Wound Medicine 20 (2018) 7–17

Contents lists available at ScienceDirect

Wound Medicine

journal homepage: www.elsevier.com/locate/wndm

Original research article

Modulation of collagen population under honey assisted wound healing in diabetic rat model

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ARTICLE INFO

Keywords:
Diabetic wound
Honey
Collagen organization
Collagen glycation
Mechanobiology

ABSTRACT

Background: Diabetic wound with impaired healing attributes owing to inappropriate synthesis and alignment of collagen fibrils with aberrant post translational modifications impose substantial economic burden. Honey: a recognized natural collagen pro-synthetic healing agent; stimulates rapid healing with its anti-inflammatory and anti-scarring approach. Present study investigates the effect of honey on collagen population in diabetic wound model.
Methodology: Full thickness wound was created on rat and divided into three groups: normal saline treated group (N), diabetic honey treated group (H), diabetic povidone iodine treated group (PI). Biopsies were collected from wound edges for collagen characterization.
Main findings: Histological attributes of collagen fibers in N and H group biopsy displayed proximity. Ultrastructure analysis using scanning electron microscope (SEM) revealed that the D-Spacing and collagen diameter under honey treatment were in close resemblance to normal skin collagen. FTIR spectra confirmed secondary structure of collagen with a greater peak height in PI group exhibiting excessive glycation than H group. Nano-indentation profiling showed decreased displacement in PI samples resulting from increased rigidity when compared to honey treated ones. Molecular expression of collagen I and III exhibited optimum ratio in H group similar to N group.
Conclusions: Based on these findings, it might be opined that honey predominantly helps in regulating synthesis, glycation and deposition followed by mechanical quality of collagen alike normal skin and its application also accelerated diabetic healing process.

1. Introduction

The rising frequency of diabetes mellitus contributes to a considerable share of global burden of diseases and reflects a major health complication for the 21st century [1]. People suffering from this chronic metabolic disorder have a 15%–25% chance of developing a diabetic ulcer during their lifetime with 50%–70% recurrence rate over the ensuing 5 years [2,3]. The identification of the underlying mechanisms of delayed healing and the quest for preventive or therapeutic strategies to address the concern is globally a demanding and continuous challenge. With no effective therapy in sight, it is a high priority to explore the new management options.

Wound healing is imputed as a complex of superimposed intrinsic and extrinsic factors that induce cumulative structural, biochemical, functional and eventually aesthetical restoration of tissue integrity. This end is acquired by the formation of connective tissue matrix [4]. Collagen is the main component that furnishes connective tissues with mechanical integrity and contributes to wound strength. In skin, collagen is produced mainly by fibroblasts in the dermis with type I and type III being the most predominant (80% type I collagen and 20% type III) [5]. The structural hierarchy of collagen tissue represents a triple helix molecule of approximately 300 nm as the basic unit. These molecules self-assemble axially in a regular pattern to form a D-periodic cross-striated fibril which are then bundled together into a collagen fiber [6]. The tight wrapping of the triple helix equips tensile strength greater than that of a steel wire of equal cross section [7]. Thus collagen organization provides a unique combination of elasticity and strength in skin.

A critical stimulus for diabetic wound healing is precise arrangement of collagen fibrils into ordered assembly with optimized post

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https://doi.org/10.1016/j.wndm.2017.12.001
Received 12 April 2017; Received in revised form 25 September 2017; Accepted 6 December 2017
Available online 07 December 2017
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translational modification. According to glycation hypothesis, hyperglycemia may cause complications through increased glycation [8] which leads to abnormalities in turnover rate, receptor recognition, enzyme activity and physicochemical properties of the wide variety of proteins and that over time these abnormalities sufficiently predispose to disease development [9]. Similarly during the course of diabetes, the collagen fibers encounter various physicochemical changes including a reduced flexibility, considerable resistance to enzymatic digestion, altered ligand binding activity and other suprastructural adjustments. As normal collagen crosslinking is a prerequisite to stabilize fibrils and provide distensibility, elasticity and mechanical strength, uncontrolled cross-links formed in diabetic collagen may have significant damaging effects [10].

The medicinal efficacy of honey in wound treatment is extracted from diverse ancient civilization [11]. It has more recently been rediscovered by medical researchers for its application in acute and chronic wounds [12]. Honey is commonly known as collection of nectars processed by honey bees. Treatment of wounds with honey is found to trigger remarkable increase in entire collagen content of the granulation tissues [13]. The amino acids (arginine and glutamic acid) in honey act as a precursor of proline resulting in enhanced collagen synthesis. Honey predominantly contains glucose, fructose and water [14]. Sugars in honey may act as fuel for ATP synthesis through glycolytic pathway, fulfilling fibroblast's energy requirement for the synthesis of collagen. Post synthetic alteration of collagen includes the formation of cross-links between peptide chains using enzyme lysyl oxidase forming aldehyde from lysyl and hydroxylysyl residues, which condense with other residues such as lysine, hydroxyproline and histidine to construct intra and inter molecular cross-links. Fe^{++} and ascorbic acid are imperative for the enzymes prolyl and lysyl hydroxylases and cupric ion (Cu^{++}) is necessitated by the enzyme lysyl oxidase, which endorse hydroxylation and cross-linking of collagen [15,16]. The quick maturation of collagen fibers may be stimulated by the presence of iron, copper, and ascorbic acid in honey. Furthermore, honey with its anti-inflammatory influence prevents scarring and hypertrophication [17] stimulating re-epithelialization phases to induce the 'partial regeneration' of cutaneous tissue [18]. Thus topical application of honey may serve as an effective strategy in wound healing with its energy-yielding, hygroscopic and bactericidal properties [19,20].

The present study investigates the effect of physicochemically characterized honey on major collagen population in diabetic wound model.

2. Materials and method

2.1. Physico-chemical characterization of honey sample

Multifloral honey was procured from local vendors of Medinipur district (West Bengal) and was physico-chemically characterized.

2.1.1. pH and conductivity measurement

pH and electrical conductivity of honey samples were quantified using pH and conductivity probe (Thermo Scientific, USA).

2.1.2. Water and total solid content measurement

Water content was measured using the following formula:

$$\text{Moisture (\%)} = (w_1 - w_2)/w_1 \times 100$$

Honey was weighed before [w_1 (g)] and after drying [w_2 (g)] (105 °C for 3 h).

Percentage of total solid content was determined using the formula:

$$\text{Total solid (\%)} = 100 - \text{moisture content}$$

2.1.3. Estimation of total phenolic and carbohydrate content

Total phenol content of the honey sample was determined by the Folin–Ciocalteau method using gallic acid as standard. Total amount of carbohydrate present in honey were estimated by anthrone method where dextrose was used as standard.

2.1.4. Fourier transform infrared analysis

FTIR analysis was performed using a 0.05 mg of honey on KBr pellets in a Nicolet 6700 spectrometer (Thermo Fisher, USA) with a frequency range of 400–4000 cm^{-1}. All measurements were done in absorbance mode at room temperature with controlled humidity conditions.

2.2. Animal model preparation

Healthy Swiss albino Wister male rats (Rattus norvegicus), 20 in number, having body weight 200–250 g and 6–7 weeks old were used as per Institutional Ethics Committee guidelines. Prior to experiment, animals were acclimatized for 7 days under controlled environmental conditions with alternate light dark cycle and access to food and water at will. All the chemicals were procured from Sigma Aldrich and were used without further modifications.

Diabetes mellitus was induced by intraperitoneal injection of freshly prepared 65 mg/kg of Streptozotocin (0.1 M, Sodium Citrate buffer) after overnight fasting of animals. After 3 weeks of injection, blood glucose measurement was performed on tail vein blood by using glucometer (Accu-Chek Aviva Nano, Roche Diagnostics, Germany) to confirm hyperglycemia. Rats whose blood glucose level exceeded 250 mg/dL (13.9 mmol/dL) were considered diabetic [21].

Animals were anaesthetized with intramuscular injection of Ketamine/Xylazine hydrochloride (90 mg/kg and 10 mg/kg) before surgical positioning for full thickness wound (6 mm diameter) creation by punch biopsy on the dorsum. Rats were randomly divided into three groups of 5 animals each: a normal saline water treated group (N), a diabetic honey treated group (H), a diabetic povidone iodine treated group (PI). Wounds were treated with physicochemically characterized honey [22] and povidone iodine in diabetic honey treated group and diabetic povidone iodine treated group respectively daily until complete healing.

2.3. Wound area measurement

The wound site of each animal was imaged with a digital camera and wound area was measured to comparatively evaluate the healing efficacy of honey at different time points (0, 5 and 10 days) using Image J software. The data from each animal was obtained as the mean of triplicate measurements.

2.4. Histopathological and immunohistochemical studies

Incisional biopsies were collected from wound edges of animals from each group under anaesthesia at different time intervals.

2.4.1. Tissue processing

The biopsy specimens were fixed in phosphate buffered formalin and after embedding in paraffin, tissue sections of 4 μm were collected on albumin and poly-L-lysine (Cat. No. P 8920 Sigma-Aldrich, St. Louis, MO, USA) coated glass slides for histological and immunohistochemical studies respectively.

2.4.2. Hematoxylin & eosin staining (H&E)

After deparaffinization, biopsy sections were rehydrated for staining with H&E. Then the sections were dehydrated through graded alcohol and finally mounted.

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