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Study on the leukocytes infiltration and association in diabetic foot ulceration patients

Raed Madhi,[1] Marwah Majeed,[1] Nidhal Abdullah Hashim[2]

[1]Department of Biology, Collage of Science, University of Misan, Maysan; [2]Southern Technical University, Amara Technical institute, Amara, Maysan, Iraq

Correspondence: Raed Madhi, Department of Biology, Collage of Science, University of Misan, Maysan, Iraq. Tel.: +964.7742017297; E-mail: raedsaddam@uomisan.edu.iq

Key words: diabetic foot ulceration, leukocytes, biochemical parameters.

Abstract

Diabetic foot ulcers are among the most common problems facing the people with diabetes. Leukocytes have been shown to penetrate the region of inflammation in order to regulate the infection. Thus, the current research was designed to study the infiltration of leukocytes in diabetic foot patients. Samples were taken from 50 healthy individuals and 100 diabetic foot ulcer patients. Levels of Fasting Blood Sugar (FBS), Hemoglobin A1C (HbA1c), cholesterol, triglycerides, urea, and creatinine were measured. Leukocytes infiltration was also evaluated in the diabetic foot patients. Levels of FBS and HbA1c were significantly increased ($p \leq 0.05$) in patients with diabetic foot ulceration. In addition, the levels of cholesterol, triglycerides and urea significantly increased ($p \leq 0.05$) in diabetic foot ulceration patients. However, the levels of creatinine greatly decreased in patients with diabetic foot ulceration. Infiltrated leukocytes were significantly increased in the ulcerated foot. Furthermore, the study found a strong association between leukocyte infiltration and HbA1c as follows: ($y = 0.6864x + 0.9455$: $R^2 = 0.776$). Thus, studying the mechanism of

controlling leukocytes in diabetic foot ulcer could greatly participate in healing of diabetic foot ulcers.

Introduction

Most non-traumatic amputations are caused by diabetic foot infections, which can be avoided. Due to aging, cultural, and demographic changes, diabetes has grown to be a serious threat. It is the leading cause of heart attacks, amputations, blindness, and kidney problems, and it has a significant financial cost. About six million amputations, five million diabetic retinopathies, and over 20 million neuropathies were recorded by the World Health Organization (WHO).[1] Among the most serious effects of diabetes are foot ulcers and gangrene, which have mortality rates nearly equal to those of cancer.[2] Furthermore, people with diabetes also have a delay in the healing of wounds. This results in diabetic foot problems that manifest in different ways.

Diabetic foot has been linked with Type 2 Diabetes (T2DM). A relative or total lack of insulin is the cause of T2DM, a metabolic disease marked by elevated blood glucose levels.[3] High blood sugar has also been linked to some illnesses, including dyslipidemia.[4] Serum levels of Low-Density Lipoprotein Cholesterol (LDL-C), Total Cholesterol (TC), Total Triglycerides (TG), and High-Density Lipoprotein Cholesterol (HDL-C) are regarded as lipid markers that may be used to predict the coronary heart disease risk.[5] A prior data investigated a positive relationship between high TG and high TC and high blood glucose levels.[6] Glycemic control level has an impact on blood lipid levels in T2DM patients. To lower mortality and complications in T2DM patients, lipid profile control is crucial.[7] Moreover, serum creatinine and urea have also been to be correlated to diabetes mellitus. For instance, it was found that low serum creatinine levels may be linked to the onset of T2DM.[8] Additionally, Blood Urea Nitrogen (BUN), which has been used for many years as a conventional indicator of renal function, has gained attention recently for its novel function in the circulatory and urinary systems, among other systems, suggesting the therapeutic significance of urea.[9] Adipokines linked to insulin resistance were raised by urea infusion in normal mice,

whereas uremic mice showed glucose intolerance, according to experimental data.[10] Increased levels of urea in the bloodstream may have a direct negative impact on the function of pancreatic β-cells by increasing islet protein O-linked-N-acetylglucosaminylation (OGlcNAcylation) and reducing glycolysis.[11] This could result in disrupted glucose homeostasis and poor glucose secretion, as well as increased risk of T2DM and worsening renal function.

Recruited leukocytes have been shown to be associated with different diseases.[12,13] Neutrophils and macrophages were demonstrated to have a substantial role in the diabetic foot ulceration. A previous study was carried out in an attempt to shed more light on the pathophysiology of diabetic wounds, emphasizing the role of inflammatory cells populations in the wound and how they are altered by diabetes.[14] The recruitment and infiltration of neutrophils into Diabetic Ulceration (DU) wounds may impede the healing process. Neutrophils Extracellular Traps (NETs) can be eliminated, releasing cytotoxic enzymes, and phagocytose pathogens as neutrophils. NETs have been shown in multiple recent studies to be essential for delayed wound healing.[15,16] Increased blood glucose stimulates neutrophils, which are significantly expressed in DU patients' wound sites, to produce NETs. I order to sustain the inflammatory response, NETs stimulate the Nucleotide-binding and Oligomerization Domain(NOD), Leucine-Rich Repeats (LRR) - and pyrin domain-containing protein 3 (NLRP3) inflammasome, and postpone the repair of DUs, and activate macrophages.[17] Thus, the current study investigated the infiltration of leukocytes in the foot of diabetic patients.

Materials and Methods

Study design

To understand the role of leukocyte infiltration in diabetic foot ulceration, the present study sought the infiltration of leukocytes in diabetic foot patients. The current project was conducted in Maysan City, Iraq. Samples of blood and tissue biopsy were taken from 100 patients who were admitted to the diabetic center in Maysan during the period from October 2023 to April 2024. Moreover, 50 blood and tissue samples were obtained from individuals in good health and served as controls.

Patients with diabetic foot ulceration were divided into four grades according to the University of Texas system.[18] Briefly, Grade 0: Pre-ulcerative lesions (No skin break);

Grade 1: Superficial wound (No penetration); Grade 2: Wound penetrating tendon or capsule; Grade 3: Wound penetrating bone or joint. Number of Patients with diabetic foot ulceration were as following: 17, 19, 26, and 38 in the Grade 0, 1, 2, and 3, respectively.

Measurement of FBS and HbA1c in patients and control group

Fasting Blood Sugar (FBS) levels in patients and control were measured using the kinetik FBS monitoring system (BG-710) (Kinetik, RH1 5DZ, London, UK). Briefly, with the test strip facing up, it was inserted into the apparatus (BG-710). Samples of blood were taken using the lancing instrument. The underside of the test strip was lightly coated with blood. Moreover, the sandwich immunodetection approach, FinecareTM HbA1c Rapid Quantitative Test, was used to calculate the levels of Hemoglobin A1C (HbA1c) in the blood of patients and control group, in accordance with the company's directives (Guangzhou Wondfo Biotech, Guangzhou, P.R. China). Briefly, the buffer tube was filled with 10 µL of blood. The tube was tapped to thoroughly mix the specimen with the buffer for one minute. The Test Cartridge's well was filled with 75 µL of the mixture. To read the sample, the test cartridge was placed into the FinecareTM FIA meter for five minutes.

Blood urea and creatinine measurement

Urea levels were measured in serum of patients and control group by using a kit (BioSystems S.A., Barcelona, Spain) measuring colored indophenol spectrophotometrically by means of an Ultraviolet–visible (UV/VIS) Spectrophotometer UV-1100 (BIOBASE, Shandong, P.R. China), in accordance with the company's instructions. Briefly, 1 mL of reagent A and 100 µL of samples and standard were pipetted into the test tube. The mixtures were mixed and incubated for 5 min at 37ºC. One mL of reagent A was pipetted into the mixtures. Following a thorough shake of the mixture, the tubes were incubated at 37ºC for five minutes. The levels of urea were measured at 600 nm. In addition, creatinine levels were also measured in serum of patients and control group by using the UV-Vis Spectrophotometer UV-1100 (BIOBASE, Shandong, P.R. China). Jaffe's description of the reaction between creatinine and sodium picrate serves as the foundation for the assay. Alkaline picrate and creatinine combine to create a crimson complex. The chosen measurement window eliminates the impact of other serum constituents. The

intensity of the color is directly correlated with the amount of creatinine in the sample (ATLAS MEDICAL, Blankenfelde-Mahlow, Germany).

Measurement of cholesterol and triglycerides

In order to evaluate cholesterol, 5 μL of sample were combined with ammonium phosphate buffer (pH 7.0) containing the enzymes methyl, hydroxy polyethoxy dodecane, acetylacetone, catalase, cholesterol oxidase, and cholesterol esterase (BIOLABO SAS, Maizy, France). Moreover, triglycerides were degraded to create glycerol in a sequence of linked processes that are used to quantify triglycerides enzymatically in serum. After oxidizing glycerol with glycerol oxidase, one of the reaction products, H2O2, is determined using the same methodology as for cholesterol. The level of triglycerides in the samples was determined by measuring the absorbance of the colored complex (quinoneimine) at 500 nm using a UV-Vis Spectrophotometer UV-1100, BIOBASE, Shandong, P.R. China).

Histological examinations

Tissue sections were prepared as outlined in a previous study.[19] Briefly, tissue samples were collected from patients with diabetic foot ulceration and control group. Samples were fixed in 10% buffered formaldehyde for 72 hours. Hematoxylin and eosin were used to stain tissue sections (6 μm). The infiltrating leukocytes were counted in blinded manner in different microscopic fields which were observed under a light microscope (Olympus CX21, New York Microscope Company, Hicksville, United States).

Statistical analysis

SigmaStat 3.5 was used to analyze the current data. The statistical analysis between the groups was performed by applying T- test and one way Analysis of Variance (ANOVA). Moreover, linear Regression was also performed to study the statistical association between the diabetic marker (FBS) and leukocyte infiltration. The mean values ± standard error of the mean were used to display the current data. A p value less than 0.05 was considered as statistically significant between the groups.

Results

Characteristics diabetic foot ulcer patients

The present study involved both sex. The percentage of men was 36%, however, the percentage of women was 64%. Moreover, the mean of age of patients with diabetic foot ulceration was 56.610±1.118 (Table 1).

Diabetic foot ulcers grading

The ulceration of diabetic foot was classified into four grades. Notably, skin penetration in grade 0 was not detected (Figure 1A). Furthermore, in grade 1, the wound was superficial and did not penetrate (Figure 1B). However, in grade 2, we could observe a puncture in the foot tendon (Figure 1C). Additionally, in individuals with grade 3, we observed a great penetration (Figure 1D).

Levels of FBS in patients with diabetic foot ulcer and control groups

Next, it was important to explore the duration of diabetes in patients with diabetic foot ulcers. Our results found that the percentage of disease duration (1-5, 6-10, and 11-15 years) was 20%, 38%, and 42%, respectively (Figure 2A). Since it is well established that the level of FBS is used as indicator for diabetes mellitus prediction, levels of FBS were measured in the patients and control groups in the current study (Figure 2B). The statistical analysis found that levels of FBS were greatly increased in grade 0, 1, 2, and 3 by 3.4 fold, 3.3 fold, 4.3 fold, and 4.2 fold, respectively, in diabetic foot ulcer patients compared to control group (Figure 2A). Moreover, the levels of HbAc1 were also examined in patients with diabetic foot ulcer and control groups (Figure 2C). We found that the levels of HbAc1 were significantly increased in grade 0, 1, 2, and 3 by 48.1%, 52.2%, 60.3%, and 60.0%, respectively, in patients with diabetic foot ulcer in compared with control group (Figure 2C).

Levels of cholesterol, triglycerides, urea and creatinine in patients with diabetic foot ulcer and control groups

Lipid profiles have shown to be one of the essential players contributing to the development of diabetes mellitus. In the current study we examined the levels of

cholesterol and triglycerides in patients with diabetic foot ulcer and control group (Figure 3A and B). Notably, the present results demonstrated that cholesterol and triglyceride levels were significantly increased ($p \leq 0.05$) in grade 0, 1, 2, and 3 in diabetic foot ulcer patients compared with control group (Figure 3A and B). In addition, the levels of urea and creatinine were also measured in patients with diabetic foot ulcer and control group (Figure 3C and D). Our results found that urea greatly increased in grade 0, 1, 2, and 3 by 2.1 fold, 2.2 fold, 3.1 fold, and 2.7 fold in patients compared with control group (Figure 3C). However, the results found that creatinine levels significantly decreased in in grade 0, 1, 2, and 3 by 56%, 62%, 70%, and 74%, respectively, in diabetic foot ulcer patients in comparison with the control group (Figure 3D).

Inflammatory cells infiltration in diabetic foot ulcer patients

It was interesting to examine the inflammatory cells in the diabetic foot ulcers. Histological examinations showed that the inflammatory cells were greatly infiltrated into the inflammatory site in the diabetic foot ulcers by 92% as compared with control group (Figure 4A, B and C). Moreover, it was important to study the association between the infiltration and diabetes. The statistical analysis showed a strong association between inflammatory cells infiltration and HbA1c (hyperglycemia severity biomarker) as follows: ($y = 0.6864x + 0.9455$: $R^2 = 0.776$). (Figure 4D).

Discussion

A limited understanding of the disease-promoting mechanism contributes to the difficulty clinicians face in managing patients with diabetic foot ulceration, who are mostly treated with supportive medications. Our study showed that high FBS and HbA1c levels were accompanied by high levels of cholesterol, triglycerides, and urea in patients with diabetic foot ulceration. Additionally, the present findings also found that diabetic foot ulcer was affected by levels of creatinine and urea. Moreover, the current data showed that the infiltration of inflammatory cells is associated with HbA1c levels.

Diabetic foot ulcers are a widespread and debilitating consequence of uncontrolled diabetes. Although the relationship between fasting blood glucose levels and the development of diabetic foot ulcers is rarely considered, knowing its predictors can help clinicians make better treatment decisions.[20] In the present study, FBS levels were significantly increased in foot ulcers patients by 69%, 70%, 77%, and 75%, respectively, in grade 0, 1, 2, and 3. Our results were in line with a recent study which found that long-term variations in fasting blood sugar were associated with an increased incidence of diabetic foot ulcers.[21] Thus, to lower diabetic foot ulcers, diabetic patients, diabetic neuropathy sufferers, and peripheral artery disease sufferers should all have their fasting blood sugar closely monitored. Moreover, it was important to examine the levels of HbA1c in diabetic foot ulcers patients. We demonstrated that HbA1c levels substantially increased in diabetic foot ulcers patients. The current findings are consistent with a recent investigation showing that the severity of diabetic foot ulcer is substantially associated with high levels of glycated HbA1c. Patients with diabetes who had an elevated HBA1c (>6.5%) demonstrated a strong link with factors such as smoking, retinopathy, ulcer length, and hypertension, as well as a higher chance of getting diabetic foot ulcers.[22] HbA1c was the only factor that statistically affected the wound healing rate of ulcers, with a 0.028 decrease in healing rate for every 1% increase in HbA1c ($p=0.027$).[23]

We also measured the levels of triglycerides and cholesterol in the current investigations. The statistical analysis found a great increase in the cholesterol and triglyceride levels in diabetic foot ulcers patients. The hallmark of diabetic dyslipidemia is high triglyceride levels and cholesterol levels.[24] Recent research has demonstrated that elevated triglyceride levels are a marker for T2DM rather than a causative factor.[24] Based on experimental findings, cholesterol levels may have a direct impact on plasma glucose levels, which could lead to a pathophysiological involvement in T2DM.[25] Parhofer, found that there may be a connection between lipid level variations and the onset of T2DM.[25] Both high and low cholesterol levels were found to be inhibitory of insulin secretion in a study conducted in an insulinoma tumor cell line which in turn can affect reproductive hormones.[5,26] Lipid fluctuations may hasten the development and susceptibility of atherosclerotic plaques by exacerbating inflammatory responses and arterial endothelial dysfunction and autoimmune thyroid

disease.[27,28] Therefore, adult patients should have their triglyceride and total cholesterol levels checked annually.

The risk of diabetes mellitus is thought to be correlated with blood urea. Thus, it was crucial to investigate the levels of blood urea in patients with diabetic foot ulcer in the current study. The current findings showed that in comparison with the control group, the diabetic foot ulcer patients' blood urea levels significantly increased. Our results are consistent with a recent study that discovered a high correlation between blood urea levels and the likelihood of developing diabetes mellitus.[29] Blood urea-induced Reactive Oxygen Species (ROS) production has believed to exaggerate inflammatory response.[30] Thus, the increased levels of blood urea could explain in part the effect of ulceration in diabetic foot.

Furthermore, numerous studies have linked lower serum creatinine levels to an increased risk of diabetes.[31,32] We examined the creatinine levels in patients with diabetic foot ulcers in the current study. The present findings demonstrated that patients with diabetic foot ulcers had considerably lower creatinine levels than the control group. According to recent studies, diabetic patients had low creatinine levels. Indeed, our results supported this assumption.[32,33] Therefore, decreased levels could also be related to diabetic foot ulcer development.

Leukocytes infiltration has been implicated in different diseases.[12,13] It was important to examine the presence of infiltrated leukocytes in diabetic foot ulcer. The histological examination observed infiltrated leukocytes in the site of ulcer. While some studies have not employed inflammatory markers, others have used them to identify diabetic foot infections.[34] Leukocytes count is one example of a typical marker. The increased synthesis and release of leukocytes in the bone marrow may be the reason for elevated leukocytes counts in cases of infection in diabetic foot, because leukocytes count is a common laboratory test indicator that can help detect infection and other nonspecific inflammations,[35] as well as because of the unique role of leukocytes in both innate immunity and acquired immunity of physiological and pathological functions,,[36] its role in the detection of infection diabetic foot has been studied. Accordingly, we also studied the correlation of infiltrated leukocytes with the HbA1c levels. By learning more about how the immune system functions, additional research on infiltrated leukocytes may be able to assist avoid diabetic foot infections.

In the present study there are some limitations. The sample size should be increased and a comparison with diabetic patients without foot ulcers should also be studied. Furthermore, the infiltrating leukocytes were not identified, therefore future studies are required to identify the infiltrating leukocytes using immunohistochemical staining.

Conclusions

The ulceration of diabetic foot of all grades was accompanied by increased levels of FBS and HbA1c. In addition, the levels of cholesterol, triglycerides, and blood urea increased concurrently with the increase of FBS and HbA1c; however, the levels of creatinine went down consistently with the increase of FBS and HbA1c in all grades of diabetic foot ulcers. Moreover, the increased levels of infiltrated leukocytes were accompanied by increased levels of FBS and HbA1c and strongly associated with levels of HbA1c. Therefore, additional studies are required to explore the exact role of infiltrated leukocytes in the ulceration of diabetic foot patients.

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Table 1. Characteristics of healthy controls and patients with diabetic foot ulcer.

Characteristics	Patients (n=100)	Healthy controls (n=50)
Age, years		
Mean ±SE	56.610±1.118	52.540±1.577
Range	30-75	30-71
Sex (%)		
Men	36%	34%
Women	64%	66%

SE, Standard Error of the mean.

Figure 1

Figure 1. Grading of diabetic foot ulceration. A) grade 0 with no skin penetration; B) grade 1, a superficial wound with no penetration; C) grade 2, a penetration in the foot tendon; D) grade 3, a huge penetration. The patients with diabetic foot ulceration were 17, 19, 26, and 38 in the Grade 0, 1, 2, and 3, respectively.

Figure 2

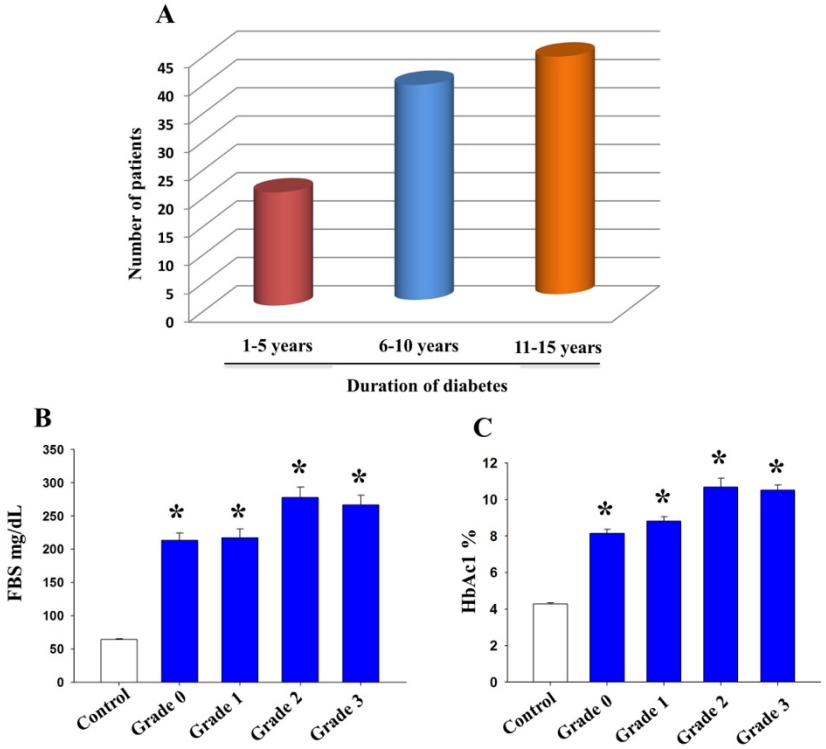

Figure 2. Levels of Diabetic parameters. A) duration of diabetes; B) Fasting Blood Sugar (FBS) levels and C) Hemoglobin A1C (HbA1c) levels in patients with diabetic foot ulcers based on their grade groups. Blue boxes represent patients with diabetic foot ulcers. The white boxes represent control groups. The patients with diabetic foot ulceration were 17, 19, 26, and 38 in the Grade 0, 1, 2, and 3, respectively. The mean values ± standard error of the mean were used to display the current data. *$p < 0.05$ versus control.

Figure 3

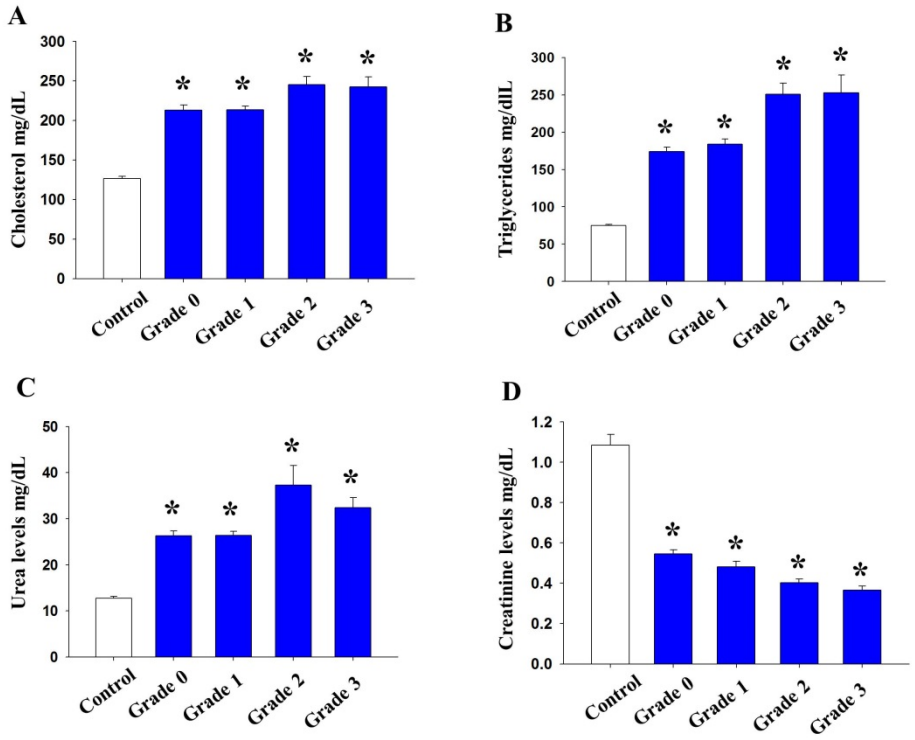

Figure 3. Levels of (A) cholesterol, (B) triglycerides (C) blood urea, and (D) creatinine in patients with diabetic foot ulcers based on their grade groups. Blue boxes represent patients with diabetic foot ulcers. White boxes represents control groups. The patients with diabetic foot ulceration were 17, 19, 26, and 38 in the Grade 0, 1, 2, and 3, respectively. The mean values ± standard error of the mean were used to display the current data. *$p < 0.05$ versus control.

Figure 4

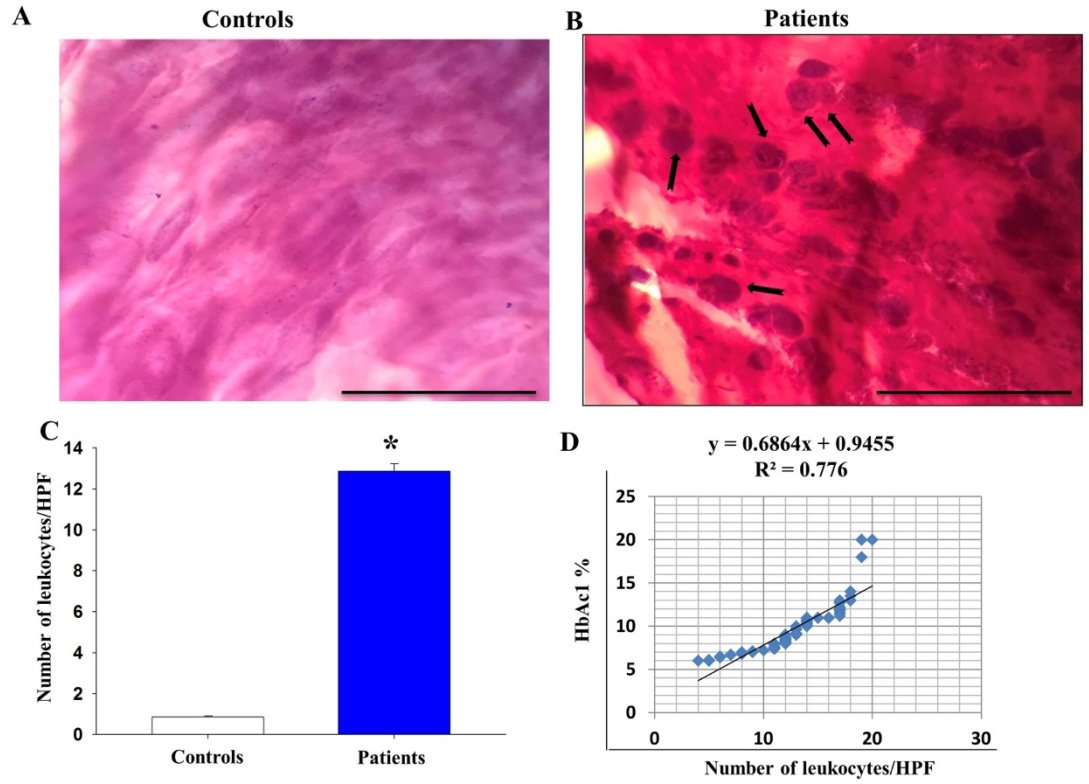

Figure 4. Infiltrated leukocytes and their association with HbAc1. Representative images for A) control group, B) leukocyte infiltration in diabetic foot patients, C) levels of infiltrated leukocytes per-field, High Powerful Field (HPF). Scale bar 200 µm. Blue box represents patients with diabetic foot ulcers. White box represents control group. The patients with diabetic foot ulceration were 17, 19, 26, and 38 in the Grade 0, 1, 2, and 3, respectively. The mean values ± standard error of the mean were used to display the current data. *$p < 0.05$ versus control. D) Association of leukocyte infiltration with HbAc1 as represented by ($y = 0.6864x + 0.9455$: $R^2 = 0.776$).

Contributions: Raed Madhi wrote the report and interpreted the findings. The experiments were conducted by Marwah Majeed and Nidhal Abdullah Hashim. Statistical analysis and data visualization were done by Raed Madhi Marwah Majeed and Nidhal Abdullah Hashim. After reading the completed manuscript, each author gave their approval.

Conflict of interest: the authors declare no conflict of interest.

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Ethical approval: the University of Misan's Ethics Committee gave its approval to the current study (approval number MA051952024 of 19/05/2024).

Informed consent: all patients participating in this study signed a written informed consent form for participating in this study.

Patient consent for publication: written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

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