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Antioxidant and anti-inflammatory potential of curcumin accelerated the cutaneous wound healing in streptozotocin-induced diabetic rats

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ABSTRACT

Prolonged inflammation and increased oxidative stress impairs healing in diabetics and application of curcumin, a well known antioxidant and anti-inflammatory agent, could be an important strategy in improving impaired healing in diabetics. So, the present study was conducted to evaluate the cutaneous wound healing potential of topically applied curcumin in diabetic rats. Open excision skin wound was created in streptozotocin induced diabetic rats and wounded rats were divided into three groups; i) control, ii) gel-treated and iii) curcumin-treated. Pluronic F-127 gel (25%) and curcumin (0.3%) in pluronic gel were topically applied in the gel- and curcumin-treated groups, respectively, once daily for 19 days. Curcumin application increased the wound contraction and decreased the expressions of inflammatory cytokines/enzymes i.e. tumor necrosis factor-alpha, interleukin (IL)-1beta and matrix metalloproteinase-9. Curcumin also increased the levels of anti-inflammatory cytokine i.e. IL-10 and antioxidant enzymes i.e. superoxide dismutase, catalase and glutathione peroxidase. Histopathologically, the curcumin-treated wounds showed better granulation tissue dominated by marked fibroblast proliferation and collagen deposition, and wounds were covered by thick regenerated epithelial layer. These findings reveal that the anti-inflammatory and antioxidant potential of curcumin caused faster and better wound healing in diabetic rats and curcumin could be an additional novel therapeutic agent in the management of impaired wound healing in diabetics.

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1. Introduction

Diabetes is a multisystem disorder and complications of diabetes induce physiological changes in tissues and cells that impair the normal healing process. The pathophysiologic relationship between diabetes and impaired healing is complex. The diabetic wounds get stuck in the inflammatory phase featured by continuing influx of neutrophils that release cytotoxic enzymes, free radicals and inflammatory mediators that cause extensive collateral damage to surrounding tissue. These destructive processes outbalance the healing process in such wounds and overproduction of free radicals that induce oxidative stress results in detrimental cytotoxic effects causing delayed wound healing [1,2]. The increased oxidative stress is one of the most common complications for the delayed wound healing in diabetics [3]. Therefore, reduction/termination of the persistent inflammation and elimination of free radicals by the introduction of an anti-inflammatory agent and antioxidant into the treatment of wounds could be an important strategy to improve healing of diabetic wounds [2].

Several studies in recent years have demonstrated curcumin as an antioxidant and anti-inflammatory agent [4,5]. Studies have also reported that curcumin alone or in combination possesses wound healing potential as well as protective effects in radiation induced endothelial injuries and mucosal damage in different animal models [6–9]. Curcumin has shown its wound healing potential in laser-induced wounds, dexamethasone-impaired healing, corticosteroid-impaired hairless rat skin, radiation-impaired healing of excisional wounds and streptozotocin induced diabetic rats as well as genetically diabetic mice [10–15].

However, to the best of our knowledge detailed time-dependent studies in diabetic rats are lacking. Hence, in view of the high level of oxidative stress and persistent inflammation associated with delayed healing in diabetic wounds, the present study was conducted to investigate the temporal wound healing potential of topically applied curcumin in diabetic rats.

Abbreviations: ANOVA, analysis of variance; ECM, extracellular matrix; ELISA, enzyme linked immunosorbent assay; GPx, glutathione peroxidase; H&E, hematoxylin and eosin; IL-10, interleukin-10; IL-1β, interleukin-1beta; MMP-9, matrix metalloproteinase-9; PF-127, pluronic F-127; SOD, superoxide dismutase; TGF-β_1, transforming growth factor-beta1; TNF-α, tumor necrosis factor-alpha.

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2. Materials and methods

2.1. Animals used and wound creation

Healthy adult male Wistar rats (170–200 g) were procured from Laboratory Animal Resource Section, Indian Veterinary Research Institute, Izatnagar (UP), India. Rats were housed in standard polycarbonate cages with ad lib access to feed and water and were maintained on a 12:12-h light dark cycle in a climatically controlled room. The experimental protocols were approved and sanctioned by the Institutional Animal Ethics Committee. All animals received humane care in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996).

2.2. Diabetes induction

After 10 days of acclimatization, a single injection of streptozotocin (60 mg kg^{-1}; Sigma-Aldrich, USA) in citrate buffer solution (0.1 M, pH 4.5) was administered intraperitoneally for the induction of diabetes. The fasting blood glucose levels of all the rats were determined using a glucometer (On Call Plus Blood glucose meter) before diabetes induction. After two days of streptozotocin injection, animals with blood glucose levels ≥300 mg/dl were kept under observation for 7 more days and rats consistently showing elevated blood glucose levels were selected for wound creation as described below.

2.3. Excision wound model

The open excision-type wound 2 × 2 cm^2 (≈400 mm^2), under pentobarbitone sodium (40 mg kg^{-1}) anesthesia, was created on the back (thoraco-lumber) region of the diabetic rats to the depth including the panniculus carnosus of rats. The wound was neither dressed nor covered. Animals after recovery from anesthesia were housed individually in properly disinfected cages.

2.4. Grouping

Three groups were used in this study and twenty diabetic rats were kept in each group. The three groups were as follows:

i) Control: Sterile normal saline was applied on the wounds once daily for 19 days.
ii) Pluronic F-127 (PF-127) gel-treated: 400 μl of PF-127 gel (25%, Sigma Aldrich, USA) was applied topically once daily for 19 days.
iii) Curcumin-treated: 400 μl of curcumin (0.3%, Sigma Aldrich) in PF-127 gel (25%) was applied topically on wounds once daily for 19 days.

2.5. Wound contraction measurements

The percentage of wound contraction was determined from the calculated wound area. During the measurement of wounds, animals were firmly held on a table top with the wound facing upwards. A firm but flexible transparent polythene rectangular (3 × 3 cm^2) sheet was held just over the wound and its margins were marked with a fine tip permanent marker on sheet and the animal was released back to the cage. The area (mm^2) within the boundaries of each tracing was determined planimetrically in which a standard quality card paper was used to convert the area of the wound on the transparent sheet into the weight of the card paper with the same area. The weight of the card paper/unit area was already known, therefore, the weight of each card paper for a particular wound was estimated easily. This method proved far more accurate than the graphical method wherein one relies on visual judgment only. The wound area on day 0 of each animal was measured at a predetermined time interval starting at 3 h post-wounding, as the delay of 3 h after the creation of wound for measurement was allowed to accommodate the wound stretching that occurs due to the struggle of the animal during recovery from the anesthesia. Subsequent measurement of wound area was taken on days 3, 7, 11, 14 and 19 post-wounding. The results of wound measurements on various days were expressed as percentage of wound contraction. The values were expressed as percentage values of the day 0 measurements and were calculated by Wilson's formula as follows:

$$\%\text{wound contraction} = \frac{\text{day 0 wound area–wound area on a particular day}}{\text{day 0 wound area}} \times 100.$$

2.6. Collection of tissue

On days 3, 7, 14 and 19, five animals from each group were killed with an overdose of diethyl ether to collect granulation/healing tissue which was immediately divided into two portions. One portion of tissue was stored in RNA stabilization reagent (RNA*later*™, Qiagen, USA) at −20 °C until RNA extraction. The second portion was preserved in 10% neutral buffer formalin for histopathological study. The third portion was homogenized in ice-cold lysis buffer [100 mg tissue in 1 ml lysis buffer: 1% Triton X 100, 10 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin and 1 mg/ml leupeptin in phosphate buffer saline (pH 7.4)] and centrifuged at 12,000 rpm for 10 min at 4 °C. The aliquots of the supernatant were prepared and stored at −80 °C till further processing for enzyme linked immunosorbent assay (ELISA).

2.7. Real-time RT-PCR

The mRNA expressions of tumor necrosis factor-alpha (TNF-α), interleukin-10 (IL-10), interleukin-1beta (IL-1β) and matrix metalloproteinase-9 (MMP-9) were determined in wound tissues with real-time RT-PCR. Total RNA was isolated from granulation/healing tissue for cDNA synthesis as described earlier [16]. An aliquot (1 μl) of cDNA was used as a template for the subsequent real time RT-PCR. The real time PCR assay was performed by using 2× QuantiTect SYBR Green PCR Master Mix (Qiagen, USA) in the 96 well plate of a Bio-Rad C-1000 thermal cycler. The real-time RT-PCR experiment was carried out according to the manufacturer's instruction and the following thermal cycling profile was used (40 cycles): 95 °C for 15 min, 94 °C for 15 s, 58–62 °C for 30 s (depending on the primers used) and 72 °C for 30 s. The primers used are given in Table 1. The $^{\Delta\Delta}$CT method of relative quantification was used to determine the fold change in expression and this was done by normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control β-actin in the same samples ($^{\Delta}CT = CT_{Target} - CT_{\beta\text{-actin}}$). Further, it was normalized with the control ($^{\Delta\Delta}CT = {}^{\Delta}CT_{Treatment} - {}^{\Delta}CT_{Control}$). The fold change in expression was then obtained as $2^{-\Delta\Delta}$CT [17].

2.8. ELISA

The supernatants of lysates were quantitatively assayed for TNF-α (Komabiotech Inc., Seoul, Korea), IL-10 (Komabiotech Inc.), superoxide dismutase (SOD) (Cayman Chemical, MI, USA), catalase (Cayman Chemical), and glutathione peroxidase (GPx) (Cayman Chemical), with levels as per the manufacturer's instructions.

2.9. Histopathological analysis

2.9.1. Hematoxylin and eosin (H&E) staining and scoring

The gross histopathological changes at the wound site were evaluated by H&E staining. The granulation/healing tissues fixed in 10% neutral buffer formalin were embedded in paraffin. 5 μm thick tissue sections were obtained and stained with H&E, as per standard method and visualized under a light microscope (Olympus CX31, Tokyo, Japan) at

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