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Bilirubin modulated cytokines, growth factors and angiogenesis to improve cutaneous wound healing process in diabetic rats

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ABSTRACT

Bilirubin has shown cutaneous wound healing potential in some preliminary studies. Here we hypothesize that bilirubin facilitates wound healing in diabetic rats by modulating important healing factors/candidates and anti-oxidant parameters in a time-dependent manner. Diabetes was induced in male Wistar rats by streptozotocin. In all diabetic rats wounds were created under pentobarbitone anesthesia. All the rats were divided into two groups, of which one (control) was treated with ointment base and other with bilirubin ointment (0.3%). Wound closer measurement and tissue collection were done on days 3, 7, 14 and 19 post-wounding. The relative expressions of hypoxia inducible factor-1 alpha (HIF-1α), vascular endothelial growth factor (VEGF), stromal cell-derived factor-1 alpha (SDF-1α), transforming growth factor- beta$_1$ (TGF-β$_1$), tumor necrosis factor-α (TNF-α) and interlukin-10 (IL-10) mRNA and proteins and the mRNA of interlukin-1 beta (IL-1β) and matrix metalloprteinase-9 (MMP-9) were determined in the wound tissues. CD-31 staining and collagen content were evaluated by immunohistochemistry and picrosirius red staining, respectively. Histopathological changes were assessed by H&E staining. The per cent wound closer was significantly higher from day 7 onwards in bilirubin-treated rats. HIF-1α, VEGF, SDF-1α, TGF-β$_1$, IL-10 mRNA and protein levels were significantly higher on days 3, 7 and 14 in bilirubin-treated rats. The mRNA expression and protein level of TNF-α and the mRNA of IL-1β and MMP-9 were progressively and markedly reduced in bilirubin-treated rats. The collagen deposition and formation of blood vessels were greater in bilirubin-treated rats. Bilirubin markedly facilitated cutaneous wound healing in diabetic rats by modulating growth factors, cytokines, neovasculogenesis and collagen contents to the wound site. Topical application of bilirubin ointment might be of great use in cutaneous wound healing in diabetic patients.

1. Introduction

The primary function of skin is to serve as a vindicatory barrier against any harmful environmental insult that may result in loss of the integrity of skin (such as wound) and ultimately lead to morbidity or even death [1]. Healing is an integral part of recovery of damaged skin [2]. Optimum healing of a cutaneous wound requires a well-orchestrated integration of complex cellular and molecular events of cell migration, proliferation, extracellular matrix deposition and remodeling [3]. Several cellular and molecular biological studies have demonstrated that many cytokines, growth factors, enzymes and cells are closely involved in the wound-healing process to complete normal tissue repair after damage [1]. The cytokines and growth factors are useful candidates and markers for determination of wound vitality and age [4].

There are a number of factors that adversely affect wound healing process, among them diabetes mellitus is one of the most inextricable and complicated impediment, wherein development of chronic non-healing foot ulcerations is one of the most serious and debilitating complications. The most common complications associated with delayed wound healing in diabetes are: reduction in chemotactic and phagocytic activities of neutrophils [5,6], decreased angiogenesis [7], decreased vasculogenesis due to decreased number of endothelial progenitor cells (EPC) [8,9], decreased endothelial nitric oxide synthase (eNOS) activity [10], increased oxidative stress [11], reduced number of growth factors like platelet derived growth factor (PDGF) [12], vascular endothelial growth factor (VEGF) [13], etc. The impairment in the healing of wounds in diabetic mice has been attributed to reduced angiogenesis, granulation tissue formation, decreased collagen formation and delay in transformation of fibroblasts to myofibroblasts [14]. Further chronic wounds seen in diabetic patients 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

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Table 1
Description of primers used.

Gene	Primer sequences	Product size	Annealing temp.	Accession number
β-actin	F: 5′-TCCTAGCACCATGAAGATCAA G-3′ R: 5′-GACTCATCGTACTCCTGCTTG-3′	132	59 °C	NC_005111
HIF-1α	F: 5′-GGAGCCTTAACCTATCTGTCAC-3′ R: 5′-AAGGGAGCCATCATGTTCC-3′	125	59 °C	NC_005105
VEGF	F: 5′-GCCAGCACATAGGAGAGATGAG-3′ R: 5′ ACCGCCTTGGCTTGTCAC-3′	234	62 °C	NM031836.2
SDF-1α	F: 5′-GAGCCAACGTCAAACATCTG-3′ R: 5′-GGCTTTGTCCAGGTACTCTTG-3′	137	59 °C	AC_000072
TGF-β_1	F: 5′-AAG TGG ATC CAC GAG CCC AA-3′ R: 5′ GCTGCACTTGCAGGAGCGCA-3′	246	62 °C	NM021578.2
TNF-α	F: 5′- GGCCACCACGCTCTTTCTGTCA-3′ R 5′-TGGGCTACGGGCTTGTCACTC-3′	153	60 °C	NM012675.3
IL-10	F: 5′-CCTGCTCTTACTGGCTGGAG-3′ R: 5′-TGTCCAGCTGGTCCTTCTTT-3′	161	60 °C	NM012854.2
IL-1β	F: 5′- GACAAGCAACGACAAAATCCC-3′ R: 5′-TGGGTATTGTTTGGGATCCAC-3′	124	58 °C	NC_005102
MMP-9	F: 5′-CTTGAAGTCTCAGAAGGTGGATC-3′ R: 5′-CGCCAGAAGTATTTGTCATGG-3′	135	59 °C	NC_005102

outbalance the healing process in such wounds and delay their repair [15]. Cutaneous wounds in diabetes, in addition to, causing pain and discomfort and predisposing the patient to superficial and chronic infection, involve significant cost associated with the long term treatment.

A large variety of treatment modalities enhance wound healing by supporting the body mechanisms. But this passive wound healing process proves inadequate for some obstinate/recalcitrant wounds or when immunity or other body functions are compromised (e.g. in diabetes). Although several substances/drugs/herbal products have been tried to fasten/enhance the wound healing process, no satisfactory therapy has been developed so far.

Bilirubin, the end product of heme catabolism in mammals, is generally regarded as a potentially cytotoxic and lipid-soluble waste product that needs to be excreted. However, bilirubin at low concentrations scavenges reactive oxygen species (ROS) in vitro thereby, reduces oxidant-mediated cellular damage and attenuates oxidative stress in vivo [16,17]. Biliverdin and bilirubin are believed to play important role in counteracting oxidative and nitrosative stress [17,18]. Reduction in ROS by bilirubin has been shown to enhance the process of wound healing in diabetes [19]. We have also shown that, intraperitonially administered bilirubin improved cutaneous wound healing in normal rats by reducing expression of pro-inflammatory tumor necrosis factor-α (TNF-α) and by increasing the expression of anti-inflammatory interlukin-10 (IL-10) [20]. Bilirubin also increased angiogenesis by increasing VEGF synthesis and by enhancing permeability of vascular walls [21]. In view of the above facts, we hypothesized that topical

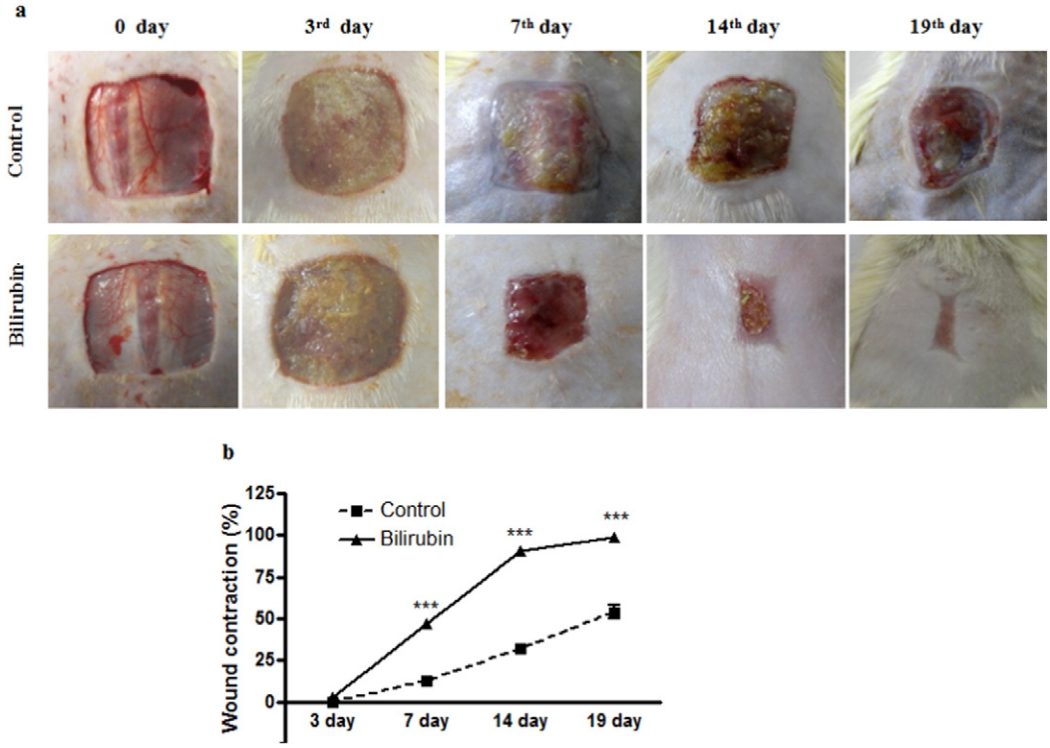

Fig. 1. Effect of bilirubin on gross appearance of healing wound and per cent wound contraction (calculated in respect to day 0) on days 3, 7, 14 and 19 post-wounding in diabetic rats. Bilirubin-treated rats showed progressively better wound closer, as compared to control (a). Per cent wound contraction in bilirubin-treated rats was greater, as compared to control (b). Data are expressed as mean ± SEM of 5 rats, ***$P < 0.001$ versus control on the same day.