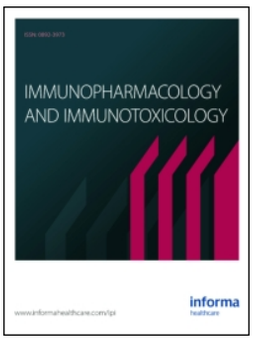

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Topical application of quercetin improves wound repair and regeneration in diabetic rats

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ABSTRACT

Purpose: There is an urgent need of effective drug/formulation to speed up the healing process in diabetic wounds. In our earlier studies, quercetin has accelerated the healing of nondiabetic wounds. So, we investigated the wound-healing potentials of quercetin in diabetic rats.

Materials and methods: A square-shaped cutaneous wound (≈400 mm^2) was created on the back of nondiabetic and diabetic rats. They were divided into three groups, viz. healthy control (nondiabetic), diabetic control and diabetic-treated group. Ointment base was topically applied for 21 days in healthy and diabetic control groups. Quercetin (0.3%) ointment was similarly applied in third group. Effects of quercetin on repair and regenerations of diabetic wounds in terms of wound closure, inflammation, angiogenesis, fibroblast proliferation, collagen synthesis, epithelialization, axonal regeneration etc was studied.

Results: Quercetin accelerated the wound closure and increased the expressions of IL-10, VEGF and TGF-β_1 in granulation/healing tissue of diabetic wound. However, quercetin decreased the expression of TNF-α, IL-1β, and MMP-9. Histopathological evaluation revealed amelioration of persistence of inflammatory cells by quercetin in diabetic wounds. There was good quality of granulation tissue, marked fibroblast proliferation, well organized collagen deposition, early regeneration of epithelial layer etc. in the quercetin treated diabetic wounds in comparison to diabetic control group. Results of immunohistochemistry showed more angiogenesis, faster phenotypic switching of fibroblast to myofibroblasts and increased GAP-43 positive nerve fibers in quercetin-treated diabetic wounds.

Conclusion: Quercetin ointment at 0.3% *w/w* concentration modulates cytokines, growth factors and protease, thereby improved repair and regenerations of cutaneous diabetic wounds in rats.

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Quercetin; diabetic rat; wound healing; cytokines; growth factors; granulation tissue

Introduction

Wound healing is a very complex and coordinated repair process, which occurs in a series of overlapping phases viz. hemostasis, inflammation, proliferation and remodeling phase. The transition of inflammatory to proliferative phase is a critical step during wound healing [1]. Numbers of cytokines, growth factors, proteases, cells (like platelets, macrophages, neutrophils, epidermal cells and fibroblasts) and other chemical mediators play an inter-dependent and pivotal role in these phases of wound healing [2]. The balancing and co-ordination among cytokines and growth factors is crucial for normal wound healing mechanisms. Diabetes mellitus is a multisystem disorder in which this co-ordination gets disturbed and resulting in impairment of the normal healing process. Impaired wound healing continues to cause significant morbidity and mortality in affected individuals. It is a serious problem of public health worldwide and it tends to increase in numbers. Diabetic wounded patients also experience physical as well as psychosocial negative effects on their day-to-day life. Further, the expenses incurred by the chronic non-healing wounds are placing a massive financial burden on the global healthcare systems. In developed countries, it has been found that 1–2% of the total population is likely to suffer from chronic wounds during their lifetime. In United States, the number of patients affected by chronic wounds is 5.7 million, which is about 2% of the total population of USA and costing the health department around US $20 billion [3].

In diabetic wounds, the persistence of inflammation stage in blunt form results in continuous influx of neutrophils, which cause production of excess amount of pro-inflammatory cytokine like tumor necrosis factor alpha (TNF-α) and down regulation of the anti-inflammatory cytokine like interleukin-10 (IL-10) [4,5]. This state of wound also leads to generation of free radicals, which further aggravate the persistent inflammatory condition in diabetic wounds. Impaired neovascularization due to decreased expressions of

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vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1 (SDF-1) have been observed to cause delayed wound healing in diabetes [6,7]. In diabetic wounds, diminished production of extracellular matrix (ECM) by fibroblasts as well as poor quality of ECM are associated with the increased oxidative stress and proteases activities at wound site [8]. Moreover, it has been observed that activities of different matrix metalloproteinase (MMP) inhibitors are lost due to decreased activity of transforming growth factor beta 1 (TGF-β_1) in diabetics [9]. Thus, impaired healing in diabetic wounds is considered multi-factorial. In recent years, many compounds with multiple actions have shown some diabetic wound healing potentials in experimental animals. However, majority of them have failed during clinical trials on diabetic or other complicated wounds, and no satisfactory therapy has been developed so far. Therefore, there is continuous global demand to develop some potent healing formulation. So, extensive research in order to develop some appropriate novel as well as alternative treatments for wounds is needed.

More than 80% of the world population has been found to use natural based drugs for curing skin-related problems [10]. The uses of the most of synthetic drugs have been resulted in some problems (like drug resistance, allergy and other side effects), which forced the scientists to seek alternative drugs [11]. Our preliminary wound-healing studies on quercetin, a flavonoid of natural origin, have showed that quercetin possess potent healing potentials [12,13]. Further, our detailed studies revealed that quercetin at 0.3% *w/w* in ointment base showed promising healing effects on the wounds of healthy rats (data under publication). Moreover, quercetin has revealed varieties of other health effects like cardiovascular protection, anticancer, anti-ulcer, anti-allergy, antioxidant, anti-inflammatory, anti-diabetic, gastroprotective, immunomodulatory and anti-infective properties [14]. Quercetin has been found to stimulate angiogenesis and proliferation of epithelial cells and fibroblasts [15]. Some other researchers have also observed the wound-healing effect of quercetin in non-diabetic and diabetic rats [16–18]. However, to the best of our knowledge, previous studies on diabetic wound-healing potentials of quercetin have some limitations (like shorter duration, type of formulation, study of systemic parameters, etc.), and the most of them lack detailed temporal investigations of quercetin effect. Considering the potentials of 0.3% quercetin ointment, as observed in our earlier studies [12,13,19], the present study was undertaken to validate its wound-healing efficacy in diabetic rats in temporal manner and investigate the possible pathways of its healing mechanisms in diabetic wounds.

Materials and methods

Animals and diabetic model

A total of 60 healthy adult male Wistar rats (150–200 g) were used in this experiment. The rats were procured from Disease Free Small Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India. Acclimatization period of one week was given to the animals. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of LUVAS, Hisar, Haryana, India [Agenda item No. 14 of letter No. VPHE/IAEC/1702-33 dated 28-05-2016]. All rats have *ad lib* access to feed and water during the entire study, and were maintained on a 12:12-h light/dark cycle in a climatically controlled room. All animals of this study received humane care in accordance with the National Institutes of Health Guide for the care and use of Laboratory animals (NIH Publication No. 85-23, revised 1996). Diabetes was induced in the 40 rats of the present study. For the induction of diabetes, single intra-peritoneal injection of streptozotocin (60 mg/kg b.wt., Sigma, USA), prepared in citrate buffer solution (0.1 M, pH 4.5), was administered. Rats were also starved for overnight and their fasting blood glucose levels were determined using glucometer (On Call Plus Blood glucose meter) before the administration of streptozotocin injection. The rats showing stabilized fasting blood glucose levels $\geq$300 mg/dl for nine days were considered diabetics.

Wound model

The nondiabetic as well as diabetic rats were anesthetized by an intraperitoneal injection of ketamine (50 mg/kg, i.p.) + xylazine (5 mg/kg, i.p.). The anesthetized rats were kept in ventro-dorsal position. The back of the rats was shaved and cleaned with antiseptic solution. Thereafter, a square shaped ($\approx$2 $\times$ 2 cm) full thickness cutaneous wound of skin was created on the back (thoraco-lumber) region of the rats to the depth including the panniculus carnosus of rats. Each wounded rat was housed individually in properly disinfected standard polycarbonate cage and wound was neither dressed nor covered.

Preparation of ointment base and quercetin ointment

Ointment base consisting of soft paraffin (90%), hard paraffin (5%) and lanolin (5%), was prepared by fusion method. This ointment base was used to prepare 0.3% *w/w* quercetin ointment by the incorporation method. The used quercetin was of $\geq$95% purity and purchased from Sigma-Aldrich, USA (cat. no. Q4951-100G). The prepared ointment base and quercetin ointment were stored at 4 °C till further uses for wound-healing studies.

Grouping

Different wounded rats were equally divided in the following three groups:

I. Healthy control (nondiabetic): The wounded area of nondiabetic rats was topically treated with ointment base once daily for 21 days.
II. Diabetic control: The wounded area of diabetic rats was topically treated with ointment base once daily for 21 days.
III. Diabetic treated: The wounded area of diabetic rats was topically treated with quercetin (0.3% *w/w*) ointment once daily for 21 days.

Photography of wounds and measurement of percent wound contraction

Wound of each rat was photographed immediately after creation of wound (day 0) and on different days, that is, 3, 7, 10, 14 and 21 postwounding. The each image of wound area of these days was further processed by using image analysis software (ImageJ, NIH), and the percent wound contraction was calculated on various days by using following formula:

$$\%\text{woundcontraction} = \frac{0\text{daywoundarea} - \text{woundareaonparticularday}}{0\text{daywoundarea}} \times 100$$

Harvesting of healing/healed tissue

Five rats from each group were killed on a day 3, 7, 10 and 21 postwounding to collect the granulation/healing tissue of each rat. The collected tissue was further processed depending upon the analysis of parameters. For the mRNA expression study, a portion of tissue was stored in RNA stabilization reagent (RNA*later*™, Qiagen, USA) at −20 °C until RNA extraction. For the histopathological and immunohistochemical evaluation, some portion of tissue was preserved in 10% neutral buffer formalin. For the western blotting and enzyme-linked immunosorbant assay (ELISA), specific weight of tissue 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)]. After homogenization, each sample was centrifuged at 12,000 *g* for 10 min at 4 °C. The aliquots of the supernatant were prepared and stored at −20 °C till further processing.

Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis

Different cytokines (TNF-α, IL-10 and IL-1β), growth factors (VEGF and TGF-β_1) and protease (MMP-9) are actively involved in different phases of healing. Their relative expressions in the healing tissues of days 3, 10 and 21 postwounding were determined by using real-time RT-PCR. Briefly, total RNA was isolated using the standard method described by Invitrogen, with Trizol reagent and quantified by Bio-spectrophotometer (Eppendorf). Following quantification, cDNA (20 µl) was synthesized from total RNA (2 µg) using cDNA synthesis kit (Bio-Rad) as per manufacturer's protocol. 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 EVA® Green qPCR Master Mix (G-Biosciences) in 96-well plate of Stratagene Mx3005P QPCR Systems, Agilent. The real-time RT-PCR experiment was carried out according to the manufacturer's instruction. The primers used are given in Table 1.

The results were expressed as threshold cycle values (CT). To study the relative change in gene expression the $2^{-\Delta\Delta CT}$ method. The gene-specific amplification was corrected for the difference in input of RNA by taking beta actin (β-actin) in account as housekeeping gene. The results were analyzed in comparison to the CT (minimum threshold of amplification) value of the target gene and the reference gene.

ELISA for TNF-α and IL-10

The supernatants of tissue lysates of days 3, 10 and 21 postwounding were quantitatively assayed for TNF-α (Komabiotech Inc., Seoul, Korea) and IL-10 (Komabiotech Inc., Seoul, Korea) levels as per the manufacturer's instructions to support the results of mRNA expression studies of these cytokines.

Western blotting for VEGF and TGF- β_1

The protein expressions for VEGF and TGF- β_1 in healing tissue lysates of day 3, 10 and 21 postwounding were determined by western blotting analysis to support the results of mRNA expression studies of these growth factors. Briefly, frozen tissue extracts were thawed on ice and mixed with equal volume of 2× Laemmli sample buffer. The western blotting of the samples was done as per the method described in our earlier study [20]. Different mouse monoclonal antibodies for VEGF (cat. no. sc-7269, 1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), TGF-β_1 (cat. no. T 0438; 1 µg/ml, Sigma Aldrich, USA) and β-actin (cat. no. sc-47778; 1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used in this study. The used secondary antibody was HRP-conjugated goat anti-mouse IgG (cat. no. sc-2005; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Each blot was scanned and band intensity was quantified by densitometry software (Image J, NIH). The western blot data for VEGF and TGF- β_1 were corrected for corresponding β-actin values and the results were expressed as normalized protein levels. A minimum of four such blots were performed for every protein analyzed.

Hematoxylin and eosin (H&E) staining and scoring

The microscopic histopathological changes at the wound site to evaluate the quality of healing were determined by the H and 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 light microscope (Olympus digital camera CX 41-Ts) at magnification 10× and 40× Specific number of random fields (40x) from different sections in each group were evaluated for different scoring. The scoring of infiltration of inflammatory cells was categorized from ranging from 0 to 4, on numerical scale [21]. Briefly, score 0 represented no inflammatory cells, score 1 represented occasional presence of cells, score 2 represented scattered cells, score 3 represented abundance of cells spread all over the section and score 4 represented confluence of inflammatory cells. Scoring for fibroblast proliferation was categorized on numerical scale from 0 to 3. The score was 0 for no formation of fibroblast, 1 for thin zone of fibroblast, 2 for thick zone of fibroblast but loosely arranged and 3 for dense zone of fibroblasts with tightly packed. The

Table 1. Description of primers used for mRNA expression studies.

Gene	Primer sequences	Product size	Annealing temp.	Accession number
GAPDH	F: 5′-CTTCAACAGCAACTCCCATTC-3′ R: 5′-GTAGCCATATTCATTGTCATACCAG-3′	106	60 °C	NC_005103
β-actin	F: 5′- TCCTAGCACCATGAAGATCAA G-3′ R: 5′- GACTCATCGTACTCCTGCTTG-3′	132	59 °C	NC_005111
VEGF	F: 5′-TTACCCTTCCCCATTTTCCC-3′ R: 5′-ACTTTCTCTTTTCTCTGCCTC C-3′	87	60 °C	NC_005108
TGF-β1	F: 5′-CCT GGG TTG GAA GTG GAT C-3′ R: 5′ TTG GTT GTA GAG GGC AAG G-3′	121	60 °C	NC_005100
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

scoring for epithelialization was graded from 0–3 as per the method described by Abramov et al. [22]. Score 0 represented no epithelialization, score 1 represented partial epithelialization, score 2 represented complete epithelialization, but immature and thin, score 3 represented complete epithelialization, but mature and thick, and score 4 represented complete epithelialization, mature, thick epithelium along with adnexa. Scoring for the overall wound maturity was done according to the methods of Greenhalgh et al. [23] and Kant et al. [24]. Briefly, score (i) 1–3 was given to none to minimal cell accumulation and granulation tissue, (ii) 4–6 to thin immature granulation tissue that is dominated by inflammatory cells, but has few fibroblasts, capillaries, or collagen deposition and minimal epithelial migration, (iii) 7–9 to moderately thick granulation tissue can range from being dominated by inflammatory cells to more fibroblasts and collagen deposition, extensive neovascularization, epithelium can range from minimal to moderate migration, (iv) 10–12 to thick, vascular granulation tissue dominated by fibroblasts and extensive collagen deposition and epithelium partially to completely covering the wound and (v) 13–15 to thick mature granulation tissue dominated by compact collagen deposition parallel to the well-formed complete epithelial layer and decreased fibroblasts and blood vessels to normal.

Immunohistochemistry for cluster of differentiation 31 (CD31), alpha-smooth muscle actin (α-SMA) and growth associated protein-43 (GAP-43)

Monoclonal mouse raised primary antibodies against CD31 (cat. no. GTX74899; 1:50, GeneTex), α-SMA (cat. no. NBP2-22120; 1:400, Novus Biologicals, Littleton, CO, USA) and GAP-43 (cat. no. NBP1-41337; 1 μg/ml, Novus Biologicals, Littleton, CO, USA) were used to assess the neovascularization, myofibroblastformation and axonal regeneration, respectively in the healing tissue. Immunohistochemical detection was done on 5-μm-thick paraffin sections and processing was carried out as per the procedure described in our previous study [25]. The biotinylated goat anti-mouse IgG secondary antibody (Sigma Chemicals, USA; 1:20 prepared in 1% BSA in PBS) was used in this study followed by incubation of slides with ExtrAvidin peroxidase (Sigma Chemicals, USA; diluted at 1:20 concentration 1% BSA in PBS) for 30 min at 37 °C. The 3-amino-9-ethyl-carbazole (AEC) chromogen substrate (AEC Staining Kit; Sigma-Aldrich) was used as per manufacturer's instructions for the color reaction, that is, red-brown (brick red) color indicating a positive reaction. Finally, the slides were covered with glycerin and examined under microscope for evaluation. Semi-quantitative scoring of reactions for different markers was done by assessing at least 20 randomly chosen high-power fields (HPFs) (40×) of each group. The micro vessel density (MVD) was assessed by counting the number of micro vessels showing positive reaction for CD31 in wounded dermis and hypodermis of different groups. The scoring of positive reactions for α-SMA was done from 0–4, on numerical scale. The score was 0 for undetected reaction, 1 for little density reaction, 2 for intermediate density reaction, 3 for intense reaction and 4 for very intense reaction. Only the fibroblasts which were showing the positive reactions for α-SMA considered for scoring and blood vessels showing strong positive reactions were omitted from the scoring criteria. The GAP-43 positive reactions were scored by counting the number of positive reactions in wounded dermis and hypodermis of different groups.

Picrosirius red staining for collagen

The synthesis and arrangement of thin as well as thick collagen fibers at wound site was determined by picrosirius red staining on days 3, 7, 10 and 21 postwounding. Picrosirius red (Direct Red 80 from Sigma-Aldrich, USA) staining was done by modified picrosirius procedure as described by Dayan et al. [26]. This was done to evaluate the collagen fraction and its thickness in healing/healed wound. The stained sections were viewed under polarized light (Leica DM 2500 P). As per the pattern of birefringence, thick and denser collagen show orange to red color, and thinner collagen fibers appear yellow to green. The quantification of the images for total collagen fraction (i.e. total percentage of collagen fibers in a particular area) was done by using the ImageJ software (NIH, USA).

Statistical analysis

Results were expressed as mean ± SEM with n equal to the number of replicates. The data were analyzed for homogeneity and normal distribution. The statistical significance between the different groups was analyzed by applying two-way analysis of variance (ANOVA) followed by Bonferroni's post-test using the GraphPad Prism v4.03 software program (San Diego, CA, USA). The differences between the different treatment groups were considered statistically significant at $p \leq .05$.

Results

Effect of quercetin on gross changes in diabetic wounds on different groups

Grossly, healing of wound in quercetin-treated diabetic rats was similar to healthy control (non-diabetic) rats and better than the diabetic control rats (Figure 1(A)). The early formation and shedding of scab was also observed in healthy control and quercetin-treated diabetic rats, as compared to diabetic control rats. During the collection of healing tissue at the time of killing of rats on different days (particularly on day 3, 7 and 10), it was distinguishable that granulation tissue was well formed, thick and red color in the healthy control group followed by quercetin-treated diabetic rats. The healing tissue of diabetic control group was thin, comparatively pale in color and of poor quality.

Quercetin increased wound contraction in diabetic rats

Topical applications of quercetin decreased the wound size by significantly increasing per cent wound contraction in diabetic rats (Figure 1(B)), as compared to diabetic control group. The healthy control group also showed significantly increased wound contraction, as compared to diabetic control group.

Quercetin decreased TNF-α, IL-1β and MMP-9, and increased IL-10, VEGF and TGF-β_1 expressions in the diabetic wound

The topical applications of quercetin in diabetic-treated group lowered mRNA expressions of TNF-α in comparison to diabetic control group, and significantly lowered expression was observed on day 3 and 10 (Figure 2(A)). The relative mRNA expression of TNF-α was also significantly lower in healthy control group during the entire duration of experiment, as compared to diabetic control group (Figure 2(A)). The quercetin-treated diabetic group also lowered the TNF-α levels in the healing tissue during the entire duration of experiment and significantly lowered levels were observed on day 10 and 21 (Figure 2(C)). The mRNA expression of IL-10 was markedly higher in healthy control and quercetin treated group on day 3 and 10, as compared to diabetic control group (Figure 2(B)). However, significantly increased expression of IL-10 in quercetin treated group was only observed on day 10. The levels of IL-10 were significantly higher in quercetin treated diabetic group only on day 3 in comparison to diabetic control group (Figure 2(D)). The IL-10 levels were also significantly higher in healthy control group on day 3 and 10, as compared to diabetic control group (Figure 2(D)).

The topical application of quercetin in diabetic-treated group up-regulated the mRNA expression of VEGF on day 3 and 10, as compared to healthy and diabetic control group (Figure 3(A)). The VEGF mRNA expression was also significantly higher in healthy control group on day 10 in comparison to diabetic control group (Figure 3(A)). The VEGF protein expression was markedly lower in diabetic control group up to day 10, and significantly lowered on day 3 and 10 in comparison to healthy control and quercetin-treated group, respectively (Figure 3(D)). The TGF-β_1 mRNA expression was significantly increased only on day 3 in quercetin treated diabetic group, as compared to other groups (Figure 3(B)). The TGF-β_1 protein expression in diabetic control group was significantly lowered on day 3 and remained markedly lower up to day 10. However, the TGF-β_1 protein expression was significantly ($p < .001$) higher on day 21 in diabetic control group, as compared to quercetin treated diabetic group (Figure 3(D)).

The relative mRNA expression of IL-1β (Figure 4(A)) was markedly higher in diabetic control group and significantly increased expression was observed on day 21, as compared to other groups. The relative mRNA expression of MMP-9 was also significantly higher in diabetic control group on day 10 and 21 in comparison to other groups, and on day 21 in comparison to healthy control group only (Figure 4(B)).

Quercetin decreased inflammatory cells, and increased fibroblast proliferation, re-epithelization and quality of healing in diabetic wounds

The representative images of H&E stained wound sections of various days of different groups are presented in Figure 5(A) (40×) and inset boxes represent lower magnification (10×). On day 3, wound sections of quercetin-treated diabetic group showed more fibroblast, mixed type inflammatory cells and blood vessels in comparison to other groups. The mixed type inflammatory cells were also more in healthy control group in comparison to diabetic control group. On day 7, the granulation tissue was not completely formed in diabetic control group and the upper area showed marked dominance of inflammatory cells within proliferative fibrous tissue. However, wound sections of quercetin-treated diabetic group showed well-formed granulation tissue and marked proliferation of fibroblasts and new as well as well-formed capillaries were evident. The healthy control group also showed the well-formed granulation tissue, but the presence of inflammatory cell was more in comparison to quercetin-treated group.

On day 10, diabetic control group still showed the marked presence of inflammatory cells in the wound area and lacking the proper proliferation of fibroblast and blood vessels. Whereas, the wound area of the healthy control and quercetin-treated diabetic group showed well-formed thick

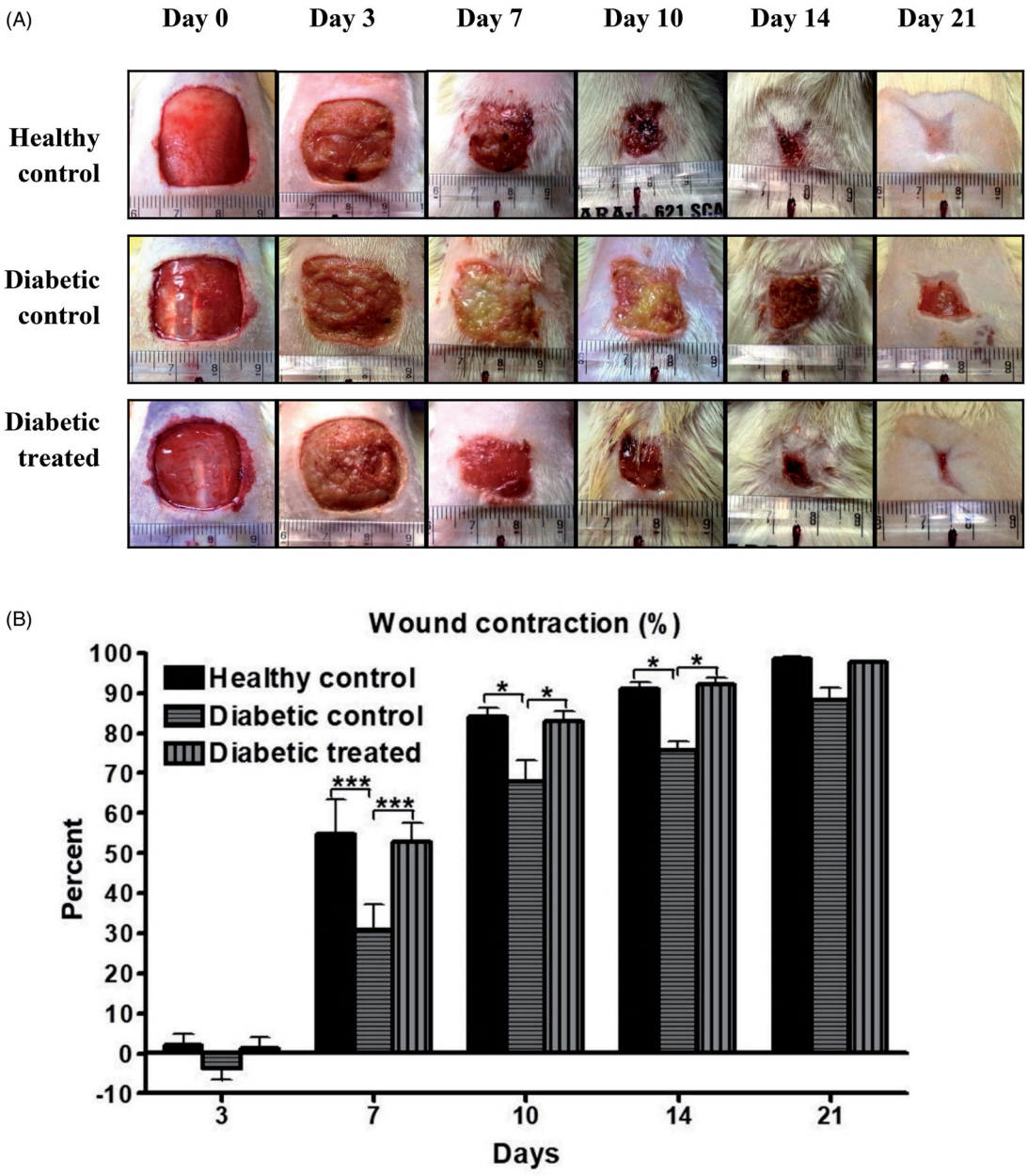

Figure 1. Effects of topical application of 0.3% quercetin on wound closure in diabetic rats. (A) Gross appearance of wound healing in different groups on different days of treatment. (B) Percent wound contraction on different days of treatment. Data are expressed as means ± SEM $^{*}p<.05$, $^{**}p<.01$; $^{***}p<.01$ versus other group(s) on the same day.

granulation tissue, collagen deposition, good number of blood vessels and few inflammatory cells. On day 21, the presence of different cells in the wound area was not uniform in diabetic control group and still showed the marked presence of inflammatory cells in many fields. The healthy control and quercetin treated diabetic group showed thick compact extracellular matrix (ECM) with well oriented collagen deposition and covered by newly regenerated epithelial layer, which were lacking in diabetic control group. The proportion of fibroblasts also decreased in the healthy control and quercetin treated group on day 21, as compared to day 10 wound of respective groups.

The score for inflammatory cell in quercetin-treated group was significantly more on day 3 and significantly less during rest of the duration of experiment, as compared to diabetic control group (Figure 5(B)). The score for inflammatory cells was also significantly lower in healthy control group on day 10 and 21, as compared to diabetic control group (Figure 5(B)). The score for fibroblast in healthy control and quercetin-treated diabetic groups in comparison to diabetic control group was significantly more during the entire duration of experiment except on day 3 for healthy control group and on day 7 and 10 for quercetin-treated group (Figure 5(C)). The score for fibroblast was also significantly more in quercetin-treated group on day 3 and 21, as compared to healthy control group (Figure 5(C)). Scoring for epithelialization revealed that the score was markedly higher in healthy control and quercetin-treated diabetic groups on day 7, 10 and 21 in comparison to diabetic control group (Figure 5(D)). The score was significantly more in quercetin treated diabetic

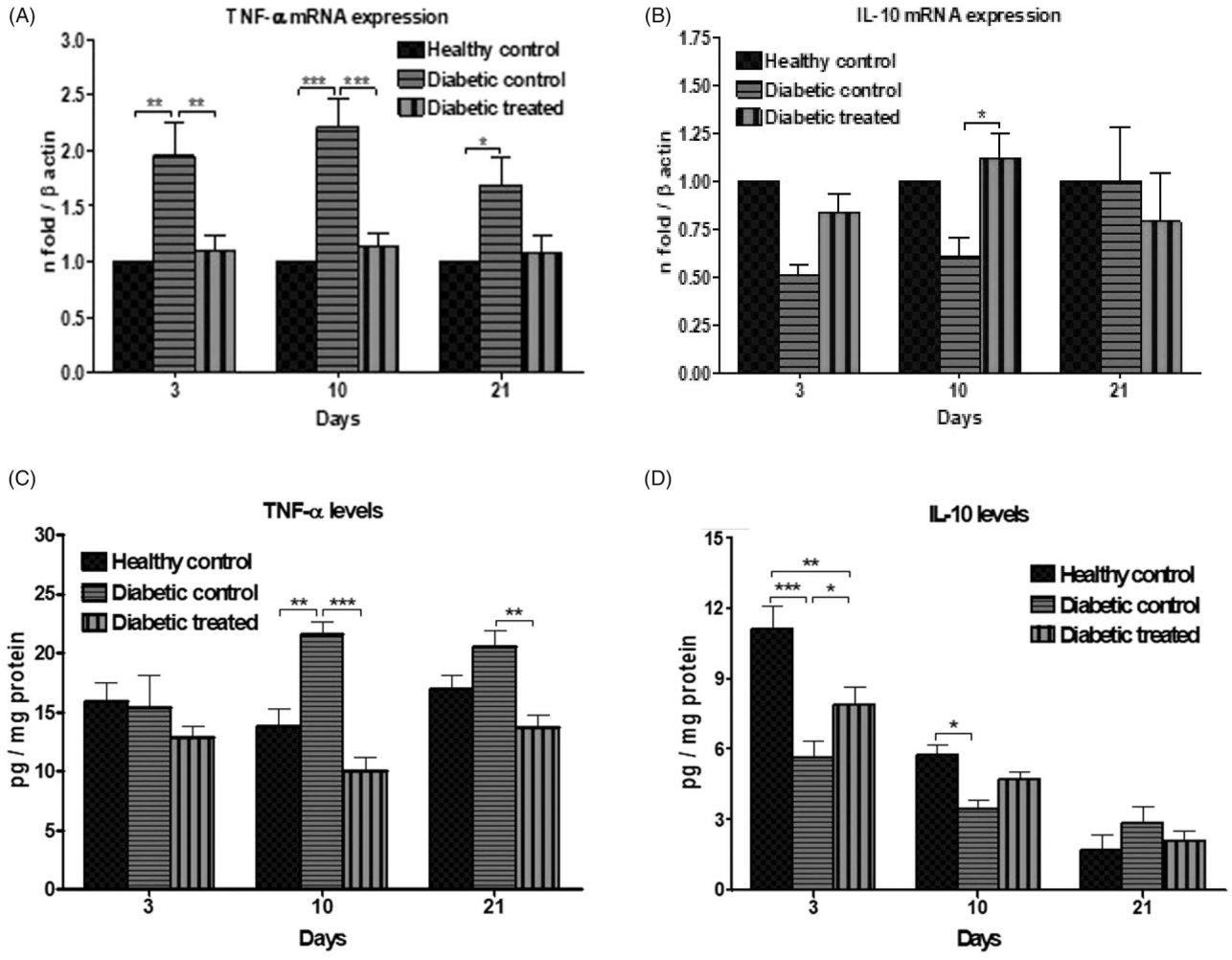

Figure 2. Effects of quercetin on the expressions and levels of TNF-α and IL-10 in the wounds of diabetic-treated rats in comparison to healthy control (HC) and diabetic control (DC) rats. (A & B) Relative mRNA expressions of TNF-α (A) and IL-10 (B) in healing tissue of excision wounds of different groups on days 3, 10 and 21 postwounding. The mRNA expressions were normalized by β-actin at each time point and data are expressed as means ± SE fold change, ($n = 5$). (C&D) ELISA analysis of TNF-α (C) and IL-10 (D) in healing tissue of rats of different groups on days 3, 10 and 21 post-wounding. Data are expressed as means ± SEM, ($n = 5$). *$p<.05$; **$p<.01$; *** $p<.001$ vs. other group(s) on the same day.

group on day 10 and 21, and in healthy control group on day 21 in comparison to diabetic control group (Figure 5(D)). The score was also significantly higher in quercetin-treated diabetic group on day 21, as compared to healthy control group (Figure 5(D)). The overall histological scoring for wound maturity of healthy control and quercetin treated diabetic groups was significantly higher in comparison to diabetic control group during the entire duration of experiment except on day 3 for quercetin-treated groups (Figure 5(E)).

Quercetin increased blood vessels formation in the diabetic wounds

The representative images of CD31-positive vessels are presented in Figure 6(A) (40×). The neovascularization was more and better in healthy control and quercetin-treated diabetic groups in comparison to diabetic control group. It was also evident in the diabetic control group that the neovascularization was aberrant as well as frustrated in nature and erratically distributed in the healing tissue. However, healing tissue of healthy control and quercetin-treated diabetic groups showed uniformly distributed newly forming blood vessels with well-marked lumen of the vessels and large perimeter. In some areas of diabetic control and quercetin-treated diabetic groups, the vascular occlusion and unmarked lumen of blood vessels was evident. The numbers of blood vessels in the diabetic control group were also more dominant at the lower part of the healing tissues on day 7, 10 and 21. The MVD was significantly more in healthy control and quercetin-treated diabetic groups in comparison to diabetic control group on day 7 and 10 (Figure 6(B)). However, the MVD was markedly higher in diabetic control group on day 21, as compared to other groups.

Quercetin stimulated the switching of fibroblast to myofibroblast in diabetic wound

The representative images (40×) of α-SMA-positive reactions in the healing tissues of different groups on various days are presented in Figure 7(A). The positive reactions for α-SMA were more apparent in the fibroblasts and blood vessels. The intensity of positive reaction in the various fibroblasts and blood vessels was also varying among their self. The number of α-SMA positive fibroblast (myofibroblast) increased in all

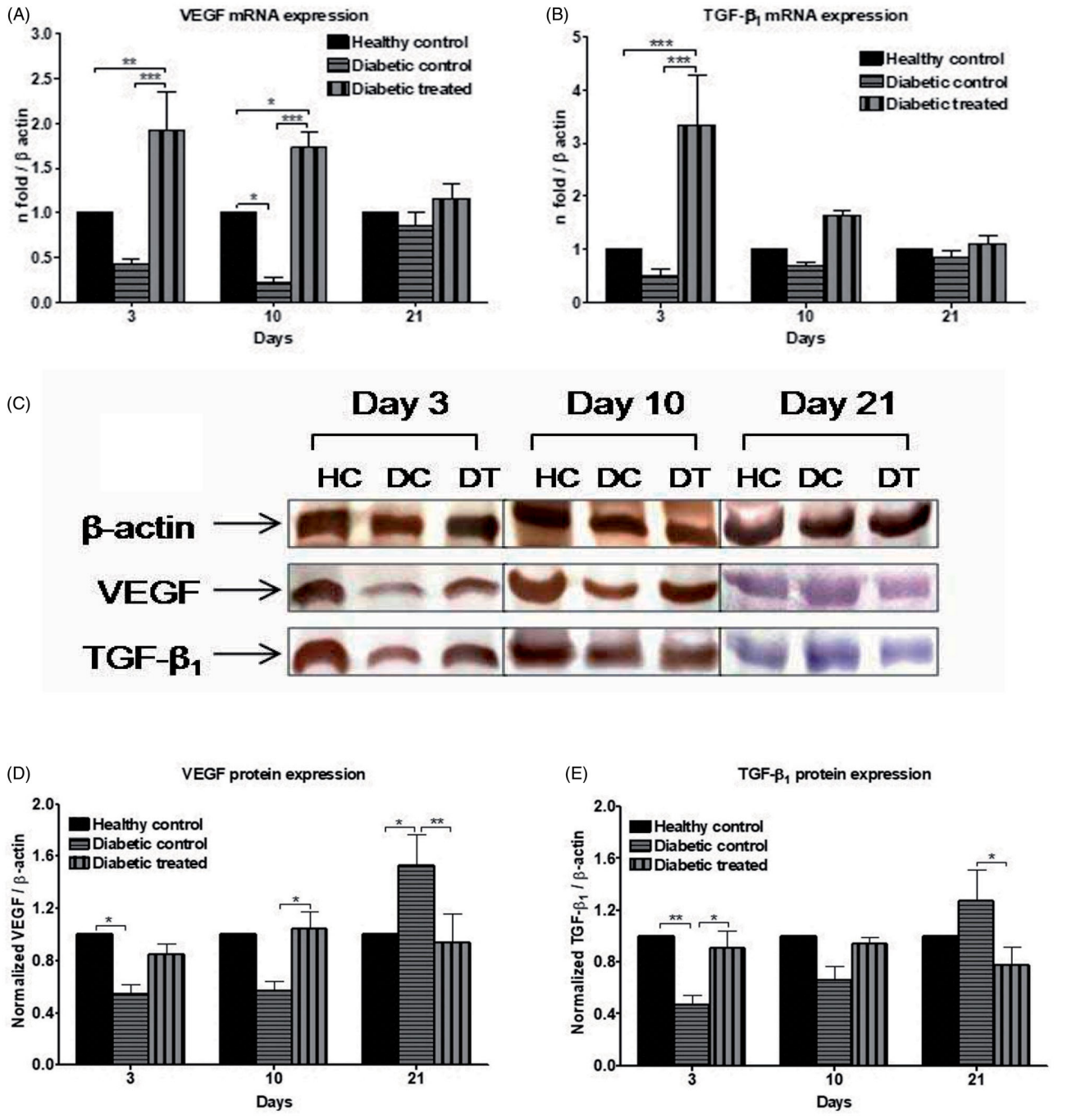

Figure 3. Effects of quercetin on the expressions and levels of VEGF and TGF-$\beta_1$in the wounds of diabetic treated (DT) rats in comparison to wound of healthy control (HC) and diabetic control (DC) rats. (A&B) Relative mRNA expression of VEGF (A), TGF-β_1 (B) in healing tissue of excision wounds in diabetic rats. The mRNA expressions of the growth factors were normalized by β-actin at each time point and data are expressed as means ± SEM fold change, ($n = 5$). (C) Representative Western blots of β-actin, VEGF and TGF-β_1 from the homogenate samples of different groups on days 3, 7, 14 and 19 postwounding. (D&E) Semi-quantitative protein expressions of VEGF (D) and TGF-β_1 (E) in different groups and data are expressed as means ± SEM, ($n = 4$). $^{*}p<.05$; $^{**}p<.01$; $^{***}p<.001$ vs. other group(s) on the same day.

the groups in time-dependent manner. Scoring for myofibroblast revealed that score was markedly higher in healthy control and quercetin-treated diabetic groups in comparison to diabetic control group during the entire duration of experiment (Figure 7(B)). The significantly higher score was observed on day 7, 10 and 21 for healthy control group, and on day 10 and 21 for quercetin-treated diabetic group, as compared to diabetic control group (Figure 7(B)). Moreover, the score was higher till day 10 and lower on day 21 in healthy control group in comparison to quercetin treated diabetic group.

Quercetin promoted the neuronal regeneration at diabetic wound site

Representative images of GAP-43 positive reactions in the healing tissues of different groups on various days are

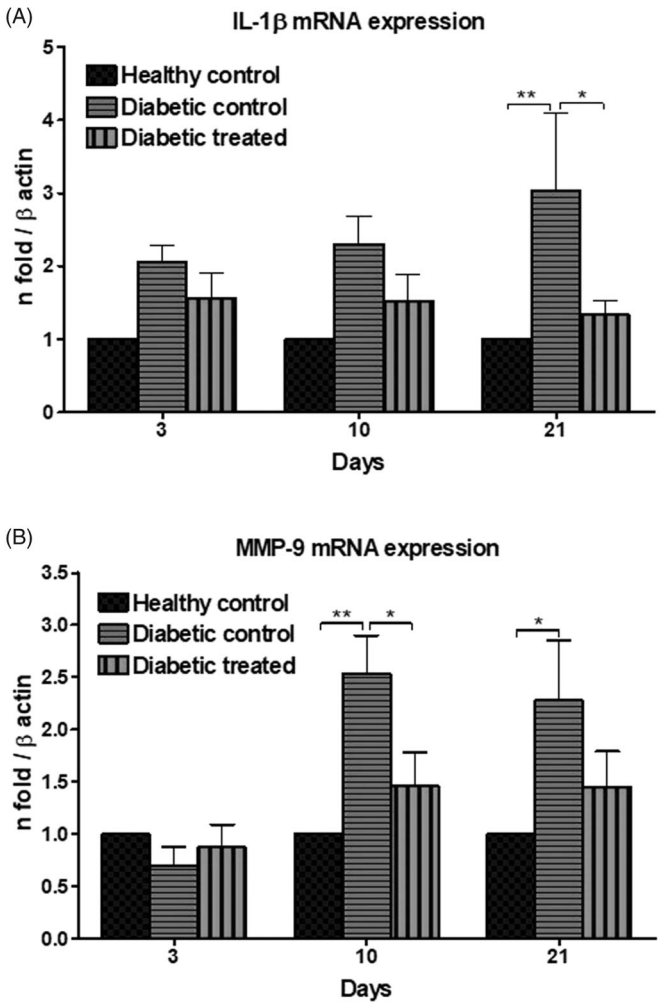

Figure 4. (A & B) The relative mRNA expressions of IL-1β (A) and MMP-9 (B) in healing tissue of healthy control, diabetic control and diabetic treated rats on days 3, 10 and 19 postwounding. The mRNA expressions were normalized by β-actin at each time point. Data are expressed as mean ± SEM, ($n = 5$). $^{*}p<.05$; $^{**}p<.01$; $^{***}p<.001$ vs. other group(s) on the same day.

presented in Figure 8(A) (40×). The positive fibers were more in the blood vessels and hypodermis. On day 10, most of the positive reactions were on blood vessels. The semi-quantitative analysis of the various field of immunostained sections revealed more GAP-43 positive reactions in the healthy control and quercetin treated diabetic groups in comparison to diabetic control group till day 10 and the reactions were significantly higher in these two groups, as compared to diabetic control (Figure 8(B)).

Quercetin increased the synthesis of good quality of collagen fibers

The representative images of picrosirius red-stained wound sections of different groups on various days are shown in Figure 9(A) (20×). It was evident from the images that the collagen content increased gradually in all the groups with the passage of time of healing. It was apparent that the collagen synthesis, deposition, compactness, orientation and organization were better in healthy control and quercetin-treated diabetic group, as compared to diabetic control group on respective days. The dominancy of yellow-green (thinner) collagen fibers were more in all the groups till day 10, but dominancy of yellow-green (thinner) collagen fibers persist till day 21 in diabetic control group. The presence of orange-red (thicker) collagen fibers was more dominant in healthy control and quercetin-treated diabetic group on day 21. Semi-quantification of collagen fiber fractions revealed less percentage of thinner collagen fibers (Figure 9(B)), thicker collagen fibers (Figure 9(C)) and total collagen fraction (Figure 9(D)) in diabetic control group, as compared to other groups. The thinner collagen fiber fraction was higher in healthy control group, as compared to quercetin treated diabetic group during the entire duration of experiment (Figure 9(B)). The thicker collagen fiber and total collagen fiber fractions were also higher in healthy control group in comparison to quercetin-treated diabetic group till day 10 (Figure 9 (C,D)).

Discussion

The results of our present investigation revealed that quercetin applications improve the wound healing in diabetic-treated rats and progressive wound closure can be visualized from the wound images of different days (Figure 1(A)). The results of this study revealed that quercetin treatment on diabetic wounds markedly decreased the expression/levels of TNF-α, IL-1β and MMP-9, and increased IL-10, VEGF and TGF-β_1 expression/levels in comparison to diabetic control group. Further, histopathological and immunohistochemical evidences confirmed better quality of healing in diabetic wounds treated with quercetin, as compared to untreated diabetic wounds. These histopathological and immunohistochemical findings were based on the better granulation tissue formation, marked fibroblast proliferation, increased blood vessels formation, optimum deposition of collagen, early formation of myofibroblasts and early as well as complete regeneration of epithelial layer in quercetin-treated diabetic wounds.

Wound healing initiates immediately after any injury and involve interaction of various cytokines, growth factors and cells in well-organized manner to complete this process. The first aim of the healing is to minimize the wound area exposed to the external environment and this occurs through centripetal movement of the wound edge toward the center of the wound for wound closure. Different processes which are involved and helpful in reducing the exposed wound area include wound contraction, granulation tissue formation, collagen deposition, epithelialization etc. The viscoelastic properties of skin cause skin to stretch beyond its normal state within a short period of time and may results into the wound contraction [27]. In the present study, wound contraction was markedly higher in quercetin treated diabetic wounds in comparison to wounds of diabetic control group during the entire experiment. This revealed that quercetin (0.3% w/w) stimulates the wound contraction process of diabetic wounds in our study and this is well supported by the earlier studies [12,17,18]. The myofibroblast formation and re-epithelialization also contribute significantly in the contraction of the wound [28]. Generally,

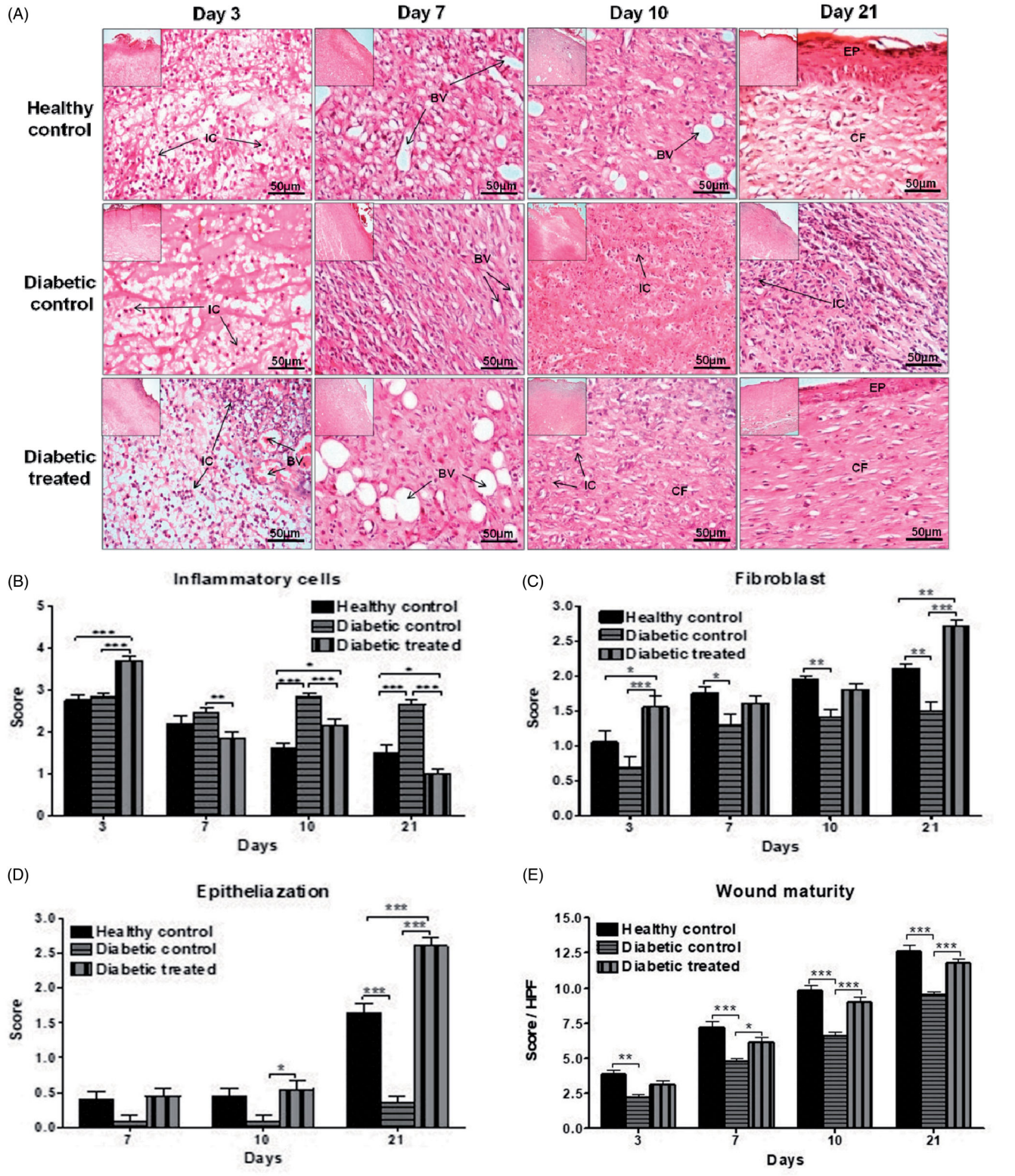

Figure 5. (A) Representative images of H &E stained histological wound sections of healthy control, diabetic control and diabetic-treated (quercetin treated) rats of different days (40× magnification and scale bar 50 µm). Low magnification (10×) images of wounds are shown in inset boxes in the left upper corner. IC: inflammatory cells; BV: blood vessels; CF: collagen fiber; EP: epithelial layer. (B–E) Histological scoring from 20 randomly selected high-power fields (40×) of H & E stained sections for: (B) inflammatory infiltrate (score from 0–4), (C) fibroblast (score from 0–3), (D) epithelialization (score from 0–3) and (E) wound maturity (score from 1–15). Data are expressed as means ± SEM, ($n = 20$) *$p<.05$; **$p<.01$; ***$p<.001$ vs. other group(s) on the same day.

myofibroblasts provide the faster contraction than re-epithelialization process [29]. Quercetin-treated diabetic wounds in our present study also showed early formation of myofibroblasts and epithelial layer in comparison to wounds of diabetic control group (Figure 7), which played role in fast contraction in quercetin-treated group. Sometimes, gross observation for wound closure does not reveal the real picture of quality of healing or healed tissue. Therefore, it

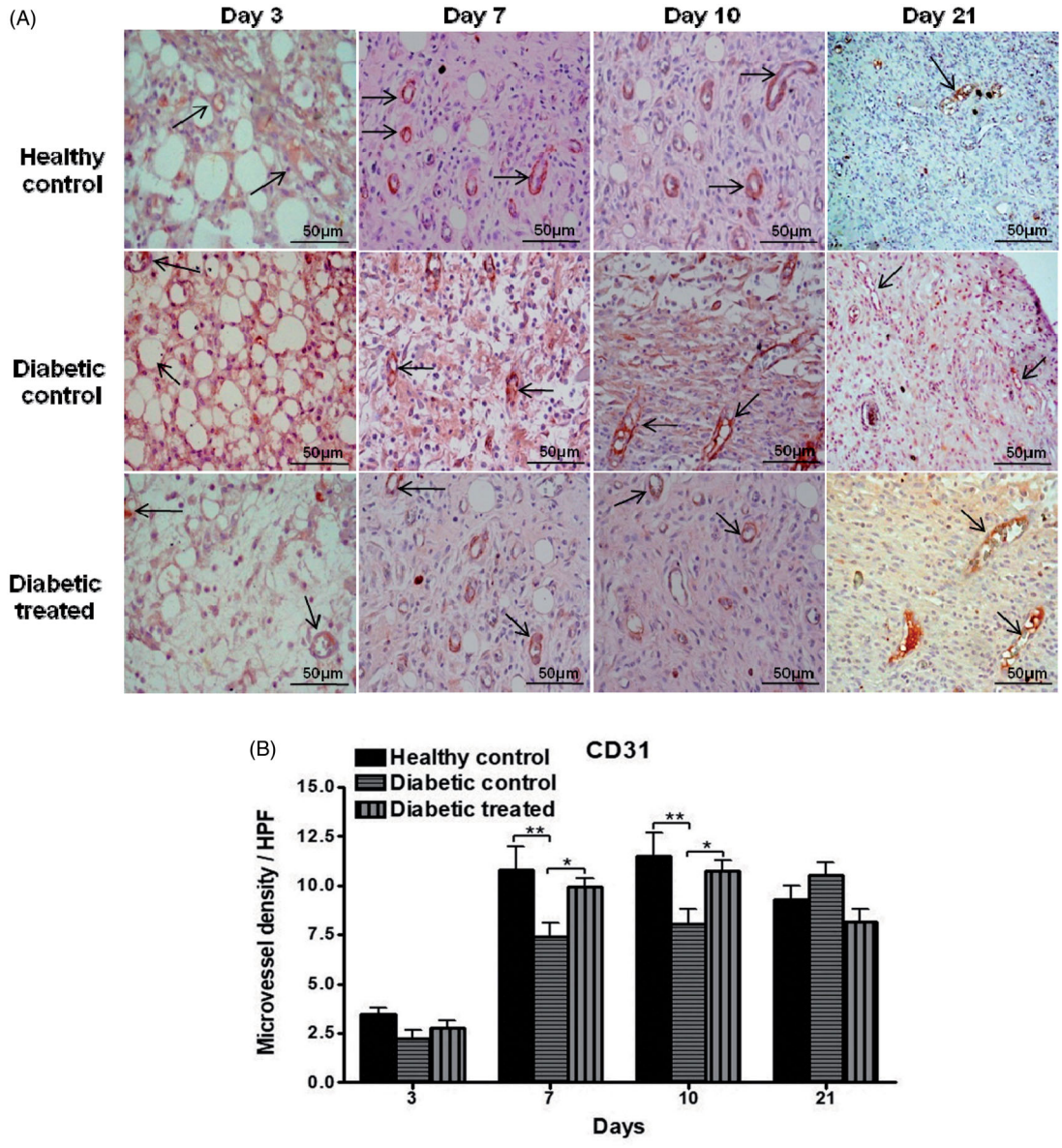

Figure 6. (A) Representative immunohistochemical CD31 staining of wound sections of healthy control, diabetic control and diabetic-treated (quercetin treated) rats on days 3, 7, 10 and 21 postwounding (40× magnification and scale bar 50 µm). Arrow indicates the positive reaction for CD31 on the endothelial cells of blood vessels. (B) Semiquantitative analysis of microvessel density (MVD) on days 3, 7, 10 and 21 postwounding. MVD was assessed by counting the number of microvessels in 20 randomly chosen high-power fields (HPFs) (40×) from minimum three sections in wounded dermis and hypodermis of healthy control, diabetic control and diabetic treated (quercetin treated) rats. Data are expressed as means ± SEM, ($n = 20$). $^{*}p<.05$; $^{**}p<.01$; $^{***}p<.001$ compared with other group(s) on the same day.

becomes essential to analyze the healing tissue in relation to important cytokines, growth factors, proteases and cells involved in the wound healing mechanisms. The histopathological and immunohistochemical studies also evidently represent the quality of wound healing.

Inflammation plays crucial role in wound healing and it should be well regulated for proper healing of wound. The shifting of inflammatory to proliferative phase is very critical step during healing. The TNF-α and IL-1β are vital pro-inflammatory cytokines, which up-regulate during inflammatory phase, and have role in initiating the early wound-healing response [30]. IL-10 is an anti-inflammatory cytokine produced by both immune and nonimmune cells [31]. Balance between proinflammatory and anti-inflammatory cytokines in the wound bed is crucial for proper wound healing. Persistent high level of TNF-α is responsible for delay in granulation tissue formation and failure of wound closure in diabetes. Thus, in the present study, persistence higher expressions and levels of TNF-α in the diabetic control group (Figure 2(A,C)) resulted in the formation of poor quality granulation tissue, which is well evident from the histopathological and immunohistochemistry results (Figures 5–9). However, these deleterious changes in the diabetic wounds were markedly ameliorated by the topical application of quercetin ointment. Further, production of inflammatory mediators is decreased by IL-10, which provide the suitable environment for the wound healing process in skin [32]. In our study, quercetin also increased the expression of IL-10 in

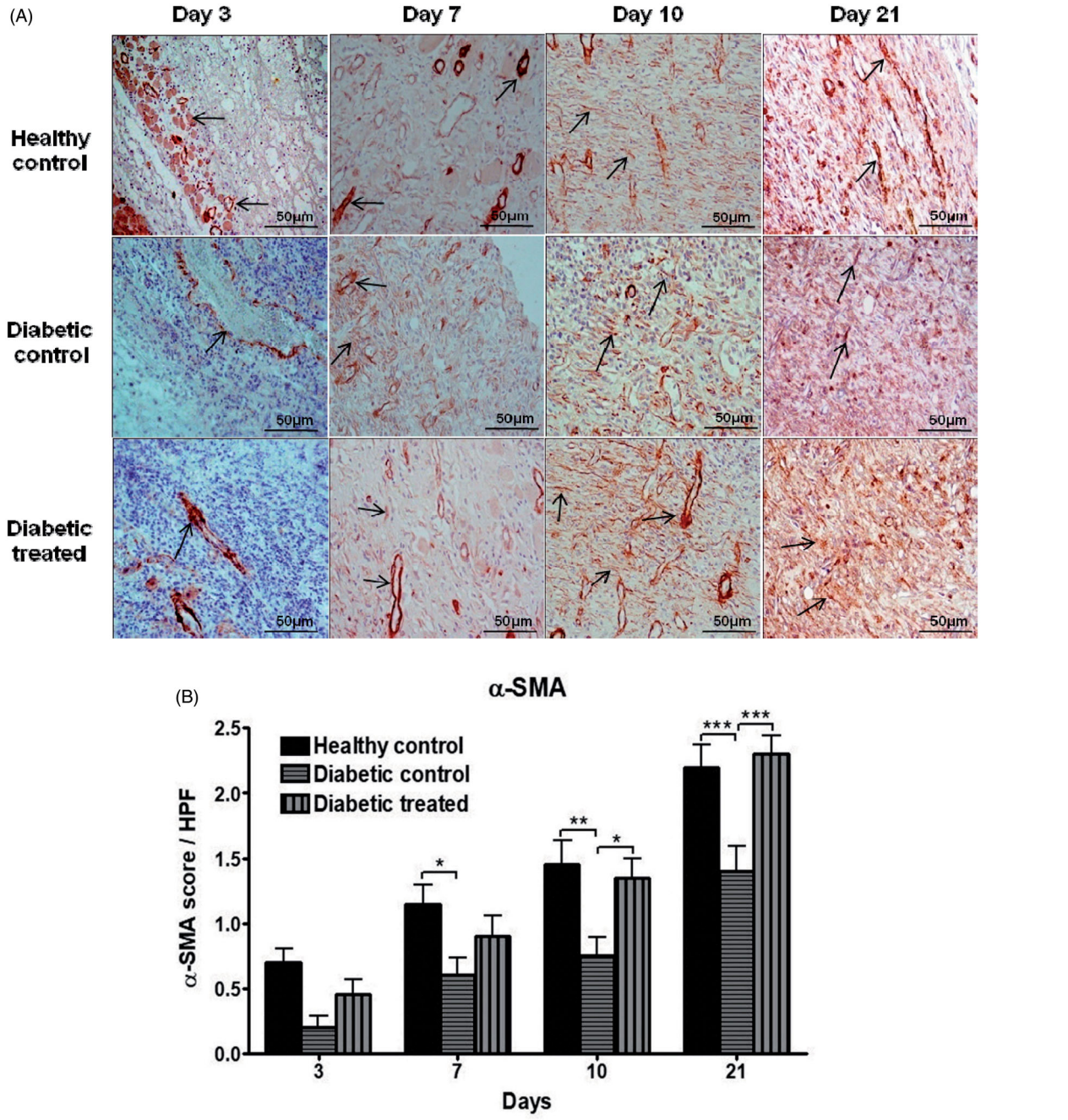

Figure 7. (A) Representative immunohistochemical α-SMA staining of wound sections of healthy control, diabetic control and diabetic-treated (quercetin treated) rats on days 3, 7, 10 and 21 postwounding (40× magnification and scale bar 50 µm). Arrow indicates the positive reaction for α-SMA on the fibroblasts and blood vessels. (B) Average score of α-SMA positive reactions in the histological wound sections of different groups on days 3, 7, 10 and 21 postwounding. Scoring was done by assessing the positive reactivity in 20 randomly chosen high-power fields (HPFs) (40×) from minimum three sections. Data are expressed as means ± SEM, ($n = 20$). $^{*}p<.05$; $^{**}p<.01$; $^{***}p<.001$ compared with other group(s) on the same day.

the diabetic wounds, which contributes in the early completion of inflammatory phase in comparison to wounds of diabetic control group. It has been reported that intravenous injection of IL-10 increased cutaneous wound healing in mice [33].

The MMPs also regulate the inflammatory conditions. Increased activity of MMPs or imbalance between MMPs and their inhibitors cause degradation of ECM and also attract more inflammatory cells, thereby worsen the chronic inflammatory conditions in the wound bed [34]. Several studies asserted the negative effect of increased levels of MMPs (mainly MMP-2 and MMP-9) on wound healing [35]. Previous report states that TNF-α stimulates its own secretion and that of IL-1β [36], and further they cause abnormal production of high levels of MMPs and free radicals to fuel the inflammatory process [37]. Therefore, in our study, the anti-inflammatory nature of 0.3% topical quercetin ointment at diabetic wound site was evident form decreased expressions of inflammatory cytokines (TNF-α and IL-1β) and MMP-9, and increased expressions of IL-10.

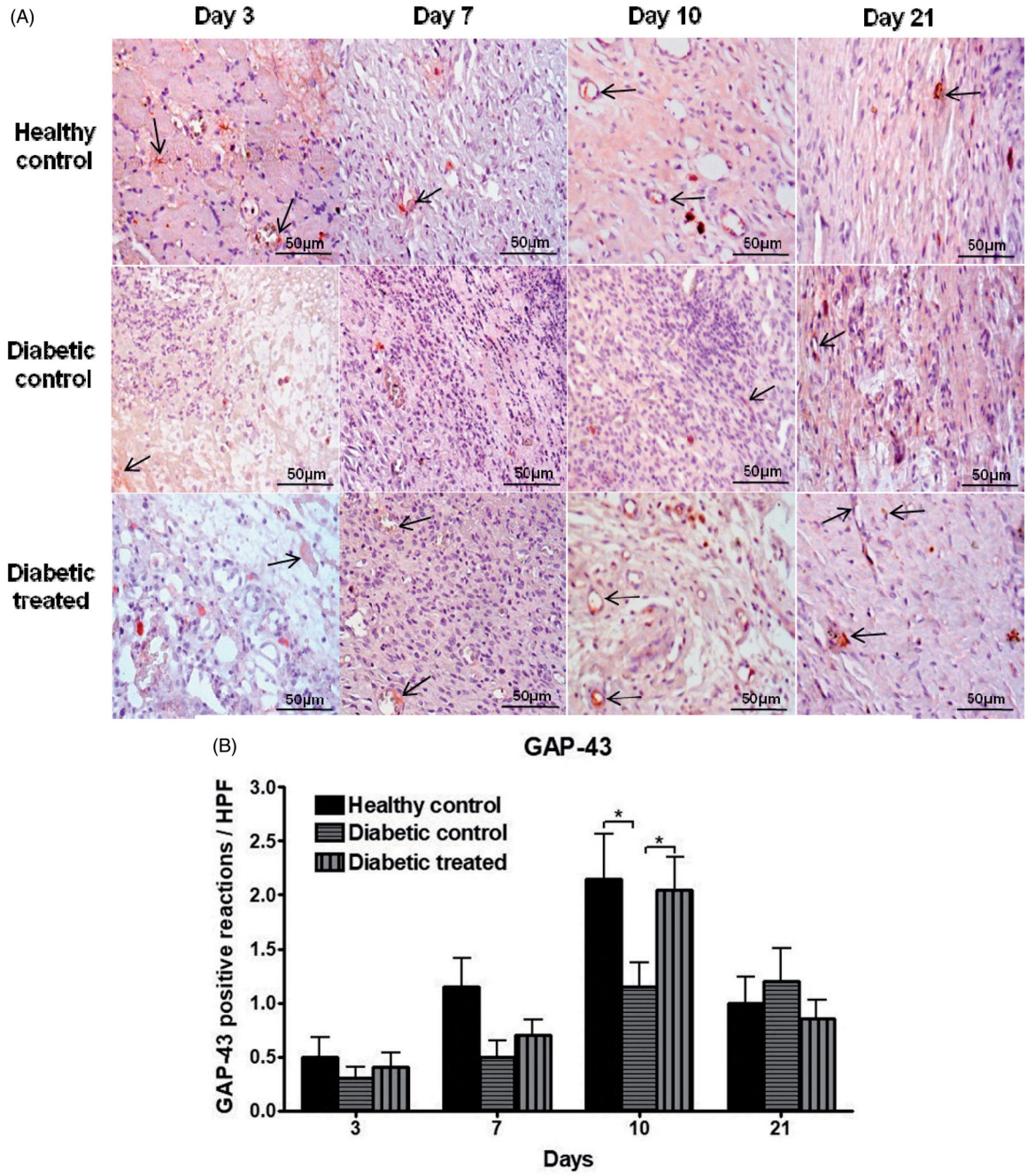

Figure 8. (A) Representative immunohistochemical GAP-43 staining of wound sections of healthy control, diabetic control and diabetic-treated (quercetin treated) rats on days 3, 7, 10 and 21 postwounding (40× magnification and scale bar 50 µm). Arrow indicates the positive reaction for GAP-43. (B) Semiquantitative analysis of GAP-43-positive nerve fibers in the stained sections of different groups on various days by assessing the positive reactivity in 20 randomly chosen high-power fields (HPFs) (40×) from minimum three sections. Data are expressed as means ± SEM, ($n = 20$). $^{*}p<.05$; $^{**}p<.01$; $^{***}p<.001$ compared with other group(s) on the same day.

Angiogenesis and neovascularization are dynamic processes of proliferative phase and regulated by multiple factors from serum and ECM [38]. Impaired angiogenesis causes further damage of tissues resulting from tissue hypoxia and impairment of micronutrients, signaling molecules and growth factors delivery to the wounded tissues [38]. There are number of pro-angiogenic growth factors like VEGF, TGF-β_1, etc., which directly or indirectly have role in the process of angiogenesis. The VEGF has involvement in the angiogenesis by stimulating endothelial cell functions needed for new blood vessel formation, such as proliferation, migration, differentiation, and survival, thus, the amount of VEGF in the healing tissue significantly impacts healing [39]. TGF-β_1 is promotor of angiogenesis along with VEGF [40], and regulator of proliferation, migration, survival and differentiation of endothelial cells [41]. In diabetes, levels of VEGF and TGF-β_1 are found to be decreased [42,43]. In the present study, we also observed the decreased expressions of VEGF

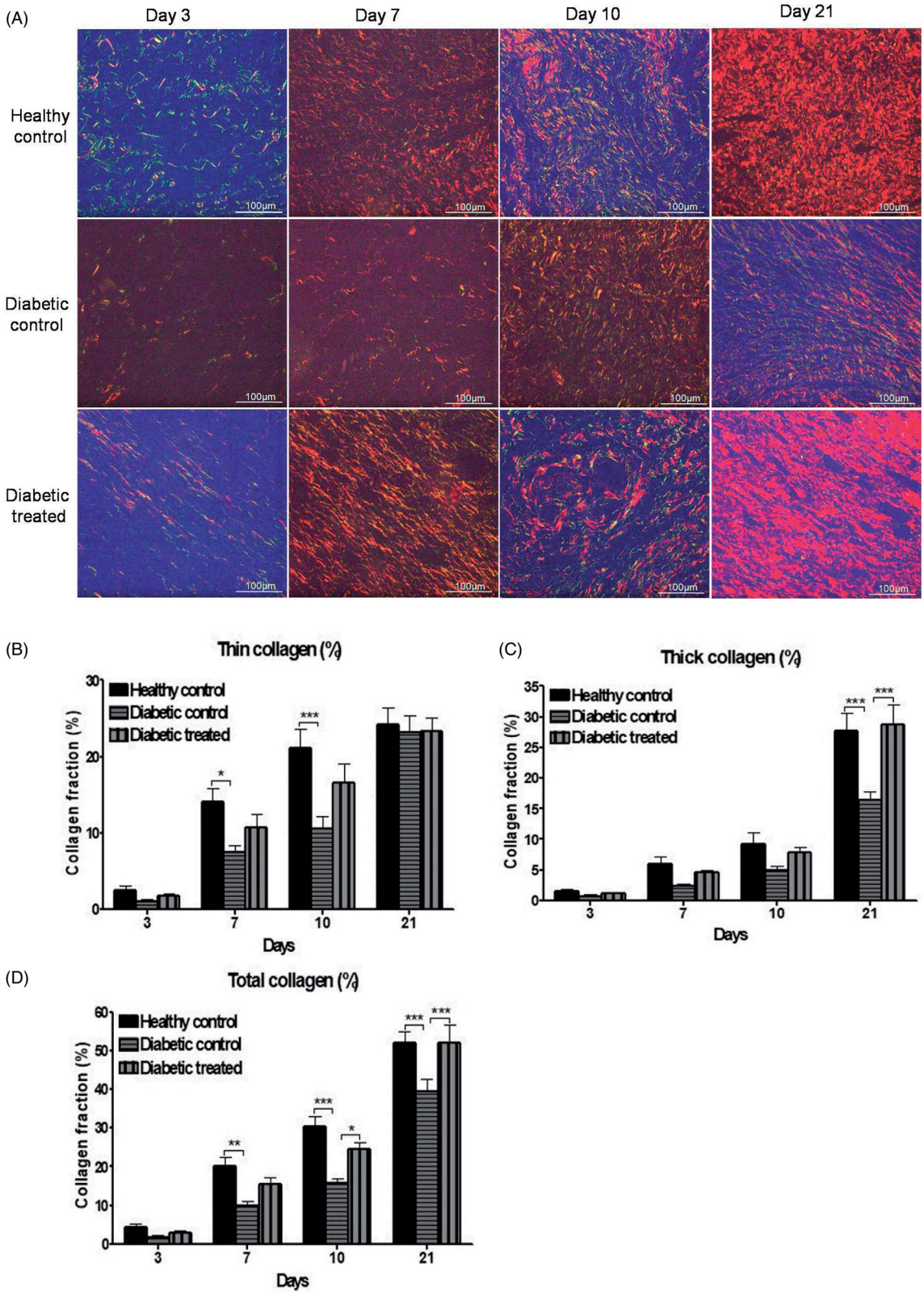

Figure 9. (A) Picrosirius red stained histological sections of healthy control, diabetic control and diabetic-treated (quercetin treated) rats on days 3, 7, 10 and 21 postwounding (20× magnification and scale bar 100 µm). Average fraction of thin (B), thick (C) and total (D) collagen fibers in picrosirius red-stained histological wound sections of different groups on various days from 10 randomly selected fields (20×) of stained sections. Data are expressed as means ± SEM, ($n = 10$). *$p<.05$; **$p<.01$; ***$p<.001$ vs. other group(s) on the same day.

and TGF-β_1 in the wounds of diabetic control group than healthy control group. However, treatment of diabetic wounds with 0.3% quercetin ointment significantly ameliorated the decreased expressions of VEGF and TGF-β_1, and even resulted in the significantly increased expressions of these growth factors in comparison to healthy control and diabetic control groups. Further, the increased expressions of these growth factors resulted in the increased number of blood vessels in the quercetin-treated diabetic wounds, which were well evident from the immunohistochemistry results for CD31 marker (Figure 6). CD31 is considered as a unique marker of a full spectrum of non-endothelial hematopoietic bone marrow cells, which is closely connected with neo-vascularization based on their high angiogenic properties, high adhesion capacity and vasculogenic ability. Increased expressions of CD31 in the healing tissue indicate more angiogenesis and neovascularization. Previous study has reported that VEGF and CD31 play a key role in physiological and pathological processes of angiogenic remodeling, and their expressions decreased in the diabetic skin [44]. Quercetin has shown the potential to up-regulate the VEGF and TGF-β_1 expressions in healing tissue of present study and some previous studies [15]. Therefore, in the present study, quercetin has shown stimulatory effect on angiogenesis by increasing the expression of VEGF, TGF-β_1 and IL-10 and decreasing TNF-α as compared to diabetic control groups.

The microscopic histological evaluation of wound sections is very vital to confirm the quality of healing or healed tissues. The histological changes in the tissue depends on the quantity and duration of production of different cytokines and growth factors and proteases, which play important role in developing suitable or deteriorating environment at wound site for the phenotypic switching, proliferation, migration, apoptosis etc. of different cells involved in the healing mechanisms. In our present study, there is absence of persistence of inflammatory cells in quercetin treated diabetic wounds, which supported the results of TNF-α, IL-1β, MMP-9 and IL-10. Fibroblasts are the primary source of extracellular matrix proteins such as collagen and fibronectin. Synthesis of collagen fibers is vital component of the extracellular matrix, which provides tensile strength to the granulation tissue. In present study, marked presence and proliferation of fibroblasts evident in quercetin treated diabetic wounds were due to increased expressions of VEGF and TGF-β_1 in this group, as the VEGF stimulates the migration and deposition of fibroblasts [45], and TGF-β_1 stimulates the fibroblast proliferation at the wound site [46]. Therefore, increased fibroblast proliferation contributes increased collagen synthesis and deposition, which was evident in picrosirius red stained sections of quercetin-treated group of present study (Figure 9). Moreover, organization and orientation of collagen fibers were better in quercetin-treated diabetic group, and there was significantly higher thick collagen fraction in this group (Figure 9(C)). Decreased TNF-α levels in quercetin-treated group were also favorable for increased synthesis of thick collagen (type I) and thin collagen (type III) in present study. As, previous studies have reported that TNF-α application decreased the expression of collagen types I and III and caused lessen tensile strength of the wound [47].

The collagen synthesis and deposition in the healing tissue are also affected by the activities of MMPs. MMP-9 expressed in skin play a role in wound healing and cell signaling [48]. Enhanced activity of MMP or imbalance between MMPs and their inhibitors have been found to be associated with extensive degradation of ECM [49]. MMP-9 interferes with the basement membrane protein structure, which in turn hinders migration as well as attachment of keratinocyte, and the reestablishment of the epidermis [50]. Our study has showed the down-regulating effect of quercetin on the expressions of MMP-9, as compared with diabetic control group, and this could be responsible for good quality granulation tissue formation for faster healing of wound in quercetin treated group. It has also been reported that the most MMP genes have TGF-β_1 inhibitory elements in their promoter regions and their expression is decreased by TGF-β_1 [9]. This observation supports that quercetin by increasing the expressions of TGF-β_1 also removed the inhibitory influence of MMP-9 on collagen synthesis and deposition.

The 0.3% quercetin application was also successful in increasing the expressions of α-SMA (marker of myofibroblast) in present study (Figure 7), which is also the reason of faster contraction in this group. The transformation of fibroblasts to myofibroblasts phenotype is essential for contraction of wounds and this conversion is triggered by TGF-β_1 [51]. Earlier studies have shown that diabetics have reduced levels of TGF-β_1 and reduced formation of myofibroblasts, which contribute to impaired wound contraction [43]. Therefore, in the present study, increased expressions of TGF-β_1 by quercetin might also contribute in early as well as more numbers of fibroblast switching to myofibroblasts. Previous study has also reported that quercetin enhanced myofibroblasts activity and increased epithelial cell growth [52].

The provisional matrix formed by fibroblasts, due to the action of TGF-β_1, acts as scaffold for the migration of the keratinocytes [40]. The migration of keratinocytes results in the epithelialization at wound site. The motility and proliferation of keratinocytes is also controlled by growth factors and contact with fibroblasts [53]. The VEGF stimulates the migration of activated keratinocytes [54] and TNF-α perturb the keratinocyte physiology [55]. Therefore, TGF-β_1 and TNF-α have opposite roles in collagen synthesis. This has been proved in a previous study, which revealed that TNF-α inhibits Smad phosphorylation through c-Jun N-terminal kinase pathway and reduces the transcription of TGF-β_1, collagen 1, fibronectin and alpha-smooth muscle actin [56]. Thus, early, thick and complete regeneration of epithelial layer in the 0.3% quercetin-treated diabetic group, as compared to diabetic control group, was due to the increased expressions of VEGF and TGF-β_1, and decreased expression of TNF-α.

GAP-43 is a marker of regeneration and remodeling of the nerve fibers [57] and its expression decreases in diabetic patients [58]. In the present study, increased expression of GAP-43 positive fibers in quercetin-treated diabetic group revealed better regeneration and remodeling of the wound. In remodeling phase of wound healing, apoptosis of blood

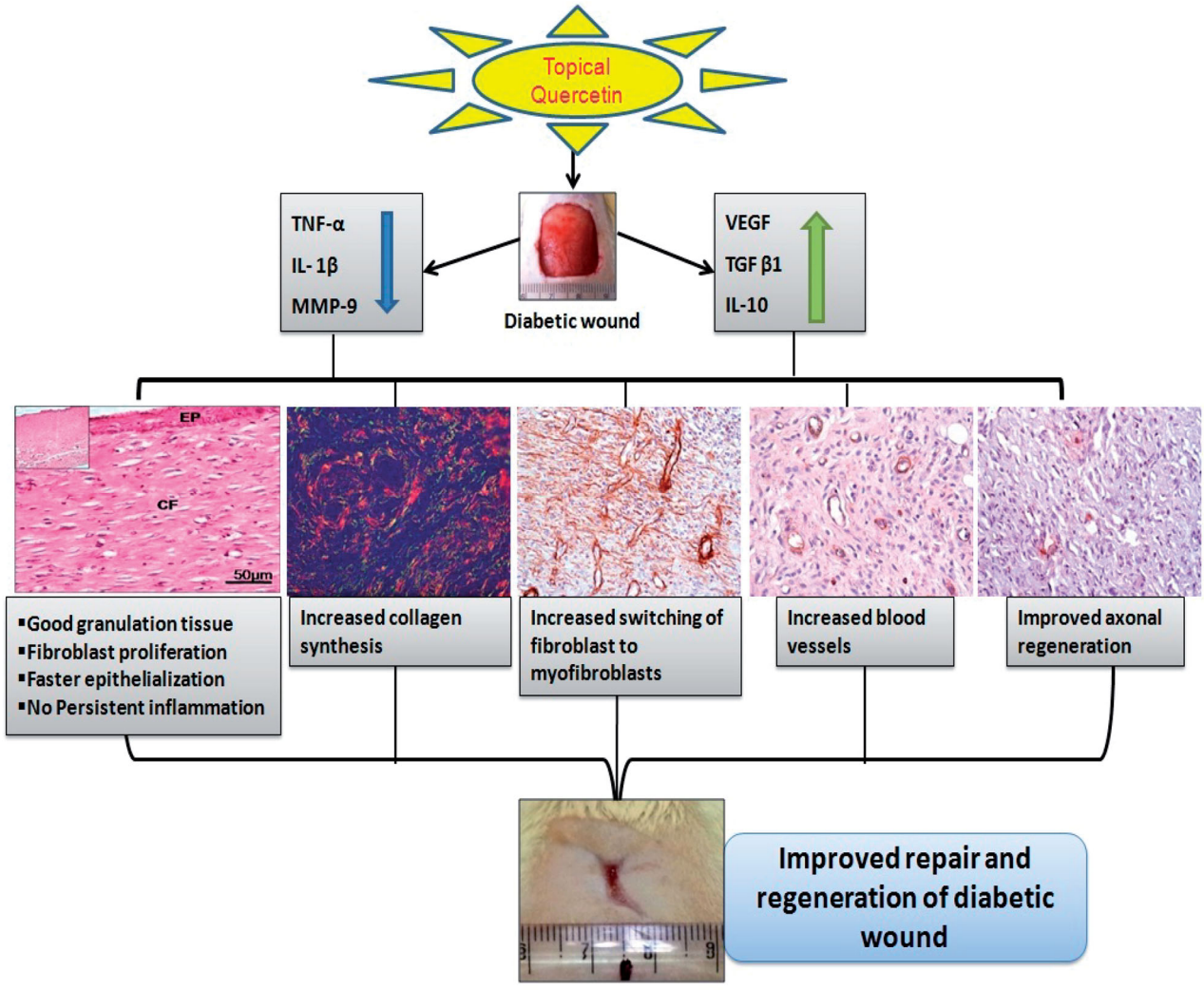

Figure 10. Graphical presentation of possible mechanisms of quercetin that converge to accelerate wound repair and regeneration in diabetic rats.

vessels or other cells occurs. Hence, marked reduction in number of blood vessels and GAP-43-positive cells in 0.3% quercetin-treated diabetic group on day 21 was might be due to their apoptosis of extra ones. The increased fraction of well-organized collagen fibers, early regenerated epithelial layer, apoptosis of extra cells etc. in the quercetin treated diabetic wounds during the later stages of wound healing accelerates the progression of the wound toward maturity. All these effects reveal that quercetin treatment facilitate a well synchronized process of wound repair in diabetic wounds. On the basis of data of present study, the possible mechanisms of quercetin that converges to accelerate wound repair and regeneration in diabetic rats is graphically shown in Figure 10.

Conclusion

It might be concluded from the present study that once daily topical application of quercetin on diabetic wounds for 21 days resulted in increased expressions of IL-10, VEGF and TGF-β_1, and decreased the expressions of TNF-α, IL-1β, MMP-9 in comparison to wounds of diabetic control group. The modulations of these cytokines, growth factors and protease led to good quality granular tissue formation, suppression of persistence of inflammatory cells, fibroblasts proliferation, better angiogenesis, thick collagen fibers synthesis, axonal regeneration and faster regeneration of epithelial layer, which significantly reflect the improvements in repair and regenerations of diabetic wounds in rats. So, as a result of this potential of quercetin (0.3%), it could be envisioned as a novel agent for the acceleration of healing in diabetic wounds or other complicated wounds of humans as well as animals. However, some additional essential elaborative studies must be carried out before its clinical use or commercial applications. Moreover, in future, quercetin nano-formulations may be prepared to evaluate their diabetic wound-healing potentials in order to exploit its healing effects in better way and at lower dose.

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Disclosure statement

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