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Naringin, a flavanone glycoside, promotes angiogenesis and inhibits endothelial apoptosis through modulation of inflammatory and growth factor expression in diabetic foot ulcer in rats

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ABSTRACT

Chronic, unhealed diabetic foot ulcer (DFU) is one of the most severe complications of diabetes mellitus (DM). Naringin, a flavanone glycoside antioxidant, was reported to have antidiabetic and anti-apoptotic properties. In the present study DM was induced experimentally by streptozotocin (STZ, 55 mg/kg, i.p.). In surgically introduced wounds on the dorsal surface of the hind paw of rats, the healing potential of naringin was investigated. Rats were treated with naringin (20, 40 and 80 mg/kg, p.o.), insulin (10 IU/kg, s.c.) and tetrachlorodecaoxide (TCDO) (1 drop, twice a day, topically) for 16 days. The wound area was measured every second day, and on day 17 various biochemical parameters were determined in serum, wound tissue, and histopathological examination of the wound was performed. Naringin (40 and 80 mg/kg) significantly ($P < 0.05$) improved wound area, serum glucose level, glycated Hb and serum insulin. Naringin treatment at 40 and 80 mg/kg resulted in significant ($P < 0.05$) up-regulation of mRNA expression of growth factor (IFG-1, TGF-β and VEGF-c), Ang-1 and collagen-1 whereas mRNA expression of inflammatory mediators (TNF-α, IL-1β and IL-6) was down-regulated. Furthermore, naringin significantly ($P < 0.05$) attenuated STZ-induced apoptosis and stimulated angiogenesis in the wound tissue. Further results suggest that angiogenesis was improved via naringin-mediated inhibition of hyperglycemia, oxidative stress, down-regulation of inflammatory mediator expression and up-regulation of growth factor expression, leading to improved wound healing of DFU.

1. Introduction

Diabetes mellitus (DM) is a serious threat for global health and its projected prevalence is around 366 million in 2030 worldwide [11,29]. DM is a complex metabolic disorder involving various organs leading to development of both macro- and micro-vascular complications, including cardiomyopathy, neuropathy, nephropathy, retinopathy and foot ulcer [53]. Amongst the various complications of DM, neuropathy and/or vasculopathy are the two major determinants of delayed wound healing [51] which is the most notorious health problem in clinical practice as most of the diabetic patients develop early diabetic neuropathy and have loss of pain sensation in the lower limbs resulting in unidentified wounds until significant infection has occurred. Healing of a diabetic wound once it developed is very difficult, which may be due to vascular ischemia and various metabolic abnormalities [17]. It has been reported that more than 15% of the diabetic patients suffer from foot ulceration and more than 3% will undergoes lower limb amputation [3]. With increase in the incidence of wound complications along with metabolic abnormalities, DM patients also suffer from general surgical risks [4].

Wound healing is a well-orchestrated and highly controlled multifactorial process characterized by four distinct overlapping phases including hemostasis, inflammation, granulation and tissue remodeling [63,77]. During the wound healing process, coordination of

Abbreviations: Ang-1, angiopoietin-1; DM, diabetes mellitus; DFU, diabetic foot ulcer; DWC, diabetic wound control; EGF, epidermal growth factor; FITC Annexin V, fluorescein isothiocyanate Annexin V; HbA1c, glycated hemoglobin A1c; H&E, hematoxylin and eosin; HDL, high-density lipoprotein; IGF-1, insulin-like growth factor-1; LDL, low-density lipoprotein; MPO, myeloperoxidase; N (20), naringin 20 mg/kg treated; N (40), naringin 40 mg/kg treated; N (80), naringin 80 mg/kg treated; ND, normal non-diabetic; NWC, normal wound control; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; GSH, reduced glutathione; RT-PCR, reverse transcriptase polymerase chain reaction; SEM, standard error of mean; STZ, streptozotocin; SOD, superoxide dismutase; TCDO, tetrachlorodecaoxide; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

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blood cells, endothelial cells, fibroblasts, keratinocytes, growth factors and cytokines play a vital role in the rate of wound repair. Although the mechanisms behind delayed wound repair in diabetic patients are not completely understood, any disruption in coordination of these cells or growth factors and cytokines may delay the wound healing process [75]. Clinical and experimental studies demonstrated the delayed character of the wound healing process in diabetic foot ulcer (DFU). It has been documented that release of reactive oxygen species (ROS) promoted apoptosis in the damaged tissue and delayed the wound-healing process [21]. Furthermore, reduced production of various growth factors including transforming growth factor-β (TGF-β), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF), or reduced collagen deposition and delayed inflammatory response, play an essential role in the delay of wound healing in DM. As per the report of The Diabetes Control and Complications Trial (DCCT) and the UK Prospective Diabetes Study (UKDPS), a tight glycemic control significantly decreases the risk of microvascular and neurological long-term complications in diabetic patients, which may help accelerate the rate of wound contraction.

The medical treatment of DFU is a major clinical problem as it is associated with some pathological conditions such as hemodynamic abnormalities, hypoperfusion, abnormal angiogenesis and neuronal ischemia, or extrinsic factors due to infection and continued trauma that delays wound healing [35]. Hence, DFU cannot be overcome by single means and there is a paucity of the standard drug treatment for it. Removal of dead, damaged, or infected tissue to improve the healing potential as well as use of antibiotics, tissue grafts, proteolytic enzymes and corticosteroids are some of the therapies that have been currently employed for the treatment of diabetic wounds. However, these therapies provide relief only to a fraction of patients and side effects limit their use [87]. The research carried out over last few decades suggests the use of various growth factors such as TGF-β, EGF, PDGF, IGF-1 and VEGF to treat DFU as they accelerate wound healing via improving cell mitosis, migration and neoangiogenesis [10]. However, this may cause improper wound healing and therefore they neither are nor routinely used. In general, cases of chronic, non-healing DFU are associated with increasing costs besides significantly affecting patients quality of life [12]. Hence, there is a need for new therapeutic agents with minimal side effects.

Now a days, plant-derived natural products with potential hypoglycemic effects received greater acceptance and prompt evidence-based clinical studies in the standard wound care practices. Plant-derived active principles including triterpenes, alkaloids, and flavonoids have been shown to possess the wound healing potential by influencing one or more phases of the healing process. The effectiveness of various herbs in promoting the healing of chronic DFU has been proven clinically. In various randomized, double-blind, placebo-controlled trials, ANGIPARS™ (herbal preparation of *Melilotus officinalis*) [61], polyherbal formulation cream (composite of *Glycyrrhiza glabra*, *Musa paradisiaca*, *Curcuma longa*, *Pandanus odoratissimus*, *Aloe vera* and *Cocos nucifera* oil) [86] as well as two Chinese herbal formulae [59] showed remarkable clinical outcomes in treatment of DFU.

Animal models have greater clinical relevance and played a vital role in the development of new therapeutic entities for DFU [50]. STZ-induced DFU in rats is a reproducible model that exhibits clinicopathological features of DFU such as hyperglycemia, elevated oxidative stress, ischemic and hypoxic factors, nerve growth factor anomalies, activation of polyol pathway and immunologic abnormalities that contribute to microvascular disease and neural dysfunction [33].

Naringin (4′,5,7-trihydroxy flavanone 7-rhamnoglucoside), a flavanone glycoside, isolated from the grape and citrus fruit species, contains an array of immense therapeutic potential [1]. Naringin is reported to possess anti-inflammatory, anti-oxidant, anti-ulcer, anticancer, antiatherogenic, hepatoprotective, and neuroprotective activities [1,7]. It is a potential metal-chelating as well as free radical scavenging agent [20]. It has been shown to modulate expression of tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) that found to be involved in the pathogenesis of lung injury and pulmonary fibrosis [19]. A report of Kumar et al. showed that naringin ameliorates experimental colitis via preventing DNA damage [55]. Moreover, two weeks oral administration of naringin (80 mg/kg) significantly inhibited 3-nitropheniolic acid-induced neuronal apoptosis in rats via inhibition of Bax-Bcl-2 pathway [30]. It has potent cardioprotective and renoprotective activity *in vivo* and *in vitro* [70,78]. A study carried out in our laboratory showed promising results obtained with naringin ointment (4% w/w) in terms of promotion of wound healing in excision and incision wound model in non-diabetic rats [40]. However, not much is known about its role in wound healing in diabetes. Hence, in the present study, the wound healing potential of naringin for DFU was investigated in an animal model by assessing changes in growth factor expression, angiogenesis, and tissue regeneration along with histopathology.

2. Material and method

2.1. Animals

Adult male Sprague Dawley rats (180–220 g) were purchased from the National Institute of Biosciences, Pune (India). They were housed at 24 ± 1 °C, with relative humidity of 45–55% and 12:12 h dark/light cycle. The animals had free access to standard pellet chow (Pranav Agro Industries Ltd., Sangli, India) and filtered water throughout the experimental protocol. All experiments were carried out between 09:00 and 17:00 h. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy, and performed in accordance with the guidelines of Committee for Control and Supervision of Experimentation on Animals (CPCSEA).

2.2. Chemicals

Naringin and streptozotocin were purchased from Sigma Chemical Co. (St Louis, MO, USA). 1,1,3,3-Tetraethoxypropane, crystalline beef liver catalase, reduced glutathione (GSH), 5,5′-dithiobis-(2-nitrobenzoic acid) were purchased from SD Fine Chemicals, Mumbai, India. Sulfanilamide, naphthalene-2,3-diamine hydrochloride and phosphoric acid were obtained from LobaChemi Pvt. Ltd., Mumbai, India. Tetrachlorodecaoxide (TCDO) (Oxoferin®) was purchased from Elder Pharmaceuticals Pvt. Ltd., Mumbai, India. Insulin injection (Mixtard®) was purchased from Novo Nordisk India Limited, Bangalore, India.

2.3. Induction and assessment of diabetes

A single dose of 55 mg/kg streptozotocin (STZ) in citrate buffer (pH 4.4, 0.1 M) was injected intraperitoneally to induce diabetes [85]. The age-matched control rats received an equal volume of citrate buffer. The blood samples were collected via retro-orbital plexus technique and serum glucose levels were estimated by GOD-POD (glucose oxidase peroxidase) diagnostic kit (Accurex Biomedical Pvt. Ltd., Mumbai, India). Rats having serum glucose levels more than 250 mg/dL were selected as diabetic and used for the present study.

2.4. Excision wound model

A previously established diabetic foot ulcer animal model was used in this study [57]. Briefly, on the day of wound creation each