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Shikonin inhibits ER stress in vascular endothelial cells, when induced by H_2O_2, and promotes vascular regeneration

Lili Guo[a,†], Yinling Li[b,c,†], Shuang Wen[b], Wen jun Liu[d], Lei Yang[a], Yu Wan[a], Jia xin Liu[a], Wen e Zhao[e,*], Shu-Wei Li[a,*]

[a] Xinjiang Production & Construction Corps Key Laboratory of Protection and Utilization of Biological Resources in Tarim Basin, College of Life Science and Technology, Tarim University, Xinjiang 843300 China
[b] Department of Immunology, Nanjing Medical University, Jiangsu 211166 China
[c] The First Affiliated Hospital of Nanjing Medical University, Jiangsu 211166 China
[d] Department of Pharmacology, School of Basic Medical Science, Nanjing Medical University, Jiangsu 211166 China
[e] School of Basic Medical Science, Nanjing Medical University, Jiangsu 211166 China

*Corresponding authors, e-mail: zwe99@njmu.edu.cn, Tarim_yfzx406@126.com
† These authors contributed equally to this work.

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ABSTRACT: Shikonin is a naphthoquinone compound. Various reports proved that shikonin not only has anticancer and antibacterial effects, but also can play a certain role in promoting wound healing. However, whether shikonin can reduce oxidative stress degree in endothelial cells of vascular and reconstruct the lining of damaged vessels is unknown. Therefore, we used a variety of biological techniques to study the function of shikonin in protecting endothelial cells at the animal and cellular levels. Our study showed that oxidative stress was significantly enriched in the endoplasmic reticulum (ER). Thus, shikonin can effectively resist ER stress and inhibit inflammation and apoptosis of endothelial cells. More importantly, we also found that shikonin can inhibit stress by regulating janus kinase2-signal transducers and activators of transcription3 (JAK2/STAT3) pathways, thereby protecting endothelial cells from H_2O_2-induced damage. In addition, the application of shikonin to skin wounds in mice can also promote the expression of vascular endothelial growth factor (VEGF) proteins in vascular endothelial cells; hence promoting the reconstruction of wounded blood vessels and providing a proper blood supply, thus accelerating wound healing. In conclusion, our research findings provide the groundwork for future clinical treatments of skin wounds and related research.

KEYWORDS: shikonin, H_2O_2, endothelial cells, oxidative stress, apoptosis

INTRODUCTION

Recently, natural drugs have become a popular research topic. Components with medicinal value extracted from plants have obvious advantages; such as tiny adverse reactions, wide therapeutic windows, and low prices; and have attracted the attention of researchers around the world. Shikonin, a naphthoquinone compound, is a crystalline powder extracted from the roots of traditional Chinese medicinal plant known as shikon [1]. Numerous studies reported that shikonin not only has anticancer [2, 3], antibacterial [4], antioxidant [5], and other effects, but can also play a role in promoting wound healing. Researchers have also reported the special role of shikonin in skin wound healing at the molecular and cellular levels [6].

Wound repair is a natural response after tissue injury, and it requires the participation of a variety of factors. Inflammatory factors play vital roles in this process. After injury, an appropriate inflammatory response begins wound repair, but an excessive inflammatory response can aggravate the injury and make the wound difficult to heal. Studies have confirmed that shikonin can effectively inhibit xylene-induced auricle swelling and acetic acid-induced increases in capillary permeability in mice in a dose-dependent manner [7]. In addition, studies also confirmed that shikonin can significantly inhibit the release of tumor necrosis factor-α (TNF-α) [8]. Fan et al [9] also proved that shikonin can downregulate interleukin (IL)-6, IL-1β, TNF-α, matrix metalloproteinase (MMP)-2, and cyclooxygenase (COX)-2 expression; thereby inhibiting the inflammatory response. Inflammasome activation can induce cell inflammation and necrosis, but a certain concentration of shikonin can inhibit inflammasome activation by acting on caspase-1 [10]. Therefore, shikonin may promote wound healing by regulating the expression of various inflammatory factors. After injury, the body produces excessive reactive oxygen species (ROS), destroying the balance of oxidation and antioxidant systems, leading to tissue damages. Excessive ROS promote the occurrence of inflammatory reactions, which stimulate the generation of oxidative stress. Oxidative stress forms a vicious cycle by promoting the continuation of the inflammatory reaction, which eventually leads to difficulties in wound healing. Guo et al [11] demonstrated that shikonin significantly decreased ROS and inflammatory cytokine levels in drug-induced liver tissues. Therefore, shikonin can reduce tissue damage and

promote wound healing through its antioxidant stress and anti-inflammatory effects.

The formation of granulation tissue plays an essential role in wound healing, and the formation of blood vessels can provide nutrients and oxygen for wound healing; thus, accelerating wound healing. Shikonin can promote neo-vascularization in mouse balloon granuloma through VEGF, which is of great significance in promoting wound healing [12]. Therefore, shikonin can promote wound healing by promoting the formation of granulation tissues and fresh blood vessels.

In our study, we used H_2O_2 to establish an injury model in cell lines to explore the protective effect of shikonin and its mechanism, providing a reference for the clinical application of shikonin in wound healing.

MATERIALS AND METHODS

Animals and treatment

C57BL6J mice, aged 8–10 weeks, were purchased from the Nanjing Medical University Animal Centre. All the mice were kept in no specific pathogen conditions in animal centres, with free access to a standard laboratory diet and water. All laboratory animal protocols have been approved by the Nanjing Medical University Committee for the care and use of laboratory animals (Ethics No.2104001). The skin and hair of the mouse ear were removed with depilation cream, and the second degree burn model of mouse ear skin was made with a burn instrument. Mice were anesthetized with ketamine (200 mg/kg) and pyrazine (10 mg/kg) intraperitoneally and euthanized by rapid neck amputation, and skin samples were obtained. After UV sterilization, the skin samples were placed at 4 °C until use.

Preparation of shikonin ointment

Two hundred milligrams of shikonin (HY-N0822, Medchem Express, China) were dissolved in 10 ml of glycerin (G8190, Solarbio, China).

ELISA

Myeloperoxidase (MPO) concentrations in ear skin were determined using an MPO Elisa Kit (ZC-38698, Shanghai Zhuocai Biotechnology Co., Ltd., China) according to the manufacturer's requirements. First, ear skin tissue was washed with 4 °C sterile phosphate buffer saline (PBS) and homogenized in Ripa buffer for 2 min. The obtained skin tissue homogenates were then centrifuged at $5000 \times g$ for 5 min and immediately put on the machine for analysis. ELISA assays IL-6(EMC004(H).96.5), IL-1β (EMC001b.96), and TNF-α(EMC102a(H).96), all from NeoBioscience (China), in cell lysis fluid were determined using the corresponding ELISA kits according to instructions provided by the manufacturer. Similarly, ELISA assays thioredoxin (TRX; Cat:A119-1-1; Njjcbio, China) and 8-hydroxy-2 deoxyguanosine (8-OHdG, Cat. H165; Njjcbio.) in cell lysis fluid were determined. Experimental data were obtained by measuring the absorbance at 550 nm with a microplate reader (Biotek Instruments, VT, USA).

Cell culture and treatment

The Bend.3 cell line (the endothelial cell line) was purchased from ATCC (USA, Cell resource bank). Dulbecco's modified eagle medium (DMEM, Cat:C11995500BT) and fetal bovine serum (FBS, Cat:10439-024ES) were purchased from Gibco company. Bend.3 cells were cultured and passaged in incubators. The endothelial cells were inoculated in a 6-well plate with an approximate density of 2×10^5 cells per well, and about 70%–90% of the cells were fused the next day for specific treatment. To induce oxidative stress, cells were treated with 700 μmol/l H_2O_2 for 12 h [13] and treated with 2, 4, or 8 ng/ml shikonin for the following 16 h.

Cell viability assay

According to the product specification, the viability of endothelial cells was measured by MTT assay (MTT Cell Growth Assay Kit, Sigma, Cat:32161000, USA). After processing, the medium was discarded. 100 μl DMEM and 10 μl 0.5% MTT solution were added into the centrifuge tube of each sample and mixed thoroughly. After 4 h incubation, the culture medium was discarded, and 100 μl DMSO was added to each well. Optical density (OD) was determined at 490 nm.

Flow cytometric analysis

The 10 × binding buffer was diluted to 1 × with deionized water. Endothelial cells were digested, collected with trypsin (Sigma, Cat:9002-07-7) without Ethylene Diamine Tetraacetic Acid (EDTA), and centrifuged at 2000 rpm for 5–10 min at 25 °C. Then, cells were resuspended in PBS (4 °C) and centrifuged at 2000 rpm for 10 min, and the endothelial cells were washed. Next, 300 μl binding buffer was added to suspend endothelial cells. Five μl Annexin V-FITC (China Shaen Bio, SNK-001) was added to the cell suspension. The mixture was incubated at room temperature for 15 min away from light, and stained with added five μl PI (China Shaen Bio, SNK-001) for 5 min. Finally, cells were analyzed using a CytoFLEX flow cytometer (Beckman Coulter, USA). The acquired all flow data were analyzed using CytExpert software.

ROS staining

The living cells were stained with 10 μmol/ml ROS-DHE (Sigma, MAK143-1KT) for 30 min and 4′,6-diamidino-2-phenylindole (DAPI, Sigma, D8417-5MG) for 5 min in a dark incubator at 37 °C. ROS levels were determined by measuring fluorescence under a fluorescence microscope (Olympus, Japan), and data were analyzed using image J software.

Localization of oxidative stress

Endoplasmic reticulum (ER) Tracker Red (Beyotime Biotech Inc., C1041, China) was used to label the ER. At the same time, the oxidative stress in endothelial cells was tracked and localized. After normal culture of endothelial cells for 12 h, cells were treated with 700 μmol/l H_2O_2 for 12 h, the ER of endothelial cells was labeled with ER probe. Oxidative stress was labeled with ROS probe for 1 h. Finally, the cell culture dishes were washed with sterile PBS at room temperature and fixed in 4% PFA for 30 min; PFA was removed, anti-fluorescence quench was added, and images were acquired by Zeiss multiphoton confocal imaging.

Cell processing and Western blot (WB) analysis

Endothelial cells were cultured for 12 h. At the same time, as H_2O_2 stimulation, shikonin or JAK2/STAT3 signaling pathway inhibitor WP1066 (MCE, HY-15312, USA) was used to intervene for 12 h. Cells were collected, and total protein was extracted. A mixture of protease and phosphatase inhibitors (ST506, st019, Beyotime Biotech Inc.) was added to the solution of radio immunoprecipitation assay (RIPA) buffer (p0013b, Beyotime Biotech Inc.). Cell proteins were extracted, separated by 10% SDS-PAGE, transferred to PVDF membranes (0.45 μM pore size), blocked in 5% milk at 24 °C for 1 h, and finally incubated with overnight at 4 °C anti-p-JAK2 (CST 3771, USA), anti-p-STAT3 (CST 9145), anti-P-PERK (AP328, Beyotime), anti-P-elF2α (CST 9721), anti-ATF4 (ab184909, Abcam, USA), anti-CHOP (15204-1-AP, Proteintech, USA), and anti-GAPDH (CST 5174). The protein bands were washed 3 times in sterile PBS, incubated with horseradish peroxidase (HRP) secondary antibody (b900210, goat anti-rabbit, Proteintech) for 2 h at 24 °C, and washed 3 times with sterile PBS. Using GAPDH as internal reference protein, protein bands on the membrane were visualized using the ECL-WB detection system; and WB protein images were acquired using Image J software. The gray value of the image was calculated, and the statistical significance was quantitatively analyzed. (National Institutes of Health, Bethesda, Maryland, USA).

Fluorescence staining and confocal imaging

The mouse ears' skin was separated with tweezers. The lateral auricular skin was preserved, washed once with sterile PBS at 4 °C, and treated as follows: fixation with 4% paraformaldehyde (PFA) for 1 h, permeabilization and blocking with 0.5% Triton X-100+10% goat serum for 4 h, washing three times with 3% BSA for 30 min each time. Next, mixing with the primary antibodies (Abcam: CD31, ab7388; VEGF-A, ab52917; VEGF-B, ab185696; VEGF-C, ab9546), and incubation overnight at 4 °C. After the incubation, the skin was washed three times with 3% BSA for 30 min each time and, then, mixed with the fluorescent secondary antibodies (Abcam: AF488, ab150077, and AF594, ab150160). The mixture was incubated in the dark for 2 h, the secondary antibody incubation solution was removed, and the skin was washed three times with 3% BSA for 30 min each time. Finally, the ear skin was carefully transferred into the confocal imaging chamber, observed and photographed with a Zeiss laser confocal microscope. For 3D imaging of skin and blood vessels, the Z-Stack mode of Zeiss confocal imaging was used; and video of the collected 3D images was made using ZEN (version 2.0) software.

Cell immunofluorescence

Cells were cultured in 24-well culture plates overnight. After fixing with 4% PFA for 2 h and washing with PBS, the cells' membrane was permeated with 0.5% Triton X-100 for 30 min, blocked with 10% goat serum for 1 h, and incubated with primary antibody (ICAM-1, USA; CST, 62133S, 1:200 PBS) overnight at 4 °C. After washing three times with sterile PBS, the cells were subjected to immunofluorescence with a secondary antibody (1:1000 in PBS, 2 h, 24 °C; Invitrogen, USA). Nuclei were stained with DAPI.

Transwell assay

Cell migration function was assessed by Transwell assays using Transwell chambers (CLS3412, Corning, NY, USA). The treated EC cells were diluted to 10×10^4/ml with serum-free DMEM medium; 200 μl cell suspension was added to the upper Transwell chamber; and 600 μl medium containing 10% fetal bovine serum was added to the lower chamber. The upper chamber was carefully immersed in the lower chamber liquid using sterile forceps. Cells were cultured for 24 h. Then, the upper chamber cells were fixed in methanol

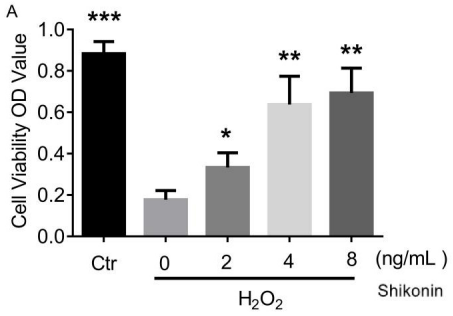

Fig. 1 Effect of shikonin on endothelial cell viability. Under H_2O_2 treatment, Bend.3 cells were stimulated with H_2O_2 (700 μmol/l) and treated with shikonin at concentrations of 0, 2, 4, and 8 ng/ml. Cell viability results of the H_2O_2 treatment with and without shikonin were compared. Ctr, the control group did not use H_2O_2 stimulation.. The data were from 5 biological independent experiments. * = $p < 0.05$, ** = $p < 0.01$, and *** = $P < 0.001$.

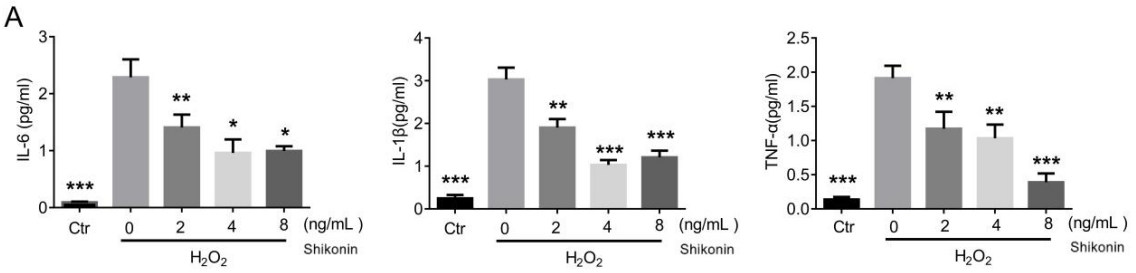

Fig. 2 Effect of shikonin on inflammation. Measurement of IL-1β, IL-6, and TNF-α in Bend.3 cells stimulated with H_2O_2 (700 μmol/l) and treated with shikonin at concentrations of 0, 2, 4, and 8 ng/ml. The data were from 5 biological independent experiments. The differences in the results of the data analysis were represented by*, * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

and stained with hematoxylin-eosin. The migrating cells were analyzed under a microscope.

Statistical analysis

All data in this study were presented as mean ± SEM. Data were analyzed and compared using SPSS Software (Version 16.0). Biological independent experiment repeats were performed for each experiment (n = 5). The differences in the results of the data analysis were represented by *.

RESULTS

Shikonin reversed the low endothelial cell viability caused by H_2O_2

To identify the effect of shikonin, we used H_2O_2 to establish a cell injury model and added shikonin at different concentrations to observe the changes in cell viability. We found that compared with the vascular endothelial cells in the normal culture group, the viability of H_2O_2 treated cells decreased significantly. Besides, the viability of endothelial cells treated with H_2O_2 and different concentrations of shikonin increased significantly, showing a concentration-dependent trend (Fig. 1). These results indicate that shikonin exerted protective effect on the viability of cells injured by H_2O_2.

Shikonin reversed inflammation of H_2O_2 treated endothelial cells

Compared with vascular endothelial cells of the normal culture group, the levels of inflammation factors in cells treated with H_2O_2 increased significantly. After adding different concentrations of shikonin, the levels of IL-1, IL-6 and TNF-α decreased in a concentration-dependent pattern (Fig. 2), indicating that shikonin had an antagonistic effect on the increase in intracellular inflammatory factors after H_2O_2 stimulation.

Shikonin reversed H_2O_2-induced apoptosis of endothelial cells

Compared with vascular endothelial cells of the normal culture group, the apoptosis rate in cells treated with H_2O_2 significantly increased. After adding shikonin at different concentrations, the apoptosis rate decreased in a concentration-dependent manner (Fig. 3). These results showed that shikonin significantly inhibited apoptosis of cells after H_2O_2 stimulation.

Shikonin reversed H_2O_2 induced oxidative stress in endothelial cells

Compared with cells in the normal culture group, the positive expression of ROS in cells treated with H_2O_2 significantly increased. After adding shikonin at different concentrations, the positive expression of ROS decreased in a concentration-dependent pattern (Fig. 4A). In addition, we investigated the effect of shikonin on oxidative stress damage, and the results showed that with the increase of shikonin concentration, the degree of oxidative stress damage of endothelial cells (TRX and 8-OHdG) was alleviated (Fig. 4B). This showed that shikonin reduced the expression of ROS in cells after H_2O_2 stimulation.

Shikonin could reduce the up-regulation of endothelial cell adhesion factors by H_2O_2

Compared with cells in the normal culture group, the H_2O_2 treated cells expressed more ICAM-1 after oxidative damage. Besides, the fluorescence intensity of ICAM-1 decreased to varying degrees after treatment with different concentrations of shikonin. The decreases showed a concentration-dependent trend (Fig. 5), indicating that shikonin could reduce ICAM-1 expression after H_2O_2 stimulation.

Shikonin could improve the migration ability of endothelial cells

Compared with vascular endothelial cells in the normal culture group, the vascular endothelial cell number crossing the transwell chamber after H_2O_2 treatment decreased significantly. However, the number of migrated cells increased significantly after adding different concentrations of shikonin, showing a concentration-dependent trend (Fig. 6), indicating that shikonin enhanced migration ability after H_2O_2 stimulation.

Shikonin inhibited ER oxidative stress in endothelial cells through regulating JAK2/STAT3 signaling pathway

We used a high-power oil microscope to locate the oxidative stress in endothelial cells, and we found that oxidative stress was mainly enriched in the ER of endothelial cells (Fig. 7A). Then, we explored whether the JAK2/STAT3 signaling pathway was regulated by shikonin. Compared with the control group, the JAK2/STAT3 signaling pathway was inhibited in endothelial cells stimulated by H_2O_2. Interestingly, shikonin could effectively activate the JAK2/STAT3 signaling pathway (Fig. 7B). In addition, the key proteins (PERK, elF2α, ATF4, CHOP) representing the increase in ER stress were also significantly down-regulated by shikonin. However, ER stress-related protein expression levels were lower in the control group (Fig. 7C). Therefore, we used WP1066, an inhibitor of the JAK2/STAT3 signaling pathway, to act on endothelial cells. The ER stress proteins were found significantly up-regulated when the JAK2/STAT3 signaling pathway was inhibited (Fig. 7D). The aforementioned data showed that shikonin could inhibit the ER stress by regulating JAK2/STAT3, thereby protecting endothelial cells.

Shikonin could promote VEGF expression and inhibit the activation of neutrophils

To identify shikonin function *in vivo*, we established a second-degree burn mouse ear skin model using a burn instrument. Compared with the burn model group (Ctr), VEGF-A and VEGF-C expressions in ear skin treated with shikonin ointment were significantly increased, as shown by fluorescence staining and confocal imaging, indicating that shikonin could promote angiogenesis and increase of blood vessel density in burned skin (Fig. 8A, Video1, Video2). Besides, compared with Ctr group, the MPO activity in ear skin treated with shikonin ointment was significantly reduced (Fig. 8B), indicating that shikonin could reduce the infiltration of neutrophils after burns.

DISCUSSION

Shikonin has a history of thousands of years [14]. Shikonin is a naphthoquinone compound extracted from shikon. Researches have mainly focused on its antitumor, anti-inflammatory, antiviral, etc; and few studies have focused on its oxidative stress and angiogenesis functions. When skin burns, large numbers of oxygen free radicals are produced, leading to oxidative stress, which is not conducive to wound healing [15]. Vascular endothelial cell damage is the pathophysiological basis for vascular injury. However, oxidative stress in burn wounds damages vascular endothelial cells, which affects wound angiogenesis and regeneration.

The activation of classical inflammatory signaling pathways increases the degree of oxidative stress and induces an increase in secretion of inflammatory cytokines, thus triggering a number of biological effects of inflammation [16]. In this study, H_2O_2 was used to establish an oxidative stress injury model in vascular endothelial cells. It was found that shikonin could reduce the levels of inflammatory factors in oxidized cells, play an antioxidant role, and protect cellular functions. Excessive ROS can cause inflammatory reactions and promote oxidative stress [17], and our study showed that treatment with three different concentrations of shikonin significantly reduced the positive expression of ROS in H_2O_2-damaged cells. In this study, ROS *in vitro* was detected, but vascular endothelial cells were stimulated by a variety of ROS with the participation of a variety of cytokines *in vivo*. Therefore, the mechanism by which shikonin reduces ROS production in cells needs to be further explored. ROS contains multiple free radicals, and H_2O_2 is a relatively stable ROS. Therefore, H_2O_2 was selected as the research object. However, whether shikonin has a protective effect against oxidative stress caused by additional free radicals in ROS requires more exploration. In addition, we found that shikonin reversed the migration ability of damaged endothelial cells. However, when cell viability is reduced, cell migration ability is also decreased. Therefore, the two experiments are mutually supportive. The decrease in cell viability is accompanied by a downregulation of coordinated motor biological events within cells, which are likely to involve the regulation of cytoskeletal proteins. So, we explored these activities in later studies.

ICAM-1 shows low expression level in vascular endothelial cells under normal conditions. Once stimulated by inflammation, infection, or trauma, ICAM-1 expression is increased, in relevance to inflammatory stimulation. Oxidative stress aggravates this inflammatory stimulation, leading to increased ICAM-1 expression. In the present study, the expression of ICAM-1 in H_2O_2 injured cells was significantly increased. H_2O_2 induces an increase in intracellular ROS, which causes an inflammatory response. The ICAM-1 expression level in injured cells significantly decreased after treatment with different concentrations of shikonin, indicating that shikonin can improve the ability of cells to clear ICAM-1, and that it has a potential protective effect on endothelial dysfunction. When oxygen free radicals and inflammation are present, ICAM-1 is produced in large quantities, which increases adhesion to inflammatory cells and induces endothelial cell apoptosis. Therefore, oxidative stress is closely associated with apoptosis. Oxidative stress causes cells to produce large amounts of ROS, damages cell DNA and proteins, and induces cell apoptosis. In this study, large amounts of ROS and ICAM-1 were produced in cells after H_2O_2 injury, accompanied by increased apoptosis

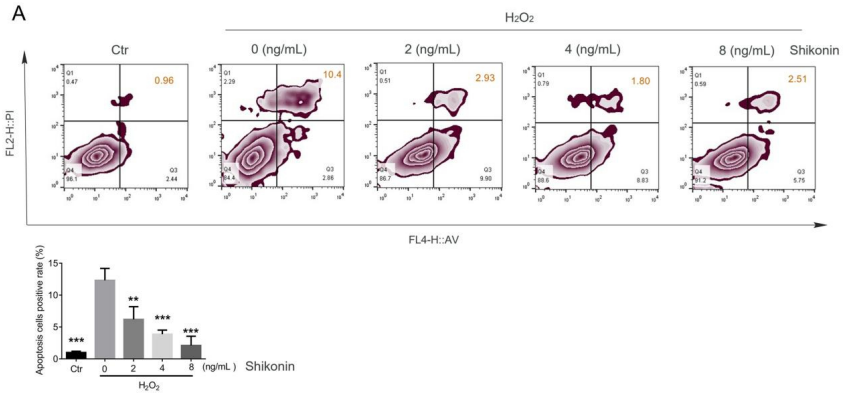

Fig. 3 Effect of shikonin on H_2O_2-induced apoptosis of endothelial cells. Flow cytometry revealed the effect of different concentrations of shikonin on the apoptosis of vascular endothelial cells. The effect of different concentrations of shikonin on the apoptosis of vascular endothelial cells was quantitatively analyzed. The data were from 5 biological independent experiments. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

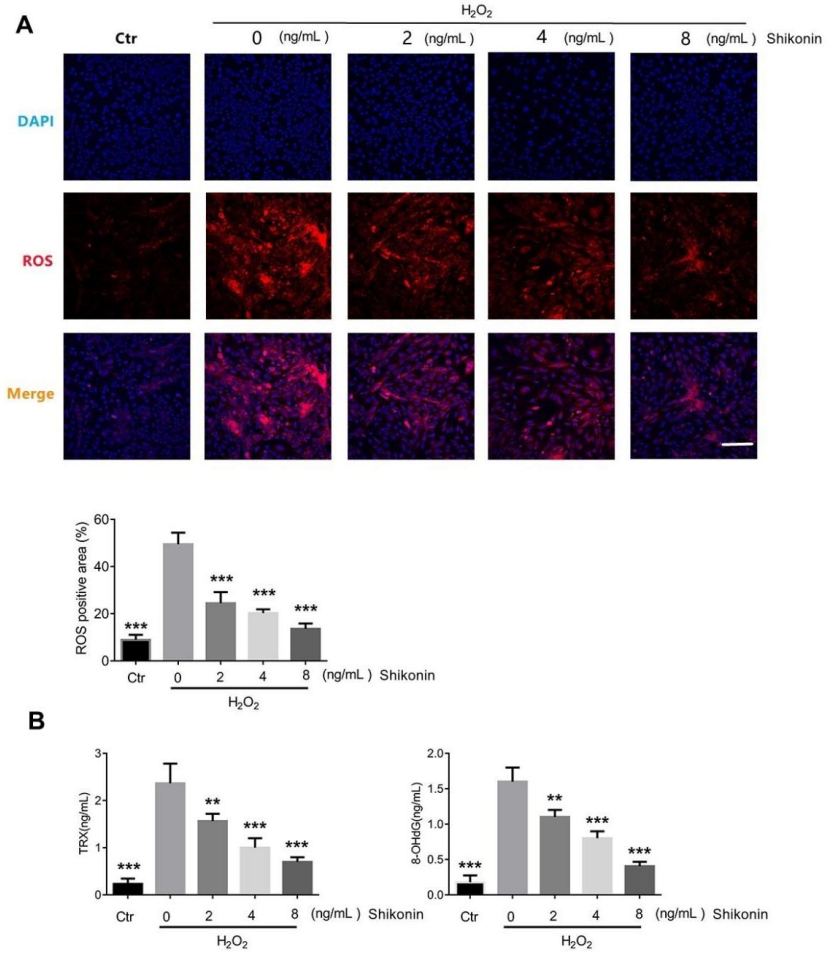

Fig. 4 Effect of shikonin on H_2O_2 induced oxidative stress in endothelial cells. A: ROS production levels in endothelial cell lines stimulated with H_2O_2 (700 μmol/l) and treated with shikonin at concentrations of 0, 2, 4, and 8 ng/ml. The data were from 5 biological independent experiments. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. B: TRX and 8-OHdG production levels in endothelial cell lines stimulated with H_2O_2 (700 μmol/l) and treated with shikonin at concentrations of 0, 2, 4, and 8 ng/ml ($n = 5$, 5 biological independent experiments). * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

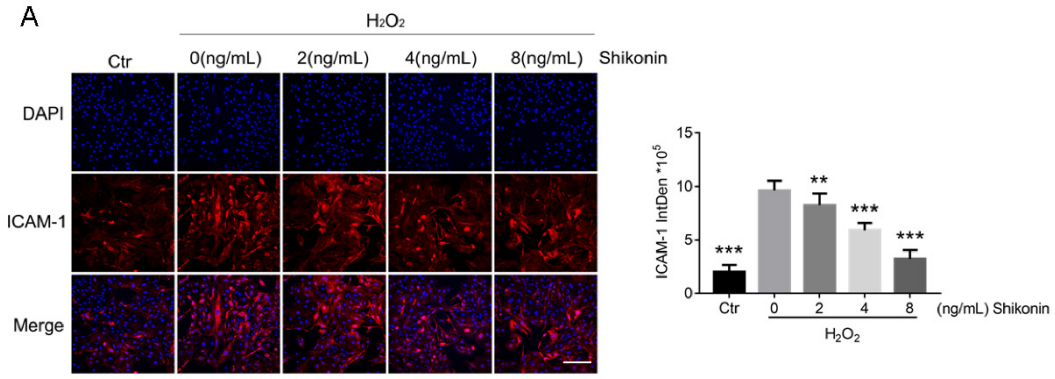

Fig. 5 Effect of shikonin on the expression of ICAM-1. Immunofluorescence showed the expression of ICAM-1 in Bend.3 cells stimulated with H_2O_2 (700 μmol/l) and treated with shikonin at concentrations of 0, 2, 4, and 8 ng/ml. The data were from 5 biological independent experiments. Blue: DAPI, red: ICAM-1. Scale bar=100 μm. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

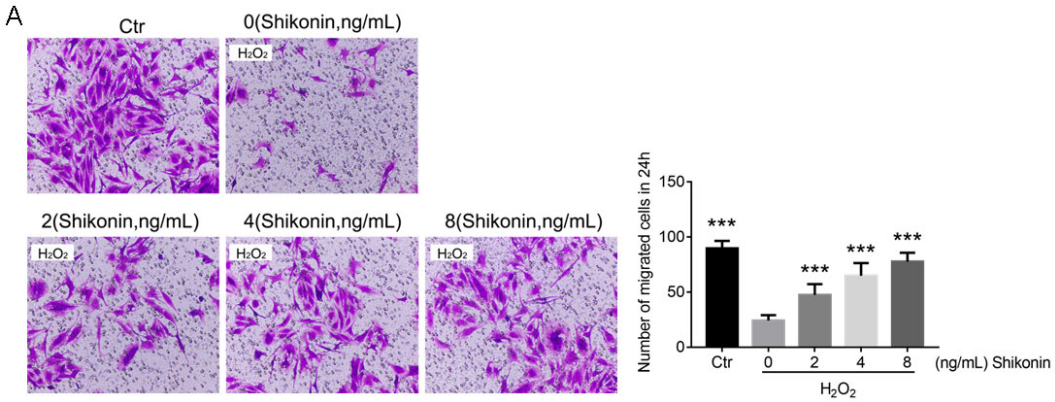

Fig. 6 Effect of shikonin on the migration ability of endothelial cells. Cell migration experiments revealed the effect of different concentrations of shikonin on the migration of vascular endothelial cells. The effect of different concentrations of shikonin on the migration of vascular endothelial cells was quantitatively analyzed. The differences in the results of the data analysis were represented by *, $p < 0.001$ with ***.

rate. After the treatment with different concentrations of shikonin, ROS and ICAM-1 expression decreased to varying degrees, accompanied by decreased apoptosis rate with concentration-dependent pattern. Shikonin showed an obvious antagonistic advantage at 8 ng/ml, indicating that shikonin could scavenge oxygen free radicals, improve antioxidant capacity, and play a protective role in apoptosis. Shikonin exerts anti-oxidative stress effects in cells; However, whether this is an ER oxidative stress or mitochondrial oxidative stress needs to be further studied. Furthermore, whether shikonin exerts its anti-oxidative stress function in cells by specifically targeting ER oxidative stress or mitochondrial oxidative stress remains to be examined. ROS induces releases of cytochrome C and apoptosis-inducing factors which can activate caspases, and the caspases induce apoptosis after a series of reactions [18]. Under normal circumstances, apoptosis is regulated by a large number of genes which can also regulate cell growth and proliferation and maintain the homeostasis of the tissue environment [19]. However, cells treated with H_2O_2 showed a high apoptosis rate and dysfunction. Shikonin reversed this apoptotic state. The oxidative stress of cells mainly comes from mitochondria and the ER. The results of our study showed that shikonin significantly reduced oxidative stress in endothelial cells. After entering the cells, shikonin accumulated in the ER and inhibited ER stress by regulating JAK2/STAT3, thus playing a role in protecting endothelial cells. Additional exploration of the relevant regulatory mechanisms of shikonin in apoptosis will provide a reference for the application of shikonin in clinical diseases.

In some cancer studies, it has been found that the migration of cancer cells can be regulated by regulating the inflammatory pathological microenvironment of cancer, thus promoting angiogenesis [20]. The study showed that angiogenesis is critical to tissue repair

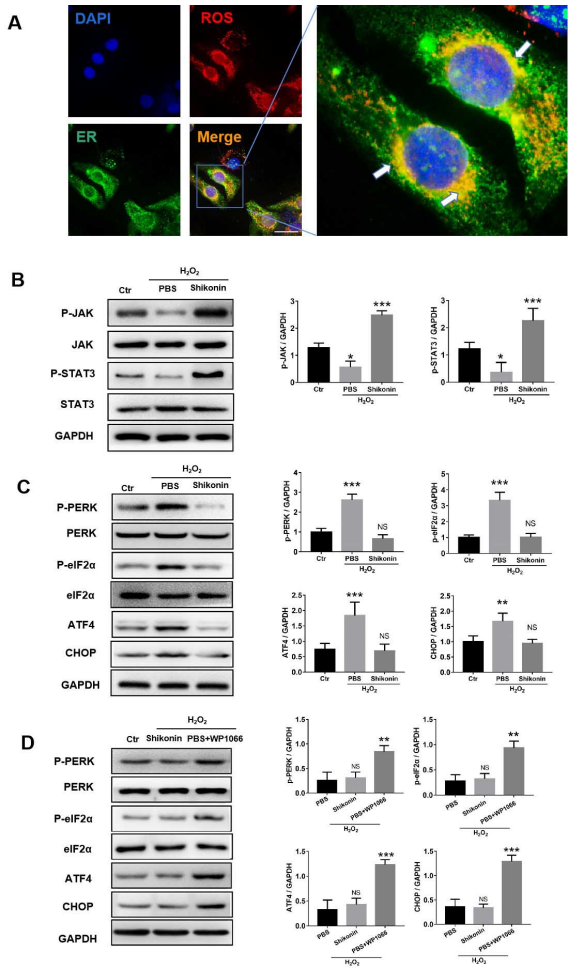

Fig. 7 Shikonin is enriched in the endoplasmic reticulum of endothelial cells. A: Representative images from ⩾ 3 independent experiments showing colocalization of oxidative stress with ER. Nuclei in blue, oxidative stress in red, endoplasmic reticulum in green. The brown arrow shows the colocalization of oxidative stress and ER. The scale is 50 μm. B: Western blot images of the shikonin-treated group showing activation of phosphorylated JAK2/STAT3 protein expression with respective quantitative analysis. C: Western blot results showing the expression of proteins representing ER stress inhibited in the shikonin treatment group, and quantitative analysis was performed. D: Western blot results showed that shikonin reduced the inhibition of ER stress protein expression after JAK2/STAT3 signaling was inhibited, and quantitative analysis was performed. All data were from 5 biological independent experiments. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

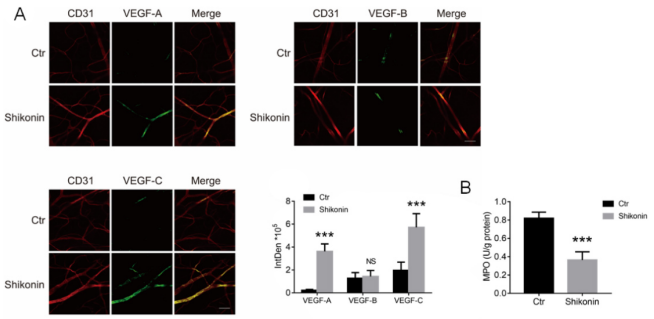

Fig. 8 Effect of shikonin on vascular endothelial cell regeneration factor. A: Immunofluorescence showing the expression of VEGFs in burned ear skin ear skin and burned ear skin treated with shikonin ointment. Red, CD31; Green, VEGFs. Scale bar= 100 μm. B: Measurement of MPO activity in burned ear skin ear skin and burned ear skin treated with shikonin ointment. All data were from biological independent experiments. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

as it perfuses blood vessels to deliver oxygen and nutrients to wounds [21]. Angiogenesis is a dynamic and complex process in which endothelial cell migration plays an essential role. Our study indicated that number of migrated cells increased significantly after adding shikonin in oxidative stress injury model established by H_2O_2, suggesting that shikonin could effectively improve the migration ability of cells and promote angiogenesis. VEGF promotes migration and angiogenesis through acting upon vascular endothelial cells [22]. All five VEGF family genes have their corresponding receptors [23]. In our study, after treatment with shikonin and compared with the Ctr, VEGF-A, B, and C expressed more in the wound tissue, and A and C were more deeply expressed than B, suggesting that shikonin could promote angiogenesis and regeneration in the wound skin. However, the effects of shikonin on different factors in the VEGF family need to be further explored.

CONCLUSION

Our study showed that shikonin can effectively resist ER-stress, inhibit inflammation and apoptosis in endothelial cells, and increase migration ability of endothelial cells. Very importantly, we also found that shikonin inhibits ER stress by regulating the JAK2/STAT3 and protects endothelial cells from H_2O_2-induced damage.

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