NEAR EAST UNIVERSITY

INSTITUTE OF GRADUATE STUDIES

DEPARTMENT OF MEDICAL BIOLOGY

MOLECULAR MEDICINE PROGRAM

THE COMPARISION OF *INTERLEUKIN-12 (IL-12)* GENE EXPRESSION ANALYSIS IN THE USE OF COLD ATMOSPHERIC NITRIC OXIDE (NO) GAS AND/OR WITH NPH INSULIN CREAM IN HEALING WOUNDS WITH TISSUE LOSS IN DIABETIC RATS.

M.Sc. THESIS

Ihab Yasir RADWAN

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M.Sc. THESIS

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Approval

We certify that we have read the thesis submitted by Ihab Yasir Radwan, "**The comparison of *Interleuken-12 (IL-12) gene* expression analysis in the use of cold atmospheric Nitric Oxide (NO) gas and/or with NPH insulin cream in healing wounds with tissue loss in diabetic rats**" and that in our combined opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Molecular Medicine sciences.

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Declaration

I hereby declare that all information, documents, analysis and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of Institute of Graduate studies, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

Ihab Yasir Radwan

31/07/2024

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I dedicate my Graduation from Master Molecular Medicine to my Department in NEU, my family, and my beautiful country Palestine.

To everyone, Thanks so much.

Ihab Yasir Radwan

ABSTRACT

The Comparison of *Interleuken-12 (IL-12) Gene* Expression Analysis in The Use of Cold Atmospheric Nitric Oxide (NO) Gas and/or with NPH Insulin Cream in Healing Wounds with Tissue Loss in Diabetic Rats.

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M.Sc., Department of Molecular Medicine

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Interluekin-12: IL-12 is one of the cytokines that play an important role in inflammation phase in the wound healing. So, when Immune cells stimulated will synthesize these Cytokines to be in the wound site. The IL-12 synthesis in high number as a results of high gene expression due to the inflammation and unable to heal the wounds. Wounds are so important to heal in healthy way and short time, and wounds in diabetic patient is a high challenge because of hyperglycemia can change to chronic wounds. Nitric Oxide (NO) can naturally produce by human body and can synthesize by most of skin cell types in healthy wounds. Many researches found that NO affect the immune cells. NO works as anti-microbial. NPH insulin cream (Neutral Protamine Hangedon) is commonly using for healing of wounds in both non diabetic and diabetic patient. And insulin is good that support the cells with nutrients at the site of wounds. So, this thesis did a gene expression analysis to IL-12 by used RT-PCR. By comparing between four groups: DC, DI, DNO, and DINO to get fully understand the effect of these treatment in the mRNA gene expression of IL-12. The total number was 24 samples from Diabetic rats divided in these four groups. And this thesis found a high significant on the DINO when compared with DC with P value< 0.0437. This found that compound therapy was better and effector more than single therapy. Also, when gene expression of IL-12 was high amount, this means that there's an inflammation and be chronic wounds so need more time in healing. But when used the NO which acts as antimicrobial, this study shown in DNO a low gene expression of IL-12 because there was no inflammation and NO made negative effect to IL-12 so better healing and faster.

***Keywords*:** Interleukin-12 (IL-12), Wounds healing, Diabetic Wound, Cold Atmospheric Nitric Oxide (NO), and NPH Insulin.

Table of Contents

Contents

CHAPTER III

CHAPTER IV

CHAPTER V

CHAPTER VI

List of Tables

List of Figures

List of Abbreviation

IL-12: Interleukin 12.

NO: Nitric Oxide.

NPH Insulin: Neutral Protamine Hagedorn insulin.

LPS: Lipopolysaccharide.

DNA: Deoxyribonucleic acid.

RNA: Ribonucleic acid.

PCR: Polymerase Chain Reaction.

cDNA: Complementary DNA.

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species.

Ct: Cycle Threshold.

qPCR: Quantitative Polymerase Chain Reaction.

VEGF: Vascular Endothelial Growth Factor.

IFN gamma: Interferon gamma.

TNF: Tumor Necrosis Factor.

CXCL-1: Chemokines Receptor.

IGF-1: Insulin Like Growth Factor 1.

IGF- beta 1: Insulin Like Growth Factor beta.

TD1: Diabetic Type 1

CHAPTER I

Introduction:

Introduction

Interleukin 12 (IL-12): is a recently discovered cytokine featuring a unique heterodimeric structure from two different protein subunits include p35 and p40, most of IL-12 is produce by monocytes, dendritic cells, natural killer cells and Macrophages resulting from a suitable stimulation. IL-12 can promote the cytolytic activity of several effector cells like natural killer (NK) cells, macrophages, Lymphokine activated killer (LAK), and T cells. In addition to that in type 2 diabetes, IL-12 plays an important role in the mechanism of arteriogenesis and ischemia angiogenesis (Brunda, 1994) (Ali, 2017) (Ali Curukoglu, 2023).

IL12 increase in many diseases for malignancy and leishmania. As a result of, IL-12 support differentiation of CD4+ T cells to TH1 effector cells that can activate natural killer (NK) also CD8+ T cells to make IFN- γ. So, this are important in the development of cell mediated immunity (Matias, 2011).

Resent researchers had discovered that giving of IL-12 after irradiation will reduces the effect and size of burn wounds from the radiation insinuation (Joanne Li, 2015).

Healing of normal wound occurs in overlapped stages: starting by haemostasis, following by Inflammation, then proliferation and at the end will be remodeling, so this will happen after injury to epithelialization be completed (Richmond NA, 2013).

Inflammatory mediators and cytokines secreted at the wound place, so they have an important role in the regulation processes by manage of cell growth. The role between growth factor and cytokines depends on deletion or addition of growth factors that have effect on wound repair. So, if happens any prolongation or failure in any phases this can be a result in over healing or delay wounds (Donna Bryan, 2005).

Nitric Oxid (NO) is a gas and can naturally produce by human body at number of tissues this production will be by enzymes from the L-arginine (amino acid), NO plays an

important role in pressure and blood flow. Also, reduce the activity of blood platelets. In addition, NO is found as Neurotransmitter. NO can bind to haem/heme (component of hemoglobin) by interaction with iron (Bruckdorfer, 2005).

NO can be synthesized by most of skin cell types examples: melanocytes, keratinocytes, and fibroblasts in healthy wounds, and several researchers studied the effect of NO on immune cells. They tried to understand how plays an important role in inflammation.

NO synthesized by NOS (Nitric Oxide Synthase) isoenzymes including NOS2 and NOS3 have an important role in the process of wound healing (Takashi Kitano, 2017).

Healing of diabetic wounds at Hyperglycemia or high blood sugar where neuropathy develop and microangiopathy, this lead to stop collagen regulation and cell proliferation, so when this stop performs, this will make wound healing delayed and increased the risk of bacterial infection (Nicholas A Richmond, 2013).

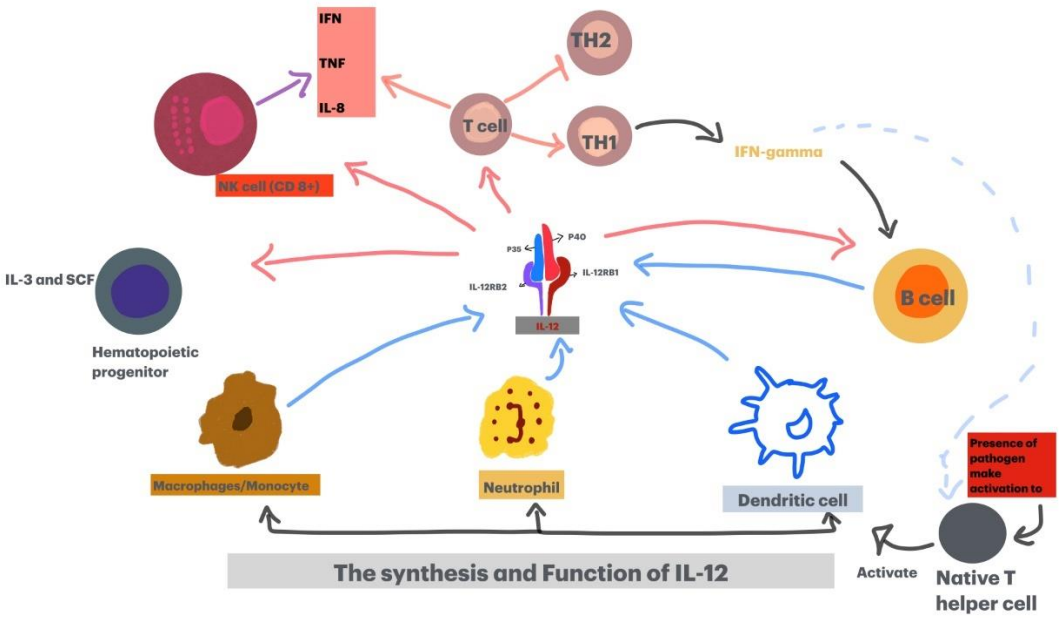
The NPH Insulin (Neutral Protamine Hagedorn) is commonly using for wounds in both non diabetic and diabetic because can make potential wound healing and also lower cost. So, when use insulin as spray, local injection or cream can decreased the time of wound healing (Lingling Zhao, 2017).

Due to the importance of treatment for diabetic wounds and preventing the danger on the health of diabetic patient so their several researchers are trying to find a good treatment. But their lots of challenges such as hypoxia and the collagen production will be decreased.

In Figure 1 bellow shown the Synthesized and the Function of IL-12 in wound healing and bacterial infection. IL-12 is a Cytokine heterodimeric structure formed from P40 and P35. And the receptor for IL-12 formed from two subunits include IL-12RB1 and IL-12RB2. And the synthesized of IL-12 performs when the Immune cells such as Monocyte, Nuetrophil and Dendritic cells activated by Native T cells due to present of Pathogens. As an immune response the immune cells synthesized IL-12 and this IL-12 when bind to the receptor this making activation to other immune cells. Like T cells that make activation to T_H1 and T_H2 and each one synthesized a different interleukin and also

IL-12 make activation to Natural Killer cells (CD8+). In addition to that, these are immune cells so the T cells and Natural Killer cells synthesized other Interleukins, TNF and IFN gamma that Play an important role in attack the microbial agents (Bram Verstockt, 2023).

Figure 1: The Pathway of IL-12 in the immune respone (adaptive form (Bram Verstockt, 2023))



Problem statement:

Diabetic patients have a high risk for several bacterial infection, long wound healing time and prolong pain. So, the study is searching how cold atmospheric Nitric oxide (NO) gas and NPH insulin affect diabetic rats. And the Inflammatory response or cytokines as Interleukin-12 (IL-12) how affect the healing process and make delaying when IL is dysregulated. Until now strategies of treatment are not completely effective. Few years ago, scientists started to see a high significant of using cold atmospheric Nitric Oxide (NO) gas as anti-inflammatory. Also, the NPH Insulin cream changed proliferation and metabolism of cells, and now using as wound healing treatment for diabetic patients. And unknown way how this makes changes in the expression level of IL-12.

This study tries to get a full understand by check the effect of cold atmospheric Nitric Oxide (NO) alone, NPH insulin cream alone, and NO with NPH insulin in IL-12 gene expression during diabetic wound healing.

The Purpose of the study:

Estimate the gene expression analysis of IL-12 in the wounds for the tissue of diabetic rats by comparing between Nitric Oxide (NO) alone, NPH insulin cream alone and both NO with NPH Insulin.

Objective for this thesis study:

1- Measure gene expression analysis for IL-12 in diabetic wound tissues which treated with NPH insulin cream alone, cold atmospheric Nitric Oxide (NO) alone or both together and found how these treatments effect on IL-12 gene expression.
2- Discover if when using cold atmospheric NO gas and NPH insulin cream together will give a good wound healing more quickly than use each of it alone.

Hypothesis for this thesis study:

Cold atmospheric NO with NPH insulin cream will give significant wound healing and less gene expression of IL-12 than each treatment alone. So, when use both of treatment together will give best healing more than use each one alone.

Significance of the study:

This study will give information how these treatments effect on the gene expression of IL-12. Diabetic patients have a delaying in wound healing so in this study may find important factors that manage wounds. In this study did comparing between three factors. And by this thesis study can determine which treatment has more effect and give good result according to the effect on gene expression of IL-12 gene.

Keywords: Interleuken-12, cold atmospheric nitric oxide (NO), NPH insulin, Wound healing, Diabetic wounds and diabetic rats.

CHAPTER II

Literature Review

Diabetes mellites:

Diabetes mellites a disorder and people with diabetes will have high blood sugar as a result of full or partial defect in production of insulin or in Function. When occurs organ damage, failure or dysfunction, this will lead to chronic hyperglycemia as nervous system, heart, and kidney. The prevalence of diabetes mellitus 366 million in 2011 according to The International Diabetes Federation, and scientists are expected in 2023 increase to 552 million. Types of Diabetes, specially most of cases of diabetes have two types include type 1 and type 2 diabetes mellitus. And there are several criteria to determine the type like age of having the diabetes, need insulin at diagnosis...ets (Alam Uazman, 2014).

And there's also other type of Diabetes called Gestational Diabetes perform with pregnant women who didn't have any previous history for diabetes. This so important to do prevention and treatment of diabetes by healthy food, without tobacco, trying to get normal body weight and physical exercise (Tao, 2015).

The function of Nitric Oxide:

Nitric Oxide (NO) has lots of Function in biological system. NO can regulate several physiological mechanisms with low concentration of NO, but also over synthesis of NO plays a role in causes of several diseases. When activation the isoenzyme for NO called NO synthase (NOS) will makes production of Nitric oxide. In addition to that, Nitric Oxide has an important function on Immune system, so the NO that production from INOS (Isoenzymes NO synthase) has antitumor and antimicrobial effect and mostly associated with free radical production. Increase the activity of INOS plays an important role in protection from several parasitic and bacterial infections. So Nitric Oxide plays a role in control immune system processes (Ali Curukoglu, 2023).

Immune system and factors play role on it:

In T cell Lymphocyte as a specific immune response. The perform of activation to lymphocyte will regulate several immune cells. And T cell lymphocyte have several

subtypes and one of these subtypes (Th-1 Lymphocyte) produce TNF-a, IL-2, IL-12 and IFN gamma (Interferon gamma). Another subtype (Th-2 lymphocyte) produces IL-13, IL-10, IL-6, IL-5, and IL4, so factors produced from Th-1 will increase production of Nitric oxide (NO), while factors from Th-2 will decrease production of Nitric Oxide (NO). So Nitric oxide (NO) can make negative effect on Th-1 lymphokines the result will be to inhibit the Th-1 cells, but for Th-2 lymphokines don't has direct effect. So there a balance of cytokines that produce from Th-1 and Th-2 (MartinaAntosoca, 2012).

How can use Cold atmospheric Nitric Oxide in wounds healing:

Cold Atmospheric Nitric Oxide. for skin infection and chronic wounds treatment can use non thermal atmospheric pressure plasma (CAP) that contain Nitric Oxide and nitrogen oxide, so a similarity in results between NO and CAP treatment. So NO has an important role in the efficiency of CAP. CAP has a good benefit on patients by protect them from bacterial infection, chronic wounds and improve wound healing. So as an example, can used CAP as antibacterial so NO can be efficient with antibiotic resistant bacteria. Several studies found that cold plasma can made wound healing better by made gene expression changing for relevant molecules (ChristophV.Suschek, 2016).

Nitric Oxide is a gas inorganic and can in aqueous solution be soluble at 2mM concentration. Nitric oxide can go through biological membranes because NO has lipophilic properties and the size of NO is small. And if made comparison between NO and other radicals at the reaction in biological system the NO had less reactivity than others. When performed oxidation this will produce unstable intermediates molecules and highly reactive, one of it: Reactive nitrogen species (RNS) as peroxynitrite ($ONNO^-$), Nitrogen dioxide (NO_2) and dinitrogen tetroxide (N_2O_4) (ChristophV.Suschek, 2016).

The synthesize of Nitric Oxide (NO) can be done by enzyme called Nitric oxide synthase (NOS). This enzyme had isoenzymes, until now they knowing three NOS isoenzymes. Two of them working in blood vessels endothelial cells to produce NO, but on immunogenic and inflammation can be done by the third iso NOS, so NO micromolar range will be high. And can be indicated as pro inflammatory stimuli in all cell types of mammals (S.Moncada, 1991).

The activity of NOS determines the concentration of NO, also in human can make production of NO from NOS independently. UV radiation between 354nm and 366nm and low pH in stomach and skin affect the NO_2^- . This gave good significant to stop the forming of capper ions and antioxidants (Christian Opländer a, 2012).

When bacteria presented in wounds this affect the healing in normal process. As a result, from here can understand the important role of using nitric oxide as antibacterial treatment which is a good and useful treatment for contaminated wounds (ChristophV.Suschek, 2016).

Physiological effect of Nitric Oxide as antimicrobial: NO is can be released from airway cells in the upper part and this happening as an immune process. And as a response of bacterial infection. There a Bitter taste receptor on the upper airway detecting the organism like *S epidermis* or others, this allows NO to go downstream (Lee RJ, 2014) (Brett PJ, 2008). The NO go to target and produce S-nitroso thiols and peroxynitrites that will be in the bacterial component region. By NO can affect the replication mechanism to DNA, factors or enzymes. So, NO is directly acts as antimicrobial (Alan D. Workman, 2017).

IL-12 structures, receptor, and role in wounds:

Interleukin-12 (IL-12) one of cytokines. Cytokines acts as markers in connection between different cells types. So can be pro inflammatory cell or anti-inflammatory, start differentiation and maturation, or can act like growth factor. And one type of cytokines is IL-12. Interleukin 12 is a heterodimer this means had two different types of protein including p35 subunit (35kD alpha chain) and p40 subunit (40KD beta chain) and linked by bond called disulphide bond. These both subunits are formed by genes on the human chromosome 3 and chromosome 5. The production of alpha-chain is less than beta-chain. That means the free p40 or beta chain can be notice. This homodimers of p40 had other functions one of these functions is acting like chemoattractant macrophages. Beta chain is found also in IL-23 (Sebastian Zundler, 2015).

In addition, IL-12 receptor formed from two different subunits, called as IL-12β1 and IL-12β2. The gene for these two subunits are on chromosome 1 and chromosome 19. Both receptor subunit made from oligomers and homodimers that mean can bind to IL-12 only with low affinity. So according to that IL-12p40 will bind to IL-12β1 but IL-12p35 will bind to IL-12β2. IL-12 the main targets are Natural killer (NK) cells and T cell. IFN-gamma production can be indication of IL-12. So, IL-18 can discover the INF-gamma activation. When IFN-gamma binds to promoter makes phosphorylation interaction of IL-12R and IL-12 (Chua, et al., 1994) (Sebastian Zundler, 2015).

They found IL-12 made inhibition to Th1 cells that synthesize IFN-gamma this can make macrophages active that can produce IL-12, So Nitric Oxide make stop to this feedback and prevent the much Th1 amplification (Fang-Ping Huang, 1998) (Nicolae Corcionivoschi, 2024).

Several researches tried to get the relation between Diabetes type 1 (T1D) and IL-12, so was observed by studies that in children with T1D had high expression of IL-12 when compared with healthy children (Sebastian Zundler, 2015) (Ashish K. Marwaha, 2014).

IL-12 related with some disorders:

There is a relation between IL-12 and Rheumatoid arthritis (RA), rheumatoid arthritis is a joints disorder due to chronic inflammation on joints. Resent researches found that IL-23 made an important role to IL-12 in making T_H1 in rheumatoid arthritis. When used mouse model the collagen induced arthritis (CIA) is the main for RA. And the animal who had deficiency in IL-12 was protestation from the CIA. So, when IL-12 given exogenous during the first stage of the disease this made the disease more sever and got lower severity when they made neutralizing antibody so did IL-12 inhibition. They found that patient with RA had more expression on IL-12 in peripheral blood and synovial tissue to these patients when compared with normal control. And the drugs used for RA treatment affect the IL-12 by making down regulation to IL-12 (Iain B. McInnes, 2011) (Sebastian Zundler, 2015).

Under research is trying to fine the role of IL-12 in cancer. the main idea, IL-12 is a anti tumorigenic cytokine so increase the immune system response against cancer. And how IL-12 acts as anti-tumor have different mechanism. And they trying to develop several models to study this and understand the role of IL-12 in different type of cancer like solid tumor or other cancers (Mario P Colombo, 2002).

Insulin plays a role in wound healing:

NPH Insulin cream, generally Insulin function allow the entry of amino acids and Glucose inside the cells and in wound cases insulin can't reach to these parts, so the cell has lack of energy this lead that these cells can't enter on mitosis and hard to do healing. According to this, the most challenges for doctors when doing surgery to diabetic patient or any surgery for open wounds and loss tissue how can doing improvement of granulation tissue then the epithelization. Newly for the chronic wound treatment especially in diabetic patients started using negative pressure therapy and hyperbaric oxygen. Several studies shown that in open wounds can used insulin for both patients with non-diabetic or with diabetic. When applied insulin in the side of wound as cream, or injection give a rapid healing for the wounds (İsa ÖZAYDIN, 2018).

Wound healing can get affected by growth factors, in addition, growth factors regulate several phases in the healing process. One of growth factors is IGF-1 (insulin like growth factor -1) activates the production of glycosaminoglycans, proteoglycan and collagens. Another growth factor is TGF-beta1 (Transforming Growth Factor beta -1) make stimulation to produce protein of ECM (Extracellular Matrix), also for mesodermal cell types start the phenotypic differentiation (İsa ÖZAYDIN, 2018) (Darby IA, 1997) (Aydin F, 2013).

In several studies, they studied the NPH insulin in the healing of wounds. They found NPH insulin gave positive effect on wound healing especially in wounds that loss tissue the result made the healing faster and finished all stages of healing for this open wounds. As conclusion they found when used NPH insulin gives you faster wound close and get healing also formation of scar tissue after healing lead to reduce period require for healing, reduce the coast of medical serves and the patient get treatment in short time so

the time in the hospital will be shorter (Bairy KL, 2014) (İsa ÖZAYDIN, 2018) (Benko, 2022).

Chronic Wounds:

When the wound unable to heal during the suspected time for healing these are chronic wounds, their no exact time for the period of healing, but in chronic wound will take too long time to get heal. The most challenges in chronic wounds are size, morbidity, body location, and pathogenesis. Adult people with neuropathies, diabetes and other diseases can get chronic wounds. Also, in other injuries caused by genetic factor or radiation. In addition, the dysfunction of the Immune system playing an important role of causing chronic wounds. Studies found in the population worldwide around 1-2% of them had during lifetime a chronic wound. And the percentage increased in less developed countries than developed one because of increase parasitic and chronic fungal infection (Vincent Falanga, Chronic wounds, 2022).

Macrophages played an important role in healing of wounds. And this important because plays a role in scar forming and phenotype healing. So, Macrophages be activated and been M1 and this secreted IL-12, Il-6, TNF, IL-1 and several cytokines. But to get more pro reparative and anti-inflammatory, will change to M2 and this synthesized IL-10, TGF-Beta and others. The appear of Macrophages as pro-inflammatory at the site of wound so important for the healing of the wound (Vincent Falanga, Chronic wounds, 2022).

When occurs Infection or hypoxia led to change the acute wound to chronic wounds, Several Factor played a role in occurred chronic wounds. The Hypoxia in the site of wounds led synthesized mediators and made activation to hypoxia inducible factor 1 (HIF1) a transcription factor that regulate re-epithelialization and angiogenesis (Gushiken, 2021).

Other type of Granulocyte that plays role in wound healing is Mast cells. These cells have function as pro-reparative so can synthesis growth factors. And in chronic wounds their high number of mast cells so inhibited repair mechanism by lead to protease activity and degranulation is increase. And in some studies, found mast cells role with fibrosis (Tellechea, 2016).

Physiological process of wound healing:

Wound healing occurs in four overlapping steps: starting with hemostasis, then following by inflammation, after that proliferation and at the end remodeling step. And these steps after injury occurred immediately, the first step is hemostasis in this phase occur blood clotting and vasoconstriction, the aim of this phase to reduce loss blood. To start the healing process the platelets, produce a cytokine and factor growth affect the endothelial cell, fibroblast, and also the immune system to do the healing. The second step is inflammation can be in 7 days. And in this phase, there phagocytic cells work on this phase like macrophages and neutrophils. To avoid contamination and make wound cleanse will secrete protease and reactive oxygen species (ROS) from Neutrophils. After that blood monocyte come to the site of wound and then form macrophages by differentiation. Then by phagocytosis clean the nonviable tissue and bacteria, in addition to that repaired blood vessels damage by synthesized more cytokines and growth factors. Then the proliferation step to start, in this step occurs angiogenesis (formation of new blood vessels), granulation of tissue and also epithelialization. At the end phase that remodeling start when close the wound and can take 1 to 2 years or more (Falanga, 2005) (Gregory S. Schultz, 2003) (Robert G. Frykberg, 2015).

Chronic wounds Pathophysiology:

When the wounds can't heal in the normal way of healing wounds in the healing steps and time of healing will call this wound as chronic wounds. In chronic wounds the inflammation phase will delay. Will make changing at molecular structure, and will produce lots amount of ROS, proteases, and cytokines. In addition to that the stem cell will be in less amount and dysfunction, also will founds lots of infection (Vincent Falanga, Chronic wounds, 2022) (Robert G. Frykberg, 2015).

Diabetes and chronic wounds:

The most worried complication with diabetes mellitus patient was the wound healing problem. And there a complication called diabetic foot syndrome, and because of increasing rates of patients with diabetes this lead to affect the public health and foot ulcers will increase in world. Several factors affect the wound healing in patients with diabetes and link to increase glucose and make complication in neurovascular. This foot ulcers in

diabetic patient been chronic. To do treatment in chronic wounds must directed treat the main factor that led to chronic wound, and the standard method to make treatment to diabetic ulcers must controlled glucose level and made sure for the status of vascular. in addition, must use antibiotic treatment to prevent infection (Elena Tsourdi, 2013).

Immune System and Chronic wound healing:

Immune system regulates the production of chemokines, growth factor and cytokines in the wound healing time. As an example: in patients with Diabetic foot ulcer will increase the production of VEGF (Vascular Endothelial Growth Factor), and IFN-gamma (Interferon-Gamma). When they did study in mice had a deficiency in receptor of IL-36 the result was delay in the healing of wounds, this occurred because of over synthesized of CXCL-1, TGF-bata, and also IL-36- gamma. In addition, the chemokines receptor called CCR4 for mice with diabetes make inhibition to expression of cytokines that important in wound healing as IL-12, IL-1 beta, IL-10, IL-6 and TNF-alpha (Kamila Raziyeva, 2021).

Family of IL-12:

Cytokines play an important role in two phases of wound healing: first one hemostatic and also in inflammation phase. These can immediately affect the cells against microbial and infection like lymphocyte. The Group of Cytokines occurs due to how can make signaling pathway, and biological structure. As Tumor necrosis factor (TNF) and with IL-1 usually using NK-$_K$B signals (Nuclear Factor $_K$B). There a cytokines Group contain Interferons (IFNs), the receptor complex for those been the gamma chain using. So, IL-23, IL-27, IL-6 and IL-12 family started the researches to focus on these. The structure of IL-12, IL-23, and IL-31 are link to this Family, the receptor complex contain gp130 on a JAK-STAT signaling pathway. In general, when the signal chain produce, cytokines will bind to receptors this receptor can be heterodimeric or homo dimeric (Elia D. Tait Wojno, 2019).

Production of IL-12 and gamma Interferon:

The effect of circulating Lipopolysaccharide (LPS) is producing the Gamma Interferon (IFN-gamma), and occurs after endotoxic shock. IFN-gamma can synthesis from in vivo cells, when they injected the mouse spleen with endotoxin injection the B

cells and T cells secreted IFN-gamma. RNA of the IFN in excessive amount in CD8+ and CD4+ of T cells in spleen. Interluekin-12 an activator to IFN-gamma that produced from NK cells and T cells. So, IL-12 makes releasing to IFN-gamma synthesized in endotoxemia (F P Heinzel, 1994).

The mRNA of p35 subunit of IL-12 is active all the times but mRNA of p40 IL-12 found that increasing when made injection of LPS. And the activator for IL-12 is heterodimer p70 when inject LPS will appear in serum between 2-4 hours. At the end, they found that IL-12 played an important role in activation of IFN-gamma when occurs endotoxemia (F P Heinzel, 1994).

IL-12 plays an important role in protection from microbial intracellular infection, and make malignancy control by the activation of effector immune cells. When occurs problem in balance and control this make an immune system disorder. So, in some autoimmune disorders will be related to cytokines one of these cytokines is IL-12 (Liu, et al., 2005).

The β – actin gene or ACTB (β-actin)

Actin is a part of cytoskeleton that plays an important role in control of processes in cell, as cell division, also in control gene expression and cell migration. Their isoforms for actin. Four of isoforms binds to smooth (alpha $_{sm}$ and gamma $_{sm}$) and (alpha $_{sk}$ and alpha $_{ca}$) started. The two isoform β- actin and γ actin are synthesize from different genes, the ACTB gene for encoding β- actin and ACTG1 for encoding γ actin. So has different amino acids and this difference made the two isoforms biochemically unique (Tina M. Bunnell, 2011).

They studied the importance for requirement of β- actin in vivo, so they made deletion in ACTB gene in location exon 2 and exon 3 and after analysis by western blotting for protein, they found that several tissues had β- actin in Hypo-morphic state and ($ACTB^{+/-}$) that mean heterozygous. And the activity of β- actin decreased in several tissues when this compared with Normal $ACTB^{+/+}$. And mice with $ACTB^{+/-}$ around 30% of them died at age between 5 week and 18 week. When occurred deletion homozygotic for ACTB the embryo will be lethality die, so no mice with $ACTB^{-/-}$ will born alive (Tina M. Bunnell, 2011).

The mRNA of β- actin is useful as a housekeeping gene for internal reference. And using in Reverse Transcriptase polymerase chain reaction (RT-PCR), and this β- actin produced in most of cells so occurred expression in most of cells. In this study require to amplify a β- actin gene in pure statues from mice then use RNA to do RT-PCR to produce cDNA then followed to do cloning. this means to check the quantify for samples, can make normalization to measures by the expression of housekeeping gene and the β- actin one of genes that can used as housekeeping, and this genome contain pseudogenes which mean can give false results (T. Raff, 1997) (S. Selvey, 2001).

Housekeeping genes needed to make adjustment for function of cells and this gene found in all cells of the organism have expression. For gene expression arrangement, used the housekeeping gene in different practical condition, so for that reason can used as internal control. The internal control must occur expression in stable state and found in all tissue and cells (Lin, 2012).

IL-12 cytokine family share a chain structure and can made interaction with several molecules, so immune cells act with similar signals that can cause of immune tolerance or inflammation. The subunit alpha and beta of IL-12 have a special biological activity (Lin Sun, 2015).

CHAPTER III:

Methodology

Material and Methods

Total samples ware 24 Wistar albino rats in this study divided them in four groups: Group 1 was a diabetic control group (DC), group 2 was diabetic nitric oxide CAP/NO group (DNO), group 3 was a diabetic NPH insulin group (DI) and last one group 4 was diabetic NO with NPH insulin group (DINO). The study got the approval for ethics from the Near East University's Animal Experiments Local Ethics Committee and the reference Number: 2021/141

This study received the samples ready to lab after they did a diabetes model and included diabetes inside rats then they did a wound model at the same time they made measurements to wound contraction rate then applied NPH insulin and/or CAP/NO according to each group, at the end they taken samples of skin from wound area and prepared samples.

Gene Expression analysis:

First was RNA Isolation and quantification: on the first day of healing wound treatment and the last day of treatment at 14^{th} day collected around 30 mg of tissue from wound area. To prepare for RNA, all tissues samples directly frozen in liquid nitrogen and saved at -80 ^{o}C. All the RNA was isolated by TRIZOL kit reagent (Invitrogen, Carlsbad, CA, USA). And used Nanodrop 1000 spectrophotometer to check the quantity of RNA and the ratio between the absorbance of Nucleic acid and the absorbance of protein should be 2.00 and this shown that study products were good and can worked with it. Then made cDNA.

Quantitative Real Time PCR (qRT-PCR) for Gen Expression Analysis:

In this thesis used the Real Time quantitative PCR Reverse Transcriptase PCR (Polymerase Chain reaction), (qRT-PCR) also used Real time PCR from the Insta-Q96 Plus (MIDC, Wagle industrial Estate, India) and SYBR kit.

Prepare for PCR and cDNA

Because in this thesis had RNA, So must be very careful and worked in cold condition because RNA can be denatured at 72 ^{O}C in 5 minutes, so for that reason in this study converted to cDNA by using **HIBRIGEN cDNA Synthesis kit**: the reaction setup as shown in Table 1 (note: This thesis worked with mRNA or cDNA because needed the coding region only exons without introns.).

Table 1: HIBRIGEN cDNA kit and SETUP

Component	**Volume**
Reaction Buffer	4µl
Enzyme Mix	1µl
Nuclease-free dH_{2O}	10µl
Total RNA	5µl
Total volume	**20µl**

Then set up the Real Time PCR device as shown in table 2 to converted the mRNA to cDNA and then can do the analysis of gene expression. Also used the Reaction Buffer to make stabilizing to PH =7 for the enzyme, and Enzyme Mix known as Reverse transcriptase enzyme that taken from viruses and this enzyme to converted mRNA to cDNA to enable worked with it and the total volume here must be **20µl.**

Table 2: PCR setup for cDNA

Step	Temperature	Time
cDNA synthesis	42^{o}C	60 minutes
Inactivation of kit	80^{o}C	10 minutes

For the PCR Machine needs to set up as in Table 2. The enzyme worked at 42^{o}C for 60 mins and the second step at 80^{o}C for 10 mins to make inactivation of kit because there an enzyme and at 80^{o}C will be denatured.

So, after that can had cDNA and can worked to do PCR, an important point must return RNA to -20^{o}C to didn't lose it. Because as mentioned RNA is very fragile.

Primers:

The important step before started PCR, must designed forward and reverse primers that required to be specific for the thesis gene IL-12 and can designed manually, by used NCBI website or by snap-gene app. So, the study the forward and reverse primer for IL-12 as shown in Table 3 below.

Table 3: Forward and Reverse primers for *IL-12*

Forward primer *IL-12*	5`- CTCATGATGGAAGAGACCCCC – 3`
Reverse primer *IL-12*	5` - AGCTCATTCTCTGCAGGC – 3`

In this study the housekeeping gene was *ACTB gene* and in Table 4 shown the primers for this gene.

Table 4: Forward and revere primer for *ACTB gene*

Forward primer *ACTB*	5'-ATCTATGAGGGTTACGCGCTC-3'
Reverse primer *ACTB*	5'-AATGTCACGCACGATTTCCC-3'

These primers came from company with concentration 100µM so when worked with primers need concentration 10 µM, so required to do a dilution reaction by put 10µl from 100 µM with 90µl of Nuclease Free dH_2O. According to equation M1*V1=M2*V2 and after that got primers with concentration 10 µM

qPCR:

The next step was preparing to qPCR so, in this thesis used kit called **2X SYBR Green qPCR Mix,** and this kit contained very important enzyme Taq Polymerase enzyme for synthesis and elongation of the DNA and the probe was SYBR Green Dye. And then added cDNA template and primers so the primer bonded to template strand and Taq polymerase made adding to nucleotides, SYPR Green dye for gave fluorescent signal in the Real time PCR. In this study had 24 rats. From every rat had two samples so this thesis had 48 samples and each one was done in duplication so prepared for 96 samples + 2 negative controls as shown in Table 5.

Table 5: preparation to 2X SYPR Green qPCR Mix

Component	**1X**	**96X**
2X SRYB Green qPCR mix	10µl	960µl
Forward Primer (10 µM)	0.8µl	76.8µl
Reverse Primer (10 µM)	0.8µl	76.8µl
dH_2O	5.4µl	518.4µl
cDNA	3µl	-

The total volume was 20µl. In negative controls must putted 3µl of dH_2O instead of cDNA.

The study used 2X SYBR Green qPCR mix that contained Taq polymerase enzyme that is heat resistance and SYBR Green as a probe for Real Time PCR. And for the Real Time PCR then added Forward primer and Reverse primer to allow the Taq polymerase to work and added nucleotides.

Then made adjustment to the PCR machine according to the 2X SYBR green kit so in PCR had three steps: first one denaturation to open the double helix cDNA then the second one was annealing when the primers attached to template and need specific temperature according to primers and the last one was elongation so used Taq polymerase

to synthesis the template and this repeated 40 cycles. So, the setup of time and temperatures shown in table 6.

Table 6: Thermal Cycle condition for PCR 40x Cycles times:

Stages		**Temperature**	**Time**
First denaturation		95ºC	1 min
40 Cycle	Denaturation	95ºC	15 sec
	Annealing	62ºC	15 sec
	Elongation	72ºC	45 sec
Melting Curve Analysis		72ºC	

Data Analysis study:

The statistical analysis was done by GraphPad Prism 10.2.3 version, the gene expression data shown as Cycle Threshold (Ct), the Ct value is the number of cycle where the logarithmic plot of PCR cross. The expression of IL-12 was compared between four groups by using (2^-ΔΔCT) method called Fold change. ΔCT= Ct of *IL-12* gene – Ct of *ACTB* (housekeeping gene). In this thesis Used **One way ANOVA** statistical analysis to compare between four groups and **T test** to compare between Diabetic Control (DC) and another group.

Statistical significance value is P<0.05

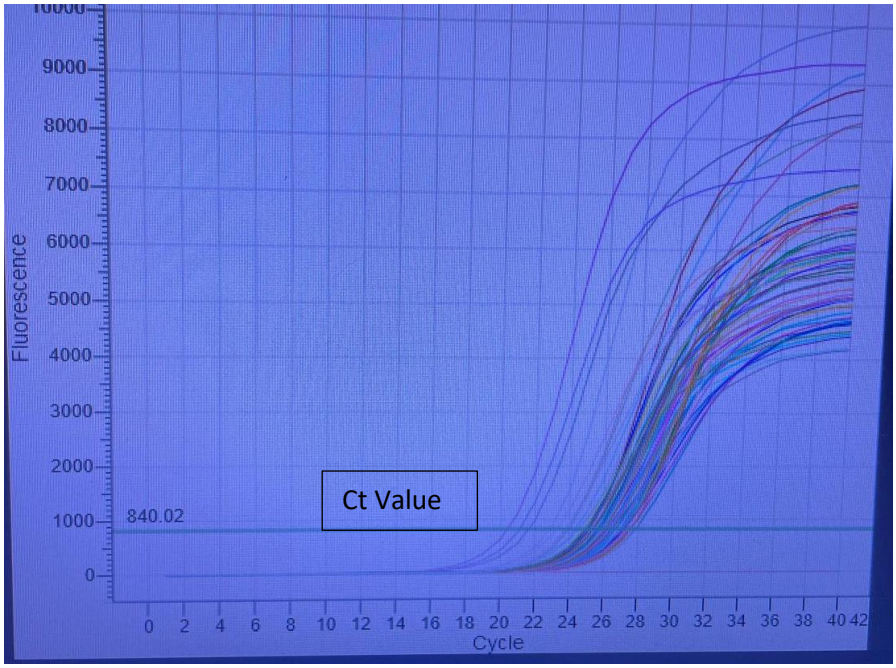
CHAPTER IV:

Results

IL-12 Gene Expression analysis in Wound tissue:

First was the result with Ct value from the qPCR for the cDNA of IL-12

Figure 2: Ct PCR for 24 rats and negative control.



So, as mentioned before in this thesis had 24 rats divided in 4 groups: DC, DNO, DI, and DINO.

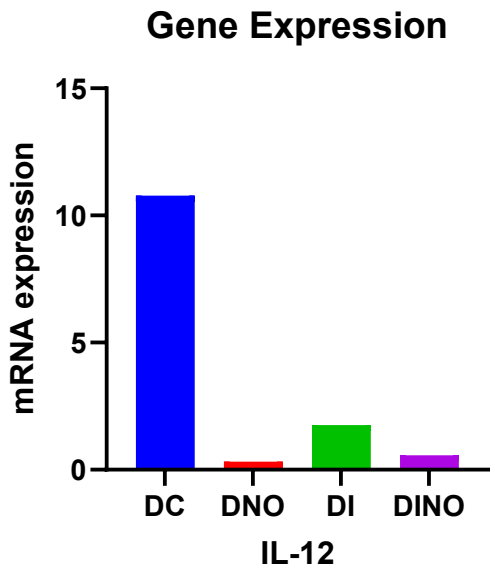
In this Figure shown the Real time-PCR curves for study samples. Real time PCR curve also known as Amplification Curve, that give information about the quality and purity of samples and should be as S shape as shown above. If become sharp not S shape this means samples dirty. But as shown in the figure 2 all of the study sample results were in S shape. Also, this curve give information about how much product has in study samples. The baseline phase: the beginning of cycles and the fluorescence is very little detect. Then when the curves start to rise, the amount of fluorescence detect is more and increase the product so this point, known as CT value (Cycle Threshold). Ct value is the number of cycle where the logarithmic plot of PCR cross. And in this study, worked with the Ct value for gene IL-12.

And the housekeeping gene as a control reference also divided in the same 4 groups in the study. And the same things for the PCR Curves and then got the CT valve. These four different groups made correlation analysis. In this thesis the targeted gene was IL-12 in these group. And the Housekeeping was ACTB (beta actin gene) in these groups. The study of applied therapies in each group will help this thesis to recognize the effect in correct way.

The thesis did data analysis to gene expression by analysis of (2^-ΔΔCT) used GraphPad prism for the four groups comparison.

The thesis aimed from this study analysis to understand the impact of therapeutic material that applied to the wound like used NPH insulin cream, cold atmospheric Nitric Oxide (NO) and both DINO for diabetic wound healing and see the effect on IL-12 gene expression and get the information from the Ct value for each sample.

Figure 3: IL-12 mRNA expression level in all groups



DC: Diabetic Control, DNO: Diabetic Nitric Oxide, DI: Diabetic Insulin, DINO: Diabetic insulin+ NO.

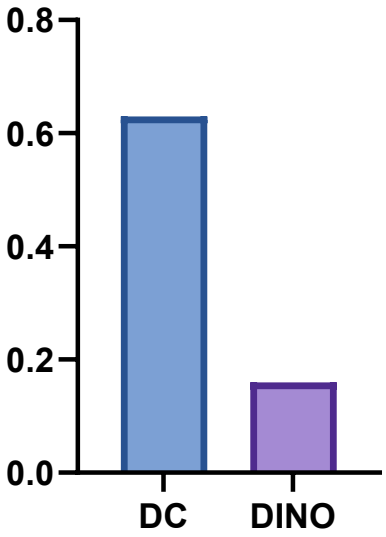
Done this statistic analysis by: ***One Way ANOVA**

In figure 3 shown the IL-12 gene expression in the four groups by the bar graph, DC showed that IL-12 mRNA expression was the highest level, then the DI showed the IL-12 gene expression in moderate level, but the DNO showed the lowest IL-12 mRNA gene expression and the fourth group DINO had a higher IL-12 mRNA gene expression more than DNO. But if made compression between the three treatment that used for wounds and gene expression of IL-12 in order from high to less: DI, DINO, and DNO.

When did comparison between **Diabetic control (DC) and Diabetic insulin + NO (DINO) by used T-Test statistical analysis:**

The P value for DC and DINO is 0.0437 so less than 0.05 so this is significant as shown in Figure 4 and Table 7. And DC had more IL-12 gene expression than DINO.

Figure 4: Compared between DC and DINO



*By T-test

Table 7: Compared between DC and DINO

P value	0.0437
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.116, df=27

The DINO had a good significant effect when compared with DC. And this due to the effect of compound treatment. P value: 0.0437 so less than 0.05 this so important in this study and gave good information for this study Hypothesis. This mean when used the compound treatment gave a good significant.

The compared between DC and DI

By used T-test for statistical analysis and the P value here is 0.2940. so not significant as shown in Table 8 and Figure 5.

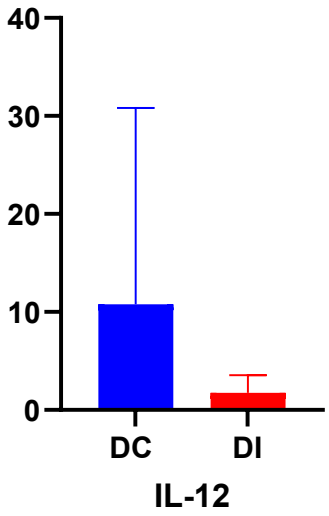
Table 8: compared between DC and DI

P value	0.2940
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.085, df=16

The DI group didn't show a significant effect when compared with DC the P value: 0.2940 so more than 0.05 so not significant. So, when used the PNH insulin alone didn't give a good significant as the DINO.

So, if compared between the three groups of treatment the DINO only gave a significant effect when compared with DC but the other groups as DNO and DI didn't give any significant. And this so important for this study.

Figure 5: Compared between DC and DI



*T-test.

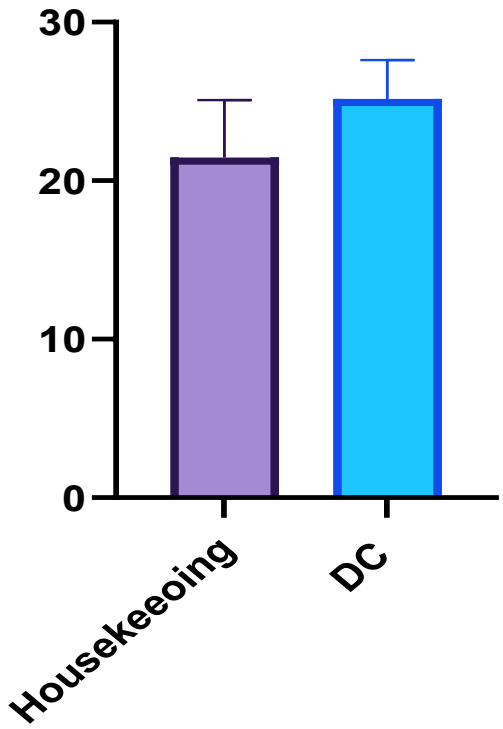
Compared DC group between the targeted gene IL-12 and housekeeping gene ACTB:

After statistical analysis by T-test found the P value is 0.0079 which mean less than 0.05 so this is significant. As shown in Table 9 and Figer 6 below.

Table 9: compared between IL-12 and ACTB

P value	0.0079
P value summary	**
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.924, df=22

Figure 6: compared the DC group between IL-12 and ACTB.



*T-test

In this study did the gene expression for both targeted gene *IL-12* and housekeeping gene *ACTB* in the four groups. The control group had the $p<0.0079$ between the IL-12 and ACTB. Which mean there a tight correlation between them.

Housekeeping gene a gene that occurred to it expression on all cells and in stable state so in this thesis used the housekeeping gene to make adjustment to the function of the cell and be sure the cell worked, because this thesis did clone gene and wanted to be sure that the cell worked normally so that's why in this thesis used the ACTB as a Housekeeping gene.

CHAPTER V:

Discussion

The diabetic wounds are type of chronic wounds, as a result of wound healing delay due to the effect of hyperglycemia. When patients have high blood sugar levels, this will be a good environment for bacteria and microbes. Leading to several complications as prolonged wound healing (Burgess JL, 2021). In this study, there are four groups of rats classified according to the type of treatment applied on them. Diabetic Control group (DC) was a group contained rats with diabetic model but without adding any treatment. Diabetic Nitric Oxide (DNO) group was contained rats with diabetic model and treated only by Nitric oxide (NO). Diabetic NPH insulin (DI) group was contained rates with diabetic model but treated only with NPH insulin. The last group was a Diabetic Nitric Oxide with NPH insulin (DINO) so both treatments applied on this groups. And this study tried to understand the effect of different treatments on the wounds healing in each group by studying the effect of each treatment on the gene expression of *Interleukin-12 gene* (IL-12).

The hyperglycemic state accompanied with reactive nitrogen species (RNS) and reactive oxygen species (ROS) production into the blood. Resulted in cessation of wound healing within inflammatory stage without completing the proliferation stage, which explain the prolongation of inflammatory stage (Bansal S, 2012). Shekhter and his team found that NO act as antimicrobial agent in wound healing. Though trying different concentration of NO on the site of wound area. Including 500 ppm for 60s once daily and they performed this trial for 6 days, in order to reduce tissue hypoxia and microbial infection (Shekhter AB, 2005). In this study, Nitic Oxide was applied in 200 ppm for 90s once daily for the DNO and for DINO groups.

NPH insulin plays an important role in metabolisms alterations and cell bioadvection within wounds area, which correct the shortage of glucose and amino acids through providing cells with nutrients resulted in cells recovery and healing in short time (Ali Curukoglu, 2023). In this study, NPH insulin was applied in DI group and DINO but

in DINO is applied after treatment with NO. On the other hand, there are several endogenous molecules like growth factors that play an important role in the regulation of cell responses and wound healing process. In chronic wounds they presented in unequal quantities in comparison to acute wounds. Which play role in prolonged wound healing and inflammatory stage (Anisyah Ridiandries, 2018).

Ali Curukoglu and his team study the same groups with focusing on different gene involved in wounds tissue histopathology, in addition to gene expression of growth factors as transforming growth factor beta (TGF-Beta) also for IL-8. They found that NO act as antibacterial agent with high expression of IL-8 (Ali Curukoglu, 2023).

Interleukin-12 (IL-12) is a type of Cytokines formed from two subunits including p40 and p35. They are produced from T cell Lymphocyte in order to attack against bacterial or microbial infections. Cytokines play an important role in two phases of wound healing such as hemostatic and inflammation phase. Several subgroups of cytokines produced in the response of signaling pathway and biological structure. So, when the wounds had a microbial infection or bacteria, the immune cells as T cell Lymphocyte synthesize the Cytokines as IL-12, which will increase the mRNA gene expression (Lin Sun, 2015). Taking an example, TH1 that induce the production of IL-12, IL-2 and INF gamma (MartinaAntosoca, 2012). Whereas, this was agreed what noticed among DC group in this study.

Nitric Oxide has a control of cell migration, inflammation and proliferation which are significant on wound healing process. In addition to that plays a regulation role of apoptosis and in vasodilator at the inflammation stage. All of these to provide more oxygen on wound area that are so important for healing process. Because the NO work as antimicrobial as a result on wound area will make cleaning from microbes and pathogens. On other hand, NO acts on behavior of immune cells as macrophages and neutrophils. When this study applied the NO on the wound site NO attacked any microbial and worked as antimicrobial so the inflammatory cells like lymphocytes and macrophages didn’t synthesis cytokines in high amount because the NO was effect as antimicrobial and prevent any infection (Bruckdorfer, 2005).

Also, T Lymphocyte have several subtypes: Th1 produce TNF gamma, IL-2, and IL-12 this increase the production of Nitric oxide. the other subtype Th2 produce IL-3, IL-10, IL-6 and IL-5 this decrease the production of Nitric Oxide. So according to that Nitric oxide will make negative effect on Th1 and Cytokines that produce by Th1. That means NO is naturally produce and when used as treatment this make high amount of NO at wounds area, this will lead to negative effect on the immune cells and cytokines that produced by it. So will lead to make negative effect on lymphocyte and IL-12.

This study results support this idea, because the lowest group with gene expression of IL-12 was the Diabetic Nitric Oxide (DNO). In this group was used only Nitric Oxide so acts as antimicrobial and prevent the prolong in inflammation stage. But have a high effect on IL-12.

Insulin function focusing on entry of nutrients like glucose and amino acids on the cells at the wound area this due to lack of nutrients on those cells. NPH insulin is using to provide cells with nutrients and improve the healing in better state, in addition cells can perform mitosis and proliferation. According to this, the most challenges for doctors during surgery to diabetic patient or any surgery for open wounds and loss tissue how can doing improvement of granulation tissue then the epithelization. Newly for the chronic wound treatment especially in diabetic patients started using negative pressure therapy and hyperbaric oxygen. Several studies shown that in open wounds can used insulin for both patients with non-diabetic or with diabetic(İsa ÖZAYDIN, 2018)(Lingling Zhao, 2017).

The usage of insulin cream plays an important role in the wound healing by support the cells with nutrients on the wound site and perform the healing better. On other hand in that area can precent microbes as a result the immune cells will still working and the inflammatory cells will synthesize the cytokines like IL-12 to attack the microbial agent. And in this thesis, the DI group had a moderate level of the gene expression of IL-12, that means insulin was good at wound healing but also needed the inflammatory cells to produce IL-12 to attack the microbes. This means the NPH insulin cream had a good effect as treatment but diabetic wounds still needed the immune system to work on microbial

agents. So NPH insulin also affected the gene expression of IL-12 but not like NO because NO worked as antimicrobial so more negative effect on IL-12.

The group that gave a good significant when compared with the DC was the DINO. DINO in this group used both Insulin and Nitric Oxide as compound treatment and this group gave a good significant P value was ($P<0.0437$). Which mean compound treatment had a good effect when compared with DC. And had a good effect on the time of wound healing due to use a compound treatment. The Nitric oxide with NPH insulin makes a compound treatment and this combination make a significant effect on the gene expression of IL-12 more than when compared with each treatment alone. this is an interesting result how can the compound treatment get a significant effect. Here in compound treatment not only one factor plays a role.

Wounds is so important especially in diabetic patients because they have a hyperglycemia so have a chance to change to chronic wounds and delay the healing for the wound and occur uncontrolled inflammation. The diabetic patient they can't get heal easily so that the importance of this study to understand how the treatment worked on wounds and gave good effect by the study of gene expression to IL-12. So according to that can decide which treatment gave good result, worked on immune cells, helped to control the infection and got the healing in short time without any delay.

When this thesis studied the IL-12 gene expression analysis in the four groups gave an idea about who can worked as anti-inflammatory, who worked only on nutrients support and who had a significant effect and better to use. So, this so important to study the effect of treatment on the IL-12 that gave an idea about how can worked and which one gave a good significant.

After these results give an idea not only about treatment and the effects on wound, in addition know how the IL-12 plays an important role and who can affect on this IL in different levels.

CHAPTER VI:

Conclusion and Recommendation

Conclusion:

In this study compared the gene expression of IL-12 between the four groups, in first group DC shown that had a highest gene expression of IL-12 that's mean there was a microbe infection and the inflammatory cell trying to increase the number of IL-12 to help in attacked the microbial and helped in healing. Also, this means when the wound occurred in diabetic patient and occurred inflammation the secretion of IL-12 will be in high level to make control to the microbial but the immune system alone in that group required more time due to hyperglycemia so make the healing need more time than normal and this was the problem in that groups and the study aimed to prevent happened this with them.

But when applied the Niric Oxide in DNO group, shown the lowest level of IL-12 gene expression, this means the NO acts as antimicrobial and prevent the bacteria to appear. on other hand, the inflammatory cells didn't synthesis IL-12. Or in other words, the NO was Naturally synthesized and when used NO as treatment increased at site of wound and led to negative effect on immune cells and make less expression to IL-12. And the NO working good as antimicrobial.

The Important group was DINO which in this group applied both insulin and nitric oxide and when compared with DC gave a good significant with P value<0.0437. this mean that when used DINO in the wound healing gave a good result and decreased the time for healing in this diabetic rats.

This study Hypothesis, suggested that when used treatment contain both Insulin and Nitric Oxide as in Group DINO will give a good results and significant effect more than when used Insulin alone and Nitric Oxide alone. And this thesis study, confirmed that when used the both treatments will give a significant effect on the wound healing for the diabetic rats, because the Nitric oxide act as anti-microbial and insulin provide nutrients to cells. In addition to that had small amount of IL-12. So, the result was Faster healing time by play a role in microbes by NO and improve the nutrients by insulin. so, in this compound treatment not only NO worked on the healing, only NPH insulin or IL-12.

No here in compound treatment all of these worked on the improvement of the wound healing that NO with IL-12 working as anti-microbial and prevent inflammation also the IL-12 is important in other steps of wound healing as mentioned before and the insulin give a good nutrition to cells there to make recovery and healing better and in less time and these points are so important for the diabetic patient.

The Highest amount of IL-12 will be in the chronic wounds because these lots of microbial so need more of cytokines to attack the microbes, as a result needs long time to healing as a perform of presence of microbial agents, so immune cells become activate and synthesize IL-12 to work against of microbial infection. But the present of hyperglycemia make wound area a good area for microbial infection and the IL-12 can't work so the immune cells synthesize more and more of IL-12 to work against microbial infection.

According to this, the IL-12 had a good effect on wound healing but in diabetic patient need more time to get the heal. When applied NO alone make negative effect on IL-12 and reduce the amount of gene expression. On other hand, the comparison with compound treatment saw a good significant because IL-12 was still synthesized in addition to NO and insulin so this makes the healing better and faster than each treatment alone.

So, in compound treatment the immune cells stay working and don't get full negative effect like when used NO alone, so the present of IL-12 with the NO and insulin make the healing better and, in this thesis, get significant result with compound treatment. The presence of IL-12 in small amount with the compound treatment make a significant effect on healing and get a good result for healing more than each treatment alone.

Also, each treatment alone affects the gene expression of IL-12 in different levels. NO make a very negative effect on IL-12 because NO produced naturally from the body and when used NO as treatment make strong effect so IL-12 was in low level of gene expression when compared with DC but in NPH insulin effected the gene expression of IL-12 but in moderate levels when compared with DC, and the compound treatment was best one for this thesis results.

Recommendation for Future Researches:

According to this thesis results and the conclusion that arrived. Can suggest to do extra studies on other Interleukins and with treatments that are helpful for wounds. And choose other medicines to see the effect on immune cells. And the wounds especially for diabetic patient is so important to do more researches and understand the most effective treatment.

Compound treatment of NO gas and NPH insulin gave a good effective wound treatment by affected several targets. The NO gas works as antimicrobial, while insulin provides the injury cells with nutrients and the small amount of IL-12 makes more balance and effect wound healing and other steps of healing.

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Ethical Approval

YAKIN DOĞU ÜNİVERSİTESİ
HAYVAN DENEYLERİ YEREL ETİK KURULU
ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi **17/01/2024**
Toplantı No **: 2024/169**
Proje Başvuru No **: 169**

Yakın Doğu Üniversitesi, Veteriner Hekimliği Fakültesi'nden, sorumlu araştırmacı Dr. Ali Çürükoğlu tarafından hazırlanan 'Diabetik ratlada doku kayıplı yaraların iyileşmesinde soğuk atmosferik Nitrik Oksit (NO) gazının tek başına ve/veya NPH insulin krem ile kullanımında *IL-1, IL-2, IL-6, IL-8 , IL-11, IL-12, Il,22 ,CD4 ,CD8* gen ekspresyon analizlerinin karşılaştırılması başlıklı araştırma önerisi kurulumuz tarafından uygun bulunmuştur.

1. Prof. Dr. Emine KOÇ (BAŞKAN
2. Prof. Dr. Tamer YILMAZ (ÜYE)
3. Prof. Dr. Nurettin ABACIOĞLU (ÜYE)
4. Prof. Dr. Dilek ARSOY (ÜYE)
5. Prof. Dr. Aysel KÜKNER (ÜYE)
6. Prof. Dr. Vedat SAĞMANLIGİL (ÜYE)
7. Prof . Dr. Ahmet Özer ŞEHİRLİ (ÜYE)
8. Vet. Hek. Ahmet SERHATOĞLU (ÜYE)
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