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Evaluation the Effect of Formulation made of Talh Honey, Whey Protein and Collagen on Acute Excisional Skin Wound Healing in Wistar Male Rat

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Abstract

Objectives: This study evaluated the ability of using formulation contains Talh honey, whey protein and collagen on wound healing using animal model.

Methods: 24 Wistar male rats were divided into four groups of 6 rats each as follow; G1 (not treated), G2 (treated with Manuka honey), G3 (treated with Povidone iodine ointment 5%), and G4 (treated with tested formulation). Excisional wound was induced in rat's dorsal skin. The duration of the study was 18 days. Morphological and histological examination was performed for all groups.

Results: The tested formula showed reduction in the duration of the inflammatory phase of wound healing when compared to other groups.

Conclusion: Results from this study indicated the therapeutic potential of the tested formula, which contains natural component, in wound healing as using other therapeutic products. The tested formula is affordable for wide range of patients. Further studies are required to investigate the different mechanisms behind the therapeutic properties of each treatment.

Keywords: Wounds and injuries, honey, whey proteins, feridas excisionais

Introduction

Skin wound is among the most common injuries globally. Wound is defined as the disruption of the tissue by chemical, thermal, physical or microbial injury.[1] Skin wound is especially important since skin function's as physical, chemical and microbial barrier. Restoration of this barrier is crucial to prevent further damage or infection. Medical conditions, wound contamination or infection can lead to impaired or prolonged wound healing. Prolonged healing process and extreme reaction of an organism may interfere with normal wound healing leading to scar formation and other complications.[2] This will increase health and economic burden.[3]

In clinical practices, wound treatment includes products that aid in creating and maintaining moist and adequate conditions for healthy healing process.[2] Managements of acute skin wound focuses on avoiding infection, mechanical protection, topical application of growth factors, etc. However, they are costly in many developing countries and could generate adverse effects. Although many pharmaceutical products are available, there is a need for wound dressing and topical products to enhance healing and reduce scarring.

For centuries, honey has been known for its therapeutic properties including wound healing. Honey has many beneficial effects, particularly, as anti-inflammatory and anti-microbial agents.[4] Different kinds of honey have been found to promote healing by providing suitable moisture in the wound bedding but clearing resident bacteria, suppressing inflammation, reducing scarring, enhancing angiogenesis, tissue granulation and re-epithelization.[5] Therefore, honey could reduce wound healing time. The healing property of honey could be through its effect on inflammatory response. It could be through reducing inflammatory cells infiltration or through stimulating normal response by enhancing proinflammatory cytokines secretion and cells proliferation.[6,7] Moreover, honey has an anti-microbial property for its high sugar concentration, low pH, hydrogen peroxide (H_2O_2), methylglyoxal (inductor), antimicrobial peptide bees defensin-1.[8] Several studies have investigated the therapeutic effect of honey on different types of wounds.[8-10] Manuka honey is well-known for its therapeutic properties.[11,12] *Acacia Gerrardii Benth* is a well-known honey in Africa and Middle East (Alqarni et al.).[13] It is known for its antimicrobial effects and has been used in traditional medicine for decades. However, to the best of our knowledge its therapeutic potential in wound healing has not been investigated.

Whey protein is one of the highest quality sources of proteins that contains different types of proteins and growth factors. Whey protein isolate contains different types of proteins and low concentration of fat and lactose. Importantly, it contains several kinds of growth factors, such as insulin-like growth factor-I (IGF-I) and transforming growth factor-beta 2 (TGF-β2).[14] Beside the important role of proteins in tissue regeneration, growth factors in whey proteins could be involved in promoting cell proliferation, differentiation thus healing process. Previous studies demonstrated the therapeutic effects of whey protein in wound healing.[15,16] Therefore, it could be a candidate component in creating topical formula with honey for wound healing.

Collagen is one of the abundant components in the skin and the main component of extracellular matrix structure. It provides the mechanical strength and elasticity of the skin. There are three types of collagens in human, I, II, and II. Type I and III are important for wound healing. Fibroblasts, in the skin, synthesize collagen fibrils. After injury, collagen III is synthesized first then replaced by collagen I. Exposed collagen during injury plays a role in recruiting platelets to form fibrin clot. During inflammatory phase, collagen is a mediator of inflammation, acting as a chemoattractant for macrophages. Degradation of collagen releases fragments that promote macrophages to combat bacterial colonization. This leads to transition to proliferation phase. During this phase collagen fragments recruits angiogenesis and re-epithelization processes.[17] Dysregulation of collagen function could lead to pathological conditions, such as scar formation.[18]

In conclusion, *Acacia Gerrardii Benth* honey, whey protein and collagen play different roles in wound healing separately. They could be investigated as a therapeutic formula for better wound healing results. The present study aimed to investigate the therapeutic effect of combining all of them for their therapeutic potential in wound healing. This study hypothesize that formulation of Talh honey (*Acacia Gerrardii Benth*), Whey protein and collagen improve acute excisional wound healing morphologically and histologically in normal healthy Wistar albino rat. Therefore, this study evaluated wound closure (contraction) in different experimental groups and compared the effect of each treatment on wound healing histologically.

Materials and Methods

Materials

The tested formula was prepared by mixing Talh honey (Thymus vulgaris), Whey protein and Collagen. The formula was prepared by mixing the various ingredients: 2 mg/ml Whey protein, 50% Talh honey and 1 mg/ml Collagen, by slow addition until all the solids were dispersed and dissolved completely.

Animals and Experimental Design

The study protocol was approved by Animal Care and Use Committee (ACUC) at King Abdul-aziz University (No. 122-19). A total of (24) healthy Wistar albino rats, weighing between (180–200 g), were housed in the animal house of King Fahad Center for Medical Research, King Abdulaziz University for 7 days to acclimatize prior to the study. They were maintained under standard laboratory conditions (25 ± 2°C; light and dark cycle of 12:12 h; relative humidity 44–56%) and fed with standard diet and water ad libitum during the study. A total of 24 rats were divided equally ($n = 6$) into four groups as follow: Group I; Negative control group that did not received any treatment, Group II; treated with Manuka honey, Group III; positive control (treated with Povidone iodine ointment 5% w/w) and Group IV; Treated with tested formulation (Tested group).

Excisional Wound Preparation

Rats in all groups were labeled and the wounds were induced as follow; The dorsum portion was shaved using depilatory cream and disinfected with the alcohol-iodine solution. Rats were anesthetized with ketamine injection (50 mg/kg, intraperitoneal (i.p) body weight) and xylazine 10 mg/kg, then marked the surgical area. A full thickness circular excision wound of (1 cm diameter) was created using forceps and pointed scissors. Wounds, except the control group, were covered after adding the formulation during the experiment. Wounds in the second group were washed with saline then covered with the control formulation. Wounds in third group were washed with saline then covered with the standard drug (Povidone iodine ointment 5% w/w). Wounds in fourth group were washed with saline then covered with tested formulation. First group did not receive any treatment. Every rat was caged individually. The standard drug and both formulations were applied over the wounds every two days post the operation until the full healing.

Wound Healing Measurement

Rats were observed daily, and wound size was measured and photographed using digital camera mounted on tripod, 20 cm above the mouse, to compare wounds between rats. Wound size was measured using image J program. Percent wound contraction was calculated by using following formula:

$$\% \text{ Wound contraction} = \frac{\text{Wound size at day 0} - \text{Wound size at specific day}}{\text{Wound size at day 0}} \times 100$$

The surgery day was considered as day 0. The end point of the treatment was defined as the complete closure of the wound (day 18th). Two rats were euthanized from each group at day 4, 11 and 18 since day 4 presents the inflammatory phase, day 11 presents proliferation phase and day 18 present the complete healing of all wounds.

Tissue Collection and Processing

The wound and the surrounding tissue were harvested and stapled onto transparent plastic sheet to prevent the over contraction of specimens. Each wound area was cut in half into two pieces; one of which was freeze under (–80) for further analysis. The other half was processed for microscopic examination. Specimens were fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer for 15 hours. Then they were dehydrated, cleared and embedded in paraffin. Sections were cut into 4 μm-thickness and mounted on saline coated slides.

Histological Examination

Sections were stained with H&E then they were scanned using digital scanner Philips. Qualitative analysis of Inflammation, neovascularization, epithelization and granulation were scored from 0 to 3 as previously described in the Table 1.[19] Samples were examined blindly.

Statistical Analysis

Two-way ANOVA and Tukey test using SPSS was used to compare all tested groups. P-value < 0.05 was considered as significance each all analysis.

Results

Macroscopic Analysis

Morphologically, excisional wound caused bleeding followed by clot formation on the surface of wounds. Scar formation was present by the end of the study for all groups. Puss formation was present in the fourth group from day 4 to 7 (Figure 1).

The duration of wounds' complete healing was similar between all groups. All groups achieved complete healing by day 18. Wounds were photographed in every two days and measured. Changes in macroscopic appearance of the wounds were monitored daily, including inflammation, puss formation, redness, odor, edema or bleeding. No adverse effects of all treatment were noticed. No significant differences were observed between all groups in day 4, 7, 9, 11, 14 and 18 (P = 0.075, 0.38, 0.88, 1.0, 1.0, 0.85, respectively) (Table 2 & Figure 1). However, on day 7 post wounding, wound contraction rate was accelerated in group four than

Table 1. **Histological criteria and scores**

Criteria	Score	Parameter
Inflammation	0	Whole skin-absence of inflammation
	1	Discrete-presence of few inflammatory cells
	2	Moderate-many inflammatory cells
	3	Sever-exaggerated inflammatory cellularity
Neovascularization	0	Whole skin-normal vascularization
	1	Discrete vascular formation
	2	Moderate vascular formation
	3	High vascular formation
Epithelization	0	Whole skin-whole epithelium
	1	Discrete-partial epithelization with a small epithelial layer (the epithelial tongue occupies, at most 1/3 of the wound gap)
	2	Moderate-partial epithlization with a longer new epithelial layer (the epithelial tongue occupies more than 1/3 of the wound gap)
	3	Complete epithelization
Granulation	0	Whole skin-absence of granulation
	1	Immature granulation tissue: loose granulation tissue (macrophages, fibroblasts) with emerging vessels
	2	Mature granulation tissue: fibroblast and spares extracellular matrix proteins forming layers, vessels running perpendicular
	3	Fibrosis: extracellular matrix proteins (mainly collagen) dominating the granulation tissue, fewer fibroblasts and vessels.

Table 2. **Percentage of wound contraction for all groups**

	Wound contraction %					
Groups	**4th day**	**7th day**	**9th day**	**11th day**	**14th day**	**18th day**
Group I	30.25 ± 9.54	35.4 ± 9.80	56 ± 10	88 ± 3	91 ± 2.5	100 ± 0
Group II	6 ± 2.94	50.25 ± 18.71	64 ± 1	88 ± 1	96 ± 1	100 ± 1
Group III	28 ± 4.81	37.25 ± 4.02	68.33 ± 12.25	88 ± 1	98 ± 2.5	100 ± 1
Group IV	47 ± 8.76*	67 ± 8.51	84 ± 4	92 ± 2	95 ± 5	100 ± 1

All values represented as Mean ± SD. $N = 2$ per group. Data were analyzed by one-way ANOVA, followed by Tukey-Kramer Multiple Comparisons Test. *: Significant difference as compared to Group II (Manuka honey) and $P < 0.05$.

group 3. On day 4, puss formation, moist necrotic tissues, was noticed in rat from group two and from group four. All samples did not show any sing of contamination or infection (Figure 1). Scabs were formed in group one and two from day 7 and in group three and four from day 11.

Histological Analysis

In day 4, the inflammatory response was initiated early by the presence of inflammatory infiltrate of neutrophils and macrophages to clear the debris and promote collagen formation in all groups. Vessel dilation was noticed in the dermis of third group. No significant differences in inflammatory infiltration were found between all treated groups (Figure 2). In day 11, mild inflammatory infiltrate was observed in all groups and formation of granulation tissue, which indicates increased number of red blood in samples from day 4. Improved organization of ECM was observed. No significant differences were observed in all groups ($P = 0.449$). In day 18, complete healing was observed in all groups with formation of epithelium and hair follicles, collagen deposition and formation of capillaries. The thickness of the formed epidermis in the second group was thicker but not statistically significant compared to other groups.

Discussion

Wound healing is an orchestrated and regulated process by different cell types and mediators interacting in temporal sequences. All wounds undergo reparative phases, including homeostasis, inflammation, proliferation and remodeling. This study aimed in investigating the efficacy of therapeutic formula containing natural products, *Acacia Gerrardii Benth* honey, whey protein, and collagen morphologically and histologically. Components of this formula were chosen according to their therapeutic potential in wound healing. To our knowledge, research has not investigated this formula as a treatment for excisional wound.

In this study, wound excision was done in Wistar rats for their feasibility and eligibility for wound testing. However, wound heals in rat's skin by contraction while in human's skin

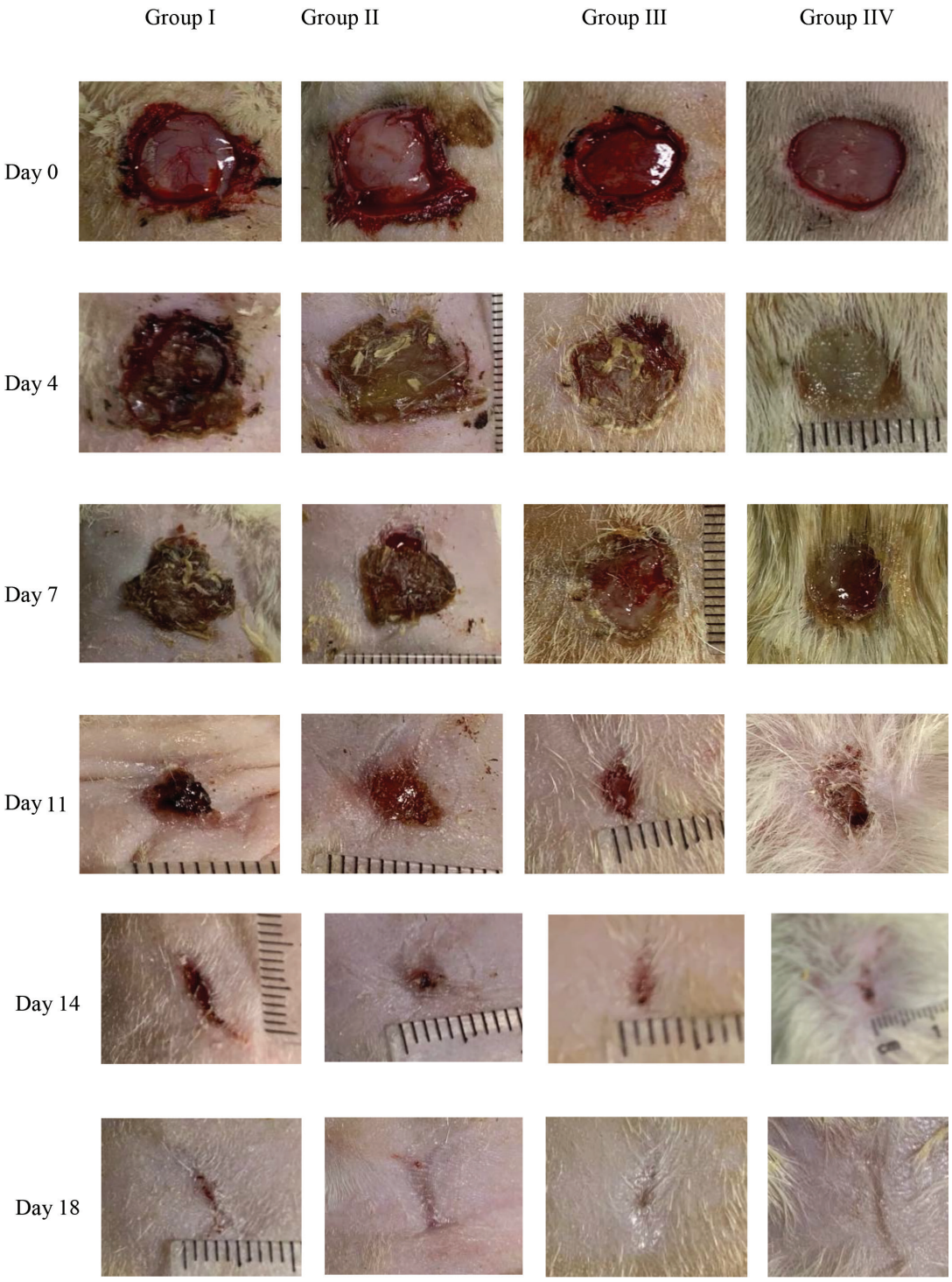

Fig. 1 **Macroscopic observation of wound contraction in all groups. Group I (negative control), group II (Manuka honey) group III (positive control), and group IV (tested formulation). *n* = 2 each group. The wound area was measured form the same distance using tripod. *P*< 0.05.**

it heals by re-epithelization. Rats were divided into four groups; group one was a negative control that did not receive any treatment after wounding; group two was treated with Manoka honey for its well-known medicinal property in wound healing; group three was positive control treated with topical standard cream for wounds (Povidone iodine ointment;) and group four was the tested formula.

Results from this study showed the safety and efficacy of applying the tested formula in wound healing. This results were in accordance with other studies used honey for its anti-microbial and anti-fungal effects in wound treatment.[9,12] Therefore, the tested formula can be applied safely on wounds and should be tested on other types of wounds, such as burn wounds.

Macroscopically, excisional wound caused bleeding followed by clot formation on the surface of wounds. Histological and morphological examination of excisional wound in Hampshire pigs showed similar results with sings of bleeding and early infiltration of inflammatory cells.[20] Moreover, results showed that wound closure was accelerated in the tested

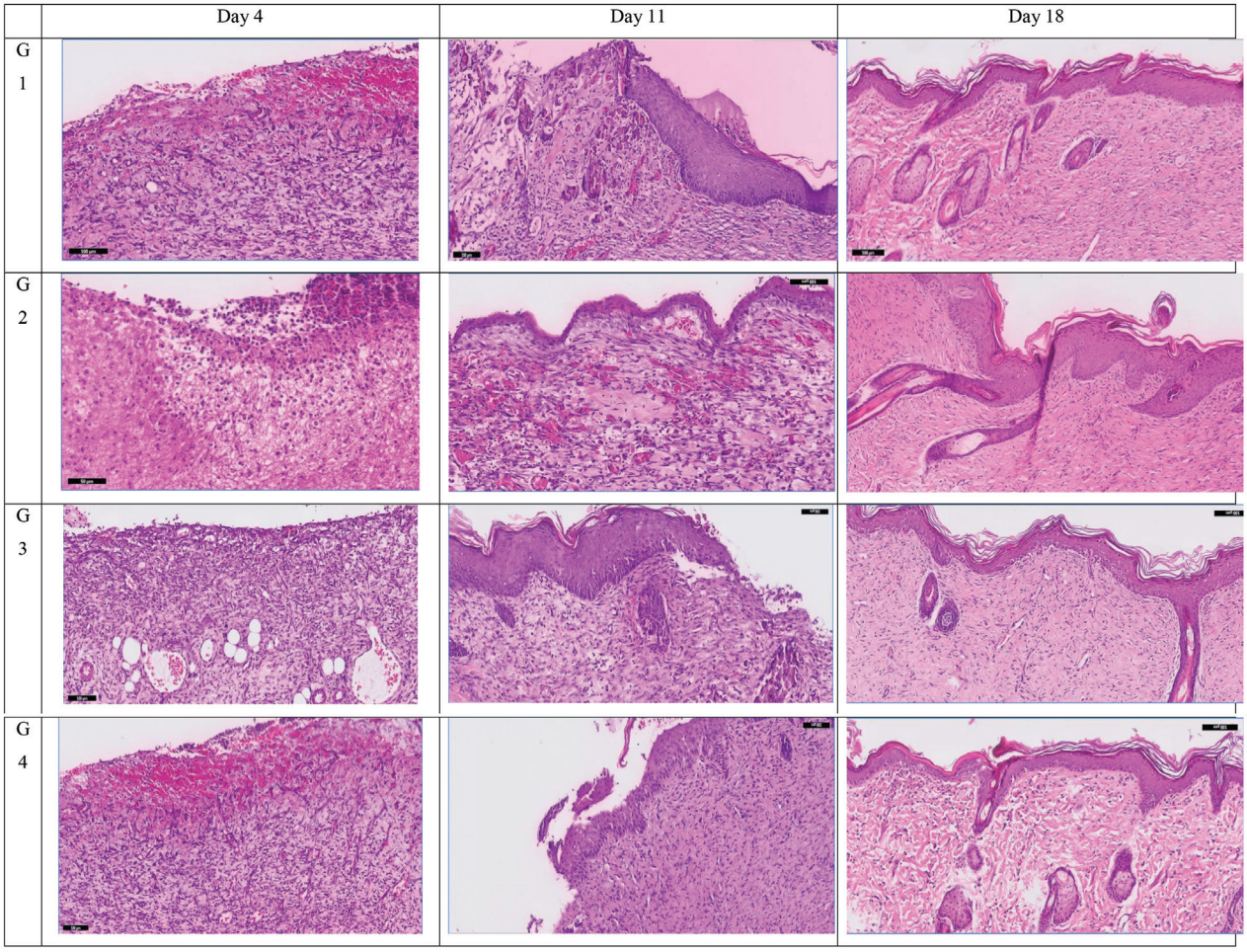

Fig. 2 **H & E staining of wounds in all groups. Scale bar = 100 µm.**

formula group compared to the third group (treated with Povidone iodine ointment) in day 7, which could represent the end of inflammatory and the beginning of proliferation phase. This could show that the formula reduces the inflammatory phase duration for its anti-inflammatory property and antibacterial properties. On day 4, puss formation, moist necrotic tissues, was noticed in rat from group two and from group four. This could show that honey-based formulas have similar healing mechanisms. By the end of the study all wounds were healed with different appearance of scars. This could be related to different mechanisms of healing properties between different treatment groups. Further studies are required to investigate the mechanism behind healing properties of the tested formula.

Histological examination showed early inflammatory infiltrate followed by formation of granulation tissues in all groups. Previous research indicated that honey enhance healing by stimulating collagen fibers formation, re-epithelization and neovascularization in rats.[21,22] Treatment with whey protein accelerated healing by decreasing free radicals and inflammatory cytokines in diabetic mice.[15,16] In day 18, complete healing was observed in all groups with formation of epithelium and hair follicles, collagen deposition and formation of capillaries. The thickness of the formed epidermis in the second group was thicker but not statistically significant compared to other groups. It was hypothesized that the epidermis's thickness correlated with scar formation or quality.[20,23] This could explain the appearance of scar in the second group compared to other groups.

Conclusion

Results from this study indicated the therapeutic potential of the tested formula, which contains natural component, in wound healing as using other therapeutic products. The tested formula is affordable for wide range of patients. Further studies are required to investigate the different mechanisms behind the therapeutic properties of each treatment.

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Nil.

Conflicts of Interest

There are no conflicts of interest.

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