Gene expression levels of inflammatory mediators in diabetic foot ulcer patients

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Abstract

Background: Diabetic foot ulcer (DFU) is a common serious complication of diabetes. Nowadays there is a suggestion that referred to the fact the chronicity of DFU is a result of the positive feedback of proinflammatory cytokines production. **Objective**: This study aimed to display the negative effects of persistent expression of proinflammatory cytokines of (IL-1β and IL-18) under hyperglycemic condition on the DFU microenvironment and clarify the role of growth factor Milk fat globule-epidermal growth factor- 8(MFG-E8) in the DFU healing process. **Materials and Methods:** Clinical data of a total of 49 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 for the measurement of the gene expression levels of (IL-1β; IL-18 and MFG-E8) by Real-Time PCR technique. **Results:** There was no significant difference in age and sex when compared with the control group at p= 0.798, p =0.514 respectively. High levels of gene expression of (IL-1β; IL-18 and MFG-E8) were shown in the patients group in comparison to the healthy control. **Conclusions:** Current data confirmed that the inflammatory mediators' (IL-1β; IL-18 and MFG-E8) have a strong association with the pathogenesis of DFU healing process.

Keywords: **cytokines, diabetic foot ulcers, wound healing, inflammatory mediators.**

Introduction

Diabetes mellitus (DM) is one of the most common metabolic disease characterized by elevated levels of blood glucose, which leads over time to serious damage of body organs, most important characters of this disease are chronic inflammation that develops from continuous inflammatory triggering involves prolonged immune response that can last for several weeks to years and is characterized by cycles of active inflammation , tissue injury ,and healing(1), endothelial dysfunction that increase risk for occurring complications such us ,diabetic foot ulcers , cardiovascular disease , neuropathy, nephropathy, and others(2).

A diabetic foot ulcer (DFU) is a major diabetes related complication. 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 (3,4).

Neutrophils, monocytes, macrophages, keratinocytes, fibroblasts, T cells, B cells, mast cells, and endothelial cells are all involved in the inflammatory response of the wound healing process. These cells are triggered by different signaling pathways and have a vital role in the production of cytokines and growth factors that initiate and regulate the inflammatory response (5-7).

The production of proinflammatory cytokines represents the first step in response to skin injury during the inflammatory phase (8). Usually, the chronic diabetic wound is accompanied by phenomenon of persistent elevation of proinflammatory cytokines such as interleukin-6 (IL-6), IL-1, IL-12, IL-18, and tumor necrosis factors (TNFs) and interferon-gamma (IFN-γ), which results in inflammation of tissues (9), IFN-γ, IL-1β, and TNF-α stimulate pyroptosis and apoptosis that mediated by the activation of innate immunity and oxidative stress (10) , the production of TNF-α and IFN-γ is stimulated by IL-12 which suppress the expression of IL-4, an anti-inflammatory cytokine, and negatively controls the expression of IFN γ(11).

Study aim

This study aimed to evaluate the role of IL-1β, IL-18, and MFG-E8 factors in the diabetic patients type 2 with chronic foot ulcer and non-diabetic patients as control by determination the gene expression levels of IL-1β, IL-18, and MFG-E8 factors.

Materials and methods

1. Clinical Specimens

This study was carried out between June 2021 and April 2022 after acquiring ethical approval that numbered (797) in the date (23/12/2020). 25 healthy volunteers and all diabetic patients with at least one active chronic foot ulcer present at the diabetic foot clinic as well as inpatients from the diabetic foot operation theatres at Al-Fayha Teaching Hospital and Al-Shifa General Hospital. 49 type 2 diabetic patients (sample number 44 was excluded because invalid for processing), aged 30-70, were included infected DFU and non-infected DFU groups. According to the detection of two or more classical indications of inflammation, such as induration, erythema, fever, increased pain and purulent discharge, the Infectious Diseases Society of America (IDSA) was chosen for the clinical diagnosis of chronic infected diabetic foot ulcers (12).

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

Peripheral blood samples were collected and examined with the informed consent of each patient and control. with the aid of a questionnaire, the research population's medical histories as well as certain crucial information—such as age, diabetes history, ulcer duration, and history of other diseases—were collected.

2. Real-Time PCR.

Up to 3 ml of EDTA blood samples were used for isolation of peripheral blood mononuclear cells (PBMCs) layer followed by total RNA extraction from this layer using (Promega ,U.S.A extraction kit),then cDNA strand synthesis and real time PCR with SYBR Green dye was applied(Promega ,U.S.A GoScript revers transcription system and

GoTaq PCR master mix) to evaluate the levels of gene expression of interest factors. β –actine gene was used as internal control as shown in the table (1).

Table (1): The primers sequence used in the RT PCR experiment

No.	Primers	Sequence	Tm	Reference
1.	**IL-1 β**	AAACAGATGAAGTGCTCCTTCCAGG	61	14
		TGGAGAACACCACTTGTTGCTCCA		
2.	**IL-18**	GGG GTA CCA TGG CTG CTG AAC CAG TAG AAG	59	15
		CCG CTC GAG AGC TAG TCT TCG TTT TGA ACA GTG		
3.	**MFGE-8**	GTGCGTGTGACCTTCTTG	60	16
		ACCTGTTACCCACATCCT		
4.	**β -actin**	CATGTACGTTGCTATCCAGGC	60	14
		CTCCTTAATGTCACGCACGAT		

The primers supplied by Alpha DNA company /Canada

Statistical Analysis

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

Results

Gene expression of pro-inflammatory cytokines (IL-1β, IL-18) and growth factor (MFGE-8).

Patients were matching to age category of control groups, statistically no significant differences at p= 0.798 between patients and control according to age, table (2) , as well as no differences were shown between the patients and control in the distribution according to sex at p =0.514 table (3) .

Table (2): 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 (3): Distribution of patients and control according to the sex

Group	Sex	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		

Real-time PCR analysis of genes encoding IL-1β, IL-18 and MFGE-8 revealed up-regulated expression of the target genes in 49 patients' samples in contrary to down-regulated expression in the 25 normal control group, For the pathway IL-1β, IL-18 and MFGE-8genes SYBR Green chemistry were used and the binding of the dye was specific to the study genes as the melting curve showed one peak for all samples as shown in Figure(4).IL-1 β, IL-18 and MFGE-8 genes recorded fold change of 2.7, 27 and 4 respectively in compression with control (expression=1) as illustrated in figures (1),(2) and (3) respectively .

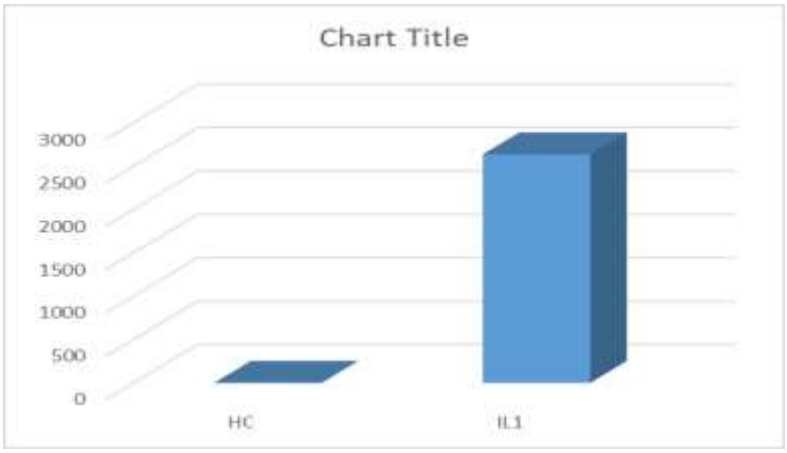


Figure (1): IL-1β gene expression levels of the patients and control group

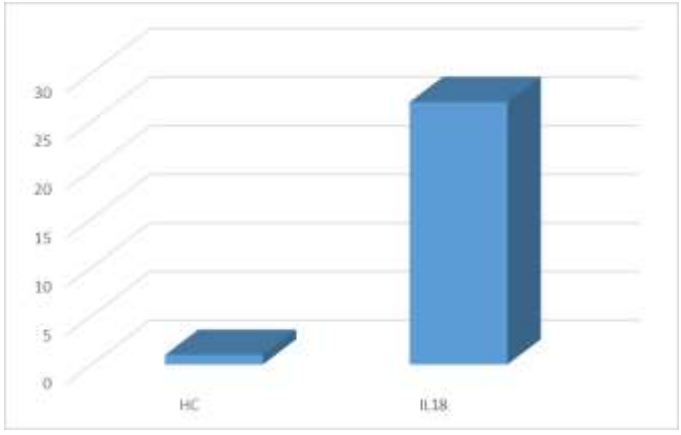


Figure (2): IL-18 gene expression levels of the patients and control group

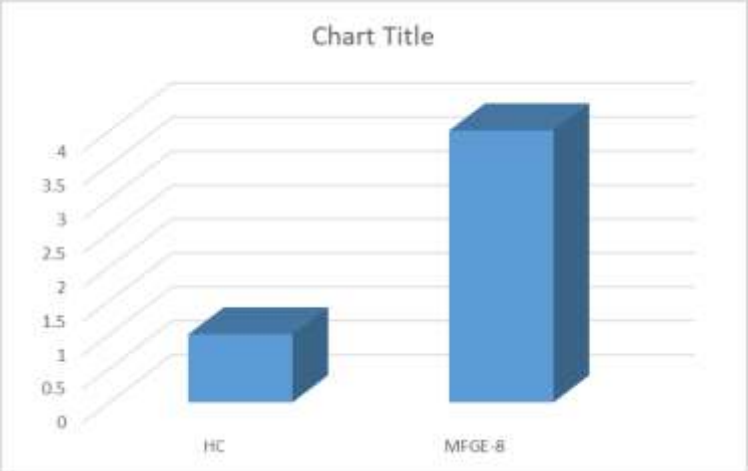
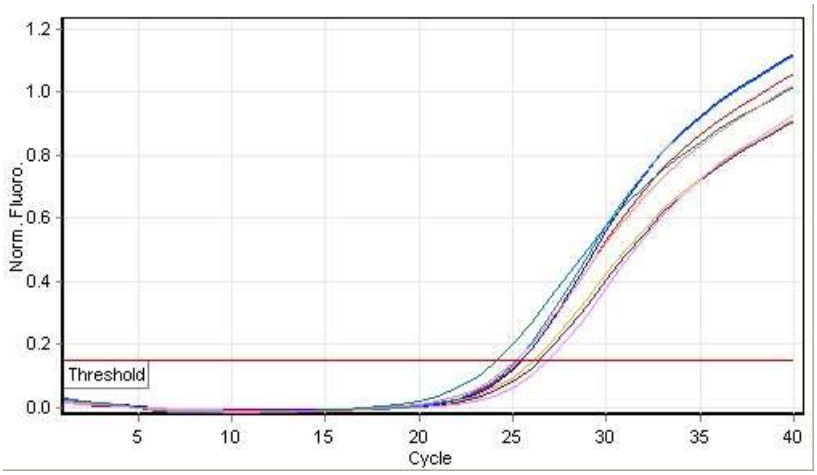
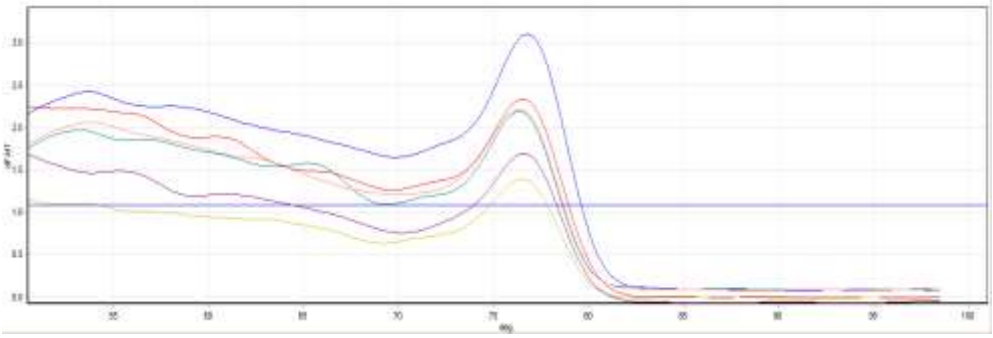


Figure (3): MFGE-8 gene expression levels of the patients and control group



A



B

Figure (4): A-The amplification plots of MFGE-8 gene for numerous samples running in the real time PCR system using SYBR Green chemistry. **B-**The melting curve of MFGE-8 gene using SYBR Green dye showed one peak representing the specific binding of the dye to the target gene in several samples.

Gene expression of pro-inflammatory cytokines (IL-1β, IL-18) and growth factor (MFGE-8) levels according to age

The current findings not indicated the presence of significant importance of age on the aimed parameters (IL-1β, IL-18) and growth factor (MFGE-8) as itemized in the table (4).

Table (4): Gene expression of pro-inflammatory cytokines (IL-1β, IL-18) and growth factor (MFGE-8) levels according to age of patients group.

Age groups	Parameters								
	IL-1β			IL-18			MFGE-8		
	Mean	S.Error	P-value	Mean	S.Error	P-value	Mean	S. Error	P-value
30-40	414.7000	159.44941	0.616	95.450	21.867	0.442	66.743	18.519	0.914
41-50	341.5333	94.22453	0.457	28.6900	4.323	0.383	45.695	9.8422	0.667
51-60	334.1500	73.81073	0.296	71.2123	12.323	0.480	65.421	17.089	0.378
61-70	412.1462	94.22453	0.457	94.4131	24.18105	0.231	52.085	15.089	0.378

The relationship between ulceration levels and studied immunological parameters

The current findings documented that the most common grade among the patients group of the study was grade 2. Statistical analysis of the results announced not the presence of the association about a pro-inflammatory cytokines (IL-1β, IL-18) and growth factor (MFGE-8) with the ulceration levels at p =0.237, 0.811 and 0.513 respectively, detailed in the table (5).

Table (5): The relationship between ulceration levels and studied immunological parameters

Parameters	Ulceration levels	No	mean	S. Error	p-value
Il-1β	2	37	380.32	33.396	0.237
	3	13	298.25	66.183	
Il-18	2	37	69.26	22.43	0.811
	3	13	59.59	22.58	
MFGE-8	2	37	64.83	13.134	0.513
	3	13	48.179	20.539	

Correlation between gene expression of pro-inflammatory cytokines (IL-1β, IL-18) and growth factor (MFGE-8)

The analysis of the statistical data documented strong correlation of IL-1 β with both of IL-18 and MFGE-8 whereas IL-18 and MFGE-8 displayed a moderate association among them as mentioned in the Table (6).

Table (6): Correlation between gene expression of (IL-1β, IL-18) cytokines and growth factor (MFGE-8)

Parameters	R-value	p-value
IL-1β	1	0.051
IL-18	0.281	
MFGE-8	0.046	

Discussion

Interlukin -1 β and Interlukin -18 (IL-1β, IL-18) are belong to the IL-1 family included eleven members, seven of which are receptor agonists (IL-1α/β, IL-18, IL-33, IL-36 α/β/γ) and anti-agonists (IL-1 receptor antagonist (Ra), IL-36Ra, IL-37, IL-38) (17).

Different reports disclosed the involvement of IL-1 in the various pathological states where in 2002 shed light on the importance of the correlation of the pathogenesis of diabetes type 2 with the levels of IL-1 β and the outcomes of these observations were the most common metabolic disorders that accompanied of diabetes is aberrant expression of IL-1 (18).

The results of the recent study and previous studies conducting by (19-21) emphasized these observations where recorded high levels of IL-1 β in the patients with DFU whether at the gene expression levels or protein levels. There were other studies that concerning IL-18 their results revealed some similarities and dissimilarities with the present findings of which study of (22-24). In the instance of MFGE-8, there is a paucity of studies about the evaluation of the role of MFGE-8 in the diabetic foot infection patients.

Indeed, there is controversial experimental and published data about the levels of MFGE-8 that documented the presence of variations in the expression and secretion of MFGE-8in the different pathological states, down-regulation found in some autoimmune diseases (25), in contrast up-regulation was noticeable in the other inflammatory diseases (26) , the recent findings recorded high levels of MFGE-8 in the DFU patients than healthy volunteers, these findings underlined the sight of the previous reports of (27).

Also this data suggest that diabetes causes overexpression of MFGE-8 either be in the norm structural state but their levels have pathological influence through the inhibition of the phagocytic action in a dose-dependent manner of MFGE-8 or diabetic conditions induce the modification of MFGE-8 molecules by glycation process, thereby production of the inactive biological molecules, this justification proved the outcomes of an animal model experiment by (28). The recent findings demonstrated that IL-1β had a strong relationship with each of IL-18 and MFGE-8 in odds IL-18 and MFGE-8 not showing linking the same strength, this result referred to the IL-1β occupying the chief role in the inflammatory response.

Conclusions

IL-1β has a strong linkage with the pathogenesis of DFU. Overexpression of MFGE-8 may exert a pathological role in the inflammatory response of the DFU healing process.

References

1. Pahwa, R., Goyal, A., Bansal, P., Jialal, I. Chronic Inflammation. [Updated 2020 Nov 20]. StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing. (2021).
2. Tuttolomondo, A., Maida, C., Pinto, A. Diabetic foot syndrome: Immune-inflammatory features as possible cardiovascular markers in diabetes. World journal of orthopedics. (2015); 6(1): 62.
3. Jeffcoate, W. J., Bus, S. A., Game, F. L., Hinchliffe, R. J., Price, P. E., Schaper, N. C., ... & European Wound Management Association. Reporting standards of studies and papers on the prevention and management of foot ulcers in diabetes: required details and markers of good quality. The lancet Diabetes & endocrinology. (2016); 4(9): 781-788.
4. D'Alessandro, S., Magnavacca, A., Perego, F., Fumagalli, M., Sangiovanni, E., Prato, M., Basilico, N. Effect of hypoxia on gene expression in cell populations involved in wound healing. BioMed Research International. (2019).
5. Moura, J., Rodrigues, J., Gonçalves, M., Amaral, C., Lima, M., & Carvalho, E. Impaired T-cell differentiation in diabetic foot ulceration. Cellular & Molecular Immunology. (2017); 14(9), 758-769.
6. Bando, T., Yokoyama, H., and Nakamura, H. Wound repair, remodeling, and regeneration. Develop. Growth Differ. (2018); 60 (6): 303–305. doi:10.1111/dgd.12566
7. Akita, S. Wound repair and regeneration: mechanisms, signaling. International Journal of Molecular Sciences. (2019); 20(24), 6328.
8. Kanji, S., Das, H. Advances of stem cell therapeutics in cutaneous wound healing and regeneration. Mediators of inflammation (2017).
9. Mende, R., Vincent, F. B., Kandane-Rathnayake, R., Koelmeyer, R., Lin, E., Chang, J., Lang, T. Analysis of serum interleukin (IL)-1β and IL-18 in systemic lupus erythematosus. Frontiers in immunology. (2018); 9, 1250.
10. Lamkanfi, M., & Dixit, V. M. Mechanisms and functions of inflammasomes. Cell. (2014);157(5): 1013-1022.
11. Rigante, D. The board ranging panorama of systematic autoinflammatory disorders with specific focus on acute painful symptoms and hematologic manifestations in children. Mediterranean Journal of Hematology and Infectious Diseases. (2018) ;10:e2018067.
12. Lipsky, B. A., Senneville, É., Abbas, Z. G., Aragón- Sánchez, J., Diggle, M., Embil, J. M., ... & International Working Group on the Diabetic Foot (IWGDF). Guidelines on the diagnosis and treatment of foot infection in persons with diabetes (IWGDF 2019 update). Diabetes/metabolism research and reviews. (2020); 36, e3280.
13. Lavery, L. A., Armstrong, D. G., Harkless, L. B. Classification of diabetic foot wounds. The Journal of Foot and Ankle Surgery. (1996); 35(6): 528-531.
14. Keita, M., Bessette, P., Pelmus, M., Ainmelk, Y., Aris, A. Expression of interleukin-1 (IL-1) ligands system in the most common endometriosis-associated ovarian cancer subtypes. Journal of ovarian research. (2010); 3(1):1-8.
15. Liu, W., Hu, M., Wang, Y., Sun, B., Guo, Y., Xu, Z., Han, B. Overexpression of interleukin-18 protein reduces viability and induces apoptosis of tongue squamous cell carcinoma cells by activation of glycogen synthase kinase-3β signaling. Oncology Reports. (2015);33(3): 1049-1056.
16. Yamazaki, M., Maruyama, S., Abé, T., Essa, A., Babkair, H., Cheng, J., & Saku, T. MFG-E8 expression for progression of oral squamous cell carcinoma and for self-clearance of apoptotic cells. Laboratory Investigation. (2014); 94(11): 1260-1272.
17. Mantovani, A., Dinarello, C. A., Molgora, M., Garlanda, C. Interleukin-1 and related cytokines in the regulation of inflammation and immunity. Immunity. (2019); 50(4): 778-795.
18. Sergeev, P., Ris, F., Oberholzer, J., Joller-Jemelka, H. I., Spinas, G.A. Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets. J Clin Invest. (2002); 110: 851-860.

19. Tiwari, S., Pratyush, D. D., Gupta, S. K., Singh, S. K. Vitamin D deficiency is associated with inflammatory cytokine concentrations in patients with diabetic foot infection. British journal of nutrition. (2014); 112(12): 1938-1943.

20. Perrault, D. P., Bramos, A., Xu, X., Shi, S., Wong, A. K. Local administration of interleukin-1 receptor antagonist improves diabetic wound healing. Annals of plastic surgery. (2018); 80(5): S317.

21. Lee, M. K., Sreejit, G., Nagareddy, P. R., & Murphy, A. J. Attack of the NETs! NETosis primes IL-1β-mediated inflammation in diabetic foot ulcers. Clinical Science. (2020); 134(12): 1399-1401.

22. Weigelt, C., Rose, B., Poschen, U., Ziegler, D., Friese, G., Kempf, K., Herder, C. Immune mediators in patients with acute diabetic foot syndrome. Diabetes care. (2009); 32(8), 1491-1496.

23. Sabuncu, T., Eren, M. A., Tabur, S., Dag, O. F., & Boduroglu, O. High serum concentration of interleukin-18 in diabetic patients with foot ulcers. Journal of the American Podiatric Medical Association. (2014); 104(3): 222-226.

24. Kaleli, S., Varım, C., Nalbant, A., & Akdoğan, M. Interleukins As a Marker of Inflammation in Diabetic Foot Syndrome and Type 2 Diabetes Mellitus. (2019).

25. Albus, E., Sinningen, K., Winzer, M., Thiele, S., Baschant, U., Hannemann, A., Rauner, M. Milk fat globule- epidermal growth factor 8 (MFG- E8) is a novel anti- inflammatory factor in rheumatoid arthritis in mice and humans. Journal of Bone and Mineral Research. (2016); 31(3): 596-605.

26. Aziz, M. M., Ishihara, S., Mishima, Y., Oshima, N., Moriyama, I., Yuki, T., Kinoshita, Y. MFG-E8 attenuates intestinal inflammation in murine experimental colitis by modulating osteopontin-dependent αvβ3 integrin signaling. The Journal of Immunology. (2009); 182(11): 7222-7232.

27. Huang, W., Jiao, J., Liu, J., Huang, M., Hu, Y., Ran, W., Zhang, D. MFG-E8 accelerates wound healing in diabetes by regulating "NLRP3 inflammasome-neutrophil extracellular traps" axis. Cell death discovery. (2020); 6(1), 1-16.

28. Das, A., Ghatak, S., Sinha, M., Chaffee, S., Ahmed, N. S., Parinandi, N. L., Roy, S. Correction of MFG-E8 resolves inflammation and promotes cutaneous wound healing in diabetes. The Journal of Immunology. (2016); 196(12): 5089-5100.

مستويات التعبير الجيني للوسائط الالتهابية في مرضى قرحة القدم السكري

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الخلاصة

الخلفية: تعد قرحة القدم السكري من المضاعفات الخطيرة الشائعة لمرض السكري ، حيث اقترح في الوقت الحاضر ان دخول قرحة القدم السكري الطور المزمن ناتج من التغذية الاسترجاعية الموجبة لانتاج السايتوكاينات الالتهابية. **الهدف:** هدفت هذه الدراسة الى عرض التاثيرات السلبية لاستمرار تعبير وانتاج السايتوكاينات الالتهابية (β1IL- وIL-18) في ظل ارتفاع مستوى السكر على البيئة الموضعية لقرحة القدم السكري، بالاضافة الى توضيح دور عامل النمو (MFG-E8) في عملية شفاء قرحة القدم السكري. **المواد وطرق العمل:** تم جمع 49 عينة دم من مرضى السكري النوع الثاني والذين يعانون من قرحة القدم السكري المزمنة عند مستوى تقرح 2 و3 تضمنت مجموعة قرحة القدم السكري المصابة (29) وغير المصابة(21) اضافة الى 25 عينة دم من الاصحاء البالغين ابتداءا من سنة 2021 لغاية 2022 .عينات الدم التي جمعت كانت لغرض قياس مستويات التعبير الجيني لجينات (IL-1β ; IL-18 and MFG-E8) بواسطة تقنية Real time PCR . **النتائج:** لاتوجد فروقات احصائية بين مجموعتي المرضى الكونترول تبعا للعمر والجنس .في حين سجلت نتائج هذه الدراسة مستويات عالية للتعبير الجيني للعوامل المستهدفة اعلاه في مجموعة المرضى عند المقارنة مع مجموعة السيطرة . **الاستنتاجات:** اكدت النتائج الحالية على الارتباط الوثيق بين مستويات الوسائط الالتهابية وامراضية عملية شفاء قرحة القدم السكري.

الكلمات المفتاحية: السايتوكاينات ، قرحة القدم السكري ، شفاء الجروح ، الوسائط الالتهابية.