

Shikonin Promotes Skin Cell Proliferation and Inhibits Nuclear Factor- κ B Translocation via Proteasome Inhibition *In Vitro*

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Abstract

Background: Shikonin is a major active chemical component extracted from *Lithospermi Radix*, an effective traditional herb in various types of wound healing. Shikonin can accelerate granulomatous tissue formation by the rat cotton pellet method and induce neovascularization in granulomatous tissue. The purpose of the study was to investigate its mechanism of action in human skin cells.

Methods: MTS assay was used to measure cell growth. The collagen type I (*COL1*) mRNA expression and procollagen type I C-peptide (PIP) production were detected by real-time quantitative reverse transcription-polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. Immunofluorescence and western blot analyses were carried out to investigate nuclear factor- κ B (NF- κ B) signaling pathway. Cell-based proteasome activity assay was used to determine proteasome activity.

Results: In this study, we found that 10 μ mol/L shikonin stimulated the growth of normal human keratinocytes and 1 μ mol/L shikonin promoted growth of human dermal fibroblasts. However, shikonin did not directly induce *COL1* mRNA expression and PIP production in dermal fibroblasts *in vitro*. In addition, 1 μ mol/L shikonin inhibited translocation of NF- κ B p65 from cytoplasm to nucleus induced by tumor necrosis factor- α stimulation in dermal fibroblasts. Furthermore, shikonin inhibited chymotrypsin-like activity of proteasome and was associated with accumulation of phosphorylated inhibitor κ B- α in dermal fibroblasts.

Conclusions: These results suggested that shikonin may promote wound healing via its cell growth promoting activity and suppress skin inflammation via inhibitory activity on proteasome. Thus, shikonin may be a potential therapeutic reagent both in wound healing and inflammatory skin diseases.

Key words: Cell Growth; Human Dermal Fibroblasts; Keratinocytes; Proteasome; Shikonin

INTRODUCTION

Lithospermi Radix (also called *Zicao* or *Gromwell*), a dried root of *Lithospermum erythrorhizon* Sieb. et Zucc., is a common herbal medicine used in Asian countries. Shikonin is a major active chemical component with a molecular weight of 288 isolated from *Lithospermi Radix*. Shikonin (R-configuration) is an enantiomer of alkannin (S-configuration), which is found mainly in roots of the plant *Alkanna tinctoria* in Europe. Shikonin possesses numerous pharmacological properties, including wound healing,^[1] anti-inflammatory effect,^[2] anti-tumor properties, and ability to mediate cellular and humoral immunity.^[3]

It has been reported that shikonin accelerated granulomatous tissue formation by the rat cotton pellet method, and

induced neovascularization in granulomatous tissue of mice.^[4,5] Meanwhile, shikonin exerted an anti-inflammatory effect by inhibiting tumor necrosis factor- α (TNF- α) and prevented activation of nuclear factor- κ B (NF- κ B) pathway.^[6,7] In addition, a recent study revealed that shikonin exerts anti-tumor activity via inhibition of proteasome activity and induction of apoptosis.^[8] However, most of these results for pharmacological mechanisms were obtained by animal model systems, and only few studies of shikonin in normal human skin cells have been reported.^[9,10] In this study, our aim was to investigate the role of shikonin in the growth of human skin cells and collagen production as an aspect of wound healing promotion by an *in vitro* study. Moreover, we studied the effect of shikonin on NF- κ B pathway in human dermal fibroblasts (HDFs) to elucidate whether NF- κ B pathway contributed to anti-inflammatory effect of shikonin.

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METHODS

Cells, reagents, and antibodies

Normal human keratinocytes (NHKs) and normal HDFs were purchased from Cell Applications Inc., San Diego, CA, USA. Epilife® medium and human keratinocyte growth supplement kit were purchased from Invitrogen, Carlsbad, CA, USA. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS, Gibco®) were obtained from Sigma-Aldrich Co. LLC, St. Louis, MO, USA, and Life Technologies Corporation, respectively. Shikonin was obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan, and ascorbic acid was from Lifeline Cell Technology, Walkersville, MD, USA. Taqman® gene expression assay was purchased from Applied Biosystems, Foster City, CA, USA. Procollagen type I C-peptide (PIP) enzyme-linked immunosorbent assay (ELISA) kit was purchased from Takara Bio Inc., Shiga, Japan. Recombinant human TNF- α was purchased from R&D Systems, Inc., Minneapolis, MN, USA, and lactacystin was from Cayman Chemical, Ann Arbor, MI, USA. Anti-NF- κ B p65 (C-20) polyclonal antibody (pAb) (sc-372) was purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG goat pAb was from DAKO, Glostrup, Denmark. Anti-inhibitor κ B- α (I κ B- α) phospho Ser 32, 36 monoclonal antibody (mAb), and nuclear extraction kit were purchased from Active Motif, Inc., Carlsbad, CA, USA. HRP-Direct-conjugated anti-GAPDH mAb was obtained from MBL, Nagoya, Japan, and alkaline phosphatase-conjugated anti-mouse IgG goat pAb was from Nacalai, Kyoto, Japan.

Cell culture conditions

Normal human keratinocytes were cultured in Epilife® medium with 0.06 mmol/L calcium chloride and human keratinocyte growth supplement, and HDFs were cultured in DMEM medium with 5% FBS. Both cells were placed at 37°C under 5% CO₂ atmosphere. Cells at early passages (up to passage four) were used for the experiments.

Cell growth assay

Cell proliferation was measured in hexad by MTS assay in 96-well plates using MTS-based CellTiter 96® Aqueous nonradioactive cell proliferation assay (Promega Corp., WI, USA) according to the manufacturer's instructions. Absorbance was read at a wavelength of 490 nm with ImmunoMini NJ-2300 microplate reader (System Instruments, Tokyo, Japan).

RNA isolation, cDNA synthesis, and real-time quantitative polymerase chain reaction

After HDFs were cultured to 70–80% confluency in DMEM with 5% FBS, cells were rendered quiescent in serum-free medium for 24 h. Then, after cells were incubated with vehicle (serum-free DMEM containing 0.1% dimethyl sulfoxide [DMSO]) supplemented with 200 μ mol/L ascorbic acid or shikonin (1 μ mol/L, 0.1 μ mol/L, and 0.01 μ mol/L) for 24 h, total RNA was extracted by RNeasy® mini kit (Qiagen, Austin, TX, USA). cDNA was synthesized by reverse transcription

using Verso cDNA synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) with random hexamers. Quantitative polymerase chain reaction (qPCR) was carried out with LightCycler® 480 II (Roche Diagnostics Ltd., Rotkreuz, Switzerland) according to the manufacturer's instructions. cDNA was amplified and detected using Taqman® gene expression assay with probes for *COL1A1* (Hs00164004_m1) and *GAPDH* (Hs02758991_g1). PCR reactions (20 μ l) was performed in triplicate at 50°C for 2 min, then incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, and then 50°C for 30 s. Relative expression of the target genes was calculated by comparing with the housekeeping gene *GAPDH* using a formula described previously.^[11]

Procollagen type I enzyme-linked immunosorbent assay

Human dermal fibroblasts were cultured to near 80% confluency, and then starved for 24 h in serum-free DMEM. After cells were treated with only vehicle (serum-free DMEM containing 0.1% DMSO), 200 μ mol/L ascorbic acid or shikonin (1 μ mol/L, 0.1 μ mol/L and 0.01 μ mol/L) for 24 h and 48 h, supernatants were collected and frozen at -80°C until use. Procollagen type I in the culture supernatants of HDFs was determined by commercially available PIP ELISA kit according to the manufacturer's instructions. Absorbance at 450 nm was read with a microplate reader. The results were compared with a standard curve, which was constructed by serial dilutions of a standard.

Immunofluorescence studies

Cells were seeded onto coverslips in six-well plates and allowed to attach overnight in a medium containing 5% FBS. After cells were starved in serum-free medium for 24 h, cells were pretreated with 1 μ mol/L shikonin or 0.1% DMSO for 2 h prior to stimulation with TNF- α (50 ng/ml). Then, after medium was removed, cells were rinsed with phosphate-buffered saline and fixed with methanol for 8 min at 4°C. A blocking step was performed with 1% BSA in PBS for 30 min at room temperature. Cells were immunostained with anti-NF- κ B p65 (C-20) antibody (1:100 dilution) in 1% BSA/PBS for 2 h at room temperature, followed by incubation with FITC-conjugated anti-rabbit IgG pAb (1:100 dilution) for 1 h at room temperature. Slides were observed with BX 51TRF fluorescence microscope (Olympus, Tokyo, Japan).

Cell-based proteasome activity assay

Approximately, 4,000 HDFs/well in a white-walled 96-well plate were treated with 0.1% DMSO, 1 μ mol/L shikonin or 10 μ mol/L lactacystin at 37°C for 2 h, and then stimulated with 50 ng/ml TNF- α for 30 min. Cells were then incubated with proteasome-Glo™ cell-based assay reagent (Promega Bioscience, Madison, WI, USA) for 15 min according to the manufacturer's protocol. Luminescence generated from each reaction was detected by Centro LB 960 microplate luminometer (Berthold Technologies GmbH and Co. KG, Bad Wildbad, Germany).

Immunoblot analysis

Human dermal fibroblasts were pretreated with 1 μ mol/L shikonin or 0.1% DMSO for 2 h, and then stimulated with

50 ng/ml TNF- α for 30 min. Then, cytoplasmic proteins were extracted by nuclear extract kit (Active Motif, Carlsbad, California, USA) according to the manufacturer's instructions. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 5–20% gradient gel (Atto Co., Tokyo, Japan), and transferred onto nitrocellulose membrane by a semi-dry transfer method using iBlot® system (Invitrogen, Carlsbad, CA, USA). After blocked with 2% BSA for 1 h at room temperature, membranes were incubated with anti-I κ B- α phospho Ser 32, 36 mAb (1:250 dilution), followed by incubation with alkaline phosphatase-conjugated anti-mouse IgG pAb (1:2000 dilution) or HRP-DirecT-conjugated anti-GAPDH mAb (1:1000 dilution). Reactions were visualized using

1-step™ NBT/BCIP (Thermo Scientific, Rockford, IL, USA) or HRP detection systems (Atto Co., Tokyo, Japan). Specific bands were scanned and analyzed by ImageQuant Las 4000 (GE Healthcare Life Sciences, Uppsala, Sweden).

Statistical analysis

Each set of experiments was performed independently at least 3 times and the results are expressed as mean \pm standard deviation (SD). Differences among quantitative parameters between two groups were assessed using paired Student's *t*-test. A *P* < 0.05 was considered statistically significant. All analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA).

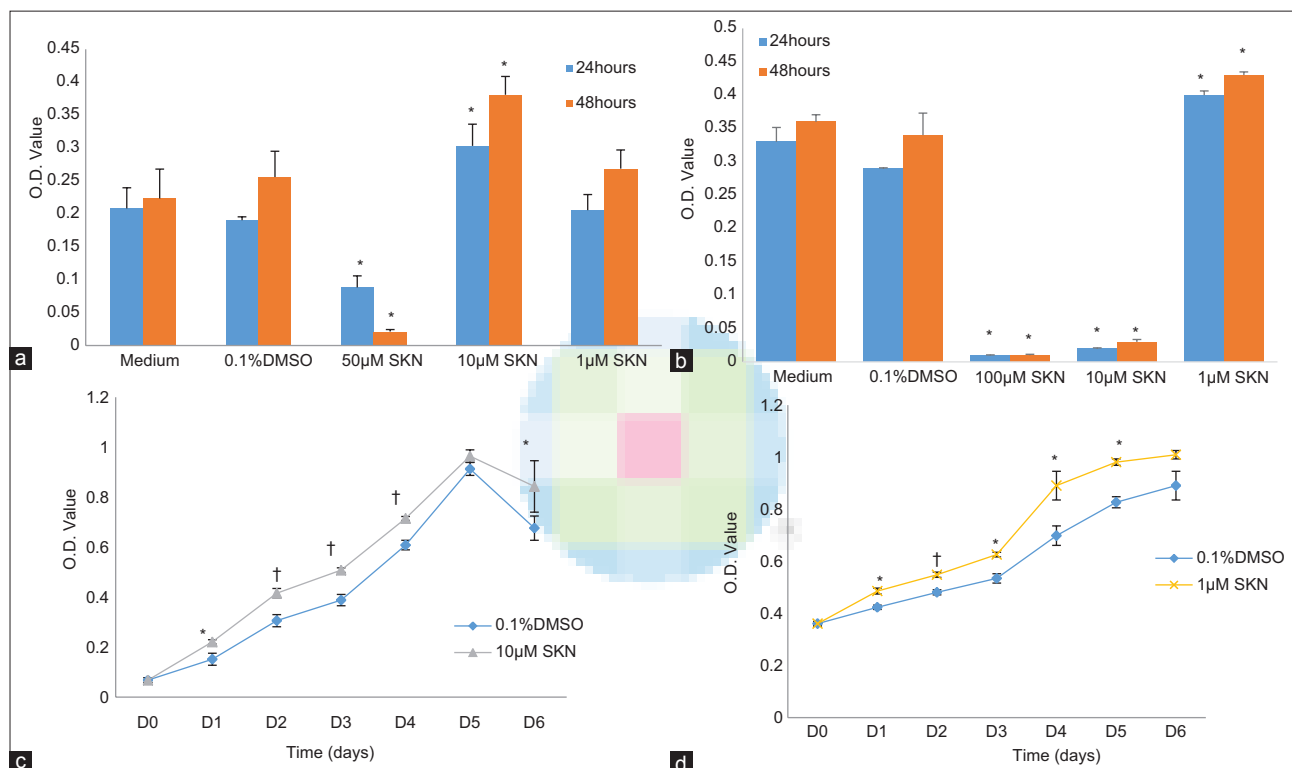


Figure 1: Effects of shikonin on viability and growth of normal human keratinocytes (NHKs) and human dermal fibroblasts (HDFs). (a) Effect of different concentrations of shikonin on NHKs; (b) Effect of different concentrations of shikonin on HDFs; (c) Effect of shikonin on the growth of NHKs; (d) Effect of shikonin on the growth of HDFs. SKN: Shikonin. Results are shown as the mean \pm standard deviation from six samples in each group. **P* < 0.05 compared with the medium group (a and b). **P* < 0.05 and †*P* < 0.01 compared with 0.1% dimethyl sulfoxide group (c and d).

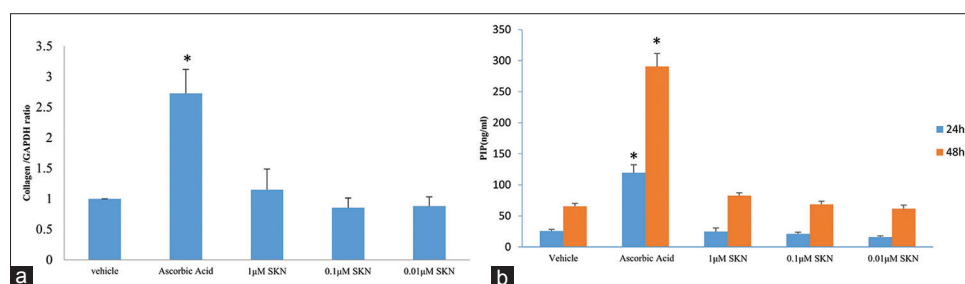


Figure 2: Effects of shikonin on collagen type I (*COL1*) mRNA level and procollagen type I C-peptide (PIP) production in human dermal fibroblasts (HDFs). (a) Expression of *COL1* mRNA in HDFs; (b) PIP production in the supernatant of HDFs. SKN: Shikonin. Results are shown as the mean \pm standard deviation from three independent experiments. **P* < 0.05 compared with the vehicle group (serum-free Dulbecco's modified eagle's medium containing 0.1% dimethyl sulfoxide).

RESULTS

Effects of shikonin on proliferation of normal human keratinocytes and human dermal fibroblasts *in vitro*

An MTS assay was applied to determine the maximum concentrations of shikonin, which did not inhibit cell viability. The nontoxic maximum concentrations of shikonin were used in further experiments. Shikonin at concentrations higher than 50 $\mu\text{mol/L}$ significantly inhibited NHKs viability, compared with that of control ($P < 0.05$) [Figure 1a]. Shikonin at concentrations higher than 10 $\mu\text{mol/L}$ significantly inhibited HDFs viability ($P < 0.05$) [Figure 1b].

In contrast, shikonin at a concentration of 10 $\mu\text{mol/L}$ promoted NHKs growth, compared with that of control ($P < 0.05$) [Figure 1a], and 1 $\mu\text{mol/L}$ shikonin also stimulated HDFs growth ($P < 0.05$) [Figure 1b].

Cell growth curves determined by MTS assay indicated an increase in average absorbance of NHKs from day 1 to day 4 [Figure 1c] and that of HDFs from day 1 to day 5 [Figure 1d]. Inter-group comparison revealed a significant difference between shikonin-treated group and control vehicle (0.1% DMSO) group ($P < 0.05$) [Figure 1c and d].

Effects of shikonin on collagen type I mRNA level and procollagen type I C-peptide production in cultured human dermal fibroblasts

To clarify whether shikonin promotes expression of collagen type I (*COL1*) in HDFs, we measured expression of *COL1* at both mRNA level by qPCR and protein level by ELISA. *COL1* mRNA expression increased significantly ($P < 0.05$) in ascorbic acid-treated group (positive control) [Figure 2a]. However, there was no significant difference in expression of *COL1* mRNA between shikonin- and vehicle-treated groups.

Procollagen type I C-peptide expression was similar to that of mRNA expression [Figure 2b]. Thus, while ascorbic acid increased production of PIP significantly ($P < 0.05$), there was no significant difference in PIP production between shikonin-treated groups (three different concentrations) and control vehicle group at 24 h and 48 h [Figure 2b].

Shikonin inhibited nuclear factor- κB translocation from cytoplasm to nucleus in human dermal fibroblasts *in vitro*

To verify the effect of shikonin on NF- κB signaling pathway, immunofluorescence was performed. Representative figures for these experiments are shown in Figure 3. In untreated cells, strong green fluorescence signal representing NF- κB p65 located mainly in cytoplasm and was almost absent in nucleus [Figure 3b]. TNF- α stimulation induced NF- κB p65 translocation to nuclei [Figure 3c and d]. However, pretreatment with shikonin for 2 h attenuated TNF- α -induced NF- κB p65 nuclear translocation [Figure 3e and f].

Shikonin inhibited proteasome activity in human dermal fibroblasts

Because NF- κB activation is regulated by ubiquitin/proteasome pathway,^[12] we considered that inhibition of NF- κB translocation in HDFs by shikonin might be due to inhibition of proteasome activity. To examine inhibition of proteasome activity in HDFs by shikonin, we performed proteasome activity assay *in vitro*. Shikonin inhibited chymotrypsin-like activity in HDFs more strongly ($P < 0.05$) than trypsin-like and caspase-like activities in HDFs [Figure 4].

Protein expression of phosphorylated-inhibitor κB - α in human dermal fibroblasts treated with shikonin and/or tumor necrosis factor- α

Levels of phosphorylated-I κB - α (p-I κB - α) were examined by immunoblot analyses, after HDFs were treated with 1 $\mu\text{mol/L}$ shikonin or 0.1% DMSO for 2 h and then with 50 ng/ml TNF- α for 30 min. Increase of p-I κB - α expression was observed in cytoplasm of TNF- α -stimulated

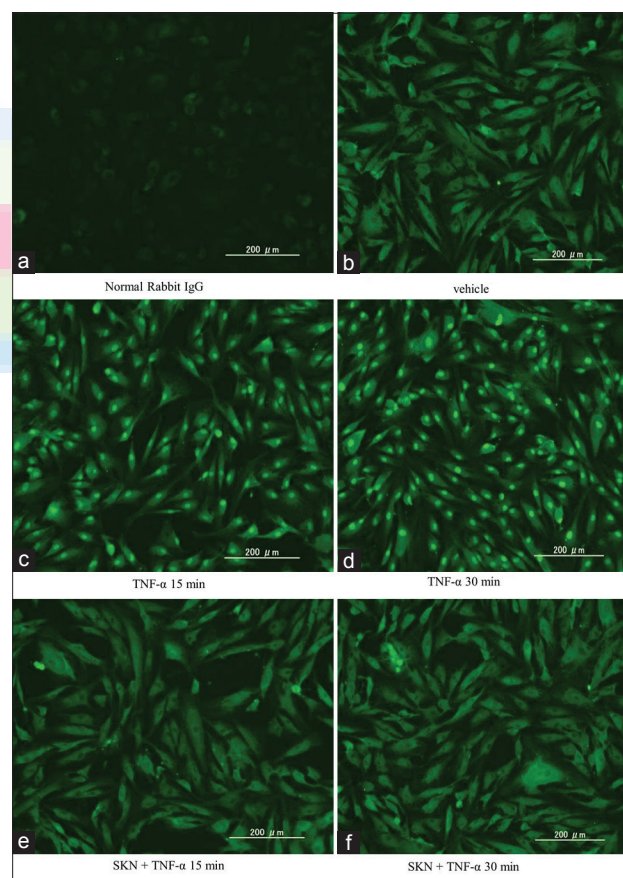


Figure 3: Immunofluorescence study of expression of nuclear factor- κB (NF- κB) in human dermal fibroblasts (HDFs). (a) Purified normal rabbit IgG (negative control); (b-d) Representative immunofluorescence images for NF- κB p65 of HDFs. Vehicle (0.1% dimethyl sulfoxide) (b). Stimulation with tumor necrosis factor- α (TNF- α) alone for 15 min (c) and 30 min (d); (e and f) Effect of pretreatment with shikonin. Stimulation with TNF- α for 15 min (e) and 30 min; (f) SKN: Shikonin, scale bars: 200 μm .

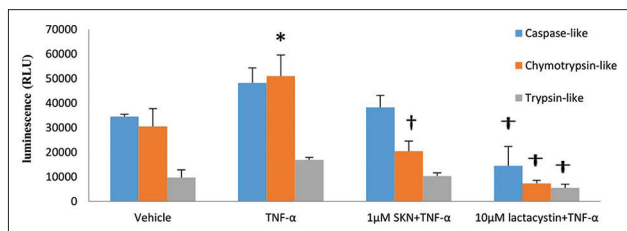


Figure 4: Effect of shikonin on proteasome activity in human dermal fibroblasts. Proteasome inhibitor, lactacystin, was used as a positive control. SKN: Shikonin. Results are shown as the mean \pm standard deviation from six samples in each group. * $P < 0.05$ compared with the vehicle group. † $P < 0.05$ compared with group treated only with tumor necrosis factor- α .

cells, compared to control groups ($P < 0.05$) [Figure 5]. Pretreatment with shikonin before TNF- α stimulation also increased p-I κ B- α level, compared to groups treated only with TNF- α ($P < 0.05$) [Figure 5].

Combined results in proteasome activity assay and expression of p-I κ B- α , pretreatment with shikonin before TNF- α stimulation inhibited degradation of p-I κ B- α . Accumulated p-I κ B- α attenuated NF- κ B p65 nuclear translocation, which resulted in decrease of p65 protein in nucleus and increase of p65 protein in cytoplasm. These results indicated that shikonin inhibited TNF- α -induced NF- κ B activation.

DISCUSSION

Wound healing proceeds through inflammatory, proliferative, and remodeling phases. During the inflammatory phase, immune cells enter into wounded site. Neutrophils respond to infection. Macrophages release inflammatory mediators, such as cytokines and growth factors, and clear dead tissues from the wound, thus facilitating fibroblast proliferative processes.^[13] Fibroblasts also secrete growth factors and extracellular matrix components, while endothelial cells contribute to the formation of new vasculature, leading to tissue regeneration.^[13]

A clinical trial for 72 patients with varicose ulcer of leg first found that alkannin esters showed excellent wound healing properties.^[14] In several *in vivo* animal studies of wound healing, shikonin and its derivatives induced granulomatous tissue formation, accelerated proliferation of fibroblasts and collagen fiber, and induced neovascularization, which eventually promoted wound healing.^[4,5] A recent study concluded that shikonin stimulated epithelial-mesenchymal transition in skin wound healing.^[15] However, mechanism of the effects of shikonin and its derivatives on human skin cells needed to be confirmed by *in vitro* experiments. Only one study demonstrated that shikonin and its derivatives stimulated the growth of cultured human amnion fibroblasts.^[16]

In the present study, shikonin promoted proliferation of cultured NHKs and HDFs in the absence of other inflammatory chemical mediators [Figure 6, left panel].

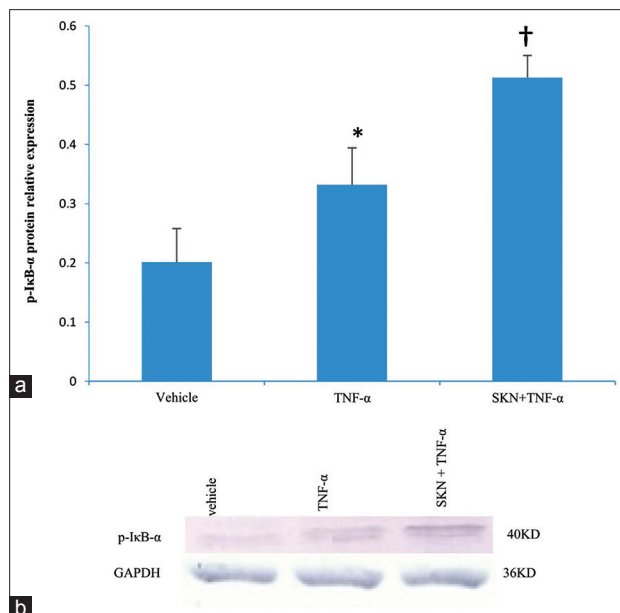


Figure 5: Effect of shikonin on tumor necrosis factor- α (TNF- α)-induced phosphorylated-inhibitor κ B- α (p-I κ B- α) protein expression in HDFs. (a) TNF- α -induced increase in p-I κ B- α expression in HDFs; (b) Representative pictures of p-I κ B- α protein expression in indicated groups detected by immunoblot analysis. SKN: Shikonin. Results are shown as the mean \pm standard deviation from three independent experiments. * $P < 0.05$ compared with the vehicle group. † $P < 0.05$ compared with group treated only with TNF- α .

Nevertheless, shikonin did not directly induce *COL1* mRNA expression and PIP production in HDFs by qPCR and ELISA. Thus, shikonin may play an essential role in promotion of wound healing via proliferation of both NHKs and HDFs but not via *COL1* production. In a previous study, although shikonin accelerated collagen fiber formation in the granulation tissue in the study by cotton pellet method,^[4] it was considered to be due to increase of fibroblasts number.

Nuclear factor- κ B is a well-known inducible transcription factor that mediates signal transduction between cytoplasm and nucleus in many cell types, including HDFs.^[17] NF- κ B in cytoplasm is normally sequestered by its endogenous inhibitor, I κ B- α . Once stimulated, I κ B- α is rapidly phosphorylated by I κ B kinase. Phosphorylated I κ B- α is transferred to proteasome and induce its degradation, while NF- κ B translocates to nucleus and mediates transcription of many pro-inflammatory genes.^[18] NF- κ B family members control promoter activity in genes of pro-inflammatory cytokines, cell surface receptors, transcription factors, and adhesion molecules, which are involved in skin inflammation.^[19]

Thus, NF- κ B pathway is an attractive target for the development of new anti-inflammatory drugs.^[20] Shikonin inhibited NF- κ B activation induced by LPS and was associated with decreased proteasome activity in rat primary macrophage culture.^[7] Our study examined for the 1st time, the role of shikonin in TNF- α -induced NF- κ B activation in HDFs *in vitro*. Immunofluorescence study of NF- κ B p65 revealed that shikonin inhibited nuclear translocation of NF- κ B p65.

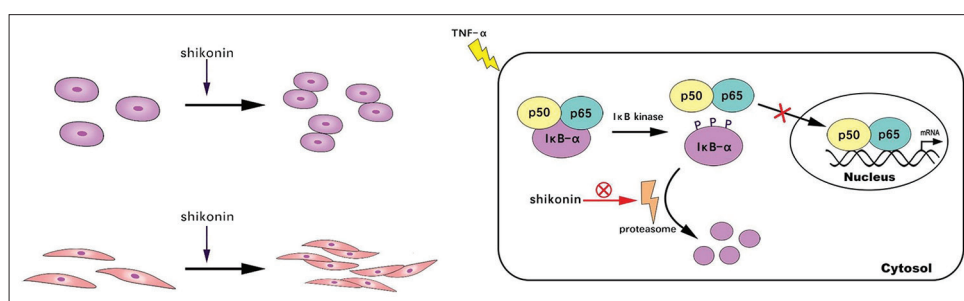


Figure 6: Schematic presentation of the results of the present study. Left panel: Effects of shikonin on the proliferation of normal human keratinocytes and human dermal fibroblasts. Right panel: Effects of shikonin on nuclear factor-κB translocation and proteasome activity.

Moreover, shikonin inhibited the chymotrypsin-like activity of the proteasome and was associated with accumulation of phosphorylated IκB-α in HDFs. These results are schematically depicted in Figure 6 right panel.

Although shikonin induced proliferation of both NHKs and HDFs, the mechanism for this effect is currently unknown. In general, inhibition of NF-κB translocation and proteasome activity was considered to inhibit cell proliferation. In this study, shikonin inhibited NF-κB translocation and proteasome activity, suggesting that inhibition of NF-κB translocation and proteasome activity did not cause inhibition of cell proliferation. Therefore, shikonin exerts the cell proliferation effects via other mechanisms, which should be clarified in future studies.

In conclusion, our findings indicated that shikonin promoted proliferation of skin cells by a currently unknown mechanism, while shikonin did not induce COL1 expression in HDFs. In addition, shikonin inhibited NF-κB signaling pathway and proteasome activity in HDFs, which suggested an anti-inflammatory effect of shikonin. Therefore, shikonin may be a potential therapeutic agent both in wound healing and in the treatment of inflammatory skin diseases. Thus, shikonin may be most suitable in the treatment of refractory inflammatory skin ulcers.

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