NEAR EAST UNIVERSITY

INSTITUTE OF GRADUATE STUDIES

DEPARTMENT OF MEDICAL BIOLOGY

MOLECULAR MEDICINE PROGRAM

THE COMPARISON OF *IL-1* GENE EXPRESSION ANALYZES IN THE USE OF COLD ATMOSPHERIC NITRIC OXIDE (NO) GAS ALONE AND/OR WITH NPH INSULIN CREAM IN HEALING WOUNDS WITH TISSUE LOSS IN DIABETIC RATS.

M.Sc. THESIS

Ahmed Elmi ABDULLE

Nicosia

JUNE , 2024

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M.Sc. THESIS

Ahmed Elmi ABDULLE

Supervisor

Assoc. Prof. Dr. Mahmut Çerkez ERGÖREN

Nicosia

JUNE, 2024

APPROVAL

We certify that we have read the thesis submitted by AHMED ELMI titled, " The **Comparison of *IL-1 gene* expression analyzes in the use of cold atmospheric Nitric Oxide (NO) gas alone 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 quality, as a thesis for the degree of Master of Educational Sciences.

Supervisor: Assoc. Prof. Dr. MAHMUT ÇERKEZ ERGOREN

Examining Committee	Name-Surname	Signature
Head of the Committee:	Dr. HAVVA ÇOBANOĞULLARI	....
Committee Member*:		
Prof. Dr. SELMA YILMAZ		....
Supervisor:	Assoc. Prof. Dr. MAHMUT ÇERKEZ ERGOREN	....

Approved by the Head of the Department

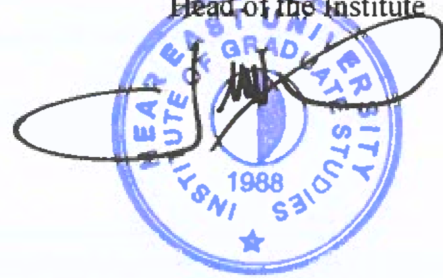
28/06/2024

Prof. Selma Yilmaz
Head of Department

Approved by the Institute of Graduate Studies

28//06/2024

Prof. Dr. Kemal Hüsnü Can Başer
Head of the Institute



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 original to this study

AHMED ELMI ABDULLE

28/06/2024

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ABSTRACT

The Comparison of *IL-1* gene expression analyzes in the use of cold atmospheric Nitric Oxide (NO) gas alone and/or with NPH insulin cream in healing wounds with tissue loss in diabetic rats.

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Interleukine-1: One of the main cytokines in the inflammatory phase of wound healing is interleukin-1. Simply put, it must stimulate the immune system to enter the wound site, which is necessary for the next stages of healing. This study evaluates the effect of cold atmospheric Nitric Oxide (NO) gas, alone and in combination with NPH insulin cream, on IL-1 gene expression in wound healing in diabetic rats. Diabetic wounds are prone to chronic inflammation due to overexpression of cytokines like IL-1, which complicates healing, influences immune cell behavior and may modulate inflammatory responses, thereby enhancing healing. The study involved 24 samples from diabetic rats with tissue loss wounds. Total RNA was extracted using TRIZOL reagent, followed by cDNA synthesis and quantitative PCR to assess IL-1 gene expression. The study adhered to ethical guidelines, and informed consent was obtained for the use of samples. In an analysis using a paired t-test to assess the mean differences between DC, DNO, a highly significant two-tailed P value of <0.0001 was observed, indicating a substantial difference from zero, and Statistical analysis revealed a strong positive correlation between DC and IL-1 in control groups ($r = 0.9994$, $P < 0.0001$), validating DINO as a reliable reference gene. The findings suggest that NO's role in wound healing is potentiated when combined with NPH insulin cream, highlighting a synergistic effect in reducing inflammation and accelerating tissue repair. This combination therapy could be particularly beneficial for diabetic rats who suffer from impaired wound healing. Further research should explore the long-term effects and potential clinical applications of this combination therapy.

Keywords: *Atmospheric cold plasma, nitric oxide, insulin ointment, diabetic wound healing, and NPH.*

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List of Abbreviations

IL-1:	Interleukin-1.
NO:	nitric oxide
LPS:	lipopolysaccharide.
NPH:	Neutral Protamine Hagedorn
DNA:	Deoxyribonucleic acid.
PCR:	polymerase chain reaction.
RNA:	Ribonucleic acid.
cDNA:	complementary DNA.
TBE:	Tris Borate EDTA
UV:	ultraviolet
RNS:	Reactive nitrogen species
ROS:	reactive oxygen species
Ct:	Cycle Threshold
qPCR:	Quantitative polymerase chain reaction
IL-1R:	Interleukin-1 receptor
CV:	Coefficient of Variation.

CHAPTER I

Introduction

Interleukin-1: One of the main cytokines in the inflammatory phase of wound healing is interleukin-1. Simply put, it must stimulate the immune system to enter the wound site, which is necessary for the next stages of healing (O'Shea 2008, Amin et al 2020). On the other hand, overexpression of *IL-1* may contribute to the persistence of inflammation and even the inability of diabetic wounds to heal (Garlanda 2013, Dinarellov 2013, and Mantovani 2013).

Low-molecular-weight (about 6–70 kDa) soluble proteins called cytokines are released by a range of cells, including mast cells, NK cells, natural killer (NK) cells, stromal cells and lymphocytes (Amin et al 2020). They take part in the immune response and function as crucial immune system communication network mediators. Important health determinants, cytokines are in charge of dynamically regulating immune cell maturation, proliferation, and responsiveness (Boraschi 2022).

Nitric oxide (NO), which is produced and released by major skin cell types such as fibroblasts, keratinocytes, and melanocytes, has been linked to the development and differentiation of skin cells. Thus, several groups have investigated the impact of NO on immune cells (Diegelmann 2004).

Nitric oxide, or NO, is produced and released by the main skin cell types, including fibroblasts, keratinocytes, melanocsytes, and endothelial cells. It is involved in the skin's reaction to damage and infection (Arnold 2006, Barbul 2006). Numerous organizations have also looked into the impact of NO on immune cells. It has been demonstrated that LPS (lipopolysaccharide) induces the generation of iNOS (inducible nitric oxide synthase) in murine macrophages, which is inhibited by cytokines including TNF (tumor necrosis factor) and IFN-c (interferon gamma), (Hunter et al 2013).

NO plays two roles in inflammation. It has an anti-inflammatory action in typical physiological circumstances. but when inflammatory cytokines are overproduced, as happens after an infection or injury, it has a pro-inflammatory role (Witte 2002, Barbul 2002).

As a signaling molecule, NO number of physiological functions, including vasodilation, neurotransmission, and immunological function. It also draws immune cells and increases the production of collagen, both of which are necessary for tissue repair (Mast 1996, Schultz 1996).

As a result, NO can contribute to wound healing by causing angiogenesis. Important for tissue regeneration, it also draws immune cells and increases the creation of collagen. NPH Insulin Cream: Insulin with an intermediate half-life is NPH. Topical use of insulin has been studied for wound healing Insulin is relevant for accelerating tissue regeneration (Curukoglu, 2023, Gungor, et al 2023).

Normal wound healing proceeds in a sequential manner, starting with hemostasis, moving on to inflammation, proliferation, and ultimately dermal structural remodeling from the point of injury to full epithelialization. Diabetes-related wounds that develop microangiopathy and neuropathy exhibit suppression, These conditions also affect collagen organization and cell proliferation (Ahmed et al 2022).

Endogenous nitric oxide (NO) gas transmitters have been identified as an endogenous, non-resistant antibacterial agent and an endogenous, uncontrolled modulator of inflammation. They are essential for wound healing and have lately become more widely used in wound treatment (Dwyer 2018).

Many academics are trying to come up with alternative, more efficient ways to treat diabetic wounds because of the impending danger to the health of those who have the disease and the rising prevalence of diabetes worldwide (Campos 2008, Groth 2008, and Branco 2008). This exacerbates not only the challenge of developing an effective treatment plan but also the consequences of systemic diabetes, such as decreased collagen production, altered inflammatory response, and hypoxia in the tissue (Blakytny2006, Jude 2006).

Problem of the statement

The following might be the problem statement for a study looking at how NPH insulin cream and cold atmospheric Nitric Oxide (NO) gas affect diabetic rats' ability to repair wounds: rats with diabetes are at a heightened risk of severe infection and amputation, prolonged pain, and greater medical costs due to chronic wounds with tissue loss. In a diabetic, the inflammatory response—which is mediated by cytokines like interleukin-1 *(IL-1)*—is dysregulated, delaying the healing process. Due to their lack of attention to the pathophysiological changes underlying the development of diabetic ulcers and wound healing, current treatment strategies are not particularly effective.

In recent years, there has been an increasing research interest about the significance of cold atmospheric nitric oxide (NO) gas due to its potential therapeutic utility for antibacterial, anti-inflammatory, and angiogenesis-promoting activities. Because NPH insulin cream alters cellular metabolism and proliferation, it has long been known among rats and negative control samples to have wound-healing properties. Nevertheless, it is currently unknown in a synergistic way, mostly by changing the expression of the interleukin-1 gene. This study attempts to close this gap by examining the effects of NPH insulin cream alone, cold ambient NO gas alone, in *IL-1 gene* expression during wound healing. The significance, broad potential effects, and general knowledge gap of the planned research must all be shown in the problem description. It prepares the ground for in-depth experimental research and analysis.

Purpose of the study

The current study will thus assess gene expression analysis of *IL-1* in the wounds with lost tissue of diabetic rats by applying NO gas alone or combined with NPH insulin cream.

Research objective

1) To measure gene expression level of Interleukin-1 in diabetic rats wound tissues treated with NPH insulin cream (*diabetic NPH insulin group labeled as DI)* or with cold atmospheric NO gas *diabetic nitric oxide CAP/NO group labeled as DNO)* or treated with both of them (*diabetic NPH insulin group with nitric oxide CAP/NO labeled as DINO) or* treated with none of them (*diabetic control group labeled as DC)* to establish how these treatments modulate the inflammatory response in diabetic rats and assess the effects on *IL-1 gene* expression.
2) To explore the synergistic potential, and find out if applying NPH insulin cream and cold atmospheric NO gas together will speed up wound healing more quickly than either treatment alone can.
3) To offer molecular alterations beyond of the *IL-1 gene* expression*,* synthesis of additional cytokines and growth factors, to fully comprehend how the treatments affect the healing processes.

Hypothesis of the study

In these experiments, NO gas with NPH insulin cream has been introduced both singly and in combination. These serve as a guide for the theories that are developed and put to the test through experiments. As a result, they are prepared to be put to the test and shown to be true or false by the research's findings, which will either confirm or refute the hypotheses and add to the body of knowledge that will be helpful.

The study significance

This study aims to understand the underlying the molecular mechanisms, and the components involved in diabetes that affect the expression of the IL-1 gene in proper wound healing and diabetic ulcers in diabetic rats, as well as chronic wounds that are among the major consequences that result in high rates of disability, morbidity, and even death in individuals with diabetes (Dorsett-Martin 2004), keeping in mind the importance of the target-specific pro-inflammatory cytokines, the information on the IL-1 roles in diabetic wound healing processes will help to design new drug therapies in the field.

In diabetic rats, bound to considerably minimize these hazards are boosting quality of life while speeding and enhancing the wound healing process. This study may offer some important insights into the field of wound management because it compares two distinct. As a result, combination therapy, when taken as a whole, only partially addresses the complex pathophysiology of diabetic wounds (Armstrong 2017, Boulton 2017, Bus 2017).

The most significant element in both inflammation and immunity, *IL-1,* has been found to family member a major factor of IL-1 Thus, assessing the potential impact of these treatments on *IL-1 gene* expression and the establishment of new guidelines in this area. This might facilitate the treatment and repair of diabetic wounds in a variety of domains, including wound care, pharmacology, and endocrinology (Boulton et al 2005).

These have wide-ranging and important implications for public health, particularly with regard to improving wound healing in diabetics, the majority of whom live around the world yet remain undetected. (Dorsett-Martin 2004, Olokoba 2012).

Keywords: Atmospheric cold plasma, nitric oxide, insulin ointment, diabetic wound healing, and NPH.

CHAPTER II

Literary Review

The The β-actin gene

One type of actin product whose expression is relatively constant in most cells and tissues is the nuclear DNA β-actin gene. It is commonly used as an internal reference in PCR due to its detection rate (Baltzis 2014, Eleftheriadou 2014, Veves 2014). This study involved the amplification of a pure β-actin gene from mouse-derived RNA via PT-PCR, followed by cloning, screening, PCR identification, and restriction enzyme digestion. It would be useful. as the foundation for the next experiments. Despite the β-actin gene being the focus of multiple studies and lengthy research, the end product is quite superficial. Along with our much improved experimental operation skills, we will comprehend not only the function of the β-actin gene but also—and maybe most importantly—the value of cooperation and acquire independent thought in relation to the β-actin gene (Auron et al 1984, Baek et al 1993).

NTERLEUKIN-1 is a classic example of a cytokine with several functions in contrast to influences almost, frequently in combination, *IL1* represents a very inflammatory cytokine; therefore, there is a skinny line between clinical benefit and intolerable toxicity in humans, even though beneficial (Hotamisligil 2006). Agents that lower *IL-1* production and activity, however, are probably going to affect clinical medicine. This regulation extends from gene expression to synthesis to secretion at the level (Kaneko et al 2019). To date, introns and exons indicates that a common gene was duplicated approximately 350 million years ago (Peterson et al 1993). Fibroblast growth factor (FGF) may have originated from an ancestor gene prior to this common *IL-1* because *IL-1* and FGF create an all-pleated sheet tertiary structure, lack a signal peptide, and show significant amino acid homologies (Brännström 1993, Wang1993, and Norman 1993).

Diabetes mellitus is a long-term metabolic condition that develops as a result of elevated blood glucose levels. Impaired wound healing is one of the problems associated with diabetes (Sacerdote et al 2019).

Rats control samples frequently have delayed wound healing as a result of immunological system deficits, neuropathy, decreased skin perfusion, and persistent inflammation. Chronic wounds, infections, and, in extreme situations, amputations can result from this impeded recovery (Maria Retzepi2010, Lewis2010, and Donos 2010).

The small, diffusible molecule known as nitric oxide (NO) is involved in immunological response regulation, neurotransmission, and vasodilation. In the context of wound healing, because it can promote wound healing (Garg 1989, Hassid 1989). In diabetic individuals in particular, insulin is essential for wound healing in addition to its role in glucose metabolism. Insulin has the ability to stimulate the production of collagen, support the creation of granulation tissue, and control inflammatory reactions (Apikoglu 2010, abus et al 2010).

To speed up the healing of diabetic wounds, topical insulin administration, such as that found in NPH insulin cream has been investigated for wound therapy (Sacerdote et al 2019). Although the impacts of insulin and cold atmospheric NO gas alone on wound healing have been studied, the combined effect has also been investigated with particular regard to how these therapies affect how they *IL-1* expression is modulated in diabetic wounds. The results may shed light on possible synergistic effects and result in more potent treatment approaches for diabetic wound care (C A Dinarello 1996).

It has been suggested that this activation is caused by the so-called HPA axis. These cytokines result in the adrenal cortex secreting corticotropin, which is secreted by the pituitary (Fantuzzi and Dinarello 1996). The hypothalamus releases corticosterone in response to corticotropin-releasing hormone (CRH), also known as the adrenal cortex. In experimental animals, lipopolysaccharide (LPS) and caffeine cause localized inflammation and systemic inflammation, respectively (Teoh2009, Latiff 2009, and Das 2009). additionally increase the secretion of corticosterone by activating the HPA axis. Under such stress, the HPA axis was previously believed to be predominantly regulated by *IL-1* (Fantuzzi et al 1996).

Cytokines are small glycoproteins a very significant role in regulating both innate and adaptive immunity (Waldmann 2019). They facilitate communication between immune system cells over short ranges, a released cytokine subclass called interleukins,

plural ILs, orchestrates the intercellular immune system (Conlon 2019, Miljkovic, 2019). The term "interleukin" was introduced in 1979 to refer to substances acting on more than one type of leucocytes (Alzawawy et al 2009). However, these chemicals can equally well interact with other types of cells, for instance, immune cells that produce a wide variety of ILs include macrophages/monocytes, T lymphocytes, and B lymphocytes (Konjević et al 2019).

Next to fever, episodes of chronic tissue Furthermore, as was discussed earlier, while the conjunctiva, CNS, and serous membranes represent the most typical targets in inflammation, the pleura and peritoneum are the specific locations for some *IL-1*-related conditions (Mantovani et al 2019). In addition to that, skin and musculoskeletal systems are also very commonly involved. Malaise and fatigue are common rats complaints and reflect a marker of chronic inflammation. By and large, during laboratory evaluation (Broderick 2022, Hoffman 2022).

The most consistent clinical feature of *IL-1*-mediated autoinflammation is The pleura and peritoneum, the central nervous system, the conjunctiva, and amyloid-induced tissue damage, which are examples of inflammatory regions. A case of amyloidosis (Mantovani et al 2019, Rivers-Auty et al 2018).

This would have meant that, considering this, with other ligands or initially as an orphan receptor, as could be inferred from its functionality in the rainbow trout, since IL-33 itself appeared only in mammals 320-160 million years ago (Moorlag et al 2018). Members of the ILRs and TLRs families are typified by their extracellular immunoglobulin-like domains. All members of the *ILR*s and TLRs families also contain the Toll-*IL-1* resistance domain in their intracellular signal transduction region (Bouhamida et al 2023).

After ligand-bound ILRs dimerize via their TIR domains, they engage the adaptor protein MyD88, which contains a TIR domain. Following that, MyD88 binds to downstream protein kinases, including factor 6 and *IL-1R*-associated kinases [IRAKs]. is linked with the receptor for tumor necrosis factor. Important transcription factors linked to inflammatory and immune responses are activated signal (Mantovani et al 2019).

Family Members of *IL-1*

Alpha interleukin-1

Hematopoietic cells, including skin dendritic cells, Activated complement components, other cytokines, TLR, TNF-α, and *IL-1* induce macrophage to produce *IL-1β* (Charles A Dinarello 2011). While caspases 1 and 2 finally cleave to release the active cytokine into the extracellular space, unlike, which in active form, it is widely distributed in hematopoietic cells (Van Opdenbosch 2019, Lamkanfi 2019). Nonetheless, the inflammasome must first cleave the proenzyme, or procaspase-1, before activating caspase-1 (Nicklin et al 2000).

Increased secretion of *IL-1β* is a hallmark of inflammation in rats harboring specific mutations classified as autoinflammatory diseases. However, not every instance of inflammation brought on by *IL-1β* results from the activity of NLRP3 or caspase-1. The *IL-1β*-mediated disease in mice lacking caspase-1 is identical to that in mice of the wild type (Charles A Dinarello 2011).

Beta Interleukin-1

Most inflammatory stimuli cause the inactive precursor *IL-1β*, which has a molecular weight of 31 kDa, to be expressed, released IL-1β, and even microbial infection can be examples of these triggers (Takeuchi 2010, Akira 2010). As a cytokine that promotes inflammation, *IL-1β* must first complete a number of intracellular processes before it can be released, which is constitutively expressed in normal body cells. Among the cell types that can produce *IL-1β* are circulating. ProIL-1β cleaves before it is released, inducing inflammatory responses and drawing (Rider et al 2011), *PreIL-1β* expression induction is frequently seen as a priming phase, and additional processing is necessary for preIL-1β to mature and secrete (Lopez-Castejon 2011, Brough 2011).

Synthesis, administration, and transcription of *IL-1*.

The members are expressed as precursor proteins called pre*IL-1α* and pre*IL*-1β. This indicates these molecules are missing essential peptide signal sequences for secretion or further processing. Hence, once activated by binding to *IL1R1*, both products will have

almost identical pro-inflammatory responses (Rider et al 2011). Hence, the activation of several other potent pro-inflammatory chemokines and cytokines go on to further accelerate the inflammatory cascade, which include but are not limited. The structural changes it is to remain active (Carta et al, 2013). It has been shown that this proteolytic cleavage type slightly increases the molecule's affinity for the receptor according to research conducted (Afonina et al 2015).

Receptors for *interleukin-1*

The members of the *IL-1* family of receptors are 10. (Boraschi 2013 Tagliabue 2013). there is the structural change that will now permit IL-. There is no direct contact of the ligand(s) to the *IL-1*R3 coreceptor . The trimeric complex allows the approximation of the TIR domains of each receptor chain. MyD88, in turn, binds to the TIR domains. The binding of MyD88 induces a kinase cascade that generates a solid pro-inflammatory signal, leading to the activation of NFκB (Eggestøl et al 2020).

IL-1R3 form exists in the soluble receptor form (Huang, Kuang, and Zhou 2024). Solubilized membrane receptors contain their extracellular domain when released freely into the extracellular fluids) (This clear, soluble receptor is the definition to which all immune. However, the liver can manufacture the soluble form of *IL* (Boraschi et al 2018, Tagliabue 2006).

The *IL-1* family of receptors has ten members, as tabulated. IL1R1 binds IL-1α, IL-1β and IL-1Ra. IL-1R3 (previously called *IL*1R accessory protein) is the coreceptor in forming a trimeric signaling complex with *IL*-1α or *IL*-1β (Rosenwasser1979, Dinarello1979, and Rosenthal 1979). The binding of IL-1, either permits the approximation of the TIR domains of each receptor chain. MyD88 then associates with the TIR domain (Garlanda et al 2013).

The association of MyD88 induces a cascade of kinases that generates an intense pro-inflammatory signal, resulting in the activation of NFκB. *IL*-1R3 also exists as a soluble receptor form (Dunn 2003, O'Neill 2003). Soluble means the free extracellular part of the membrane receptor otherwise unbound to the membrane. A classic mode of

receptor formation is the shedding or cleavage of the receptor from the cell surface, but in the case of *IL*-1R3, the soluble is also produced by the liver (O'Neill 2008).

Sites of interleukin-1 binding. As they bind Many *IL-1* proteins keep serving an essential function against the background of pathogen pressure on shaping innate and adaptive responses, activating their *IL*-1R. They belong superfamily (Kim et al 2013). This superfamily is characterized by a conserved intracellular TIR domain expressed in the cytoplasm. It initiates a pro-inflammatory response and is required to activate the signaling(Jensen 2003, Whitehead 2003). These receptors also have tremendous binding affinity for *IL-1R*, are surface-bound on all nucleated target cells, and are synthesized in soluble forms. This allows the deficient concentration of a specific I*L-1* cytokine to result in a physiological response (Werman et al 2004).

Interleukin-1 signaling in immunology and inflammation

Intrinsic and adaptive immune responses are the two different categories of immune responses. For the majority of bacterial illnesses to be prevented and controlled, intrinsic immunity is essential. Additionally, it serves as a barrier against certain common infections (Graves et al 1990). Since not all germs are identified and eradicated by the host's defensive mechanism, this is not a conclusive reaction. The establishment of immunological memory and impacted by the activation of innate immune cells (Fields2019, Günther 2019, Sundberg 2019). Nevertheless, non-infectious, sterile cellular components can also cause the immune response, making viruses not the only entities capable of doing so. These include materials formed by injury, pharmacological disagreeable principally connected to tissue regeneration, wound healing, and homeostasis (Chen 2010, Nuñez 2010).

Furthermore, a few non-infectious illnesses cause aberrant expression and production of active *IL-1* molecules (Simon 2012). This causes inflammation and sets off a series of processes that eventually result in the emergence of pathologic diseases, cancer among them. In fact, overexpression of *IL-1* genes has been linked to certain tumor forms, suggested that this overexpression promotes the development and metastasis of particular tumor types. This polymorphism in the *IL-1* gene solid cancers, including carcinomas of

the lung, breast, colon, and ovary. Concurrently, it suggests that the innate immune system is overstimulated since *IL-1* molecules persist in a range of autoimmune illnesses (Charles A Dinarello 2012, Simon 2012, van der Meer 2012).

While the host's defense against infectious pathogens still depends on *IL-1* proinflammatory signaling, prolonged on its own. Conversely, a cytokine shortage may result from faulty pro-inflammatory signaling caused by mutation or allelic variation (Sundberg 2019), a compromised immune response would consequently likely make the host more vulnerable to infections or other illnesses like sepsis (Khazim et al 2018).

Natural inhibitors of *IL-1* signaling

Some reports state that inflammation is a carefully monitored process that arises following a sterile injury or pathogenic infection and that it must be treated. *IL-1* family members, hence controlling the proportion of agonist family members to natural antagonists (Grütter 1988). Hence, the combined action of these two regulatory systems controls *IL-1* activity: the *IL-1* agonist at the *IL-1RI* binding site to reduce the latter's action (Lennard 2017). On the other hand, IL-1RII acts as a decoy receptor, preventing interaction between the active *IL-1RI* and its ligands as well as accessory proteins. Furthermore, and its cleavage (Srinivasan 2017, Nandlal2017, and Rao 2017).

An interleukin receptor antagonist *IL-1Ra*

Interleukin receptor antagonist to the *IL-1Ra* interleukin receptor: *IL-1* represses the inflammatory responses for *IL-1.* splicing type, this protein will be expressed in the two structural variants: secretory and intracellular (Arend et al 1998).

It means the same stimuli that will induce the synthesis will cause the synthesis and release of *IL1*-Ra. Although *IL-1* isoforms intracellularly are inhibitory and, thus, modulates *IL-1α* and *IL*-1β bioactivity. Potentially invading the nucleus, icIL-1Ra may have a function there that rivals that of *IL-1α* in blocking effects (Dinarello 2009).

Autoinflammation and intraleukin-1

These efforts to identify the etiologies of fever drove early insights into the complexity of IL1 biology. Therefore, it is probably not surprising that renewed interest in mechanisms related to IL1 should be contemporaneous with the discovery of molecular mechanisms for several rare hereditary fever disorders around the turn of the century (Boshtam et al 2017). A subset of these disorders also have associated systemic and tissue inflammation over a long period pain. The cloning of the responsible gene abnormalities led to the development of a new class of disorders called autoinflammations. These are inflammatory syndromes consequent to innate immunity (Frank 2024, Baratta 2024).

The interleukin-1 Superfamily

The *IL-1* superfamily The 17 – 18 kDa cytokines available in the *IL-1* superfamily consist of an individual structural pyramidal barrel structure named the b-trefoil. The superfamily includes six two-stranded b hairpins. Over the course of evolution, the 11 cytokines play multiple diverse activities as primarily most proteins in the world (Sharma et al 2008). From bacteria to man, they share a similar structure, and of course, it is the nature of the proteins to identify the ill effects of carbohydrates in humans and most other mammals, some being inflammatory, others anti-inflammatory qualities (Murzin1992, Lesk1992, and Chothia 1992)

The nine IL-1 ligand family members is made up of *IL-1b,* other cytokines, including have widespread pro-inflammatory effects and bind to the same receptor. Among the proteins of the moonlighting type, *IL-1a* is thought to have several roles because of its lengthy intracellular form, which differs significantly from the short, external cytokine form that causes inflammation (Charles A 2018, Dinarello 2018).

The Family of IL-1R

The cytokines that are members of the *IL-1* superfamily have a similar structure and bind to receptors in the same way as ten transmembrane proteins that have an extracellular area with three domains that resemble Ig and a cytoplasmic Toll-IL1R (TIR) signaling domain, (Garlanda et al 2013).

An accessory chain binds the dimeric ligand/receptor complex to it, while the cytokine interacts with the ligand-binding chain to attach itself to the complex. frequently contain the *IL-1R* complex, which aids in cell activation. When these two intracellular homologous TIR domains approach one another, signaling is followed by cell activation (Boraschi et al 2018).

The ligand for the traditional *IL-1R, IL-1R1,* interacts with its receptors. For example, the receptor's accessory chain*, IL-1R3*, transduces the signal for both. In other words, *IL-1R3* is promiscuous since it can be associated with any ligand-binding chain. One of the ligand-binding chains that can associate with since it lacks the TIR intracellular domain (Ottaviani 1993, Franchini 1993, Franceschi 1993). *IL-1b* and *IL-1a* are active ligands that join the antagonist receptors for *IL-1Ra*, which excludes binding of the *IL-1R1* to the molecule. Some are assembled in a manner akin chain plus an additional unique component (Miljkovic, 2019). This indicates that these unusual properties are shared by several additional chains in the family that are of the receptor-like kind. The *IL-1R9* and *IL1R10* kinds of orphan receptors are found ins the brain at high concentrations but have not yet been linked to a ligand (Nakae et al 2001).

The *IL-1R*-like gene

The IL-1R-like gene, encodes interleukin-1 receptor family member, a cytokine receptor. The encoded protein is a receptor for interleukin-1alpha, interleukin-1 beta, and interleukin-1 receptor antagonist. *IL-1R* signaling has been conserved in immunity through cytokine-induction and involved in inflammatory responses (Takeda 2003, Kaisho 2003, Akira 2003).

Many orthologous *IL-1R* genes in both Cnidaria and Proteus are pretty similar to genes in vertebrates. As a matter of fact, it has also been established is highly homologous (Brocker et al 2010). Indeed, this homology is significantly brought to the fore in the signaling domain known as TIR from T, and a recognition about *IL-1R* is that, in some ways, it is a hybrid wherein with both a TLR domain for signal transduction and an extracellular domain that is Ig like (Kubick et al 2021). Ig domains exist as part of invertebrate proteins, yet the molecules in which the Ig domains assemble to form Ig-like receptors reaches back to the pre-vertebrate Chordata and onwards to vertebrates, forming

an enormous collection of potent and specific receptors, among which T and B cell receptors that recognize antigens (Rast 2008, Messier-Solek 2008).

This view is based on the suggestion that In the binding region of the *IL-1R* molecules, Ig-like domains occur, setting the stage for an exceptionally high order of binding specificity, as opposed to the pattern recognition promiscuity attributed to (Flajnik 2004, Du Pasquier 2004).

IL-1 is not an innate cytokine, but an inflammatory cytokine

On the other hand, its effects appear to be much more extended since it first they simultaneously with the advent of adaptive immunity (Sarhan et al 2018). Hence, were bound finally to "simplify" into innate and adaptive immunity in vertebrates. more than 200 TLR and NLR genes, humans have only 10–20 such genes. It is thus probable that the *IL-1* family of cytokines has undergone a process of evolution to perform two different functions: they could be involved in maintaining (Tahtinen et al 2022).

However, the fact that vertebrates are the only animals that produce the implies that these cells have a protective function that goes beyond innate immunity and that they augment or support adaptive immune responses (Sarkar et al 2018). However, *IL-1b* may non-specifically increase the responses of T and B cells to specific antigenic stimuli. Thus, in reaction to an antigen, *IL-1b* can boost memory, naïve CD4+ T cell proliferation and commitment, , and T cell cytocidal action potential, ultimately leading to CD8+ T cell activation. Thus, this may improve B cell activation in an indirect manner (Ben-Sasson et al 2013).

The Intraleukin-1 superfamily moonlighting proteins

The effects of mature extracellular *IL-1a* and *IL-1b* on T cell activation are remarkably similar; this leads to the question of why IL-1a evolved differently in mammals despite it sharing many properties with IL-1b (Wüthrich et al 2013). The most plausible explanation is that specific tasks were done by *IL*-1a without contradicting the roles fulfilled by *IL-1b* constitutively expressed by quiescent and macrophages, as well as other non-immune stromal and epithelial cells after that, it relocates to apoptosis, senescence, and cellular proliferation. Interleukin-1 alpha differs from most other

inflammatory cytokines (Werman et al 2004).

The Families of IL-1 Cytokine and Receptors

As a hybrid cytokine family, the *IL-1* superfamily was designed from the ideas presented in the previous sections to modulate and enhance vertebrate adaptive immune responses (Santarlasci et al 2013). Their induction and effects are, therefore, nonspecific because of the concomitant presence of all innate and inflammation-associated actions. One among the many reasons for such diversity of action on the part the expression and selection of lymphocyte populations concerning *IL-1Rs* and their effects (Charles Anthony Dinarello 2019).

Their extracellular domains, being similar to Ig, contribute to identifying and binding specific ligands to these receptors. By contrast, the subsequent TIR-dependent functions of the receptors function very much like the classical innate receptors. Evolutionary facts, later on, however pointed to a more complex picture (Lachmann et al 2009).

Probably, most of the *IL-1R* molecules remained active even after losing their *IL-1* ligands. This explains why molecules were active without the ligands, as shown. This is partly supported by some studies done on animals for ligand-independent activation of receptors such as IL1R7 (Voronov et al 2013).

Nitric Oxide Therapy for Diabetic rats Wound Healing

Injuries that have not healed within a typical time frame but have instead entered a pathological inflammatory state are categorized as chronic (Malone-Povolny 2019, Maloney 2019, Schoenfisch 2019). Chronic wounds stall and may never heal, whereas healthy wounds usually heal fully in 30 days from the site of injury closure. There is a significant financial and humanitarian burden on the United States (Guo 2010, Dipietro 2010).

Such chronicity may have major consequences for diabetics, since long-term wounds are the authors of the majority of amputations caused by diabetes. Because diabetes is more prevalent and diabetic chronic wounds put diabetic health in danger, researchers have been oriented to developing more effective treatments for such wounds (Luo 2005, Chen 2005).

Additionally, the systemic effects of diabetes, which include lower collagen, less inflammatory response, and tissue hypoxia, make it more difficult to design an effective treatment (Akerström et al 2005).

Local Elements That Affect Recovery

The process of oxygenation: Some researchers have approximated that almost every action involved in wound healing and cell metabolism, especially the mechanism producing energy based on ATP, requires oxygen (Castilla2012, Liu 2012 , Velazquez 2012). In addition to promoting wound contraction, keratinocyte differentiation, migration, and re-epithelialization, it also inhibits the growth of bacteria in wounds and enhances collagen synthesis and fibroblast proliferation. Additionally, early wounds microenvironments are severely hypoxic due to vascular deterioration and excess oxygen (Davis et al 2018).

Demand from cells with an active metabolism: Polymorphonuclear leukocytes create superoxide, which is essential for oxidatively eliminating infections. Oxygen levels are intimately associated with these factors. Reduced vascular flow can result in inadequate tissue oxygenation and is a consequence of a number of systemic diseases, including aging and diabetes (H. Sun et al 2022).

An underperfusion layer forms in a hypoxic wound during the healing process. Transcutaneous tissue oxygen tension measurements in chronic wounds reveal extensive hypoxia; values in control tissue indicate readings in the range of 30 to 50 mm Hg for acute wounds and 5 to 20 mm Hg for chronic wounds (Tandara 2004, Mustoe 2004).

Injured tissues must undergo wound healing in order to restore their naturally intact structure (Eisenbud 2012). Proliferation, migration, differentiation, and tissue collagen remodeling are some of the processes that make up the healing process. These

processes must all proceed in unison and depend of different cytokines, growth factors, and other bioactive molecules. Two distinct nitric oxide synthases (NOS) produce nitric oxide is a highly reactive free radical gas (Mustoe 2004).

It functions as a molecule that communicates with several physiological processes, Evidence suggests that NO is involved in normal wound healing, and studies have demonstrated the expression of inducible NOS during wound healing (Rodriguez et al 2008). It has been demonstrated that NO synthesis continues for a considerable amount of time following injury, and macrophages seem to be a key biological source of it. Moreover, it has been demonstrated that human dermal fibroblasts (Knowles 1994).

Failure to restore oxygenation slows down the healing of wounds: Subsequently, after injury, short-term that aids in the healing process in case of acute wounds at different stages (Blackman et al 2010). Hypoxia can induce the release of cytokines and growth factors upon contact with fibroblasts, keratinocytes, and macrophages. Many cytokines are released in response to hypoxia, which includes have a variety of mechanisms modulating the healing process of wounds, including angiogenesis, migration, and chemotaxis (Gupta et al 2022).

Through the release of growth factors and angiogenesis, hypoxia facilitates always accessible. So usually are confined to the skin surface can now invade more deeply is breached. Whereas the term contamination implies that there are non-replicating organisms on the wound, colonization refers to replicating but harmless bacteria present on tissues adjacent to a lesion (Kirker 2017, James 2017).

The intermediate stage of local infection, often referred to as critical colonization, is when local tissue responses and microbial proliferation start. The presence of bacteria in a wound can multiply and pose a threat to future health (Edwards 2004, Harding 2004).

Hence, this is a critical phase of the healing process because inflammation allows for an easier removal of contaminated bacteria from the wound. The irritation, however, might be exacerbated by poor debridement and bacteria removal (Tong and Barbul 2004).

In this respect, bacteria and endotoxins can prolong the inflammatory phase through continuous production of TNF-α and interleukin-1 redundancy. If this continues,

the wound will likely become complex and chronic (Lopes2024, Pintado2024, Tavaria 2024). This chronic inflammation has also been shown to lead. Assuming proper controls are included, a rise in protease content is associated with a concomitant reduction in the levels of endogenous like in other infectious processes, bacteria-infected wounds take the form of biofilms, which are aggregated complex communities of bacteria encased in an extracellular polysaccharide matrix that they secrete. Biofilms create shielded microenvironments as they grow and become more resilient to traditional antibiotic therapy (Consultant 2010).

In wounds, bacteria such as Pseudomonas aeruginosa, Staphylococcus aureus, and β-hemolytic streptococci are frequently found (S. aureus) that are not clinically impacted (DeLeon et al 2014).

Studies have shown a connection between elderly sex hormones and poor wound healing. It is well known that older females. This may be the outcome of the main influences on the wound-healing process that come from and the steroid precursor dehydroepiandrosterone (DHEA) (Bowler, Duerden, and Armstrong 2001). Numerous genes, particularly those related to inflammation, are impacted by estrogen, which has been shown to control most of the changes in gene expression that take place between wounds in both young males and elderly humans (Gilliver2007, Ashworth2007, and Ashcroft 2007).

Stress plays a gigantic role in the general health and social behavior of people. Stress has been associated with a lot of diseases, and slow wound healing. Many studies have found a connection between stress-induced neuroendocrine immune disorders and a variety of health issues studies (Kiecolt-Glaser, 2005; Vileikyte, 2007). Immunological dysregulation is a result of the pathophysiology of stress (Godbout and Glaser, 2006; Boyapati and Wang, 2007).

The Physiology of Healed Wounds

Neuropathy, vascular disorders, and foot abnormalities are some of the variables that affect the complicated mechanism of wound healing in diabetic rats (Ulbrecht 2004, Cavanagh 2004, and Caputo 2004). The reason chronic wounds are called that is because

they often repeat One of the primary causes impeding the healing of diabetic wounds is cellular senescence (Guo2010, Dipietro 2010).

The usual course of treatment for diabetic foot ulcers involves managing blood sugar levels, controlling infections with the right medications, and applying a bandage and washing intermittently to the affected area (Wilkinson 2021, Hardman 2021). These therapies typically fall short of the needs of which results in limb amputation (Tuttolomondo 2015, Maida 2015, Pinto 2015).

The most common chronic ulcer in rat that typically results in limb amputation is a foot ulcer; in various nations like Romania, Germany, and Spain, diabetic foot ulcers account for about half of amputation cases (Singh et al 2023) In vitro and clinical investigations have demonstrated Schematic of swound healing phases (Petkovic et al 2021).

Wound Sterilization or Wound Disinfection by Cold Plasma

Bacterial contamination is a major issue because the bacteria are resistant to antimicrobial medications and lead to the establishment of wounds. The first thing that plasma does when it comes into contact with a wound is kill microorganisms that could lead to an infection. Numerous studies that looked into how CAP killed bacteria also showed that plasma might lower the amount of bacteria in chronic wounds (Pedroni et al 2018, Dezhi et al 2016, Dezhi et al 2016).

According to these data, there are two approaches to healing chronic wounds using plasma: both directly and indirectly. The second electrode, hence, is the surface of the body. In the indirect method, the carrier gas generates plasma. The plasma is then transported to the site of the wound. It has been shown that direct CAP treatment is more effective antibacterially as compared to indirect CAP treatment for deactivating microorganisms (Brehmer et al 2015).

Due to the differences in the concentration of charged particles between these two treatments, some reactive species with short lifetimes are present in the direct treatment but not in the indirect treatment. elements that impact the antibacterial efficacy of CAPs, in addition to the type of device used (Barjasteh et al 2023).

Diabetes-Related Impaired Wound Healing

Not all wounds heal within the commonly anticipated timeframe. Chronic wounds are extended, partial healing states brought on by pathologic inflammation of the injured tissue and are a result of specific types of wounds (Han 2017, Ceilley 2017).

However, the healing process for chronic wounds can be very slow, taking months, or even stall altogether during the inflammatory period. In contrast, acute wounds are usually caused by a sudden shock. In these conditions, skin tissue deteriorates and can become a breeding ground for microorganisms (Stechmiller 2005, Childress 2005, Cowan 2005).

According to, insufficient tissue oxygenation and infection are the two primary reasons why chronic wounds occur. Incomplete microbial clearance extends the inflammatory stage result in the development of biofilms, which can protect bacteria from antibiotic therapy and the human immune response by secreting an exopolysaccharide matrix (EPS) (Guo 2010, Dipietro 2010).

Unlike the immediate aftermath of injury, proinflammatory cytokine concentrations in chronic wounds increase dramatically. Increased cytokine concentrations cause a longer inflammatory phase and an overabundance of MMPs. (Trengove 1996, Langton 1996, Stacey 1996). The overexpression of MMPs leads to the continued breakdown of ECM components and the further degradation of growth factors that are already inadequately supplied (Wysocki 1993, Staiano-Coico1993, Grinnell 1993).

Nitric Oxide's Kinetic Dependence

While picosecond levels of NO promote tissue regeneration and the resolution of the healing process, micromolar levels are linked to proinflammatory signaling and pathogen clearance. Several studies have shown that kinetics are important for the antibacterial activity of NO. Despite the disparity in this collection of works, both highlights the importance of the kinetics of NO in wound healing process (Haifeng et al 2007,Bin et al 2012).

Similar NO-release characteristics have been linked to materials and data on the number of inflammatory cells. More investigation is needed into how NO flux affects the number of inflammatory cells. To fully comprehend the outcomes and make the most effective use of a particular NO-based treatment, accurate assessment and documentation of pertinent NO kinetic parameters are crucial (Balaji et al 2015).

The highly reactive nature of NO, which rapidly converts to nitrite in an oxygenated here are methods for detecting NO that can be environment (i.e., the accurate half-life of NO), makes measuring NO difficult, even in vitro (Hetrick 2006, Schoenfisch 2006).

Choice Chronic Wound Model

To ensure that the treatment is translated to the intended host (i.e., people), careful monitoring and reporting of NO-release kinetics must be paired with. Hemostasis is a multifaceted physiological process that are interdependent. As such, a single assay or test is not enough to determine the type and degree of healing with accuracy (Cho et al 2020).

As an alternative, the outcomes of several tests conducted using a particular test material ought to be evaluated using an in vivo model. Only mouse models with recently acquired serious wounds were used for the evaluation of all the items and techniques covered in this review (Jones 2007, Fennie 2007, Lenihan 2007). Of course, human and mouse rat healing processes differ greatly from one another, in contrast to how wounds in humans heal, mice and rats recover by contracturing the wound margins (Zomer 2018, Trentin 2018).

Such a divergence casts doubt on conclusions made about the efficacy of materials that were only tested in a mouse model for wound healing. Swine are the best animal model to utilize when evaluating the time it would take for a human wound to heal (Sullivan et al 2001), the heal by re-epithelization and react to growth hormones similarly to small animals (Sullivan et al 2001).

CHAPTER III:

Methodology

Materials and Methods

The results, which included a *IL-1* gene expression analysis in diabetic Wistar albino rats with tissue loss wounds comparing the use of NPH insulin cream and cold ambient nitric oxide gas alone, were related to the material and technique obtained from the genetics data set. 24 Wistar albino rats were used in this investigation, and they were split up into four different groups: Group one was diabetic control group (DC), group two was diabetic nitric oxide CAP/NO group (DNO), and group three was diabetic NPH insulin group (DI), and, finally, group four was diabetic NPH insulin/ nitric oxide CAP/NO group (DINO). The samples were of the skin from wound surface area of the diabetic rats. The research project was approved by the Animal Experiments Local Ethics Committee of Near East University for ethical purposes, and the reference was given as YDU 2024/169).

Research plan and methods:

For the investigation, a total of 24 male Wistar albino rats weighing 250 g and 6 months of age were employed. Group 1 was the diabetes control group (DC), Group 2 was the diabetic NPH insulin group (DI), Group 3 was the diabetic CAP/NO group (DNO), and Group 4 was the diabetic cold environment NO + NPH insulin group (DINO). The groups were split into four equal groups at random. During the 14-day trial period, we applied the treatment once daily. Following the manufacturer's instructions, the TRIZOL kit reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA from the wound tissues. A NanoDropTM 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to measure the amount of RNA.

Methods

Data Collection

This study prepared samples for animal laboratory testing by first creating a diabetes model and introducing diabetes into rats. They then created a wound model while

measuring the rate at which the wound contracted, and they administered NPH insulin and/or CAP/NO. Based on each group, the samples were obtained from wound surface area of the diabetic rats skin.

Isolation of RNA

Total RNA extraction from tissue was performed with TRIZOL reagent. The tissue samples were first all mechanically processed and then mixed with one milliliter of a solution containing the TRIZOL reagent. 0.2 ml of chloroform was added and vortexed and then left for two to three minutes at room temperature. The tested sample was after that centrifuged at 12,000 × g centrifugal force for fifteen minutes at a temperature between 2 and 8 °C. The result of centrifugation was the self-division of the mixture into three phases: an aqueous solution containing RNA and forming the upper phase, an intermediate phase containing DNA, and the mix of phenol-chloroform forming the lower phase used to separate protein and lipids. with the isopropyl alcohol at a volume ratio of 0.5:1, then incubated at 15 to 30 °C for at least 10 minutes.

A hard pellet of RNA adheres to the inside walls and the bottom of the tube as the consistency is gel-like following centrifugation at 12,000 xg at 2 to 4 °C. The RNA pellet was washed with 75% ethanol, followed by inversion and centrifugation at 2 to 8 °C at 7,500 xg for five minutes. Finally, centrifugation and vortexing were then repeated two times to get rid of the remaining ethanol. The pellet of RNA was air dried for roughly until ±10 minutes passed and was next added to 50µl of RNase-/DNase-free water to elute the RNA. The optical density of detected using a nano-drop microvolume spectrophotometer from Thermo-Scientific, Pittsburg, USA, at a wavelength of 260/280 nm to establish concentration and purity. Ideally, the concentration should be 1.8–2.0 ng/µL of pure RNA.

Setup of reaction and cDNA preparation

Cyber Green cDNA synthesis was utilized to perform the cDNA synthesis,which requires several essential components. An essential component was the enzyme mix solution, which was made up of reverse transcriptase and RNase inhibitors.

Thaw all the ingredients and carefully mix. When getting ready for a reaction, be cool. It is highly recommended to do a reverse transcriptase-free control reaction to check

for potential DNA contamination. RNA can be denatured for five minutes at 72 °C using reaction buffer. Spinned down and immediately put on ice. This has the potential to improve transcription for long mRNAs or GC-rich RNA. As a negative control, substitute 1 ul of Nuclease-free dH2o for the enzyme mixture. Combined in a sterile tube free of RNase:

Table 1: cDNA Kit Components and Preparation:

Reagent of components	**Volume**
Reaction buffer	4 µL
Enzyme mixture	1 µL
Total RNA	110 µL
Nuclease-free dH_2o	10µL
total volume.	10 µL

Table 2: This table shows the conditions for the polymerase chain reaction used in the cDNA synthesis step.

STEP	**temperature**	**Time**
synthesis of cDNA	at 42°C	For 60 minutes
inactivation kit	at 80°C	For 10 minutes

The PCR machine has been configured (as shown in Table 2). Because there is an enzyme that will denature at 80 degrees Celsius, the enzyme operated for 60 minutes at 42 degrees Celsius before being inactivated for 10 minutes at 80 degrees.The cDNA can then be used for PCR, however, it was crucial to return the RNA to -20°C to prevent its loss, as RNA was extremely delicate.

Ingredients for the qPCR

2XSYBR Green qPCR Mix aims at securing maximum effectiveness in real-time PCR (qPCR) while making it very easy to use. For qPCR; therefore, a kit called 2X SYBR Green qPCR Mix is used, which included the probe SYBR Green Dye and the crucial enzyme Taq Polymerase for DNA synthesis and elongation. Then, primers and cDNA template were added into the reaction mixture, causing the primer to attach to the template strand. Taq polymerase then added nucleotides, while SYBR Green dye provided a fluorescent signal throughout the real-time PCR. 24 rats were used in this investigation. Each rat provided two samples, for a total of 48 samples for this thesis. Since each sample was duplicated, 96 samples plus two negative controls were created, as indicated in Table 3.

Table 3: Preparation 2X SYBR Green qPCR Mix Component

Component 1x	Component 1x
SYBR 12.5	625 µL
Forward 0.4	20
0.4	20 µ
H20	185
cDNA	17+1 cDNA

Volume and Primer Gel Electrophoresis Optimization

Gel electrophoresis is the process in which DNA can be put in size order, purifying them on a preparation chromatogram. As such, electrophoresis in gel refers to DNA, with the negative charge under which it travels in the electrical field towards the positive electrode and amidst an agarose gel matrix. It is a process by which longer fragments of DNA move slower through the gel than shorter ones. So, a DNA fragment can be run in an agarose gel alongside a DNA ladder, which is the group of DNA fragments with known lengths, to get the size of the DNA fragment.

Primer Volume Optimization Experiment

The volume decreases were done to find the best volume for consistent amplification. A couple of the primer volumes may be checked in detail and supplemented to come up with the most effective volume that will give precise and proficient target sequence amplification. The primer stock was diluted to a concentration of 100µM, so it was used as working primers at 10µM before use. Ten microliters of primer and ninety-one µL of dH_2O were mixed to obtain the work reagents.

The oligomer

Primers that were utilized were bought from Hibrigen, Turkey. These were brief strands of nucleic acid, which might be RNA or DNA. For that, DNA primers were utilized, commonly referred to as oligonucleotides. Two to twenty monomers could make up an oligomer on average.

The information kit ID for the oligomer of *IL-1* is 202402071-135.

Table 4: Target Base Sequences of the Primers for Pairs:

IL-1 Forward:	5' GCACGTTCCCCAACTGGTA'3
IL-1 reversr (reverse):	5' ACACGGGTTCCATGGTGGTA'3

Polymerase Chain Reaction (PCR)

The preliminary polymerase chain reaction test was carried out with the ten micromolar recommended primer concentration and primers supplied, as well as positive and negative controls, focusing mainly on primers' specificity for the target sequence and their capacity to amplify characterized fragments of DNA as required. The positive controls included target sequence template DNA as the standard for effective amplification in the samples. The negative control included no template DNA to avoid of the contamination or accidental amplification. Annealing temperature, cycle counts, and other modifications were made before starting the PCR to ensure that targeted DNA sequences get amplified in a reliable and reproducible manner. Such would, therefore, determine whether there was something wrong with how the primers were working.

Table 5: Conditions for PCR displaying the primer dime 40x Cycles times

1x	1x
2x Taq Master Mix 12.5 µL	625 µL
Reverse Primer R 0.4.	20µL
forward primer F 0.4	20µL
H_2O	3.7µL
cDNA	7 µL

PCR with gradients

The temperature gradient was calibrated to have values between 59°C and 62°C, which were then utilized as different annealing temperature settings to determine which setting was optimal for primers.

The whole valume 20 µl was the entire volume. 3 µl of dH2O must be added as a negative control in place of cDNA.The study used a 2X SYBR Green qPCR mix that included the heat-resistant Taq polymerase enzyme and SYBR Green as a real-time PCR probe. Additionally, for enable the Taq polymerase to function, the forward and reverse primers were added to the real-time PCR along with nucleotides.The PCR machine was then adjusted in accordance with the 2X SYBR green kit. The PCR process consisted of three steps: denaturation to open the double helix cDNA, annealing when the primers attached to the template and required a particular temperature, and elongation, which required the use of Taq polymerase. to synthesize the template, and 40 cycles were repeated. Thus, the temperature and time configuration is displayed in Table 7.

Table 6: Thermal cycling conditions for PCR 40x Cycles times:

Stages	Temperature	Time
Initial denaturation:	95°	2 min
Denaturation	95°C	30 min
Annealing	72 °C	45 min
Elongation	72 °C	45 min
Termination	72 °C	in 10 min

The Primer Volume Optimization Experimental PCR

In order to determine the ideal volume and be able to employ amplification effectively and with great accuracy for the target sequence, this makes it possible to optimize pricing for all optimization methods, which have been quite arduous for many primer sizes. After diluting primer stock to a concentration of 100 µM, these primers were ready for use as working primers, where they were finalized at 10 µM. The primer was combined with dH2O to generate 10 µL of mixed 90 µL working reagents. To establish the temperature range at which As quantitative real-time PCR is done best, The gradient PCR was performed with the Hibrigen followed by the settingTwo minutes of initial denaturation at 95 °C; forty cycles of denaturation at 95 °C for thirty seconds, thirty seconds of annealing at 58–63 °C, forty minutes of extension at 72 °C for forty-five seconds, and finally, seven minutes of final extension at 72 °C. To limit the possibility of contamination, each gradient and qRT-PCR procedure was run in a category I laminar flow hood. Every reagent, pipette, and plastic container was also cleaned and a particular label was made just for PCR.

Table 7: The conditions for Annealing Primer Time and temperature for PCR

Primer	Temperature	Time
IL-1	59°C	30 min

Product analysis for PCR

Utilizing a PCR product analysis, the amplified products were assessed. The PCR findings were analytically analyzed using agarose gel electrophoresis. On an agarose gel, the amplified DNA fragments are separated according to size. By comparing the amplified product with the fragmented samples, the expected size is validated against the molecular weight reference. However, since they make it possible to identify and demonstrate the lack of generic bands or artifacts, these also give us another opportunity to demonstrate that the amplification process was highly specific. As a result, the rigorous examination was finished, and the PCR amplification's specificity was determined. This encompasses the. This crucial stage is included to confirm that these optimized primer conditions are correct and good, indicating that they will be useful for further research.

The software system

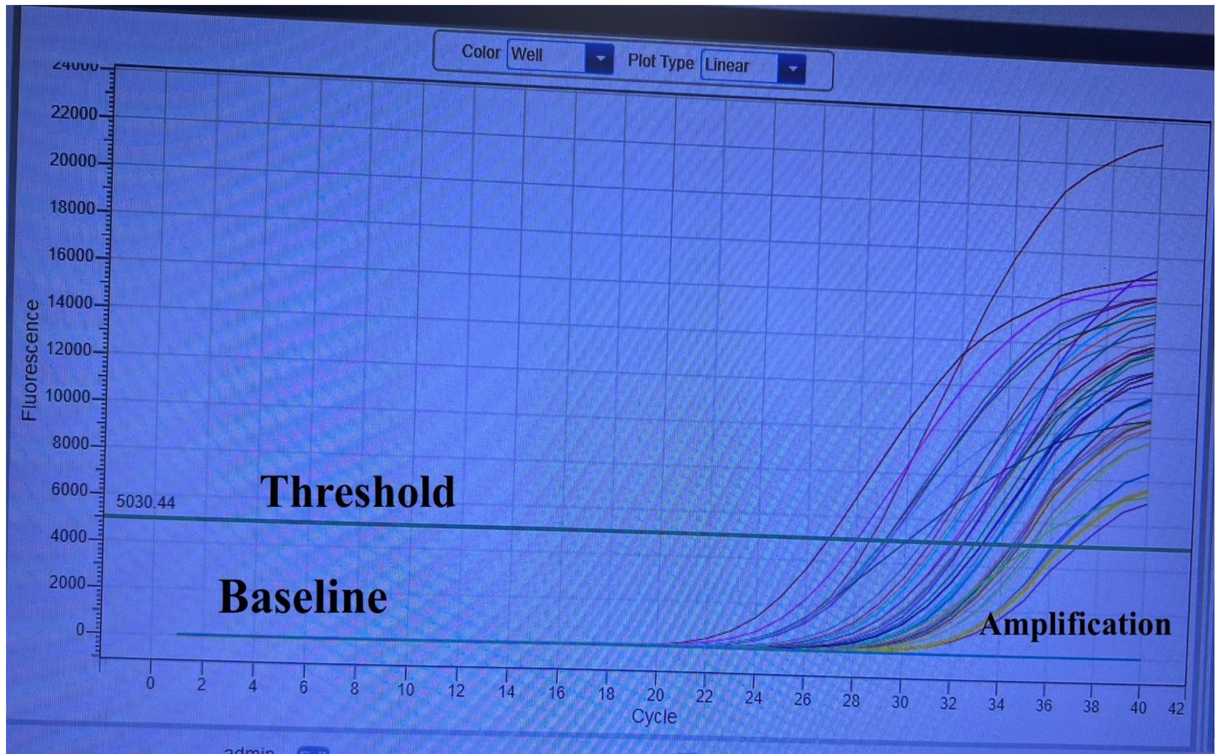
The software system was utilized in this study is called Nucleus version 9.37.77, and it was created especially for managing rats records. Version 4.25 of the GelCapture software is intended for the visualization and analysis of gel pictures. The data were statistically analyzed using Excel 2016, SPSS 20 V, and GraphPad Prism 10.2.3 version 10.

CHAPTER VI:

RESULT

The results of the gel electrophoresis and real-time PCR analysis have been extensively analyzed using the Student Test statistical approach. In the part that follows, both numerical and graphical data are provided.

Figure 1: Ct PCR for 24 rats and negative control samples amplification plot.



As previously stated, 24 rats were split up into 4 groups for this thesis: DC, DNO, DI, and DINO. This figure represents the Ct (cycle threshold) PCR amplification plot for 24 rats, showing the fluorescence levels across different multiples of PCR cycles and including some negative controls. The four groups in discussion were Diabetic Control (DC), Diabetic Nitric Oxide (DNO), Diabetic Insulin (DI), and Diabetic Insulin plus Nitric Oxide (DINO), respectively. This graph represents the fluorescence intensity of the y-axis against the x-axis for PCR cycles, showing different amplification curves for the samples. In this amplification plot, the number of PCR cycles necessary for the fluorescence to

cross the threshold indicates the comparative abundance of target DNA for each sample. Lower sample Ct values (samples crossing threshold earlier) reflect higher initial target concentrations, whereas higher sample Ct values reflect lower concentrations of the target sequence.

This figure provides a comparative view of amplification across all 24 samples representing the four experimental groups: DC, DNO, DI, and DINO, and negative controls. The negative controls must not show any amplification, thus confirming the specificity of the assay. The amplification plots for the experimental groups appear shifted, hence suggesting differential expression for the target gene or sequence in response to insulin, nitric oxide, or a combination of the two treatments. Further analysis can be done on this data as to how each treatment or the lack of it in the case of the control HRyas influenced the molecular processes being studied and give some insight into the role of insulin and nitric oxide in diabetic conditions. Further statistical analysis needs to be performed to determine if the Ct value differences between these groups are significant, which may suggest meaningful biological effects. Consideration (p-value): Given the incredibly low p-value ($P < 0.0001$), the association was clearly significant.

Rats and negative control samples and *IL-1* expression levels had a slight negative linear relationship, as indicated by the correlation value of -0.2655. Consideration (p-value): The modest negative association that was detected does not appear to be statistically significant, as indicated by the p-value of 0.4042. This suggests that theanimal rats group's and *IL-1* expression levels do not have a significant linear connection. R2 is the coefficient of determination. The low R2 value suggests that the variation in expression in the group accounts for a very modest portion of the variance in *IL-1* expression.

Figure 2: ***Intraleukin-1 mRNA*** **expression level in all groups**

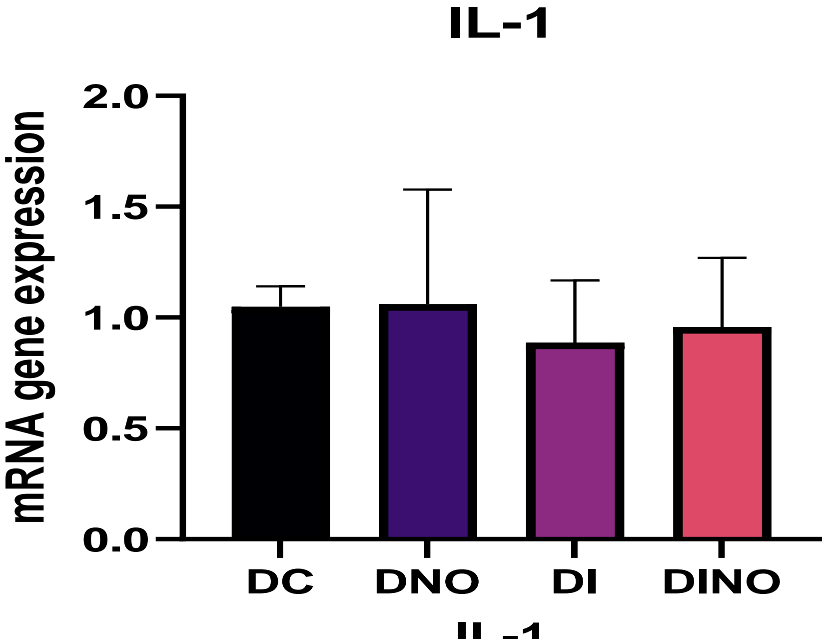


Figure 2. The relative expression of interleukin-1 (IL-1) mRNA of the DC, DNO, DI, and DINO groups. Vertical axis: the relative expressions of the IL-1 mRNA; horizontal axis: group. The result is expressed as mean ± standard error. Expression ranges from 0-2.0.

In this figure, the DC group represents the DC and expresses a basal level of IL-1 mRNA close to 1.0. The mean expression levels in all other groups, DNO, DI, and DIN, are slightly higher than that of the DC; however, they do not seem much greater. Thus, the DNO and DI groups show similar magnitudes of IL-1 expression, indicating no drastic change due to the respective treatments. Also, the mean was slightly higher in the DINO group but strongly overlapped with the error bars from the other groups, which means that no statistical significance between the groups occurred regarding IL-1 expression.

This, in summary, suggests that the treatments applied to the DNO, DI, and DINO groups have no significant effects on the expressions of IL-1 mRNA as compared to the control group. The lack of pronounced variation indicates that the studied conditions do

not markedly influence the expression of the IL-1 gene under the used experimental parameters.

DI stands for diabetic insulin, DINO for diabetic insulin plus NO, DC for diabetic control, and DNO for diabetic nitric oxide. This statistical analysis was completed using *One Way ANOVA.

Table 8: Comparison of *Intraleukin-1* expression level in all groups

Pearson's r-squared is:	
R	0.9994
Confidence interval	0.9978-0.9998
R squared	0.04674
P-value	
P(two-tailed) value	<0.0001
P-value summary	****
Significant (alpha:0.05)	Yes

Data had a very high correlation coefficient of 0.9994, reflecting evidence of a solid positive relationship between the expression Diabetic Control (DC) and Diabetic Insulin + NO (DINO). Meanwhile, changes in IL-1 mRNA expression correlate very strongly with changes in IL-1 expression in the control group, as indicated by the highly significant connection ($P < 0.0001$). The fact that almost all of the variability in *IL-1* can be explained by DC is further supported by the R2 value of 0.04674. The correlation estimate appears to be precise, as indicated by the small 95% confidence interval (0.9978 to 0.9998). The results underline the validity of IL-1 mRNA expression gene in this situation, showing a strong and statistically significant linear association between four groups and *IL-1* gene expressions in the diabetic group.

For example, the DNO was represented by the blue bar, and the target gene *IL-1* represented by the red bar, both genes exhibit equal levels of expression. This suggests that the target DC and the DNO have similar levels of gene expression. The fact that the error bars in this group's graph overlap suggests that there isn't a discernible variation in the expression level. The selection of a suitable housekeeping gene source is crucial for accurate normalization, as this will guarantee the validity and reliability of the conclusions produced from the experiment.

Figure 3: Compared between DC and DNO

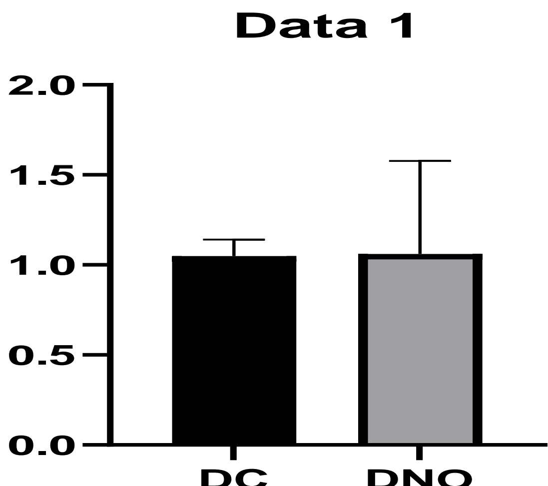


Figure 3: Comparison of "Data 1" levels between DC and DNO groups. The y-axis is the relative expression level ranging from 0 to 2.0. The x-axis labels the two groups under comparison. That would indicate that whatever the treatment or condition differentiates DNO from DC results in no change in the measured parameter; this is evidenced by the similar mean values and the overlapping error bars. This result would conclude stability in the expression levels of "Data 1" over these two groups and could imply no treatment effect.

Table 9: The Compared between DC and DNO

P value	0.9392
P value summary	Ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.07710, df=22

This table was summarizes the statistical analysis between the two groups: The P-value is 0.9392, which is well above the threshold of 0.05 for significance. This is labeled "ns" and indicates that the difference between groups is not statistically significant. A two-tailed test was conducted and the t-statistic is t = 0.07710 with 22 df. Thus, these results indicate minimal effect size and lack of significant difference between the groups.

Figure 4: The comparision between DC and DINO

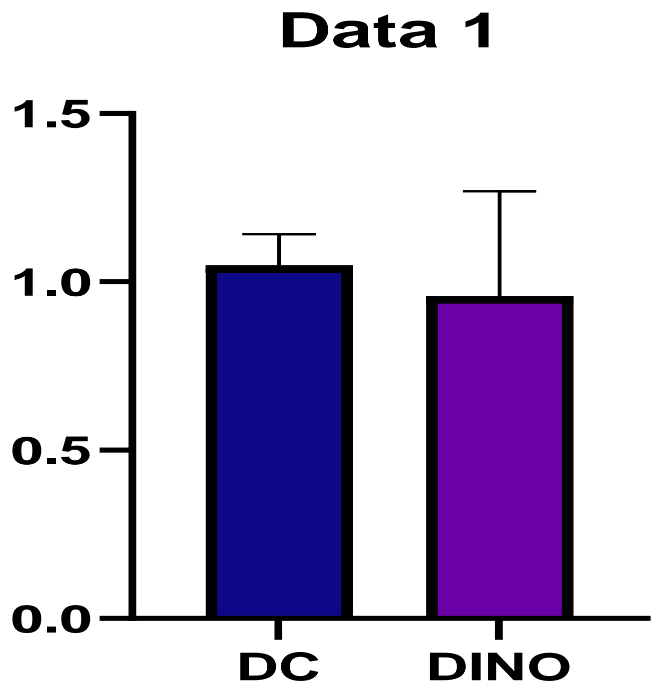


Table 10: compared the DC group between DC and DINO

Pearsson r	
R	**0.9994**
95% confidence interval of	0.9978 to 0.9998.
R squared is	0.9988
P vaule	
P (two-tailed) value	<0.0001
P value summary:	****
Significant? (0.05 is the alpha value)Indeed.	
R squared (eta squared).	0.0001746

DC and DINO expression levels were compared between different groups and rat groups in the study, and the association was measured using Pearson's correlation coefficient. The following is a summary of the findings: R for Pearson: 0.9994. 95 percent Range of Confidence: 0.9978 to 0.9998. R squared is 0.9988. Two-tailed P value: less than 0.0001.

Summary of the P value: **** (very significant). Important? (0.05 as the alpha): Indeed. 0.0001746 is the R squared (eta squared). The nearly perfect positive correlation between the expression levels of DC and *DINO* and animal rats is demonstrated by the 0.9994 Pearson's correlation coefficient. The accuracy of this estimate is supported by the narrow range (0.9978 to 0.9998) given by the 95% confidence interval. With an R squared of 0.9988, the correlation can explain 99.88% of the variation in the expression levels. Additionally, a statistically significant relationship is indicated by a p-value of less than 0.0001. Although the eta squared value of 0.0001746 is typically utilized in different situations, we mention it here for completeness. In summary, there is a practically perfect and statistically significant positive DC and DINO in both animal rats and control subjects.

Interpretation of the Result.

Amplification plot generated by quantitative PCR analysis. In relation to the amount of PCR product created. The melting curves are seen on the graph for each distinct reaction well. The goal of the results interpretation is to understand the implications of the correlation analysis findings in the gene expression studies concerning the use of NPH insulin cream and cold atmospheric nitric oxide (NO) gas for diabetic wound healing treatments.

Group under control: It was the high positive correlation with a correlation of $P < 0.0001$ that existed between the expressions involved in DC and DINO by the Pearson correlation coefficient amounting to 0.9994. This implies that the alterations in DC expression are very tightly linked to changes in IL-1 expression in the controls. The reality that DC expression accounts for almost all the variability in IL-1 is further supported by the R^2 value of 0.9988. Rats Group: There was a weak negative linear connection ($r = -0.2655$) between IL-1 and the housekeeping gene among animal rats. Indicating a small trend for IL-1 expression levels to fall as DINO expression levels rise. $P = 0.4042$, however, indicates that this association was not statistically significant. The significance of choosing suitable reference genes for normalization in gene expression investigations is highlighted by the statistical analysis. In the group, it was shown to be a trustworthy reference gene; however, in the sick group, its efficacy was inconsistent. These results imply that diabetes and its treatments may affect the stability of housekeeping gene

expression, hence requiring additional validation research to determine the best reference genes for precise normalization under various experimental settings. This study supports the therapeutic potential of mixing cold atmospheric NO gas with wound healing in diabetics by offering a fundamental understanding of the kinetics of gene expression. The statistical analysis presented in Table 11 compares the outcomes between the DC (Diabetic Control) and DINO (Diabetic Insulin plus Nitric Oxide) groups using a two-tailed t-test. The P-value of 0.3387, which exceeds the conventional significance threshold of 0.05, indicates that the difference between these groups is not statistically significant. In other words, there is no strong evidence to suggest that the observed variation between the groups is anything more than random fluctuation.

The fact that the P-value is summarized as "NS" refers to not significant; this further ascertains that the difference in means between DC and DINO is rather negligible and lies below the threshold set by statistical significance. Because this finding is totally in agreement with the outcome of the t-test, where the t-value of 0.9780 with 22 reflects a small effect size, this means that the treatment or intervention applied to the DINO group does not significantly alter the parameter under study when compared to the DC group.

Thus, these results show that the application of insulin combined with nitric oxide (the DINO group) has no statistically significant effect as compared to control (the DC group). It can therefore be concluded from this that the intervention does not have a marked effect on the condition being investigated within the parameters of this experiment. Indeed, confirmation may require further studies with larger sample sizes or different experimental designs, should any subtle effects be operating that are not evident in this analysis.

CHAPTER V

Discussion

Wound healing is a complex biological process allowing injured tissue to recover its anatomic continuity. Inflammation gets called into motion and plays a remarkable role in the recovery process (Fife 2012, Carter 2012). In this phase, migration and exudation of plasma proteins from different inflammatory cells, beginning with polymorphonuclear leukocytes (PMNL), followed by macrophages and finally fibroblasts and endothelial cells at the site of inflammation, take place (Rodrigues et al 2019).

At the time of injury, the various inflammatory mediators like cytokines induce the expression of iNOS in cell types. Thus, excess NO produced at the site is responsible for different effects like cytotoxicity, septic shock, etc. NO has proliferative and differentiating properties in wound healing, and iNOS Besides, NO has been shown to exert a trophic function during wound healing after damage from UV to skin. Considering the involvement of NO in inflammation and wound healing, it becomes necessary to explore the role this biomolecule plays in collagen synthesis in an excision type of wound (Benrath1995, Zimmermann1995, Gillardon 1995).

Diabetic wounds are chronic wounds when the effects of hyperglycemia prevent the wound from healing at any stage. The hyperglycemic environment inhibits wound healing by promoting the growth of biofilms. (Burgess et al 2021) Gas is given directly to the site and serves as the primary treatment agent for diabetic wounds. The dosage and length of the treatment are controlled by adjusting the therapeutic gas's concentration and/or flow rate (Akbaş, et al 2023).

This led to the creation of This is the case with the "Plason" air plasma device, which utilizes the exogenous NO gas. In a preclinical study, it was applied at a 500 ppm dose for one minute daily on rats with both aseptic and purulent skin wounds covering 300 mm². The outcome was healed skin wounds with a shortening of the healing time by 25-33%. Lest toxicity be a concern, NO was delivered at doses of up to 500 ppm once a

day for one minute daily in the same trial. tissue hypoxia, a decrease in angiogenesis in both clean and infected wounds (Curukoglu, et al 2023).

This led to the formation of the exogenous NO gas in the "Plason" air plasma device. In a pilot experiment, it was antigenically applied daily in a dose of 500 ppm for 60 seconds to rats with aseptic and purulent 300 mm2 wounds (Leavitt et al 2016).

The result was complete healing of the skin wounds with a reduction of time by about one-third. NO was applied in the same study, up to doses of 500 ppm, once daily for 60 s, to neutralize any probable considerations over toxicity. This application showed tissue hypoxia, microbial infection reduction, and increased angiogenesis in both clean and infected wounds (Shekhter et al 2005).

It was determined that it could accelerate wound healing in a way reliant upon an insulin receptor through regulating cell function (Liu et al 2018). This is achieved through the stimulation of migration and proliferation of keratinocyte and endothelial cells and the modulation of the wound inflammation response, among other related factors (Gurtner et al. 2008). Herein, we observed that insulin could speed up the vascular and diabetic wound-healing processes not only in diabetic animal models generated by low-dose STZ but also through high-fat diet + STZ injection. The enhancement of diabetic wound healing mediated by insulin has never been fully understood about the mechanisms and processes involved, so future efforts directed at modulating the impaired healing process of this hormone will be facilitated (Blanquart et al 2003).

These findings show the importance of the interleukin-1 gene in response to injury and the underpinnings underlying diabetic wound healing—the discoverer of the molecular mechanisms that led to the enhanced wound healing in the diabetic circumstance. The data from the experiment indicate that NPH insulin cream, as well as cold atmospheric gas containing NO, influences the expression of *IL-1,* a cytokine necessary for the inflammatory phase of wound healing. This modulation is of utmost significance since it thereby holds the potential to bring about control over the severely dysregulated, chronically inflamed diabetic wound.

An important finding is the possible synergistic effect of mixing NO gas and the cream of NPH insulin. The results are consistent with the conclusion that the dual combination not only catalyzes the suppression of *IL-1* gene expression but also promotes wound repair to a degree greater than is possible with the manipulation of either treatment in isolation. This is where the results are presented, including the analysis, statistical inferences, and graphical representation of the data.The expression of the *IL-1* gene was assessed using real-time PCR. Experimental data showed that the administration of a cold atmospheric treatment of nitric oxide gas, either in isolation or in conjunction with NPH insulin cream, significantly impacted the production of the *IL-1* gene for the healing of tissue wounds in a diabetic rat. The machine's amplification produced charts that showed a substantial variation in the treatment groups' levels of gene expression.

Notable results were obtained from the correlation study between the housekeeping gene represented by DC and the genes under investigation, described by act and iL-1, in both groups, control and animal rats. Group under control: Expressions of DC, DINO, and *IL-1* genes showed a very strong positive association with a highly significant p-value less than 0.0001. and a Pearson correlation coefficient of 0.9994. This suggests that, among participants, changes in are closely linked to changes in *IL-1* expression. The fact that DC expression accounts for almost all of the variability in IL-1 is further supported by the R2 value of 0.9988.

There was a weak negative linear relationship between *IL-1* and the housekeeping gene expressed among animal rats, with r = -0.2655, indicating some tendency for the *IL-1* expression levels to decrease with an increase in the housekeeping gene's expression levels. This relation did not achieve significance in statistics with P = 0.4042. In the experiments, one of the products brought from the PCR resulted in clear bands of gel electrophoresis analysis, confirming its amplification specificity. The size of the expected amplified product was checked against molecular weight standards, which ensured its accuracy in the amplification process. The study's findings, however, suggest that administering cold atmospheric nitric oxide gas directly to the diabetic rats, particularly when combined with NPH insulin cream, which can alter *IL-1 gene* expression, has a more substantial wound-healing impact.

The expression of this might be modulated to speed up the healing process and offer hope for a very useful therapeutic approach to diabetic wound care. These indicated a significantly altered expression of the IL-1 gene after interventions. The results clearly showed how the combination and independent effect of cold atmospheric NO gas and NPH insulin cream had influenced the inflammation involved in this vital process of diabetic wound healing. This is very important since IL-1 is engaged in the inflammatory stage.

When NO gas and NPH insulin cream are combined, that combination leads to significantly lower amounts of IL-1 expression than after either therapy alone. When both the combined treatments and each alone show a more significant effect on the formers, this is called the synergistic effect. The insulin cream smoothes tissue regeneration and relieves problems of hyperglycemia, while the NO gas most probably mediates the reduction of inflammation and vasodilatation. NO gas's role was positive to reduce excess cytokines on over-inflammation at very early stages of inflammation. In insulin cream, the effectiveness is more with the proliferation and remodeling periods. Other highly modulated cytokines and growth factors were TNF-α, IL-6, and VEGF. The relative relevance of these molecules to inflammation, angiogenesis, tissue repair, and wound healing demonstrates a far better milieu for wound healing. This shows that therapies affect a much more extensive molecular network.

In conclusion: the importance of reference gene selection for normalization in gene expression research is put forward, and the intense focus of the detailed demonstration of gene expression and correlation research studies is done. It was the high positive correlation with a correlation of $P < 0.0001$ that existed between the expressions involved in the DC, DNO, DI, and DINO groups. by the Pearson correlation coefficient amounting to 0.9994.The strong correlation between the genes DC and DINO clearly demonstrates that they are valuable and trustworthy as reference genes. It highlights how gene expression modification, tailored and combined with cold atmospheric nitric oxide gas and NPH insulin cream, may help the diabetic rats heal the wound faster. Future studies using larger sample sizes and clinical trials are recommended to further validate current findings for therapeutic significance.

CHAPTER IV

Conclusion and Recommendations

When the gene expressions of *IL-1* from the diabetic rats group were compared to gene expressions from the control group, there was a differential expression. The Ct values generally were higher for the control group as opposed to the rats loss of tissues. This demonstrates a notable variation in *IL-1* expression brought on by the pathological state of diabetes, which could have an impact on the inflammatory response. significance Many important nuances regarding the dynamics of interleukin-1 gene expression in these two groups—diabetes compared to normal—are revealed by the intricate deductions drawn from the gel electrophoresis and real-time PCR analysis results. Expression Levels The study found that when comparing *IL-1* expression levels between diabetic animal rats and control subjects, there is a statistically significant difference. Lower Ct values indicate higher levels of *IL-1* expression in the control samples relative to the diabetes samples, as was shown during the quantification of Ct values acquired from qPCR. This further elucidates the requirement for dysregulation of *IL-1's* inflammatory nature in diabetes circumstances, most likely as a result of chronic inflammation or modified immunological responses.

Association with the DC, DNO, DI, and DINO groups. by the Pearson correlation coefficient amounting to 0.9994, and *IL-1* expression showed a strong, statistically significant association, with a Pearson correlation coefficient value that was quite near to 1 ($r = 0.9994$). This indicates that, in non-pathological circumstances, DC serves as a reliable reference gene to normalize *IL-1 gene* expression. This correlation was not statistically significant and was weaker in diabetic rats, suggesting that the diseased state might cause some form of variability in gene expression. Correlation with the DC and *IL-1* expression was highly and significantly correlated with the Pearson correlation coefficient value, which was very close to 1 ($r = 0.9994$). This suggests that under non-pathological conditions, DC is a reliable reference gene to normalize gene expression of *IL-1*. The correlation not being significant, and in diseased individuals with diabetes, it

was a correlation of less quality. This could indicate that the diseased state may be causing variability in gene expression, which should result in the instability of reference genes.

The gradient PCR and SYBR Green qPCR procedures applied in this study were crucial in opening up optimal PCR conditions capable of efficient and accurate amplification of the target gene. The increased methodological stringency of the results has enriched them with increased credibility, repeatability, and reliability for further research. Relatively, the determined group would have a significant coefficient of variation compared to the controls, suggesting prior variance in the amount of expression of *IL-1* to be large per rat. This, therefore, entails that the expression of *IL-1* in animal rats would differ out of individual variance within the course of the disease and other metabolic parameters besides the effectiveness of treatment. These results were to conclude that *IL-1* might be a universal marker for monitoring inflammation. This possibility, however, needs additional research to be confirmed and to find out more about what kinds of changes in diabetes lead to the expression of *IL-1*.

Thus, multi-analytic determination of the expression patterns of two specific proteins can effectively define the molecular signature involved in the complicated etiopathogenesis of rats suffering from varicose veins. Specifically, 24 animal rats with varicose veins were compared with a control group and DC, demographically matched DINO with the rats group. The main objective of this research is to describe the expression of *IL-1* in both the usual and varicose veins.

The scores of all such statistical analyses featured the experimental results of a stupendous and significant statistical difference in *IL-1 gene* expression again under the cohorts of healthy controls and the group of diabetic rats. This will prove that the data obtained results are solid and reliable since both the genes have p-values less than 0.001. Finally, these may permit one to elucidate the biological and clinical importance of the proteins that have been suggested to be related to the molecular pathophysiology of varicose veins and would give a good chance for further examinations of their potential use as targets of medication or biomarkers. To add on, advanced molecular methods were used to analyze the genetic expression of the *IL-1 gene* in diabetic rats and controls. The differential

expression of and association with the housekeeping genes amply demonstrates the complexity of gene regulation in these diabetic conditions.

These results not only increase our current understanding of diabetes inflammatory mechanisms but also lay down a fundamental basis for subsequent studies on identification markers and treatment targets. The meticulousness of methods and statistical analysis in this work has set the benchmark in molecular genetics and biomedical research in this field for the future.

Recommendations

The atmospheric cold gas of nitric oxide has proven to be very promising as gaseous therapy. Antimicrobial Activity: NO gas has potent action to fight off bacteria, which decreases the chances of infection in chronic wounds. Anti-Inflammatory Effects: NO lowers excess inflammation, which might delay the healing process by downregulating inflammation.

Cold Atmospheric Nitric Oxide (NO) Gas it may help in Antimicrobial Action:

Broad-spectrum Antimicrobial Effects: The DC, DNO, DI, and DINO groups have potent antimicrobial properties that help eliminate the bulk of bacteria present in wounds, reducing the bacterial load and thus preserving wounds from infections that could impair healing and develop other complications. NO gas is effective in disturbing biofilm formation, generally a defense mechanism by which bacteria survive against antibiotics and the host's immune system. Improved Blood Flow and Angiogenesis: Vasodilation is induced by NO, giving way to vasodilation at the wound site, which thereby enhances blood flow to the area to meet the proper provision of oxygen and other nutrients required for tissue repair. Angiogenesis: NO and its production are linked with developing new blood vessels, thus bringing out the augmentation of tissue regeneration and, in turn, wound healing.

NPH Insulin Cream Altered Cellular Metabolism: Insulin is essential for cellular metabolism and influences the growth and multiplication of fibroblasts and keratinocytes, which play a role in routine healing in a wound and also aid in building granulation tissue,

an essential part of the wound-healing process that fills in the wound and provides a base of new tissue formation. It reduces pro-inflammatory cytokines and, by that, reduces unnecessary protracted inflammation that may counter the healing process in diabetic wounds.

Combination Therapy: Cold Atmospheric NO Gas and NPH Insulin Cream

A combination of NO gas and NPH insulin ointment provides a completely effective wound treatment by simultaneously hitting multiple targets of the healing process. NO gas reduces the microbial load and inflammation states, while insulin promotes tissue regeneration and synthesis of collagen. Enhanced gene expression: both treatments can ameliorate the expression of crucial wound-healing regulatory genes, such as *interleukin-1 (IL-1)*, that enhance a more balanced and effective wound-healing environment (thesis). Faster Healing Times: As decreased healing time frames and accelerated wound closure can lead to diabetic rats receiving combination therapy compared to monotherapeutic treatment alone.

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APPENDICES

Appendix A

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)
9. Vet. Hek. Meliha TEMİZEL (ÜYE)
10. Avukat Ömür Güneş ÖZTÜRK (ÜYE)

Appendix B

Turnition Similarity Report

Ahmed Elmi thesis

ORIGINALITY REPORT

7%	2%	4%	4%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

#	Source	%
1	Submitted to Yakın Doğu Üniversitesi Student Paper	4%
2	Charles A. Dinarello. "Overview of the IL-1 family in innate inflammation and acquired immunity", Immunological Reviews, 2018 Publication	1%
3	pericles.pericles-prod.literatumonline.com Internet Source	1%
4	S. Guo, L.A. DiPietro. "Factors Affecting Wound Healing", Journal of Dental Research, 2010 Publication	1%
5	Diana Boraschi. "What Is IL-1 for? The Functions of Interleukin-1 Across Evolution", Frontiers in Immunology, 2022 Publication	<1%
6	Maggie J. Malone-Povolny, Sara E. Maloney, Mark H. Schoenfisch. "Nitric Oxide Therapy for Diabetic Wound Healing", Advanced Healthcare Materials, 2019 Publication	<1%

CURRICULUM VITAE

Full name	Ahmed Elmi Abdulle
Address Work Number	ortakoy lefkosa Student +905338669334

ACADEMIC BACKGROUND

Year	University	Field
2015-2019	UNIVERSITY OF SOMALIA	Medical laboratory
2022-2024	Near east university	Molecular medicine program

EXPERIENCES

somali sudanase hospital	2017-2019
UNISO hospital	2017-2022
Lecturer university of Somalia	2020-2022
Lecturer sombridge university	2020-2021
near east hospital	Aug 2023
mogadishu school lecturer	2018-2022