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A Randomized Controlled Trial on the Effect of Local Insulin Glargine on Venous Ulcer Healing

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ABSTRACT

Introduction: To determine whether the local administration of insulin glargine compared with placebo in nondiabetic patients with venous ulcers (VUs) leads to increased wound healing.
Methods: A randomized controlled trial using a split-plot design was performed in 36 adults with leg VUs >25 cm^2 and more than 3 mo of evolution. Each hemi-wound received either 10 UI insulin glargine or saline solution once a day for 7 d. Size of the wounds, thermal asymmetry, the number of blood vessels, and the percentage area of collagen content in wound biopsies were assessed at baseline and after 7 d of treatment. Blood capillary glucose was monitored once a day after the insulin injection.
Results: After 7 d of treatment, the hemi-wounds treated with insulin glargine were significantly smaller, had less thermal asymmetry, more blood vessels, and more collagen content than the saline-treated side. Correlation between thermal asymmetry and the number of blood vessels was also found ($r^2 = 66.2$, $P < 0.001$). No patient presented capillary glucose levels ≤3.3 mmol/L nor any adverse effects.
Conclusions: In nondiabetic patients with chronic VUs, the topical administration of insulin glargine seems to be safe and promotes wound healing and tissue repair after 7 d of treatment.

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Introduction

Venous ulcers (VUs) are the most severe and debilitating complication of chronic venous insufficiency and account for nearly 80% of all lower extremity ulcers.[1] These lesions are characterized by having poorly defined margins, a granulating wound bed, moderate to high exudate and having a recurring nature.[2] Despite venous valve incompetency and ensuing venous hypertension being the main driver for VU formation, fibrinogen leak from the capillaries has also been proposed as a contributing factor. The fibrinogen leak forms a pericapillary fibrin cuff that inhibits the diffusion of oxygen and nutrients, accumulation of white blood cells and redox dysregulation, which ultimately lead to hypoxia, tissue damage, and impaired wound healing.[3] The mainstay of the current treatment for VUs is compression bandaging and ablation of incompetent superficial veins because they are associated with an increase in the number of new blood vessels, which in turn are associated with healing wounds.[4,5] Nonetheless, for these treatments to be effective, a high level of patient compliance is needed, and a prolonged time period to see changes is often required, which negatively impacts the former.[6]

Because microangiopathic improvements are associated with healing ulcers, there is a pressing need for the search of novel treatment strategies to promote neovascularization of the wound. One such innovative treatment may be the topical use of insulin, as several studies have demonstrated that insulin is effective in promoting wound healing. The topical use of this drug is associated to increased rate of healing, increased capillary density, and increased granulation tissue.[7-11] However, no studies have been done to date on the effectivity of topical insulin application for VUs. A previous study demonstrated that the topical administration of neutral protamine Hagedon (NPH) insulin, an intermediate-acting insulin, in acute wounds of non-diabetic patients was associated with increased angiogenesis without any patient presenting hypoglycemia;[10] nonetheless, this remains as a potential risk. Longer-acting insulin formulations, such as insulin glargine, which has a half-life of approximately 12 h, an onset of action of approximately 2 h and a duration of action of approximately 24 h, are associated with fewer hypoglycemia events in diabetics while retaining a long-acting effect.[12-14] Therefore, the objective of this study was twofold. First, to determine whether the local administration of insulin glargine compared with the placebo in nondiabetic patients with VUs would lead to increased healing of the wound; and second, to determine the safety profile of insulin glargine in this population.

Materials and Methods

Patients

A prospective randomized controlled trial (RCT) was performed on adult patients with chronic VUs secondary to varicose veins and venous hypertension in their lower extremities referred to the vascular surgery outpatient clinic of Hospital Central "Dr. Ignacio Morones Prieto," in San Luis Potosi, Mexico. Inclusion criteria were the presence of full-thickness VUs $\geq$25 cm^2 with an evolution of at least 3 mo. All patients received compression bandaging since their first visit to the clinic and ablation of incompetent superficial veins at least 4 wk prior to being enrolled in the study. Exclusion criteria were the suspicion of active infection of the ulcer, a prior diagnosis of post-thrombotic venous syndrome, deep vein thrombosis, diabetes mellitus, Hb-A1c >6.5%, presence of any comorbidity that would affect wound healing such as malnutrition, cancer or collagen disease, active smoking, or peripheral arterial disease.

Approval for the study was granted from the Hospital's Research Ethics Board (registry 70-19). The study was performed in accordance with the Declaration of Helsinki. Written informed consent was provided by all participants prior to any study procedures. The study is registered at clinicaltrials.gov under entry number NCT04310280.

Study design

After consenting to participate in the study, the patient's VUs were divided into two equal parts and randomization using the randomizeR package[15] from the R statistical software was used to select on which side insulin glargine was going to be applied and which side would receive placebo (0.9% saline solution). This split-plot experimental design offers the advantage of making each patient its own control and thus, is well suited for small sample sizes and has previously been used to assess wound healing in acute wounds.[10,11] The dose and application of insulin or placebo was done following a previous study.[11] Briefly, 0.1 mL (10 UI) of insulin glargine was diluted in 0.9 mL of saline solution. Afterward, this preparation or the same volume of saline solution was injected into the wound bed at a depth of 3 to 4 mm on each wound half once a day for 7 d using a tuberculin syringe needle after administration of 2% lidocaine spray for pain control. Half of the dose was applied in the center of the hemi-wound and the rest in at least three radial points between the center and the wound edge. No additional doses were applied on the wound borders nor systemically. In all cases, we ensured that the point of administration of either the insulin or placebo were at least 1 cm away from the other wound half or from the wound border. The patients were blinded to which wound side was the active treatment applied. The treatment was applied in the outpatient clinic immediately after the patients received a luncheon. Capillary blood glucose was measured before and 3 h after the treatment was administered, and if hypoglycemia ($\leq$3.3 mmol/L) was ruled out, the patients were sent home until the next day. The patients were instructed on how to recognize signs and symptoms of hypoglycemia and to record them if present. VUs were treated with compression stockings, daily cleanse with sterile saline solution and alginate dressings. All patients were scheduled for wound grafting after completion of the treatment protocol.

Infrared thermography

The temperature of a wound, particularly the difference between the temperature of the wound and the surrounding

healthy skin, also known as the temperature asymmetry, has been correlated with blood flow and blood supply to the wound,[16] and has previously been used as an indirect marker of neovascularization.[11,17] To assess the temperature asymmetry, infrared thermography imaging was done by a blinded to treatment observer on day 0 and on day 7 using an FLIR One Pro mobile camera (FLIR Systems, Wilsonville, OR) attached to an iPhone. For image acquisition, the camera was left on for 3 min before acquiring the images to allow stabilization of the sensor. Afterward, auto-calibration of the instrument was done following the manufacturer's instructions. Before imaging, the dressing was removed, and the wound was cleaned with a 5% chlorhexidine solution, rinsed with 0.9% saline, dried with sterile gauze and the wound was left undisturbed for 3 min to reach room temperature. All the temperature measurements were taken following the Thermographic Imaging in Sports and Exercise Medicine (TISEM) check list,[18] at a distance of 0.5 m and at an angle of 90° relative to the wound, in a closed room under controlled conditions of light and external radiation exposure, controlled room temperature (23°C) and atmosphere humidity of 40%.

Thermographic analysis of the images was performed using the FLIR Tools Quick-Report v.1.2 software (FLIR Systems, version 5.70, 2016) by a researcher blinded to the treatment. The skin emissivity was set at 0.98 for all acquired images. The investigator delineated a region of interest (ROI) corresponding to half of the ulcer and the software was used to obtain the average temperature of the ROI. A 25 cm^2 region was delineated on healthy skin adjacent to the wound and the same measurement was performed. The healthy skin temperature was then compared with both wound halves and the thermal asymmetry between them obtained.

Wound measurement and biopsy

Measurement of the hemi-wound area was done using a transparent film overlying the VU by an observer blinded to the treatment at days 0 and 7. Tissue biopsies at the center of each wound half were also obtained on day 0 and on day 7 and 4 μm tissue sections were prepared. Hematoxylin and eosin staining were used to assess blood vessels, and the percentage of collagen content in the samples was determined through Masson's trichrome stain. The number of blood vessels and the percentage of collagen content area was counted by a trained observer blinded to the treatment received using the ImageJ v1.44 software (National Institutes of Health, Bethesda, MD) in three randomly selected fields at a 10× magnification and the results for each individual patient were averaged. Intra- and interrater agreement for the blood vessel quantification and collagen area estimation were 0.98 and 0.99, respectively.

Statistical analysis

The main outcome of interest for this study was the change in the number of blood vessels in tissue biopsies. Secondary outcomes included the change in wound size, temperature of the wound, and content of collagenous tissue. Data are presented as mean and standard deviation or proportions for continuous or categorical data, respectively. Statistical analysis was performed using the statistical package R v.3.4.1 and RStudio at the 95% confidence interval. After assessment and confirmation of normal data distribution through Shapiro–Wilk tests, mixed-effects models were used to assess the differences in wound size, temperature asymmetry, number of blood vessels, and percentage of fibrosis area between wound sides according to treatment. This statistical method considers individual patient responses to treatment and is particularly well suited to analyze differences over time, especially when a natural change of the outcome if interest is expected and therefore, the interaction of time and treatment needs to be assessed.[19] Moreover, by clustering the individual patients' responses, any potential risk of contamination between treatments in the hemi-wounds is also controlled by this statistical method. For evaluation of the correlation between temperature asymmetry and the number of blood vessels, a linear regression analysis was done. *P*-values <0.05 were considered statistically significant for all analyses.

The sample size was calculated using data for blood vessel quantity from a previous study[10] using the program GLIMMPSE (University of Colorado Denver, URL: http://glimmpse.samplesizeshop.org/).[20] For a statistical power of 80%, a type I error rate of 0.05, measurement of the primary outcome variable (number of blood vessels) for two times, and assuming linear exponent first-order autoregressive (LEAR) correlations at a decay rate of 0.5, we calculated that to detect a treatment-by-time interaction of at least 30% in the number of blood vessels after 7 d of treatment, a minimum sample size of 18 patients would be needed.

Results

A total of 36 patients, eight men and 28 women, were enrolled into the study. No patients were lost to follow-up. The patient's clinical characteristics are presented in Table. The study's CONSORT flowchart is presented in Figure 1.

The patient's wounds were divided in two, and each half received either daily insulin glargine or saline solution injections, thereby making each patient its own control (Fig. 2A and B). Infrared thermograms obtained at baseline (day 0, Fig. 2A1) showed a practically identical temperature difference between the wound area and the surrounding healthy tissue (temperature asymmetry) between hemi-wounds (2.79 ± 1.3 *versus* 2.81 ± 1.3°C, $P = 0.91$). After 7 d, the hemi-wounds treated with insulin glargine showed

Table – Patient characteristics.

Variable	Value
Age (y)	60.2 ± 11.7
Gender	Female = 18 (69%) Male = 8 (31%)
Wound size (cm^2)	62.6 ± 35.9
Wound evolution (mo)	18 (24)*

* Median (IQR).

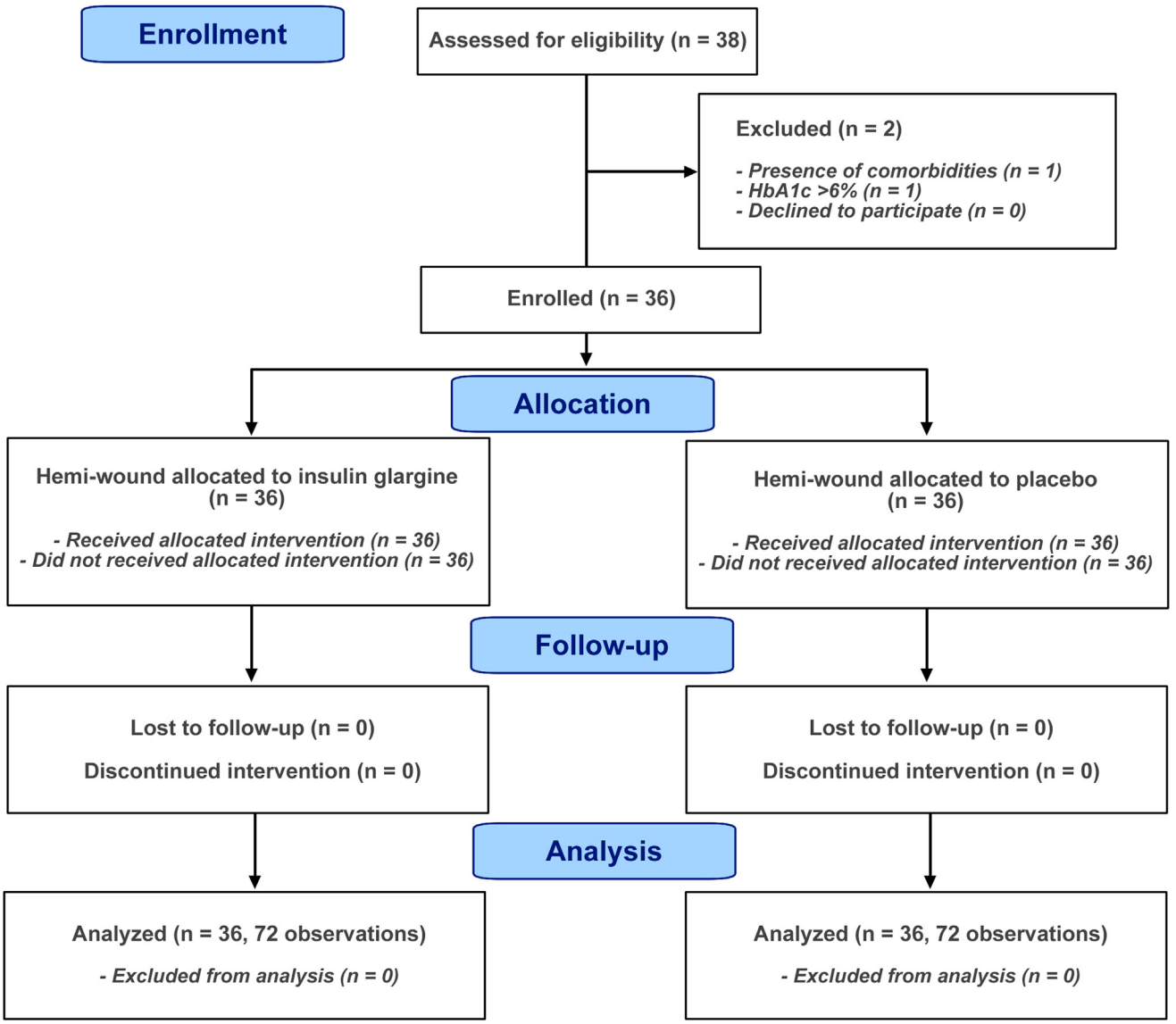

Fig. 1 – Study flowchart.

significantly lower temperature asymmetry than the placebo-treated hemi-wounds (1.41 ± 0.77 *versus* 2.38 ± 1.03°C, $P < 0.001$) (Fig. 2B1). Significant interaction between the treatment and time was observed in the mixed-effects model ($P = 0.018$), with lower thermal asymmetry in the insulin glargine treated hemi-wounds (Fig. 2C). Likewise, the hemi-wounds treated with insulin showed a reduction of size from 31.3 ± 18.2 cm^2 to 26.15 ± 15.0 cm^2, compared with 31.1 ± 18.2 cm^2 to 28.21 ± 16.4 cm^2 in the placebo side ($P < 0.001$). This represents a reduction of 16.3 ± 4.5% *versus* 10.0 ± 3.5% ($P < 0.001$) in the size of insulin treated hemi-wounds compared with saline.

The number of blood vessels in the wound biopsies increased from 32.7 ± 18.6 to 77.1 ± 21.6 ($P < 0.001$) in the insulin glargine group and from 30.7 ± 19.1 to 51.8 ± 18.7 ($P < 0.001$) in the placebo group. Significant interaction between treatment and time was observed ($P = 0.013$), with higher blood vessel number in the insulin glargine treated hemi-wounds (Fig. 3A). Likewise, the percentage of collagen area in the biopsies was found to be significantly higher in the insulin glargine-treated hemi-wounds ($P = 0.004$ for the treatment by time interaction). Collagen content area increased from 15.7 ± 20.5% to 66.5 ± 15.1% in the insulin group ($P < 0.001$) compared with 17.0 ± 16.4% to 44.1 ± 16.3% in the placebo group ($P = 0.011$) (Fig. 3B). Significant negative association was found between the number of blood vessels in the tissue biopsies and the thermal asymmetry values ($\beta = -0.025$, $r^2 = 0.662$, $P < 0.001$) (Fig. 4).

Over the course of the study, no patient presented with signs or symptoms suggestive of hypoglycemia, adverse events, or with capillary blood glucose levels ≤3.3 mmol/L.

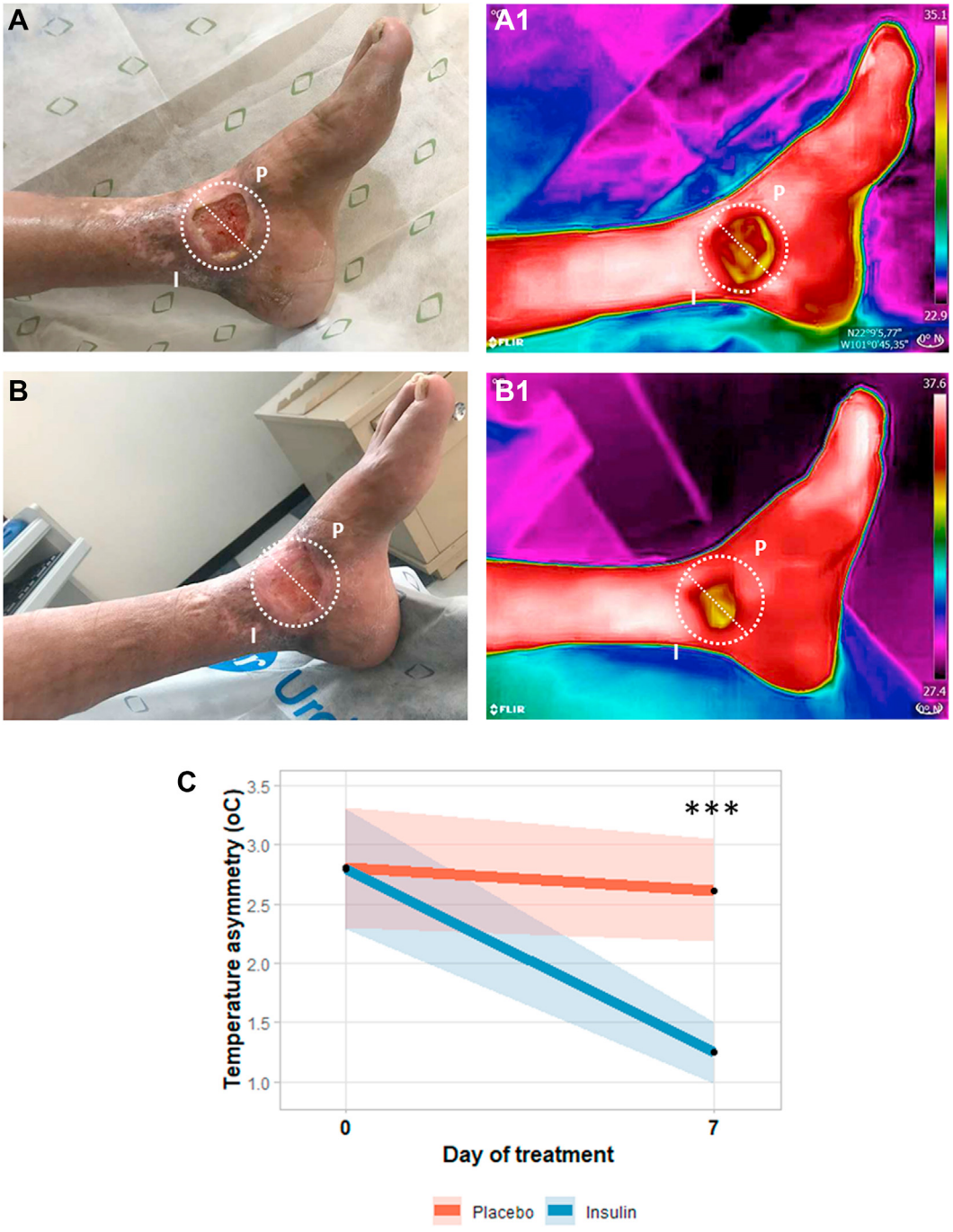

Fig. 2 – Wound healing and temperature asymmetry. The patient's wounds (circles in A-B) were divided into halves (dotted lines in A-B) and each hemi-wound was randomly assigned to receive 0.1 mL daily insulin glargine injections (I) or the same volume of normal saline (placebo, P). Infrared thermograms (A1, B1) were used to assess the thermal asymmetry between the hemi-wound and surrounding healthy tissue. After 7 d of treatment, insulin-treated hemi-wounds showed significantly lower thermal asymmetry (C), which suggests increased neo-vascularization of the wound. Shaded areas in C represent 95% CI. ***$P < 0.001$.

Glucose measurements before insulin application (5.5 ± 0.6 mmol/L) were not significantly different to those obtained 3 h later (5.4 ± 0.7 mmol/L, $P = 0.86$). No other adverse events, including pain in the site of administration, allergic reactions, or infection were recorded. Regardless of the trial result, after competition of the 7 d of treatment, all patients received an autologous skin graft covering both hemi-wounds.

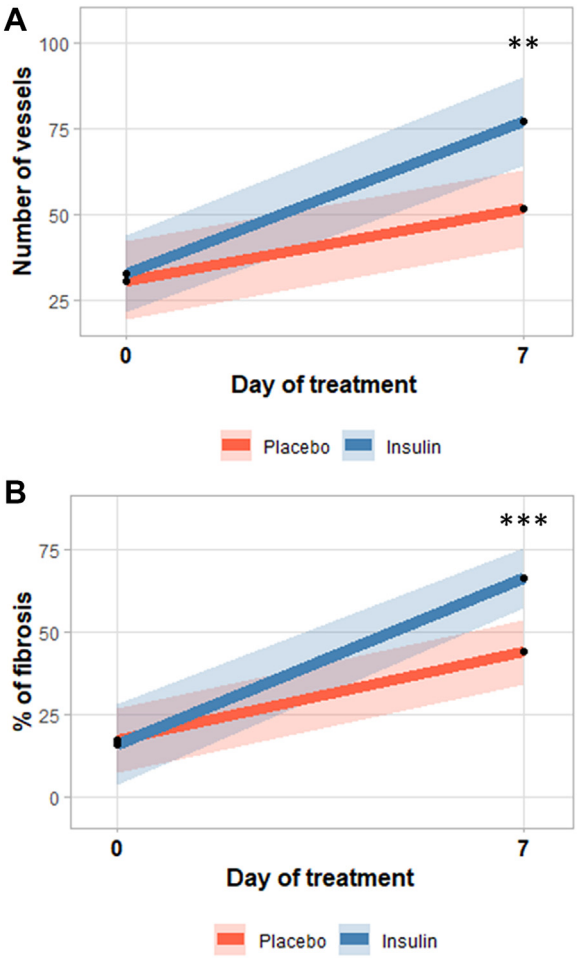

Fig. 3 – Wound biopsy results. The number of blood vessels (A) and the percentage of collagen content (fibrosis) area (B) were found to be significantly higher in the insulin-treated hemi-wounds after 7 d of treatment. A significant interaction of treatment by time ($P < 0.01$ in both cases) was detected. Shaded areas represent 95% CI. **$P < 0.01$, ***$P < 0.001$.

Discussion

Wound healing is a complex and dynamic biological process that involves the subsequentially but overlapping phases of hemostasis, inflammation, proliferation, and remodeling of tissue.[21] This process involves the restoration of lost or damaged skin layers through the effect of cytokines and growth factors on keratinocytes, fibroblasts, immune cells, and endothelial cells to promote extracellular matrix (ECM) deposition and remodeling.[22-24] In the case of chronic VUs, the deposition and maturation of granulation tissue is impaired due to the reduced activation and migration of fibroblasts and low levels of ECM production, which ultimately lead to diminished wound contraction.[25,26] This condition is further compromised by ensuing hypoxia due to venous stasis, fibrin cuffs, microangiopathy, and inflammatory cell entrapment and dysregulation, which ultimately lead to tissue necrosis, full-thickness skin loss, and recurring wounds.[3]

Our results demonstrate that in nondiabetic patients with chronic VUs, the topical administration of insulin glargine is safe and effective to promote wound healing, tissue neovascularization and ECM deposition prior to skin grafting. The use of topical insulin glargine compared with placebo led to an increase of 25.3 vessels/cm^2 in the hemi-wound, which was our primary outcome, an increase in ECM deposition as measured by Masson's trichrome staining of 22.4%, a 4.95 cm^2 or 6.3% reduction in the hemi-wound size, and a 0.97°C increase of the hemi-wound's thermal load. Importantly, these results were achieved with a mean change in blood glucose in non-diabetic subjects of 0.1 mmol/L, highlighting the safety profile of the treatment. The reason for selecting the number of blood vessels as our primary endpoint was that an increase in neo-angiogenesis marks the shift from a wound's inflammatory phase into the proliferative one[21,27] and that this is in turn followed by deposition of ECM and reduction in wound size. Furthermore, as supported by our findings, we also hypothesized that the increase in blood flow into the wound could be measured by the wound's thermal load.

Insulin is a pleiotropic anabolic agent for wound healing. In addition to promoting the expression of VEGF in keratinocytes through the expression of the HIF-1α pathway,[28,29] insulin promotes fibroblast and endothelial cell survival through its effects on the AKT and ERK pathways.[30] Furthermore, insulin has also been tied to immune cell regulatory activity through modifying the content of CD68 positive cells and the expression of TNF- α and IL-6.[31] Taken together these findings with our own results, we believe insulin can be used as a potent agent to promote wound healing in chronic VUs, as it modifies most of the dysregulated pathways of the wounds. In accordance with this line of thought, here we demonstrate that the topical administration of insulin glargine leads to increased angiogenesis and collagenous tissue in VUs wound bed. This is in stark contrast to our previous finding of no increase in the fibrous tissue deposition of acute wounds in non-diabetic patients treated with NPH insulin, despite similar results on neo-vascularization.[10] In contrast, diabetic foot ulcer wounds treated with insulin also show significant increase in neovascularization and ECM deposition.[11] Because venous and diabetic foot ulcers are trapped at the inflammatory and proliferative phases of wound healing,[3,32] insulin therapy most likely promotes a major positive effect on wound regulation and shifts the balance into a pro-regenerative state.

Despite the promissory results of our trial, it has several limitations. The most important one is its small sample size, which warrants a larger trial to confirm the results. Another limitation is that because it was a split-plot design, measurement of the wound size to assess the effect of insulin on this outcome was not feasible. However, based on our observations (Fig. 1B), it is very likely that it will lead to decrease in the size of the wound. Finally, there are still several major hurdles for the translation of our findings into major widespread clinical practice. First, the optimal dose of insulin for wound healing has not yet been established. Although there seems to be a dose-dependent effect of insulin application and acute wound healing where lower doses of insulin promote better healing than higher ones,[33] a consensus has not been reached. Second, there is heterogeneity in the way insulin is applied to wound beds.

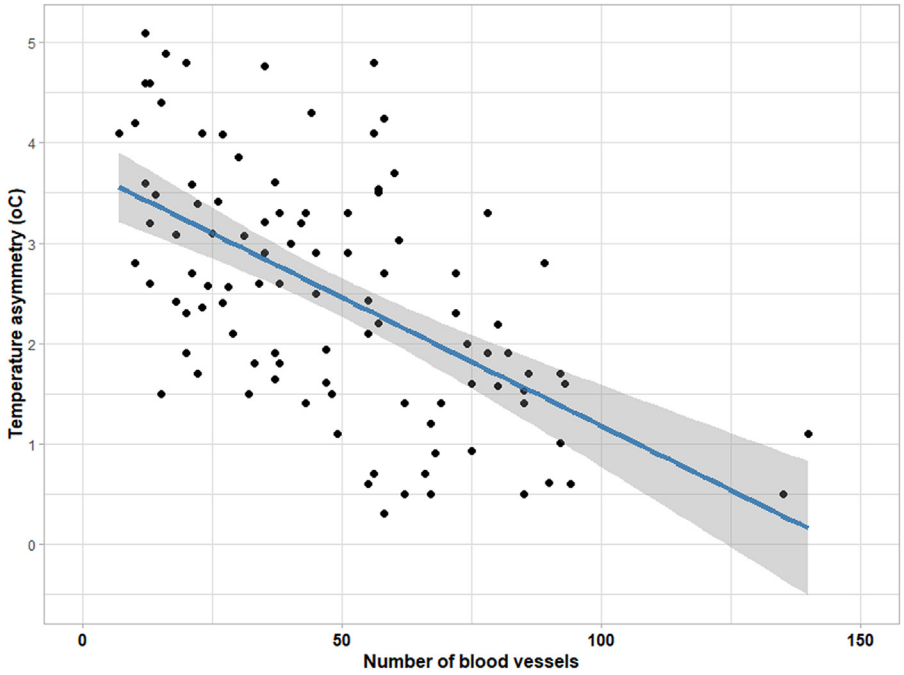

Fig. 4 – Correlation between the number of blood vessels and temperature asymmetry. The number of blood vessels and thermal asymmetry showed a significant negative correlation. For each increase in 1 blood vessel in the tissue biopsy, there is a decrease of 0.02 (95% CI 0.01 to 0.03, $P < 0.001$) °C in the thermal asymmetry. This result highlights the intimate relationship between the wound's vascularity and its relative temperature. Shaded area represents 95% CI.

Although most studies have used a similar approach to ours, a recent paper demonstrated that the topical use of insulin in wound dressings also lead to enhanced wound healing of diabetic foot ulcers.[34] If this finding can be replicated in other settings and other wound types, it would pave the way for a less invasive way to administrate insulin to patients. Finally, it should be noted that the use of a 7-d course of topical insulin did not lead to complete wound healing as this time point was too short to achieve this. The optimal duration of the topical insulin treatment has not been found. Although most papers suggest a course of 14 d of treatment, here we demonstrate that longer acting insulins may reduce the treatment time by half. However, replication of this finding in other wounds needs to be carried before a definitive recommendation can be done.

In addition to the aforementioned results, our study shows a high degree of correlation ($r^2 = 0.662$) between temperature asymmetry wound measurements and the number of blood vessels in the wound bed. Infrared thermography is a noncontact, noninvasive imaging modality that provides significant insight into a wound's depth and healing potential,[35] thereby potentially aiding in the selection of the optimal treatment strategy for wound care.[17] Our results suggest that in the absence of tissue biopsies, thermograms can be used to monitor and track wound healing as an adequate indirect measurement to blood vessel density. However, caution must be taken for interpreting the images, as numerous external factors, including the quality of the image acquisition protocol, patient activity, and the presence of inflammation or infection might alter the results.

In conclusion, here we demonstrate that in nondiabetic patients with chronic VUs, the topical administration of insulin glargine leads to increased tissue neo-vascularization and collagen content deposition after 7 d of treatment, compared with placebo. This strategy can be deployed for patients with VUs as an attempt to modify the wound microenvironment and promote healing or prior to skin grafting. Furthermore, thermographic measurements are well correlated with wound vascularity, opening the possibility to indirectly monitor the blood vessel supply to healing tissue in a noninvasive manner.

Author Contributions

This work was conducted in San Luis Potosi, Mexico. MMJ, FA, and SKM conceptualized and designed the study. MMJ performed all procedures. MGG and GDR performed data collection. SKM, LCOD, and FGG performed data analysis. FVC performed the follow-up of patients and paid for the medication. JRGL performed the statistical analysis plan, statistical analysis, and supervision of the study. All authors contributed to manuscript writing.

Disclosure

MMJ declares that he has no conflict of interest. FA declares that he has no conflict of interest. SKM declares that she has no conflict of interest. LCOD declares that he has no conflict of

interest. GDR declares that he has no conflict of interest. MGG declares that she has no conflict of interest. FGG declares that he has no conflict of interest. FVC declares that he has no conflict of interest. JRGL declares that he has no conflict of interest.

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RCT Registration

The study was registered in clinicaltrials.gov with the id NCT04310280.

Registration

This study is registered at clinicaltrials.gov, registration number: NCT04310280 (March 17, 2020).

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee (Registry 70-19) and with the 1964 Helsinki declaration and its later amendments.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

Ethics Disclosure Statement

Approval for the study was granted from the Hospital's Research Ethics Board (registry 7013). The study was performed in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines of the International Conference on Harmonization. All patients provided written informed consent.

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