The Inflammatory Role of Netosis Markers (Cit-H3 And MPO) in the Patients with Chronic DFU

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Abstract
Background: Diabetic foot ulcer (DFU) is a much more common serious complication of diabetes and a recently proposed that DFU could be a result of the interference of metabolic disorders with an inflammatory response which firstly triggers by the involvement of neutrophils. Objectives: This study was carried out to investigate the immunological role of NETosis specific markers (cit Histon 3 and MPO)and their reflection on the inflammatory response in the DFU healing process and to evaluate which of these markers have a close association with the development of DFU. Approach: Clinical data of a total of 50 type 2 diabetic patients have chronic diabetic foot ulcer grade 2 and 3 (infected DFU n= 29 and non-infected n=21) in addition to the control group (n=25) were participated in the study since June 2021 to April 2022, venous blood samples were collected to the estimation of NETs markers levels by flow cytometry technique. Results: There was no significant difference in age and gender when compared with the control group at p= 0.798, p =0.514 respectively. High levels of NETs markers (cit Histon 3 and MPO) were shown in the patients group in comparison to the control (p=0. 000). While no statistical significance within patients group at P =0.108,0 .923 of cit H3 and MPO levels respectively. Conclusions: The results proved that diabetes serves as a stimulating factor for NETosis, and citH3 more specific factor than MPO in the development of DFU.
Keywords: neutrophil extracellular traps, diabetic foot ulcers, wound healing.

1. Introduction

Diabetes-related diabetic foot ulcers are a common exacerbated consequence that may be a medical and social issue that ultimately reflects their detrimental effects on the patient's quality of life (1).

It is distinguished by the appearance of an open wound on the uncontrolled diabetes patient's foot. Diabetes inhibits the healing process by affecting hemostasis, inflammation, proliferation, and remodeling during each stage of wound healing (2,3).

The presence of collaboration between inflammatory cells and biochemical mediators stimulated by various factors is necessary for the wound healing process to be successful. Any alteration of the cellular and biochemical factors and their activities has been linked to the failure of wound healing in diabetics, as this diabetic foot infection reverses the altered immune state.

The inflammatory response of the wound healing process involves a variety of cells, including neutrophils, monocytes, macrophages, keratinocytes, fibroblasts, T cells, B cells, mast cells, and endothelial cells. These cells are stimulated by many signaling pathways and are essential for producing the cytokines and growth factors that start and control the inflammatory response (4-6).

Additionally, the inflammatory response is initiated when neutrophils are drawn to the wound site during the initial stages of wound development. These cells increase netosis or net formation when the blood sugar is high. A network of chromatin, histones, and non-histone proteins, including proteinase 3, cathepsin G, different defensins, azurocidin, neutrophil elastase (NE), and others, make up neutrophil extracellular traps (NETs).

These net components act as a cytotoxic mediator through inflammation and augmented inflammatory response, NETosis is consider a developed form of the innate immune mechanisms, in recent days has taken a new and different trace in the defective wound healing process of diabetic foot ulcer patients (7,8).

The NET formation was discovered by Takei et al (9). in 1996 as a pathway of cellular death different from apoptosis and necrosis, Finally, in 2004, Brinkmann et al (10). further detailed this process and named it NETosis.

To date, there are three models of NETosis description: suicidal NETosis, vital NETosis with the release of nuclear DNA, and vital NETosis with release of mitochondrial DNA (11).

Current work aims to

Evaluate the role of NETosis components (cit Histon 3 and MPO) in the diabetic patients type 2 with foot ulcer and non-diabetic patients in addition to investigating the difference in the levels of the target factors between infected diabetic foot ulcers and non-infected diabetic foot ulcers.

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2. Materials and Methods

-Study population

This study had been conducted since June 2021 to April 2022. Venous blood samples were collected from 25 healthy volunteers and all diabetic patients with at least one active foot ulcer who were attending the diabetic foot clinic and inpatients from operation theaters of diabetic foot of Al-Fayha Teaching Hospital and Al-shifa general hospital. 50 patients with type 2 diabetes aged between 30-70 years divided into two groups infected and non-infected. the Infectious Diseases Society of America (IDSA) are chosen for clinical diagnosis of chronic infected diabetic foot ulcers according to the recognition of 2 or more classical signs of inflammation including induration, erythema, raised temperature, increased pain, and purulent discharge) (12).

Ulceration levels were selected according to the university of Texas diabetic foot ulcer classification system (grade2 and 3stage A and B) (13).

Each patient and control gave their own informed consent for the collection and examination of peripheral blood samples. with a questionnaire were used to acquire the medical histories of the study population as well as some necessary data, including age, history of diabetes, duration of ulcer, and history of other diseases.

-samples collection

Two ml of blood were collected in a tube containing ethylene diamine tetra acetic acid (EDTA) to estimate the intracellular levels of NETosis specific markers (cit H3 and MPO) of the studied groups.

Immunological study

Flow cytometry

the intracellular levels of NETosis specific markers (cit H3 and MPO) were determined for 50 patients and 25 healthy control using BD Accrui C6 flow cytometry. The experiment was accomplished by utilizing four monoclonal fluorescent-labeled antibodies as reagents such as anti –MPO-FITC, Rat anti –Histon-H3 Alexa Fluor 647, FITC- Mouse IgG1, k Isotype control and Alexa Fluor 647 Mouse IgG2,k Isotype control in addition to the 7-ADD and PMA figure(1).

3. Statistical Analysis

The data were statistically analyzed using SSPS software and the significance was determined at p-value < 0.05. t-test t was used to investigate the significant differences between parameters, one way ANOVA test was used to investigate the differences between the age groups.

4. Results

Patients and control groups were subjected to same age period category, statistically no significant differences at p= 0.798 between patients and control according to age, table (1) , as well as no differences were shown between the patients and control in the distribution according to gender at p =0.514 table (2) .

Table (1): Distribution of patients and control according to age range groups

Group	Age range groups (Years)	NO.	P-value
Patients(N=50)	30-40	4(8%)	0.798
Control(N=25)		7(28%)	
patients(N=50)	41-50	17(34%)	
control(N=25)		10(40%)	
patients(N=50)	51-60	18(36%)	
control(N=25)		7(28%)	
patients(N=50)	61-70	11(22%)	
control(N=25)		1(4%)	
Total=75			

Table (2): Distribution of patients and control according to the gender

Group	Gender	NO.	P-value
Patients (N=50)	Male	28(56%)	0.514
	Female	22(44%)	
Control (N=25)	Male	16(%64)	
	Female	9(%36)	
Total=75			

Scientific experiment

In this study, a highly significant elevation was found at P =0 .000 in the levels of cit H3 and MPO respectively of the patients group in comparison with healthy individuals, table (3) but no significance between infected DFU and non- infected DFU in the patients group at P = 0.108,0 .923 of cit H3 and MPO levels respectively table (4) ,besides the statistical analysis of the results study announced the presence of the association between NETosis marker His3with ulceration level (3) at p = 0.000 whereas no association was present about MPO at P=0.86 table (5).

Table (3): Levels of cit H3 and MPO in the blood of patients and control					
Parameters	Groups	No.	Mean	S. error	P-value
cit H3	Patients	50	206.56	24.673	.000
	Control	25	5.80	2.007	
MPO	Patients	50	1314.64	71.946	.000
	Control	25	672.68	41.809	

Table (4): Levels of cit H3 and MPO in the blood of patients groups					
Parameters	Patients group	No.	Mean	S. error	P-value
cit H3	Infected DFU	29	240.34	35.901	.108
	Non- infected DFU	21	159.90	29.524	
MPO	Infected DFU	29	1308.62	87.928	.923
	Non -infected DFU	21	1322.95	123.455	

Table (5): The relationship between ulceration levels and immunological studied parameters					
Parameters	Ulceration levels	No	mean	S. error	p-value
cit H3	2	37	180.81	25.285	0.000
	3	13	1293.54	155.03	
MPO	2	37	1322.1	81.855	0.864
	3	13	1293.54	155.03	

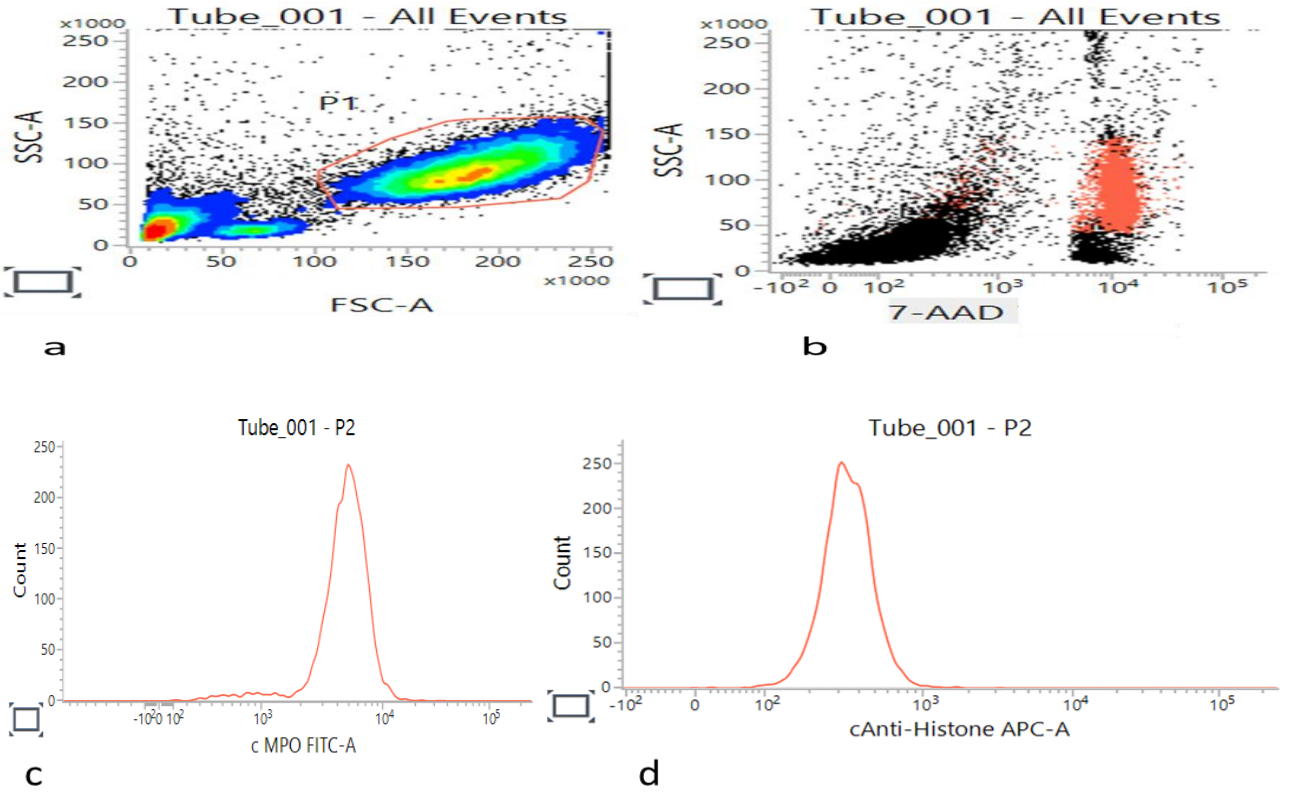

Figure (1): Gating of neutrophil cells by flow cytometry. A- Determination of granulocyte B- Detection of total neutrophil cells C-Measurement of MPO expression levels. D- Measurement of cit H3 expression levels.

5. Discussion

According to the unique phagocytic action of neutrophils in the innate immune system, several studies were conducted through different intervals of time and divided their orientations between previous studies that concern the abnormal situations of neutrophil function as well chemotaxis, phagocytosis and intracellular reactive oxygen species (ROS) and more recent research that focused on neutrophil ROS generation, pro-inflammatory cytokine production and aberrant neutrophil cell death mechanisms which are confirmed to be critical agents causes inefficient inflammatory response (14), in order to this study aims to support the new directions of the negative reflections of the innate defense mechanisms of neutrophils on the inflammatory response in diabetes through the evaluation of the eccentric role of NETosis markers (citH3 and MPO) in the immune response of the diabetic patients .

The onset of a multistep NETosis process from the response of neutrophil to hyperglycemia (15) and the production of excessive amounts of NETs componentes such as nuclear proteins and histones in the extracellular environment, which serve as typical damage-associated molecular patterns (DAMPs).

DAMPs are molecules typically generated by the intracellular activity (16,17), when were DAMPs distinguished by PRRs in the extracellular environment consequently inducing the immune response to proceed with the removal of cellular debris, phagocytic function of macrophages is well organized under a steady state but is disruptive under diabetes (16). Where glucose not only exert the pro-inflammatory action increased in T1D and T2D (18,19) but acts as a pro-survival factor of neutrophils to maintain the persistent accumulation of their at the wounded site without undergoing apoptosis or clearance through the macrophage is characterized by the diminished phagocytic function under these conditions (20) resulting in the damage of the host tissue ,release of pro-inflammatory cytokines and amplify inflammation through the positive feedback (21,22)

Indeed, there is abroad acceptance regarding the critical etiological role of NETosis markers in the chronicity of DF ulceration, as well as the finding of the present study go along with the same line with these facts where support and agreed the results of other studies that were conducted in this scope such as Yang et al.,2020(23) their results revealed the elevated levels of circulating citH3 and nucleosomes in the DFU patients than from diabetic patients without foot ulcer and healthy controls

As expected in the Huang et al.,2020(24) their study detected the high expression levels of citH3 and MPO in the isolated neutrophil from peripheral blood of DFU patients than from diabetic patients and healthy controls besides this study and other studies like Menegazzo et al.2015 (25) documented high concentrations of other types of NETs constitute as neutrophil elastase (NE) and cell-free dsDNA in patients with DFU when compared with healthy individuals group.

The current study didn't found any a significant difference in the levels of the target NETosis components (citH3 and MPO) among infected DFU and non-infected DFU even between ulceration levels (grade 2 and 3) regarding MPO in contrast citH3 proved its relation to the severity of disease through its recording of high expression levels with grade 3, may be increasing the number of participants in this study find the differences between infected and non -infected ulcer, existence of other subclinical disease or interference of the anti-glycemic drugs with the effects of the inflammatory response on the DFU microenvironment in general, these results firmed diabetes as major driver of NETosis either in the presence or absence of the microbial infection.

6. Conclusions

NETosis marker MPO is a biomarker of chronicity of DFU and the levels of cit H3 could form a specific indicator of the progression of DFU.

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