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A Study On Investigation Of Variations In Certain Proinflammatory Markers And Cd34 In Chronic Diabetic Wounds Upon Exposure Of Topical Insulin

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Abstract

Background: Chronic ulcers that occur because of diabetes, a significant complication seen in diabetic patients, with untreated or poorly managed conditions lead to serious outcomes such as chronic infection and limb amputation.

Aim Of The Study, To test the hypothesis that administering premix insulin injection directly into chronic diabetic wounds can improve wound management in the South-Indian population by investigating the levels of proinflammatory markers (IL-6 and TNF-α) and CD34 expression.

Results: In this study found that wound closure rates significantly improved in the test subjects after the 12th and 16th days of treatment compared to the 4th and 8th days. Additionally, the levels of hydroxyproline in both the control and insulin-treated groups significantly varied between day 0 and day 16. The tissue samples collected from the insulin-treated group showed significantly varied levels of IL-6 during 8th and 16th day of treatment.

Conclusion :The results suggest that local insulin treatment can accelerate wound healing by improving blood flow, reducing inflammation, and stimulating the production of new blood vessels and collagen. This finding indicates that local insulin could be a valuable addition to wound healing treatment options for diabetic patients.

Keywords: Diabetic wound; topical insulin; proinflammatory markers; immune histochemistry

Introduction

Diabetic mediated ulcer is a common complication of diabetes and can lead to serious infections and sometimes amputation of lower extremity. [1] The underlying cause of diabetic foot ulcers is peripheral neuropathy, which results in the loss of sensation and impaired circulation in the feet. Other risk factors for developing diabetic foot ulcers include high blood sugar levels, poor foot hygiene, and foot deformities such as hammertoes or calluses. Treatment of diabetic ulcers requires careful management of blood sugar levels, offloading of pressure from the affected area to promote healing, wound care to prevent infection, and in some cases, surgical intervention. In severe cases, patients may need to be hospitalized for intravenous antibiotics and wound management.[2]There are many overlapping mechanisms reported in relation to diabetic wounds, among which endotheliopathy was found to be most contributing factors which resulted in development of micro and macrovascular changes (Richard et al., 2012). [3]It can lead to development of several neuropathic complications; this significantly increases the risk of wound formation and delay in wound healing. [4]Revascularization is the only

option when microangiopathic and macroangiopathic wound progression has reached a certain stage. Aside from thrombotic and embolic closure techniques, no vascular surgery techniques have been developed to restore microcirculation vessel flow (known as resistance subcutaneous arteries)[5] Although there is not a single known cause of these ulcers, a number of elements are thought to be crucial in separating chronic wounds from their acute counterparts. These wounds are primarily distinguished by abundant granulation tissue, enhanced fibrosis, and markedly elevated levels of proinflammatory cytokines, proteases, and neutrophil elastase.[6] Additionally, it has been hypothesized that a hyper stimulated neutrophil response, which results in excessive inflammation, may contribute to the chronicity of a wound. The vulnerability of exposed wounds to infection may make this worse. [7] Insulin is a dipeptide hormone and growth regulator have several important biological functions. Its beneficial effects towards the wound healing were known for a decade apart from the functions like regulation of carbohydrate and lipid metabolism. Local use of long-acting insulin containing Zn ions has been proved to accelerate the healing of diabetic wound without any major adverse events .[8]Intralesional insulin administration during the earlier stage of wound healing, showed restoration of collagen synthesis and formation of granulation tissue, thus helped in progress of healing process [9]Few studies evidenced that improvement in matrix formation and simulate the formation of fibroblast and keratinocyte and increase in angiogenesis upon exposure to insulin [10]The present study was framed to test the hypothesis that local administration of premix insulin injection into chronic diabetic wound. The factors like proinflammatory markers (IL-6, and TNF-α) and CD34 expression level was investigated to explore the role of insulin in chronic wound management of South-Indian population.

Materials And Methods

This prospective study was conducted at Rajah Muthiah Medical College, Annamalai University, Tamilnadu, India, between May 2022 and September 2022, comprising 10 cases (insulin administrated) and 5 cases (placebo). Before recruiting the individual for this study, the study plan was reviewed by institutional ethical committee and approved (IHEC/0401/2018) was obtained. Informed written consent was obtained from all the individuals who participated in this study.

Treatment Plan

Before injection of insulin, dead tissue or eschar was removed carefully to order provide the suitable environment to enable insulin injection. The wound was cleaned with sterile saline (0.9% NaCl) to avoid spread of further infection. Treatment was initiated by provide single or multiple injection of premix insulin (Huminsulin 30/70; Eli Lilly and Company India Pvt Ltd); twice daily based on the body weight of the patients as recommended by International Diabetic Federation. This product consisted of 30% of rapid acting and 70% intermediate acting, recombinant conventional human insulin.

Assessment of wound diameter

In this study wound size was measured, to access the rete of wound healing upon insulin treatment and wound size was recorded every four days of time interval from the 0^{th} to 16^{th}.

Histological investigations

Small piece of wound sample was cut on the day 0 and 16, at once fixed in 10% buffered formalin for histological investigations. Further, fixed tissue samples were dehydrated using gradient ethanol treatment and samples were embedded in paraffin wax allowed to solidify. The tissue blocks were subjected to slicing with the help of microtome to get the tissue sections around 5 µm. Sections of the tissues were placed in clean glass slide then stained with hematoxylin and eosin (to evaluate the certain parameters related to tissue regeneration) and another section of the tissue was stained using Masson's trichrome stain (to evaluate the collagen level in the samples).

Immunohistochemistry (IHC)

Further to evaluate the treatment progress, expression level of CD34 was measured with the help of proper antibodies. The majority of hematopoietic progenitor cells and endothelial cells in blood arteries display the surface antigen CD34. The detection of CD34 positive cells in tissue samples using CD34 immunohistochemistry (IHC) is a technique that can be helpful for the diagnosis of some blood vessel-related disorders as well as for locating cells that have the ability to develop into different blood cell

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types. The following steps are commonly included in the general CD34 IHC protocol: The tissue samples are divided into thin sections after being fixed in paraffin. After that, the portions are set up on glass slides. In order to get rid of the paraffin and rehydrate the tissue, the sections are also treated with xylene and a number of alcohol solutions. The sections are treated with a solution of a mild acid or base (such as citrate or EDTA) at high temperature to retrieve the antigen. Blocking of non-specific binding, the sections are incubated with a blocking solution, such as normal serum, to reduce non-specific binding of the primary antibody. Then the sections are incubated with a primary antibody that specifically binds to CD34. The primary antibody is typically a monoclonal antibody raised against the CD34 antigen.

Detection of primary antibody: The sections are incubated with a secondary antibody conjugated to a reporter enzyme, such as horseradish peroxidase (HRP), which binds to the primary antibody. The presence of the primary antibody is then detected by incubating the sections with a substrate that produces a colored reaction product when it binds to HRP. Counterstaining: The sections are counterstained with a nuclear dye, such as hematoxylin, to visualize the nuclei of the cells. Finally, the sections are viewed under a microscope and analyzed.

Determination of hydroxyproline content

The level of hydroxyproline from the wound samples before and after treatment was estimated according to the method described by Boakye et al., with slight modifications (Boakye et al., 2016). Tissue samples from the wound was collected during 0^{th} and 16^{th} day of treatment and stored at -20℃ until further analysis. The tissue samples were thawed to room temperature and dried at 60℃ using hot air oven, the dried samples hydrolysed with the help of 6 M HCl for 130℃ for 3 h and neutralized to pH 7 with 2.5 M NaOH. Two millilitre off the solution was mixed with chloramine-T oxidation for 20 min at 25℃, reaction was terminated by addition of 1 mL of 3.15 M per chloric acid at 25℃ for 10mins. To this 1ml Ehrlich reagent was added, shaken, and incubated at 60℃ for 20 min. The development of pink was indicative positive reaction, intensity was measured at 557 nm. The concentration of hydroxyproline was measured against the standard graph plotted using L-hydroxyproline.

As part of this investigation, the collected tissues were immediately stored at -20℃ (8^{th} and 16^{th} day) immersed in physiological saline. The frozen tissues were thawed and homogenised using buffer containing cocktail of protease inhibitors, the homogenate was separated by centrifugation at 14000 RPM for 15mins at 4℃ (El-Samad et al., 2022). Further, level of proinflammatory cytokines like TNF-α and IL-6 was determined by enzyme linked immunosorbent assay (ELISA) method. The analytical limit of the kit was 7.89 pg/ml (intra- and inter-assay CVs (%) were 7.4 and 6.5, respectively) and 0.15 ng/ml (intra- and inter-assay CVs (%) were 5.4 and 4.2, respectively) for IL-6 and TNF-α respectively (Zubair et al., 2012).

Results

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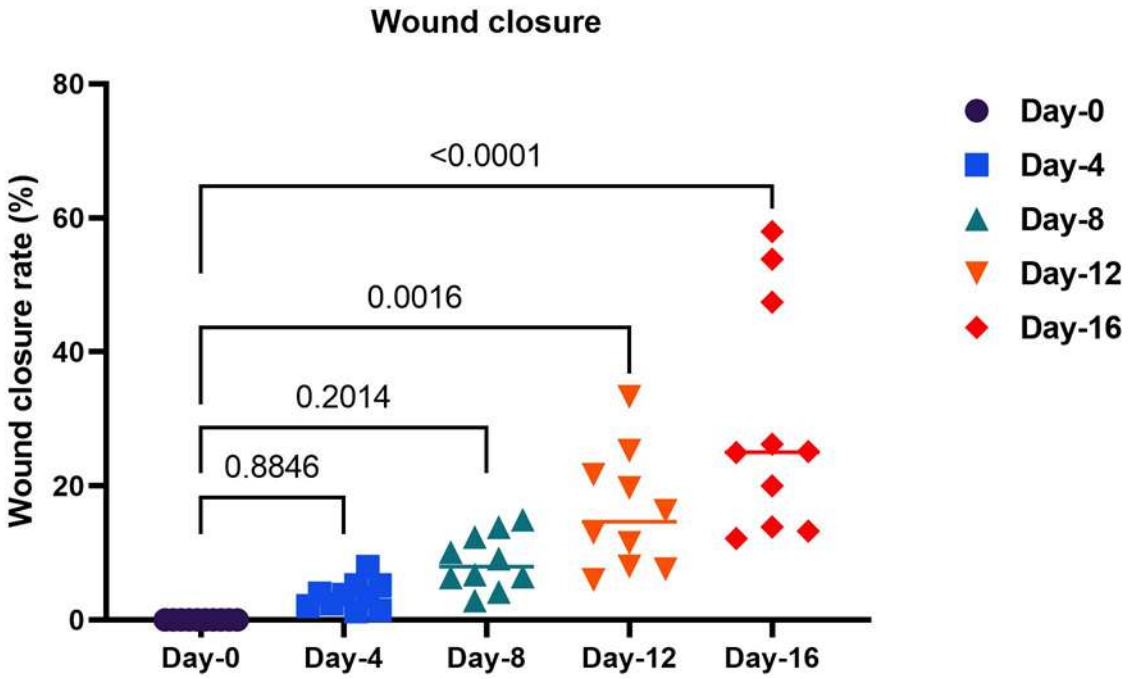
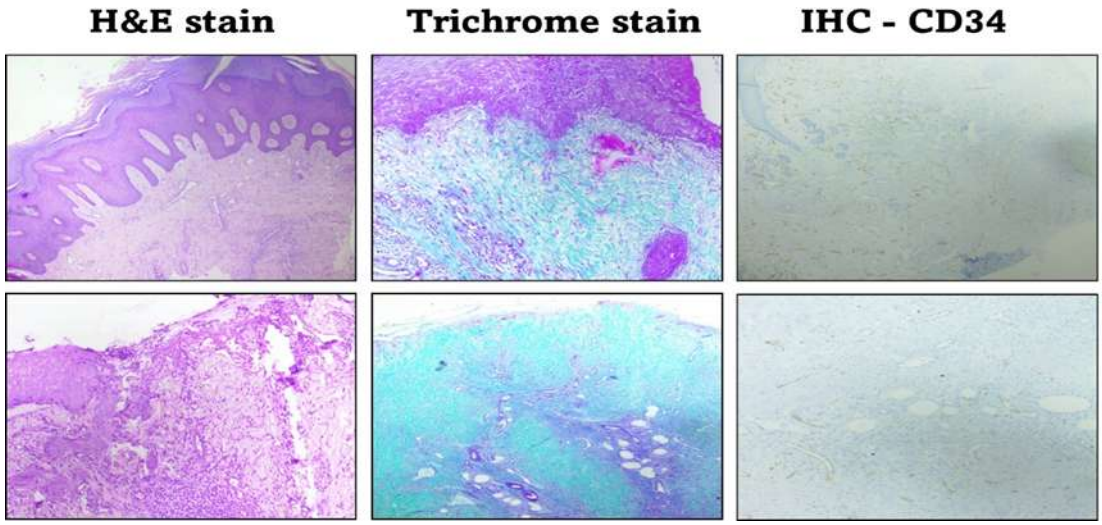
Figure :1 Effect of insulin injection on wound closure



Morphometric image analysis revealed that administration of local insulin (premix) has the positive impact on wound closure which faster the wound healing process at given time interval. During the treatment there was no complete wound closure was seen since this study was restricted within 16 days. However, there were significant variation in the rate wound closed noted in all the test subjected after 12^{th} ($p=0.0016$) and 16^{th} day ($p<0.0001$) while it was delayed during 4^{th} ($p=0.88$) and 8^{th} ($p=0.20$) day of the investigation (**Fig 1**).

Figure :2 Histological investigation



Histological analysis of wound tissues by haematoxylin and eosin staining has shown tissue reconstruction and collagen deposition toward the wound area. Some hallmarks of wound healing (angiogenesis, scab formation, reduction in inflammatory cells and coarse collagen fibres) are noted in the samples treated with insulin. In contrast, this event has not been observed in the control group. Further, the rate of collagen fibres present in the samples was evaluated using modified trichrome staining. The intensive increase in blue-coloured fibres is directly indicative of the rapid deposition of collagen fibres, this was evidenced by insulin injection (**Fig 2**).

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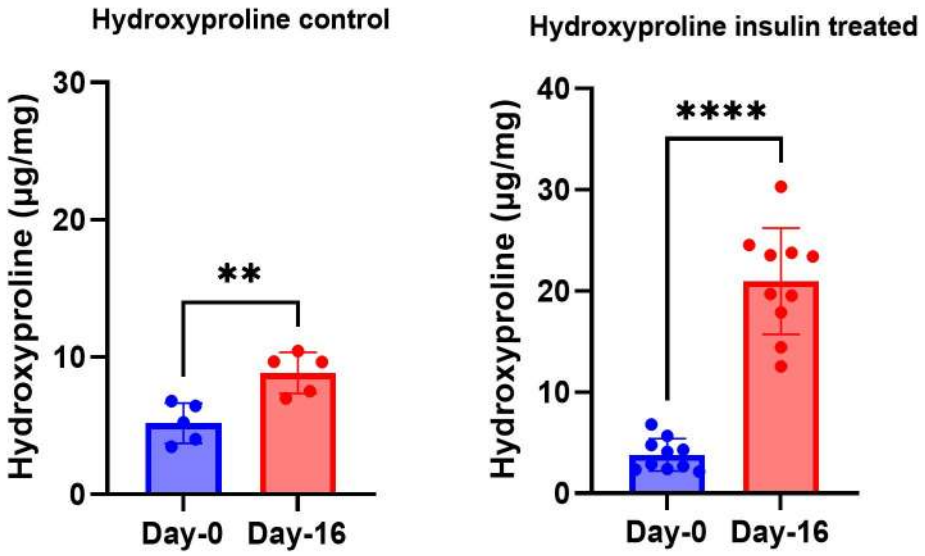
Immuno-histochemistry

Immunohistochemical analysis CD34 marker of both insulin and saline treated samples are shown in **Figure 2**, CD34 is a market used to study the progress of wound healing. By analysis 0^{th} and 16^{th} day sample from both groups showed that elevation in the level of CD34 in all the samples treated with insulin whereas it was significantly lesser in control groups. Zhang et al., 2015 observed that the injection of local insulin promotes the healing of chronic wound in patient presenting with uncontrolled diabetics, during the treatment period on 5^{th} day onwards increase in formation new blood vessel was noted with respect to control groups. In the insulin treated group the level of microvessel density significantly varied this directly evidenced that formation of new blood vessels. The same phenomena were observed in our study, where CD34 expression was significantly improved at day 16^{th} day of treatment.

Figure :3 Effect of insulin on hydroxyproline



The level of hydroxyproline in the tissues of treated and untreated groups are presented in **Fig 3**, the level was significantly varied among the group. This behavior of the experiment implicated that local administration of insulin has the impact on increase the level of hydroxyproline whereas it was lesser in control group. However, the level of hydroxyproline in both control ($p=0.0002$) and insulin demonstrated groups ($p<0.0001$) were significantly varied when tested 0^{th} and 16^{th} day of treatment. Content of hydroxyproline from tissues are directly proportional to synthesis of collagen, hydroxyproline was the only amino acid abundantly found in collagen. Quantitative measurement of hydroxyproline was used to understand the progress of wound healing. In this study high concentration of hydroxyproline in insulin treated group indicated faster healing process of diabetic wound in contrast to saline dressed tissues. Collagen not only provide the structural integrity to the tissue matrix, but also play an important role in homeostasis and epithelization during the latter phase of wound healing (Iyyam Pillai et al., 2010; Enoch & Leaper 2008).

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Figure :4 The level of proinflammatory markers

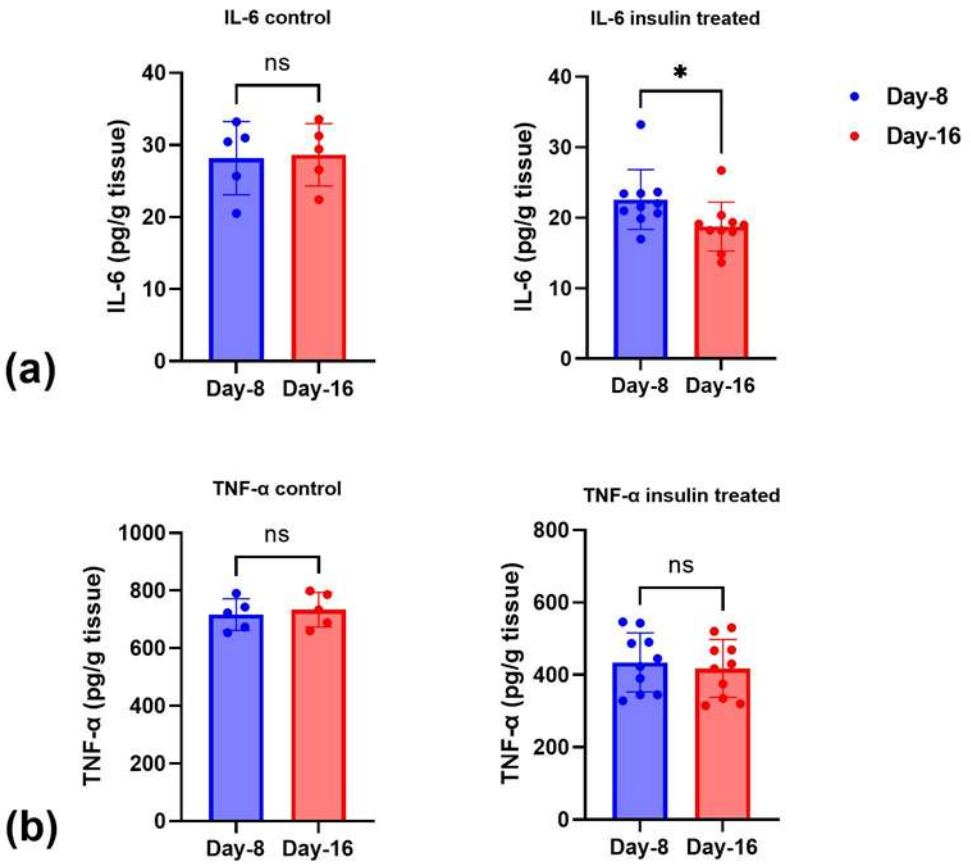

Fig 4 describes the level of proinflammatory markers such as IL-6 and TNF-α, as shown in **Fig 4a and 4b**, concentration of these markers found during this investigation was varied with respect control group. The level of IL-6 of tissue samples collected from insulin treated group was significantly varied ($p=0.04$) between 8^{th} and 16^{th} day of treatment and concentration range between 13.65 to 33.23pg/g of tissue. However, there is no significant difference found between samples of control groups ($p=0.87$). Considering TNF-α, the level was upregulated between 8^{th} and 16^{th} day, but they are not varied significantly ($p=0.65$). IL-6 and TNF-α are the most common proinflammatory markers extensively used in various diagnosis purposes. Here, we evaluated the level of both markets upon treatment with insulin to chronic wounds in line with previous investigations. Recently, El-Samad et al., found that administration of some nano formulations induce reduction of all the tested proinflammatory markers like IL-1, IL-6, and TNF-α in comparison with control group (El-Samad et al et 2022)

Discussion

Wound healing is a complex process which has several specialized characteristics that includes hemostasis, inflammation, re-epithelialization, granulation, remodelling of the extracellular matrix, and scar formation .Insulin was found to have the potency to accelerate the chronic cutaneous wound healing and remodulate the structure of wound as normal one. Likely, it enhances the proliferation of dermal cells which enables the tissue remodelling.[11] Local injection of insulin on chronic wounds showed significant variations in wound contraction rate in comparison to normal dressing, which are indicative of high rate of wound closure.[12] Wound contraction is a healing response of wound tissue either by exposure of any healing agent or through the normal physiology, but there are many factors control the rate of wound contraction.[13] Likely, size of the wound was reduced and decreases the number of damaged tissues around the wound. Besides many substances has the

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role of wound healing by inducing wound closure. [14]Local insulin versus placebo in treating diabetic ulcer revealed that significant improvement in parameters associated with wound healing process among the 110 patients who presented with diabetic foot ulcers. End of this investigation they found that reduction in wound diameter which are significantly different from the control groups P=0.022 local injectable insulin produced better wound healing than control groups, evidenced by 79.4% of reduction in size and depth of the wound [15]

Further, the remarkable reduction in wound area was seen upon insulin injection, they were confirmed by histopathological investigation where we can find several distinguished morphological features.[16] Through this investigation the cellular proliferation, angiogenesis, and granulation tissue formation, evidence of marked re-epithelization, and accumulation of collagen around the wound area was noted. [17]Surprisingly, the results of present study were correlated to earlier study where they addressed role of plant extract .[18]Granulation of tissue formation is one among prime factor controlling the healing process of acute and chronic wounds. Local use of insulin in diabetic patients improves fibrosis and angiogenesis. There were significant variations in number of blood vessels formed during the study, in comparison to saline treated group [19]

Recently, insulin loaded chitosan gel was evaluated for their efficacy of wound healing results showed that reduction in wound size upto 2.46±0.57 cm .Similarly, mucoadhesive liposomal gel loaded with insulin improved the wound healing rate 36.67±12.179 mm after 8 weeks daily treatment with no sign of hypoglycemia [20]Collagen is a major component of extracellular matrix, which is stimulated by fibroblasts located around the wound. Collagen eases the migration of endothelial cells, allows the formation of new blood vessels in order to enhance granulation, tissue formation, thus helps in improving in improving the wound healing and reduce the wound area. [21]

Histopathological studies, which examine the microscopic structure of tissues, have revealed that insulin therapy can result in several alterations in the wound tissue of people with diabetes. For example, insulin has been shown to improve blood flow to the wound site, resulting in increased oxygen and nutrient delivery and improved tissue repair. Additionally, insulin therapy has been shown to reduce oxidative stress and inflammation in the wound, which can help to reduce the risk of infection and promote faster healing. It has also been shown to stimulate the growth of new blood vessels, which is critical for wound healing, and to enhance the production of growth factors that promote tissue repair.[22,23,24].Compared the efficiency of local insulin versus topical phenytoin in diabetic wound cases. This investigation consisted of 60 individuals of both sex and separated into three groups insulin, normal saline, and phenytoin. Upon application of the insulin and phenytoin produced significant variations in wound size, depth, formation of granulation tissue and time taken to heal the wound. Meanwhile all the individuals are maintaining their normal glycaemic level during entire study period [25].

Conclusion

In conclusion, the study provides evidence that local insulin administration is an effective treatment option for improving wound healing in diabetic patients. The results of the study show that local insulin treatment can accelerate wound healing by increasing blood flow to the wound, reducing inflammation, and stimulating the production of new blood vessels and collagen. These findings suggest that local insulin may be a valuable addition to the wound healing treatment options for diabetic patients. However, it's important to note that more research is needed to replicate the results and to investigate the long-term safety and efficacy of using local insulin to treat chronic diabetic wounds.

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