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Assessment of IL-6, IFN-Y and CRP levels in patient with diabetic foot

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Abstract

Diabetic foot is one of the main causes of mortality and morbidity among people with diabetes. Its include an injury to all layers of skin, necrosis or gangrene that usually occur on the soles of the feet, as a result of peripheral neuropathy(PN) or peripheral arterial disease (PAD) in diabetes patients , diabetes have about a 25% chance of developing a foot ulcer in their lifetime, This study aimed to investigate the role of some immune parameters such interlukin-6 (IL- 6) and interferon-y (IFN-y) among patients with diabetic foot . This study was conducted on a total of (150) individual in different sex and age group cases (48 males + 92 females) including (50) patients with diabetic foot (DF) and (50)diabetic patient without foot syndrome and (50) healthy individuals. DM patients were recruited at specialist center for endocrinology and diabetic in Baghdad governorate, through the duration of the beginning of September 2019 till the end of December 2019. All patients diagnosed with DM by HBA1c test. The age range of the study population was from (15-75). Blood was withdrawn from a vein, the serum was used for immunological tests including IL-6 ,IFN-y and HSCRP by ELISA technique. The study showed that DF more common in male (64.0%) rather than female (36.0%), with incidence among age groups (>60), (38.0%), and in rural (66.0%) rather than urban (34.0%) areas. The Study also showed DF significantly common among type 2 diabetes than type1 (68.0%) , (32%) respectively . and (52%) of DF patient had disease duration longer than 10 years. The findings revealed that diabetic foot patients had a significantly higher mean of IL-6 than the DM patient and healthy Group, 123.1 vs. 104.7 and 86.0 respectively (P. value <0.05) DF group also had a significantly higher mean of IFN-Y than the DM patient and healthy Group, 44.8 vs. 37.8 and 23.6 respectively as (P. value < 0.05). DF group had a significantly higher mean of CRP than DM patient and control, 67.1 vs. 58.9 and 32.1 respectively (P. value <0.01), There was a significant direct (positive) correlation between IL-6 and CRP, IFN-Y (p < 0.05).

Keywords: *peripheral neuropathy (PN), diabetic patient, peripheral arterial disease*

I. Introduction

Diabetic foot syndrome (DFS) is defined, 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 in diabetic person

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(Tuttolomomdo etal., 2015) The lifetime risk of adiabetic patient for development of achronic foot wound has been estimated to reach 15–25% . Some Cytokines such as IL-6 and Ifn-y play important roles in the immune pathogenesis of diabetic foot , some studies suggest that IL-6 could contribute to development of micro- and macro vascular complications in diabetic patients. It could be due to the fact that IL-6 participates in pathogenesis of endothelial dysfunction by stimulation of monocyte chemotactic protein-1 and cell adhesion molecules, such as intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 in endothelial cells (Wegner *et al* ., 2013). Moura *et al* ., (2017) show that the effector T cells, which tend to accumulate in diabetic foot patients, are the major producers of the IFN-γ and TNF-α inflammatory cytokines that, in turn, enhance naive T-cell activation and differentiation also shown that the concomitant and continuous accumulation of both CD4+ and CD8+ 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 chemokine receptor (CHR) expression, which could possibly affect T-cell migration into inflamed tissues.

II. Materials and Methods

Patients Group

The collection of blood specimens was carried out during the period from the beginning of September 2019 till the end of December 2020 from100 diabetic patient 50 patient with diabetic foot and 50 without diabetic foot whose ages ranged between (27-75) years . DM patient were recruited of the specialist center for endocrinology and diabetic in Baghdad . First, patients were interviewed directly by using an anonymous questionnaire which included the details and history of the patients.This study was in agreement with the ethics of specialist center for endocrinology and diabetic and verbal informed consent were obtained from all participants.

Control Group

The control group was composed of 50 randomly healthy persons with the age ranging between (15-60) years This control group was examined by ELISA All control group was asked to fill a questionnaire and all had no family history of disease .

Blood Collection

Seven milliliters of venous blood sample was taken from the patients and control group Then the blood samples were divided into two portions The first portion (2 ml) was transferred into an anticoagulant tube from both study groups and immediately stored for use in the hematological analysis The other portion (5ml) was transferred into a Gel tube for serum separation, the blood was left for about30 minutes in room temperature for clotting and then centrifuged at 3000 g for 2minutes Then the serum was collected in a sterile eppendrofe tube in three repeaters and kept frozen at -20 C for the determination of C-reactive protein(CRP),interlukine -6 (IL-6) And interferon gama (ifn-y) (Lima-Oliveira *et al*., 2017).

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III. Diagnosis

Diagnostic Test

HbA1c

HbA1c test used fluoresce immunoassay technology, (Sandwich immune – detection method). the detector antibody in buffer bind to antigen in sample , forming antigen-antibody complex , and migrate onto nitrocellulose matrix to be capture by the other immobilize antibody on test strip the more Ag in sample form more the antigen –antibody complex and lead to stronger intensity of flurescence signal on detector antibody, the result displayed on Ichroma M Reader in unit of % (Mohammed, 2015).

Immunological test

Interleukin-6

This kit uses enzyme-linked immune sorbent assay (ELISA) based on biotin double antibody sandwich technology to assay Human Interleukin 6(IL-6) Add Interleukin 6(IL-6) to wells that are pre-coated with Interleukin 6(IL-6) monoclonal antibody and then incubate After incubation, add anti IL-6 antibodies labeled with biotin to unite with streptavidin-HRP, which forms the immune complex Remove unbound enzymes after incubation and washing, then add substrate A and B The solution will tur```n blue and change to yellow with the effect of acid(stop solution that terminated the reaction) absorbance is measured at 450 nm. The OD value is proportional to the concentration of Human IL-6 You can calculate the concentration of Human IL-6 in the samples by comparing the OD of the samples to the standard curve (Abe *et al*., 2013).

Interferon-gamma IFN-Y

This kit uses enzyme-linked immune sorbent assay (ELISA) based on biotin double antibody sandwich technology to assay Human Interferon gamma (INF-γ) Add Interferon gamma (INF-γ) to wells that are pre-coated with Interferon gamma(INF-γ) monoclonal antibody and then incubate After incubation, add anti INF-γ antibodies labeled with biotin to unite with streptavidin-HRP, which forms the immune complex Remove unbound enzymes after incubation and washing, then add substrate A and B The solution will turn blue and change to yellow with the effect of acid(stop solution that terminated the reaction) The shades of solution and the concentration of Human Interferon gamma (INF-γ) are positively correlated .

c- reactive protein (CRP)

The (CRP) ELISA kit is a solid phase direct sandwich method Microliters strips coated with anti-CRP antibody are incubated with diluted standard sera and patient samples During this incubation step CRP is bound specifically to the wells After removal of the unbound serum proteins by a washing procedure, the antigen-antibody complex in each well is detected with specific peroxidase-conjugated antibodies After removal of the unbound conjugate, the strips are incubated with a chromogen solution containing tetramethylbenzidine and hydrogen peroxide: a blue color develops in proportion to the amount of immunocomplex bound to the wells of the strips The enzymatic reaction is stopped by the addition of 0 5M H2SO4 and the absorbance values at 450 nm are determined A standard

curve is obtained by plotting the absorbance values versus the corresponding standard values The concentration of CRP in patient samples is determined by interpolation from the standard curve (Koivunen, Krogsrud, 2006)

IV. Statistical Analysis

Data of the study participants, DFpatients, DM group and Healthy group, were entered , managed and analyzed using the Statistical Package For Social Sciences (SPSS) version 25 software for windows, IBM, US, 2017. All variables were checked for errors or inconsistency prior to the analysis process. Continuous variables included interleukin-6 (IL-6), interferon-y (IFN-Y) and HSCRP were tested for statistical normality distribution using histogram and normal distribution curves and they all appeared to follow the statistical normal distribution. (LSD) t. test used to compare mean levels of these parameters. Level of significance (P. value) of 0.05 or less considered significant. Finally, results and findings presented in tables and or figures accordingly, using the Microsoft Word application 2010 for windows (Al-Rawi, 2000).

V. Result and discussion

Demographical Profile of diabetes mellitus Patients

The study had shown that DF disease was most common in age >60 years(38.0%) followed in age groups 51-60 years (36.0%) with significant difference within DF and DM groupe according to age (p=0.001) DF patients were older than DM mean age was 58.9 , 46.4 respectively . Physiological increasing age is accompanied by decline in immune system function and immune alteration during ageing increases susceptibility to infections and disease (Valiathan *et al*., 2016)

The majority of DF patient were male 32 (64%) as compared to female 18 (36%) .wounds found in females may have healed better due to the existence of estrogen receptor β, while male androgen was considered harmful for wound healing (Wang *et al*.,2014).

The residence of DF was more common in rural ,66% compared to 30% in DM patient with statistical significant differences (P = 0.001) diabetic patients who lived in the rural area had poor awareness about personal hygiene and foot self-care practice, and they often walk with bare feet. This may expose their feet to harm and lead to the development of foot ulcer (Mariam *et al*., 2017).In both diabetic groups, type I DM represented 32% (16/50), (P= 1.00) no significant difference between groupe while under the DF group T2DM is consist 34 (68%) higer than T1DM 16 (32%) as show in table (3-2).

In compare The duration of DM among patient groups the duration of DM was significantly longer in DF group where 26 (52%) of patients in this group had a disease duration of >10 years compared to only 8 (16%) patients in DM group, (P < 0.005) longer duration of diabetes increase the chances of foot ulcer due to the fact that the long term diabetic vascular complications and neuropathic complications developed with passage of time which could predispose to the occurrence of foot ulcer (Danmusa *et al*., 2016)

Table (3-1) demographic characteristics of DF, DM , and control

Variable		Group				P. value between group
		DF		DM		
		No.	%	No.	%	
Age (year)	≤ 30	0	0.0	2	18.0	0.23 Ns
	31 – 40	1	2.0	3	12.0	
	41 – 50	12	24.0	17	34.0	
	51 – 60	18	36.0	16	24.0	
	> 60	19	38.0	12	12.0	
	Mean	58.9		55.6		
Statistical test and p. value within group		X=16.4 df=4 P = 0.001		X= 20.2 df=4 P value <0.001		
Sex	Male	32	64	35	70	0.523 Ns
	Female	18	36	15	30	
Statistical test and p. value within group		X=3.92 df=1 P=0.04		X=8.00 p.= 0.005		
Residence	Urban	17	34	35	70	0.001 Sig
	Rural	33	66	15	30	
Statistical test and p. value within group		X=5.12 df=1 P=0.02		X=8.00 P=0.005		

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Table (3-2) Distribution and comparison of diabetic related variables among diabetic groups with and without DF.

Variable		Diabetic group				P. value
		DF		DM		
		No.	%	No.	%	
Type of DM	Type 1	16	32.0	16	32.0	1.00 NS
	Type 2	34	68.0	34	68.0	
Duration of DM In years	< 5	8	16.0	28	56.0	0.001 Sig
	5 – 10	16	32.0	13	26.0	
	> 10	26	52.0	9	18.0	

Table 2: Comparison of mean values of IL-6, IFN-Y and HSCRP of DF patients, DM group and controls

Parameter	Group						P1	P2	P3
	DF (n=50)		DM (n=50)		Control (n=50)				
	Mean	SD*	Mean	SD*	Mean	SD*			
IL-6	123.1	43.7	104.7	38.9	86.0	11.9	0.02 Sig	0.001 Sig	0.002 Sig
IFN-Y	44.8	16.2	37.8	17.6	23.6	7.17	0.04 Sig	0.001 Sig	0.001 Sig
HSCRP	67.1	10.0	58.9	17.6	32.1	5.87	0.001 Sig	0.007 Sig	0.001 Sig

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P1: P. value for comparison of DM with DF vs. DM no DF groups

P2: P. value for comparison of DM with DF vs. control groups

P 3: P. value for comparison of DM no DF vs. control groups foot

Interleukin-6 (IL-6)

The mean Interleukine-6 in DF patients was significantly higer than that of DM patients and control 123.1 , 104.7 and 86.0 , respectively, as shown in table (3-5), figure (3-4) P value < 0.05.

IL-6 is produced by neutrophils and monocytes and has been shown to be important in initiating the healing response, Its expression is increased after wounding and tends to persist in older wounds(Barrientos *et al*., 2019) . Patients with diabetes, irrespective of their risk for developing foot ulcers, have been found to have an increased number of inflammatory cells in the dermis and around vessels as indicator of chronic inflammation, both pre- and post-injury (Baltzis *etal*.,2014).

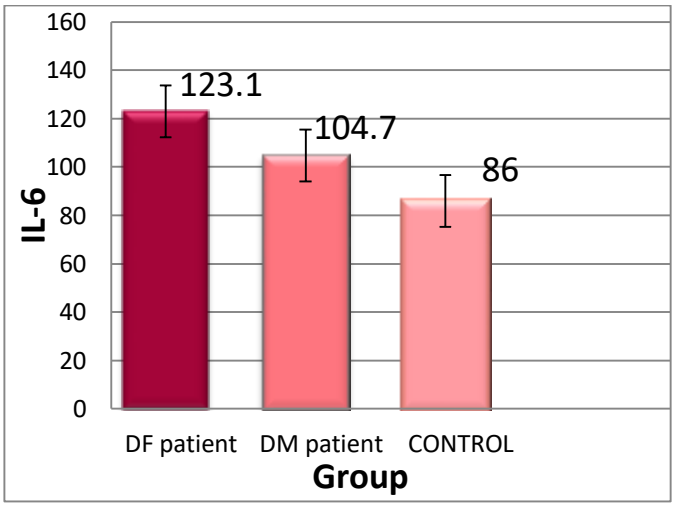

Figure 3: Graphical comparison of the mean IL-6 level of DF patients, DM group and Healthy Group

Interferon-y (IFN-Y)

The mean of IFN –Y in DF patient was 44.8 and it was significantly higer than that of DM patient which was 37.8 (p value <0.05) Weigelt et al ., (2009) obtain that IFN-Y level were elevated in DF patient . Beidler et al (2012) explain that pro-inflammatory cytokine IL-1α, IL-1β, IFN-γ, IL-12 protein levels were elevated in ulcer tissue compared to healthy tissue, and indicated that cytokines may provide novel therapeutic approaches to leg ulcer healing. a lot of data suggests that Th1-associated cytokines induce hyper inflammatory response and subsequently lead to

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progressive innate immune response It is reported that the circulating levels of IFN-Y increased in diabetic patients (Francisco *et al* ., 2016)

Hight sensitivity C-reactive protein (HSCRP)

The mean value of CRP was significantly higher in DF group compared to both DM and controls , 67.1, 58.9 and 32.1 respectively (P<0.05). Also the mean value of CRP of DM patient was significantly higher than that in controle , (P<0.05), as show in table (3-5), figure (3-3). Elevated level of circulating plasma HSCRP frequently clusters with risk factors of T2DM such as obesity and insulin resistance (Pick up *et al* .,2004).

CRP is an acute-phase protein and its synthesis by the liver is rapidly dysregulated in various conditions, including tissue damage and infection . local factors that affect wound healing such as tissue necrosis and infection may be better reflected by the systemic CRP levels than any other inflammatory cytokine DFU patients had higher levels of a number of inflammatory cytokines, including IL-8, TNFα and CRP but only lower levels of CRP were associated with complete wound healing (Tecilazich *et al* ., 2013)

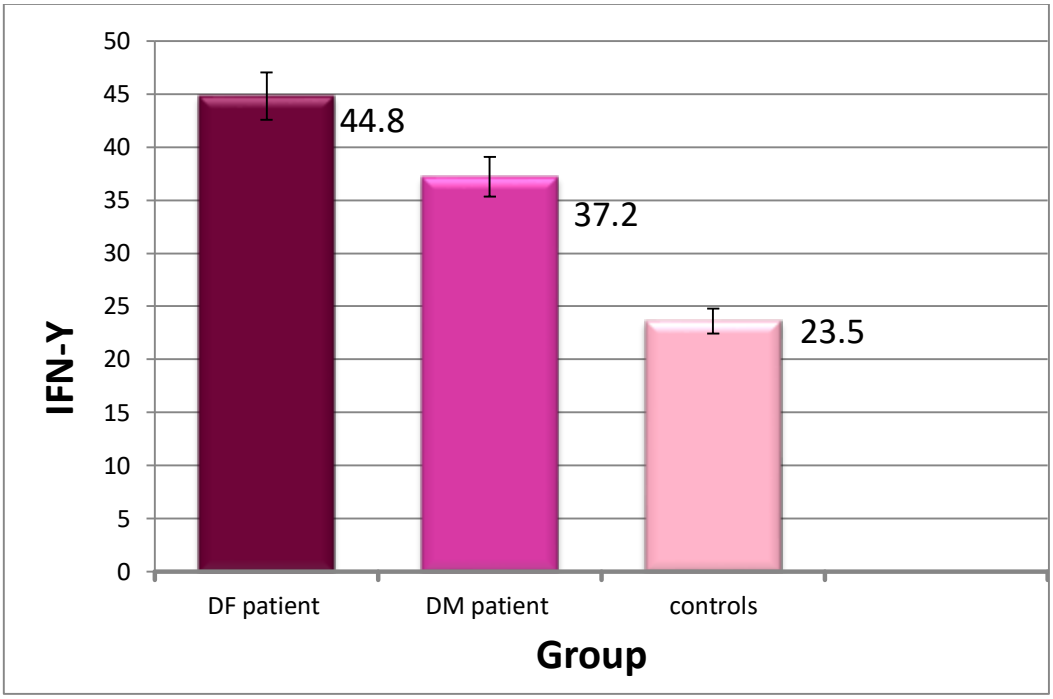

Figure 4: Graphical comparison of the mean IFN-Y level of DF patients, DM group and Healthy Group

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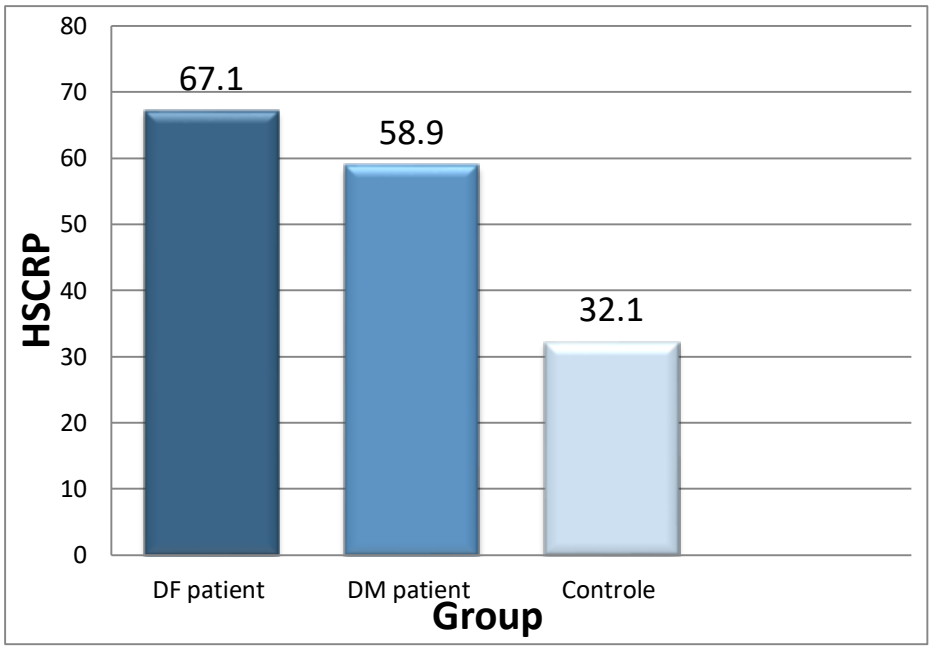

Figure (3-3) graphical comparrision of the mean HSCRP level of DF patient , DM patient and controls

VI. Correlation Analysis among The immunological parameters

Results of Bivariate correlation analysis is shown in the Table(3-6).Among DF patient three significant correlation had been found the first was a direct (positive) between HSCRP and IL-6 (R=0.429 , P.value = 0.002) .

CRP is a principal downstream mediator of the acute phase response and is primarily derived via IL-6 dependent hepatic biosynthesis (Pradhan *et al*., 2001) .CRP plays important roles in inflammatory processes and host responses to infection including the complement pathway, apoptosis, phagocytosis, nitric oxide (NO) release, and the production of cytokines, particularly interleukin-6 and TNF-α (Sproston&Ashworth, 2018).

The second significant correlation was adierct (positive) found between IL-6 and IFN-Y (R=0.461 , P.value =0.001) . Pangrazzi *et al*.,(2017) show that IFN-γ and IL-6 were required for the expression of each other, IFN-γ stimulates IL-15 as well as IL-6 production by CD11c, In addition, the expression of genes regulated by either IFN-γ was reduced by the absence of IL-6. the absence of IFN-γ reduced IL-6 expression supporting a suspected regulatory linkage between these 2 cytokines (Cauvi *et al*., 2017).

IFN-y contributes to skin inflammatory and immune responses by amplify IL-6 production in epidermis. IL-6 produced from keratinocytes can activate lymphocytes, leading to a further production of IFN-y (Chatzigeoragiou *et al* ., 2012).

The last sigmificant correlation was direct (positive) found between IFN-Y and CRP (R= 0.422 , Pvalue =0.002)Inflammation is emerging as an important mechanism for micro- and macrovascular complication of diabetes.

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The macrophage plays a key role in the chronic inflammatory response in part by generating particular cytokines. IL-1β, IL-6, IL12, IL-18, TNFα, and IFN-Y are produced primarily in macrophages (Wen *et al*., 2006).

Table (3-6) result of multiple correlation analysis among the immunological parameters

Group	Parameters	Correlation statistics	HSCRP	IL-6
DFpatient (n =50)	IL-6	R*	0.429**	
		P. value	0.002	
	IFN-Y	R*	0.422**	0.461**
		P.value	0.002	0.001
DM patient (n =50)	IL-6	R*	0.291*	
		P. value	0.04	
	IFN-Y	R*	0.178	0.229
		P.value	0.21	0.11

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Ethical approval

All authors hereby declare that all actions have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Conflict of Interests

The authors did not declare any conflict of interest.

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