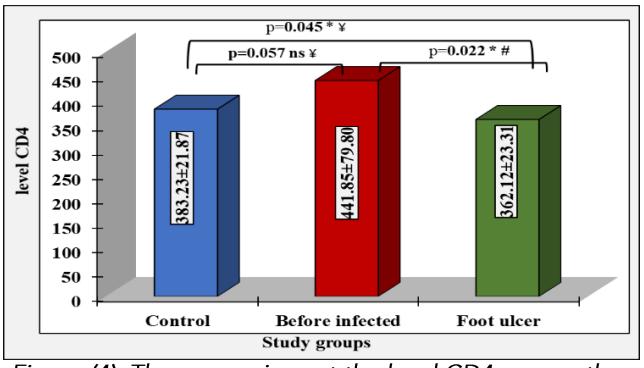


*Figure (4): The comparison at the level CD4 among the three study groups in the rabbit, (*Significant differences at p value <0.05. ns: non-significant. #: Paired samples T-test. ¥: independent samples T-test).*

4. Discussion

A total of 60 specimens was collected from diabetic mellitus patients with foot ulcer 42(70%) males and 18(30%) females. The ages of the patients were ranged from (30 – 80) years for both sexes. There were several previous studies mention that diabetic foot ulcer has polymicrobial infection like *S. aureus*, *Streptococcus* and *Pseudomonas*, while *S. aureus* was a most frequent among them [8, 9]. In this study, *Staphylococcus aureus* is the most isolated organisms commonly recorded in 37 (61.1%). These results are consistent with previous results of Bansal et al. [10].The isolation results in current study were similar to those gathered by many studies [11, 12]. In the present study with regard the level of CD4 was significantly lower in patients with diabetic with foot ulcers than in both diabetic non foot ulcer and healthy control groups (p<0.05).This was agreed with Moura et al. [5] who similarly observed a significant decrease in the percentage of naïve CD4+ T cells between controls and all diabetic patient groups.

The result of the current study showed that significantly reduced in CD4 level is due to the concomitant and continuous accumulation of both CD4+ effector T cells may be responsible for the abnormally high IFN-γ and TNF-α levels observed in diabetic patients, and may lead to a reduction of the inflammatory CHR expression, which could possibly affect T-cell migration into inflamed tissues. These agreements with several studies of Moura et al. [5], Dahiru et al. [13].

The present results observe is no effects of sex in the level CD4 in each (DM- foot ulcer and DM - non foot ulcer, healthy controls), that was agreement with other studies by Duffy et al. [14], that showed there is no effect of sex on levels, However, other variables, such as stress, aging, and potentially diabetes mellitus alone, can diminish serum lymphocyte counts by impairing the immune system.

The present results showed that there is no effect (comorbidity, type DM, and treatment had been found within study groups on level CD4. (P. Value >0.05), but another study by Chawla et al. [15] demonstrated that chronic hyperglycemia was significantly associated with defects in complement receptors and Fcγ receptors on isolated leukocytes, resulting in impairment phagocytosis and CD4+T, CD8+T cells,β-cell was significantly reduced.

Further more, Ghobadi et al. [16] showed cardiovascular complications more in DFU patients. Also results of current study showed the age group > 61 years within the diabetic foot ulcer patients group was the CD4 level significantly decreased in the DM-foot ulcer group compared to both DM-non foot ulcer and controls healthy P-value <0.05, the decrease in the CD4 level with age, consistent with the study of Frasca et al. [17] that although CD4 T cell defects occur, defects in B cells play a significant role in age-related humoral immune changes. The reduction of level CD4 in older patients with diabetic foot ulcer agreement with studies Walter et al. [18] found absolute numbers of human B cell precursors in the bone marrow have been shown to decline moderately with age. Current results showed Patients with DFU were the highest percentage of infected patients appeared with Grade 2 (43.3%), followed by Grade 3 (23.3%) and finally Grade 5 (6.7%) with Significant difference,P-value <0.05, these results were consistent with the results of Zubair et al. [19] show the Grade 2 most prevalence was 35%, then Grade 3 (22.7%). The current result also confirmed the result of the studies done in Nigeria by Ogbera et al. [20] reported that grade III and II Wagner lesions were the most frequently noted grades of foot ulceration in Unachukwu et al. [21] reported grade III as the most common in Port Harcourt.

This study was conducted to confirm the role and relationship of bacteria *S. aureus* and the immunological aspect with DM- foot ulcer. In rabbits, the layer of skin that covers the feet is very thin in rabbits, resulting in susceptibility of the hind feet to pododermatitis (hock sores or sore hocks) or injury wounds [22]. The result of the current study was shown that the level of CD4 level in rabbits groups (after inducing the infection by *S. aureus* that isolate from diabetic foot ulcer patients) showed significantly decreased with significant difference compared with both the healthy control rabbits and rabbits groups (before the infection), P <0.05 while we observe that there was no significant difference between, the rabbits groups healthy control and before infected. CD4 levels in rabbits decreased after infection was induced because CD4 (+) T cells expanded robustly when exposed to bacteria during acute infection, but this response was totally eliminated during chronic infection. Not only was the anti-*S. aureus* T cell response harmed, but so was CD4(+) T cell reactivity in general [23]. The current results were consistent with the results of Viana et al. [24], that the rabbits infected by strain *S. aureus* presented the lowest counts of total lymphocytes, CD4 and CD19, CD5and CD8 lymphocytes during chronic infection,the decline in CD4- T cell due to depletion of CD4+ T-cell after accumulation it in chronic infection.Finally, the results for the animal side rabbit and human were identical.

5. Acknowledgements

Conflicts of interestThere are no conflicts of interestThe

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