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RESEARCH ARTICLE

Effect of Topical Insulin and Ozonized Cream for the Treatment of Full-thickness Dermal Burn Injuries: A Clinical and Histopathological Study in Diabetic Rats

Gül Çıray Akbaş Güngör[1], Çağrı Gültekin[1], Aysel Kükner[2], İlker Etikan[3], Meliha Temizel[4] and Fatma Eser Özgencil[1]

[1]Department of Surgery, Faculty of Veterinary Medicine, Near East University, Nicosia/TRNC; [2]Department of Histology, Faculty of Medicine, Near East University, Nicosia/TRNC; [3]Department of Biostatistics, Faculty of Medicine, Near East University, Nicosia/TRNC; [4]Experimental Animals and Research Center, Near East University, Nicosia/TRNC
*Corresponding author: gul.akbas.91@gmail.com; gulciray.akbas@neu.edu.tr

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ABSTRACT

Diabetic wound healing is a major health problem encountered in both human and veterinary medicine. In this study, 30 Wistar albino rats were experimentally induced diabetes having bilateral full-thickness tissue loss and second-degree burns onto the skin and divided into three groups. The effects of a 14-day-wound treatment with NPH insulin cream (G1), ozonized cream (G2), and ozonized cream + NPH insulin cream (G3) was investigated. Photographic evidence of wound healing was measured on 3, 7 and 14 days. On the fourteenth day, the rats were euthanized, and scar tissue samples were collected for histopathological examination. It was observed that topical NPH insulin alone acted as a strong immunomodulator in preventing long-term inflammation that could lead to delayed wound healing. In G2 and G3 groups, the epithelization score was increased, additionally in G3 the fibroblast activation score was increased as well. In conclusion, a combination of equal amounts of NPH insulin cream and ozonized cream contributed to wound healing by decreasing inflammation score and increasing epithelization and fibroblast activation.

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INTRODUCTION

Diabetic wounds causing microangiopathy and neuropathy are chronic and persistent wound types, in which one or more steps of the normal healing process are disrupted. Healing of such wounds is interrupted due to impaired local circulation and infection, where healing becomes hampered or impossible. This is because insulin acts as a receptor for the entrance of glucose and amino acids into the cell, it cannot access the size of the wound and thus the cell becomes insufficient in energy. To maintain the balance in the steps of healing, developing topical agents that positively affect the wound is one of the essential duties (Mervis, 2018). Insulin is a peptide hormone and a growth factor that can repair the damaged skin, and the insulin receptor has been demonstrated to be expressed extensively from the healthy skin around the wound, and the potential role of the insulin signaling pathway in wound healing has been shown by Abdelkader *et al.* (2016). Therefore, addition of insulin to both diabetic and non-diabetic wound dressings has become an accepted method to facilitate wound healing (Zhao *et al.*, 2017; Özaydın *et al*., 2018). Furthermore, it has been reported that topical application of insulin in the form of a local injection, spray, cream, and controlled release of bioactive insulin, has reduced healing time, accelerated epithelization and neovascularization through inflammation, and supports wound remodeling (Jiao and Jixiong, 2020).

Ozone (O_3) has been frequently used in diabetic wound healing in humans, but it has limited use in the veterinary field. It is a strong antioxidant and triatomic form of oxygen which supports antioxidant enzymes. Exposure of the dermal ischemic area to O_3 has been reported to reduce wound bacterial infections through its bactericidal, antiviral, and antifungal effects in trophic, ischemic, and diabetic wounds (Silva *et al.*, 2021). Ozon increases the local peripheral vasodilatation and subsequent oxygenation, modulates the inflammatory phase, stimulates angiogenesis, prevents pain, and releases endothelial cellular factors when it comes in contact with a biological fluid. Since it accelerates wound healing and

has antimicrobial, immunomodulator, antioxidant and oxygenation properties, it appears as a particular prominent treatment method in diabetic wound healing (Anzolin *et al.,* 2020). Ozon has been reported to have beneficial effects on the healing of connective and muscle tissues together with its regenerative processes (Üstebay *et al.,* 2017). Similar to insulin, there are different commercial pharmaceutical forms of O_3 in the market such as ozone water, ozone oil, O_2-O_3 gas mixture, cream, injectable forms, paillette, foam, bolus forms for local use in wound healing (Đuričić *et al*., 2012).

The aim of this study was to investigate and compare the effects of Neutral Protamine Hagedorn (NPH) insulin cream prepared in the laboratory, a commercial ozonized cream and the combination of NPH insulin cream and ozonized cream on early-term wound healing in rats with experimentally induced diabetes.

MATERIALS AND METHODS

Ethical statement and animals: This study was approved by the local ethics committee of Laboratory Animals Research Center of XXXX (Approval no: 2020/106). A total of 30 female, 6–8-week-old Wistar albino rats weighing 250-300g were used in the study. The rats were classified into three groups: NHP insulin cream group (n=10, G1), ozonized cream group (n=10, G2) and insulin+ozonized cream group (n=10, G3).

Study design: Diabetes was induced through an intraperitoneal injection of a single dose @50mg/kg streptozotocin (STZ) solved in pH=6, 0.07 *M* citrate buffer (Sigma, St Louis, MO, ABD) (Ataroalsadat *et al.*, 2016). Rats were kept on fasting for 12h prior and 4h after STZ administration. The blood glucose levels of all rats were measured from the blood samples obtained from tail veins on 1st, 2nd and 14th day using a digital glucose measuring device (HMD GlucoLeader Enhance v2, HMD BioMedical Inc.). The water was provided in the form of 15% dextrose for the first two days. The rats were accepted as diabetic when the blood glucose level reached constant levels over 200mg/dL following STZ injection.

The rats were anesthetized by using Xylazine HCl @10mg/kg (2% VETAXYL® 50ml vial, VET-AGRO) and 100mg/kg Ketamine HCl (Ketamine 10%, 25ml flacon, Dutch Farm®) intraperitoneal. A full-thickness cutaneous incision > 1cm diameter, comprising the circular panniculus carnosus muscle was made on both sides of the dorsal area. The wound bed was even complicated by forming a second degree burn on the wound using an iron rod. The iron rod was heated for 30 seconds until it turned violet red or violet blue (300-400 °C) (Dorsett-Martin, 2004).

Insulin cream was prepared by homogeneously mixing 5 ml insulin (Humulin®N NPH 100 IU/ml, Lilly) in NPH form with 95 g of solid medical Vaseline (Özaydin *et al.,* 2018). As ozonized cream, Vaseline, ozonized olive oil and an O_3 preparation Aktifoks (Aktifoks®, Işık Kozmetik) (Varol *et al.,* 2017). Medical Vaseline was administered to the left dorsal area (LS) of all rats for 14 days, and the right dorsal area (RS) underwent application of thin layers of NPH insulin cream in group 1, ozonized cream in group 2, and NPH insulin cream + ozonized cream in group 3. The creams were applied in a thin layer and gently by massaging until they were absorbed by the wound.

Photographs were taken on the day of wound formation, and additionally on days 3, 7 and 14 by the same observer. These images then transferred to the Digimizer® (Version 5.6.0, 2005-2021 MedCalc Software Ltd) software program to measure. The photographs were obtained using a stable tripod attachment from a distance of 20cm. Calibration of the measurement was carried out for each image using a ruler. The sizes of the wounds were then measured and recorded as mm^2. Wound area reduction rate (%) was calculated by using Anuk *et al.* (2016) equation.

Histopathological examinations: At the end of the study period rats were euthanized with an overdose of Xylazine and Ketamine anesthesia. The wound area and surrounding full-thickness skin were dissected on both sides and were then fixed with 10% formaldehyde. After the fixation period, the tissues were taken to the automatized follow-up instrument and prepared for paraffin blocks sections (3-5µm thickness). The sections were stained using hematoxylin eosin. Microscopic examinations and scorings were performed analyzing the levels of inflammation, angiogenesis, fibroblast activation and epithelial regeneration. Scoring was performed according to the scoring of Rahman *et al.* (2019). According to this, inflammation cell score defined 0: 1-5 cells/area, 1: 6-8 cells/area, 2: 9-15 cells/area, 3: > 15 cells/area. Angiogenesis, congestion, and hemorrhage score defined 0: Angiogenesis including congestion is mild, 1: 2-4 vessels/area angiogenesis including congestion and hemorrhage, 2: 5-6 vessels/area and mild congestion, 3: 7-8 vessels/area and vessels extending towards epithelium. Fibroblastic activation score defined 0: Collagen fibers are scattered and segmented, 1: Mild fibroblast increase and thin collagen fibers, 2: Moderate fibroblast increase and incomplete collagen organization, 3: Significant-thick fibroblast layer and ≥80% organized collagen fibers. Epithelization score defined 0: Not present, 1: ≥50% epithelization, 2: ≥60% epithelization and moderate healing, 3: ≥80% epithelization and complete healing.

Statistical analysis: The Wilcoxon Sign test and the Friedman test were used for comparisons between RS and LS and for periodic comparisons. The inter-group comparisons were made using the Kruskal-Wallis Variance Analysis test. Groups with significant differences were compared using the Conover post-hoc test. Statistical significance was accepted as $p \leq 0.05$. SPSS 23 (IBM®, SPSS®, demo version:2021) statistical program package was used for these calculations.

RESULTS

In-group statistical analysis of wound size comparison: A significant difference in the LS was observed between day 0 and day 14 ($P<0.001$) and day 3 and day 14 ($P<0.001$) in group G1. A significant difference in the RS was observed between day 0 and day 7 ($p=0.006$), day 0

and day 14 (P<0.001) and day 3 and day 14 (p=0.006) in G1. A significant difference in the LS was observed between day 0 and day 14 (p=0.006), and day 3 and day 14 (P<0.001) in group G2. A significant difference in the RS was observed between day 0 and day 7 (p=0.003), day 0 and day14 (P<0.001) and day 3 and day 14 (p=0.01) in G2. A significant difference in the LS was observed between day 0 and day 14 (p=0.001) and day 3 and day 14 (P<0.005) in G3. A significant difference in the RS was observed between day 0 and day 7 (p=0.05) and day 0 and day 14 (P<0.001) and day 3 and day 14 (p=0.005) in G3 (Fig. 1).

Intergroup statistical analysis of wound size comparison: No significant difference was observed in the wound size measurements of the LS between the groups according to Kruskal-Wallis test on the 3rd day (p=0.437), whereas the difference in the RS was significant between the G1 and the G3 (p=0.020). On day 7, no significant difference was observed in wound size measurements on the LS between the groups (p=0.067), whereas the difference was significant between the G1 and the G3 groups on the RS (p=0.024). On day 14, no significant difference was observed in the wound size measurements of both LS (p=0.159) and the RS (p=0.989) (Table 1).

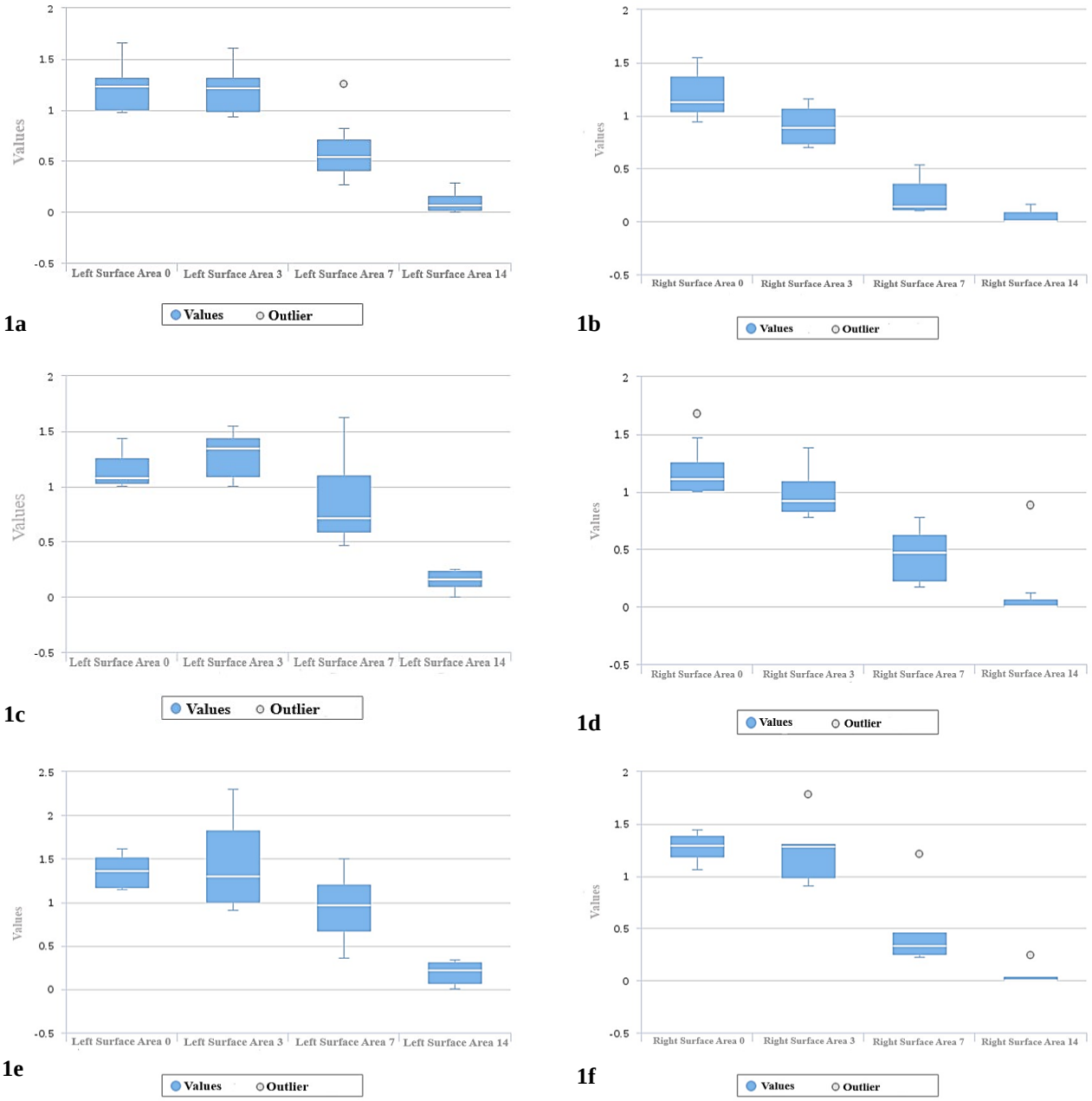

Fig. 1: In-group statistical comparison of wound areas. The Nemenyi test was used in all statistically analysed area calculation tests. A significant difference was observed between LS in G1 at the days 0-14 (P<0.001) and 3-14 (P<0.001) in terms of wound area closure (1a). A significant difference was observed between RS at the days 0-14 (P<0.001), at 0-7 (p=0.006), and 3-14 (p=0.006) in terms of wound area closure (1b). A significant difference was observed between LS in G2 at the days 0-14 (p=0.006) and 3-14 (P<0.001) in terms of wound area closure (1c). A significant difference was observed between RS at the days 0-14 (P<0.001), at 0-7 (p=0.003), and 3-14 (p=0.010) in terms of wound area closure (1d). A significant difference was observed between LS in G3 at the days 0-14 (p=0.001) and 3-14 (p=0.005) in terms of wound area closure (1e). A significant difference was observed between RS at the days 0-14 (P<0.001) and 3-14 (p=0.005) in terms of wound area closure (1f).

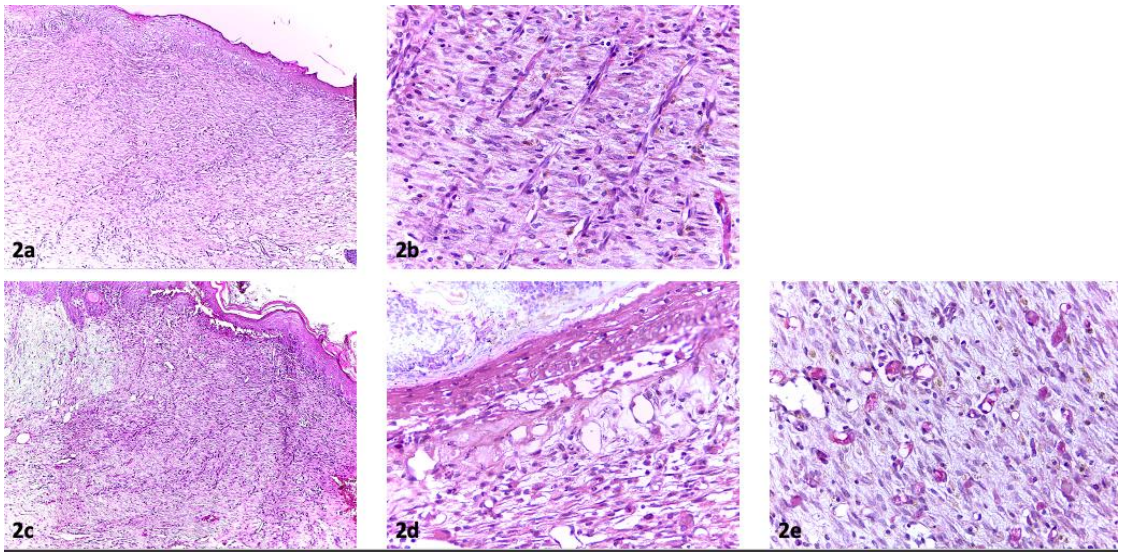

Fig. 2: Significant epithelial regeneration and subepithelial fibroblastic tissue formation (2a x10) where an increased number of blood vessels are extended towards the epithelium and the number of inflammatory cells is low (2bx40) in G1 RS. Detachments are seen in the epidermis-dermis junction in areas with no treatment, hemorrhage (2cx10), increased inflammatory cells in the subepithelial sizes with edema (2dx40) and moderate congestion (2ex40) in G1 LS. Hematoxylin & Eosin.

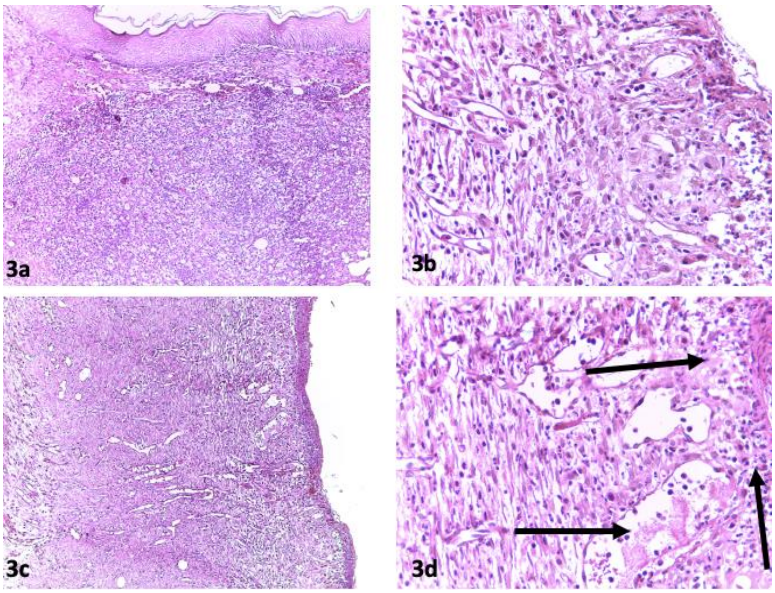

Fig. 3: Regenerated thick epidermal layer, many inflammatory cells, and very few collagen fibers (3ax10), many blood vessels related to angiogenesis (3bx40) in G2 RS. In the LS epithelial regeneration is not complete compared to the RS and many capillaries (3cx10), disorganized collagen fibers, neutrophils in wide capillaries and connective tissue (arrows).

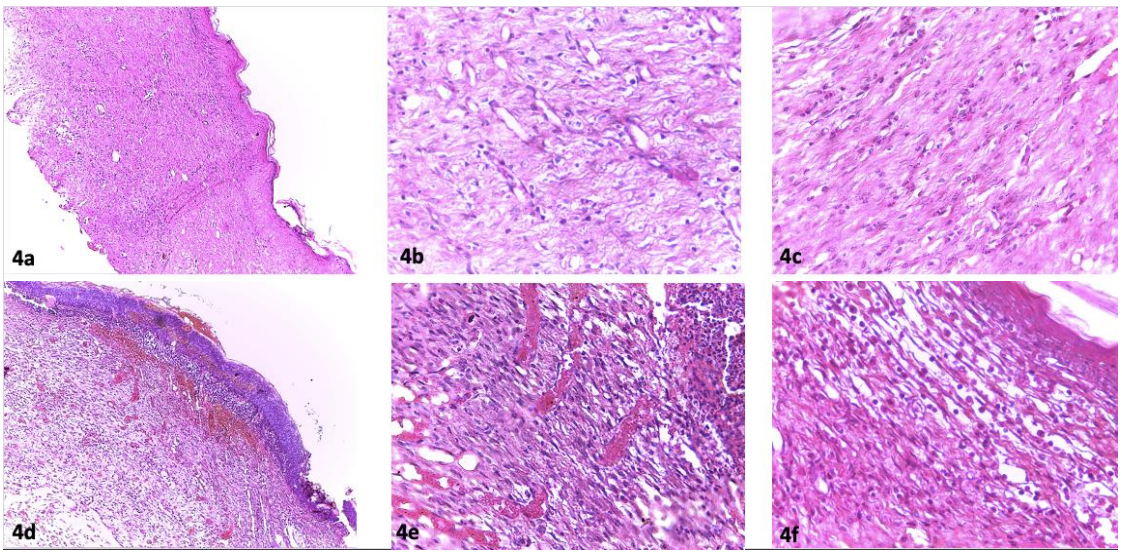

Fig. 4: Epithelium is substantially regenerated (4ax10), increased number of blood vessels extending towards the epithelium (4bx40), significant subepithelial edema, decreased inflammatory cells were seen in the healing area (4cx40) in G3 RS. Delayed wound healing, clot formation (4dx10), angiogenesis with congestion (4ex40), edema and inflammation (4f x40) in G3 LS. Hematoxylin & Eosin.

Table 1: Median values that belong to area measurements in the RS and LS region of all groups in four different time periods.

Groups	LS				RS			
	0.day	3.day	7.day	14.day	0.day	3.day	7.day	14.day
G1	1.228	1.211	0.534	0.065	1.127	0.88	0.144	0
G2	1.0715	1.340	0.708	0.160	1.1125	0.9185	0.473	0
G3	1.355	1.300	0.965	0.219	1.289	1.283	0.331	0

G1: NPH insulin cream group; G2: ozonized cream group; G3: NPH insulin + ozonized cream group; LS: Left side; RS: Right side.

Histopathological findings

In G1 RS: Significant epithelial regeneration and significant subepithelial fibroblastic tissue formation (Fig. 2a), increased number of blood vessels extending towards the epithelium and inflammatory cells low in number (Fig. 2b).

In G1 LS: Detachments in the epidermis-dermis border, hemorrhage (Fig. 2c), increased number of inflammatory cells in the subepithelial regions with edema (Fig. 2d), and mild congestion (Fig. 2e). No hair follicle or fat glands.

In G2 RS: Regenerated thick epidermal layer, edema in the dermis, many inflammatory cells, several collagen fibers (Fig. 3a), many blood vessels related to angiogenesis (Fig. 3b).

In G2 LS: Incomplete epithelial regeneration, many capillaries (Fig. 3c), disorganized collagen fibers, increased neutrophils within wide capillaries and connective tissues (arrows) (Fig. 3d).

In G3 RS: Substantially regenerated epithelium (Fig. 4a), increased number of blood vessels with angiogenesis extending towards the epithelium (Fig. 4b), increased inflammatory cells and significant edema in the subepithelial region (Fig. 4c).

In G3 LS: Delayed wound healing, clot formation (Fig. 4d), angiogenesis with congestion (Fig. 4e), edema and inflammation (Fig. 4f).

Comparison of histopathological parameters between the LS and RS: A significant difference was observed in the inflammation scores between the RS and LS in G1 (p=0.026). No significant difference was observed between both sides in the inflammation scores in G2 (p=0.102), whereas a significant difference was observed in the epithelization scores (p=0.026). In G3, a significant difference was observed between the sides regarding inflammation scores (p=0.017), epithelization scores (p=0.038) and fibroblast activation scores (p=0.046).

Comparative findings of RS of all groups: A significant difference was observed between the groups regarding inflammation scores of the RS (p=0.045). The mean inflammation score in group G2 was significantly higher compared to that of group G1; however, the differences in the remaining parameters were not significant.

DISCUSSION

Persistent chronic inflammation causes obstinacy in the wound, excessive scarring, and delayed healing which leads to pathogenic characteristic of diabetic wound healing (Mervis, 2018). Topical use of insulin reduced the duration of healing, accelerate epithelization and neovascularization through altering the levels of inflammation, supporting remodeling, and increased keratinocyte migration, fibroblastic activity, monocyte/ macrophage chemotaxis and collagen accumulation (Zhao *et al.,* 2017; Liu *et al.,* 2018; Wang *et al.,* 2019; Jiao and Jixiong, 2020). NHP insulin pomade applications have a positive effect on diabetic wounds by providing a balance in the steps of inflammation (Özaydin *et al.,* 2018; Yu *et al.,* 2019). Recently, topical ozone application has been indicated as a treatment method in diabetic healing through increasing the local peripheral vasodilatation and oxygenation, modulating the inflammatory phase, stimulating angiogenesis, preventing pain and as a bioregulator that releases endothelial cellular factors when in contact with biological fluids (Anzolin *et al.,* 2020). This study investigated and compared the effects of NPH insulin cream, a low-cost ozonized cream, and the combination of NPH insulin cream and ozonized cream on short-term wound healing in rats with experimentally induced diabetes, tissue loss and secondary-degree burns.

Özaydın *et al.* (2018) observed that a significant reduction in wound size and a beneficial effect on wound depth due to its collagen ingredients with the NPH insulin cream application on day 3, 7 and 14 in rats with experimentally induced diabetes, and also stated that angiogenesis was quicker. Similarly in our study, the reduction in wound size was observed in the LS-G1 on day 3 and 14 significant (respectively, p=0.006 and P<0.001).

Furthermore, the reduction was significant on days 0-7 in the G1 (p=0.006), which led us believe that NPH insulin cream contributed to wound healing at an important extent starting with day 7.

Topical use of ozonized oil has been carried out widely in cutaneous wounds where conventional treatment methods were unsatisfactory, such as cases with persistent wounds, ulcers, or cutaneous losses. Whereas it had no effect on wound contraction; it led to thrombus formation in the hypodermis and cellular infiltration on the next day and increased the formation of granulation tissue by day 10 (Zeng and Lu, 2018). Ozonized ointment, 2% nitrofurazone pomade and their combination were studied for the healing of excisional wounds formed on the dorsal areas of diabetic rats and it was observed that ozonized ointments had anti-inflammatory and wound healing effects. Furthermore, more favorable anti-inflammatory effects were observed in partially ozonized ointments. It was concluded that in animals with no infectious findings during the treatment, ozonized ointments with low level of ozonization had promising outcomes compared to those observed after application of commercial medications (Guerra-Blanco *et al.,* 2017). According to our clinical observations, there was no healing on day 3, crust formation on the wound on day 7, increase in the wound size and incomplete healing even

on day 14 in the LS-G2 (P<0.001, p=0.006). On the other hand, in the RS-G2 on 3rd(p=0.006) and 14th(P<0.001) days, there was a reduction in the wound size starting on day 3, almost complete healing on day 7 (p=0.003), closure in the form of a thin line and furring on day 14. These findings were consistent with Zeng and Lu (2018), and Guerra-Blanco *et al.* (2017) Furthermore, the reduction was significant on day 7 in the G2, which made us believe that it contributed to wound healing on day 7, as previously described by different authors. On the other hand, a significant reduction was observed in the G3, was studied for the first time, in the LS on day 0 and 14 (p=0.005), and day 3 and 14 (p=0.001), and in the RS, a significant reduction was observed on day 3 and day14, and on day 0 and day 7 as well (p=0.05). According to these data, it was concluded that the combined use of the two creams led to early wound healing and complete closure.

The inter-group comparisons revealed no significant difference in wound size measurements of the LS (p=0.437) and a significant difference in group G1 and G3 (p=0.02) on day 3, no significant difference in LS (p=0.067) and a significant difference in groups G1 and G3 (p=0.024) on day 7, and no significant difference in both LS (p=0.159) and RS (p=0.989) on day 14. Significant wound site reduction was observed in G1 and G3 on day 3 compared to LS (p=0.024). This demonstrates that NPH insulin cream and ozonized cream together were significantly superior to ozonized cream alone regarding reduction in wound size, with no detrimental effects.

It has been reported that topical insulin induces the early accumulation of neutrophils to eliminate the degraded tissues, conversely, it has shown an anti-inflammatory effect and healing of chronic wounds in diabetes by increased re-epithelization and fibroblastic activity. (Chen *et al.,* 2012; Kakanj *et al.,* 2017; Liu *et al.,* 2018; Yu *et al.,* 2019; Wang *et al.,* 2019). Avezado *et al.* (2016), investigated the effect of insulin cream applied for 26 days on second degree burns formed on diabetic rats, they demonstrated an increase in the inflammatory cell infiltration and collagen accumulation in the study group compared to the control group. O_3 is known to contribute to wound healing by increasing the local peripheral vasodilatation and oxygenation, modulating the inflammatory phase, stimulating angiogenesis, preventing pain and as a bio-regulator that releases endothelial cellular factors when in contact with biological fluids (Anzolin *et al.,* 2020). The in-group comparison of histopathological examination in G1, revealed a significant difference and reduction in the inflammation scores (p=0.026) between both sides, which suggested that NPH insulin cream reduced the long-term inflammation in diabetic wounds by modulating the inflammation process. In G2, there were no significant differences between the right and the left sides regarding inflammation (p=0.102), whereas in G3, a significant difference was observed between both sides (p=0.017), with the mean scores obtained from the RS being significantly lower. These data suggest that combination of NPH insulin cream and ozonized cream lead to anti-inflammatory effects and promote wound healing.

Specific receptors that are carrying insulin on their nuclear sheath provided repair by promoting epithelization in diabetic wounds. The exposure of O_3 is related to the activation of transcription factors which leads to an inflammatory response and subsequent completion of wound healing, and act by increasing the levels of some cytokines, which is known as a critical factor in epithelization and tissue remodeling (Travagli *et al.,* 2010; Lee *et al.,* 2019). A significant difference was observed between both sides in G2 (p=0.026) and G3 (p=0.038) regarding to epithelization, with the mean score of the RS being significantly higher. This suggests that ozonized cream alone or in combination with NPH insulin cream significantly contributed to epithelization.

It has been reported that wound dressings loaded with insulin-chitosan nanoparticles contributed to wound healing in rats, as well as fibroblast proliferation (Ehterami *et al.,* 2018). Topical insulin application to cutaneous wounds has been reported to increase reepithelization and fibroblastic activity (Liu *et al.,* 2018; Wang *et al.,* 2019). The ozon treatment has been reported to be effective in restoring the number of fibroblasts and angiogenesis in the buccal mucosa of rats (Pchepiorka *et al.,* 2020). Ozonized ointments have been reported to be effective in wound healing by activating many cellular components including fibroblasts and signal pathways. Kim *et al.* (2009), applied topical ozonized olive oil onto 6 mm full-thickness excisional cutaneous wounds formed on the dorsal areas of guinea pigs, this was observed to promote fibroblast proliferation, collagen synthesis, and increased the expression of growth factors on day 7 in the wound site, leading to an acceleration of cutaneous wound healing. In our study, a significant difference was determined in the fibroblast activations scores between the RS and LS in G3 (p=0.046). In other words, G3 significantly increased the fibroblast score in the RS. In G1 and G2, significant differences were observed in all three parameters of inflammation (p=0.017), epithelization (p=0.038) and fibroblast activation scores (p=0.046). A significant difference was observed between the groups of the RS regarding inflammation scores (p=0.045). The mean inflammation score was significantly higher in G1 compared to that of G2 (p=0.045). According to these data, it was concluded that topical insulin is a strong immunomodulator for the prevention of long-term inflammation that leads to delayed healing in diabetic wounds.

Exposure of a dermal ischemic area to O_3 has been reported to reduce bacterial infection in the wound by its bactericidal, antiviral, and antifungal effects in trophic, ischemic, and diabetic wounds. Furthermore, ozonized creams have been reported to provide a slow release of O_3 and reduce the pain during re-dressing of the wound (Silva *et al.,* 2021). Some wounds in G1 showed wound infection, while G2 did not. Complete wound closure was observed in RS at 14 days in both groups, but not in LS. Early wound healing was also demonstrated in the G1 and G2 groups. Furthermore, in G3, which was studied for the first time, supported wound healing, and reduced the pain due to the pain-relieving effect of ozone, especially in infected wounds.

Conclusions: In summary, the combined use of NPH insulin cream and ozonized cream was beneficial regarding the reduction in wound size and histological wound healing parameters. Equal amounts of topical NPH insulin cream and ozonized cream contributed to a reduction in the inflammation score and increased epithelization-fibroblast activation. The combined use of NPH insulin and ozonized cream in diabetic wound healing has been evaluated as a useful and alternative approach, considering the antibacterial and pain-relieving effects of ozone.

Authors contribution: Concept – GÇAG, EÖ, ÇG; Design – GÇAG, EÖ, ÇG; Supervision – EÖ; Resources – GÇAG, AK, MT; Materials – GÇAG, EÖ, ÇG, İE; Data Collection and/or Processing – GÇAG, EÖ, ÇG, AK, İE, MT; Analysis and/or Interpretation – GÇAG, EÖ, İE, AK; Literature Search - GÇAG, EÖ, ÇG; Writing Manuscript - GÇAG, EÖ, ÇG; Critical Review - GÇAG, EÖ, ÇG; Other – MT.

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