Quercetin accelerated cutaneous wound healing in rats by increasing levels of VEGF and TGF-β1

A Gopalakrishnan, M Ram, S Kumawat, SK Tandan & D Kumar*
Division of Pharmacology and Toxicology, Indian Veterinary Research Institute, Izatnagar 243 122, Uttar Pradesh, India

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Quercetin (3,3',4',5,7-pentahydroxyflavone)-induced biological effects have been beneficial in various disease conditions. In this study, wound healing potential of quercetin was evaluated in a time-dependent manner in open excision wounds in adult Wistar rats. Experimentally-wounded rats were divided into two groups namely, control and quercetin-treated. Wounds were photographed and the area was measured on the day of wounding and on days 3, 7, 11 and 14 post-wounding. The granulation/healing tissue was collected on days 3, 7, 11 and 14 post-wounding for cytokine/growth factor measurements and histology/immunohistochemistry studies. There was significant time-dependent increase in wound closure in quercetin-treated rats. Vascular endothelial growth factor and transforming growth factor-β1 expressions were significantly upregulated in quercetin-treated rats, whereas tumor necrosis factor-α level was markedly reduced. Interleukin-10 levels and CD31 stained vessels were markedly higher on day 3 and on day 7, respectively, in quercetin-treated rats. In H & E stained sections, quercetin-treated group showed less inflammatory cells, more fibroblast proliferation, increased microvessel density, better reepithelialization and more regular collagen deposition, as compared to control. The results suggest that topical application of quercetin promotes wound healing by effectively modulating the cytokines, growth factors and cells involved in inflammatory and proliferative phases of healing.

Keywords: Cytokines, Endothelial cells, Growth factors, Interleukin

Quercetin (3,3',4',5,7-pentahydroxyflavone), the most abundant dietary flavonoid, possesses adaptogenic/antistress, anticancer, antidiabetic, anti-inflammatory, antimicrobial, antimutagenic, atminousetive, antioxidant, antiulcer, cardioprotective, cytoprotective, hepatoprotective, hypolipidemic, neuroprotective and vasoprotective effects and application in pain management, particularly thermal hyperalgesia and cold allodynia. It exhibits considerably high antiradical property. It is also known to attenuate pesticide toxicity. Natural sources of quercetin (‘quercus’ means oak) include oak, red grapes, apples, onions, green tea, citrus fruits and other leafy green vegetables. Chouhan and Flora have also, in their review, discussed the potential benefits of quercetin including arsenic and fluoride poisoning. It is regarded as a useful therapeutic agent for colitis and gastric ulcer since it potentially stimulates gastric epithelial proliferation and has been suggested as an effective promising new treatment for healing common mouth ulcers. In a preliminary study, application of quercetin-incorporated collagen

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*Correspondence:
Fax: +91 581 2303284
E-mail: dineshks17@ivri.res.in; dineshks17@gmail.com
increased hydroxyl-proline contents in dermal excisional wounds in rats and in bone regeneration in rabbits. Vascular endothelial growth factor (VEGF), was upregulated by quercetin via activating the key transcription factor of VEGF gene, the hypoxia inducible factor-1α (HIF-1α). In view of the above, here we evaluated time (phase)-dependent wound healing potential of quercetin in excisional wound model in rats. Wound area was measured on different days post-wounding and major cytokines and growth factors that participate in wound healing were measured to support gross healing effects of quercetin.

Materials and Methods

Animals
Healthy adult male Wistar rats (150-170 g) were procured from the Laboratory Animal Resource Section, Indian Veterinary Research Institute, Izatnagar (UP), India. The experimental protocols were approved by the Institutional Animal Ethics Committee (number: F.1-53/2012-13-J.D. (Res), 10th September 2012) and conforms to the guidelines for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

Drug preparation
Quercetin (Q 4951-10G, ≥98% HPLC solid) purchased from Sigma-Aldrich, USA was used to prepare 0.1% quercetin ointment in soft paraffin.

Creation of excision wound
The rats were anaesthetized using sodium pentobarbital (40 mg/kg i.p.) and a 2x2 cm full thickness wound up to the depth of loose subcutaneous tissue was created on the dorsal thoracic region of rats. The wounded rats were divided into two groups, viz., control and treatment, of 20 each, and housed in individual cages. In control group, soft paraffin and in treatment group, 0.1% quercetin ointment were topically applied twice daily for 14 days. Positive control group was not used as universally accepted standard wound healing agent was not available.

Wound area measurement and photography
Margins of the wounds were traced on a transparent sheet using a fine tip marker pen. The area (mm²) within the boundaries of each tracing was determined planimetrically starting from the day of creation of wound and on days 3, 7, 11 and 14 post wounding. Along with area measurement, photograph of each wound was taken.

Tissue harvesting
Five animals from each group were sacrificed on days 3, 7, 11 and 14 with an overdose of diethyl ether and the granulation/healing tissue was carefully excised out. The tissue collected was immediately divided into three portions. Tissue homogenate was prepared with one portion in ice-cold lysis buffer and centrifuged at 12000 rpm for 10 min at 4°C. The supernatant obtained was aliquoted and stored at −80°C till further processing for Western blotting and ELISA. Second portion was stored in RNA stabilization reagent (RNAlater™, Qiagen, USA) at −20°C for real time-PCR studies. The third portion was preserved in 10% neutral buffer formalin for histological evaluation and for immunohistochemical studies.

mRNA expression studies (Real-time RT-PCR)
Total RNA was isolated from the granulation tissue using the standard method described by Amresco, USA with Ribozol™ RNA extraction reagents and cDNA was synthesized using cDNA synthesis kit (Thermo Scientific RevertAid First Strand cDNA Synthesis Kit), as per standard protocol. The real time PCR assay was performed by using 2x SYBR Green master mix Universal (KAPA BIOSYTEMS, USA) in CFX96 real time PCR DET SYS (C-1000 thermal cycler, BIO-RAD laboratories India). The following thermal cycling profile was used (40 cycles): initial incubation at 95°C for 3 min followed by 95°C for 3 s, 59-62°C (depending on primer) for 30 s and 72°C for 30 s. The ∆∆CT method of relative quantification was used to determine fold change in gene expression and was obtained as 2−∆∆CT.

SDS-PAGE and Western blot analysis
The expression levels of VEGF and TGF-β1 were determined by Western blot analysis. Equal protein concentrations of tissue homogenates were resolved by 15% SDS polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride (PVDF) membrane at 30V for 1 h 45 min. The membranes were blocked in 3% BSA for 1 h at 37°C followed by overnight incubation at 4°C with the respective primary antibodies separately: goat polyclonal antibodies of VEGF & TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:500 dilutions) and mouse monoclonal antibody of β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000 dilutions). After washing with Phosphate Buffered Saline with Tween 20 (PBS-T), blots were incubated with secondary antibody; horseradish


peroxidase (HRP)-conjugated chicken anti-goat IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; 1:1000 dilutions) for VEGF & TGF-β and with goat anti-mouse IgG (1:1000 dilutions) for β-actin, at 37°C for 2 h. Following successive washes, the blots were developed using the 3', 3'-Diaminobenzidine (DAB) system (GeNei, Bangalore, India). The membranes were subsequently scanned and band intensity was quantified using densitometry software (Image J, NIH). The western blot data for VEGF and TGF-β1 were corrected for corresponding β-actin values and the results were expressed as normalised protein levels. A minimum of three such blots were performed for every protein analyzed.

ELISA for IL-10 and TNF-α
ELISA assay of the tissue lysate was conducted for TNF-α and IL-10 as per the manufacturer’s instructions (Abcam UK, Catalogue Nos. ab100785 for TNF-α rat ELISA kit, and ab100764 for IL-10 rat ELISA kit).

Histological study by haematoxylin and eosin (H&E) staining
About 5 µm thick tissue sections were stained with H & E as per standard method and visualized under light microscope at magnifications of 10x and 40x and photographed. Inflammatory cell infiltration and fibroblast proliferation in the granulation tissue sections of two groups of rats on various days were assessed by using the 0-4 numerical scale method described earlier39. Ten random fields (40x) from three stained sections (at least two field from each section) of each group were scored. The scores assigned were 0 for absence, 1 for occasional presence, 2 for light scattering, 3 for abundance and 4 for confluence of cells.

Immunohistochemistry for CD31
On the 7th day, skin sections were immunolabeled for CD31 marker for angiogenesis, by incubating with primary mouse monoclonal CD31 antibody (Novus Biologicals, Littleton, CO, USA) and HRP-conjugated goat anti mouse IgG (Santa Cruz Biotechnology). The aminoethylcarbazole (AEC) was used as chromogen substrate (AEC Staining Kit; Sigma-Aldrich, USA). Microphotographs were captured under light microscope at 10x magnification. Ten random fields were observed (40x) for the semi-quantitative analysis of microvessel density (MVD).

Statistical analysis
Results are expressed as mean ± standard error (SE) with ‘n’ equal to number of animals. The statistical significance between the experimental and control values was analyzed by applying unpaired ‘t’ test using the Graphpad Prism v4.03 software program (San Diego, CA, USA), and the differences were considered statistically significant at P <0.05 or lower.

Results
The representative photographs of wounds of the two groups of rats taken on days 0, 3, 7, 11 and 14 post-wounding, are given in Fig. 1. The absolute wound surface area of two groups calculated on respective days are presented in table 1. The reduction in wound surface area was in a time-dependent manner in both the groups. On 7th day onwards, both the groups showed an increased reduction in wound area wherein wound area was significantly (P <0.05) lower in quercetin-treated group (165.80 ± 10.28) as compared to the control (203.70± 11.78). The absolute wound area in quercetin-treated group remained significantly lower till 14th day post-wounding (22.14 ± 1.59), as compared to control (33.69 ± 2.06).

The relative VEGF mRNA expression in response to quercetin treatment is presented in Fig. 2A. The
expression of VEGF was upregulated significantly ($P < 0.05$) in quercetin group to approximately $2.24 \pm 0.41$ fold on 3rd day and $2.11 \pm 0.20$ fold on 7th day, as compared to control. The relative TGF-$\beta_1$ mRNA expression in response to quercetin treatment, as compared to the control group is presented in the Fig. 2B. There was significant upregulation of TGF-$\beta_1$ mRNA in wounds treated with quercetin, approximately $1.45 \pm 0.11$ and $2.12 \pm 0.21$ fold compared to control group on day 3 and 7, respectively.

The representative Western blots of $\beta$ actin, VEGF and TGF-$\beta_1$ are presented in Fig. 3A. The band intensity of VEGF blot was more on days 3 and 7 in quercetin-treated group, as compared to that of control. The VEGF protein expression was significantly increased by $1.17 \pm 0.05$ fold on day 3 in quercetin treated group (Fig. 3B) and on day 7, $1.41 \pm 0.14$ fold increase noted in quercetin-treated group which was significantly higher, as compared to control. The intensity of TGF-$\beta_1$ protein bands was more in quercetin-treated group, as compared to control on days 3 and 7. On day 7, $1.50 \pm 0.27$ fold significant increase in expression was noted in treatment group as compared to control (Fig. 3C).

On all the days, TNF-$\alpha$ mRNA expression was found to be down-regulated in quercetin-treated group, as compared to control group (Fig. 4A). On day 3 there was significant ($P < 0.001$) reduction in expression (0.26 $\pm$ 0.05 fold) of TNF-$\alpha$ gene in quercetin group and on day 7 also there was 0.79 $\pm$ 0.08 fold reduction in expression noted in quercetin group, which was significantly ($P < 0.05$) less as compared to control. The relative IL-10 mRNA expression in response to quercetin treatment as compared to the control group is presented in the Fig. 4B. On day 3, IL-10 expression was increased by $1.30 \pm 0.10$ fold in quercetin-treated group which was significantly ($P < 0.05$) higher as compared to control.

The TNF-$\alpha$ protein levels remained lower during the entire experiment in the quercetin-treated group as compared to control group (Fig. 4C). The levels were significantly lower on days 3 (1940 $\pm$ 161 pg/mg protein, $P < 0.01$) and 7 (1052 $\pm$ 48 pg/mg protein, $P < 0.05$) in quercetin-treated group as compared to control. The IL-10 levels by ELISA on different days of post-wounding in both the groups are presented in Fig. 4D. IL-10 level in quercetin-treated group was significantly higher on day 3 (1154 $\pm$ 31 pg/mg protein, $P < 0.05$) as compared to control.
On day 3 (Fig. 5 A,a) H & E stained sections of control group showed presence of more inflammatory cells and very few fibroblast infiltration. In quercetin-treated group (e) showing less number of inflammatory cells and more fibroblast infiltration compared to control (a). On day 7: quercetin group (f) showing more fibroblast proliferation, less inflammatory cells and more blood vessels compared to control (b). On day 11: quercetin group (G, g) showing well formed granulation tissue and regular collagen deposition compared to control (C, c). On day 14: quercetin group (H, h) showing complete superficial epithelial layer and regular collagen deposition compared to control (D, d). BV: blood vessels, C: collagen, F: fibroblasts, I: inflammatory cells, G: granulation tissue, N: necrotic tissue. (I, J) Histological scoring for inflammatory cells and fibroblast proliferation (score from 0-4). Data are expressed as mean ± SE, n= 10; *P<0.05; **P<0.01 vs. control on respective day

On day 3 (Fig. 5 A,a) H & E stained sections of control group showed presence of more inflammatory cells and very few fibroblast infiltration. In quercetin-treated group, there was considerably less number of inflammatory cells and more fibroblast infiltration noticed (Fig. 5 E,e), as compared to control. The wound sections on day 7 (Fig. 5 B,F,b,f) showed appearance of granulation tissue, in both the groups. Control group still showed presence of more inflammatory cells, fibroblast proliferation was evident with few capillaries and mild collagen deposition. In quercetin-treated group inflammatory cells were less in number and more fibroblast proliferation observed. Histological scoring of wound sections for inflammatory cells (Fig. 5I) and fibroblast proliferation (Fig. 5J) revealed significant difference between the groups on days 3 and 7. Capillary density was more in quercetin group. On day 11 (Fig. 5 C,C,G,g), collagen deposition was evident in both the groups. In quercetin-treated group deposition of collagen was regular and throughout the thickness of granulation tissue as compared to control. On 14th day (Fig. 5 D,d,H,h),
sections of both groups showed well formed granulation tissue with collagen deposition and superficial epithelialisation. Quercetin-treated group showed more compact, and regular collagen deposition and complete superficial epithelialisation as compared to control group.

The representative images of CD31-positive vessels of both groups are presented in Fig. 6A and 6B. The neovascularization was better in quercetin-treated group where well marked lumen of blood vessels with large perimeter noticed as compared to control. The MVD in quercetin-treated group was significantly ($P < 0.05$) increased on day 7 (Fig. 6C) compared to control.

**Discussion**

Plant derived agents have been attempted for decades to attain the goal of perfect aesthetic healing of skin wounds. In the present study, quercetin caused significant time-dependent increase in wound closure. VEGF and TGF-$\beta_1$ expressions were significantly upregulated in quercetin-treated rats, whereas TNF-$\alpha$ level was markedly reduced. IL-10 level and CD31 stained vessels were markedly higher on days 3 and 7, respectively, in quercetin-treated rats. In H & E stained sections, quercetin-treated group showed less inflammatory cells, more fibroblast proliferation, increased microvessel density, better re-epithelialization and more regular collagen deposition.

In the process of healing, wound contraction occurs due to the activity of myofibroblasts. It begins soon after wounding and peaks at 2 weeks with significant reduction of wound area especially in loose skinned animals. In this study, quercetin-treated wounds showed significantly higher reduction in wound area from day 7 to 14, which is suggestive of an increased myofibroblast activity, as quercetin has been reported to enhance myofibroblast activity and to increase epithelial cell growth in oral ulcers.

**Fig. 6**—Immunohistochemistry depicting for the expression of CD31 in granulation tissues of control (A) and quercetin-treated (B) rats on day 7 post-wounding. (C) Graphical representation showing semiquantitative analysis of MVD on day 7 post-wounding. The MVD was assessed by counting the number of microvessels in 10 randomly chosen high-power fields in stained wound sections of both control and quercetin-treated rats. *$P < 0.05$ vs. control ($n = 4$).
VEGF is the major angiogenic agent in wound that stimulates migration, proliferation and differentiation of endothelial cells. Reduced expressions of VEGF-A gene or mRNA or its accelerated degradation were found to be associated with wound healing defects. Quercetin has been shown to influence the expression of some growth factors and cytokines in studies related to soft tissue healing. It upregulates the VEGF expression in colon epithelial cell lines and in the inflamed colonic tissue, most likely, by activating the HIF-1α pathway. In our study, VEGF mRNA and protein levels were significantly higher in quercetin treated group on days 3 and 7, indicating prohealing effect of quercetin at the wound site. The increased MVD noted in quercetin-treated group on day 7, also suggests the angiogenic potential of quercetin in skin.

TGF-β1 having varied functions in the healing wounds is an important growth factor modulator of cell growth and differentiation. It is also involved in upregulating the angiogenic growth factor VEGF. TGF-β1 can be both inhibitory and stimulatory in nature. Several studies suggest that quercetin modulates TGF-β1 expression and its signalling pathways and many of them emphasise that quercetin has an inhibitory effect on TGF-β1 expression in conditions like pulmonary fibrosis, liver fibrosis and keloids and hypertrophic scars, where tissue function and cosmeses are compromised by excess TGF-β1 activity. There are also reports of quercetin enhancing TGF-β1 expression in ovarian cancer cell line and in leukemic blasts, where TGF-β1 is supposed to act selectively as a negative regulator on early hematopoietic progenitor cells and, thereby, checking angiogenesis and tumour progression. However, there is a lack of information about expression pattern of TGF-β1 in skin wounds. In the present study, TGF-β1 levels furnish an indication that quercetin can enhance TGF-β1 expression in healing wounds in early proliferative phase, thereby, favouring the fibroblast activity and better extracellular matrix (ECM) deposition and granulation tissue formation. We also observed a decreasing trend in TGF-β1 expression in quercetin-treated rats by day 11 onwards, which might have balanced ECM deposition and degradation. Thus, quercetin might also reduce scar formation.

The maximum gene expression of proinflammatory cytokine TNF-α was observed at 3 h post wounding and the same was maintained until 120 h post-wounding. Elevated levels of TNF-α and IL-1 noted in several wounds can cause increased synthesis of matrix metalloproteinase and decreased expression of tissue inhibitors of metalloproteinase and ECM proteins which can hamper smooth progress of healing. Throughout the course of present study, TNF-α level was lower in quercetin-treated rats and on day 3 and 7 it was significantly lower. In earlier reports, quercetin pretreatment inhibited the secretion of TNF-α and IL-1β by almost 40% in bone marrow derived macrophages through inhibition of the NF-κB pathway in a dose-dependent manner. Additionally, IL-10, the major anti-inflammatory cytokine produced in the early phases of healing by keratinocytes and infiltrating mononuclear cells, is known to limit and terminate the inflammatory responses and renders the wound to evolve into a more proliferative healing phase. In this study, IL-10 mRNA expression was found to be enhanced on day 3 in quercetin group and rest of the days both the groups followed almost a similar decreasing trend in expression. In an earlier study on acute pancreatitis in mice, it has been reported that quercetin treatment increased the serum IL-10 levels. In yet another study, quercetin stimulated the expression of the anti-inflammatory cytokine IL-10 at low concentrations. But some reports suggest that quercetin does not affect or might even decrease IL-10 levels. By analysing the results of TNF-α and IL-10 mRNA expression and protein levels, the present study proposes that quercetin might ameliorate inflammatory response in wounds mainly by downregulating the expression of proinflammatory cytokines like, TNF-α rather than by upregulating anti-inflammatory cytokines like, IL-10. Evidently, decreased inflammatory cell density in H & E stained wound sections of quercetin-treated rats strongly supports its anti-inflammatory nature. Furthermore, histological data also revealed better epithelialisation and collagen deposition in later phases of healing in quercetin-treated rats favouring better wound closure in these rats.

In conclusion, the present study suggests that quercetin has marked beneficial effects in healing of cutaneous wounds by effectively modulating the expression of cytokines and growth factors involved in multiple phases of healing.

**Conflict of interest**

No conflict to disclose.
References


