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Efficacy of Talh Honey, Whey Protein, and Collagen Based Novel Formulation Against Wound-Associated Skin Microbiota

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Abstract

Objective: This study aims to evaluate the efficacy of a novel wound-healing formulation composed of honey, whey protein, and collagen against the skin microbiota associated with delayed wound healing.

Methods: A rat excisional model was employed to assess the wound-healing formulation. Pathogen contraction rates were measured on various days following the onset of wounds to investigate the formulation's impact on microbial infection. The formulation, known for its antimicrobial properties and rich in growth factors, was applied to promote cell proliferation, differentiation, and tissue repair.

Results: The tested wound-healing formulation demonstrated significant effectiveness in promoting the wound-healing process in rats. The pathogen contraction rates exhibited positive outcomes, indicating the formulation's potential in addressing microbial infections associated with delayed wound healing.

Conclusion: This study underscores the promising therapeutic potential of the wound-healing formulation comprising honey, whey protein, and collagen. The observed positive effects on microbial infection and wound recovery suggest that this formulation could serve as a valuable intervention in managing delayed wound healing, particularly in conditions such as diabetes, immunosuppressive treatments, and obesity.

Keywords: Wound healing, excisional wound, honey, whey protein, skin microbiota, collagen

Introduction

Wound healing is a dynamic and complex biological process characterizing skin and tissue recovery after the injury. Hemostasis, inflammation, proliferation, and remodeling are the four main phases of this process. Delayed or completely failed wound healing leads to chronic wound formation, which exerts an economic burden of over 25 billion USD/year. The rising prevalence of chronic diseases (diabetes) has significantly increased the number of such patients to more than 6.5 million/year.[1,2] Skin microbiota (fungi, viruses, bacteria, and archaea) infect the underlying tissues by penetrating through skin injuries. Fungal and bacterial infections are considered the main influential factors in delaying wound healing. Several studies have investigated the survivability, infectivity, and inter-microbial interactions of human skin microbiota.[3-6] The recent novel techniques have enabled us to reassess the human microbiota. High-throughput DNA sequencing can provide individual genomes of human microbiome. Furthermore, it facilitates the characterization of individual microbes and communities.[7] Genomic characterization of skin bacteria has revealed a broad diversity of microorganisms in comparison to traditional culture-based procedures.[8-10]

The debilitated skin allows exogenous microbiota to access the warm, humid, and nutrient-rich environment of the primary tissues.[11,12] The diversity of skin microbiota and cutaneous microenvironment (sebaceous, moist, and dry) could cause skin infection and affect the wound healing process.[11,13] The delayed healing alters the normal wound microbiota and favors the growth of more aggressive microbes.[11,14] The common applications of antibiotics have reduced honey usage in modern medicine.[15] However, the higher medicinal potential of honey can be useful against antibiotic-resistant bacteria.[15,16] Several studies have demonstrated numerous medicinal characteristics of honey without any reports of bacterial resistance.[15] Contrarily, chemical antibacterial agents often face microbial resistance, which could be detrimental to the wounded tissue.[17-19]

Protein plays a crucial protective and repairing role for the body tissues. The lower protein levels impair collagen development to further delay the wound healing process.[20] Whey proteins contain almost all the non-essential and essential amino acids and are considered "excellent nourishing protein" for humans.[21-25] Multiple studies have reported the advantages of whey protein in chronic wound treatment.[26-28] Collagen is crucial for each wound healing phase. The chemotactic features of collagen attract fibrotic cells for enhancing wound healing and guide the fibroblasts for better nucleation and vascularization. Furthermore, exogenous collagen participates in wound healing as the neutral salt molecules prefer fibrillation production.[29-30] Consequently, the fibrillated collagen network serves as a sponge to support the deposition of new collagen, which induces capillary growth and tissue repair.[30] Therefore, this study elaborates on the efficacy of a novel wound-healing formulation (honey, whey protein, and collagen) against skin microbiota associated with delayed wound healing.

Materials and Methods

Animals

The current study was conducted at King Fahd Center for Medical Research, King Abdul-Aziz University, Jeddah, KSA. Healthy male Wistar albino rats (24, 170–230 g) were used during the study. Rats were acclimatized for 7 days in an animal house before the initiation of the experiments. During the study, rats were kept under standard laboratory conditions

(25 ± 2°C, 12:12 h (light and dark cycle), and 44–56% relative humidity) and fed with a standard diet, water, and libitum. Animal Care and Use Committee (ACUC) approved the study protocol (Reference No 122–19). Rats were equally divided (n = 6) into four groups (Group I) negative control group without any treatment, (Group II) Manuka honey-treated rats, (Group III) positive control (rats treated with Povidone iodine ointment 5% w/w), and (Group IV, Tested group) rats treated with the prepared formulation.

Preparation of Wound Healing Formulation

The tested formulation was comprised of Talh honey (*Thymus vulgaris*), Whey protein, and Collagen. Whey protein (2 mg/ml), Talh honey (50%), and Collagen (1 mg/ml) were slowly added and mixed until completely dispersed and dissolved.

Excision Wound Model

Rats of all the groups were labeled and excision wound was individually induced in each animal. Briefly, depilatory cream was used to shave the rats' dorsum portion followed by disinfection with an alcohol-iodine solution. Xylazine (10 mg/kg) and Ketamine injection (50 mg/kg, intraperitoneal body weight) was used to anesthetize the rats, and the surgical area was marked. A circular (1 cm) full-thickness excision wound was induced using toothed forceps, a surgical blade, and pointed scissors. Wounds of the Group I (negative control) were left undressed throughout the experimental period. A saline solution was used to wash the wounds of Group II and covered them with Manuka honey. A saline solution was used to wash the wounds of Group III and covered with Povidone-iodine ointment (5% w/w). Similarly, a saline solution was used to wash the wounds of Group IV and covered with the prepared formulation of Talh honey, Whey protein, and collagen. Rats of all the groups were separately caged. All the treatments were performed on alternate days until the complete healing of the wounds.

Sample Collection for Bacterial Identification

The wound samples were collected at various intervals (days 2, 4, 7, 9, 14, and 18). The wounds were superficially pre-cleansed with distilled water and a sterile cotton swab was rotated across the wound surface from the center toward the outer side of the wound (zig-zag motion). Then, the swab was placed in a tube with a transport medium and sent to the Microbiology laboratory of King Fahd Medical Research Center for further culture analysis.

Sample Culturing

The wound samples were directly cultured in NB media (2 ml) and incubated for 24 hrs at 37°C. Then, the serial dilution method was performed with the incubated bacterial samples.

Storage of Pure Isolates

Purified bacterial colonies were inoculated in tubes containing NB medium (3 ml) followed by overnight shaking incubation. Then, 500 µl of these isolates was mixed with 500 µl of glycerol (50 %) and stored at –20°C until used (Add Gene Organization, 2020).

Bacterial Identification

Gram staining method

The gram-positive and gram-negative bacteria respectively produced a positive and negative reaction to the gram staining.

Biochemical catalase test

The catalase test differentiated the catalase-positive (staphylococci) and catalase-negative (streptococci) bacteria in the culture. To perform the test, several colonies from pure culture were added onto a clean microscope slide. Then, H_2O_2 (a few drops) was added and mixed by a loop. A rapid O_2 evolution confirmed the positive results.[31]

Molecular identification of bacterial isolates

The method of Azcárate-Peril and Raya (2001)[32] was followed to extract the total genomic DNA with minor modifications. An overnight NA-grown pure bacterial culture (1 ml) was transferred to a tube (1.5 ml) and centrifuged (10000 rpm) at 4°C for 5 min. The supernatant was discarded followed by the addition of TES buffer (200 µl) and vortexed. Then, lysozyme (20 µl, 10 mg/ml) was added and thoroughly mixed using a vortex. The mixture was incubated in a water bath for 1 hour at 37°C. Proteinase K (20 µl, 10 mg/ml) was added and mixed by vortexing. The mixture was again incubated in a water bath for 1 hour at 37°C followed by cooling for 5 min and sodium acetate (250 µl) was added. Then, the mixture was centrifuged (8000 rpm) for 5 min at 4°C. The supernatant was carefully transferred to a new Eppendorf tube. Chloroform: isoamyl (250 µl, 24:1) mixture was added and mixed by stirring between the fingers followed by centrifugation (8000 rpm) for 5 min at 4°C. Then, the aqueous phase was transferred to a new Eppendorf tube and an equal isopropanol volume was added. The mixture was overnight stored at –20°C. The solution was centrifuged (10000 rpm) for 5 min on the following day to discard the liquid zone. The pellet was dried for 10 min at room temperature and re-suspended in distilled water (50 µl). Gel electrophoresis was carried out to examine the DNA quality. The first lane contained the DNA ladder (1 kb). DNA samples (3 µl) were mixed with loading dye (1µl of 6X) and added to the wells. Gel electrophoresis was carried out at 120 V and bacterial DNA was examined under UV light.

PCR amplification of 16S rRNA genes

Bacterial DNA served as templates for the 16S rRNA gene amplification with universal bacterial Primers [Forward primer 5′-AGAGTTTGATCMTGGCTCAG-3′ (27) and Reverse Primer; 5′-GWATTACCGCGGCKGCTG-3′ (519)]. PCR mixture consisted of primers (2 µl of each forward and reverse primer (10 pmol)), Master Mix (25 µl) (GoTaq® Green Master Mix, 2X, Promega), DNA template (2 µl), and water to make a final volume of 50 µl. An Applied Biosystems™ Veriti™ 96-Well Thermal Cycler was used for the amplification. PCR conditions were adjusted as one cycle of initial denaturation (94°C for 5 min), 35 cycles (94°C for 30 sec, 58°C for 30 sec, and 70°C for 1.30 min), and final elongation at 70°C for 10 min. Gel electrophoresis

was carried out and a DNA ladder (100 bp) was used to compare the PCR products. A gel documentation system was used to observe the target bands under UV light. Macrogen services were acquired to sequence the PCR products and identify the isolated strains. MEGA (Molecular Evolutionary Genetics Analysis) software was used to perform the data analysis and sequences were compared at the NCBI database.

Results

16S rRNA gene sequencing revealed the nucleotide similarity with the known species in the NCBI database (97%–100% similarity ratio). The results revealed that rat-wound bacteria mainly belonged to five genera such as *Enterobacter*, *Staphylococcus*, *Enterococcus*, *Escherichia*, and *Bacillus*. Table 1 presents the bacterial species detected in Group I (control) wounds on various sampling days. *Staphylococcus aureus* and *Enterobacter ludwigii* were detected on day 2 whereas *Enterobacter cloacae*, *Staphylococcus aureus*, and *Enterococcus sp.*, were found on the 4th day. The presence of *Staphylococcus chromogenes, Enterococcus faecalis*, and *Staphylococcus aureus* was noted on the 7th day and *Staphylococcus aureus* and *Escherichia coli* were detected on day 9. The samples of the 14th day revealed the presence of *Staphylococcus saprophyticus*, *Staphylococcus sp.*, *Staphylococcus aureus,* and *Staphylococcus agentis* in rat wounds. *Staphylococcus aureus*, *Enterococcus massiliensis*, *Bacillus sp*., and *Staphylococcus chromogenes* were detected on day 18. Table 2 demonstrates the bacterial species detected in Group II (Manuka honey) wounds at different intervals. The presence of various bacterial species was noted on the sampling days such as day 2 (*Staphylococcus lentus, Staphylococcus agentis*, and *Staphylococcus chromogenes*), day 4 (*Enterococcus faecalis* and *Staphylococcus chromogenes*), day 7 (*Enterococcus faecalis*, *Staphylococcus chromogenes*, and *Enterococcus sp.*), day 9 (*Escherichia fergusonii*, *Escherichia coli* and *Staphylococcus aureus*), day 14 (*Staphylococcus sp.,* and *Staphylococcus chromogenes*), and day 18 (*Staphylococcus chromogenes* and *Enterococcus faecalis*).

Table 1. **Bacterial species in the control group at different intervals**

Sampling days	Bacteria	Gram stain
Day 2	*Enterobacter ludwigii*	Gram-negative
Day 2	*Staphylococcus aureus*	Gram-positive
Day 4	*Enterobacter cloacae*	Gram-negative
Day 4	*Enterococcus* sp.	Gram-positive
Day 4	*Staphylococcus aureus*	Gram-positive
Day 7	*Enterococcus faecalis*	Gram-positive
Day 7	*Staphylococcus aureus*	Gram-positive
Day 7	*Staphylococcus chromogenes*	Gram-positive
Day 9	*Staphylococcus aureus*	Gram-positive
Day 9	*Escherichia coli*	Gram-negative
Day 14	*Staphylococcus saprophyticus*	Gram-positive
Day 14	*Staphylococcus* sp.	Gram-positive
Day 14	*Staphylococcus agnetis*	Gram-positive
Day 14	*Staphylococcus aureus*	Gram-positive
Day 18	*Staphylococcus aureus*	Gram-positive
Day 18	*Enterococcus massiliensis*	Gram-positive
Day 18	*Staphylococcus chromogenes*	Gram-positive
Day 18	*Bacillus* sp.	Gram-positive

Table 2. **Bacterial species in the Manuka honey group at different intervals**

Sampling days	Bacteria	Gram stain
Day 2	*Staphylococcus agentis*	Gram-positive
Day 2	*Staphylococcus chromogenes*	Gram-positive
Day 2	*Staphylococcus lentus*	Gram-positive
Day 4	*Enterococcus faecalis*	Gram-positive
Day 4	*Staphylococcus chromogenes*	Gram-positive
Day 7	*Staphylococcus chromogenes*	Gram-positive
Day 7	*Enterococcus* sp.	Gram-positive
Day 7	*Enterococcus faecalis*	Gram-positive
Day 9	*Escherichia fergusonii*	Gram-negative
Day 9	*Escherichia coli*	Gram-negative
Day 9	*Staphylococcus aureus*	Gram-positive
Day 14	*Staphylococcus* sp.	Gram-positive
Day 14	*Staphylococcus chromogenes*	Gram-positive
Day 18	*Enterococcus faecalis*	Gram-positive
Day 18	*Staphylococcus chromogenes*	Gram-positive

Table 3. **Bacterial species in the standard group at different intervals**

Sampling days	Bacteria	Gram stain
Day 2	*Staphylococcus chromogenes*	Gram-positive
Day 4	*Staphylococcus aureus*	Gram-positive
Day 4	*Staphylococcus chromogenes*	Gram-positive
Day 7	*Staphylococcus chromogenes*	Gram-positive
Day 7	*Enterococcus faecalis*	Gram-positive
Day 9	*Staphylococcus aureus*	Gram-positive
Day 9	*Enterococcus faecalis*	Gram-positive
Day 9	*Bacillus* sp.	Gram-positive
Day 14	*Enterococcus faecalis*	Gram-positive
Day 18	*Enterococcus faecalis*	Gram-positive

Table 3 reveals the presence of bacterial species in Group III (standard drug) wounds at different intervals. *Staphylococcus chromogenes* was noted on day 2 whereas *Staphylococcus chromogenes* and *Staphylococcus aureus* were found on the 4th day. *Staphylococcus chromogenes* and *Enterococcus faecalis* were found to infect the rat wounds on day 7 and *Bacillus sp*., *Staphylococcus aureus*, and *Enterococcus faecalis* were detected on the 9th day. Days 14 and 18 featured the presence of only *Enterococcus faecalis*. Table 4 depicts the bacterial presence in Group IV (tested formulation) wounds at different intervals. The sampling days exhibited bacterial presence on day 2 (*Enterobacter asburiae*), day 4 (*Enterococcus sp*., *Bacillus cereus*, and *Enterobacter cloacae*), day 7 (*Staphylococcus chromogenes*

Table 4. **Bacterial species in the tested formulation group at different intervals**

Sampling days	Bacteria	Gram stain
Day 2	*Enterobacter asburiae*	Gram-negative
Day 4	*Bacillus cereus*	Gram-positive
Day 4	*Enterobacter cloacae*	Gram-negative
Day 4	*Enterococcus* sp.	Gram-positive
Day 7	*Staphylococcus chromogenes*	Gram-positive
Day 7	*Enterococcus* sp.	Gram-positive
Day 9	*Staphylococcus lugdunensis*	Gram-positive
Day 9	*Enterococcus faecalis*	Gram-positive
Day 14	*Staphylococcus rostri*	Gram-positive
Day 14	*Staphylococcus aureus*	Gram-positive
Day 18	*Enterococcus faecalis*	Gram-positive
Day 18	*Staphylococcus chromogenes*	Gram-positive

and *Enterococcus sp.*), day 9 (*Staphylococcus lugdunensis* and *Enterococcus faecalis*), day 14 (*Staphylococcus rostri* and *Staphylococcus aureus*), and day 18 (*Staphylococcus chromogenes* and *Enterococcus faecalis*).

Discussion

Wound healing is a multiphase complicated process in animals and humans. Different phases are referred to as hemostasis, inflammatory, proliferation, and remodeling phases.[33,34] Diseases (obesity, diabetes, arterial and venous diseases) and other factors (smoking, microbial infection, and old age) severely impair the wound-healing process. Delayed wound healing becomes prone to bacterial infections. The development of honey, whey protein, and collagen-based novel antimicrobial formulation could enhance the wound healing process. During this study, the presence of different bacterial species (16) was detected in the rat wounds. Most of the bacteria were gram-positive and *Staphylococcus chromogenes, Staphylococcus aureus*, and *Enterococcus faecalis* were commonly detected. The predominant presence (40–60% of the total microbial population) of *Staphylococcus aureus* in wounds has been reported in multiple studies.[35-40] *Staphylococcus aureus* is a common part of the normal healthy skin microbiota and is an opportunistic pathogen of immunocompromised individuals.[41,42] Higher morbidity and mortality rates are associated with this pathogen in tropical countries.[43] *Enterococcus faecalis* is also commonly detected in all wound types.[44-46] *Staphylococcus chromogenes* colonizes human and animal mucous membranes and skin.[47]

Other identified gram-positive bacteria were related to skin microbiota. *Staphylococcus saprophyticus* is a non-hemolytic coccus and coagulase-negative gram-positive opportunist bacteria of skin microflora.[48,49] *Staphylococcus lentus* is a *Staphylococcus sciuri* group-associated coagulase-negative bacteria.[50] It is a component of the normal mucosal and skin flora in various animals and can also colonize wound infections and humans.[50-54] *Staphylococcus lugdunensis*, a coagulase-negative bacteria, is a part of normal skin flora.[55] The presence of *S. lugdunensis* has been reported in cutaneous abscesses (13%), pustules (4.5%), and other infections (≤1%) including incisional surgical and unspecified wound infections and impetigo.[56,57] Wound-colonizing gram-negative bacteria such as *Escherichia fergusonii*, *Enterobacter cloacae*, and *Escherichia coli* were also detected in rat wounds during the current study. *Enterobacter cloacae* is a non-spore-forming, rod-shaped, and facultatively anaerobic gram-negative bacteria that belongs to the *Enterobacteriaceae* family.[58] *E. cloacae* is associated with a wide range of wound infections.[59] The gram-negative *Escherichia coli* can severely infect the surgical sites and joins other bacteria to colonize open wounds.[60-64] Rod-shaped gram-negative *Escherichia fergusonii* is a member of the *Enterobacteriaceae* family, which is associated with human wound infections.[65-67]

Conclusion

The results of rat-model experiments revealed the effectiveness of the tested formulation in promoting the wound-healing process. Talh honey and whey protein did not cause any side effects during the experimental period and honey's anti-microbial properties efficiently prevented bacterial infection during the healing process.

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Nil.

Conflicts of Interest

There are no conflicts of interest.

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