Archives of Razi Institute, Vol. 77, No. 5 (2022) 1673-1681

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DOI: 10.22092/ARI.2022.357191.1991

Original Article

Amplification of Wound Healing by Propolis and Honey Ointment in Healthy and Diabetic Rat Models; Histopathological and Morphometric Findings

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Received 25 December 2021; Accepted 20 February 2022
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Abstract

Skin wound healing, especially in diabetic patients, has been a major medical challenge for decades. In the meantime, the use of traditional medicine has always been questioned. Propolis (resin and wax) is one of the most likely solutions to this problem. The present study aimed to establish an animal model for healing skin wounds and diabetic ulcers. To this aim, rats were randomly allocated into two healthy and diabetic groups (50 mg/kg streptozotocin resulted in diabetes with high BSL to 300 mg/dL), which were divided into four subgroups. The 7 mm full-thickness skin wounds were created on the abdomen region in 80 male Wistar rats using paunch. In the subgroups, the wounds were cleaned with normal 0.9% saline as the control subgroup and dressed with Eucerit, 1.5% honey+eucerit, and 3% propolis +1.5% honey+eucerit, once daily for 14 days in other subgroups, respectively. On days 1, 3, 5, and 7 after the intervention, wound and area contractions were calculated using digital photographs measurement. The histopathological and semi-quantitative studies were performed on days 7 and 14 after wounds creation. The microscopic findings demonstrated that the granulation tissue, fibroblasts, re-epithelization, and angiogenesis increased ($P \leq 0.05$) in the subgroups treated by propolis and honey combination in healthy and diabetic rats within 7 and 14 days post-injury. Also, less inflammation and a significant reduction in wound contraction were observed in the same subgroups on days 3, 5, and 7 compared to other subgroups ($P \leq 0.05$). The results indicated that significant healing quality and acceleration were affected by propolis and honey compared to other subgroups on days 3 and 5 ($P \leq 0.05$).

Keywords: Animal Model, Diabetes, Honey, Propolis, Wound Healing

1. Introduction

Healing is a dynamic method which involves simultaneous biochemical and physiological stages and can be summarized in 4 phases: coagulation and hemostasis, inflammatory, proliferative, and remodeling (1). The cutaneous tissue is exposed to various chronic injuries such as burns, ulcers, and erosions that may not heal or be delayed (2). Nowadays, diabetes mellitus is a chronic metabolic disorder in which blood glucose level rises due to deficiency or resistance to insulin (3, 4). One of the most common problems in these patients is lower-limbs ulcers, with about 15% prevalence in diabetic patients, and 85% of them lose their legs by amputation. Furthermore, the etiology of these types of ulcers contains peripheral nerve neuropathy, decreased organ

perfusion, and trauma (3, 5). Diabetic Ulcer (DU) healing is a complex, lengthy and costly process which is one of the major health challenges of diabetic patients (6).

The positive effects of honey and propolis on human health have always been considered throughout the history of medicine. Although honey is often known for antimicrobial effects (7), it contains compounds that have other therapeutic effects, such as hydrogen peroxide inducing angiogenesis by stimulating the vascular endothelial growth factor (VEGF) (8) or proline which has antioxidant and anti-inflammatory effects (9). Also, the acidity and osmotic effects of honey stimulate the activity of phagocytes and lymphocytes at the wound site (10). Additionally, propolis is a natural lipophilic resins mixture made by bees from different sections of plants, buds, and gum trees. Propolis contains vitamins such as B complex, C, E as well as minerals such as iron, copper, calcium, potassium, cobalt, and zinc (11) and other substances including flavonoids, caffeic acids, terpenes, and esters (12) which cause antioxidant, anti-inflammatory, restorative, and anesthetic effects of propolis (13).

The present study aimed to evaluate the role of propolis and honey in healing skin and diabetic wounds in rat models by histopathological and morphometric approaches.

2. Materials and Methods

2.1 Animals

Eighty male Wistar rats, 4-6 weeks old and 250-300 g in weight were selected by simple randomization and maintained in standard animal housing conditions (12/12hr light/dark cycles, 30% humidity, and 23°C), and then equally allocated into healthy and diabetic groups (n=40). The animals were fed with standard rat chow and had free access to water. The animal adaptation period was performed for a week before experimental procedures.

2.2 The Propolis Ointment Formation

The fresh propolis was collected by mesh from hives and kept in a dark place under lab conditions. To prepare the propolis extract, 20 g of fresh and raw propolis was dissolved in 50 ml of 95% ethanol (v/v). The solution was then stored in a dark glass bottle for one week. For best results, the bottle was shaken twice a day. After ethanolic extraction, the solution was filtered and the excess ethanol was evaporated and dried extract with the following compound was used (14). Two types of ointment were made as follows:

1. To prepare the propolis + honey ointment (PHO): 3 g of propolis was mixed with 1.5 g of honey and 6.5 g of Eucerin to obtain 3% propolis+1.5% honey ointment.

2. To prepare the honey ointment (HO): 1.5 g of honey was mixed with 8.5 g of Eucerin to obtain 1.5% honey ointment.

2.3 Rat Model of Diabetic Ulcer

The fasting blood sugar level (BSL) was measured for each rat before induction of diabetes by glucometer (IME-DC Blood Glucose Meter System, Int. Medical Equipment Diabetes Care, Germany), and blood samples were taken from their tails to ensure their health. The normal BSL was considered to be about 80-100 mg/dL. Afterward, Streptozotocin (STZ) (50mg/kg; Sigma-Aldrich, St. Louis, MO) was used to induce diabetes in 40 rats allocated to the diabetic group. For this purpose, 1g of STZ was dissolved in ice-cold citrate buffer (pH=6) and administered subcutaneously. For the next 24 hrs, the rats were given 5% glucose water to counteract hypoglycemia by STZ injection. After five days, the BSL was measured in animals, and the rats were considered diabetic with a BSL above 300 mg/dL. The rats also showed clinical signs such as polydipsia, polyuria, and weight loss. The BSL of these rats were repeatedly monitored weekly for two weeks to ensure the progression of their disease.

2.4 Creation of Skin Wounds

The rats were anesthetized by intraperitoneal injection of 10% Ketamine (90 mg/kg, Alfasan, Netherlands) and 2% Xylazine (10 mg/kg, Alfasan, Netherlands). All rats were given subcutaneous Tramadol (10 mg/kg, Exir Pharmaceutical Co, Iran) just before induction of

anesthesia. The integrated circle wound was created by a 7 mm skin punch on the abdominal skin of rats under surgical asepsis, and then the rats of each group were divided into subgroups (n = 10).

2.5 Animal Grouping

Healthy and diabetic groups of rats were randomly divided into four subgroups. Healthy rats were divided into a, b, c, and d groups. The diabetic rats were divided into a', b', c', and d' ones. In the a and a' subgroups, the wounds were rinsed with normal 0.9% saline as diabetic and healthy control groups, in b, b', c, and c' groups the wounds were also rinsed with normal saline. Then in b and b' groups, the wounds were treated with Eucerit alone as an ointment base, c and c' groups were treated with 1.5% honey ointment, and d and d' groups were treated daily with 3% propolis+1.5% honey ointment in 14 days. These wounds were dressed with cotton bandages daily to heal by secondary intention.

2.6 Wound Assessment

2.6.1. Morphometric Evaluation

The wound area measurement (cm2) was checked on days 0, 3, 5, and 7 post-injury by digital photographing. The digital photos were taken of the wound with a 1 cm2 piece of marker once daily for 7 days post-injury. The photos were processed by ImageJ (Version 1.52o, 23 April 2019, LOCI, and University of Wisconsin). The measurement was performed by another veterinarian who was blinded to the design of the experimental group. The percentage of wound contraction on days 3, 5, and 7 post-injury was calculated as follows (3):

Wound contraction percentage (%)= (A0− At)/A0×100)

A0= wound area at the day 0, *At*= wound area on the day of evaluation

2.6.2. Histopathological Evaluation

Five rats were sacrificed from all groups on days 7 and 14 after surgery, and full-thickness wound tissues were isolated along with surrounding skin to an area of 1×1 cm. The samples were kept in 10% phosphate-buffered formaldehyde and embedded in paraffin wax for sectioning following fixation. Then, 5 µm sections were taken and stained with hematoxylin and eosin. The light microscope was used to evaluate the histopathological section blindly. Re-epithelization, polymorphonuclear leucocytes, fibroblasts, and angiogenesis were assessed with a semi-quantitative method, based on Gal study (Table 1) (15). The averages of values were used for statistical evaluation.

Table 1. Clarification of semi-quantitative evaluation scale for histological sections [surrounding tissue (ST), demarcation line (DL), subcutaneous tissue (SCT), granulation tissue (GT)]

Scale	Re-Epithelization	Polymorphonuclear Leucocytes	Fibroblasts	Angiogenesis
0	Thickness of cut edges	Absent	Absent	Absent
1	Migration of cells (< 50%)	Mild ST	Mild ST	Mild SCT
2	Migration of cells (≥ 50%)	Mild DL/GT	Mild GT	Mild GT
3	Bridging the excision	Moderate DL/GT	Moderate GT	Moderate GT
4	Keratinization	Marked DL/GT	Marked GT	Marked GT

2.6.3 Statistical Analysis

The mean ± SEM was used to compare different groups. The non-parametric Kruskal-Wallis test was used to assign the statistical difference. The semi-quantitative evaluation scales of the histopathological study were compared with ANOVA and Tukey-Kramer multiple comparisons using SPSS software, version 22 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1 Healthy Rat

3.1.1 Morphometric Examination

The entire wound surface (mm^2) was measured, and changes in wound contraction (%) were calculated as shown in figure 1. In the morphometric examination, the mean post-surgery wound size was 61.17 mm^2. Despite numerical differences between different study

subgroups, significant differences were observed between a and d subgroups on days 3, 5, and 7 after surgery (Figure 1).

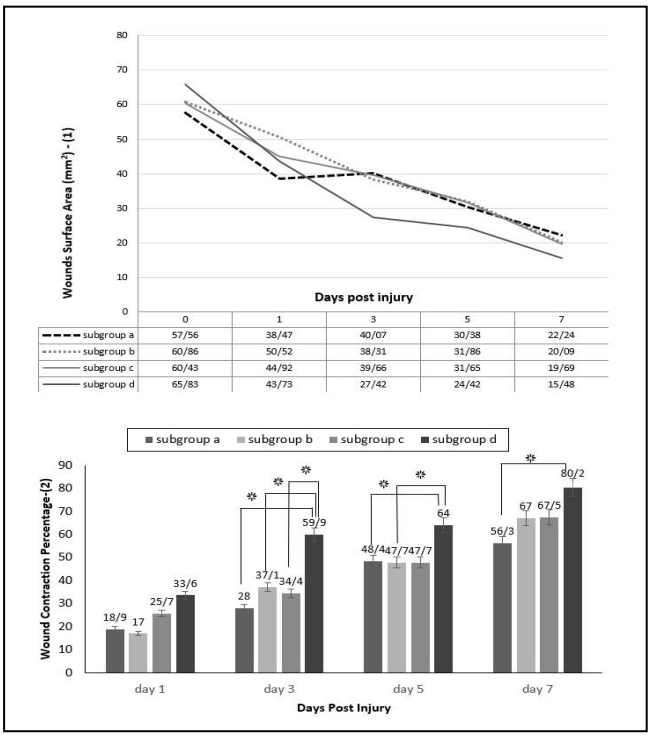

Figure 1. Compartment of histopathological scale (Re-Epithelization, Polymorphonuclear, Leucocytes, Fibroblasts, and Angiogenesis) of a-d subgroups in 7 days (1), and 14 days (2) after wounding
* Significant difference in results between different subgroups ($P \leq 0.05$)

3.1.2. Histopathological Examination

In the microscopic study of tissue samples of the wound site in a subgroup, re-epithelization was observed only at the wound margin, inflammation and polymorphonuclear leucocytes were detected with angiogenesis, and mild level of fibroplasia with a weak healing procedure by end of the 14 days. In the b subgroup, the proliferative phase began; however, re-epithelization was similar to a subgroup with a scab, and angiogenesis and fibroplasia were also observed, although there was no serious difference in 7 days and 14 days post-injury. In the c subgroup, the inflammatory phase was finished, and re-epithelization was observed at the wound margin, under scab without a complete covering of the wound surface for 14 days with no mature granulation tissue. In the d subgroup, re-epithelization was well developed, covered the wound surface, and was clearly seen under the scab, and the healing process mostly finished 14 days post-injury (Figure 2).

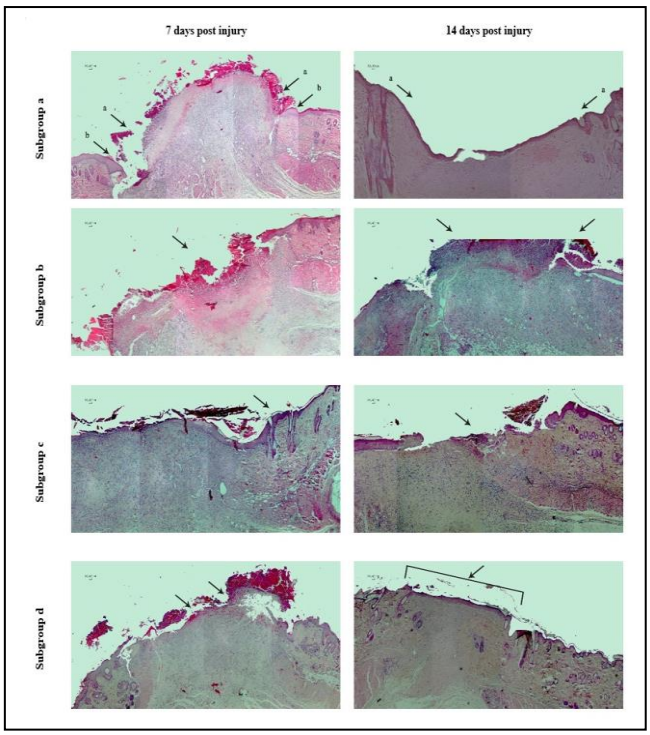

Figure 2. Subgroup a: 7 days post-injury, arrows (**a**) = the granulation tissue found in the center of the wound is fully grown, arrows (**b**) = re-epithelization at the wound margin. Subgroup a: 14 days post-injury, arrows = very weak and thin layer of epithelium. Subgroup b: 7 days post-injury, arrows= no re-epithelization on the wound surface, with massive granulation tissue under the epidermis. Subgroup b: 14 days post-injury, arrows = re-epithelization absence was continued. Subgroup (**c**): 7 days post-injury, arrows = no mature granulation tissue in wound margin. Subgroup c: 14 days post-injury, arrows= moderate granulation tissue growth in wound center, re-epithelization was started, with some angiogenesis center. Subgroup **(d**): 7 days post-injury, arrows = re-epithelization was seen on all surfaces of the wound. Subgroup d: 14 days post-injury, arrows = complete wound contraction

3.1.3. Semiquantitative Examination

The histopathological stricture scales of subgroups which include re-epithelization, polymorphonuclear leucocytes, fibroblasts, and angiogenesis were analyzed in 7 days and 14 days as presented in figure 3. The statistical difference between a and d subgroups was observed 7 days after surgery in all measured scores. By reducing the size of the wounds and the passage of

time and healing of the wounds, a significant difference was created between the different subgroups 14 days after surgery (Figure 3).

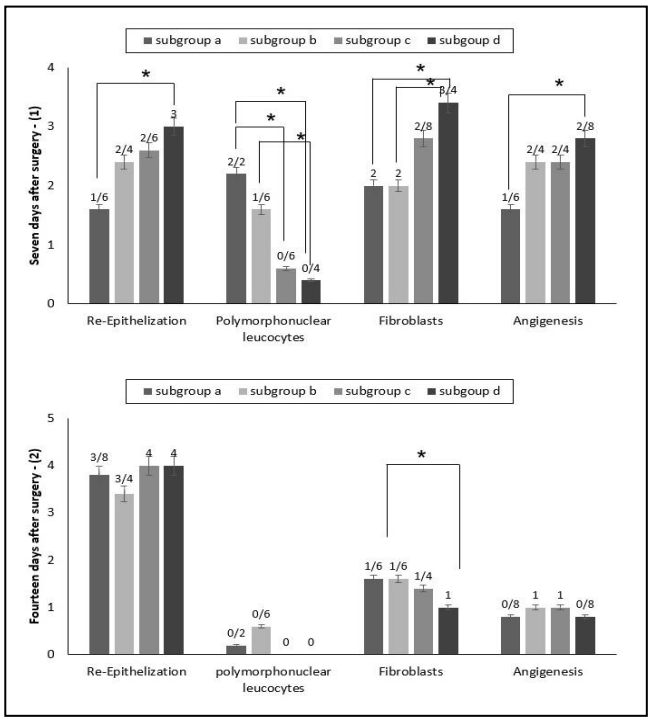

Figure 3. Evaluation of scale for histological morphometric strictures (Re-Epithelization, Polymorphonuclear, Leucocytes, Fibroblasts, and Angiogenesis) in a-d subgroups in 7 days (1), and 14 days (2) post-injury .
* Significant difference in results between different subgroups ($P \leq 0.05$)

3.2 Diabetic Rat

3.2.1 Morphometric Examination

Wound area decreased in all groups during the study; however, it was significantly more prominent in the d' subgroup. Also, the wound contraction percentage in this subgroup was significantly higher than that of the a' and c' subgroups, 3 days after wounding, and subgroups b' and c', 5 days after wounding (Figure 4).

3.2.2. Histopathological Examination

In the a' subgroup, seven days after wounding, the formation of granulation tissue was observed in the middle of the wound and almost the entire wound surface lacked epithelial tissue. Moreover, the angiogenic foci were observed with a few fibroblasts. Inflammatory cells and leucocytes could also be observed. The healing process was not complete and granulation tissue remained for 14 days. In the b' subgroup, despite the improvement of healing compared to that of a' subgroup, it was significantly lower in quality, compared to that of d' subgroup. In c' subgroup, the granulation tissue, and inflammatory cells presented 7 and 14 days after wounding. Furthermore, the quality of healing was better compared to a' and b' subgroups, but far from ideal. Less granulation tissue and angiogenic foci existed in d' subgroup on day 14 compared to day 7. No inflammation was present in the wound area and new epithelium completely covered the wound surface after 14 days. However, angiogenic foci were more on day 7 than on day 14 (Figure 5).

3.2.3 Semi-Quantitative Examination

Histopathological scores concerning re-epithelization, polymorphonuclear leucocyte, fibroblasts, and angiogenesis were performed and compared 7 and 14 days after surgery (Figure 6).

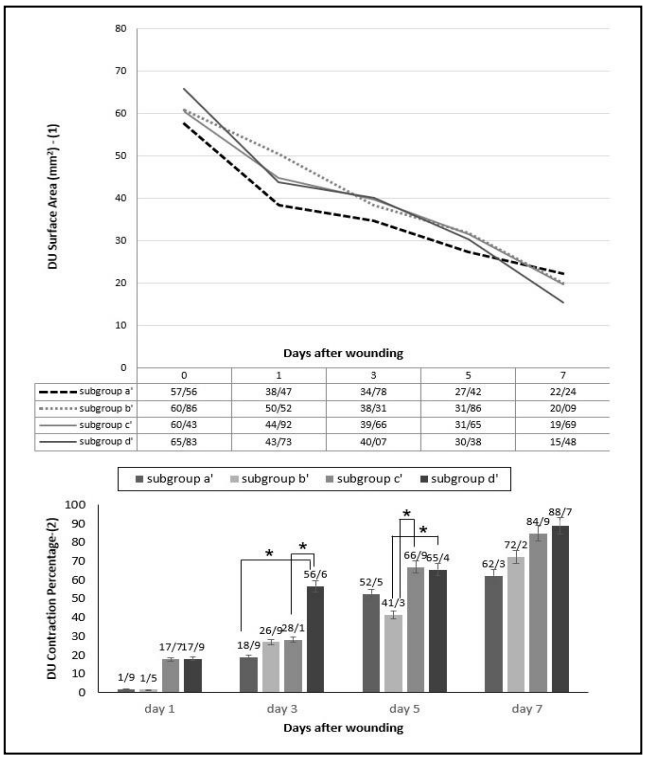

Figure 4. 1; DU surface area (mm2) in 0, 1, 3, 5, and 7 days after wounding – 2; percentage of DU contraction in 1, 3, 5, and 7 days after wounding.
* Significant difference between subgroups ($P \leq 0.05$)

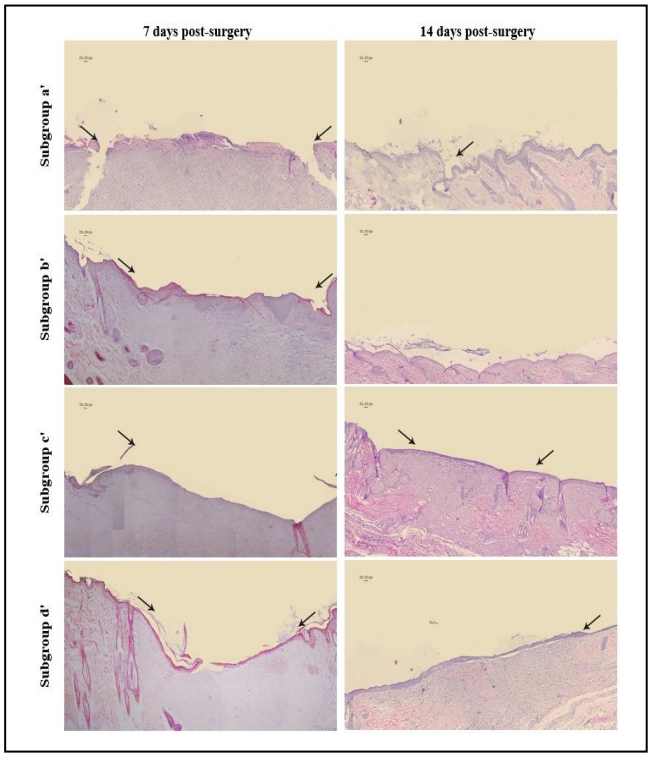

Figure 5. Subgroup a': 7 days after wounding, arrows = the granulation tissue in the DU area and inadequate formation of the epidermis. Subgroup a': 14th day, arrows = incomplete re-epithelization and the scar presence. Subgroup b': 7th day, arrow = re-epithelization has not been completed. Subgroup b': 14th day, arrows = re-epithelization absence was continued. Subgroup c': 7th day, arrows = the granulation tissue formation was located and inflammatory cells were presented but lower than that of the previous two groups. Subgroup c': 14th day, arrow = moderate re-epithelization with a thin layer of the epidermis covering the wound. Subgroup d': 7th day, arrows = re-epithelization was started. Subgroup d': 14th day, arrows = new epidermis was covered all wound surface and almost was near to normal skin tissue

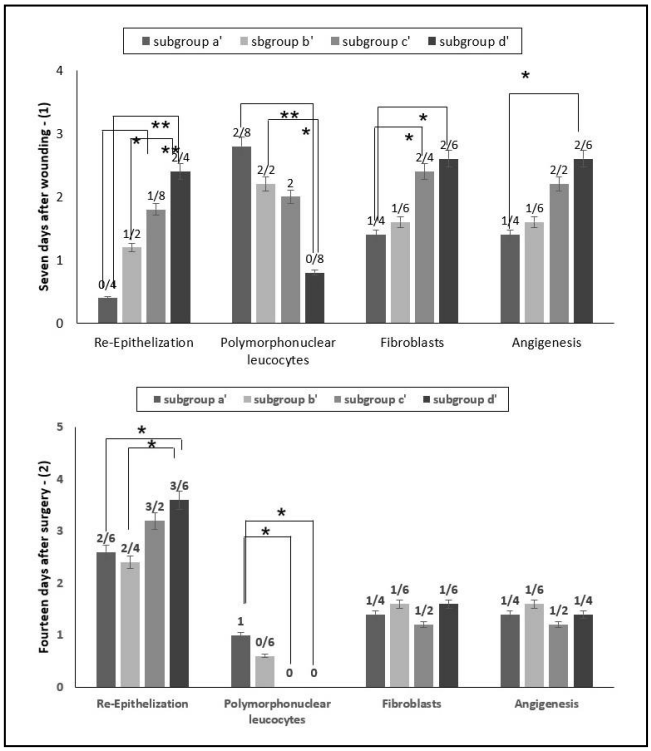

Figure 6. Compartment of histopathological scale (Re-Epithelization, Polymorphonuclear, Leucocytes, Fibroblasts, and Angiogenesis) of a'-d' subgroups in 7 days (1), and 14 days (2) after wounding
* Significant difference in results between different subgroups ($P \leq 0.05$)

4. Discussion

In the present study, the effects of propolis and honey were investigated on wound healing in experimental healthy and diabetic rat models using a standard and iterative method. Skin wound healing has four regular, overlapping, and continuous phases: coagulation, inflammation, proliferation, and remodeling (16-19). This procedure involved various types of cells and structures such as granulation tissue, collagen, fibroblasts, keratinocytes, endothelial and inflammatory cells (20).

DU is a serious issue with negative effects on quality of life that are associated with high economic costs, and its management is a major challenge. Dysregulation of wound healing phases is an important variance between diabetics and non-diabetic patients. The highest difference is in the persistence of the inflammatory phase in DU (21). In diabetic patients, wound healing is inhibited by several mechanisms such as a prolonged inflammatory phase due to decreased chemotaxis and macrophagic activities of neutrophils and macrophages (22).

A wide variety of natural products have been used for wound healing due to ease of use, convenient access, economic efficiency, and antibacterial effects (5). Propolis and honey have been shown therapeutic applications, especially in the wound healing process

(23). Based on previous studies, honey is known as a biological dressing with some bioactive ingredients such as oligosaccharides, minerals, carbohydrates, enzymes, flavonoids, and ferulic and caffeic acids (24).

The therapeutic effects of propolis are more relevant to its anti-inflammatory and antioxidant effects (13). Other possible effects of propolis on wound healing include reducing the recovery period, increasing wound contraction, and accelerating tissue healing (11).

Sutta (25) evaluated the alcoholic solution of propolis in wound healing of animals in a clinical and experimental study and reported that reducing the inflammatory phase by propolis affected wound healing quality. McLennan, Bonner (26) indicated that the anti-inflammatory characteristic of propolis is probably the main reason which affects wound healing in the diabetic models (26). The anti-inflammatory properties of propolis are due to its flavonoid and caffeic acid which inhibits the inflammatory process and accelerates healing by inhibiting the production of prostaglandins by the lipoxygenase pathway (27). According to the results obtained in the d subgroup, the rate of wound contraction has significantly decreased compared to that of other subgroups in the healthy group due to the time of the inflammatory phase and the initiation of tissue repair. Furthermore, reducing inflammation increases collagen fiber production, sedimentation, and the speed of healing (13). The results of histopathology in our study indicated that dressing wounds with propolis and honey ointment significantly reduced inflammation in cutaneous tissue.

According to Abu-seida and Saleh (2), the healing rate in the experimental skin ulcer of a dog treated with propolis was significantly higher than that of the control group on days 14, and 21 after injury. Also, no side effects were recorded after using propolis (2). Based on the results of the present study, the rate of wound contraction in the d and d' subgroups was almost twice that of the a and a' subgroups.

Propolis acts as an antioxidant that inhibits angiogenesis by modulating angiogenesis and inflammatory stimulants (28,29). Honey has positive effects on increased angiogenesis in open cutaneous wound healing (30), therefore the combination of honey and propolis in the d and d' subgroup significantly increased this critical factor in wound healing compared to that of the a and a' subgroups.

However, Olczyk, Komosinska-Vassev (31) claimed that propolis accelerates wound healing by stimulating the accumulation of glycosaminoglycan at the wound bed and creating a favorable wound matrix for re-epithelization of burn wounds in pigs. Also, changes in extracellular matrix glycosaminoglycan in wound healing as well as its possible effect on the acceleration of healing have been described in the previous study (31). The findings of the present study revealed that simultaneous use of propolis and honey accelerates the healing of DU at least on day 3 compared to that of other studied subgroups which can be attributed to changes in the extracellular matrix or other unknown factors. Since honey has positive effects on increased angiogenesis in open cutaneous wound healing (30), the combination of honey and propolis in the d and d' subgroups significantly increased this vital factor in wound healing compared to the a and a' subgroups.

Based on McLennan, Bonner (26), a single treatment with propolis could increase the healing of full-thickness wounds in a diabetic rat model. According to their study, epithelial closure failed to happen through re-epithelialization, and instead, wound contraction indicated the anti-inflammatory effect of propolis (26). Concerning the results of the present study, simultaneous use of propolis with honey could increase its effects and produce a better combination to accelerate wound closure by re-epithelization. Also, PHO can normalize polymorphonuclear leucocytes count in DU compared to the a and a' subgroups after 7 and 14 days of wounding which can be attributed to their antibacterial effect (24). In a semi-quantitative pathological study, the increase in the re-epithelization and also the decrease in the fibroblast number, like the morphometric result, indicates the positive effect of

PHO on superficial wound healing. In addition, increasing wound contraction in the PHO treatment subgroup can be attributed to the activity of collagen fibers, which can be the result of the effect of PHO in improving their function. Re-epithelization, polymorphonuclear leucocytes, fibroblasts, and the presence of angiogenesis in different phases of skin repair play a critical and decisive role in the quality of healing and newly formed tissue (32).

5. Conclusion

The findings of the present study revealed that skin wound and DU treatment with propolis and honey ointment can increase wound contraction and healing quality by increasing fibroblasts and angiogenesis. Also, re-epithelization and tissue maturation were accelerated.

Authors' Contribution

Study the concept and design: M. F. A. and A. A. S.
Data acquisition: A. B. and M. M.
Analysis and interpretation of data: A. B. and S. D.
Draft the manuscript: A. A. S.
Critical revision of the manuscript for important intellectual content: M. F. A. and M. M. O.
Statistical analysis: M. M. and M. F. A.
Administrative, technical, and material support: M. M. O. and S. D.
Study supervision: M. F. A. and A. A. S.

Ethics

The present study was approved by the Research Ethics Committee of AJA University of Medical Sciences, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

The authors received no financial support for the research, authorship, or publication of this study.

Acknowledgment

The present study is approved by the Research Ethics Committee of AJA University of Medical Sciences, Iran. (IR.AJAUMS.REC).

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