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Evaluation of angiogenesis in diabetic lower limb wound healing using a natural medicine: A quantitative approach

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ABSTRACT

Increasing incidents of diabetes (mellitus) induced non-healing lower extremity wounds and disease associated amputations have raised significant concerns related to quality of life of afflicted patients. High glucose level in diabetic wounds inhibits the transactivation of angiogenesis related molecules resulting delayed healing progression. Present study investigates the impact of a natural medicine like honey in angiogenesis of non-healing diabetic lower limb wounds. Quantitative assessment of different vessel parameters was performed on *in vitro* CAM model for validation of angiogenic potential of honey. Further the upregulation of angiogenesis related prime molecular markers like HIF-1α, VEGFA, VEGFR2 is under the therapeutic intervention of honey indicated improved angiogenesis which in turn promote the healing rate. These results may facilitate in determining the healing impact of this natural product in treatment of diabetic wounds and it may also help in developing alternative cost effective therapeutic modality.

1. Introduction

Successful management of diabetic chronic foot ulceration is an unmet clinical challenge and often leads to disease-associated amputations. In diabetic patients, impaired healing process has been attributed to micro and macro vascular alterations causes peripheral neuropathy and tissue hypoxia while abnormalities in inflammatory pathways lead to development of infectious non-healing foot wounds [1]. In such situations, besides anti-bacterial activity, stimulation of angiogenesis becomes necessary pre-requisite to promote adequate healing. Though hypoxic chronic wound ambience induces hypoxic-inducible factors-α (HIFα) that stimulates new blood vessels formation, high glucose level in diabetic wound prevents transactivation of HIF-1α and impairs angiogenesis by blocking the transcription of cascade of angiogenic activators like vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), stromal-derived factor-1(SDF-1), *etc.* [2,3]. Dysfunction of HIF-1α is majorly caused by reactive oxygen species which modify its co-activator p300 [4].

In wound beds angiogenesis is mainly controlled by the balance between angiogenic (like VEGF) and anti-angiogenic factors (like endostatin) [5]. VEGF signaling is a critical rate limiting step in sustained neo-vascularization. Its isoforms especially VEGF-A stimulates the angiogenesis process in paracrine manner after cutaneous injury and have been detected on the blood vessels of newly formed granulation tissues [6]. VEGF signaling is interceded by activation of transmembrane tyrosine kinase receptors – VEGFR-1 and VEGFR-2. During angiogenesis VEGF binds to VEGFR-2 leading to formation of new vasculature [7].

To manage chronic diabetes complications, different therapeutic strategies have been employed [8–10]. However, no single therapy have been shown to adequately support the multi-dimensional requirements of diabetic wound beds. In this direction, there is relentless search for appropriate therapeutic modalities that promote rapid healing as well as substantially provide a cost effective holistic support. Different alternative approaches for diabetes therapy includes various herbal preparations, dietary components and other natural products [11]. Amongst them, honey has been used as natural therapeutic agents as wound dressing for over 100 years. Its healing role as a topical agent for both infected and non-infected wounds [12] has been reported. In last few years, increased evidence-based reports on

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beneficial effects of honey encourage its therapeutic applications in diverse disease conditions including diabetes mellitus [13]. Amongst other therapeutic activities of honey, broad spectrum antimicrobial effects of honey are variably ascribed to acidic pH (3.2–4.5), H_2O_2 content and osmotic properties in addition to role of other components in low doses [14,15]. Different *in vitro* as well as *in vivo* studies have revealed interesting results that administration of honey along with other anti-diabetic drugs (like glibenclamide or metformin) can more efficiently reduce the serum glucose level in diabetic rats [16]. Moreover, honey is known to substantially decrease the serum fructosamine concentration which is not observed with standard anti-diabetic drugs. Combination of honey and anti-diabetic drugs are shown to improve the antioxidant defenses and reduce oxidative damage [17] which has impact in angiogenesis. Hydrogen peroxide present in honey plays stimulatory role in angiogenic which further facilitates granulation tissue formation. Cumulative effect of low pH and angiogenesis help to release oxygen in wound bed that stimulates tissue regeneration process [14,18]. Clinical evidences confirm rapid healing impact of honey with substantial angiogenesis role in diabetic wound repair process. However lack of scientific validations limits its application in contemporary medicine. Though some recent *in vitro* and *in vivo* studies have been scientifically interpreted the healing mechanism of honey but as the molecular basis mainly examined in animal models, they have a limited translational potential. Moreover, as origin of honey is a vital issue for quality control and to ensure consistent healing outcome, hence quantitative validation is necessary for developing a standard assessment process. This study intends to bridge the gaps between experimental outcome and clinical observations through quantitatively analyzing the different aspects of angiogenesis and expressions of related molecular markers under honey in lower limb diabetic wounds.

In this current study we sought to evaluate the angiogenic potential of physico-chemically characterized honey on treatment of non-healing diabetic foot wounds. In order to examine the vascular adaptation of this therapeutic agent, in *vitro* CAM assay has been performed and quantitative evaluation of vessel structure may predict the stimulatory role of honey in neo-vascularization. Further the modulation in the expressions of angiogenesis related prime molecules (like HIF1α, VEGF-A, VEGFR-2) during healing of diabetic wounds under therapeutic intervention of honey may provide scientific validation of its well known healing potential. However, further studies are required to elucidate in depth molecular mechanism that may answer the angiogenesis paradox of diabetic wounds and to establish therapeutic impact of honey in diabetic wound healing.

2. Materials and methods

2.1. Collection of honey

Honey (ripe and dark amber color honey) collected from bee keepers of greater Kolkata, India [19].

2.2. Physico-chemical characterization

2.2.1. pH, electrical conductivity and water content assessment

Electrical conductance and pH of honey was measured at 37 °C temperature with pH-conductivity meter (420A, Orion, UK). For pH measurement ROSS ultra glass combination glass electrode was used (Orion 8102BNUWP). Electrical conductivity was determined through Orion DuraProbe 4-Electrode Conductivity Cells (013605MD) with 0.55 cm^{-1} cell constant.

To measure the free water content, frozen honey was vacuum dried by lyophilizer (Laboratory freeze dryer, IIC Indus. Corp.). Water loss of the sample was measured at different time intervals (*i.e.*, 30, 60, 90, 120 min) up to the constant weight reached.

2.2.2. Estimation of total phenolic content

Total phenolic content in honey was determined by the method using Follin–Ciocalteu's phenol reagent (Fluka analytical, Germany). Aqueous solution of raw honey (*i.e.*, 5 g of honey dissolved in 50 ml of distilled water) was initially filtered (millipore filter −22 μm) and 500 μl of diluted honey was treated with 2.5 ml of Follin–Ciocalteu's phenol reagent (0.2 N) for 5 min. 2 ml sodium carbonate solution (75 g/l) was added to the above mixture and incubated for 2 h at room temperature in dark [20]. Absorbance at 760 nm was measured through UV–vis spectrophotometer (V-1601, UV-Visible Spectrophotometer, Shimadzu, Japan) against the blank methanol without honey. The concentration of phenolic compound was determined from the standard curve of gallic acid.

2.2.3. Estimation of catalase enzyme activity (H_2O_2 activity) and DPPH radical scavenging activity

For determination of catalase activity, 2 ml of 0.02 M hydrogen peroxide (H_2O_2) was added in 0.5 ml of honey dilutions (v/v in 2.5 ml PBS). 1 ml of this mixture was further mixed with 2 ml 5% potassium dichromate/acetic acid solution at time intervals of 0, 60 and 120 s. On addition of potassium dichromate, the enzyme begins to react with the honey forming a deep blue color and also bubbling of the liquid was seen due to the breakdown of hydrogen peroxide into hydrogen and oxygen. The solution was incubated in a boiling water bath for 10 min and OD was taken at an absorbance of 620 nm.

Radical scavenging activity was determined by 2,2-diphenyl-1-picryylhydrazyl (DPPH). 0.5 ml of different honey dilutions, 1 ml of methanol and 100 μl of DPPH was added to test tubes. Blank was prepared by adding 0.5 ml PBS, 1 ml of methanol and 100 μl of DPPH. The tubes were kept in the dark and absorbance was takes after 60 min at 517 nm in UV–visible spectrophotometer. Ascorbic acid was used as positive control. Changing of color indicates the reducing activity of DPPH to DPPHH. Yellow color indicated the scavenging efficiency of samples.

$$\text{Scavenging activity in } \% = A - B/A \times 100$$

A – is the absorbance of DPPH; B – is the absorbance of DPPH and honey combination.

2.3. Biological characterization

2.3.1. Anti-microbial activity

Bacterial strains like *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Klebsiella pneumonae* and *Escherichia coli* were collected from B S Medical College, West Bengal, India. Disk diffusion test and minimum inhibitory concentration (MIC) assays were performed to determine the anti-bacterial activity of selected honey against these bacterial strains.

In disk diffusion technique, 100 μl of the standard bacterial nutrient broths of above mentioned isolates (cultures of the isolates at the density of 1.5×10^8 CFU/ml) were spread on Muller Hinton agar (HiMedia) plates. Honey impregnated filter paper disk (8 mm in diameter) was placed on each types of bacteria inoculated plates. Commercially available tetracycline disk (30 mcg) and sterilized distilled water were set as positive and negative control respectively. Plates were incubated at 37 °C for 24 and 48 h under aerobic condition. After incubation period, the inhibition zones around discs were measured in millimeters (mm). Tests were performed in triplicate with fresh subcultures.

In MIC assay, honey was serially diluted with de-ionized sterilized distilled water [concentration of 70%, 60%, 50%, 40%, 30%,