Small Molecules Accelerate Skin Wound Healing: Shikonin Efficacy and Mechanism of Action in Mice

Las Moléculas Pequeñas Aceleran la Curación de Heridas en la Piel: Eficacia de Shikonin y Mecanismo de Acción en Ratones

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SUMMARY: The objective of this study was to investigate the therapeutic wound healing potential and molecular mechanisms of shikonin as small molecules *in vitro*. A mouse burn model was used to explore the potential therapeutic effect of shikonin; we traced proliferating cells *in vivo* to locate the active area of skin cell proliferation. Through the results of conventional pathological staining, we found that shikonin has a good effect on the treatment of burned skin and promoted the normal distribution of skin keratin at the damaged site. At the same time, shikonin also promoted the proliferation of skin cells at the damaged site; importantly, we found a significant increase in the number of fibroblasts at the damaged site treated with shikonin. Most importantly, shikonin promotes fibroblasts to repair skin wounds by regulating the PI3K/AKT signaling pathway. This study shows that shikonin can effectively promote the proliferation of skin cell, and local injection of fibroblasts in burned skin can play a certain therapeutic role.

KEY WORDS: Shikonin; Small molecules; Fibroblasts; Burned skin; PI3K/AKT; Wound Healing.

INTRODUCTION

Shikonin is a natural naphthoquinone-based small molecule, which has anti-tumor, anti-virus, and anti-inflammatory properties, and thus it is considered an effective wound-healing agent (Papageorgiou *et al*., 2008). Shikonin has shown excellent pharmacological properties in many therapeutic fields, such as inflammation, wound healing, oxidative stress, and oncology. Shikonin is a potential new drug to treat colon cancer as well, inducing colon cancer cell apoptosis by stimulating the generation of reactive oxygen species (ROS) and activating the mitochondrial pathway regulated by the Bcl2 protein family (Liang *et al*., 2017). In addition, shikonin is also a potential candidate drug for the treatment of acetaminophen (APAP)-induced acute liver injury. Shikonin can maintain the dynamic balance of cell redox and downregulate tumor necrosis factor-a and other cellular inflammatory factors to inhibit the level of oxidative stress and inflammation, thereby reducing the acute liver injury induced by APAP (Guo *et al*., 2019). In addition, a large number of biological experiments have also confirmed that preparations containing shikonin and its derivatives have good clinical effects in treating burns, wounds, and ulcers (Lee *et al*., 2021; Han *et al*., 2021). According to statistics, there are about 100 million patients in China who need wound conditioning every year, and about 30 million patients with serious conditions; in the United States, the annual cost of refractory wounds is close to 30 billion US dollars. With the acceleration of population aging in the future, the incidence of related chronic refractory wounds will further increase. Therefore, how to repair chronic wounds quickly, safely, and perfectly is a clinical problem to be solved urgently.

Wound healing is a complex process involving the interaction among different types of cells, growth factors and extracellular matrix proteins (D'Amico *et al*., 2022). Generally, the process of wound healing is divided into three consecutive and overlapping stages: the inflammation stage, proliferation stage and remodeling stage (Karayannopoulou *et al*., 2011; Revi *et al*., 2013; Md Fadilah *et al*., 2022). In recent years, relevant research has mainly focused on the effectiveness and mechanism of shikonin in various aspects of wound treatment and it was

found that shikonin-containing wound healing drugs mainly act on the inflammation and proliferation stages (Papageorgiou *et al.*, 2008). Indeed, our research group found that, after applying shikonin-based human ointment to dogs with second-degree trauma, shikonin could significantly promote angiogenesis, collagen generation and epithelialization of damaged skin in an acute noninfectious state (Karayannopoulou *et al.*, 2011). Also, after shikonin was applied externally to the injured skin of mice, shikonin significantly stimulated the epithelial mesenchymal transformation of skin tissue during wound healing (Yin *et al.*, 2013), inhibited the expression of related microRNAs, and activated some anti-inflammatory response-related genes mediated by IL-10 (Sun *et al.*, 2017). In addition, it was demonstrated that shikonin can promote a variety of growth factors involved in wound healing, such as vascular endothelial growth factor (VEGF) (Xu *et al.*, 2014), basic fibroblast growth factor (bFGF) and transforming growth factor-b (TGF-b) (Li & Zeng, 2020). All these factors are related to the wound healing proliferative activity of shikonin.

However, so far, research on the mechanism of shikonin in treating skin trauma has not been fully clarified yet, and the therapeutic effect of shikonin on skin trauma needs further research. Therefore, in this study, we verify whether shikonin has a certain protective effect *in vivo*. Our study clarified the specific mechanism by which shikonin promotes skin wound healing through a series of research models and experimental methods. Keeping the above points in view, this study provides a certain theoretical and experimental basis for the later development of shikonin ointment that promotes skin wound repair and provides also pharmacological basis for clinical application.

MATERIAL AND METHOD

Animals and treatment. C57BL6J mice aged 8-10 weeks were purchased from the Nanjing Medical University Animal Centre. All mice were kept in non-specific pathogen conditions in animal centers, with free access to a standard laboratory diet and water. All laboratory animal protocols were approved by the Nanjing Medical University Committee for the Care and Use of Laboratory Animals (Ethics No. 2104001). 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, skin samples were placed at 4 °C until use.

Preparation of shikonin ointment. Two hundred milligrams of shikonin (HY-N0822, MedChemExpress) were dissolved in 10 ml of glycerin (G8190, Solarbio).

Specimen preparation. After anesthetization with pentobarbital sodium, the hair on the backs of the mice was shaved and removed with sodium sulfide (B7250, Solarbio) until it was hairless. The limbs and tail of the mice were fixed on a clean table and arranged at intervals. Water soaked paper was placed on the back of the mice (2.0 cm¥3.5 cm = 7.0 cm2, accounting for 10 % of the body surface area). Ethanol was added using a dropper until the skin was completely soaked. Ignited the lighter and started a stopwatch to count for 5 sec. Once a second degree burn is achieved the mice were put back into the cage. Shikonin ointment and excipient ointment were used to smear the burn wounds and the same dosage was used throughout the experiment.

In addition, 40 ml of cell proliferation marker 5-Ethynyl-2'-deoxyuridne (EdU) (20 mmol/L, ST067, Beyotime Biotech Inc.) diluted with 0.01 mol/L PBS was injected subcutaneously into the model site of each group. PI3K inhibitor (LY294002, MCE, CAS No.154447-36-6) was injected subcutaneously on the wound surface of mice. According to the experimental design duration, skin samples were taken on the 3rd and 7th day after drug application, and then embedded in OCT (4583, SAKURA), frozen for 1h using liquid nitrogen, and frozen at -80oC for 48h. Slices of 8 mm thickness were made using a Lycra cryoslicer. The frozen slices were absorbed by positive charge anti-stripping slides and frozen in -20°C refrigerator.

Pathological staining. Routine pathological staining of skin tissue sections was performed using hematoxylin and eosin staining (H&E, G1120, Solarbio), fixed with neutral glue (Gibco, 100 ml) and observed under a Motic digital microscope.

Immunofluorescence (IF) staining. The frozen sections were naturally dried for 30 min, fixed with 4 % paraformaldehyde, and washed with sterile phosphate buffered saline (PBS) 3 times, permeating the membrane with 1 % Triton-X-100 (T8200, Solarbio) for 20 min, adding 250 ml PBS containing 3 % bovine serum albumin (BSA, ST025, Beyotime Biotech Inc.) and 10 % normal goat serum (PROTEINTECH, B900780) for 1 h at 4 °C. The incubation solution was removed, and the primary antibodies (CK14 (ab119695, Abcam), 1:150; CK19 (ab52625, Abcam), 1:150; a-SMA (ab150301, Abcam) 1:150) were added. PBS was used as a negative control and incubated overnight at 4 °C. Then, cells were washed threetimes with PBS, and fluorescent secondary antibody (1:200) and 4'6-diamidino-2-phenylindole (DAPI (C0065, Solarbio) were added and incubated at 4 °C for 1 h. Cells were then washed three times with PBS, sealed with anti-fluorescence quenching agent, observed and photographed in a dark room under a Zeiss inverted fluorescence microscope (Oberkochen, Germany).

EdU staining. Four percent paraformaldehyde was dropped on frozen sections of skin tissue labeled with the cell proliferation marker EdU, fixed at room temperature for 30 minutes, rinsed three times with 3 % BSA for 5 min each, infiltrated with 0.5 % Triton X 100 for 30 mins and washed 3 times with 3 % BSA for 5 mins each time. Finally, KeyFluor594 Click-iT reaction mixtures were added and incubated at 25 °C for 30 mins in the dark. PBS was used as a negative control instead of KeyFluor594 Click-iT. The Click-iT reaction mixture was discarded and the tissue was washed three times with 3 % BSA, and the reaction mixture was removed. DAPI (1:1000) was added, incubated for 30 mins in the dark, and washed 2 times with 3 % BSA. The reaction solution was removed, anti-fluorescence quencher was added, and images were captured in a dark room under a Zeiss inverted fluorescence microscope.

Flow sorting. 10X Binding Buffer was diluted to 1X with deionized water. The skin of suckling mice was digested, collected with ethylene diamine tetraacetic acid (EDTA)-free collagenase (Sigma, Cat: C0130), and centrifuged at 2000 rpm for 5-10 minutes at 25°C. Then, the cells were resuspended in PBS (4°C) and centrifuged at 2000 rpm for 10 min, and the skin cells were washed. Next, 300 µL of binding buffer was added to suspend the skin cells. 5 mg a-SMA was added to the skin cells, mixed and incubated at room temperature for 1 h in the dark. Then, 5 mL of AF-488 was added for staining 30 min before flow cytometry. Finally, cells were sorted using a CytoFLEX flow cytometer (Beckman Coulter).

Protein extraction and Western blot (WB) analysis. A mixture of protease and phosphatase inhibitors (ST506, ST019, Beyotime Biotech Inc.) was added to the radio immuno-precipitation assay (RIPA) buffer (P0013B, Beyotime Biotech Inc.) solution. The proteins of the cells were extracted and separated by 10 % SDS-PAGE. Extracted proteins were transferred to the Poly (vinylidene fluoride) (PVDF) membrane (0.45 mm pore size) by a constant current electrophoresis apparatus, and blocked with 10 % skimmed milk at room temperature for 1 hours. Finally, the primary antibodies Anti-PI3K (Abcam, ab101606), Anti-Akt (CST, 4691), Anti-p-PI3K (Abcam, ab182651), Anti-p-AKT (CST, 4060), and Anti-GAPDH (CST, 5174) were incubated at 4°C for overnight. The protein bands were washed 3 times with sterile PBS and incubated with horseradish peroxidase (HRP) secondary antibody?PROTEINTECH, B900210, Goat anti-rabbit) at room temperature for 2 hours, and finally washed 3 times with sterile PBS. GAPDH serves as an internal reference protein. Visualize protein bands on the membrane using the ECL WB Detection System, and using Image J software to acquire WB protein image. The gray value of the image was calculated and quantitatively analyzed for statistical significance. (National Institutes of Health, Bethesda, MD).

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

RESULTS

Effect of shikonin on wound healing in mice with second-degree burns. Histological observation showed that in the Control group, developed hair follicles and sebaceous glands were visible, and the internal cellular structure of the appendages was complete on the cross section of the skin. In the Vaseline group, the skin collagen fibers were swollen, some fibers were necrotic, and the outer root sheath cells of hair follicles and some cells close to the hair follicle center exhibited coagulative necrosis. In addition, some dermal sebaceous gland cells were necrotic, and no new tissue appeared. Compared with the Vaseline group, the collagen fibers in the shikonin group were less swollen, and new hair follicles and sebaceous glands were formed (Fig. 1A).

The longitudinal section of skin showed that the structure of the epidermal layer in the control group was complete, the morphology and structure of the squamous epithelial cells were normal and closely arranged, and the dermis was rich in collagen fibers. Hair follicles, sebaceous glands and other accessory organs were visible, the structural boundaries of each tissue layer were clear, and no obvious inflammation was found. The epidermis of the Vaseline group was significantly thinner, with pyknosis and fragmentation of epidermal nuclei, cytoplasmic fusion, severe dermal edema, a large amount of inflammatory cell infiltration, poor skin structural integrity and only a small amount of connective tissue. In the shikonin group, a large number of epithelial cells differentiated from the wound skin, and a thicker neoepidermal layer was visible. There were more sebaceous glands and hair follicles in the dermis, which was close to the structure in the control group (Fig. 1B).

Effect of shikonin on the proliferation of wounded skin with second-degree burns. To study the mechanism of shikonin in accelerating the wound healing of II-degree burn skin, the effect of shikonin on the proliferation of skin cells *in vivo* was observed. EdU immunofluorescence staining showed that compared with the control group, there was only a small amount of red fluorescence in the Vaseline group, while the red fluorescence in the shikonin group was intense, and the cell proliferation ability was active, indicating that shikonin can promote wound healing by promoting the proliferation of skin cells (Fig. 2A).
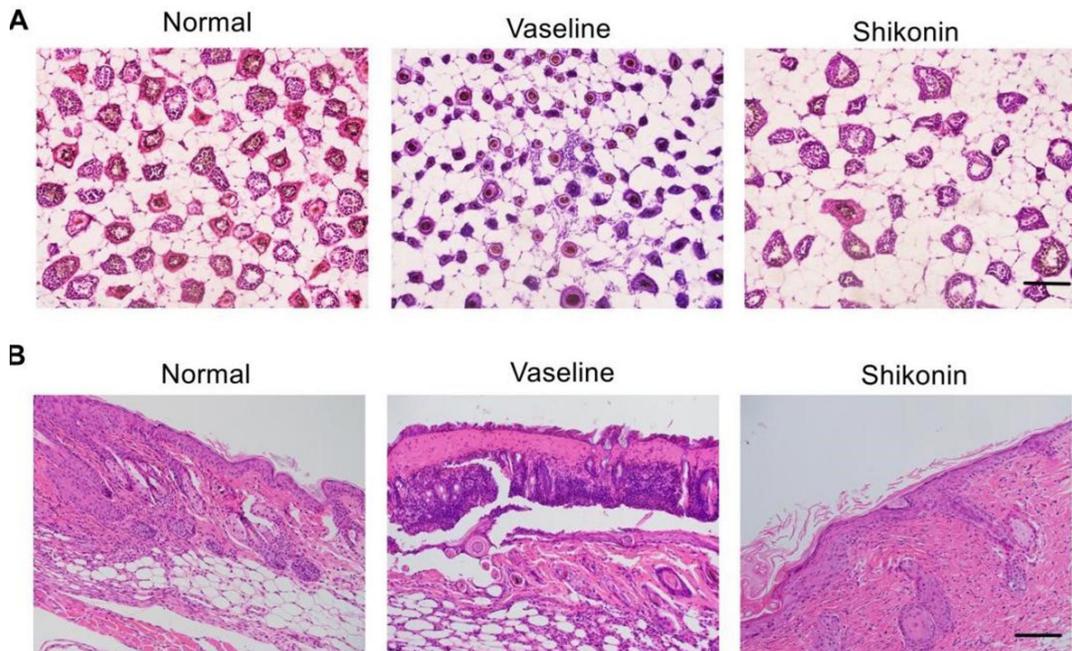
Fig. 1. H&E staining of skin with second-degree burns treated with different ointments. **A.** Representative images of H&E staining in transverse sections of mouse skin treated with vehicle, Vaseline and shikonin ointment (n=5). **B.** Representative images of H&E staining in longitudinal sections of mouse skin treated with vehicle, Vaseline and shikonin ointment (n=5). Scale bar = 100 µm.
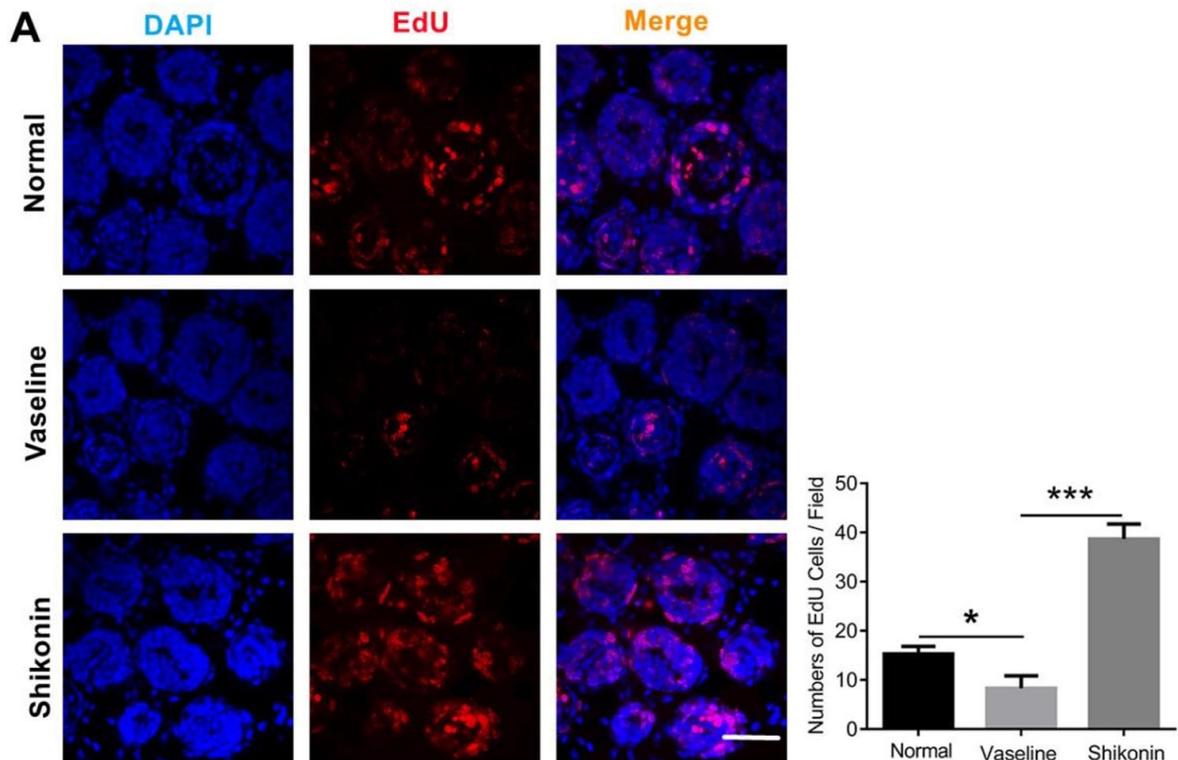
Fig. 2. EdU staining of skin treated with different ointments. **A.** Representative image of EdU staining on mouse skin treated with vehicle, Vaseline and shikonin ointment (n=5). Blue indicates the nucleus, and red indicates proliferating hair follicle cells. Scale bar = 100 µm. Data were statistically analyzed and presented as the mean ± SEM. *p <.05, **p <.01, and ***p <.001 were obtained using a two-tailed unpaired Student's t test.

Effect of shikonin on keratin in wound skin with second-degree burns. Under normal conditions, basal cells gradually migrate to the superficial skin and differentiate into mature cells until they fall off, maintaining the self-renewal of the epidermis. When the skin is burned, the cells in the basal layer of the epidermis proliferate greatly, accelerating the re-epithelialization and tissue remodeling of the wound. Immunofluorescence staining showed that CK19 was highly expressed in keratinocytes in the epidermis, fibroblasts in the dermis and skin appendages in the control group; CK14 was stably expressed in the dermis of normal tissues. In the Vaseline group, the fluorescence intensity of CK19 and CK14 was significantly lower than that in the control group due to the loss of skin epidermis and the damage to the dermal structure caused by the II-degree burns. Compared with the Vaseline group, the skin tissue structure of the shikonin group tended to be complete, the expression range of CK19 and CK14 was significantly increased, and the expression level was close to the level of the control group, which was superior to that of the Vaseline group (Fig. 3A).

CK19 was mainly expressed in the hair stem and hair matrix in normal hair follicles, and CK14 was mainly concentrated in the hair matrix. Compared with the control group, the positive expression of CK19 and CK14 in the Vaseline group was weak. The positive reaction of CK19 and CK14 in the shikonin group was stronger, suggesting that shikonin could promote cell proliferation and accelerate wound repair by increasing the expression of CK14 and CK19 in wounds (Fig. 3B).
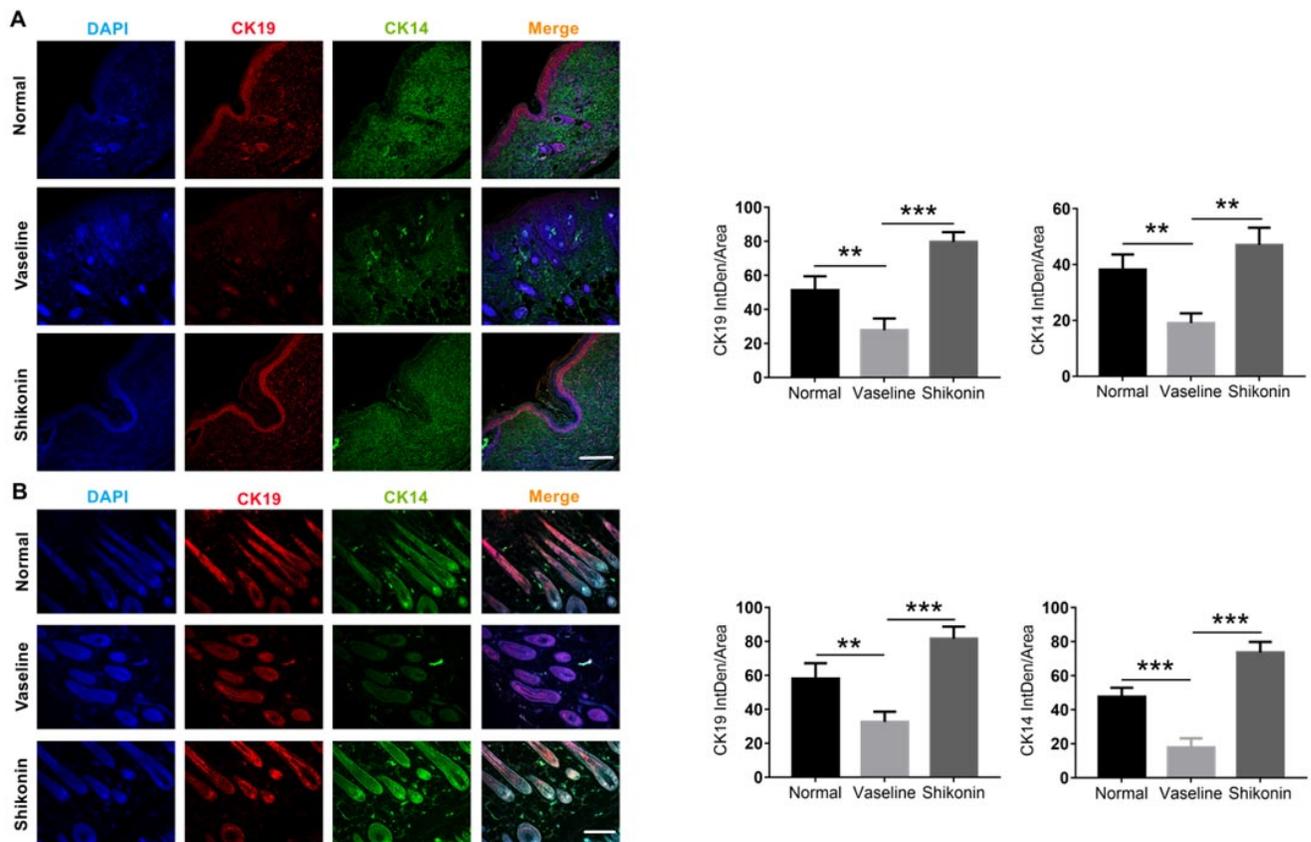
Fig. 3. Immunofluorescence staining of skin treated with different ointments. **A.** Representative immunofluorescence-stained images of longitudinal sections of mouse skin treated with vehicle, Vaseline and shikonin ointment (n=5). Blue is the nucleus, red is CK19, and green is CK14. **B.** Representative immunofluorescence-stained images of transverse sections of mouse skin treated with vehicle, Vaseline and shikonin ointment (n=5). Blue is the nucleus, red is CK19, and green is CK14. Scale bar = 100 µm.

Shikonin increases fibroblasts in mouse skin wounds. We performed immunofluorescence staining of a-SMA protein on the skin of the burn wound site to locate the fibroblasts in the skin wound site. The results showed that in the mouse skin of the shikonin treatment group, we found a large number of fibroblasts, while the number of fibroblasts in the mouse skin of the control group and the Vaseline control group did not increase significantly (Fig. 4A). The data show that in shikonin, fibroblasts may play an important role in promoting skin wound healing.
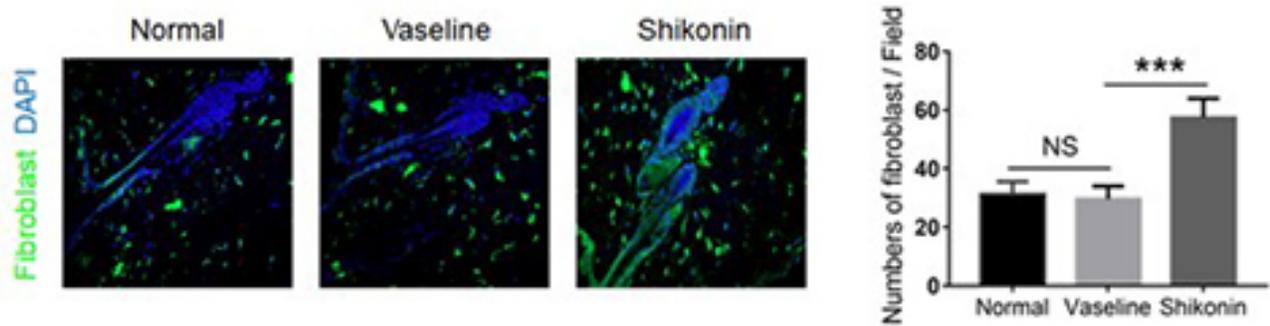
Fig. 4. Shikonin increases fibroblasts in mouse skin wounds. Representative immunofluorescence-stained images of longitudinal sections of mouse skin treated with vehicle, Vaseline and shikonin ointment (n=5). Blue is the nucleus, and green is a-SMA. Data were statistically analyzed and presented as the mean ± SEM. * p <.05, ** p <.01, and ***p <.001 were obtained using a two-tailed unpaired Student's t test.

Fibroblasts promote skin wound healing and morphological recovery. We sorted the fibroblasts in the skin of novice mice by flow cytometry. First, we carried out immunofluorescence identification of a-SMA on the sorted cells to clarify that the sorted cells were skin fibroblasts (Fig. 5A). We treated burn model mice with shikonin + fibroblasts for 1 week by local injection of fibroblasts, and under the protective effect of shikonin, we found that, compared with the blank control and Vaseline control mice, under shikonin + fibroblast treatment, the skin wounds of the mice healed better, the wounds of the mice desquamated earlier, the skin wounds healed better, there were no obvious skin lesions, and the skin edema was significantly improved. However, after one week of treatment for the wound skin of the mice in the blank control group and the Vaseline control group, the morphology of the mouse skin wounds was still incomplete, the skin healing was poor, and the conventional pathological staining showed significant accumulation of inflammatory cells (Fig. 5B); the above data showed that shikonin combined with fibroblasts has a good supporting effect on the healing of skin wounds.

Shikonin promotes fibroblasts to repair skin wounds by regulating the PI3K/AKT signaling pathway. We performed different interventions on mice treated with shikonin combined with fibroblasts and detected the PI3K/
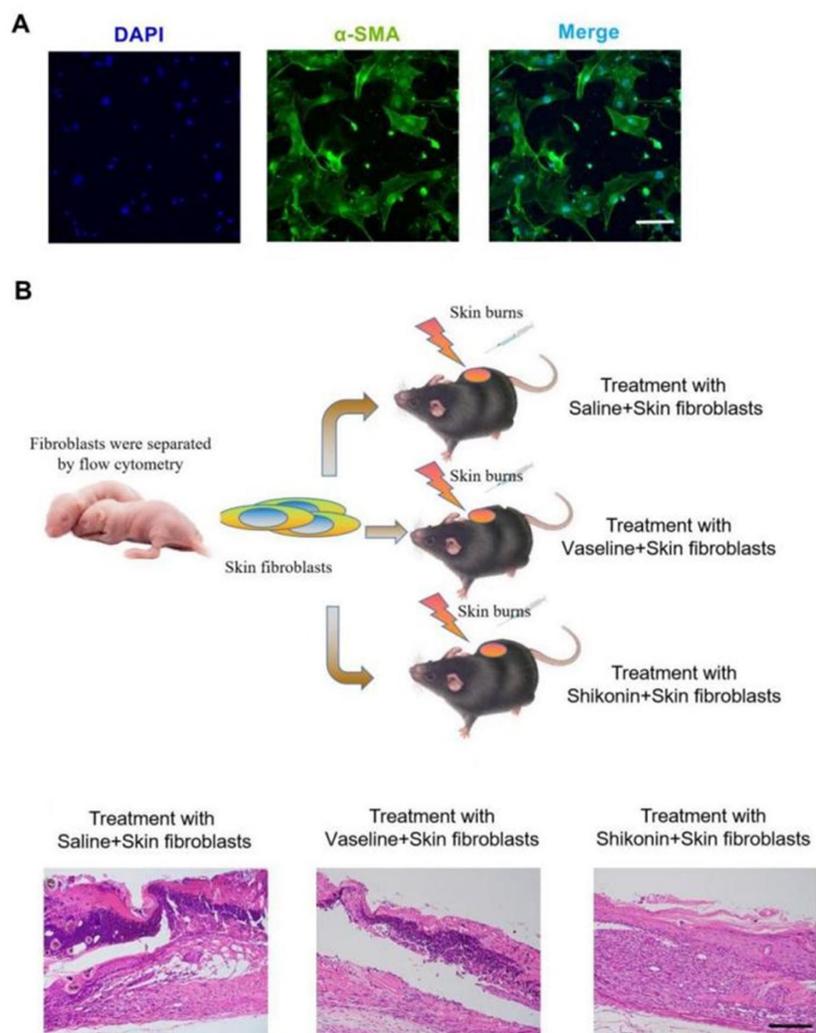
Fig. 5. Fibroblasts promote skin wound healing and morphological recovery. **A.** Representative image from the immunofluorescence identification of a-SMA in fibroblasts sorted by flow cytometry. DAPI in blue is the nucleus, and a-SMA in green. Scale bar: 100 μm. **B.** Schematic diagram of flow-sorted fibroblasts combined with different treatment methods to treat burn skin of mice; representative images are from routine pathological staining of mouse skin under different treatment conditions. Scale bar: 100 μm.

AKT signaling pathway by lifting the protein in the skin wound site. We found that shikonin significantly activated the PI3K/AKT signaling pathway (Fig. 6A). Next, we injected LY294002, an inhibitor of the PI3K/AKT signaling pathway into the wounds of the mice. We found that with the inhibition of the PI3K/AKT signaling pathway, the wound healing status of the mouse skin became worse (Fig. 6B). Based on the above data, we know that shikonin can regulate the PI3K/AKT signaling pathway, thereby promoting fibroblasts to repair skin wounds.
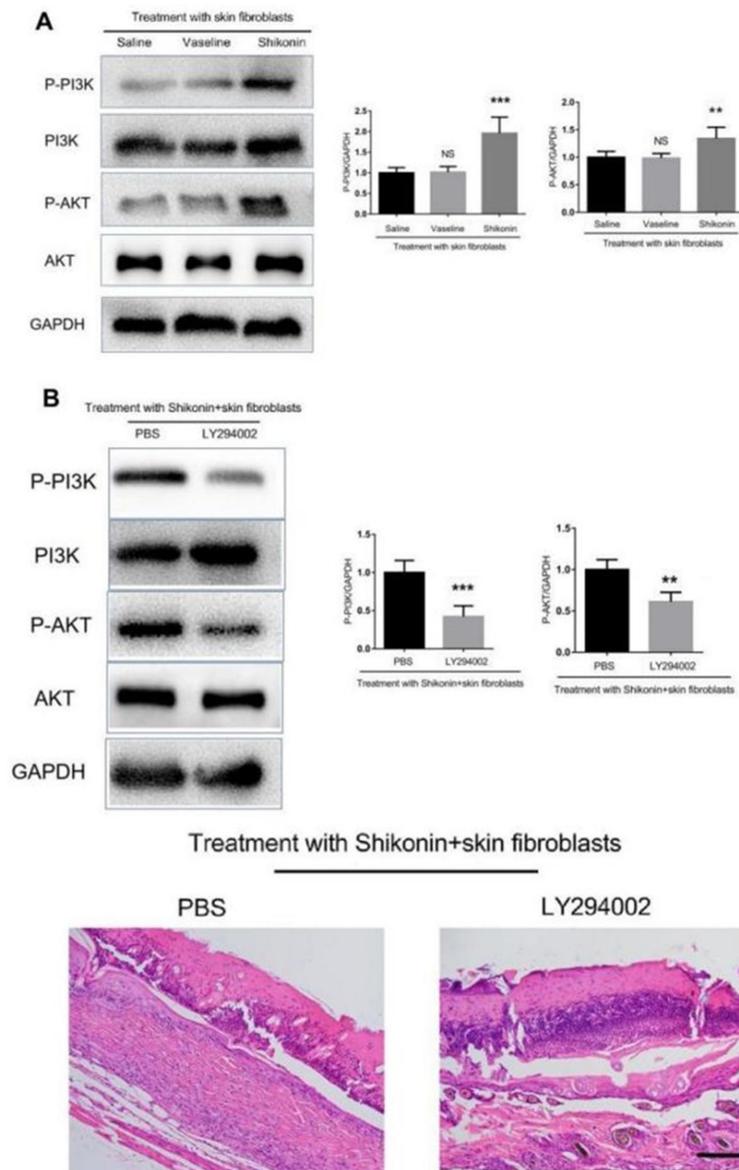
Fig. 6. Shikonin promotes fibroblasts to repair skin wounds by regulating the PI3K/AKT signaling pathway.

A. Representative images derived from detecting changes in the PI3K/AKT signaling pathway, and to quantify the changes in PI3K/AKT signaling pathway proteins. The gray intensity of the protein was statistically analyzed and expressed as mean ± SEM. *p <.05, **p <.01, and ***p <.001 were obtained using a two-tailed unpaired Student's t-test.

B. Representative images derived from detecting changes in the PI3K/AKT signaling pathway following LY294002 inhibitor treatment and quantifying changes in PI3K/AKT signaling pathway proteins. Statistical analysis was performed on the gray intensity of proteins and expressed as mean ± SEM. *p <.05, **p <.01, and ***p <.001 were obtained using a two-tailed unpaired Student's t-test. Representative images from routine pathological staining of LY294002-treated mouse skin samples. Scale bar: 100 µm.

DISCUSSION

Shikonin, a natural naphthoquinone organic derivative, separated and purified from the traditional Chinese medicine Alkanna, has excellent antitumor, antioxidant, anti-inflammatory and other biological functions (Wu *et al.*, 2022). Among them, it seems to promote wound healing by means of different mechanisms, for example, increasing fibroblast proliferation (Imai *et al.*, 2019).

Shikonin, an active ingredient of Lithospermum erythrorhizon, exerts anti-inflammatory and antibacterial effects, and promotes wound healing. Fibroblasts are the main cell type of the dermis and play a leading role in the process of skin repair (Bohari *et al.*, 2015). In the past, researchers have mainly focused on keratinocytes in the epidermis in research on wound healing. However, an increasing

number of studies have shown that dermal fibroblasts play an important role in the process of skin wound healing. The processes of wound contraction, tissue repair, scar formation and reconstruction are closely related to the function of fibroblasts (Stunova & Vistejnova, 2018). Dermal fibroblasts are divided into papillary fibroblasts and reticular fibroblasts; reticular fibroblasts are mainly involved in human skin wound healing, and their main functions are cytoskeleton remodeling and cell movement (Janson *et al.*, 2012). Changes in fibroblast phenotype, quantity and function regulate and affect the whole process of wound repair. Damage to the skin causes cell degeneration, necrosis, and tissue defects to varying degrees. With the cooperation of various cells and cytokines, fibroblasts will proliferate, migrate, activate, and undergo apoptosis.

In this study, a II-degree burn model was successfully established. Histopathological sections showed that the epithelial cells of wound skin in the shikonin group were largely differentiated, and a thicker neoepidermal layer was visible. There were more sebaceous glands and hair follicles in the dermis. The effect in the shikonin group was obviously better than that in the Vaseline group, and the structure and function of skin in the shikonin group were closer to normal. Therefore, we speculated that shikonin could accelerate the regeneration of injured skin in the epidermis and promote tissue and functional repair in the dermis. In recent years, some studies have confirmed that epidermal stem cells have a strong ability to proliferate and differentiate in multiple directions. They can proliferate and differentiate according to the needs of the body to meet the needs of self-renewal of the epidermis and play a key role in the process of wound repair. In this study, we found that the proliferation ability in skin cells of the shikonin group was significantly stronger than that of the Vaseline group, indicating that shikonin could promote wound healing by activating the proliferation of skin cells at the wound. To verify the effect of shikonin on skin repair and hair follicle regeneration during wound healing, we detected the expression of the fibroblast marker keratins CK14 and CK19 in the skin and hair follicles of II-degree burn mice. Immunofluorescence results showed that the positive reaction of CK19 and CK14 in the shikonin group was stronger than that in the control and Vaseline groups. Compared with normal hair follicles, CK19 was mainly enriched from the hair stem of hair follicles to the concentric circle formed by the outer root sheath and inner root sheath of hair follicles, and CK14 was also enriched in the hair papilla of hair follicles in the shikonin group. This indicates that shikonin can promote the healing of hair follicles in the wound skin by increasing the proliferation of fibroblasts and hair follicle stem cells. It can be inferred that shikonin can not only accelerate the repair of the epidermis but also repair the function of skin tissue.

In the inflammatory stage of wound repair, fibroblasts enter the mitotic proliferation phase through paracrine and autocrine pathways and promote the transformation of mesenchymal cells into fibroblasts under the action of growth factors and other chemokines, thus increasing the number of fibroblasts in the wound (Malcherek *et al.*, 1993). At the early stage of temporary matrix formation, fibroblasts begin to proliferate and migrate. Inflammatory cells secrete growth factors such as epidermal growth factor (EGF) and bFGF, which promote proliferation, as well as TGF-b and IL-6, which drive fibroblasts and epithelial cells to migrate to the wound (Malcherek *et al.*, 1993; Johnson *et al.*, 2020). After the fibroblasts migrate to the temporary wound matrix, the cells begin to proliferate and produce matrix metalloproteinases (MMPs) and other proteases to degrade the temporary matrix (Gill & Parks, 2008; Zhang & Huang, 2020). Researchers have found that activation of the Wnt/b-catenin pathway can increase the proliferation of fibroblasts, thus promoting remodeling of the extracellular matrix in skin tissue (Collins *et al.*, 2011). Epidermal growth factor, basic fibroblast growth factor and hepatocyte growth factor can enhance the proliferation and migration of fibroblasts, keratinocytes, and other skin cells by activating the PI3K/ Akt or FAK/ERK1/2 signaling pathways, promote the process of re-epithelialization, and finally accelerate wound contraction (Park *et al.*, 2018).

The formation of granulation tissue and remodeling of the extracellular matrix are related to the abnormal activation and proliferation of fibroblasts. Fibroblasts can differentiate into myofibroblasts that express a-SMA (Bainbridge, 2013). First, fibroblasts are activated into myoblasts under the stimulation of TGF-b and express integrin through the TGF-b/Smad signaling pathway, completing the connection between proto myofibroblasts and vascular endothelial cells. At the same time, type I collagen is secreted to fill the cells (Darby & Hewitson, 2007). Then, in the presence of TGF-b1, TGF-b2 and rigid matrix, proto myofibroblasts are further activated into myofibroblasts, transforming type I collagen into type III, synthesizing and secreting a large number of collagen fibers, MMPs, TIMPs and other protease components, and reconstructing the extracellular matrix (Bainbridge, 2013). Myofibroblasts form a "fiber connection network" to connect microfilaments in cells, muscle fibroblasts and the surrounding extracellular matrix and cause contraction of the wound through contraction of a-SMA. When healing is completed, myofibroblast apoptosis occurs. During this stage, type III collagen generated rapidly in the extracellular matrix is replaced by type I collagen (Witte & Barbul, 1997). Increasing the apoptosis of fibroblasts at the later stage of repair can accelerate the repair of skin structure. If the apoptosisof myofibroblasts is incomplete, scar and other

fibrosis diseases will occur (Bainbridge, 2013; Lagares *et al*., 2017). In our study, we found that excessive injection of fibroblasts increased the burden of skin healing and easily led to skin scarring (Vairo *et al*., 2019).

Oxidative stress is the result of an imbalance between excessive production of reactive oxygen species (ROS), inability to remove oxides and damage to tissue oxidative repair (Zhou *et al*., 2016; Zhang & Huang, 2020). Once ROS cannot be removed by the antioxidant system in time, they will damage the cell membrane, nucleic acids, lipids, proteins, etc., and then cause damage to fibroblasts. However, there are still some deficiencies at this stage, a certain level of ROS in normal cells participates in multiple signal transduction pathways that can promote cell proliferation, facilitate cell survival and function, and maintain the balance between oxidation and antioxidation in the body. ROS produced in large quantities after burn injury can cause extensive damage to body tissues and cells through the mitochondrial pathway, NADPH oxidase pathway and other pathways. Therefore, to further study the effect of shikonin on -burn injury induced oxidative stress in fibroblasts, we will detect redox indicators in fibroblasts and explore possible pathways in further studies.

CONCLUSION

Through animal models, we have confirmed that shikonin small molecules can repair burnt skin and promote skin cell proliferation. At the same time, at the wound healing site, fibroblasts are significantly enriched under shikonin treatment; therefore, we injected sorted fibroblasts into mouse skin wounds and applied Shikonin ointment to the wounds. After 7 days of treatment, the wound skin of mice was well repaired, and the skin structure was restored. In conclusion, this study showed that shikonin can effectively promote the proliferation of skin cell, and local injection of fibroblasts+ shikonin in burned skin can play a role in a certain therapeutic effect.

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RESUMEN: El objetivo de este trabajo fue investigar el potencial terapéutico de cicatrización de heridas y los mecanismos moleculares de la shikonina como moléculas pequeñas *in vitro*. Se utilizó un modelo de quemaduras en ratones para explorar el posible efecto terapéutico de la shikonina; Rastreamos las células en proliferación *in vivo* para localizar el área activa de proliferación de células de la piel. A través de los resultados de la tinción para patología convencional, encontramos que la shikonina tiene un buen efecto en el tratamiento de la piel quemada y promueve la distribución normal de la queratina de la piel en el sitio dañado. Al mismo tiempo, la shikonina también promovió la proliferación de células de la piel en el sitio dañado. Es importante destacar que encontramos un aumento significativo en la cantidad de fibroblastos en el sitio dañado tratado con shikonina. Lo más importante es que la shikonina promueve la función reparadora de fibroblastos en las heridas de la piel regulando la vía de señalización PI3K/AKT. Este estudio muestra que la shikonina puede promover eficazmente la proliferación de células de la piel y que la inyección local de fibroblastos en la piel quemada puede desempeñar un cierto papel terapéutico.

PALABRAS CLAVE: Shikonin; Moléculas pequeñas; Fibroblastos; Piel quemada; PI3K/AKT; Cicatrización de la herida.

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