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Estimation of Some Cytokines and Haematological Parameters in Diabetic Foot Ulcer Patient's Treated with Superoxidized Water

A Thesis

Submitted to the Council of the College of Medicine, University of Kerbala in Partial Fulfilment of the Requirements for the Degree of Master in Medical Microbiology

By

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بسم الله الرحمن الرحيم

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Supervisors Certification

We certify that this M.Sc. thesis titled:

Estimation of Some Cytokines and Haematological Parameters in Diabetic Foot Ulcer Patient's Treated with Superoxidized Water

Was prepared under our supervision in the College of Medicine/ University of Kerbala, as a partial fulfillment of the requirements for the Degree of in Medical Microbiology.

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In view of the available recommendation, we forward this thesis for debate by the examining committee

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Estimation of Some Cytokines and Hematological Parameters in Diabetic Foot Ulcer Patient's Treated with Superoxidized Water

We have examined the student **(Fatima Fadel Saeed Hammadi)**

In its contents. In our opinion it meets the standards of thesis for the degree of Master in Medical Microbiology and Immunology.

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Dedication

To the one who missed her warm applause for the joy of my achievement, to the one who wished to see me always superior, to the soul of my mother, may God have mercy on her.

To those who had a great role in achieving my dream, my brother, the martyr, may God have mercy on him.

To the one who embraced me and became my second mother and faced the odds for me "dear sister"

With my honest affection for them, I make this modest effort.

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List of Abbreviations

Code	Words
CBC	Complete blood count
CRP	Carbohydrate reactive protein
DFU	Diabetic foot ulcer
DM	Diabetes mellitus
GDM	Gestational diabetes mellitus
HbA1C	Hemoglobin glycemic A1C
HCT	Hematocrit
Hb	Hemoglobin
IL-17	Interleukin 17
IL-1 β	Interleukin 1beta
LYM	Lymphocyte
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MMPS	Matrix metalloproteinase
MPV	Mean platelet volume
NEU	Neutrophil
PCT	Plateletcrit
PDW	Platelet distribution width
PLT	Platelet
P-LCC	platelet large cell count
P-LCR	platelet large cell ratio
RBC	Red blood cell
RDW	Red cell distribution width
SD	Standard deviation
SOW	Super oxidized water
TGF- β	Transforming growth factor beta
TIMP	Tissue inhibitor of metalloproteinase
WBC	White blood cell

Summary

Diabetic Foot ulcers (DFU) are a common and important complication of diabetes mellitus and the most common foot condition leading to lower extremity amputation. DFU is characterized by delayed healing and this delay is hypothesized to be a result of dysregulated immunity. Superoxidaized water (SOW) showed potential to cure DFU in relatively short time. The aim of this study was to characterize the DFU patients in terms of some blood and immunological indices and to study the effect of superoxidized treatment on those indices.

This study includes 58 patients with type 2 diabetes mellitus and have DFU (43 males and 15 females). This study was performed in Iraq in 2022 AD. Patients attending to Al-Kafeel Super Specialty Hospital in Karbala and private clinic in the period extending from 9 April 2021 to 6 September 2021. All patients were diagnosed with diabetic Foot ulcer based on clinical diagnosis and X-ray by specialists. In this study diabetic Foot ulcer patients had visited the clinic regularly and received regular treatment by Superoxidized water. Of the 58 patients, 26 patients had results before and after treatment, out of those 26 patients, who received treatment with SOW 24 had recovered completely and only 2 did not recover.

The demographic and clinical data were collected from patients and /or their families through a questionnaire. Sera and whole blood were collected from each participant noting that the sera were used to determine serum IL-1β, IL-17 and TGF-β levels for all samples, while whole blood was used for complete blood count, glycosylated haemoglobin and C-reactive protein and the data were statistically analysed by software SPSS version 25.

Most of the patients (79.3%) were above the age of 50 years and most of them (74.1%) were male. Most of the patients (93.1%) had poor glycaemic control.

These results indicate that DFU is largely of older male patients with poor glycaemic control. Regarding the characteristics of ulcers, most of patients (81%) had ulcers for more than 20 weeks' duration. Most of the ulcers (65.5%) associated with swollen feet and the area of ulcers in most of the patients (69%) were equal or less then15cm^2. Most of the patients (87. 9%) present with single ulcers and ulcers were new (72.4%) were painless (79.3%) and non-bleeding (62.1%).

This study showed an increase in both total WBCs counts and neutrophils counts in ulcer patients with bone involvement (13.9×10^3 cell/mm^2, 10.19 $\times10^3$ cell /mm^2 respectively) and ulcers with gangrene (14.59×10^3 cell/mm^2, 11.35×10^3 cell/mm^2 respectively) in comparison to superficial ulcers (7.39 $\times10^3$ cell/mm^2 and 7.15 $\times10^3$ cell/mm^2 respectively).

However, the increasing lymphocyte counts were significantly associated with increasing in the duration of ulcers (p=0.001). Volar site of ulcers was associated with significant lower haemoglobin and haematocrit in comparison to dorsal site of ulcers (p=0.025).

Interestingly, ulcers with gangrene were associated with increase in RBC count (mean= 9.91 $\times10^{12}$ cell/L). Mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and red cell distribution width (RDW) are affected by the area of ulcers. Indeed, there was inverse relation between ulcer area and MCH (p=0.007), MCHC (p=0.025)

and RDW (p=0.005). Furthermore, ulcers with bad smell were Significantly associated increased MCH (p=0.004).

Regarding the platelets indices volar site of ulcers was associated with lower platelet large cell count (P-LCC) (p=0.038) Interestingly, increasing ulcer duration was associated with significant increase in platelets count (p= 0.013). Furthermore, higher PCT was associated with presence of multiple ulcers (p=0.005) and recurrence of the ulcers(p=0.05) In addition presence of painful ulcer was associated higher mean platelet volume (MPV) (p=0.025).

High levels of HbA1c were found to be associated with increasing in the ulcer area (p=0.016).

Regarding the correlation of the studied cytokines with the ulcers characteristics, only IL-1β levels were found to be correlated with depth of ulcers. In this regard, higher IL-1β levels were significantly increase with the increase in the depth of ulcer (p=0.049). Regarding the correlations of the cytokines levels and blood indices, this study reported several significant associations That could be good subjects for further studies. High IL-1β levels were significantly associated with low MCHC (p=0.026) but with high levels of RDW (p=0.052). These results shows That IL-1β has direct effects on multiple blood indices in DFU patients. on the other hand, a significant negative correlation was seen in this study between IL-17 levels and HbA1c concentration.

In this study 26 patients were successfully followed up until the final outcomes of The treatment by superoxidized water, 24 of them were cured completely. The data before treatment were compared to that after treatment.

In this regard total WBCs counts and neutrophils count were significantly reduced after the treatments, whereas lymphocytes were significantly elevated.

Cytokines were also reduced after treatment however this reduction was only significant in case of IL-1β ($p=0.043$).

In addition, in this study duration of recovery was reported to be affected by several factors. Duration of recovery reduced in association with reduced ulcer duration ($p= 0.001$) and increase in lymphocyte count ($p=0.001$).

Chapter One

Introduction & literature Review

1.1. Introduction

Diabetic foot ulcers are an injury to all layers of skin, necrosis or gangrene that usually occur on the soles of the feet of people with diabetes (Rosyid 2017). Diabetics face a 25% chance of developing a diabetic foot ulcer (Haji Zaine, Burns *et al*. 2014). There are multiple causes that lead to the break in the skin, and once the ulcer has developed, several factors impede its prompt healing. The causes of the break in the skin will vary from person to person, and the causes of the delay in healing will not only vary between people but also vary with time, different factors may be dominant in delaying healing at different stages in the healing process (Monteiro-Soares, Boyko *et al*. 2020).

Effective treatment of any one ulcer depends on the clinician being aware of which causes are most important at any given time and selecting an appropriate management strategy (Monteiro-Soares, Boyko *et al*. 2020).

DFU is characterized by poor wound healing, which can lead to infection and, finally, amputation. DFU is the cause of 85 percent of amputations in the developed world (Morey, O'Gaora *et al*. 2019).

Generally, every wound goes through various stages of healing, depending on the type of wound and its severity. In DFU, because of inability to use glucose properly, circulation and nerve are damaged and can cause wounds to heal slowly. Wound healing is a multi-phased process that includes inflammatory, proliferative, and remodeling phases. These stages function differently and have separate histological properties. To accomplish regulatory activities, they rely on the interplay of cytokines, growth factors,

chemokines, and chemical mediators from cells. (Eming, Martin *et al*. 2014, You and Han 2014). Tissue development, increased mechanical stress, and cytokine expression, such as TGF- β, cause fibroblasts to differentiate into myofibroblasts, which express a smooth muscle actin with contractile function, allowing these cells to migrate from the edges to the center of the wound for wound contraction. (Penn, Grobbelaar *et al*. 2012).

Cytokine-based strategies are suggested to resolve chronic wounds. Cytokines are obviously critical during the time course of wound healing, making them an attractive target for therapeutic development. For example, IL-1 β is part of a proinflammatory positive feedback loop that sustains a persistent proinflammatory wound macrophage phenotype, contributing to impaired healing of diabetic wounds(Mirza, Fang *et al*. 2013, Zhao, Liang *et al*. 2016). IL-1 β increases leukocyte recruitment, the release of acute phase proteins, and the permeability of blood arteries. (Dinarello 1984, Basbaum and Julius 2006, Verri Jr, Cunha *et al*. 2006). Inflammation and wound healing may be delayed if the IL-17 family signaling is disrupted (Tanno, Kawakami *et al*. 2017). Chronic wounds may be linked to persistently increased levels of IL-17 family members. Indeed, investigations have demonstrated that IL-17 levels in wound fluid from venous ulcers are higher than the patients' contemporaneously obtained venous blood levels (Bellaver, Bobermin *et al*. 2016, Marzano, Damiani *et al*. 2017).

Superoxidized water (SOW) is a relatively new formulated pH-neutral solution that has a potent disinfectant activity (Aras, Karaman *et al*. 2017). SOW does not cause harmful effect on the human tissues and used for various conditions like wound, skin infections, ulcers and diabetic foot (Aras,

Karaman *et al*. 2017). A SOW debrides necrotic tissue, reduces microbial load, stimulates granulation, and shortens healing time in diabetic foot ulcers without harming normal tissue or causing problems. This hyper oxidized solution is a recommended alternative for diabetic foot ulcer care because of its moistening effect and low toxicity. New controlled trials, however, are needed to thoroughly prove the antibacterial, anti-inflammatory, and wound-healing properties (Chittoria, Yootla *et al*. 2007).

Aim of The Study

The aim of this study was to characterize the inflammatory status of the diabetic foot ulcer patients in terms of some blood inflammatory markers and cytokines and to study the effect of superoxidized water on those parameters.

Objectives

1. Study the relationship of characteristics of ulcer with all of total and differential WBCs counts, RBCs indices, PLTs indices, CRP, HbA1c and some cytokines (IL-1β, IL17 and TGF- β).
2. To detect the correlation for each of total and differential WBCs counts, RBCs indices, PLTs indices, CRP and HbA1c with peripheral cytokine levels.
3. Determine and comparing the total and differential WBCs counts, RBCs indices, PLTs indices, CRP and some cytokines (IL-1β, IL17 and TGF- β) before and after using superoxidized water.
4. Study the ulcer recovery according to duration of treatment with superoxidized water.

1.2. literatures Review

1.2.1. Diabetes Mellitus

Diabetes mellitus (DM) is a metabolic disease, involving inappropriately elevated blood glucose levels. Diabetes is a worldwide epidemic. With changing lifestyles and increasing obesity, the prevalence of DM has increased worldwide. The global prevalence of DM was 425 million in 2017. According to the International Diabetes Federation (IDF), in 2015, about 10% of the American population had diabetes. Of these, 7 million were undiagnosed. With an increase in age, the prevalence of DM also increases. About 25% of the population above 65 years of age has diabetes. DM is caused by, in the islets of Langerhans in the pancreas, there are two main subclasses of endocrine cells: insulin-producing beta cells and glucagon secreting alpha cells. Beta and alpha cells are continually changing their levels of hormone secretions based on the glucose environment. Without the balance between insulin and glucagon, the glucose levels become inappropriately skewed. In the case of DM, insulin is either absent and/or has impaired action (insulin resistance), and thus leads to hyperglycemia (Lenzen, 2021).

1.2.2. Types of Diabetes Mellitus

1.2.2.1. Type 1 Diabetes Mellitus

Type 1 diabetes is an autoimmune condition. This means the immune system mistakenly attacks and destroys the beta cells in pancreas that produce insulin. The damage is permanent (Roep, Thomaidou *et al*. 2021). The condition is usually diagnosed in children and young people, so it used to be called juvenile diabetes. A condition called secondary diabetes is like type 1,

but beta cells are wiped out by something else, like a disease or an injury to pancreas, rather than by immune system (Gupta, Manchanda *et al*. 2021).

1.2.2.2. Type 2 Diabetes Mellitus

One of the most common metabolic illnesses, Type 2 Diabetes Mellitus (T2DM), is caused by a combination of two basic factors: inadequate insulin secretion by pancreatic -cells and a failure of insulin-sensitive tissues to respond adequately to insulin. Because insulin release and activity are critical for glucose homeostasis, the molecular mechanisms involved in insulin synthesis, release, and detection are all closely controlled. Defects in any of the mechanisms involved in these processes might cause a metabolic imbalance, which is what causes the disease to develop (Galicia-Garcia, Benito-Vicente *et al*. 2020).

T2DM is a rapidly rising epidemic worldwide owing to a multifactorial combination of genetic and environmental factors (Sirdah and Reading 2020). Following exposure to an environment characterized by sedentary behavior and high calorie intake, genetic variables take influence. Genome-wide association studies have revealed common glycemic genetic variations for T2DM, but they only account for 10% of overall phenotypic variance, implying that rare variants are essential (Grarup, Sandholt *et al*. 2014).

T2DM accounts for around 90% of all cases of diabetes. The response to insulin is diminished, and this is defined as insulin resistance. During this state, insulin is ineffective and is initially countered by an increase in insulin production to maintain glucose homeostasis, but over time, insulin production decreases, resulting in T2DM. T2DM is most commonly seen in persons older

than 45 years. Still, it is increasingly seen in children, adolescents, and younger adults due to rising levels of obesity, physical inactivity, and energy-dense diets (Goyal and Jialal 2018).

1.2.2.3. Gestational Diabetes Mellitus

Gestational diabetes mellitus (GDM) is characterized by glucose intolerance first manifesting during pregnancy and is an important driver for fetal macrosomia, birth injury, and neonatal metabolic alterations--particularly when persistent maternal hyperglycemia exists (Cleary, Thung *et al*. 2021). The prevalence of GDM is rapidly rising, ranging from 9 to 26% of pregnancies throughout the world, and is highest in ethnic groups that have a higher incidence of Type 2 diabetes mellitus (Johns, Denison *et al*. 2018). GDM is caused by carbohydrate intolerance due to abnormalities in at least 3 aspects of fuel metabolism: insulin resistance, impaired insulin secretion, and increased hepatic glucose production (Mottola and Artal 2016). Although diabetes usually remits after pregnancy, up to 70% of individuals diagnosed with GDM go on to develop T2DM later in life, particularly if obesity is present (Cleary, Thung *et al*. 2021).

1.2.3. Complications of Type 2 Diabetes Mellitus

A- The Microvascular complication of diabetes (Retinopathy, Neuropathy and Nephropathy)

B- The Microvascular complication of diabetes (Peripheral artery disease, coronary artery disease and cerebrovascular disease)

C- Another type of diabetic complication (Diabetic cardiomyopathy and Diabetic foot) (Mezil and Abed 2021)

1.2.4. Diabetic Foot Ulcer

The term “diabetic foot ulcer” (DFU) is imprecise. It describes the presence of a break in the skin of the foot in a person with diabetes (Monteiro-Soares, Boyko *et al*. 2020). The most common, painful, and disabling effects of diabetes mellitus are DFU (Coffey, Mahon *et al*. 2019). Each year, these illnesses impact 3% of the diabetic community, and this number is predicted to climb as diabetes becomes more prevalent (Krzyszczyk, Schloss *et al*. 2018). Diabetics have a 25% chance of acquiring DFU during their lifetime (Aras, Karaman *et al*. 2017). Every 20 seconds a limb is amputated somewhere in the world due to diabetes (Cassidy, Reeves *et al*. 2021). DFU is the result of various factors like peripheral vascular disease, peripheral neuropathy, trauma, foot deformities, arterial insufficiency and impaired resistance to infection. It can be caused by both type 1 and type 2 diabetes)Noor, Zubair *et al*. 2015). DFU is associated with impaired wound healing, due to inappropriate cellular and cytokines response, infection, poor vascularization, and neuropathy (Ezhilarasu, Vishalli *et al*. 2020).

1.2.5. Epidemiology of DFU

Diabetic foot illness is a serious consequence of diabetes that causes significant morbidity, death, financial expenditures, and a lower quality of life. In patients with diabetes, diabetic foot disease often manifests as ulcers, infection, and Charcot foot in the presence of peripheral neuropathy or peripheral vascular disease, and is the most common cause of lower-extremity

amputations (Martins-Mendes, Monteiro-Soares *et al*. 2014, Jupiter, Thorud *et al*. 2016, Zhang, Lu *et al*. 2017).

According to the International Diabetes Federation's 2015 prevalence figures, foot ulcers affect 9.1 million to 26.1 million people with diabetes globally each year. The proportion of people with diabetes who have a history of foot ulceration is higher than the proportion who have an active ulcer; 3.1 to 11.8 percent of people with diabetes, or 12.9 million to 49.0 million people worldwide, have a history of foot ulceration, with 1.0 million to 3.5 million in the United States alone. The lifetime incidence of foot ulcers in people with diabetes was previously expected to be 15 to 25 percent, but new evidence shows that between 19 and 34 percent of people with diabetes are likely to be affected (Atlas 2015, Armstrong, Boulton *et al*. 2017). Several prior studies in Indonesia found the rate to be between 17 and 32 percent, with amputations accounting for 15 to 30 percent of the total. The one-year survival rate after amputation can be as high as 14.8 percent, but the percentage rises to 37 percent in the following three years (Syafril, 2018).

Chronic wound complications are becoming more of a global concern, and their impact is posing a threat to public health and the economy. Obesity, decreased wound healing, and chronic DFU development are all linked to the rising global prevalence of diabetes, which affects all populations. Even if obesity levels remain constant, the number of individuals with diabetes is expected to climb from 171 million in 2000 to 366 million in 2030, with this "diabetic epidemic" continuing.

Soft tissue and boney wound infections, increasing tissue loss, lower extremity amputations, hastened cardiovascular disease, and patient mortality are all complications of diabetic wounds. The lifetime risk of lower extremity ulceration in diabetic patients is as high as 25%, with approximately 7% of diabetic neuropathic foot ulcers leading to amputation (Hoke, Ramos *et al*. 2016).

1.2.6 Etiology of DFU

Complex metabolic pathways contribute to the development of neuropathy and peripheral artery disease in people with uncontrolled diabetes (Tripathi and Gupta 2018). Foot ulcers can be caused by a loss of feeling induced by peripheral neuropathy, ischemia caused by peripheral artery disease, or a combination of these factors. According to a systematic review (78 papers from 84 cohorts), diabetes-related peripheral neuropathy has a prevalence of 0.003-2.8% while diabetes-related peripheral arterial disease has a prevalence of 0.01-0.4% (Lazzarini, Hurn *et al*. 2015). The factors that contribute to foot problems are depicted in Figure 1-1. Diabetes is also linked to Charcot arthroplasty, a condition in which the bones, joints, and soft tissues of the ankle and foot deteriorate over time. Charcot's arthropathy is a diabetes-related arthropathy with a reported prevalence of 0.08 percent to 13%, however there are no high-quality epidemiological research on Charcot's foot (Frykberg, Belczyk *et al*. 2008, Rogers, Frykberg *et al*. 2016). Inflammation is caused by a combination of neuropathy, improper foot loading, recurrent micro trauma, and metabolic abnormalities of bone, resulting in osteolysis, fractures, dislocations, and deformities (Mishra, Chhatbar *et al*. 2017).

Management of DFU focused on prevention of amputation in lower limb with 3 strategies, namely: identification of risk factor of DFU, acute treatment and prevent complication. Therapy performed on the wound of the DFU is carried out constantly with the type of action that depends on the severity of the ulcer and the presence or absence of ischemia. The basis of DFU therapy are necrotomic/debridement, reducing offloading, managing the infection by diagnosing the type of bacteria and providing an adequate antibiotic, ulcer treatment using wound dressing clean and moist (Rosyid 2017).

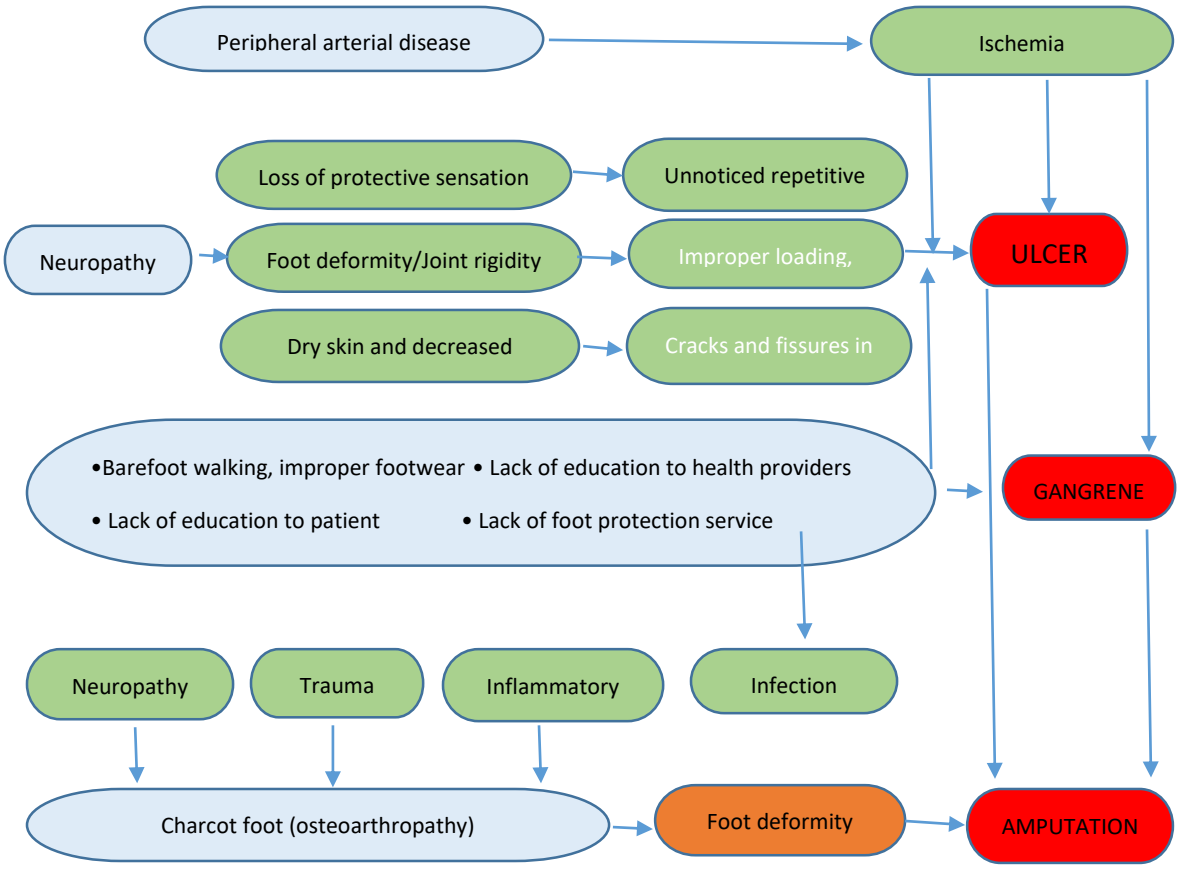

Figure 1-1 Risk factors and mechanism for foot ulcer amputation (Lazzarini, Hurn et al. 2015).

1.2.7. Risk Factor of DFU

Because everyday tissue hypoxia can promote vascular and neuropathic issues in diabetic patients' lower extremities, previous research have identified smoking as a risk factor for diabetic foot ulcers (Zhang, Lu *et al*. 2017). The study also found that diabetic foot was more common in male diabetes patients than female diabetic patients. One explanation for this gender disparity could be males' involvement in more physical labor (Moura Neto, Zantut-Wittmann *et al*. 2013, Ibrahim ,2017).

1.2.8. Pathophysiology

Diabetic foot ulcers (DFUs) have a variety of causes. A foot ulcer is caused by a combination of factors. Several factors must come together to generate a strong enough impact for ulceration to occur (Syafril, 2018). A diabetic ulcer normally progresses in three phases. The formation of a callus is the first stage. Neuropathy generates callus, motor neuropathy causes physical deformities, and sensory neuropathy causes sensory loss, which leads to continued damage. Another significant factor is skin drying caused by autonomic neuropathy. Finally, repeated stress to the callus causes subcutaneous bleeding, which leads to the callus eroding and becoming an ulcer (Oliver and Mutluoglu 2019).

Diabetes mellitus causes severe atherosclerosis of the tiny blood arteries in the legs and feet, leading to vascular compromise and diabetic foot infections. Healing is slowed as a result of the lack of blood supply to the wound, eventually leading to necrosis and gangrene (Oliver and Mutluoglu 2019).

1.2.9. Diagnosis of DFU

Patient workup for diabetic ulcers includes blood tests, radiography, ankle-brachial index and toe pressure, pulse-volume recording, ultrasonography, computed tomography (CT) scanning or magnetic resonance imaging (MRI), bone scans, and angiography(Stacy, 2021).

Examining the peripheral pulses of the feet, checking for any anatomical anomalies, the presence of callus, evidence of vascular insufficiency, which may suggest hair loss, muscular atrophy, and the location of the ulcer are all part of the clinical examination. Examine with a monofilament for the presence of purulence, scabs, and indications of neuropathy (Oliver and Mutluoglu 2019).

1.2.10. Classification of DFU

The ulcer should be staged after it has been diagnosed. The diabetic foot has been classified in a variety of ways. However, the Wagner-Ulcer Classification System and the University of Texas Wound Classification System are the most often used classification systems (Syafril, 2018). Wagner divides wounds into six categories based on their depth: Ulcer on the surface, A deep ulcer that affects a tendon, bone, or joint. Osteomyelitis or a deep ulcer with an abscess, Gangrene of the forefoot and Gangrene of the Whole Foot (Monteiro-Soares, Boyko *et al*. 2020).

This categorization has been criticized for grading only the depth of the ulceration and not taking into account other known parameters that affect the outcome. The University of Texas classification is based on the wound's final

outcome, which includes not only the depth but also the type of infection and ischemia (Bravo-Molina, Linares-Palomino *et al*. 2018).

1.2.11. Treatment of DFU

DFU treatment accounts for almost one-third of the total cost of diabetes care, which in 2012 was projected to be $176 billion in direct healthcare spending in the United States (Everett and Mathioudakis 2018). Despite the high cost of healthcare, about 20% of patients have unhealed DFUs after a year (Prompers, Schaper *et al*. 2008). Following wound clearance, DFUs are prevalent, with a recurrence rate of around 40% of patients within one year (Najafi, Reeves *et al*. 2020).

Local wound care with surgical debridement, dressings that promote a moist wound environment, wound off-loading, vascular assessment, active infection treatment, and glycemic control are among the therapeutic pillars (Lipsky, Berendt *et al*. 2012, Bakker, Apelqvist *et al*. 2016, Lavery, Davis *et al*. 2016).

Multidisciplinary diabetic foot care is also becoming a mainstay of therapy in addition to these principles. Surgical debridement entails the removal of any necrotic and devitalized tissue, as well as the callus that surrounds it, that is incompatible with recovery. This procedure aids in the production of granulation tissue and re-epithelialization of callused areas while also lowering plantar pressures (Charles, Falabella *et al*. 2012).

There are various non-surgical wound debridement alternatives available that may be beneficial, but there is currently insufficient data to endorse one procedure above others (Everett and Mathioudakis 2018).

Autolytic debridement using hydrogels, enzymatic debridement, bio surgery, and mechanical debridement with hydrotherapy are all examples of non-surgical debridement (Everett and Mathioudakis 2018).

Other methods for diabetic foot care include dressing selection, wound off-loading, and shoe modification. Assessment of the vascular system, Treatment of an active illness, Glycemic control is a term used to describe how well a person's Care that is multidisciplinary, Dressings and topical products, adjuvant therapy Human growth factors, oxygen therapies, negative-pressure wound therapy, acellular bio products Bioengineered skin and skin grafts Systemic therapy and energy-based therapies (Everett and Mathioudakis 2018).

1.2.12. Diabetic Ulcer Healing Process

1.2.12.1. Wound Healing

Generally, in the wound healing process, neutrophils are the first inflammatory cells to move to the wound tissues. They sterilize wounds by killing microbes, and they stimulate other immune cells to protect the host from infection. In contrast, neutrophil-derived proteases cause damage to host tissues, so neutrophils play dual opposite roles in wound healing. The body is a complex and remarkable machine, and the dynamic process of wound healing is a great example of how our body’s different systems, along with the proper wound care products, work together to repair and replace

devitalized tissues. But how, exactly, does our body heal? When the skin is injured, our body sets into motion an automatic series of events, often referred to as the "cascade of healing," in order to repair the injured tissues. The cascade of healing is divided into these four overlapping phases: Hemostasis, Inflammatory, Proliferative, and Maturation as in Figure 1-2 (Ziv-Polat, Topaz *et al*. 2010).

Phase 1: Hemostasis Phase

The goal of hemostasis, the first step of healing, is to stop the bleeding as soon as possible after an injury. The body's emergency repair system, the blood clotting system, is activated during this period, and a dam is formed to prevent the drainage. Platelets come into touch with collagen during this process, causing activation and aggregation. At the center is an enzyme called thrombin, which starts the development of a fibrin mesh, which strengthens the platelet clumps into a stable clot.

Phase 2: Defensive/Inflammatory Phase

If phase 1 is largely concerned with coagulation, the Defensive / Inflammatory Phase is concerned with eliminating bacteria and clearing debris, effectively prepping the wound bed for new tissue growth. Neutrophils, a type of white blood cell, enter the wound during Phase 2 to destroy bacteria and remove debris. The population of these cells peaks between 24 and 48 hours after damage, and then rapidly declines after three days. As the white blood cells depart, macrophages, specialized cells, enter to continue removing debris. These cells also emit growth hormones and proteins that attract immune system cells to the wound, allowing tissue repair

to proceed more quickly. This phase usually lasts four to six days and is characterized by edema, erythema (skin reddening), heat, and pain.

Phase 3: Proliferative Phase

After the wound has been treated, it enters phase 3, the Proliferative Phase, in which the goal is to fill and cover the wound.

There are three distinct steps in the Proliferative phase: 1) filling the wound, 2) contraction of the wound edges, and 3) covering the wound (epithelialization).

Shiny, deep red granulation tissue fills the wound bed with connective tissue and new blood vessels during the first stage. The wound margins compress and pull toward the wound core during contraction. Epithelial cells emerge from the wound bed or borders in the third stage and proceed to migrate across the wound bed in a leapfrog pattern until the wound is covered in epithelium. The proliferative phase usually lasts four to twenty-four days.

Phase 4: Maturation Phase

The regenerated tissue gradually increases in strength and flexibility during the Maturation period. Collagen fibers restructure, the tissue remodels and matures, and the total tensile strength increases (though maximum strength is limited to 80 percent of the pre-injured strength). The Maturation phase might range anywhere from 21 days to two years, depending on the damage. The healing process is impressive and complex, and it can be disrupted by local and systemic factors such as wetness, infection, and maceration (local) and age, nutritional condition, and body type (systemic).

When the right healing environment is created, the body heals and replaces devitalized tissue in amazing ways (Ziv-Polat, Topaz *et al*. 2010).

As the skeleton regenerates after a bone damage, developmental mechanisms such as endochondral and intramembranous ossification are replicated. Skeletal healing, in contrast to development, involves inflammation. A variety of inflammatory cells penetrate the wounded region during the early phases of healing, debride the wound, and stimulate the repair process (Lange, Sapozhnikova *et al*. 2010).

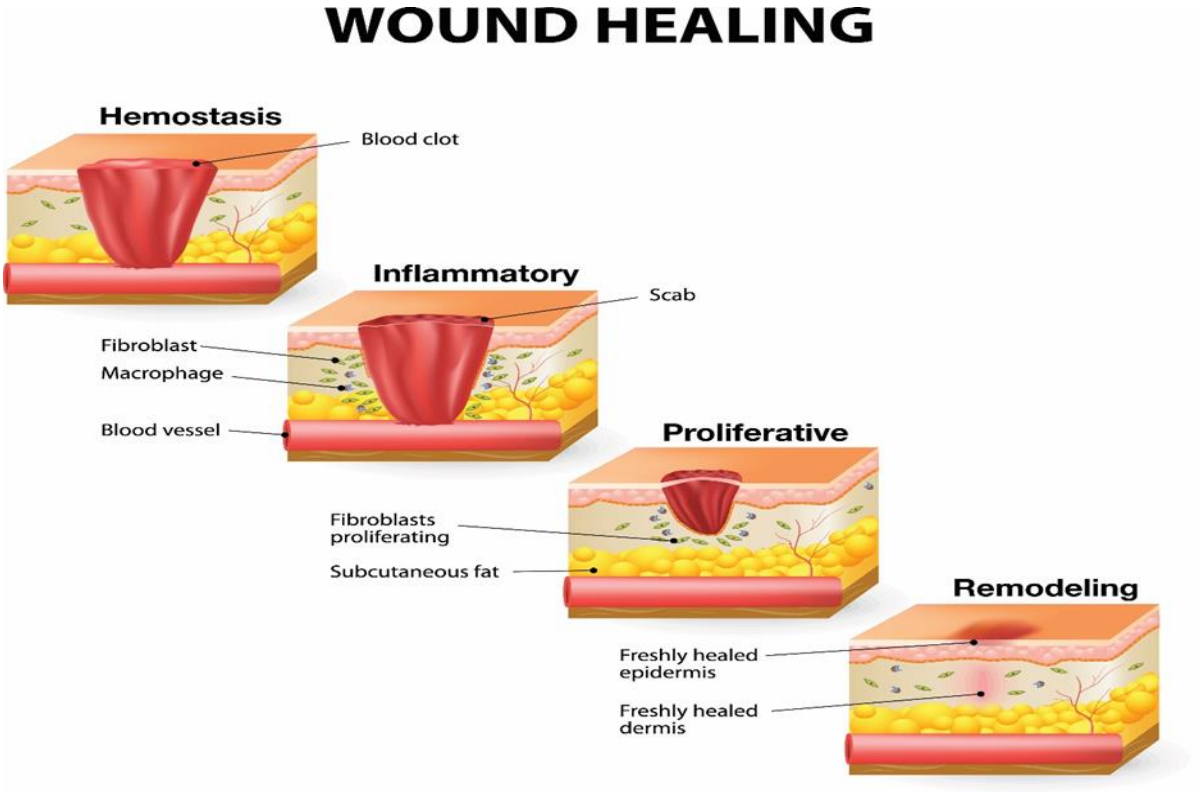

Figure 1- 2 Phases of wound healing (Ziv-Polat, Topaz et al. 2010).

Table 1-1 Factors that Influence Wound Healing (Guo and DiPietro 2010)

Local Factors	Systemic Factors
Oxygenation	Age and Gender
Infection	Sex hormones
Foreign body	Stress
Venous	Ischemia
Sufficiency	Diabetes, keloids, fibrosis, inherited healing abnormalities, jaundice, and uremia are some of the diseases.
	Obesity
	Glucocorticoids, nonsteroidal anti-inflammatory medicines, and chemotherapy are some of the medications used
	Alcoholism and Smoking
	Cancer, radiation therapy, and AIDS are all immune-compromised disorders
	Nutrition

1.2.12.2. Factors Affecting Wound Healing

Many variables can affect wound healing, producing inappropriate or compromising tissue restoration by interfering with one or more phases of the wound healing process (Guo and DiPietro 2010).

Wound healing can be hampered by a variety of circumstances. The elements that influence repair can be divided into two categories: local and systemic. Local factors are those that have a direct impact on the wound's features, whereas systemic factors are those that affect an individual's overall health or illness status and hence his or her ability to heal. (Table 1-1) shows

many of these elements are interconnected, and systemic factors influence wound healing via local consequences (Guo and DiPietro 2010).

1.2.12.3. Healing of DFU

Many patients with diabetes suffering from wounds that take a long time to heal, heal poorly, or never heal at all. Foot ulceration is among the most common health issues, and its prevalence has increased recently. It is one of the major causes of amputations, particularly in patients with uncontrolled diabetes (Boulton, Armstrong *et al*. 2008).

According to the findings, persons who had surgery for chronic diabetes wounds were more likely to heal completely if they had good blood glucose control at the time of surgery. The function of white blood cells is harmed when blood glucose levels remain consistently high. The immune system's role relies heavily on white blood cells. When white blood cells don't work properly, the body has a harder time fighting infections and healing wounds. Diabetes that is not well controlled might lead to poor circulation and nerve damage. Blood travels more slowly when circulation declines, making it more difficult for the body to provide nutrients to wounds. As a result, the injuries may take a long time to heal or may not heal at all (Das, Pande *et al*. 2013).

Many people who have diabetes also have problems with immune system activation. The number of immune fighter cells sent to heal wounds, and their ability to take action, is often reduced. If the immune system cannot function properly, wound healing is slower and the risk of infection is higher (Frydrych, Bian *et al*. 2018).

1.2.14. Immunopathology

A diabetic's immune system is substantially lower than that of a healthy person (Syafril, 2018) . Hyperglycemia causes macrophages to produce more pro-inflammatory cytokines and chemokines and reduces their ability to phagocytose, which is necessary for inflammation resolution. The cytokines involved in wound healing, on the other hand, were unaffected by the elevated glucose concentration (Morey, O'Gaora *et al*. 2019).

The immune system is compromised by lowered leukocyte activity, inappropriate inflammatory response and the disruption of cellular immunity (inhibition of fibroblast proliferation and impairment of the basal layer of keratinocytes, reducing epidermal cell migration) (Atlas 2015). Leukocyte phagocytosis was significantly reduced in patients with poorly controlled diabetes, and improvement of micro biocidal rates was directly correlated with correction of hyperglycemia (Hobizal, Wukich *et al*. 2012).

Reduced growth factor and cytokine chemotaxis, combined with an excess of metalloproteinase, obstructs normal wound healing by prolonging the inflammatory state. A catabolic condition is created by fasting hyperglycemia and the presence of an open wound. Insulin deficiency leads to negative nitrogen balance, which is generated by gluconeogenesis from protein breakdown. This metabolic inefficiency inhibits protein, fibroblast, and collagen synthesis, leading to severe systemic deficits and nutritional compromise. Serum glucose levels of less than 150 ml/dl have been linked to immune system dysfunction in studies. Infection is poorly tolerated by diabetic patients, and infection has a negative impact on diabetic control. This

cycle results in uncontrolled hyperglycemia, which worsens the host's response to infection (Clayton and Elasy 2009).

Muscle sheaths, tendons, soft tissues of the foot, such as the plantar aponeurosis, and fascia are all susceptible to infection. Furthermore, various parts of the foot are interrelated, making it impossible to prevent infection from spreading from one to the other. Soft tissue infection spreads to the bones, resulting in osteitis. As a result, if an ulcer on the foot is not treated properly, it can lead to gangrene and osteitis/osteomyelitis (Singh, Pai *et al*. 2013).

1.2.15. Cytokines Playing Role in Healing of DFU

1.2.15.1. Transforming Growth Factor Beta(TGF- β)

TGF-beta is a type of cytokine that controls proliferation, cellular differentiation, and other functions in most cells. There are some other names of TGF-beta, for example, Platelet Transforming Growth Factor; Bone-Derived Transforming Growth Factor; Milk Growth Factor and so on. TGF-beta, is a factor synthesized in a wide variety of tissues. It acts synergistically with TGF-alpha in inducing phenotypic transformation and can also act as a negative autocrine growth factor (Malik, Mohammad *et al*. 2013).

TGF-beta has a potential role in embryonal development, cellular differentiation, hormone secretion, and immune function (Khalil, 1999). Increased level of TGF-β is prominent in the proliferative phase of wound healing (Malik, Mohammad *et al*. 2013). TGF- β is a chemoattractant for neutrophils and monocytes early in the healing process, as well as a stimulator of monocyte-to-macrophage differentiation, fibroblast proliferation, and

extracellular matrix formation later on. (Klass, Grobbelaar *et al*. 2009). Platelets, keratinocytes, resident macrophages, and fibroblasts all secrete it after an injury (Faler, Macsata *et al*. 2006). As a result, TGF- β has a biphasic expression pattern during normal wound healing, peaking within a few hours and then declining after 5 days (Brunner, Blakytny *et al*. 2004).

This cytokine works by attaching to, heterodimer zing, and phosphorylating its receptor, which then phosphorylates Smad proteins, which subsequently translocate to the nucleus and regulate target gene expression by binding to promoter regions (Shi and Massagué 2003).

As a result, the reduced concentration of TGF- seen in diabetic wounds may delay wound healing by inhibiting the proliferative phase from growing and allowing the matrix metalloproteinase to disintegrate the extracellular matrix. Studies have revealed that injecting a TGF- β expressing plasmid into the skin promotes wound closure considerably (Chesnoy, Lee *et al*. 2003, Lee, Chesnoy *et al*. 2004). TGF- β applications corresponded with better healing in a blinded trial of human diabetic neuropathic ulcers when compared to controls alone. (Jeffcoate, Price *et al*. 2004). Because the latent form of TGF-β attaches to the extracellular matrix, it has been claimed that it may be a more effective in vivo therapeutic than active TGF- β due to its longer half-life. (Mi, Rivière *et al*. 2007).

The remodeling phase manifests itself most visibly at the conclusion of the granulation tissue production step. Tissue development, increased mechanical stress, and cytokine expression, such as TGF-β, cause fibroblasts to differentiate into myofibroblasts, which express a smooth muscle actin with

contractile function, allowing these cells to migrate from the edges to the center of the wound for wound contraction (Penn, Grobbelaar *et al*. 2012). TGF-β, regulates tissue remodeling as part of its function (Yamamoto, Eckes *et al*. 2001). TGF- β inhibits matrix protein breakdown while enhancing TIMPs formation and lowering MMPs synthesis. Low TGF- β concentrations or inhibition can have a negative impact on the repair process, signaling that something is wrong (Vanezis 2000).

1.2.15.2. Interleukin 1 beta (IL-1 β)

IL-1beta is a pro-inflammatory cytokine and is expressed by many cells including macrophage, NK cells, monocytes, and neutrophils. It belongs to the IL-1 family cluster that includes the IL-1a, and IL1-RN genes. The caspase 1 (CASP1/ICE) gene proteolitically activates IL-1β. This gene is involved in the proliferation, differentiation and apoptosis of cells. Inflammatory hypersensitivity has been found to be the result of IL-1β activation of cyclooxygenase-2 (PTGS2/COX2). IL-1beta has also been associated with wound healing (Lopez-Castejon, Brough *et al*. 2011).

Besides, caspase 1 and IL-1β signaling, as the downstream effector of absence in melanoma 2 (AIM2), enhances the migration of iSCs and accelerates epithelialization (Xiao, Yan *et al*. 2020).

In diabetes, increased IL-1β has been linked to the development of insulin resistance and abnormal healing (Dai, Shen *et al*. 2021).

IL-1β levels are higher in diabetic foot ulcers in humans, but they decrease when the ulcers heal (Oncul, Yildiz *et al*. 2007). Topical IL-1β

therapy on normal tissue linked with increased CXCR2 expression and delayed wound closure in skin explants (Zaja-Milatovic, Richmond *et al*. 2008). Through 10 days of recovery, isolated wound macrophages from diabetes humans and db./db. mice show elevated IL-1β and NALP3 inflammasome components, and inhibiting the inflammasome corresponded with enhanced wound healing (Mirza, Fang *et al*. 2014). Researchers have been attempting to limit the effects of IL-1β for the past decade in the hopes of reducing the chronically inflamed condition of diabetic wounds.

The use of neutralizing antibodies or a receptor antagonist to limit IL-1β function in diabetic wounds has the potential to improve diabetic wound healing (Finucane, Lyons *et al*. 2015). Since IL-1β has a very short half-life in plasma, it makes sense to suggest that protected IL-1β is destined for sites distant to the inflammatory lesion (Lopez-Castejon, Brough *et al*. 2011).

1.2.15.3. Interleukin 17 (IL-17)

IL-17 is one of the best-studied cytokines in immunology, at least in part owing to its involvement in inflammatory pathology(Gooderham, Posso-De Los Rios *et al*. 2015). IL-17 is a pro-inflammatory cytokine secreted by CD4 Th17 and CD8 Tc17 cells (Soutto, Saleh *et al*. 2017). Originally IL-17 was thought to be produced exclusively by T cells (Fossiez, Djossou *et al*. 1996), but it is known to be secreted by a variety of innate cells including macrophages, dendritic cells (DC), natural killer, natural killer T, lymphoid tissue inducer and γδ-T cells (Korn, Bettelli *et al*. 2009).

IL-17 has one of the most restricted synthesis profiles among the cytokines (Mak and Saunders 2006). It is an ancient pro-inflammatory

cytokine with important roles in host defense as well as inflammatory and autoimmune disease (DeFilippo, Ebersole *et al*. 2018). IL-17 is associated with tissue inflammation by release of pro-inflammatory cytokines and stimulation of neutrophil chemotaxis (Moghbeli, Khedmatgozar *et al*. 2021). It is a cytokine that stimulates the recruitment of neutrophils and monocytes into inflamed areas (Mizoguchi and Andoh 2013). IL-17 may be related with diabetic disease and can be used as a marker in diagnosis of DM. Additionally, higher level of IL-17 in DFU group suggests that IL-17 can be used as a pro-inflammatory marker for diabetic foot. High IL-17 levels in patients with DFU may be due to inflammation in the ulceration, deterioration of skin integrity, and various types of bacteria causing infections (Kaleli, Varım *et al*. 2019).

Depending on the cytokine subtype, members of the IL-17 family are released from cells in both the innate and adaptive arms of immunity, as well as a wide spectrum of non-hematopoietic cells such as epithelial cells (Steck, Ritzmann *et al*. 2019). IL-17 family members are secreted in the early stages of inflammation in a variety of tissue types after tissue injury (Isailovic, Daigo *et al*. 2015, MacLeod and Mansbridge 2016). Members of the IL-17 family may initially aid in acute wound healing by stimulating keratinocyte proliferation and antimicrobial peptide synthesis (Reich and Venereology 2012, Isailovic, Daigo *et al*. 2015, Wu, Chen *et al*. 2015, MacLeod and Mansbridge 2016).

Inflammation and wound healing may be delayed if the IL-17 family signaling is disrupted (Tanno, Kawakami *et al*. 2017). Chronic wounds may be linked to persistently increased levels of IL-17 family members. Indeed, investigations have demonstrated that IL-17 levels in wound fluid from

venous ulcers are higher than the patients' contemporaneously obtained venous blood levels, and IL-17 levels in lesion tissue samples from patients with pyoderma gangrenous are higher than normal skin (Ligi, Mosti *et al*. 2016, Marzano, Damiani *et al*. 2017).

1.2.16. Role of Super Oxidized Water (SOW) in Treating DFU

Super-oxidized water (SOW) is a relatively new formulated pH-neutral solution that has a potent disinfectant activity(Aras, Karaman *et al*. 2017). SOW does not cause harmful effect on the human tissues and used for various conditions like wound, skin infections, ulcers and diabetic foot (Altamirano 2006, Eftekharizadeh, Dehnavieh *et al*. 2016).

SOW is generated by exerting electrical current on salty water and conduct electrochemically in aqueous solutions from water and sodium chloride. Water is reduced to oxygen, ozone and other reactive species however, the major ingredient formed during this process is hypochlorite and hypochlorous (Gunaydin, Esen *et al*. 2014). The final result is a blend of high reactive species of chloride and oxygen as depicted in Fig 1-3 (Bergstrom, Abdelkhalek *et al*. 2018).

On marketing, there are a variety of compounds that have different concentrations and pH values (Gunaydin, Esen *et al*. 2014). Although SOW is used mainly for hospital wall’s surfaces and metal health’s equipment, many published data investigated its effect on external surface of body such as in diabetic foot ulcers, burn wounds and skin infections(Aras, Karaman *et al*. 2017). This solution being used for many conditions including nonorganic and organic surfaces(Garg, Rose *et al*. 2007).

The new generation SOW solutions have a pH-neutral with a longer shelf-life (>12 months) than the former super-oxide solutions. These new generation SOW are intended for the topical treatment of infective chronic and acute wounds like diabetic ulcers(Aras, Karaman *et al*. 2017).

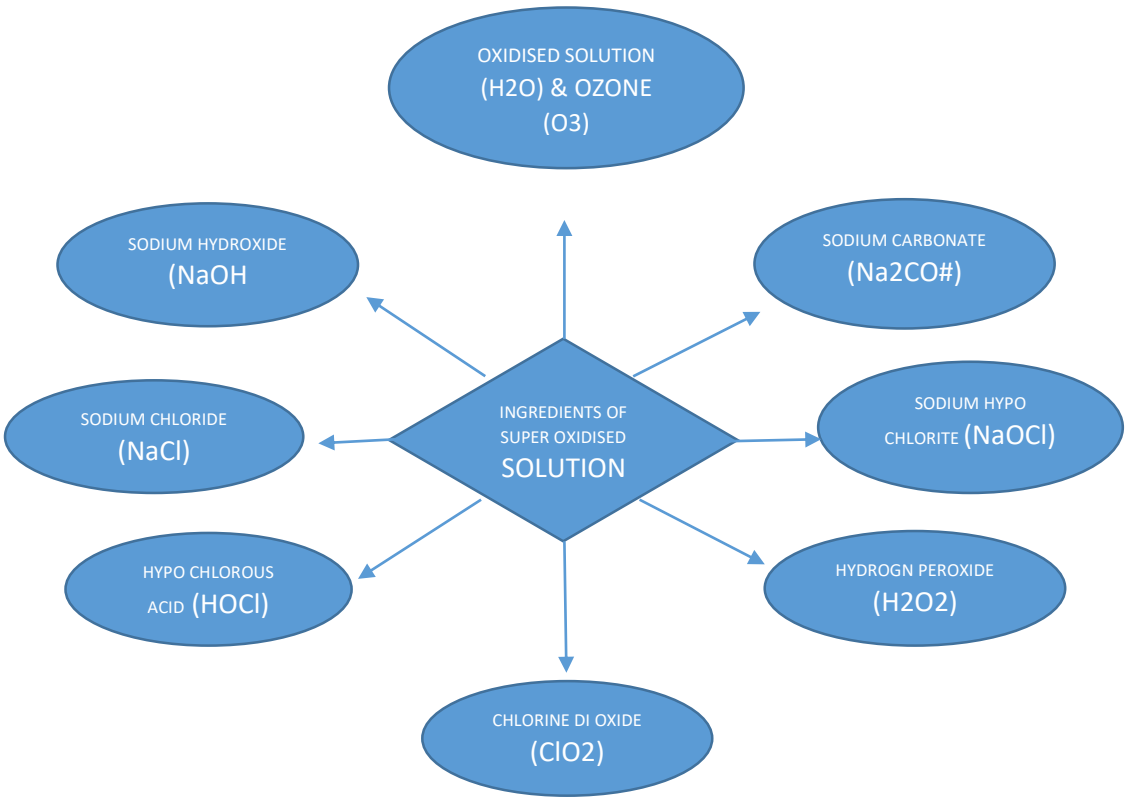

Figure 1-3 Ingredients of Superoxidized water (Bergstrom, Abdelkhalek et al. 2018).

A SOW debrides necrotic tissue, reduces microbial load, stimulates granulation, and shortens healing time in diabetic foot ulcers without harming normal tissue or causing problems. Patients with minor superficial ulcers or those who are not candidates for surgery can be treated conservatively with Super Oxidized Solution alone. This hyper oxidized solution is a

recommended alternative for diabetic foot ulcer care because of its moistening effect and low toxicity. New controlled trials, however, are needed to thoroughly prove the antibacterial, anti-inflammatory, and wound-healing properties (Chittoria, Yootla *et al*. 2007).

Many practitioners have used this solution to treat wounds of diverse etiologies with favorable outcomes. In the literature, no reaction or problem has been recorded (Landa-Solis, Gonzalez-Espinosa *et al*. 2005, Chittoria, Yootla *et al*. 2007). Superoxidized solution has shown early epithelialization process and speedy granulation tissue formation, less time to lesion healing and earlier asepsis (Meera 2014).

Superoxidized water is a very new concept in wound dressing, having first been introduced in 2003. The promising effects on antisepsis, faster wound healing and non-irritable nature of Superoxide solutions have prompted more people to use this solution for dressing diabetic foot ulcers than other treatment such as povidone (Wolvos Tom 2006, Hadi, Khaliq *et al*. 2007).

Chapter Two

Materials and Methods

2. Materials and Methods

2.1. Subjects and Study Design

2.1.1. Subjects

Patients with type 2 diabetes and have DFU who visited Al-Kafeel Super Specialty Hospital in Karbala and private clinics between April and September, 2021 were included in this study. A specialist diagnosed all of the patients with DFU based on clinical diagnosis and X-ray.

In this study، 58 patients with type 2 diabetes and have DFU had visited the clinic regularly and received regular treatment of SOW. Their ages were ranged between forty-five to sixty-one years old.

2.1.2. Inclusion and Exclusion criteria

2.1.2.1. Inclusion criteria: patients with type 2 diabetes and have diabetic foot ulcer

2.1.2.2. Exclusion criteria: patients with diabetic foot ulcer, but they are using superoxidized water or using immunotherapy treatments، or they have another infection or ulcer in another position in their body

2.1.3. Study Design

There are 58 DFU patients in this cross-sectional study (43 males and 15 females). A study design is enrolled in this study is shown in figure (2-1).

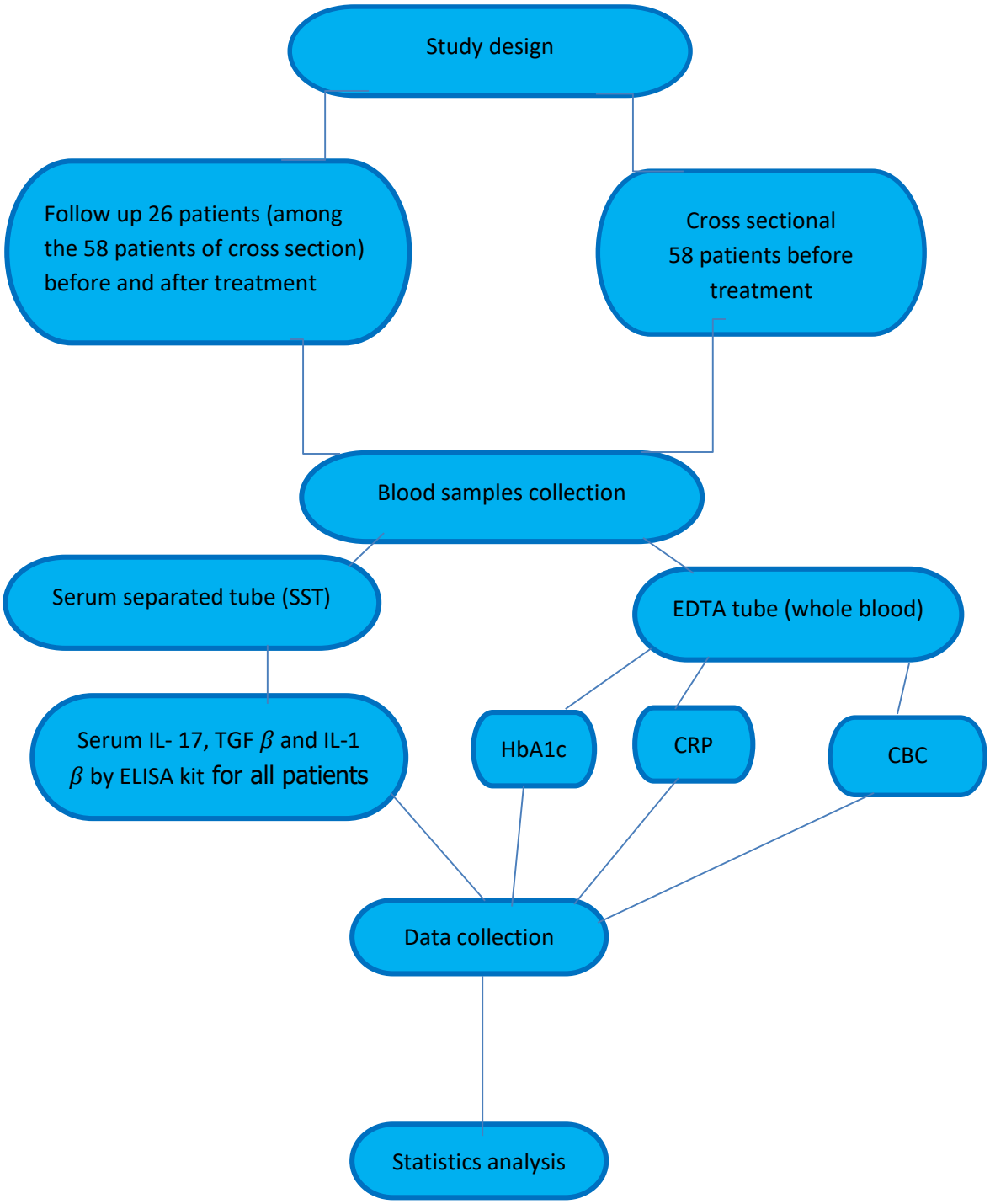

Figure 2-1: A flowchart depicting the study's methodology.

2.2. Ethics Approval

Ethics approval was obtained from Kerbala Health Directorate. In addition, verbal consent was taken from patients. When sampling, health and safety precautions were implemented.

2.3. Data collection

The demographic and clinical data were acquired through a questionnaire-based interview with patients.

2.3.1. Questionnaire

In Al-Kafeel Super Specialty Hospital and private clinic, questionnaires were designed to record and detect how many people were diagnosed with DFU disease, and the Research Committee used worldwide and local standards to collect data from DFU patients.

The questionnaires asked about sex, age, diabetes duration, ulcer features (duration, size, smell, color, and kind), blood pressure, body temperature, temperature and swelling of the foot, and smoking, among other things.

2.4. Blood Samples Collection

Each participant had approximately 4 ml of venous blood collected after cleaning the antecubital fossa with 70% ethanol and then puncturing veins with disposal syringes after applying a tourniquet. For the haematological testing. Two millilitres of blood were discharged into an

EDTA tube. Two millilitres of blood were injected into an SST tube, allowed to clot, and then the serum was separated by centrifugation at 3000 RPM for 15 minutes. The serum was then transferred to an Eppendorf tube and kept at -20°C for use in immunological tests.

2.5. Materials

2.5.1. Equipment and Instruments

The following devices, equipment and instruments were utilized in this study: (Table 2-1 and Table 2-2).

Table 2-1: Manufacturing companies and countries of origin for devices.

Instruments and Equipment	Manufacturing Company	Country
Centrifuge	Hettich	Germany
I Chroma 2	Boditech	Korea
I chamber	Boditech	Korea
Haematology analyser	Swelab Alfa	China
Freezer	Sanyo	Japan
Refrigerator	Panasonic	Korea
Combi wash	Human	Germany
Thermo shaker	Biosan	Latvia
Reax top vortex	Heidolph	Germany
Human reader HS	Human	Germany
Printer	HP laser jet p 2035	Vietnam

Table 2-2: Country of Origin of Equipment and Instruments

Instruments and Equipment	Country
Cold medical box	China
EDTA tube	China
Gel tube	China
Eppendorf tube (0.5 ml & 1.5 ml)	China
Syringes	China
Gloves	China
Face mask	China
Micropipettes (different size)	Japan
Tips (Yellow & Blue)	China
Ruler	China

2.5.2. Biochemical Kits

Table 2-3: Biochemical Kits used in the study

Biochemical Kit	Manufacturing Company	Country
CRP	Boditech	Korea
HbA1c	Boditech	Korea

2.5.3. ELISA Kit

Table 2-4: ELISA Kits used in the study

ELISA Kit	Manufacturing Company	Country
Human Interleukin 17(IL-17) ELISA Kit Cat.No.: YHB1710Hu	YEHUA	China

Human Transforming growth factor β (TGF- β) ELISA Kit Cat.No.:YHB3050Hu	YEHUA	China
Interleukin -1 β (IL-1 β) Human ELISA Kit Cat.No.:DE4437	Demeditec Diagnostics GmbH	Germany

2.5.3.1. ELISA Kit Content of human IL-17

Table 2-5: ELISA kit for detection of human IL-17

Configuration	96 wells	48 wells	Preservation
Instruction	1	1	
sealplate membrane	2	2	
Hermetic bag	1	1	
Coated ELISA plate	12- well * 8 tubes	12- well * 4 tubes	2-8 ˚c
Standard solution (640ng/L)	0.5ml×1	0.5ml×1	2-8 ˚c
Streptavidin-HRP	6ml×1	3ml×1	2-8 ˚c
Stop Solution	6ml×1	3ml×1	2-8 ˚c
Chromogenic reagent A	6ml×1	3ml×1	2-8 ˚c
Chromogenic reagent B	6ml×1	3ml×1	2-8 ˚c

Anti IL-17 antibodies labelled with biotin	1ml×1	1ml×1	2-8 ˚c
Standard dilution	3ml×1	3ml×1	2-8 ˚c
Washing concentrate	(20ml ×30)×1	(20ml ×20)×1	2-8 ˚c

2.5.3.2. ELISA Kit Content of human IL-1 β

Table 2-6: ELISA kit for detection IL-1 β

Reagents	96 test kit	Reconstitution
SORB MT Microtiterplate with 96 anti-IL1β(monoclonal antibodies)coated wells	96 wells	Ready for use
ENZ CONJ Conjugate: HRP labelled anti-IL-1β (monoclonal antibodies)in TRIS-Maleatel buffer with bovine serum albumin and thymol	1 vial 6ml	Ready for use
CAL 0 – 5 Calibrator 0 to 5 (see exact values on QC data sheet) in human serum , benzamidin and thymol	6 vials Lyophil .	Add 2ml distilled water

SAM DIL Specimen Diluent : human serum , benzamidin and thymol	3 vials Lyophil .	Add distilled water (see on the QC data sheet for the exact volume)
WASH SOLN 200X Wash solution (Tris-HCL)	1 vial 10ml	Dilute 200 × with distilled water (use a magnetic stirrer).
CONTROL 1&2 Controls 1 and 2 in human serum , benzamidin ,and thymol	2 vials Lyophil .	Add 2ml distilled water
SUB TMB Chromogen TMB (Tetramethylbenzydine)	1 vial 25ml	Ready for use
STOP SOLN Stopping solution : HCL 1.0N	1 vial 12ml	Ready for use

2.5.3.3. ELISA Kit Content of human TGF-β

Table 2-7: ELISA kit for detection TGF-β

Configuration	96 wells	48 wells	Preservation
Instruction	1	1	
Seal plate membrane	2	2	
Hermetic bag	1	1	
Coated ELISA plate	12- well * 8 tubes	12- well * 4 tubes	2-8 ˚c

Standard solution (4800ng/L)	0.5ml×1	0.5ml×1	2-8 ˚c
Streptavidin-HRP	6ml×1	3ml×1	2-8 ˚c
Stop Solution	6ml×1	3ml×1	2-8 ˚c
Chromogenic reagent A	6ml×1	3ml×1	2-8 ˚c
Chromogenic reagent B	6ml×1	3ml×1	2-8 ˚c
Anti TGF-β antibodies labelled with biotin	1ml×1	1ml×1	2-8 ˚c
Standard dilution	3ml×1	3ml×1	2-8 ˚c
Washing concentrate	(20ml ×30)×1	(20ml ×20)×1	2-8 ˚c

2.6. Methods

2.6.1. Complete Blood Count (CBC)

Blood samples were collected in EDTA tubes, samples were shaken up, and Swelab Alpha automated haematology analysers were used to analyse them as soon as feasible (Swelab, China) to determine the number of white blood cells, red blood cells, and platelets.

2.6.2. Biochemical Tests

In this investigation, a variety of biochemical materials were found. As a result, the I Chroma 2 boditch method was used to detect C-reactive protein (CRP) and glycated haemoglobin (HbA1c).

2.6.3. ELISA

2.6.3.1. Measuring The Concentration of Serum IL-17 in Patients' Blood

The serum IL-17 concentration was determined using the YEHUA Human Interleukin 17(IL-17) ELISA Kit on a BioTek ELx800 automated immunoassay analyzer (BioTek, USA) (Cat.No.: YHB1710Hu).

2.6.3.1.1. Principle of the Test of IL-17

This kit uses enzyme-linked immune sorbent assay (ELISA) based on biotin double antibody sandwich technology to assay Human Interleukin 17(IL-17). Add Interleukin 17(IL-17) to wells that are pre-coated with Interleukin 17(IL-17) monoclonal antibody and then incubate. After incubation, anti IL-17 antibodies labelled with biotin are added to unite with streptavidin-HRP, which forms the immune complex. Then the unbound enzymes are removed after incubation by washing, then substrate A and B are added. The solution colour will turn blue and change to yellow with the effect of acid. The shades of solution and the concentration of Human Interleukin 17(IL-17) are positively correlated and will determine the IL-17 concentration.

2.6.3.1.2. Procedure of The Test of IL-17

a) Dilution of standard solutions: following the chart below dilutions are prepared.

320ng/L	Standard No.5	120ul Original Standard + 120ul Standard diluents
160ng/L	Standard No.4	120ul Standard No.5 + 120ul Standard diluents
80ng/L	Standard No.3	120ul Standard No.4 + 120ul Standard diluent
40ng/L	Standard No.2	120ul Standard No.3 + 120ul Standard diluent
20ng/L	Standard No.1	120ul Standard No.2 + 120ul Standard diluent

b) Sample injection:

1) Blank well: no samples are added, anti-IL-17 antibody labelled with biotin and streptavidin-HRP, adding chromogen reagent A & B and stop solution, each other step operation is the same.

2) Standard solution well: 50ul of standard and streptomycin-HRP 50ul are added.

3) After adding 40ul of sample, 10ul of IL-17 antibodies, and 50ul of streptavidin-HRP, the sample well tube was examined before being capped with a seal plate membrane. To mingle, shacked lightly. After that, it was incubated for 60 minutes at 37°C.

d) Preparation of the washing solution: for later use, the washing concentration is diluted (30X) with distilled water.

e) Cleaning: carefully remove the seal plate membrane, drain liquid, and shake off the rest. Fill each well with washing solution, set aside for 30 seconds, and

then drain. This method should be performed five times before the plate is blotted.

f) Color development was accomplished by adding 50ul of chromogen reagent A to each well, followed by 50ul of chromogen reagent B to each well. To blend, gently shake the container. For color development, incubated for 10 minutes at 37°C away from light.

g) Stop: 50ul of Stop Solution was applied to each well to bring the reaction to a halt (color changes from blue to yellow immediately at that moment).

h) Assay: use blank wells as a control and measure the absorbance (OD) of each well one at a time under a 450 nm wavelength within 10 minutes of adding the stop solution.

i) The standard curve's linear regression equation is computed using the concentrations of standards and their related OD values. The concentration of the related sample is then computed based on the OD value of the samples.

2.6.3.1.3. Reading Results of IL-17

The concentration of standards makes the value OD and abscissa ordinate. Drawing the standard curve on graph paper. According to the OD value of the sample, we have located its corresponding Concentration (which is the concentration of the sample); or the linear regression equation of the standard curve calculated according to the standard concentration and the OD value. Then substitute with the OD value of the sample to calculate its concentration.

2.6.3.2. Measuring The Concentration of Serum IL-1β in Patient's Blood

2.6.3.2.1. Principle of Test of IL-1β

The IL-1β-ELISA is a micro-titer plate-based solid phase Enzyme Amplified Sensitivity immunoassay. The technique employs monoclonal antibodies (MAbs) directed against specific IL-1 β epitopes. The capture monoclonal antibody (MAb 1) coated on the microtiter well and a monoclonal antibody (MAb 2) tagged with horseradish peroxidase react with the calibrators and samples (HRP). The microtiter plate is washed to eliminate unbound enzyme labelled antibody after an incubation period allowing the development of a sandwich: coated MAb 1- human IL-1 β MAb 2 HRP.

A chromogenic reaction is used to quantify bound enzyme-labeled anti bodies. TMB is added to the chromogenic solution and incubated. With the addition of Stop Solution, the reaction is halted, and the microtiterplate is read at the appropriate wavelength. By measuring the absorbance, which is proportional to the IL-1 β concentration, the amount of substrate turnover is estimated calorimetrically.

A calibration curve is produced, and the concentration of IL-1 β in samples is calculated using interpolation from the calibration curve. The combination of an ELISA reader (linearity up to 3 OD units) and a sophisticated data reduction technology (polychromatic data reduction) yields a high sensitivity in the low range and a wide calibration range.

2.6.3.2.2. Procedure of The Test of IL-1β

1. After selecting the required number of strips for the run. The unused strips were resealed in the bag with a desiccant and stored at 2-8°C.
2. The strips are secured into the holding frame.
3. 200 ul of each calibrator, control and sample pipetted into the appropriate wells.
4. Then 50 ul of anti-IL-1 β-HRP conjugate Pipetted into all the wells.
5. Then the wells are incubated for 2 hours at 18-25°C on a horizontal shaker set at 700 rpm ± 100 rpm.
6. Then the liquid is aspirated from each well.
7. Then the plate is washed 3 times by: dispensing 0.4 ml of Wash Solution into each well then aspirating the content of each well
8. Then 200 ul of the chromogenic solution Pipetted into each well within 15 minutes following the washing step.
9. Then microtiter plate incubated for 15 minutes at 18-25°C on a horizontal shaker set at 700 rpm 100 rpm, avoid direct sunlight.
10. Then 100 ul of stop solution pipetted into each well.
11. The absorbencies read at 450 nm and 490 nm within 3 hours and the results were calculated.

2.6.3.2.3. Reading results of IL-1β

A. Polychromatic Reading

1.In this case, the software will do the data processing.
2.The plate is first read at 450 nm against a reference filter set at 650 nm (or 630 nm).

3.A second reading is performed at 490 nm against the same reference filter.

4.The software will drive the reader automatically and will integrate both readings into a polychromatic model. This technique can generate OD's up to 10.

5.The principle of polychromatic data processing is as follows:

- Xi = OD at 450 nm
- Yi = OD at 490 nm
- Using a standard unweighted linear regression, the parameters A & B are calculated: Y= A*X +B
- If Xi< 3 OD units, then X calculated = Xi
- If Xi> 3 OD units, then X calculated = (Yi-B)/A
- A 4 parameter logistic curve fitting is used to build up the calibration curve.
- The IL-1β concentration in samples is determined by interpolation on the calibration curve.

B. Bichromatic Reading

1. Read the plate at 450 nm against a reference filter set at 650 nm (or 630 nm).

2. Calculate the mean of duplicate determinations.

3. On semi-logarithmic or linear graph paper plot the OD values (ordinate) for each calibrator against the corresponding concentration of lL-1 β (abscissa) and draw a calibration curve through the calibrator points by connecting the plotted points with straight lines.

4. Read the concentration for each control and sample by interpolation on the calibration curve

5. Computer assisted data reduction will simplify these calculations. If automatic result processing is used, a 4-parameter logistic function curve fitting is recommended.

2.6.3.3. Measuring The Concentration of Serum TGF-β in Patient's Blood

2.6.3.3.1. Principle of Test of TGF-β

This kit uses enzyme-linked immune sorbent assay (ELISA) based on biotin double antibody sandwich technology to assay Human Transforming Growth factor BCTGF-beta). Add Transforming Growth factor beta (TGF- β) to wells that are pre-coated with Transforming Growth factor β (TGF- β) monoclonal antibody and then incubate. After incubation, add anti TGF- β antibodies labelled 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. The shades of solution and the concentration of Human Transforming Growth Factor beta (TGF-β) are positively correlated

2.6.3.3.2. Procedure of The Test of TGF-β

a) Dilution of standard solutions: following the chart below dilutions are prepared.

2400ng /L	Standard No.5	120ul Original Standard + 120ul Standard diluents

1200ng/L	Standard No.4	120ul Standard No.5 + 120ul Standard diluents
600ng/L	Standard No.3	120ul Standard No.4 + 120ul Standard diluents
300ng/L	Standard No.2	120ul Standard No.3 + 120ul Standard diluents
150ng/L	Standard No.1	120ul Standard No.2 + 120ul Standard diluent

b) Sample injection:

1) Blank well: no samples are added, anti TGF-β antibody labelled with biotin and streptavidin-HRP, then adding chromogen reagent A & B and stop solution, each other step operation is the same.

2) Standard solution well: 50ul standard and streptomycin-HRP 50ul are added.

3) After adding 40ul of sample, 10ul of TGF-β antibodies, and 50ul of streptavidin-HRP, the sample well will be examined. The seal plate membrane was then applied. To mingle, shacked lightly. Incubated for 60 minutes at 37°C.

d) Preparation of the washing solution: for later use, the washing concentration is diluted (30X) with distilled water.

e) Cleaning: carefully remove the seal plate membrane, drain liquid, and shake off the rest. Fill each well with washing solution, set aside for 30 seconds, and then drain. This method should be performed five times before the plate is blotted.

f) Color development: 50ul chromogen reagent A was applied to each well first, shacked gently to mix, and then 50ul chromogen reagent B was added to each well. Then, for color development, incubated for 10 minutes at 37°C away from light.

g) Stop the reaction by adding 50ul Stop Solution to each well (color changes from blue to yellow immediately at that moment)

h) Assay: using blank wells as a baseline, measure the absorbance (OD) of each well one by one using a 450 nm wavelength within 10 minutes of adding the stop solution.

i) The standard curve's linear regression equation is computed using the concentrations of standards and their related OD values. The concentration of the related sample is then computed based on the OD value of the samples.

2.6.3.3.3. Reading Results of TGF-β

Make concentration of standards the value OD and abscissa ordinate. Draw the standard curve on the graph paper. According to the OD value of the sample, locate its corresponding Concentration (which is the concentration of the sample); or calculate the linear regression equation of the standard curve according to the standard concentration and the OD value. Then substitute with the OD value of the sample to calculate its concentration.

2.7. Statistical Analysis

To conduct statistical analysis, data was entered into the Specific Software Statistical Package for the Social Sciences (SPSS) version 25 for Windows (GraphPad Software, San Diego, California, USA), while figures

were created using the Microsoft Office 2016 EXEL software (GraphPad prism Microsoft).

The data was presented as a mean standard deviation. T tests were used to compare two means, while ANOVA was used to compare multiple means. A p value of 0.05 was judged statistically significant, while a p value of 0.001 was regarded very significant. The Chi-square (x2) statistic is used to compare two categorical variables. The pearson correlation was also employed to explain the relationship between IL-17, IL-1 β, and TGF-βlevels and several hematological and biochemical markers.

Chapter Three

Results

3. Results

3.1. Characteristics of the diabetic foot ulcer patients

This study included 58 patients with type 2 diabetes and have DFU. Of the 58 patients, 26 patients had results before and after treatment by SOW, Out of the 26 patients, only 2 patients did not recover.

These patients were attending to Al-Kafeel Super Specialty Hospital in Kerbala and private clinic holy kerbala, in the period extending from 9 April 2021 to 6 September 2021. Patients were diagnosed with DFU based on clinical diagnosis and X-ray by a specialist. DFU patients had visited the clinic regularly for treatment of the wound with Super oxidized water.

Table 3-1 shows the age of the patients which was ranged between 42 and 78 years old. According to age, the patients were grouped into two age groups (≤50 and >50years). The results revealed that the DFU was more frequent in the age group >50 years (79.3%) than other age group.

According to gender, DFU was more prevalent in males, who were 43 (74.1 %), whereas the females were 15 (25.9%).

Regarding smoking habits, 50% of DFU were smoking.

Hypertension was found in 41.4% and those with no hypertension was 58.6%.

DFU patients with insulin treatment were more, 33(56.89%), whereas, the non -insulin dependent patients were 25(43.1%).

DFU patients with poor control were more, 54(93.1%), whereas, only 4 (6.9%) of DFU patients were good control.

Diabetic duration was ranged between 1 and 35 years. This duration was grouped into two groups (≤15 and >15years), DFU patients 31(53.4%) had diabetes for more than 15 years' duration and 27(46.6%) had diabetes for less than 15 years' duration.

DFU patients with swollen feet were more, 38(65.5%), whereas, patients with no swollen feet were 20(34.5%).

Regarding ulcer duration, DFU patients 47(81%) had ulcer for more than 20 weeks' duration and only 11(19%) had ulcer for less than 20 weeks' duration.

DFU patients who had ulcer on volar side of foot were 31 (53.4%), whereas, 27 (46.6%) of DFU patients were having on dorsal side of foot.

Ulcer type is classified into superficial ulcer, deep ulcer, ulcer with bone involvement and gangrene (15.5% ,41.4%, 20.7%, 22.4%) respectively.

Ulcer area ranged between 3 to 50 cm^2, these areas were grouped into two area groups (≤15 and > 15cm^2), the results revealed that most patients (69%) had ulcer area of ≤15 cm^2.

The colour of the ulcer was classified into red, white and black (14 (24.1%), 20 (34.5%), 24 (41.4%)) respectively.

About ulcers smell, 50% of DFU were have a bad smell in their ulcers.

The number of ulcers is classified into single and multiple, a higher prevalence single ulcer was seen.

Table 3-1: Description of characteristics of DFU patients (N=58).

Variables		Frequency	Percentage
Age	≤50	12	20.7%
	>50	46	79.3%
Gender	Male	43	74.1%
	Female	15	25.9%
Smoking status	Smoker	29	50%
	Non smoker	29	50%
Blood pressure	Hypertension	24	41.4%
	Normal	34	58.6%
Insulin	Insulin dependent	33	56.9%
	Insulin non dependent	25	43.1%
HbA1c glycaemic	Good control ≤6.8%	4	6.9%
	Poor control >6.8%	54	93.1%
Diabetic duration/year	≤15	27	46.6%
	>15	31	53.4%
Swollen foot	Present	38	65.5%
	Absent	20	34.6%
Ulcer duration/week	≤20	47	81%
	>20	11	19%
Ulcer sites	Volar	31	53.4%
	Dorsal	27	46.6%
Ulcer type	Superficial ulcer	9	15.5%
	Deep ulcer	24	41.4%
	Ulcer with bone involvement	12	20.7%
	gangrene	13	22.4%
Ulcer area cm^2	≤15	40	69%
	>15	18	31%
Ulcer color	Red	14	24.1%
	White	20	34.5%
	Black	24	41.4%
Ulcer smell	Normal	29	50%
	Bad	29	50%
Ulcer number	Single	51	87.9%
	Multiple	7	12.1%
New or recurrent ulcer	New	42	72.4%
	Recurrent	16	27.6%
Ulcer bleeding	Present	22	37.9%
	Absent	36	62.1%
Ulcer Pain	Present	12	20.7%
	Absent	46	79.3%

Ulcers that occurs for the first time were 42 (72.4%) and recurring ulcers were 16 (27.6%).

In connection with ulcers bleeding, 22(37.9%) of DFU patients were having bleeding in their ulcers, whereas, 36 (62.1%) had with no bleeding in their ulcers.

DFU patients with no pain in foot were more, 46(79.3%), whereas, patients with pain were 12(20.7%).

3.2. Correlation between characteristics of ulcer with total and differential WBCs counts.

Table 3-2 shows the relationships between characteristics of ulcers with total and differential WBCs counts. A significant correlation between ulcer type and total WBC count (p=0.019), where higher WBC counts were found in ulcer with bone involvement (mean=13.9×10^3cell/mm) and ulcers with gangrene (mean=14.59×10^3cell/mm) in comparison to superficial ulcers (mean=10.4×10^3cell/mm), Regarding the differential WBCs counts, neutrophils were significantly correlated with ulcer types (p=0.025), where higher neutrophils counts were found in ulcers with gangrene (mean=11.35×10^3cell/mm) and ulcer with bone involvement (mean=10.19×10^3cell/mm) in comparison to superficial ulcers (mean=7.39×10^3cell/mm) and deep ulcers (mean=7.15×10^3cell/mm).

There was an increase in mean of WBC count in patients with ulcer area more than 15cm^2 compared to patients with ulcer area less than 15cm^2 (14.07×10^3 cell/mm versus 10.89×10^3 cell/mm), however the difference does not reach the statistical significance (p=0.065). A similar trend was seen in neutrophils count, where mean count was higher in patients with ulcer area

more than 15 cm^2 compared to the count in patients with ulcer area less than 15cm^2(10.73×10^3 cell/mm versus 7.87×10^3 cell/mm).

Regarding the ulcer duration calculated by weeks, the duration of an ulcer and the number of lymphocytes in the blood were discovered to have a highly significant relationship (p=0.001), where patients with duration of ulcers more than 20 weeks showed a higher lymphocyte counts than patients with duration of ulcers less than 20 weeks (2.27×10^3 cell/mm versus 3.09×10^3 cell/mm).

There was an increase in mean of WBC count in patients with black ulcer color compared to patients with white and red ulcer color (13.37×10^3 cell/mm, 11.44×10^3 cell/mm and 9.89×10^3 cell/mm) respectively, however the difference does not reach the statistical significance (p=0.084). A similar trend was seen in neutrophils count, where mean count was higher in patients with black ulcer color compared to the count in patients with white and red ulcer color (10.22×10^3 cell/mm, 8.46×10^3 cell/mm and 6.67×10^3 cell/mm) respectively.

Regarding the ulcer smell there was an increase in mean of WBC count in patients with bad smell of ulcer compared to patients with normal smell of ulcer (14.07×10^3 cell/mm versus 10.89×10^3 cell/mm), however the difference does not reach the statistical significance (p=0.065).

The results illustrated that there was an increase in mean of WBC count in patients with multiple ulcers compared to patients with single ulcer (13.04×10^3 cell/mm versus 11.70×10^3 cell/mm), however the difference does not reach the statistical significance (p=0.059). A similar trend was seen in neutrophils count, where mean count was higher in patients with multiple

ulcers compared to the count in patients with single ulcer (10.26×10^3 cell/mm versus 8.55×10^3 cell/mm).

Table 3-2: Relationship between characteristics of ulcer with total and differential WBCs counts.

Variables		WBC $\times10^3$cell/mm		LYM $\times10^3$cell/mm		NEU $\times10^3$cell/mm	
		Mean(±SD) (n=58)	*p* - value	Mean(±SD) (n=58)	*p* - value	Mean(±SD) (n=58)	*p* - value
Ulcer site	Volar (N=31)	12.54±6.68	0.374	2.48±0.79	0.602	9.29±6.15	0.451
	Dorsal (N=27)	11.09±5.45		2.37±0.75		8.14±5.23	
Ulcer type	Superficial ulcer (N=9)	10.4±3.2	0.019	2.54±0.8	0.841	7.39±3.26	0.025
	Deep ulcer(N=24)	9.89±5.91		2.25±0.75		7.15±5.8	
	Ulcer with bone involvement(N=12)	13.9±6.62		2.78±0.76		10.19±6.24	
	gangrene(N=13)	14.59±6.55		2.35±0.76		11.35±5.65	
Ulcer area (cm^2)	≤15(N=40)	10.89±4.76	0.065	2.45±0.78	0.744	7.87±4.3	0.077
	>15(N=18)	14.07±8.16		2.38±0.76		10.73±7.82	
Ulcer duration (week)	≤20(N=47)	11.89±6.54	0.967	2.27±0.74	0.001	8.94±6.1	0.626
	>20(N=11)	11.79±4.18		3.09±0.5		7.99±3.81	
Ulcer color	Red (N=14)	9.89±3.25	0.084	2.69±0.81	0.554	6.67±2.68	0.061
	White (N=20)	11.44±6.54		2.21±0.75		8.46±6.26	
	Black (N=24)	13.37±6.85		2.46±7.45		10.22±6.3	
Ulcer smell	Normal (N=29)	10.4±3.64	0.069	2.5±0.77	0.458	7.32±3.12	0.055
	Bad (N=29)	13.3±7.67		2.35±0.78		10.19±7.25	
Ulcer number	Single (N=51)	11.7±6.39	0.059	2.45±0.8	0.503	8.55±5.94	0.064
	Multiple (N=7)	13.04±3.93		2.24±0.44		10.26±3.67	
New or recurrent ulcer	New (N=42)	11.49±5.81	0.456	2.41±0.78	0.718	8.47±5.35	0.542
	Recurrent (N=16)	12.84±7		2.49±0.76		9.51±6.72	
Ulcer bleeding	Present (N=22)	12.31±7.73	0.667	2.22±0.84	0.114	9.32±7.1	0.557
	Absent (N=36)	11.59±5.02		2.55±0.71		8.41±4.75	
Ulcer pain	Present(N=12)	12.68±8.15	0.606	2.35±0.61	0.699	9.55±7.67	0.594
	Absent(N=46)	11.65±5.58		2.45±0.74		8.55±5.18	

3.3. Correlation of characteristics of ulcer and RBCs indices.

Table 3-3 shows the correlation between ulcer characteristics and RBC indices. A significant correlation between ulcer site and haemoglobin (*p* =0.046), where higher haemoglobin was found in dorsal site of ulcer (mean=11.23g/dl) in comparison to volar site of ulcer (mean=9.16g/dl),

Regarding the differential RBCs indices, haematocrits percentage were significantly correlated with ulcer site (p =0.025), where higher haematocrits percentage were found in dorsal site of ulcers (mean=34.73%) in comparison to volar site of ulcers (mean=30.43%).

The results illustrated that there was an increase in mean of RBCs in patients with gangrenes ulcer type (mean=9.91×10 ^{12} cell/L) in compared to patients with superficial ulcer, deep ulcer and ulcer with bone involvement (4.26×10 ^{12} cell/L, 4.24×10 ^{12} cell/L and 3.85×10^{12} cell/L), however the difference does not reach the statistical significance (p =0.091).

Regarding ulcer area, a highly significant correlation between ulcer area and mean corpuscular haemoglobin MCH (p =0.007), where higher MCH was found in ulcer less than 15 cm ^{2} (mean=27.9pg) in comparison to ulcer more than 15 cm ^{2} (mean=24.55pg), As well in mean corpuscular haemoglobin concentration MCHC appear a significant correlation with ulcer area (p =0.025), where higher MCHC was found in ulcer less than 15 cm^2 (mean=33.56g/L) in comparison to ulcer more than 15 cm^2 (mean=31.03g/L), Also a similar trend was seen in red cell distribution width RDW, a highly significant correlation with ulcer area (p =0.005), where higher RDW was found in ulcer less than 15 cm^2 (mean=50.67) in comparison to ulcer more than 15 cm^2 (mean=41.92).

The study showed a highly significant correlation between ulcer smell and mean corpuscular haemoglobin concentration MCHC (p =0.004), where higher MCHC was found in ulcer with bad smell (mean =35.98g/L) in comparison to ulcer with normal smell (mean =31.19g/L). Also a significant correlation between ulcer smell and red cell distribution width RDW (p

=0.048), where higher RDW was found in ulcer with bad smell (mean =50.87) in comparison to ulcer with normal smell (mean =45.04).

The results showed that there was an increase in mean of RBCs indices in patients with single ulcer compared to patients with multiple ulcers (4.14×10^{12} cell/L versus 3.28×10^{12} cell/L). However, the difference does not reach the statistical significance (p =0.098).

Table 3-3: Relationship between characteristics of ulcer and RBCs indices.

Variables		RBC $\times10^{12}$ cell/L		HGB g/dL		MCV fL		HCT %		MCH pg		MCHC g/L		RDW fL	
		Mean (±SD) (n=58)	p - value	Mean (±SD) (n=58)	p - value	Mean (±SD) (n=58)	p - value	Mean (±SD) (n=5)	p - value	Mean (±SD) (n=5)	p - value	Mean (±SD) (n=58)	p - value	Mean (±SD) (n=58)	p - value
Ulcer site	Volar (N=31)	3.96 ±0.69	0.129	9.16 ±2.09	0.046	79.7 ±9.05	0.241	30.43 ±5.85	0.031	25.79 ±3.46	0.319	32.13 ±2.46	0.874	49.22 ±12.17	0.363
	Dorsal (N=27)	4.24±0.69		11.23 ±1.86		82.29 ±7.29		34.73 ±5.41		26.62 ±2.7		32.03 ±2.42		46.5 ±10.18	
Ulcer type	Superficial ulcer (N=9)	4.26±0.66	0.091	11.33 ±1.95	0.166	81.62 ±5.79	0.843	34.76 ±6.23	0.082	26.67 ±1.85	0.934	32.59 ±1.29	0.875	50.39 ±9.88	0.849
	Deep ulcer(N=24)	4.24 ±0.83		10.79 ±2.19		81 ±8.07		34.15 ±6.49		26.1 ±3.37		32.16 ±2.46		46.67 ±9.3	
	Ulcer with bone involvement(N=12)	3.85 ±0.54		9.67 ±1.73		80.05 ±8.63		30.68 ±4.04		25.2 ±2.92		30.9 ±2.58		46.23 ±13.98	
	gangrene(N=13)	9.91 ±0.53		10.5 ±1.98		81.07 ±10.56		31.65 ±5.3		26.87 ±3.61		32.69 ±2.66		50.23 ±13.32	
Ulcer area (cm^2)	≤15(N=40)	4.39 ±0.68	0.451	10.82 ±1.95	0.378	82.23 ±7.25	0.073	33.12 ±5.6	0.764	27.9 ±2.92	0.007	33.56 ±2.35	0.025	50.67 ±11.52	0.005
	>15(N=18)	4.19 ±0.74		10.3 ±2.25		78 ±9.9		32.62 ±6.51		24.55 ±3.06		31.03 ±2.3		41.92 ±8.11	
Ulcer duration (week)	≤20(N=47)	4.05 ±0.73	0.443	10.71 ±2.1	0.672	81.85 ±7.71	0.078	33.05 ±5.92	0.830	26.53 ±3.06	0.069	32.13 ±2.53	0.751	49.39 ±11.65	0.044
	>20(N=11)	4.23 ±0.56		10.42 ±1.82		76.95 ±9.93		32.8 ±5.73		25.3 ±3.11		32.5 ±1.99		41.83 ±7.03	
Ulcer color	Red (N=14)	4.47 ±0.59	0.194	11.31 ±1.75	0.382	80.52 ±7.74	0.936	35.56 ±5.12	0.221	25.65 ±2.73	0.831	31.66 ±1.94	0.944	49.14 ±12.6	0.297
	White (N=20)	4 ±0.86		10.75 ±1.88		81.67 ±6.42		31.5 ±5.57		26.7 ±2.41		32.73 ±2.05		49.86 ±9.24	
	Black (N=24)	4.05 ±0.54		10.58 ±2.29		80.52 ±10.12		32.67 ±6.17		26.04 ±3.85		31.8 ±2.9		45.68 ±12.05	
Ulcer smell	Normal (N=29)	4.19 ±0.69	0.279	10.85 ±2.12	0.484	82.09 ±7.9	0.287	34.21 ±5.73	0.104	25.98 ±3.03	0.637	31.19 ±2.51	0.004	45.04 ±11.26	0.048
	Bad (N=29)	3.99 ±0.7		10.47 ±1.97		79.75 ±8.68		31.71 ±5.77		26.39 ±3.27		35.98 ±2.01		50.87 ±10.67	
Ulcer number	Single (N=51)	4.14 ±0.71	0.098	10.74 ±2.12	0.391	80.47 ±8.7	0.273	33.24 ±6.03	0.337	26 ±3.2	0.261	32.05 ±2.38	0.767	47.82 ±11.09	0.812
	Multiple (N=7)	3.28 ±0.41		10.03 ±1.25		84.17 ±3.37		30.96 ±4.01		27.43 ±2.4		32.34 ±2.92		48.91 ±13.45	
New or recurrent ulcer	New (N=42)	4.12 ±0.62	0.538	10.68 ±1.85	0.900	79.63 ±8.48	0.055	32.76 ±5.28	0.677	26.02 ±3.42	0.550	32.33 ±2.52	0.213	48.86 ±11.72	0.328
	Recurrent (N=16)	3.99 ±0.89		10.6 ±2.54		84.3 ±7.01		33.49 ±7.28		26.58 ±2.24		31.44 ±2.1		45.59 ±9.95	
Ulcer bleeding	Present (N=22)	4 ±0.88	0.479	10.49 ±2.08	0.636	81.67 ±7.81	0.592	32.45 ±6.44	0.605	26.51 ±3.12	0.532	32.39 ±2.18	0.463	50.41 ±12.77	0.198
	Absent (N=36)	4.14 ±0.57		10.76 ±2.04		80.45 ±8.68		33.28 ±5.51		25.97 ±3.16		31.9 ±2.57		46.46 ±10.15	
Ulcer pain	Present(N=12)	3.81 ±0.71	0.121	10.19 ±2.25	0.381	84.29 ±5.87	0.115	32.16 ±6.38	0.596	26.71 ±2.81	0.510	30.88 ±2.75	0.053	47.98 ±14.69	0.992
	Absent(N=4)	4.16 ±0.68		10.78 ±1.99		80.04 ±8.67		33.17 ±5.75		26.03 ±3.22		32.4 ±2.26		47.95 ±10.4	

3.4. Correlation of characteristics of ulcer with total and differential PLTs indices

Table 3-4 shows the relationship between characteristics of ulcers and platelets indices. The current study showed that there was an increase in mean of plateletcrit (PCT) in patients with dorsal site of ulcers compared to patients with volar site of ulcers (2.15mL/L versus 0.52mL/L). However, the difference does not reach the statistical significance (p =0.194). A significant correlation seen in ulcer site with platelet large cell count P-LCC (p =0.038), where higher P-LCC was found in dorsal site of ulcer (mean =43.48×10 ^{9} cell/L) in comparison to volar site of ulcer (mean =35.42×10 ^{9} cell/L).

Regarding the ulcer duration, a significant correlation was found between ulcer duration and PLT indices (p =0.013), where patients with duration of ulcers more than 20 weeks showed a higher PLT indices than patients with duration of ulcers less than 20 weeks (389.73×10 ^{9} cell/L versus 290.53×10 9 cell/L). Also there was an increase in mean of platelet-large cell ratio P-LCR in patients with ulcer duration less than 20 weeks compared to patients with ulcer duration more than 20 weeks (19.4% versus 16.49%), however the difference does not reach the statistical significance (p =0.299).

However, the decrease in mean of PLT indices in patients with red ulcer color compared to patients with white and black ulcer color (107.71×10 ^{9} cell/L, 308.2×10 ^{9} cell/L and 311.25×10 ^{9} cell/L) respectively. Never the less, the difference does not reach the statistical significance (p =0.926). Regarding PCT, there was higher in patients with black ulcer color in compared with red and white ulcer color (2.23mL/L, 0.99 0.34mL/L and 0.34mL/L) respectively. However, the difference does not reach the statistical significance (p =0.391).

Table 3-4 Relationship between characteristics of ulcer with PLTs indices.

Variables		PLT $\times 10^9$ cell/L		MPV fL		PDW		PCT mL/L		P-LCR		P-LCC	
		Mean (±SD) (n=58)	*p* - value	Mean (±SD) (n=58)	*p* - value	Mean (±SD) (n=58)	*p* - value	Mean (±SD) (n=58)	*p* - value	Mean (±SD) (n=58)	*p* - value	Mean (±SD) (n=58)	*p* - value
Ulcer site	Volar (N=31)	307.13 ±136.86	0.883	8.47 ±1.34	0.174	12.71 ±2.49	0 .118	0.52 ±0.66	0.194	18.49 ±8.76	0.724	35.42 ±9.87	0.038
	Dorsal (N=27)	311.89 ±101.77		8.95 ±1.35		13.73 ±2.36		2.15 ±7.5		19.27 ±7.91		43.48 ±18.31	
Ulcer type	Superficial ulcer (N=9)	336.11 ±101.24	0.097	8.16 ±1.1	0.883	12.59 ±2.36	0.811	0.62 ±1.08	0 .131	15.68 ±6.26	0.886	38.67 ±11.36	0.828
	Deep ulcer(N=24)	253.46 ±100.06		8.87 ±1.37		13.29 ±2.43		0.53 ±0.68		19.13 ±8.11		38.13 ±10.74	
	Ulcer with bone involvement (N=12)	344.75 ±121.9		9.31 ±1.28		14.48 ±2.02		0.79 ±0.1		24.12 ±8.5		42.42 ±26.9	
	gangrene(N =13)	361.31 ±136.83		8.16 ±1.36		12.21 ±2.63		3.57 ±10.78		15.68 ±7.95		38.46 ±7.99	
Ulcer area (cm^2)	≤15(N=40)	306.08 ±114.36	0.762	8.59 ±1.32	0.400	12.9 ±2.47	0.195	1.64 ±6.19	0.435	18.55 ±8.13	0.679	39.65 ±16.84	0.718
	>15(N=18)	316.61 ±137.22		8.92 ±1.45		13.81 ±2.37		0.48 ±0.42		19.53 ±8.9		38.11 ±9.34	
Ulcer duration (week)	≤20(N=47)	290.53 ±107.24	0.013	8.79 ±1.29	0.280	13.23 ±2.34	0.754	1.33 ±5.7	0.882	19.4 ±8.06	0.299	39.81 ±15.99	0.505
	>20(N=11)	389.73 ±146.59		8.29 ±1.59		12.97 ±3.04		1.07 ±1.3		16.49 ±9.32		36.45 ±8.44	
Ulcer color	Red (N=14)	107.71 ±91.14	0.926	8.66 ±1.45	0.912	13.34 ±2.57	0.880	0.99 ±1.21	0.391	17.49 ±8.33	0.633	41.21 ±11.31	0.396
	White (N=20)	308.2 ±116.42		8.79 ±1.42		12.86 ±2.36		0.34 ±0.32		19.56 ±8.32		40.15 ±22.4	
	Black (N=24)	311.25 ±142.33		8.63 ±1.3		13.37 ±2.55		2.23 ±7.95		19.09 ±8.55		37.17 ±7.23	
Ulcer smell	Normal (N=29)	297.41 ±113.11	0.457	8.98 ±1.43	0.110	13.99 ±2.6	0 .011	2.08 ±7.23	0.241	20.41 ±9.11	0.155	39.66 ±9.71	0.807
	Bad (N=29)	321.28 ±128.88		8.41 ±1.23		12.38 ±2.05		0.48 ±0.71		17.39 ±7.25		38.69 ±18.82	
Ulcer number	Single (N=51)	304.35 ±118.59	0.400	8.74 ±1.35	0.473	13.26 ±2.47	0.561	0.59 ±0.77	0.005	19.12 ±8.25	0.509	39.41 ±15.56	0.744
	Multiple (N=7)	345.71 ±140.17		8.34 ±1.45		12.67 ±2.51		6.32 ±14.62		16.89 ±9.19		37.43 ±8.66	
New or recurrent ulcer	New (N=42)	305.14 ±123.35	0.672	8.54 ±1.25	0.171	13.01 ±2.39	0.384	0.47 ±0.63	0.053	18.17 ±8.02	0.317	39.74 ±16.81	0.642
	Recurrent (N=16)	320.38 ±116.93		9.09 ±1.58		13.64 ±2.65		3.39 ±9.66		20.64 ±9.04		37.69 ±7.89	
Ulcer bleeding	Present (N=22)	221.32 ±100.66	0.060	8.7 ±1.37	0.986	13.19 ±2.47	0.988	0.57 ±0.56	0.417	18.14 ±7.69	0.615	38.55 ±10.93	0.804
	Absent (N=36)	332.58 ±127.34		8.69 ±1.37		13.18 ±2.48		1.71 ±6.52		19.29 ±8.75		39.56 ±16.94	
Ulcer pain	Present(N=1 2)	289.19 ±103.68	0.517	9.47 ±1.25	0.025	14.08 ±2.26	0.157	0.7 ±0.84	0.666	21.53 ±7.62	0.214	41.17 ±9.14	0.606
	Absent(N=4 6)	314.65 ±125.35		7.49 ±1.32		12.95 ±2.48		1.43 ±5.78		18.15 ±8.42		38.65 ±16.05	

The study showed a highly significant correlation between ulcer number and PCT (p =0.005), where higher PCT was found in multiple ulcers (mean =6.32mL/L) in comparison to single ulcer (mean =0.59mL/L).

By the way new or recurrent ulcers, a significant correlation between new or recurrent ulcers PCT (p =0.053), where higher PCT was found in recurrent ulcers (mean =3.39mL/L) in comparison to new ulcer (mean =0.47mL/L).

The study showed that there was an increase in mean of PLT indices in patients with nonbleeding ulcer compared to patients bleeding ulcers (332.58×10^{9} cell/L versus 221.32×10^{9} cell/L). However, the difference does not reach the statistical significance (p =0.060).

A significant correlation was found between painful ulcer and mean platelet volume MPV (p =0.025), where patients with pain of ulcers showed a higher MPV than patients with no pain of ulcers (9.47fLversus 7.49fL).

3.5. Correlation of characteristics of ulcer and both of CRP and HbA1c.

Table 3-5 shows the correlation between characteristics of ulcer with serum CRP and HbA1c levels. The results illustrate that there is an increase in mean of C- reactive protein CRP in patients who have ulcer with bone involvement (mean=141.61mg/L) compared to patients with superficial ulcer, deep ulcer and ulcer with gangrene (73.16mg/L, 71.75mg/L and 97.21mg/L). However, the difference does not reach the statistical significance (p =0.235).

It was noticed that CRP, the mean was higher in patients with ulcer area which was more than 15cm ^{2} compared with ulcer area less than 15cm ^{2} (115.54mg/L versus 81.59mg/L). However, the difference does not reach the statistical significance (p =0.226).

A similar orientation was noticed in new or recurrent ulcers, where mean of CRP was higher in patients with recurrent ulcer in comparison with new ulcer (118.81mg/L versus 81.96mg/L), however the difference does not reach the statistical significance (p =0.204).

Like that was seen in ulcer bleeding, where mean of CRP was higher in patients with bleeding ulcer compared with nonbleeding ulcer (111.02mg/L versus 80.58mg/L). Still, the difference does not reach the statistical significance (p =0.255).

Additionally, a similar orientation was seen in ulcer pain, where mean of CRP was higher in patients with painful ulcer compared with no pain in ulcer (113.87mg/L versus 86.46mg/L). Yet, the difference does not reach the statistical significance (p =0.393).

Regarding the HbA1c level, a significant correlation was found between ulcer area and HbA1c level (p =0.016), where patients with ulcer area which are more than 15 cm^2 showed a higher HbA1c level than patients with ulcer area which are less than 15 cm^2 (12.07% versus 10.19%).

Table 3-5: Relationship between characteristics of ulcer and biomarkers CRP and HbA1c.

Variables		CRP mg/L		HbA1c %	
		Mean(±SD) (n=58)	*p* -value	Mean(±SD) (n=58)	*p* -value
Ulcer site	Volar (N=31)	96.23±96.39	0.736	10.53±2.9	0.479
	Dorsal (N=27)	87.41±101.59		11.06±2.67	
Ulcer type	Superficial ulcer (N=9)	73.16±94.27	0.235	10.58±2.93	0.693
	Deep ulcer(N=24)	71.75±100.53		10.35±2.69	
	Ulcer with bone involvement(N=12)	141.61±100.5		12.23±2.28	
	gangrene(N=13)	97.21±86.88		10.36±2.97	
Ulcer area (cm^2)	≤15(N=40)	81.59±89.48	0.226	10.19±2.74	0.016
	>15(N=18)	115.54±114.2		12.07±2.5	
Ulcer duration (week)	≤20(N=47)	94.37±100.56	0.722	10.51±2.7	0.129
	>20(N=11)	82.54±90.4		11.93±2.97	
Ulcer color	Red (N=14)	97.24±112.56	0.950	11.36±2.83	0.306
	White (N=20)	83.1±97.99		10.82±2.93	
	Black (N=24)	96.68±92.91		10.4±2.69	
Ulcer smell	Normal (N=29)	86.18±102.15	0.648	11.15±2.77	0.307
	Bad (N=29)	98.08±95.23		10.4±2.8	
Ulcer number	Single (N=51)	93.93±101.93	0.710	10.76±2.85	0.921
	Multiple (N=7)	79.03±67.38		10.87±2.45	
New or recurrent ulcer	New (N=42)	81.96±95.37	0.204	10.8±2.75	0.918
	Recurrent (N=16)	118.81±103.1		10.71±2.95	
Ulcer bleeding	Present (N=22)	111.02±111.8	0.255	10.64±2.85	0.777
	Absent (N=36)	80.58±88.35		10.85±2.78	
Ulcer pain	Present(N=12)	113.87±115.3	0.393	11.44±2.58	0.356
	Absent(N=46)	86.46±93.95		10.6±2.81	

3.6. Correlation of characteristics of ulcer and peripheral cytokine levels (IL-1β, IL17 and TGF- β).

Table 3-6 shows the correlation between characteristics of ulcer with peripheral cytokine levels (IL-1β, IL17 and TGF- β). According to ulcer type, a significant difference was found between ulcer type and IL-1β levels (p =0.049), where it was (mean=1.76pg/ml) in superficial ulcer, (mean=2.03pg/ml) in deep ulcer, (mean=2.45pg/ml) in ulcer with bone involvement and (mean=5.74pg/ml) in ulcer with gangrene.

There was seen in IL-1β, where mean was higher in patients with black ulcer color in compared with white and red ulcer color (4.57pg/ml, 1.35pg/ml and 2.29pg/ml) respectively. However, the difference does not reach the statistical significance (p =0.125).

A similar orientation in ulcer bleeding, where mean of IL-1β was higher in patients with nonbleeding ulcer in compared with bleeding ulcer (3.46pg/ml versus 1.99pg/ml), however the difference does not reach the statistical significance (p =0.305).

Also a similar trend was seen in ulcer pain, where mean of IL-1β was higher in patients with painful ulcer in compared with no pain in ulcer (4.97pg/ml versus 2.37pg/ml). Still, the difference does not reach the statistical significance (p =0.127).

IL-17 and TGF-β did not show correlation with ulcer characteristics.

Table 3-6: Relationship between characteristics of ulcer and peripheral cytokine levels (IL-1β, IL17 and TGF-β).

Variables		IL-1β pg/mL		IL-17 ng/L		TGF-β ng/L	
		Mean(±SD) (n=58)	*p* - value	Mean(±SD) (n=58)	*p* - value	Mean(±SD) (n=58)	*p* - value
Ulcer site	Volar (N=31)	2.95±4.44	0.948	26.62±10.13	0.626	195.14±89.21	0.493
	Dorsal (N=27)	2.86±6.13		28.11±12.9		212.33±100.4	
Ulcer type	Superficial ulcer (N=9)	1.76±1.59	0.049	22.47±8.56	0.494	152.26±53.3	0.486
	Deep ulcer(N=24)	2.03±3.95		29.35±12.05		222.6±99.67	
	Ulcer with bone involvement(N=12)	2.45±3.75		25.03±13.17		197.48±118.5	
	gangrene(N=13)	5.74±8.65		29.02±10		207.66±74.51	
Ulcer area (cm^2)	≤15(N=40)	2.49±3.39	0.376	26.82±11.71	0.625	198.65±97.6	0.593
	>15(N=18)	3.82±8.03		28.42±11.14		213.12±87.8	
Ulcer duration (week)	≤20(N=47)	2.84±5.57	0.841	26.88±11.61	0.555	197.18±92.96	0.323
	>20(N=11)	3.2±3.76		29.17±10.95		228.61±99.44	
Ulcer color	Red (N=14)	2.29±3.33	0.125	27.25±11.54	0.895	197.64±91	0.775
	White (N=20)	1.35±1.83		26.94±13.2		202.65±99.4	
	Black (N=24)	4.57±7.35		27.66±10.21		206.76±95.3	
Ulcer smell	Normal (N=29)	3.73±6.6	0.233	26.05±9.97	0.403	192.06±93.57	0.375
	Bad (N=29)	2.08±3.32		28.58±12.77		214.22±95.05	
Ulcer number	Single (N=51)	2.95±5.49	0.880	27.25±11.83	0.909	197.89±93.78	0.255
	Multiple (N=7)	2.62±3.27		27.78±8.65		241.4±94.7	
New or recurrent ulcer	New (N=42)	2.72±5.65	0.659	27.25±12.7	0.942	199.16±101.3	0.607
	Recurrent (N=16)	3.4±4.1		27.49±7.46		213.58±74.07	
Ulcer bleeding	Present (N=22)	1.99±3.93	0.305	28.83±11.58	0.434	205.65±96.09	0.876
	Absent (N=36)	3.46±5.89		26.39±11.4		201.61±94.28	
Ulcer pain	Present(N=12)	4.97±8.61	0.127	27.33±7.53	0.995	199.74±86.58	0.890
	Absent(N=46)	2.37±3.9		27.31±12.31		204.03±96.91	

3.7. Correlation of total and differential WBCs counts with peripheral cytokine levels.

Table 3-7 shows the correlation of total and differential WBCs counts with peripheral cytokines levels (IL-1β, IL17 and TGF- β). There was no significant correlation between total and differential WBCs counts with peripheral cytokine levels.

Table 3-7: Correlation of total and differential WBCs counts with peripheral cytokine levels.

		IL-1β pg/mL	IL-17 ng/L	TGF-β ng/L
WBC $\times 10^3$cell/mm	Pearson Correlation	-0.084	0.034	-0.004
	p -value	0.528	0.798	0.978
	N	58	58	58
LYM $\times 10^3$cell/mm	Pearson Correlation	-0.091	0.192	0.177
	p -value	0.498	0.150	0.183
	N	58	58	58
NEU $\times 10^3$cell/mm	Pearson Correlation	-0.081	0.005	-0.017
	p -value	0.545	0.968	0.896
	N	58	58	58

Table 3-8: Correlation of RBCs indices with peripheral cytokine levels.

		IL-1β pg/mL	IL-17 ng/L	TGF- β ng/L
RBC $\times 10^{12}$ cell/L	Pearson Correlation	-0.076	-0.155	-0.052
	p -value	0.568	0.245	0.696
	N	58	58	58
HGB g/dL	Pearson Correlation	0.025	-0.070	-0.038
	p -value	0.851	0.600	0.776
	N	58	58	58
MCV fL	Pearson Correlation	0.208	0.062	-.0026
	p -value	0.118	0.646	0.844
	N	58	58	58
HCT %	Pearson Correlation	0.057	-0.127	-0.089
	p -value	0.671	0.343	0.509
	N	58	58	58
MCH pg	Pearson Correlation	0.124	0.143	0.073
	p -value	0.355	0.283	0.588
	N	58	58	58
MCHC g/L	Pearson Correlation	-0.293	0.200	0.189
	p -value	0.026	0.131	0.154
	N	58	58	58
RDW fL	Pearson Correlation	-0.208	0.162	0.267
	p -value	0.116	0.224	0.043
	N	58	58	58

3.8. Correlation of RBCs indices with peripheral cytokine levels.

Table 3-8 reveals the correlation of RBCs indices with peripheral cytokines levels (IL-1β, IL17 and TGF- β). There was a significant negative correlation between MCHC and IL-1β (p =0.026). As well a significant positive correlation between RDW and TGF- β (p =0.043).

3.9. Correlation of PLTs indices with peripheral cytokine levels.

Table 3-9 shows the correlation of PLTs indices with peripheral cytokines levels (IL-1β, IL17 and TGF- β). There was a significant positive correlation between PDW and IL-1β (p =0.052).

Table 3-9: Correlation of PLTs indices with peripheral cytokine levels.

		IL1- β pg/mL	IL-17 ng/L	TGF- β ng/L
PLT $\times 10^9$ cell/L	Pearson Correlation	-0.029	0.052	0.139
	p -value	0.829	0.697	0.300
	N	58	58	58
MPV fL	Pearson Correlation	0.245	-0.157	-0.195
	p -value	0.064	0.240	0.142
	N	58	58	58
PDW	Pearson Correlation	0.250	-0.252	-.0250
	p -value	0.052	0.057	0.058
	N	58	58	58
PCT mL/L	Pearson Correlation	-0.021	0.141	0.088
	p -value	0.875	0.289	0.514
	N	58	58	58
P-LCR	Pearson Correlation	0.226	-0.194	-0.237
	p -value	0.088	0.145	0.073
	N	58	58	58
P-LCC	Pearson Correlation	0.039	-0.126	-0.045
	p -value	0.770	0.347	0.737
	N	58	58	58

3.10. Correlation of HbA1c and CRP levels with peripheral cytokine levels (IL-1β, IL17 and TGF- β).

Table 3-10 express the correlation of HbA1c and CRP levels with peripheral cytokines levels (IL-1β, IL17 and TGF- β). There was a significant negative correlation of HbA1c with IL-17 level (p =0.036), and there no significant with other cytokines IL1- β and TGF- β.

Regarding CRP, no significant correlation exists with anyone of cytokines IL-1β, IL17 and TGF- β

Table 3-10: Correlation of HbA1c and CRP levels with peripheral cytokine levels (IL-1β, IL17 and TGF- β).

		IL1- β pg/mL	IL-17 ng/L	TGF- β ng/L
HBA1C %	Pearson Correlation	0.150	-0.277	-0.185
	p -value	0.261	0.036	0.165
	N	58	58	58
CRP mg/L	Pearson Correlation	-0.115	0.117	0.030
	p -value	0.389	0.382	0.826
	N	58	58	58

3.11. Comparison of total and differential WBCs counts before and after treatment with SOW for DFU patients who are recovery.

Table 3-11 shows the comparison of total and differential WBCs counts before and after treatment with SOW. Total WBC count and neutrophils were significantly reduced, whereas, lymphocytes were significantly elevated (p = 0.024, 0.026 and 0.020) respectively.

Table 3-11: Comparison of total and differential WBCs counts between before and after treatment with SOW.

Parameters	Normal value	Pre-treatment mean(±SD) (n=24)	Post-treatment mean(±SD) (n=24)	*p* -value
WBCs×10^3cell/mm	3.5-10	13.38±6.96	9.9±2.78	0.024
LYM×10^3cell/mm	0.9-5	2.41±0.86	2.87±0.9	0.026
NEU×10^3cell/mm	1.2-8	10.1±6.6	6.55±2.4	0.020

3.12. Comparison of RBCs indices before and after treatment with SOW for DFU patients who are recovery.

Table 3-12 shows the comparison of RBCs indices before and after treatment with SOW. There was no significant difference in anyone of RBCs indices before and after treatment with SOW.

Table 3-12: Comparison of RBCs indices between before and after treatment with SOW.

Parameters	Normal value	Pre-treatment Mean(±SD) (n=24)	Post treatment Mean(±SD) (n=24)	*p*-value
RBC×10^{12} cell/L	3.5-5.5	3.96±0.76	3.99±0.9	0.753
HGB g/dl	11.5-16.5	9.93±2.19	9.95±2.14	0.949
MCV fL	75-100	78.8±9.7	78.6±7.04	0.859
HCT%	35-55	31.035±6.13	31.39±7.1	0.671
MCH pg	25-35	25.26±3.874	25.1±2.99	0.675
MCHC g/L	31-38	31.5±2.93	31.46±2.97	0.937
RDW fL	0.1-250	47.71±12.97	46.7±9.23	0.673

3.13. Comparison of PLT indices before and after treatment with SOW for DFU patients who are recovery.

Table 3-13 shows the comparison of PLT indices before and after treatment with SOW. It was noted mean of MPV was decreased after the

treatment with SOW (8.4 versus 7.5), however, this decrease was statistically not significant (p =0.095).

Regarding PCT, a significant reduced concerning PCT (p =0.036).

Table 3-13: Comparison of PLTs levels between before and after using treatment SOW.

Parameters	Normal value	Pre-treatment Mean(±SD) (n=24)	Post-treatment Mean(±SD) (n=24)	p -value
PLT $\times 10^9$ cell/L	150-400	348.86±136.41	354.35±145.31	0.861
MPV fL	6.5-11	8.4±1.55	7.5±1.25	0.095
PDW	0.1-30	12.81±2.72	12.89±2.53	0.855
PCT mL/L	0.01-9.99	1.9±7.67	0.5±0.41	0.036
PLCR	0.1-99.9	16.45±8.74	16.17±7.7	0.846
PLCC	1--1999	36.9±8.74	36.81±12.62	0.977

3.14. Comparison of CRP level before and after treatment with SOW for DFU patients who are recovery.

Table 3-14 shows the comparison of CRP level before and after treatment with SOW. It was noted a highly significant reduced concerning CRP level (p =0.001).

Table 3-14: Comparison of CRP before and after treatment with SOW.

Parameters	Normal value	Pre-treatment Mean(±SD) (n=24)	Post-treatment Mean(±SD) (n=24)	p -value
CRP mg/L	<6.0	118.28±101.09	40.135±45.55	0.001

3.15. Comparison of cytokines levels before and after treatment with SOW for DFU patients who are recovery.

Table 3-15 shows comparison of cytokines level (IL-1β, IL-17 and TGF-β) before and after treatment with SOW. It was noted a significant

reduced of IL-1β level (p =0.043). The other two cytokines were also reduced, but their decreased levels did not reach statistical significant.

Table 3-15: Comparison of cytokines levels before and after treatment with SOW.

Parameters	Normal value	Pre-treatment Mean(±SD) (n=24)	Post-treatment Mean(±SD) (n=24)	p - value
IL-1β pg/mL	0-13.6	3.74±6.6	2.07±2.42	0.043
IL-17 ng/L	2-600	30.38±13.17	29.05±13.8	0.456
TGF-β ng/L	10-4000	236.05±113.87	231.4±112.33	0.800

3.16. Ulcer recovery according to duration of treatment with SOW

Figure 3-1 shows the patients response to treatment with SOW by measuring the ulcer area every five days, and the results reveal that every ten days the ulcer area was decreased at least 4.5 cm ^{2} (LSD = 4.623). This figure indicates that no relationship between ulcer area and recovery period.

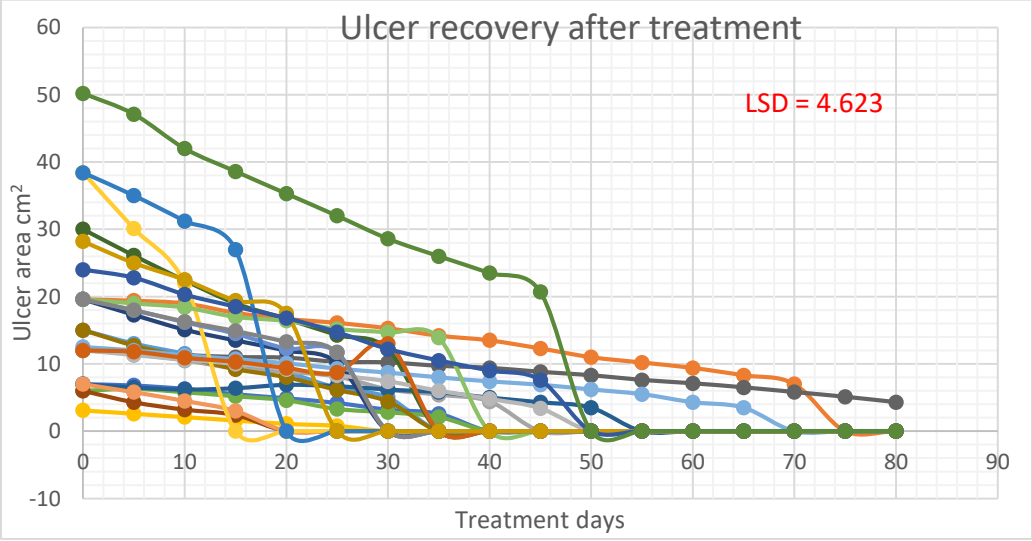

Figure 3-1 showed the behaviour response of each patient foot ulceration to SOW treatment for 5 days periodic follow up.

3.17. Correlation between recovery duration and characteristics of DFU patients.

Table 3-16 shows correlation between recovery duration and characteristics of DFU patients. It was noted a significant correlation between recovery duration and glycaemic control (p =0.038). The other characteristics of DFU patients did not reach statistical significant.

Table 3-16: Correlation between recovery duration and characteristics of DFU patient.

		Age	Gender	Smoking	Insulin	Glycaemic control	DM duration
Recovery duration (week)	Pearson Correlation	-0.125	-0.145	0.059	-0.296	-0.426	0.299
	p -value	0.561	0.499	0.784	0.161	0.038	0.156
	N	24	24	24	24	24	24

3.18. Correlation between recovery duration and characteristics of ulcer.

Table 3-17 shows correlation between recovery duration and characteristics of ulcer. A highly significant correlation between recovery duration and ulcer duration (p =0.001). The other characteristics of ulcer did not reach statistical significant.

Table 3-17: Correlation between recovery duration and characteristics of ulcer.

		Ulcer site	Ulcer type	Ulcer area (cm^2)	Ulcer duration (week)	Ulcer color	Ulcer smell	Ulcer number	New or recurrent ulcer	Ulcer bleeding	Ulcer pain
Recovery duration (week)	Pearson Correlation	-0.239	0.209	-0.060	0.612	0.329	0.237	-0.079	0.062	0.125	0.000
	p -value	0.261	0.327	0.781	0.001	0.117	0.266	0.713	0.772	0.561	1.000
	N	24	24	24	24	24	24	24	24	24	24

3.19. Correlation of recovery duration with total and differential WBCs counts before treatment with SOW.

Table 3-18 shows correlation of recovery duration with total and differential WBCs counts before treatment with SOW. It was noted a significant correlation between recovery duration and lymphocyte before treatment (p =0.021).

Table 3-18: Correlation of recovery duration with total and differential WBCs counts before treatment with SOW.

		Pre -treatment		
		WBC $\times 10^3$cell/mm	LYM $\times 10^3$cell/mm	NEU$\times 10^3$cell/mm
Recovery duration (week)	Pearson Correlation	0.090	0.470	0.034
	p -value	0.676	0.021	0.873
	N	24	24	24

3.20. Correlation of recovery duration with RBCs indices before treatment with SOW.

Table 3-19 shows correlation of recovery duration with RBCs indices before and after treatment with SOW. No significant correlation of recovery duration with anyone of RBCs indices before treatment with SOW.

Table 3-19: Correlation of recovery duration with RBCs indices before treatment with SOW.

		Pre -treatment						
		RBC $\times 10^{12}$ cell/L	HGB g/dL	MCV fL	HCT %	MCH pg	MCHC g/L	RDW fL
Recovery duration (week)	Pearson Correlation	0.010	-0.041	-0.303	-0.162	-0.131	0.201	-0.170
	p -value	0.962	0.848	0.151	0.450	0.541	0.347	0.427
	N	24	24	24	24	24	24	24

3.21. Correlation of recovery duration with PLTs indices before treatment with SOW.

Table 3-20 shows correlation of recovery duration with PLTs indices before treatment with SOW. No significant correlation of recovery duration with anyone of PLTs indices before treatment with SOW.

Table 3-20: Correlation of recovery duration with PLTs indices before treatment with SOW.

		Pre -treatment					
		PLT×10 ^{9} cell/L	MPV fL	PDW	PCT mL/L	P-LCR	P-LCC
Recovery duration (week)	Pearson Correlation	0.100	-0.240	-0.256	-0.155	-0.089	-0.103
	p -value	0.642	0.259	0.227	0.469	0.679	0.633
	N	24	24	24	24	24	24

3.22. Correlation of recovery duration with CRP level before treatment with SOW.

Table 3-21 shows correlation of recovery duration with CRP level before and after treatment with SOW. No significant correlation of recovery duration with CRP level before treatment with SOW.

Table 3-21: Correlation of recovery duration with CRP level before treatment with SOW.

		Pre -treatment
		CRP mg/L
Recovery duration (week)	Pearson Correlation	-0.083
	p -value	0.701
	N	24

3.23. Correlation of recovery duration with peripheral cytokines levels before treatment with SOW.

Table 3-22 shows correlation of recovery duration with IL1- β, IL-17 and TGF- β levels before treatment with SOW. No significant correlation of recovery duration with anyone of cytokines was noticed before treatment with SOW.

Table 3-22: Correlation of recovery duration with peripheral cytokines levels before treatment with SOW.

		Pre -treatment		
		IL1- β pg/mL	IL-17 ng/L	TGF- β ng/L
Recovery duration (week)	Pearson Correlation	0.124	-0.032	-0.061
	p -value	0.564	0.882	0.777
	N	24	24	24

Chapter Four

Discussion

4. Discussion

In this study, patients with diabetic foot ulcers were enrolled. These patients used Super Oxidized water as local agent. All patients were newly diagnosed and did not receive any treatment for the DFU. The study parameters and specimens were obtained before the commencement of treatment with SOW. The study parameters and specimens were also obtained for 26 patients after the finish of treatment period. Of those 26 patients, 24 had fully recovered while 2 patients failed to recover.

The patient's groups were divided into two categories according to their age ranges. The highest frequency of patients fell in age group above 50 years (n=46 (79.3%)) and lowest frequency at ≤50 years which were 12(20.7%). These results indicate that DFU is more common in older patients and old age is possibly a risk factor for DFU. This finding was almost comparable with Al-Rubeaan, Al Derwish *et al*. (2015), who found that Older patients face significantly more complications than younger ones. This result may be because most elderly people have weak immunity.

Regarding gender, higher prevalence of DFU in male, was reported in this study. Indeed, three quarters of DFU patients in this study were males. This study was comparable with Ahmad, Khan *et al*. (2013). They found the majority of patients with diabetes who develop foot ulcers are male (more than two-thirds). This result may be because males are more likely to injure their feet, due to their frequent going out and exposure to many obstacles, as well as due to the large amount of time they wear their shoes. In addition, another study that contradicts our study reported by Magalhães and Cardoso (2018)

who showed that the incidence of DFU in female (58.6%) is higher than male. This difference may be due to difference ethnicities and geographical location.

This study suggested that smoking did not represent a risk factor for DFU as half of patients were non-smokers. This study was comparable with Abeer Elnour (2021), who found only 12% from DFU patients were smokers.

The present study showed that only 24(41.4%) of the diabetic foot patients were hypertensive. This result almost matches with Syauta, Hendarto *et al*. (2021), they found hypertension affected (43%) of the diabetic foot.

The study result revealed that DFU patients who using insulin 33(58.89 %) were more than those who using oral agent or just having lifestyle modification (43.1%). This result was similar to previous studies (Yusuf, Okuwa *et al*. 2016).

The result of this study showed that DFU patients who have poor glycemic control were more than those who have good control, in which patients with poor control were (93.1%). This result was similar to the result reported by Purwanti, Yetti *et al*. (2016).

The current study showed that the older people were with diabetes, the more likely they were to develop diabetic foot. This result was convenient with study of Bi, Zhang *et al*. (2016).

Swollen feet was found in most of the patients (65.5%). Poor blood circulation often causes swollen feet when person have diabetes. Swelling in the feet is caused by excess fluid that builds up in the body tissue (Crocker, Palmer *et al*. 2021).

Ulcer duration ranged between 3 to 48 weeks distributed into two groups. Most of DFU patients had ulcer less than 20 weeks 47(81%), where, 11(19%) had ulcer more than 20 weeks, as shown in table 3-1. This result corresponds with (Johani, Malone *et al*. 2017).

Regarding ulcer site, DFU patients have an ulcer in volar site of foot more than in dorsal site of foot. These results are comparable to the results reported by Driver, Lavery *et al*. (2015).

Ulcer type is classified into superficial ulcer, deep ulcer, ulcer with bone involvement, and ulcer with gangrene, the majority of patients have deep ulcer. This result identical with study of Driver, Lavery *et al*. (2015). However, this finding is inconsistent with the finding of another study done by Gezawa, Ugwu *et al*. (2019), One-third of the subjects had gangrene of toe or forefoot. The possible reason for this discrepancy that the sample of the different study is later than the sample of current study.

Ulcer area ranged between 3 to 50 cm^2 distributed into two groups, the results revealed that most of ulcer area of DFU patients less than 15cm^2. There was a similar study reported by (Pinto, Ubilla *et al*. 2018) but it measures the size of ulcers, not the area of ulcers. Still, it is almost the same.

In this study, ulcer color was classified into red, white and black, most of the patients have black ulcer. One of the most common signs of diabetic foot ulcers is black tissue called eschar that often appears around the wound because of a lack of blood flow to the feet (Kirsner, R. *et al*.2015)

In this study there was no difference in proportions between ulcers with bad smell and ulcer with normal smell. Similar to this study, another study

(Gillespie, Carter *et al*. 2019) showed there is no difference in the ratio between normal and foul-smelling ulcers. Color and smell of the ulcer are among the signs of ulcer evaluation.

DFU patients with single ulcer were more than those who have multiple ulcers. Also this study included 42(72.4%) of patients have ulcer occur first time and 16(27.6%) have recurrent. This results contradicts with the results of another study reported by Khalifa (2018). Another study in European that is run against this study is where the patients who have recurrent ulcers are more than the patients who occur with them for the first time (Dubský, Jirkovská *et al*. 2013).

The results also showed that only small proportion of patients had pain in their feet. The result indicate absence of protective sensation due to peripheral neuropathy, The motor neuropathy causes physical deformity of the foot, and sensory neuropathy causes sensory loss (Armstrong, Boulton *et al*. 2017). These results indicate the DFU are chronic and not healing for long time and painless non-bleeding.

In this study a significant difference between ulcer type and total WBCs and neutrophils counts (P-values=0.019, 0.025 respectively), where there was an increase in total WBC and neutrophils counts in patients with gangrenous ulcer and ulcer with bone involvement. These results indicate that inflammation is stronger in these types of ulcer compared to superficial and deep ulcers. These results are comparable to the results reported by Zhang, Ding *et al*. (2021).

The results showed the direct relationship of white blood cell (WBC) total and neutrophils with ulcer area, as the WBC total and neutrophils increases as the area of ulcer increases. These results are comparable to the results reported by Wang, Aiping, *et al*. (2014), there are showed, patients with larger wound size their WBC is high. Another study indicates that neutrophils -specific markers were significantly higher in DFU patients than in diabetic patients without DFU or healthy controls (Yang, S., Gu, *et al*. (2020)). These results indicate the total count of WBCs in addition to neutrophils count increase with increase in the depth of the ulcers.

Regarding the ulcer duration, a highly significant correlation was found between ulcer duration and lymphocyte count, where, patients with duration of ulcers more than 20 weeks showed a higher lymphocyte counts than patients with duration of ulcers less than 20 weeks. These result similar to study reported by Yunir, Tahapary *et al*. (2021). This results could be explained on the basis that lymphocytes increase in chronic infection.

The results showed that WBCs and neutrophils count are highest in patients with black ulcers followed by patients with red ulcers. These results further support the finding that most visible sign of a serious foot ulcer is black tissue (called eschar) surrounding the ulcer. Eschar is developed because of an absence of healthy blood flow to the area around the ulcer (Armstrong, Boulton *et al*. 2017).

Concerning the ulcer smell, there was an increase in mean of WBC count in patients with bad smell of ulcer compared to patients with normal smell of ulcer. A similar study reported by Cooney and Cooney (2011), who reported a relationship between high WBC and foul smelling.

Presence of multiple ulcers was reported to be associated with high WBCs and neutrophils count.

The results illustrated that there was a significant difference of haemoglobin (HGB) and haematocrit (HCT) with ulcer site, where's, HGB and HCT in patients with dorsal site of ulcer higher than patients with volar site of ulcer. In general and most studies concluded that most DFU patients suffer from anaemia (Cooney and Cooney 2011).

The results showed that red blood cells (RBC) counts are higher in ulcer with gangrene than other ulcer types. Previous study included that red blood cells are differ in DFU patients from diabetic patients only, these showed RBCs are less in DFU patients in compare with diabetic patients (Mushlih 2020).

Mean corpuscular haemoglobin concentration (MCHC) is a measure of the average concentration of haemoglobin inside a single red blood cell. Mean corpuscular haemoglobin (MCH) is the average quantity of haemoglobin present in a single red blood cell. The current study showed a highly significant negative correlation between ulcer area and both of MCH and MCHC, As the ulcer area increase, the MCH and MCHC decreases. A previous study showed a similar result about MCH with DFU, whereas, the MCH range is similar to range in this study (Wright, Oddy *et al*. 2014).

Red cell distribution width (RDW) is a measure of the range of variation of RBC volume. In this study a highly significant correlation between RDW and ulcer area, as the ulcer area increase, the RDW decreases.

Regarding ulcer smell, a highly significant difference between ulcer smell and MCHC and also a significant correlation between ulcer smell and RDW, where there was an increase in MCHC and RDW in patients who have a foul-smelling ulcer.

DFU with single ulcers were found to have higher RBC counts than those with multiple ulcers, and patients with gangrene also showed higher RBC counts. A study of Cahn, Livshits *et al*. (2016) showed a significant rise in the percentage of minimally deformable RBCs in diabetic foot patients compared with the patients with no complications was observed. In current study, it was found that RBCs were high in patients with single ulcer. These results indicate that all of ulcer site, area and smell had effect on red blood cell indices.

According to previous study reported by Mardia, Gatot *et al*. (2018), generally, there was an increase in platelet count, PDW and PCT levels in diabetic patients with diabetic foot ulcers, it indicates that platelet function becomes more reactive and aggregately.

Plateletcrit (PCT) is the volume occupied by platelets in the blood as a percentage. In this study noticed PCT more in patients with dorsal site of ulcers compared to patients with volar site of ulcers.

A significant difference noticed in ulcer site with platelet large cell count (P-LCC) where a higher P-LCC was found in dorsal site of ulcer in comparison to volar site of ulcer. Moreover, a significant correlation was found between ulcer duration and platelet (PLT) counts, as a duration of ulcer longer, the PLTs higher in counts. For platelet large cell ratio (P-LCR),

showed an inverse relationship with duration of the ulcer, as the shorter duration of ulcer, is the more P-LCR will be.

The results found out that PLTs were decreased in patients with red ulcer color compared to patients with white and black ulcer color. But the plateletcrit (PCT), there was higher in patients with black ulcer color in compared with red and white ulcer color.

A highly significant correlation exists between ulcer number and PCT, where, PCT was higher in patients with multiple ulcers than in patients with single ulcer. Also the PCT was higher in recurrent ulcers in comparison to new ulcer.

The study showed that there was an increase in mean of PLT counts in patients with nonbleeding ulcer compared with patients bleeding ulcers. To the best of our knowledge this is the first study to correlate some PLTs indices with DFU.

Mean platelet volume (MPV) is a measure of the average size of platelets. A significant correlation was found between painful ulcer and MPV, where patients with painful ulcers showed a higher MPV than patients with no pain of ulcers. The previous study reported by Mardia, Gatot *et al*. (2018) showed the relationship between PLTs counts and DFU, where it was found an increase in platelet count, Platelet distribution width (PDW) and PCT levels in patients with diabetic foot ulcers. The elevated MPV levels in diabetic foot ulcers patients have been reported by Gunes, Eren *et al*. (2017). These results highlight the importance of platelets indices in the pathology and pathogenesis of diabetic foot ulcer.

This study showed an association between C-reactive protein (CRP) levels depth of ulcers. There was an increase in CRP mean in patients having ulcers with bone involvement. This result disagrees with a study achieved by Hadavand, Amouzegar *et al*. (2019) who found that CRP was significantly higher in patients with class IV foot ulcers (whole foot gangrene) compared to those with class III ulcers (deep ulcer with abscess or osteomyelitis) ($p<0.001$). This might be cause CRP had poor accuracy, in detecting the diabetic foot cases with osteomyelitis.(Moallemi, Niroomand *et al*. 2020).

Regarding CRP, a higher CRP is noticed in patients with ulcers' area more than 15cm^2. CRP level was higher in patients with recurrent ulcer in comparison with new ulcers, This might be as Wang, Shao *et al*. (2021) demonstrated that serum CRP levels may be biomarkers of DFU. Also, it was higher in patients with bleeding ulcers compared with nonbleeding ulcers. Like that was seen in association between ulcer pain and CRP, where, the CRP level is higher in patients with painful ulcer compared with no pain in ulcer.

This study showed a significant difference between elevated Haemoglobin glycaemic A1C (HbA1C) levels and patients with ulcer area more than 15 cm^2. These results indicate the effect of poor diabetic control on the area of ulcers. To the best of our knowledge this is the first study to correlate HbA1C with ulcer area.

The current study revealed that a significant difference was found between ulcer type and IL-1β levels, where, the IL-1β level was higher in

gangrenous ulcer than other types of ulcer In another study showed the IL-1 is decreased in grade 2 (deep ulcer) (Amini, Sheikh Hosseini et al. 2020).

In this study, there was increase in IL-1β levels, in patient's black ulcer color than white and red ulcer. Since IL-1β levels is higher in patients with gangrenous ulcers, it is expected to be higher in patients with black coloured ulcers. To the best of our knowledge this is the first study to correlate IL-1β levels with ulcer color.

Regarding ulcer bleeding, there was raise in IL-1β levels in patients with nonbleeding ulcer comparison with bleeding ulcer, also in association with pain in ulcer, whereas, ulcers with pain have higher IL-1β levels than those without pain. This study contradicts with Paulsen, Laird *et al*. (2017), that found IL-1β levels decreases with presence of pain.

The study showed there was a significant negative correlation between MCHC and IL-1β (P=0.026). These results gave further evidences to support to the suggested role of IL-1β in the delayed healing of ulcer.

The results revealed that a significant positive correlation between RDW and TGF- β.

Platelet distribution width (PDW) is an indicator of volume variability in platelets size. In current study reported positive correlation between IL-1β and PDW. The previous study reported by Sağ, Sağ *et al*. (2018) finding there was no correlation of IL-1β with hematologic markers.

This study showed a significant negative correlation of IL-17 level with HbA1c. In diagnosing diabetic foot according to DM type 2 group, the performances of IL-17 in diagnosis of DFU (Kaleli, Varım *et al*. 2019).

In this study, the levels of several parameters were compared before and after treatment with SOW. Comparable to the result reported by Lee, Kim *et al*. (2013). Total WBC counts and neutrophils counts were reduced while lymphocytes were elevated. These results indicate recovery from the inflammation and restoring the normal WBC counts.

The current study showed a significant difference of PCT between patients of DFU before and after treatment with SOW.

In this study noted the MPV was decreased after the treatment with SOW. This results corresponds with Yan, Wan *et al*. (2021). These result that complete blood count was restored to normal after treatment.

The result illustrated that there was a highly significant difference concerning CRP between patients of DFU before and after treatment with SOW, whereas, initial CRP level was higher in comparison with after treatment with SOW. High level of c-reactive protein (CRP) the main risk factors predictive of diabetic foot treatment failure and considered to be risk factors for amputation (Lee, Kim *et al*. 2013).

Among the cytokines studied in this study, only The current study IL-1β levels were shown to be significantly reduced after treatment with SOW. Indeed, IL-1β levels were reported by other researchers to be elevated in diabetes (Xue, Dai *et al*. 2017), and its increased levels appears to mediates delayed wound healing in diabetic patients (Dai, Shen *et al*. 2021). Therefore,

treatment with SOW may be able to reduce IL-1β levels and thus promote wound healing.

In the management of diabetic foot ulcer, a SOW debrides necrotic tissue, reduces microbial load, promotes granulation and decreases the healing time, without damaging the normal tissue or complications(Chittoria, Yootla *et al*. 2007). The effectiveness of ulcer healing according to the use of SOW is very clear in this study, as there is a significant change in the area of the ulcer every five days, and the results showed that, as an average for all healed patients every ten days, the area of the ulcer decreased 4.6 cm^2 (LSD =4.623). This result was convenient with Chittoria, Yootla *et al*. (2007).

A significant correlation was obtained between glycemic control and recovery duration. The finding of this study is in line with the study of Fernando, Crowther *et al*. (2016). Control of hyperglycaemia may be important in the healing of ulcers. Another Cochrane review assessing effects of glycaemic targets in type 2 diabetes found that those with intensive glycaemic control had a 35% reduction in risk of lower-extremity amputation (Hemmingsen, Lund *et al*. 2013). Elevated HbA1c was associated with slower and incomplete healing of foot ulcers in diabetic patients. Given their reliability as tools to diagnose and monitor diabetes and its related complications, HbA1c parameter can be used as dependable predictors of foot ulcer healing in the diabetic (Kumar and Manjunath (2018)).

Regarding ulcer duration, there was a highly significant correlation between recovery duration and ulcer duration. A similar result reported by Smith-Strøm, Iversen *et al*. (2017), early detection and referral by both the patient and general practitioner are crucial for optimal foot ulcer healing.

Ulcer grade and severity are also important predictors for healing time, and early screening to assess the severity and initiation of prompt treatment is important.

In this study, a significant difference was noticed between recovery duration and lymphocyte before treatment. this study identical with Seraphim, Leal *et al*. (2020), the decrease in lymphocytes, further impair the wound healing conditions.

Conclusion &Recommendations

Conclusion

1- Diabetic foot ulcers are common in diabetic males above the age of 50 years with poor glycaemic control, in whom the duration of the diabetes is more than 15 years

2- Most of the patients show swollen feet, and most of the ulcers show duration of more than 20 weeks and are mostly single, deep, less than 15cm ^{2}, white or black.

3- Increase in WBC count and neutrophils are in DFU patients bone involvement or gangrene and ulcer area of more Than 15 cm ^{2}. Lymphocyte count increased when the duration exceeds 20 weeks, volar site of ulcers associated with decreased haemoglobin. Increased DFU area was associated with decreased MCHC

4- Dorsal site of ulcer was associated with platelets counts which was high when the disease duration is longer.

5-DFU with bone involvement shows higher CRP, Whereas HbA1c was higher in patients with higher ulcer area.

6- IL-1β was increased in association with increasing the depth of the ulcer and with gangrene. IL-1β was negatively correlated with MCHC.

7- IL-17 levels were negatively correlated with HbA1c.

8- Treatment of DFU with superoxidized water caused significant decrease in total and differential WBC count in addition to the CRP serum levels.

9- Treatment of DFU with superoxidized water caused significant decrease in the serum levels of IL-1β.

10- There is positive correlation between glycaemic control (HbA1c) and recovery duration, and between recovery duration and ulcer duration.

Recommendations

1- A further a greater sample size study is recommended to verify the results of this study.

2- Studying the local expression of cytokines in association with treatment by superoxidized water.

3- Use The superoxidized water in earlier time to treat the diabetic foot ulcers.

4- Focusing of glycaemic control to accelerate the response to treatment with superoxidied water in DFU patients.

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Appendix

Appendix I: DFU patient's questionnaires

DFU QUESTIONNAIRE

No		Name		Age		Date	
Gender		female	male	No. of phone			
Smoking		Yes	No				
Diabetes duration		≤15years	>15 years	15			
Insulin therapy		Yes	No				
Types of ulcer		Intact skin	Superficial ulcer	Deep ulcer	Ulcer with bone involvement	gangrene	
Ulcer duration		≤20 week	>20 week				
Ulcer color		Red	White	Black			
Ulcer dimensions		Length	Width	Depth			
Ulcer site		Volar	Dorsal				
Smell ulcer		Normal	Bad				
Ulcer no.		Single	multiple				
New or Recurrent ulcer		New	Recurrent				
pain		Present	Absent				
Swelling foot		Present	Absent				
Blood pressure		Hypertension	Normal				

Parameter:

Complete blood count…….

C- reactive protein ……...

Haemoglobin A1c…….

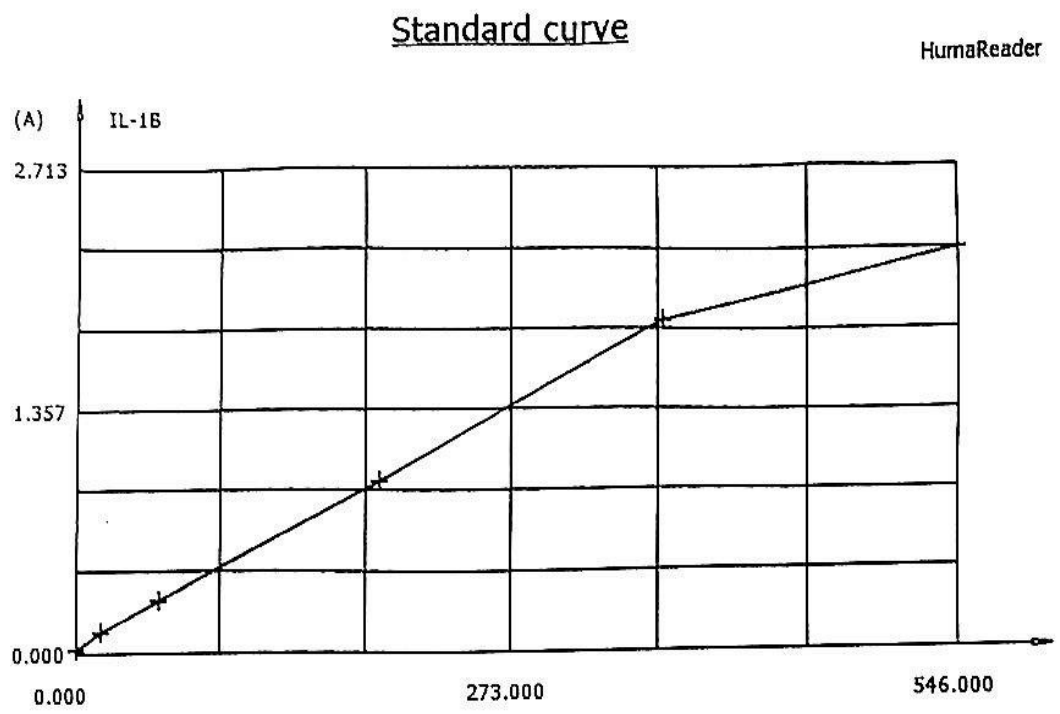
Serum IL-1beta ……

Serum IL-17 ……

Serum TGF-beta ……

Appendix II: Standard curve of cytokines

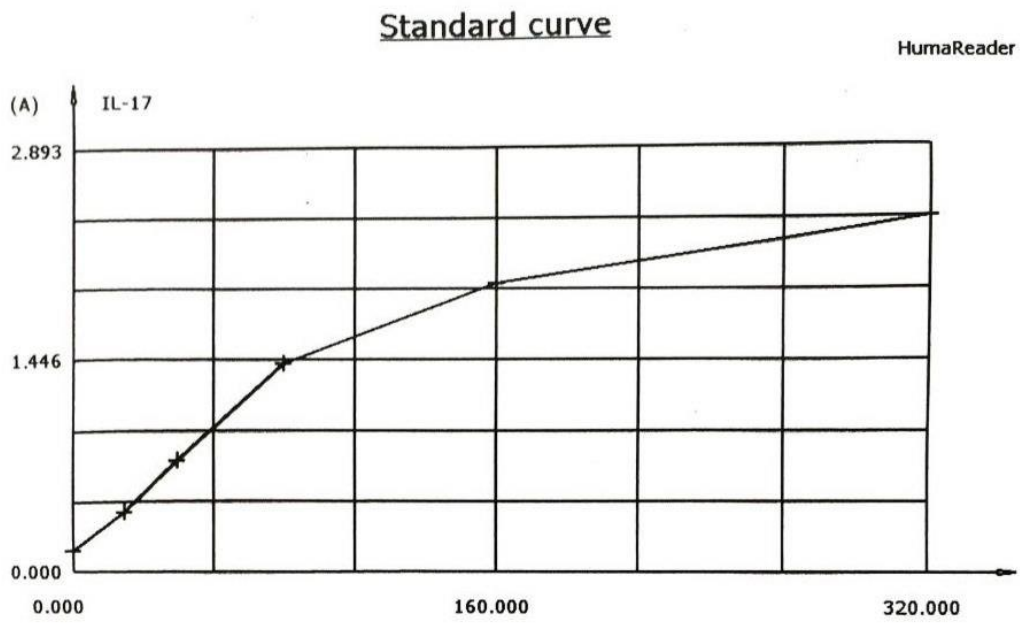
Interleukin-1beta



Program: IL-1B IL-1B Caculation: Curve Wavelength(nm): 450/630

Standard sample	#1	#2	#3	#4	#5	#6
Concentration(pg/ml)	0.000	16.300	52.700	191.000	368.000	546.000
Abs(A)	0.018	0.112	0.283	0.946	1.856	2.261

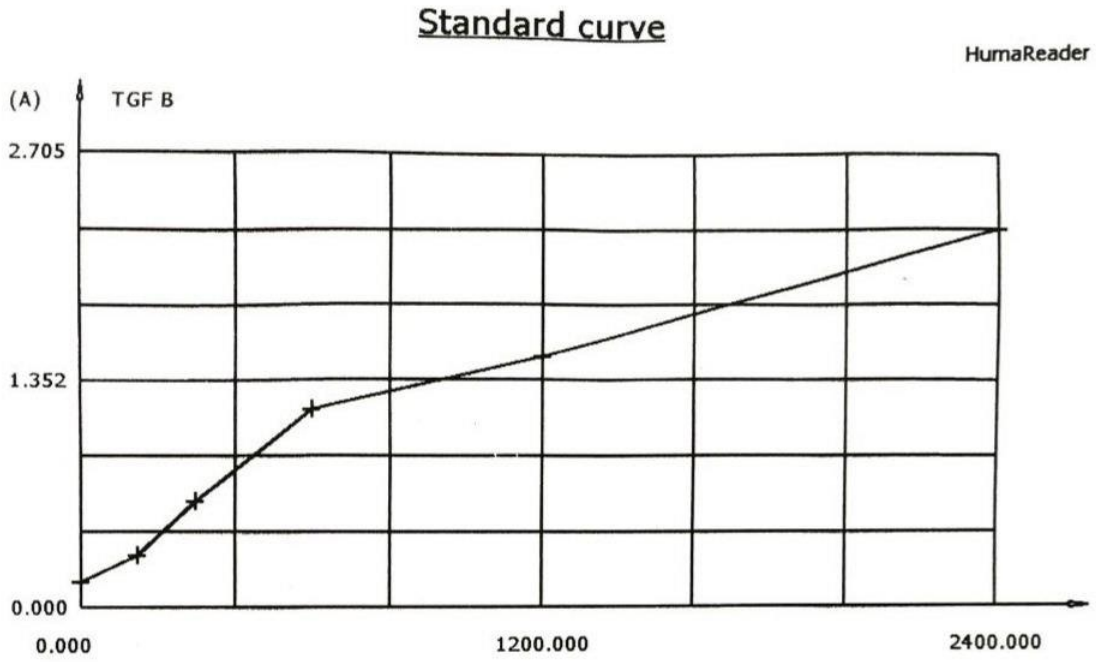
Interleukin-17



Program: IL-17 IL-16 Caculation: Curve Wavelength(nm): 450

Standard sample	#1	#2	#3	#4	#5	#6
Concentration(pg/ml)	0.000	20.000	40.000	80.000	160.000	320.000
Abs(A)	0.144	0.406	0.758	1.413	1.958	2.410

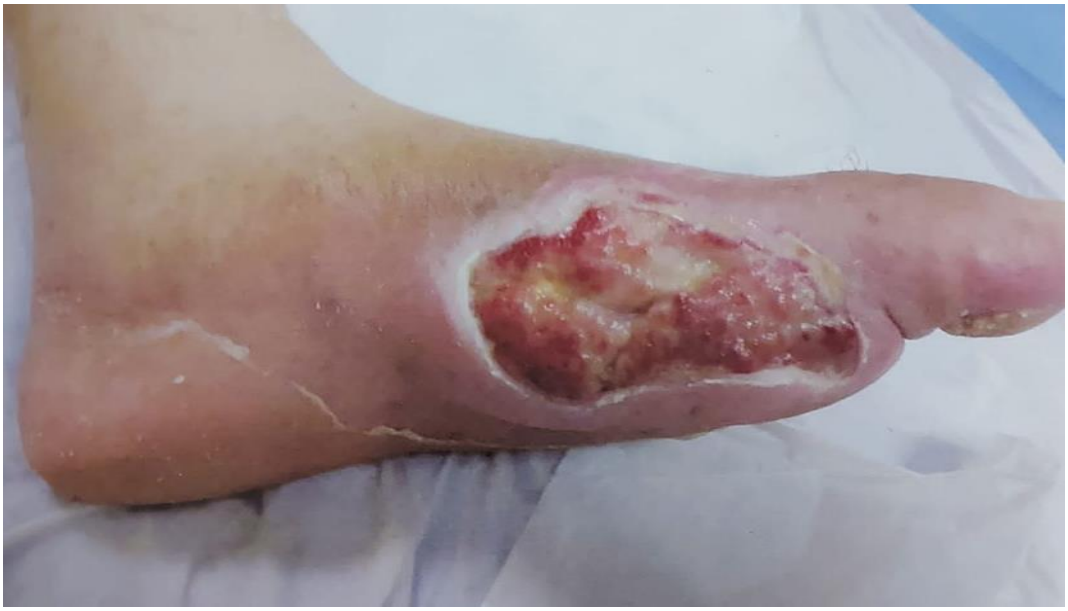
Interleukin-TGF



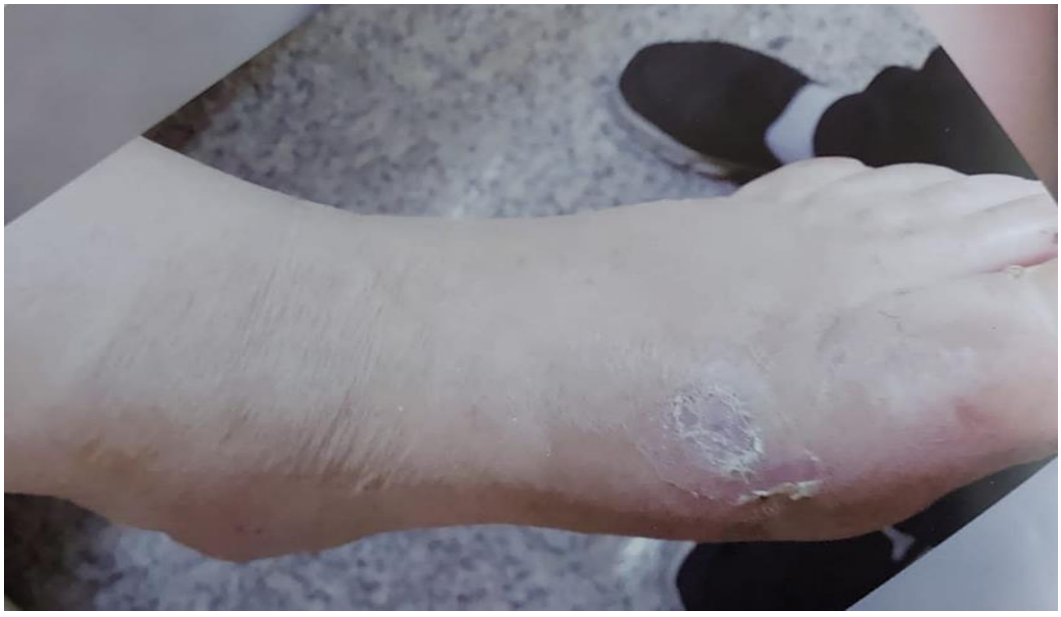
Program: TGF B TGF B Caculation: Curve Wavelength(nm): 450

Standard sample	#1	#2	#3	#4	#5	#6
Concentration(ng/l)	0.000	150.000	300.000	600.000	1200.000	2400.000
Abs(A)	0.143	0.303	0.623	1.168	1.484	2.254

Some cases of recovered patients

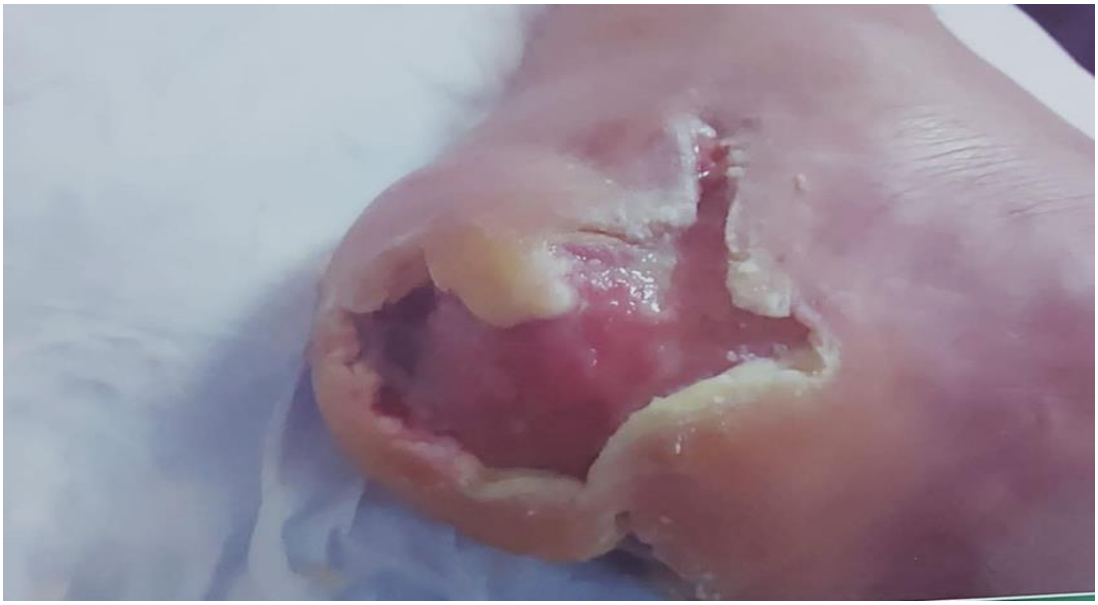


Before treatment with Superoxidized water

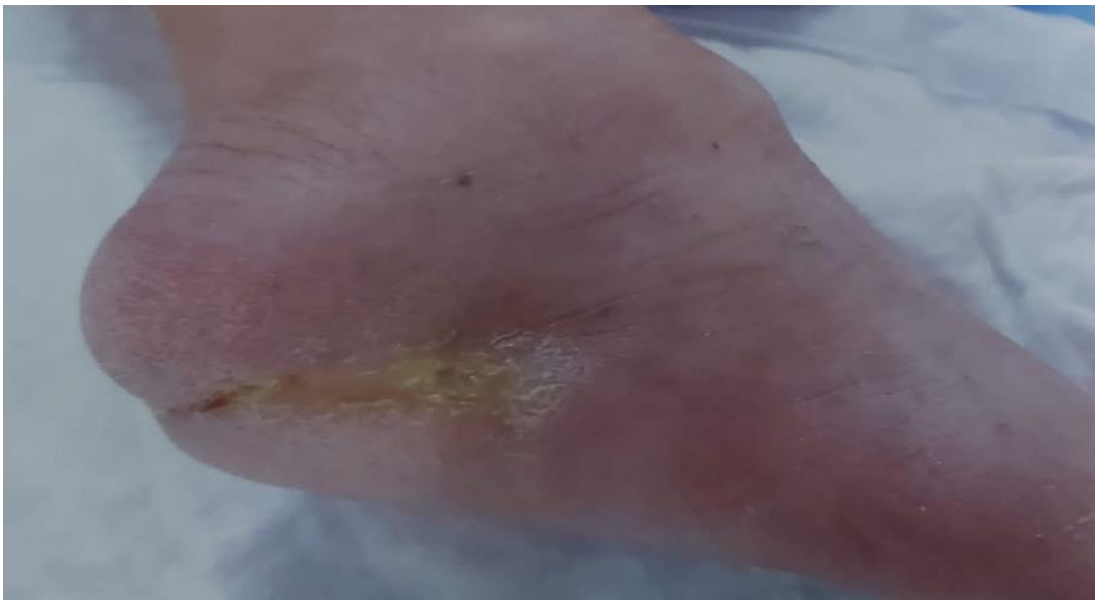


After treatment with Superoxidized water

Some cases of recovered patients



Before treatment with Superoxidized water



After treatment with Superoxidized water

الخلاصة

تعد قرح القدم السكري من المضاعفات الشائعة والمهمة لمرض السكري ومن أكثر أسباب بتر الأطراف السفلي الأكثر شيوعًا. تمتاز قرحة القدم السكري بتأخر الشفاء وأن هذا التأخر في الشفاء يعتقد انه متسبب عن سوء تنظيم مناعي.

لقد بينت الدراسات ان العلاج بـ الماء الفائق الأوكسيد له إمكانية الشفاء وخلال فتره قصيره نسبياً. الهدف من الدراسة الحالية هو لتوصيف مرضى قرحة القدم السكري خلال التركيز على المؤشرات الدموية من والمناعية وكذلك دراسة تأثير الماء الفائق الأوكسيد على هذه المؤشرات.

شملت هذه الدراسة 58 مريضاً يعانون من مرض السكري من النوع الثاني ومصابين بـ قرحة القدم السكري، من ضمنهم 43 ذكور و 15 إناث.

أجريت هذه الدراسة في العراق عام 2022 ميلادي في مستشفى الكفيل التخصصي في كربلاء وعيادات خاصة في الفترة الممتدة من 9 نيسان إلى 6 ايلول لسنة 2021 .

تم تشخيص جميع المرضى بـ قرحة القدم السكري بناءً على التشخيص السريري والأشعة السينية من قبل أخصائيين. المرضى كانوا يؤدون زيارات منتظمة الى العيادات التخصصية لغرض الرعاية والعلاج بـ الماء الفائق الأوكسيد.

من ال 58 مريضاً. 26 مريض حصلوا على نتائج قبل وبعد العلاج. من بين هؤلاء المرضى البالغ عددهم 26 مريضاً الذين تمت معالجتهم بـ الماء الفائق الأوكسيد، 24 مريضاً تعافوا تماما وفقط مريضين لم يتعافوا.

تم جمع البيانات الديموغرافية والسريرية من المرضى و / أو عائلاتهم من خلال ورقة استبيان خاصة. كما تم جمع الأمصال والدم الكامل من كل مشارك مع ملاحظة أنه تم استخدام المصل لتحديد مستويات عدد من السايتوكينات في الدم (انترولوكين 1 بيتا، انترلوكين 17 وعامل النمو المتحول بيتا).

بينما تم استخدام الدم الكامل لتعداد الدم الكامل، والسكر التراكمي وبروتين سي التفاعلي لجميع العينات وتم تحليل البيانات إحصائيًا بواسطة برنامج SPSS الإصدار 25.

كان معظم المرضى (79.3٪) فوق سن الخمسين وكان معظمهم (74.1٪) من الذكور. كان معظم المرضى (93.1٪) يعانون من عدم انتظام السكر

تشير هذه النتائج إلى أن قرح القدم السكري تصيب إلى حد كبير المرضى الذكور الأكبر سنًا الذين يعانون من ضعف انتظام نسبة السكر في الدم. فيما يتعلق بخصائص القرحة، فإن معظم المرضى (81 ٪) يعانون من القرحة لمدة تزيد عن 20 أسبوعًا. معظم القرح (65.5٪) مصحوبة بتورم القدمين وكانت منطقة القرحة لدى معظم المرضى (69٪) متساوية أو أقل من 5سم مربع. معظم المرضى (87.9٪) مصابون بـ قرحة مفرده ولأول مرة (72.49٪)، غير مؤلمة (79.3 ٪) وغير نازفة (62.1٪).

أظهرت هذه الدراسة زيادة في كل من إجمالي عدد كريات الدم البيضاء وعدد العدلات في القرح مع إصابة العظم (13.9 × 10^3 خلية / مم '، 10.19 ×10^3 خلية / مم^2 على التوالي) والقرح المصابة بالغرغرينا (14.59 × 10^3 خلية / مم'، 10.19 × 10^3 خلية / مم^2 على التوالي) بالمقارنة مع القرحات السطحية (7.39 × 10^3 خلية / مم'، 7.15 × 10^2 خلية / مم^2 على التوالي).

ومع ذلك، فإن زيادة تعداد الخلايا الليمفاوية أظهرت ارتباطًا معنويًا عاليًا بزيادة مدة القرحة (p=0.001) .هناك ارتباط معنوي بين القرحة الموجودة في أخمص القدم وفقر الدم مقارنةً بالموقع الظهري للقرحة (p=0.025).

ومن المثير للاهتمام أن القرحات المصحوبة بالغرغرينا كانت مصحوبة بزيادة في عدد كريات الدم الحمراء (9.91 × 10^{12}خلية / لتر). يتأثر متوسط الهيموغلوبين في الجسم (MCH) ومتوسط تركيز الهيموغلوبين في الجسم (MCHC) وعرض توزيع الخلايا الحمراء (RDW) بمساحة القرحة. في الواقع، كانت هناك علاقة عكسية بين مساحة القرحة و MCH (p=0.007)، (p=0.025) MCHC، RDW (p=0.005). علاوة على ذلك، فإن القرح ذات الرائحة الكريهة كانت مرتبطة بزيادة MCH (p=0.004) .

فيما يتعلق بمؤشرات الصفائح الدموية، فقد ارتبط موقع القرحة في أخمص القدم مع انخفاض عدد الصفائح الدموية الكبير (p =0.038) (P-LCC). ومن المثير للاهتمام، أن زيادة مدة القرحة كانت مرتبطة بزيادة معنوية في عدد الصفائح الدموية (p =0.013) علاوة على ذلك، ارتبط ارتفاع PCT بوجود القرح المتعددة (p= 0.005) وتكرار القرحة (p= 0.05) بالإضافة إلى ارتباط آلم القرحة بارتفاع حجم الصفائح الدموية (p= 0.025) (MPV). تم العثور على مستويات عالية من السكر التراكمي تترافق مع التقليل في مساحة القرحة (p=0.016).

فيما يتعلق بعلاقة السايتوكينات المدروسة بخصائص القرحة، وجد أن مستويات انترلوكين 1 بيتا فقط مرتبطة بعمق القرحة. في هذا الصدد، زادت مستويات انترلوكين 1 بيتا بشكل ملحوظ مع زيادة عمق القرحة (p=0.049).

فيما يتعلق بالارتباطات بين مستويات السايتوكينات ومؤشرات الدم، أبلغت هذه الدراسة عن العديد من الارتباطات المهمة التي يمكن أن تكون موضوعات جيدة لمزيد من الدراسات. ارتبطت مستويات انترلوكين 1 بيتا المرتفعة بشكل كبير مع انخفاض MCHC (p=0.026) ولكن مع مستويات عالية من RDW (p=0.052). تظهر هذه النتائج أن انترلوكين 1 بيتا له تأثيرات مباشرة على مؤشرات الدم المتعددة في مرضى قرحة القدم السكري. من ناحية أخرى، لوحظ وجود ارتباط سلبي معنوي في هذه الدراسة بين مستويات انترلوكين 17 وتركيز السكر التراكمي.

تمت متابعة 26 مريضًا في هذه الدراسة حتى النتائج النهائية للعلاج بالمياه الفائقة الأكسدة، شُفي 24 منهم تمامًا. تمت مقارنة البيانات قبل وبعد العلاج. في هذا الصدد، انخفض عدد كريات الدم البيضاء الكلي وعدد العدلات بشكل ملحوظ بعد العلاج، في حين ارتفعت الخلايا الليمفاوية بشكل ملحوظ. هذه النتيجة تبين عودة تعداد الدم الكامل إلى طبيعته بعد العلاج

تم أيضًا تقليل السايتوكينات بعد العلاج، لكن هذا الانخفاض كان مهمًا فقط في حالة انترلوكين 1 بيتا (p=0.043) بالإضافة إلى أن مدة التعافي في هذه الدراسة تم الإبلاغ عنها بواسطة عدة عوامل. تقليل مدة التعافي ترتبط مع تقليل مدة القرحة (p =0.001) وزيادة في عدد الخلايا الليمفاوية (p =0.001) .

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فرع الاحياء المجهرية

قياس بعض الحركيات الخلوية والمعايير الدموية في مرضى قرحة القدم السكري المعالج بالماء الفائق الأوكسيد

رسالة مقدمة

الى مجلس كلية الطب - جامعة كربلاء

في استيفاء جزئي لمتطلبات درجة ماجستير في علم الأحياء المجهرية الطبية

مقدمة من قبل

فاطمه فاضل سعيد الموسوي

بكالوريوس علوم – قسم التحليلات المرضية- جامعة الكوفة

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