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RESEARCH ARTICLE
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The Comparison of *IL-12 Gene* Expression Analysis in the use of Cold Atmospheric Nitric Oxide Alone and Combination with NPH Insulin on the Full-thickness Excisional Wound Healing in a Diabetic Rat Model

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ABSTRACT

This study was designed to compare the effects of different treatments on the expression of the IL-12 gene and its role in the healing of diabetic wounds. An experimental approach was employed to evaluate a novel treatment combining atmospheric nitric oxide (NO) and NPH insulin cream, which has been shown to impact IL-12 gene expression and enhance diabetic wound healing. A total of 24 diabetic rat samples were divided into four groups based on the treatment received: a) DC (Diabetic Control), b) DI (Diabetic + Insulin), c) DNO (Diabetic + Nitric Oxide), d) DINO (Diabetic + Insulin + Nitric Oxide) Gene expression analysis of the IL-12 gene was performed using RT-PCR. Comparisons were made among the four groups (DC, DI, DNO, and DINO) to understand better the impact of these treatments on IL-12 mRNA expression. The result was a statistically significant decrease in IL-12 expression in the DINO group compared to the DC group (P=0.0437). Since higher expression of the IL-12 gene is associated with inflammation and chronic wounds, its reduction indicates improved healing. Notably, nitric oxide (NO), which has antimicrobial properties, appeared to suppress IL-12 expression in the DNO group, suggesting that NO contributes to reduced inflammation and promotes faster wound healing.

Keywords: Interleukin-12; IL-12; Wound healing; Diabetic wound; Cold atmospheric nitric oxide; NPH Insulin.

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INTRODUCTION

Healing of a normal wound occurs in overlapping stages, starting with hemostasis, followed by inflammation, and then proliferation and remodeling, which occur after injury to epithelialization is completed (Richmond et al., 2013; Cai et al., 2023). Healing of diabetic wounds in the presence of hyperglycemia or high blood sugar, where neuropathy and microangiopathy develop, leads to the disruption of collagen regulation and cell proliferation. Consequently, when this process stops, it delays wound healing and increases the risk of bacterial infection (Richmond et al., 2013; Goyal et al., 2023).

Inflammatory mediators and cytokines are secreted at the wound site, playing a crucial role in regulating cell growth processes. The role of growth factors and cytokines depends on the deletion or addition of these factors, which affect wound repair. Therefore, if any prolongation or failure occurs in any phase, it can result in over-healing or delayed wound healing (Bryan et al., 2005; Chen et al., 2022). Interleukin 12 is a recently discovered cytokine characterized by a unique heterodimeric structure formed from two distinct protein subunits, p35 and p40. Most IL-12 is produced by monocytes, dendritic cells, natural killer cells, and macrophages in response to suitable stimulation. IL-12 can promote the cytolytic activity of several effector cells, including natural killer (NK) cells, macrophages, lymphokine-activated killer (LAK) cells and T cells.

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Additionally, in type 2 diabetes, IL-12 plays a crucial role in the mechanisms of arteriogenesis and ischemia angiogenesis (Brunda & Michael, 1994; Ali & Maha, 2017; Curukoglu et al., 2023). Recent research has demonstrated that administering IL-12 after irradiation reduces the severity and size of radiation-induced burn wounds (Li, 2015; Kielbowski et al., 2025).

Nitric oxide (NO) is a gas that the human body can naturally produce in various tissues. This production is by enzymes from the L-arginine (amino acid). NO plays a crucial role in regulating pressure and blood flow. Also, it reduces the activity of blood platelets. In addition, NO is found as a Neurotransmitter. NO can bind to haem/heme (a component of hemoglobin) by interaction with iron (Bruckdorfer & Richard, 2005; Tatlıcıoğlu et al., 2023). NO can be synthesized by NOS (Nitric Oxide Synthase) isoenzymes, including NOS2 and NOS3, which have an important role in the process of wound healing from most skin cell types, examples: melanocytes, keratinocytes, and fibroblasts in healthy wounds, and several researchers studied the effect of NO on immune cells (Takashi Kitano, 2017; Kitano et al., 2017). The NPH Insulin (Neutral Protamine Hagedorn) is commonly used for wounds in both non-diabetic and diabetic patients, as it can promote wound healing while also lowering costs. When using insulin as a spray, local injection, or cream can decrease the time required for wound healing (Zhao et al., 2017).

Due to the importance of treatment for diabetic wounds and preventing the danger to the health of diabetic patients, several researchers are trying to find a good treatment. However, there are numerous challenges, such as hypoxia, which can lead to decreased collagen production.

The objective of this study was to measure the gene expression level of IL-12 in diabetic wound tissues treated with NPH insulin cream alone, cold atmospheric Nitric Oxide (NO) alone, or both together and to determine how these treatments affect IL-12 gene expression—discover that when using cold atmospheric NO gas and NPH insulin cream together will give a good wound healing more quickly than using each of them alone.

MATERIALS & METHODS

The total samples were 24 Wistar Albino rats in this study divided into four groups, and in each group 6 rats: Group 1 was a diabetic control group (DC), group 2 was a diabetic nitric oxide CAP/NO group (DNO), group 3 was a diabetic NPH insulin group (DI), and last one group 4 was diabetic NO with NPH insulin group (DINO). This division was based on the type of treatment applied to each group. The study got approval for ethics from the Near East University's Animal Experiments Local Ethics Committee, with reference Number 2021/141—sample collection.

This study received the samples, ready for laboratory adaptation, from Ali Curukoglu and his team (Curukoglu et al., 2023) after they developed a diabetes model in rats. They created a wound model and simultaneously measured the wound contraction rate. Then, they applied NPH insulin and/or CAP/NO according to each group. Ultimately, they took samples of skin from the wound area and prepared them.

Gene Expression Analysis

On the first day of wound healing treatment and the last day of treatment, approximately 30mg of tissue was collected from the wound area on the 14th day. To prepare for RNA, all tissue samples were directly frozen in liquid nitrogen and stored at −80°C. All the RNA was isolated using the TRIzol kit reagent (Invitrogen, Carlsbad, CA, USA). Moreover, we used a Nanodrop 1000 spectrophotometer to check the quantity of RNA and the ratio between the absorbance of Nucleic acid and the absorbance of protein, which indicates that the study products were suitable and could be used with them.

The gene expression of the IL-12 gene as a target gene and the ACTB gene (The – actin gene) as a housekeeping gene were detected by Real-Time quantitative PCR (Reverse Transcriptase) (qRT-PCR) following Kit procedure of HIBRIGEN cDNA Synthesis kit (Gebze – KOCAELI- Turkey), in order to prepare 20µL of cDNA from 4µL of mRNA, then Real-time PCR was used from the Insta-Q96 Plus (MIDC, Wagle Industrial Estate, India) following kit procedure of HIBRIGEN 2X SYBR Green qPCR Mix (Gebze – KOCAELI-Turkey), in order to get the gene expression levels, in addition to use forward and reverse primers from Oligomer company. In Fig. 1 below, the results of PCR Curves for the IL-12 gene, as a target gene, and the ACTB gene (the β-actin gene) are shown.

Data Analysis Study

The statistical analysis was performed using GraphPad Prism 10.2.3. The gene expression data are presented as Cycle Threshold (Ct) values, where the Ct value represents the number of cycles at which the logarithmic plot of PCR amplification crosses the threshold. The expression of IL-12 was compared between four groups by using the (2^-ΔΔCT) method called Fold change. ΔCT= Ct of *IL-12* gene – Ct of *ACTB gene* (housekeeping gene). In this study, we used one-way ANOVA statistical analysis to compare the four groups and a t-test to compare the Diabetic Control (DC) group with the other groups. The statistical significance value was $P<0.05$.

RESULTS

The gene expression of the IL-12 gene for all study groups is shown in Fig. 2 below. In Fig. 2A, the IL-12 gene expression levels in the four groups are shown by a bar graph, indicating that the DCs exhibited the highest level of IL-12 mRNA expression. The DI showed moderate IL-12 gene expression, while the DNO exhibited the lowest IL-12 mRNA gene expression. In contrast, the fourth group, DINO, had a higher IL-12 mRNA gene expression than DNO. The comparison between the three treatments used for wounds and gene expression of IL-12, in order from highest to lowest, was DI, DINO, and DNO.

This study aims to analyze the impact of therapeutic materials applied to diabetic wounds, including NPH insulin cream alone, cold atmospheric Nitric Oxide (NO) alone, and both DINO, on diabetic wound healing by examining the effect on IL-12 gene expression. The study obtained information from the Ct values for each sample.

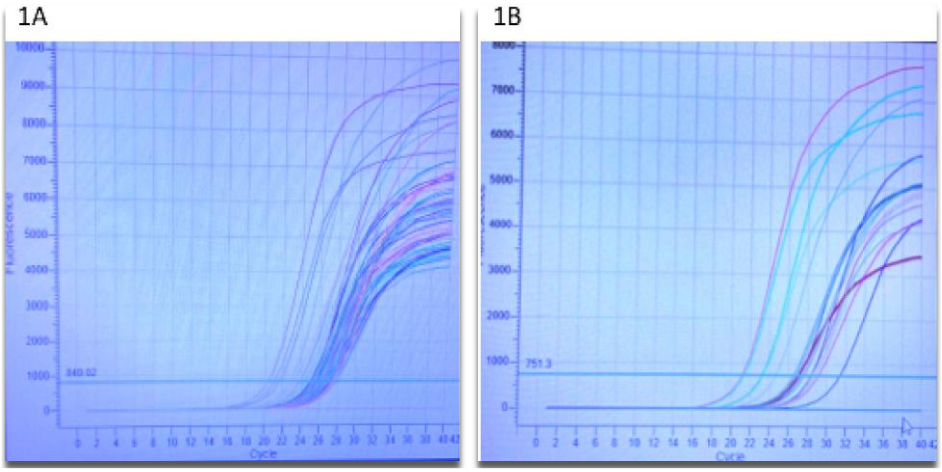

Fig. 1: RT-PCR results for samples. 1A) RT-PCR result for the ACTG gene. 1B) RT-PCR results for the IL-12 gene.

When comparing Diabetic control (DC) and Diabetic insulin + NO (DINO) using a t-test (Fig. 2B), the difference was significant (P=0.0437). DC had higher IL-12 gene expression than DINO; thus, DINO showed significant differences (P<0.05). On the other hand, when comparing DC and DI using the T-test statistical analysis (Fig. 2C), the difference was non-significant (P>0.2940). NPH İnsulin alone did not provide a significant as compared to DINO. A comparison between the three treatment groups and the DC group using a t-test revealed a significant effect only in the DINO group. In contrast, the DNO and DI groups did not show a significant effect. This is particularly important for this study.

Housekeeping gene: a gene that is expressed in all cells and maintained in a stable state. In this study, we cloned a gene and aimed to modify its function to ensure the cell was working correctly. In this study, ACTB was kept as a housekeeping gene (as a control reference). There was a significant difference (P<0.0079) comparing the DC group between the targeted gene IL-12 and the housekeeping gene ACTB (Fig. 2D).

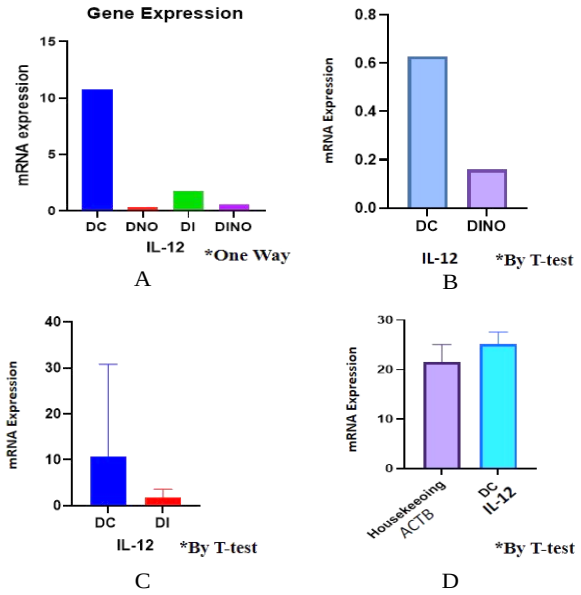

Fig. 2: A) IL-12 mRNA expression level in all groups, B) Comparison between DC and DINO, C) Comparison between DC and DI, and D) Comparison of the DC group between IL-12 and ACTB. Statistical analysis: A) One-way ANOVA; B-D) T-Test. DC: Diabetic Control, DNO: Diabetic Nitric Oxide, DI: Diabetic Insulin, DINO: Diabetic insulin+ NO.

DISCUSSION

Diabetic wounds are a type of chronic wound resulting from delayed wound healing due to the effects of hyperglycemia. When patients have high blood sugar levels, it creates a favorable environment for bacteria and microbes, leading to several complications, including prolonged wound healing (Burgess et al., 2021). In this study, four groups of rats were classified according to the type of treatment they received. The Diabetic Control (DC) group consisted of rats with a diabetic model that received no treatment. The Diabetic Nitric Oxide (DNO) group consisted of rats with a diabetic model and treated only with Nitric oxide (NO). The diabetic NPH insulin (DI) group contained diabetic model rates but was treated only with NPH insulin. The last group was a Diabetic Nitric Oxide with NPH insulin (DINO), so both treatments were applied to this group. This study aimed to investigate the impact of various treatments on wound healing in each group by examining the effect of each treatment on the gene expression of the Interleukin-12 gene.

The hyperglycemic state is accompanied by the production of reactive nitrogen species (RNS) and reactive oxygen species (ROS) in the blood, resulting in the cessation of wound healing within the inflammatory stage without completing the proliferation stage. This explains the prolongation of the inflammatory stage (Bansal, 2012; Zhang et al., 2024; Hegazi et al., 2024). Shekhter and his team found that NO acts as an antimicrobial agent in wound healing. However, we tried different concentrations of NO on the wound site. Including 500 ppm for 60 seconds once daily; they performed this trial for 6 days to reduce tissue hypoxia and microbial infection (Shekhter et al., 2005).

NO is a multifunctional gasotransmitter known for its antimicrobial, vasodilatory, and immunomodulatory effects. As Xia et al. (2025) emphasized, NO participates in angiogenesis, collagen deposition, and infection control—all of which are critical in chronic wound healing. Notably, NO-releasing systems that enable controlled release via stimuli such as light, pH, and enzymatic activity have been developed to enhance therapeutic outcomes and minimize side effects. In our study, 200 ppm cold atmospheric NO was applied for 90 seconds daily, leading to a marked reduction in IL-12 levels in the NO-treated groups (Xia et al., 2025).

NPH insulin plays a crucial role in metabolic alterations

and cell bioadvection within the wound area, correcting the shortage of glucose and amino acids by providing cells with necessary nutrients, thereby facilitating cell recovery and healing in a shorter timeframe (Curukoglu et al., 2023; Emad-Eldin et al., 2024). In this study, NPH insulin was applied in the DI group and DINO, but in DINO, it was applied after treatment with NO. On the other hand, several endogenous molecules, such as growth factors, play a crucial role in regulating cell responses and the wound healing process. In chronic wounds, they were present in unequal quantities in comparison to acute wounds, which plays a role in prolonged wound healing and the inflammatory stage (Ridiandries et al., 2018; Bahadoran et al., 2025).

Curukoglu et al. (2023) investigated various genes involved in wound tissue by histopathology, as well as the gene expression of growth factors such as transforming growth factor-beta (TGF-beta) and IL-8. They found that NO acts as an antibacterial agent with high expression of IL-8.

Interleukin-12 is a type of cytokine formed from two subunits, including p40 and p35. T-lymphocytes produce them to attack bacterial or microbial infections. Cytokines play a crucial role in two phases of wound healing: the hemostatic and inflammatory phases. Several subgroups of cytokines are produced in response to specific signaling pathways and biological structures. When wounds have a microbial infection or bacteria, immune cells, such as T-lymphocytes, synthesize Cytokines, including IL-12, which increases mRNA gene expression (Sun et al., 2015; Yamamoto et al., 2020). For example, TH1 induces the production of IL-12, IL-2, and IFN-γ (Antosova et al., 2012). Whereas this was agreed upon, what was noticed among the DC group in this study?

Zaborova et al. (2025) further elaborated on the dermatological relevance of NO. Its lipophilic nature allows it to penetrate the skin barrier and support microcirculation while promoting a healthy epidermal environment. This underscores NO's suitability for topical applications, especially in patients with impaired barrier function, such as those with diabetes. However, the clinical use of NO still faces challenges, such as instability, dosing variability, and control of penetration, all necessitating the development of stable delivery platforms and patient-specific treatment regimens (Zaborova et al., 2025).

Perhaps most compellingly, Davis et al. (2025) demonstrated NO's superior antimicrobial efficacy compared to silver sulfadiazine in a porcine model of second-degree burn wounds. Their results showed a microbial reduction of greater than 99.7% in wounds inoculated with *MRSA*, *A. baumannii*, and *C. albicans* following treatment with the NO-releasing compound NVN4000. This directly supports our findings by providing robust in vivo evidence that NO not only modulates inflammation but also exerts powerful antimicrobial effects, critical in diabetic wounds where infection is a major obstacle to healing (Davis et al., 2025).

When used in combination with insulin, as in our DINO group, NO amplified the anti-inflammatory benefits while insulin supported tissue regeneration and metabolic balance. This synergistic effect resulted in the lowest IL-12 expression levels among all groups (P=0.0437), supporting the therapeutic benefit of a dual-modality approach.

Looking forward, integration of controlled-release NO formulations—such as hydrogels, microneedles, or transdermal patches—could offer long-term and targeted delivery options. Coupled with real-time monitoring systems and personalized dosing strategies (Zhang et al., 2022; Wang et al., 2024; Zaborova et al., 2025), NO-based therapies have strong potential for translation into routine clinical practice (Zaborova et al., 2025).

Nitric oxide plays a crucial role in regulating cell migration, inflammation, and proliferation, all of which are essential to the wound-healing process. Additionally, it plays a regulatory role in apoptosis and acts as a vasodilator during the inflammatory stage. All of these are designed to provide more oxygen to the wound area, which is crucial for the healing process. Because NO works as an antimicrobial, it helps clean the wound area and prevents microbial and pathogenic infections. On the other hand, NO acts on the behavior of immune cells, such as macrophages and neutrophils. When this study applied NO to the wound site, NO attacked any microbial pathogens, acting as an antimicrobial. Consequently, inflammatory cells, such as lymphocytes and macrophages, did not synthesize cytokines in high amounts, as NO effectively prevented infection (Bruckdorfer & Richard, 2005).

Additionally, T-lymphocytes have several subtypes. Th1 cells produce TNF gamma, IL-2, and IL-12, which increase the production of Nitric oxide. the other subtype Th2 produce IL-3, IL-10, IL-6 and IL-5 this decrease the production of NO. According to this, NO will hurt Th1 and the cytokines produced by Th1. That means NO is naturally produced, and when used as a treatment, it creates a high amount of NO at the wound site, which can harm the immune cells and the cytokines they produce. So, it will lead to a negative effect on lymphocytes and IL-12. The results of the present study support this idea, as the DNO group had the lowest gene expression of the IL-12 gene. In this group, only NO acts as an antimicrobial, preventing the prolongation of the inflammation stage. However, it has a significant impact on the IL-12 gene.

Insulin function focuses on facilitating the entry of nutrients, such as glucose and amino acids, into cells in the wound area, which are often deficient in these nutrients. NPH insulin is used to provide cells with nutrients and enhance the healing process; additionally, cells can undergo mitosis and proliferation. According to this, the most challenging situation for practitioners during surgery on diabetic patients or any surgery for open wounds and loss of tissue is improving the granulation tissue before epithelialization. Recently, for chronic wound treatment, especially in diabetic patients, negative pressure therapy and hyperbaric oxygen have been started. Several studies have shown that insulin can be used in open wounds for both patients with non-diabetic and diabetic conditions (Zhao et al., 2017; Özaydın et al., 2018; Liu et al., 2021).

The use of insulin cream plays a crucial role in wound healing by supplying the cells at the wound site with necessary nutrients and promoting better healing. On the other hand, in that area, the microbes can be prevented. As

a result, the immune cells will continue to function, and the inflammatory cells will synthesize cytokines, such as IL-12, to attack the microbial agent. Moreover, in this study, the DI group exhibited a moderate level of gene expression for the IL-12 gene, indicating that insulin was effective in wound healing but also required inflammatory cells to produce IL-12 to combat microbes. This means the NPH insulin cream had a beneficial effect as a treatment, but diabetic wounds still required the immune system to combat microbial agents. So, NPH insulin also affected the gene expression of the IL-12 gene, but not like NO, because NO acts as an antimicrobial, resulting in a more negative effect on the IL-12 gene. Similarly, Özaydın et al. (2018) conducted a study demonstrating that topical NPH insulin application significantly accelerated the healing of full-thickness open wounds in both diabetic and non-diabetic mice. This study emphasized that insulin not only provides cellular nutrient support but also regulates inflammation and healing processes at the histopathological and immunohistochemical levels (Özaydın et al., 2018).

The group that provided significant (P=0.0437) results when compared with the DC was the DINO. DINO in this group used both Insulin and NO as compound treatment. This means compound treatment had a good effect when compared with DC. Moreover, it had a positive effect on wound healing due to the use of a compound treatment. The combination of NO with NPH insulin forms a compound treatment, and this combination has a significant effect on the gene expression of the IL-12 gene, greater than when compared with each treatment alone. This was an interesting result.

Wounds are so important, especially in diabetic patients, because they have hyperglycemia, so they have a chance to change to chronic wounds, delay the healing process, and lead to uncontrolled inflammation. The diabetic individual cannot heal easily; thus, the importance of this study lies in understanding how the present treatment affects wounds and has a beneficial effect, as demonstrated by the study of gene expression of the IL-12 gene. According to this, we can determine which treatment yielded the best results, specifically by targeting immune cells, which helps control the infection and leads to healing in a short time without delay.

When this research analyzed IL-12 gene expression in the four groups, it provided insight into which could serve as anti-inflammatory agents, which required only nutritional support, and which had a significant effect and were more suitable for use. Therefore, it is crucial to investigate the impact of treatment on the IL-12 gene to gain insight into how it works and which one yields a significant result. These results provide not only insight into the treatment and its effects on the wound but also an understanding of how IL-12 plays a crucial role and who can be affected by this IL at different levels.

Conclusion

In this study, the gene expression of IL-12 was compared between the four groups. In the first group, DC showed the highest gene expression, indicating a microbial infection and the inflammatory cells were attempting to increase the number of IL-12 to aid in attacking the microbe, which facilitated healing. However, the immune system alone in that group required more time, and this was the problem in that group; the study aimed to prevent this from happening to them. However, when nitric oxide was applied in the DNO group, it showed the lowest level of IL-12 gene expression. The NO was naturally synthesized, and when used as a treatment, it increased at the site of the wound, leading to an adverse effect on immune cells and reducing the expression of the IL-12 gene. Moreover, nitric oxide (NO) serves as an effective antimicrobial agent. The important group was DINO, which applied both insulin and NO in this group, and when compared with DC, showed a significant difference (P=0.0437). This means that when DINO was used in wound healing, it yielded a good result and decreased the time required for healing in diabetic rats.

DECLARATIONS

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Data Availability: The data are available upon request.

Ethics Statement: The study got the approval for ethics from the Near East University's Animal Experiments Local Ethics Committee, with reference Number 2021/141.

Author's Contribution: Conceptualization, A.C. and M.C.E.; RNA extraction, I.Y.R.; Investigation and conducting experiments, I.Y.R., A.C., M.C.E.; Formal analysis, I.Y.R..; Visualization, I.Y.R., A.C., M.C.E.; Writing—original draft preparation, I.Y.R.; Review, M.C.E.; Writing—review and editing, M.C.E.; Supervision, M.C.E.; Project administration, M.C.E.; Funding acquisition, M.C.E. All authors have read and agreed to the published version of the manuscript.

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