Burn Ointment Promoted Cutaneous Wound for Burns and Scalds by Modulating the PI3K/AKT/mTOR Signaling Pathway

Da li Gan
south-central university for nationalities
Yan Yao
Wuhan University Renmin Hospital
Qi yuan Su
University of Illinois at Urbana-Champaign
Su qin Yang
South-central university for nationalities
Li Wu
South-central university for nationalities
Han wen Su
Wuhan University Renmin Hospital
mei xiang (756626131@qq.com)
South-central university for Nationality

Research

Keywords: Burn Ointment, burn wounds healing, analgesia, anti-inflammatory, cytokines, PI3K/AKT/mTOR

Posted Date: July 20th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-42754/v1

Abstract

Background: Burn injury is common, burn ointment (BO) is a common preparation used to treat burns and scalds in folk as an effective remedy for burn healing. The present study was undertaken to evaluate the healing effect and related underlying mechanisms of BO in a model of deep second-degree thermal burn by animal experiments.

Methods: BO is was made up of a combination of extracts from several traditional Chinese medicine and borneol, solid paraffin, rapeseed oil, and and its quality control was assessed. The acute toxicity test and skin irritation test were evaluated by rats. The anti-inflammatory effect was revealed by using inflammation animal models, including the xylene-induced auricle swelling in mice and carrageenan-induced toe swelling in rats. The hot plate test was used to evaluate its analgesic activity. Moreover, the experiments of knife and a deep second-degree burn wound were used to explore the effect of BO in promoting wound healing. On days 7, 14 and 28, the wounds were digitally photographed by a camera and after sacrifice of the SD rats, skin samples were obtained for performing H&E staining, immunohistochemical staining and Western Blotting examination. In addition, The expressed of TNF-α, TGF-β1 and VEGF in serum were detected by ELISA kits.

Results: BO had no toxicity or side effects on the skin and liver or kidney function. BO could significantly inhibit auricular swelling in mice, paw welling in rats and markedly prolonged the latencies of licking paws in mice; it also could accelerate the process of wound healing and repair scar by promoting the formation of new epithelial tissue. In addition, BO significantly reduced the content of TNF-α and markedly increased the content of VEGF and TGF-β1 in the serum. Moreover, BO promoted the expression of collagen I. Furthermore, it increased the ratio of p-PI3K/PI3K, p-AKT/AKT and p-mTOR/mTOR in the PI3K / AKT / mTOR pathway.

Conclusions: BO could effectively reduce inflammation and pain, and effectively accelerate the healing process of deep second-degree burn wounds. And the mechanism of BO promoting wound healing may be related to activate PI3K / AKT / mTOR pathway. therefore, it may be recommended as a promising topical medication for treating burn wounds in the future clinical trials.

Background

Burns, one of the worst forms of skin injury, are increasing in worldwide incidence annually[1]. The number of burn injury victims in the United States alone is estimated to be 1.2 million per year. Among these injuries, on average 50,000 burn patients were severely burned and required medical treatment in hospitals[2]. The skin is the largest organ of the human body, and it is able to resist external infections and maintain the stability of the internal environment of our body[3]. Mild scalding will damage the skin tissue, but severe burns will be life-threatening. Additionally, burns can cause inflammation, pain and other complications[4]. There is an urgent need for medical workers to solve the problem of repairing and

healing wounds, reducing scars, and relieving the inflammatory response and pain to improve the patients' quality of life.

Many research studies had indicated that several key cell factors were involved in the process of burn repair, such as tumor necrosis factor-alpha (TNF-α), vascular endothelial growth factor (VEGF) and transforming growth factor-beta1 (TGF-β1)[5, 6]. If the body acquires an infection when the skin is damaged, the body is stimulated to secrete the proinflammatory cytokine TNF-α. Then, this cytokine initiates an inflammatory cascade to remove necrotic tissues and cells[7]. However, overactive inflammation can cause systemic inflammatory response syndrome and immune dysfunction in patients, which will endanger their lives[8]. Therefore, anti-inflammatory treatments are very important for burn wound healing processes. Along with the inflammation subsides, macrophages and other cells begin to secrete growth factors, such as VEGF. VEGF is an essential cell factor for forming new blood vessels, and it can promote vascular endothelial cell proliferation to produce new blood vessels[9]. The new blood vessels can provide nutrition to the injured area and can accelerate wound healing[10]. In addition, TGF-β1 is also a necessary cell factor for the healing process, as it promotes fibroblasts to proliferate and differentiate, forms granulation tissues and synthesizes type I collagen[11]. Collagens help with wound contraction and restore the elasticity of skin to heal the burn wound by filling the space between cells[12].

At present, most known drugs had shown limitations in treating burns and scars, such as leaving scars easily. Epidemiological studies had suggested that components of traditional Chinese medicine (TCM) had become important sources of potential therapeutic agents with tremendous diversity[13]. Many studies had shown the great potential of TCM in treating burns, such as many chemicals of *Rheum palmatum* L and *Angelica sinensis* (Oliv.) Diels that could reduce the release of pro-inflammatory cytokines,including IL-1β, IL-6, IL-18, TNF-α and IL-33 et al[14, 15]. They had the effect of anti-inflammatory and analgesic[16], induced cell proliferation[17] and protected cells from injuries[18]. As a self-developed compound Chinese medicine ointment which contains *Rheum palmatum* L., *Angelica sinensis* (Oliv.) Diels, *Radlx Codonopsis pilosula* (Franch.) Nannf., *Asari Radix et Rhizoma*, Borneolum, and Hg_2Cl_2 powder, it is still unclear that the activity of BO in promoting skin wound healing, anti-inflammation and its specific mechanism. Therefore, this study aimed to preliminarily elucidate the efficacy and the involved molecular mechanisms of BO in the healing of burn wounds and their complications. This research might contribute to the development and application of new drugs for the treatment of burns and scalds.

Methods

Materials and reagents

The following reagents and materials were used: burn moisturizing scald (BMS; Shantou Meibao Pharmaceutical Co., Ltd.); ELISA Kit (Neobioscience Technology Company); Collagen I/HRP-labeled goat anti-rabbit antibody (Servicebio); Microscope (XSP-C204); Cognex camera; Toe volume measuring instrument (PV-200; TAIMENG Co., Ltd.); Autoclave (LDZX-50KBS; Shanghai Shenan Medical Instrument

Factory); Full-wavelength microplate reader 1510 (Thermo Fisher Scientific). Chinese herbal medicine pieces of Rheum palmatum L., Angelica sinensis (Oliv.) Diels, Radlx Codonopsis pilosula (Franch.) Nannf., Asari Radix et Rhizoma, Borneolum, and Hg2Cl2 powder et al were bought from Hubei Tianji Pieces Factory, they were identified by Dr Dingrong Wan's laboratory, School of Pharmaceutical Science, South-Central University for Nationalities, China. Voucher samples (No. SC-20191022-20191027) were deposited at the Herbarium of Medical Plants located in the College of Pharmacy, South-Central University for Nationalities, China.

Animals

Kunming (KM) mice (20 ± 2 g) and Sprague Dawley (SD) rats (200 ± 20 g) were purchased from the Experimental Animal Center (Certificate of experimental animals: SYXK 2016-0089), Institute of Health and Epidemic Prevention (Wuhan, China) and housed in the standard specific pathogen-free (SPF) environment. All animals were allowed free access to food and water.

HPLC analysis of components of BO

A total of 10 mg of BO was extracted with 10 mL methanol by ultrasonic for 20 min, then the resulting extract was filtered and transferred into 10 mL volumetric flask and kept at a constant volume of 10 mL with methanol. By this procedure a concentration of crude medicine equivalent to 1 mg/mL was obtained. For HPLC analysis, the mobile phase was acetomitrile (Aladdin, Shanghai, China): water (20: 80) mixture and the preparative column was Super Co. Inc. Water S Spheripor ODS (particle size: 5 μm, diameter: 4.5 mm, length: 265 nm). Flow rate was set at 1.0 mL/min, and injected 20 μL/ time; the detection was performed at 254 nm. The temperature of the column oven was set at 35℃

Skin irritation experiment in rats

On the day before the experiment, 30 SD rats were shaved with pet electric scissors, and the shaving area was approximately 4×2 cm^2. The shaved part was then flushed with saline. Rats with no skin wounds were selected. Twenty-four hours later, the rats were randomly divided into two groups: the control group with canola oil treatment (Con) and the test group with BO treatment. The application times were 10:00, 14:00, and 19:00, and the skin was applied to 0.5 g of the drug each time for 3 days. One hour after the last treatment, the treated skin was cleaned with cotton and water. At 0 h, 12 h, 24 h, 48 h, and 72 h, the allergic reactive changes were recorded.

Physical and chemical indicators test

Ten male and female SD rats were randomly selected. After anesthetization with ether anesthesia, blood samples were collected from the abdominal aorta, serum (3000 rpm, 10 min) was taken and sent to the Hubei Provincial People's Hospital for liver and kidney function tests according to the basic standards. Samples from the experimental animals were collected and tested with the same procedure.

The test of rat's toe swelling

Thirty male and female rats (200 ± 20 g) were randomly divided into three groups: the negative Con group and the BO and BMS treatment groups. The animals were treated with 0.5 g drugs on the toes 3 times per day for 5 days. On the sixth day, the right hind foot ankle of each rat was marked, and the volume before and after inflammation was measured by toe volume measuring instrument, and the average of three measurements was recorded. The right foot was injected with 0.1 ml carrageenan (1%), and the foot volume was measured at 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 h after inflammation began. The foot was still treated with the drug every time after the volume was measured. The swelling volume (ΔV) = The predose (V2) – The postdose (V1)

The test of the xylene-induced mouse ear swelling

Thirty male and female mice (20 ± 2 g) were randomly divided into three groups: the negative Con group and the BO and BMS treatment groups. The animals' ears were treated with 0.5 g of the drugs 3 times per day for 5 days. On the sixth day, 100 μl of xylene was dripped on both sides of the right ear with a pipette. After the model was established successfully, the animal was immediately sacrificed, and both ears were cut off along the root of the ear. The sample was immediately weighed after being punched. The degree of ear swelling: Δm = The right ear mass - The left ear mass

The hot-plate test of mice

Twenty-seven KM mice were screened out of 40. The index of the test was the time that the mouse licked its toe with its tongue at 54 ± 1 °C, which was the mean of 3 times (the interval was repeated every 30 min) to obtain the mouse's pain threshold for 5–40 s. The mice were randomly divided into the following groups: the negative Con group and the BO and BMS treatment groups, which were coated with 0.5 g drug each time, 3 times a day for 7 days. The test method of the mouse's pain threshold was same as that 7 days prior. Pain threshold change = After treatment - Before treatment

The test of knife wound

Thirty mice were randomly divided into two groups. The skin of the wound was approximately cut 1.5 cm in size after anesthetized by ether anesthesia. The Con group and the BO treatment group were coated with 0.5 g drug 3 times a day for 16 days.

The test of scalds in rats

Thirty-five SD rats were anesthetized, and their backs were shaved. The mice were burned with a soldering iron (1.5 cm^2) for 10 s. After half an hour, all animals were awakened and randomly divided into the negative Con group, the BO treatment group and the positive BMS group, which were coated with 0.5 g drug each time and 3 times a day for 28 days. After the model was established on the second, seventh, fourteenth, and twenty-eighth days, the skin of wound took off and infiltrated by a paraformaldehyde fixative, and the animals' blood was centrifuged at 3000 rpm for 10 min. The serum was preserved at -80 °C.

The test of H&E staining

The pretreated sample tissues from rats were embedded in paraffin wax. The embedded wax blocks were cut into slices with a thickness of 4 μm. The sections were routinely dewaxed with xylene and washed with ethanol at every step. The reagent incubation order was as follows: the samples were incubated with xylene twice for 10 min each time, followed by incubation with anhydrous ethanol twice for 10 min each time, 95% ethanol for 5 min, 90% ethanol for 5 min, 80% ethanol for 5 min, 75% ethanol for 5 min and distilled water for 5 min. These sections were soaked for 2–5 min in hematoxylin and rinsed with tap water. Then, the cells were differentiated in 1% hydrochloric acid ethanol for 30 s. After differentiation, the cells were submerged in tap water. Next, their differentiation was performed in 0.6% ammonia water for 30 s. The sections were rinsed with tap water 1 h after differentiation and submerged for 30 s. The samples were incubated with eosin for 2 min and rinsed with tap water. Then, the samples were dehydrated to transparency and sealed: the handled samples were placed into 95% ethanol, anhydrous ethanol, and dimethylbenzene, and every step was repeated twice for 5 min each; then, the samples were sealed with neutral resin. Finally, the pathological sections were placed under a positive fluorescence microscope for observation, photographing, describing and comparing with normal tissues. The observations included the epidermis, dermis, blood vessels, cells and organelles. Another two evaluators used the histological changes in burn wound healing scoring system[19] to perform blind histological evaluations.

The test of immunohistochemistry

The prepared paraffin tissue sections were placed in a wet box filled with citric acid antigen repair buffer (pH 6.0). The box was placed in a microwave to boil for 8 min, allowed to cool for 8 min, kept warm for 8 min and then boiled for 7 min. The sections were washed with PBS three times after naturally cooling. The sections were incubated with 3% hydrogen peroxide protected from light for 25 min and then rinsed with PBS three times for 5 min each. Then, 3% BSA was added, and the solution was kept at room temperature for 30 min. The blocking solution was discarded, and the primary antibody diluted in PBS was added to the box, followed by an incubation at 4 °C overnight. Next, the sections were washed with PBS, and the appropriate secondary antibody was mixed with horseradish peroxide and incubated at room temperature with the sections for 50 min. Then, the sections were cleaned with PBS and excess moisture was removed. The DAB staining solution was prepared in a staining box and added dropwise onto the tissues, and the reaction was monitored by microscopy. The nuclei were counterstained with hematoxylin. Finally, the cover slips were dehydrated with ethanol, anhydrous ethanol and xylene twice for each liquid, and then the tissue sections were sealed. The sections were placed under a positive fluorescence microscope for observation, photographing, describing and comparing with normal tissues. Then we used Image J to semi-quantitative and analysis the results.

ELISA analysis

The contents of TNF-α, VEGF, and TGF-β1 were measured in the sera of SD rats from the burn test by ELISA following the kit instructions.

Western-blot analysis

The samples were prepared as follows: the skin tissue was removed from the burn wounds in rats, cut into small fragments, placed into a mortar, ground into powder in liquid nitrogen (200μL lysis solution per 20 mg tissue), and centrifuged (12,000 rpm/min, 5 min), to obtain the samples (the supernatant). The proteins from tissue lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto PVDF membranes. Then, the membranes were blocked with a Western blocking solution and incubated using primary antibodies, followed by an incubation with the appropriate secondary antibodies. Finally, the protein levels were normalized against that of GAPDH.

Statistical analysis

The data were presented as the mean ± SD. Statistical analysis was performed via Student's t test and one-way ANOVA (SPSS Program, version 11.5; SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a significant difference between the groups.

Results

The result of HPLC analysis

HPLC is used to determine the content of active ingredients present in the formula, When the sample passes HPLC, the active ingredients in the formula are separated according to their polar size. The content of Emodin, Rhein, chrysophanol, ferulic acid, Lobetyolin, Asarinin, Borneol was quantitated by HPLC (Fig. 1).

The safety assessment of BO

No difference was noted in skin appearance in terms of inflammation or color change before and after treatment (Fig. 2). In the physical and chemical indicator testing, it was shown that there was no change in either the liver or kidney function (Table 1), as there was no increase in either the aspartate transaminase (AST) or creatinine (Cr) level. In contrast, the levels were decreased. The cause was unclear, and we hypothesize that this phenomenon may be caused by hemolysis or an insufficient sample size.

Table 1
The results of liver and kidney function (n = 10, `x ± s)

Index	I_1	I_2
ALT	65.00 ± 13.22	37.00 ± 3.00
AST	354.58 ± 109.31	98.00 ± 3.00*
TP	59.73 ± 3.05	58.10 ± 1.80
ALB	38.32 ± 2.35	37.85 ± 1.45
Cr	31.80 ± 3.42	22.50 ± 0.50*

Legend: I_1 = before dosing,I_2 = after dosing, the data are expressed as the mean ± SD (n = 30), compared with before administration, *P < 0.05, **P < 0.01.

BO inhibits inflammation and upregulates the pain threshold

Inflammation is very common in tissue injuries, especially in burn and scald wounds. The common inflammation models include carrageenan-induced toe swelling in rats and xylene-induced ear swelling in mice. Carrageenan stimulation can cause swelling of the foot, and xylene stimulation can cause swelling of the ear, and this swelling gradually alleviates over time. To understand the anti-inflammatory effect of BO, we chose to use these two models. In the model of carrageenan-induced toe swelling, we observed that the animals treated with BO demonstrated a significant reduction in swelling after the 3rd hour (Fig. 3C). In the second model, auricle swelling was significantly reduced in the BO-treated group (Fig. 3B). These results showed that BO could inhibit inflammation.

Burns cause not only inflammation but also pain, and hot-plate experiments can evaluate the effect of BO analgesia. The time that the mouse was kept on the hot-plate meter before and after was recorded and analyzed. As revealed in Fig. 3D, on the seventh day, the pain threshold of the BO group was significantly increased compared with that of the Con group. These results showed that BO had an analgesic effect.

BO promotes wounds healing

After cutting, the healing process of the skin was observed and photographed on the 1st, 5th, 10th and 16th days. On the 5th day, there was no significant difference between the Con group and the BO group. On the 10th day, the scar was mainly in the Con group animals, whereas the scars tended to gradually disappear in the treatment group. On the 16th day, the scars in the Con group were still predominant, while the scars became lighter in the treatment group and disappeared. Newly grown hair grew vigorously in the original wound areas, which were covered completely by the epidermis (Fig. 4A).

After the scald, on the 1st day, the surface of the scald wound was slightly yellowish, and the edges of the wound became hardened. On the 7th day after modeling, the wounds in the Con group and the treatment group were still swollen and hardened; however, the degree of scalding in the BO group was reduced. On the 14th day, the wounds of the Con group were red and swollen and were full of secretions. In the BO

group, however, the secretions had ceased, and the eschar had completely disappeared. The eschar even began to shed. On the 28th day, compared with the Con group, the BO group was mainly present with scar formation, and new fur had been growing on the scar (Fig. 4B). These results suggest that BO could promote wound healing.

BO promotes wound differentiation from the epidermis to the dermis

To determine the extent to which the skin heals after burns, the H&E staining was used. On the first day, the epidermis was fragmented, the cells of the blood vessels and subcutaneous tissues under the dermis were seriously damaged, and large hairless areas appeared. On the 7th day, the epidermis of the Con group was severely damaged, and there were more blank areas in the dermis. Compared with the Con group, the treatment group and the positive group had reduced blank areas, indicating that the area of injury was reduced. On the 14th day, the cells of the epidermis began to appear at the edge, but there was still a blank area between the epidermis and the dermis in the Con group, especially in the dermis. In the BO and BMS groups, the dermis cells advanced slowly toward to the epidermis for preparing cell keratinization. The dermis of the treatment group, in which the blank area was much less, was darker at the rims and had a greater tendency to be keratinized than the positive group. On the 28th day, there was a tendency for the epidermal cells to keratinize. Compared with the Con group, the BO group had wound edges that were neat with no extraneous dye, and the junction between the epidermis and dermis layers was tight and seamless. Moreover, the epidermis of the BO group was much more uniform in thickness than that of the positive group (Fig. 5A). The results of pathological score were shown in the Fig. 5B, it showed that BO group was significantly difference with Con group at 28th day (*$P < 0.05$).This result showed that BO could promote differentiation from the epidermis layer to the dermal layer and had the effect of promoting wound healing.

BO promotes increase of type I collagen

Small molecule amino acids form the biological macromolecule collagen, the main component of connective tissue in animals, which helps to protect cells against external pressure and elasticity. On the first day, the results showed that the skin tissue was severely destroyed. The gap between cells (the negative staining area (blue) represents the nucleus) was larger, and the interstitial area [the positive staining area (brown and yellow) represents collagen] was sparsely distributed. On the 7th, 14th and 28th days, the irregular brown-yellow areas began to appear between cells. Within the same field of vision, the irregular brown-yellow areas in the tissues from the BO and BMS groups were increased compared with those from the Con group. During dosing, the brown-yellow color of the same group increased between the cells and was arranged closely and evenly over time (Fig. 5C). The semi-quantitative data were shown in Fig. 5D, and the results showed that compared with the control group, the expression of collagen I in the BO groups increased significantly at 7th day (*$P < 0.05$). The results indicated that BO played an impartment role in promoting an increase in cellular type I collagen.

BO regulates the cytokine and growth factor contents

TNF-α is a representative anti-inflammatory substance. The TNF-α content (52.77 ± 6.53 pg/ml) in the BO group was significantly decreased compared to that in the Con group (222.14 ± 3.74 pg/ml) (Fig. 6A). The results demonstrated that BO could reduce the blood TNF-α content after scalding.

VEGF is an important cell factor involved in epidermal recovery. On the 14th day, compared to the content of VEGF in the Con group (108.19 ± 2.69 pg/ml), the content in the BO group (116.08 ± 1.07 pg/ml) was significantly increased. On the 28th day, the content in the treatment group (131.59 ± 5.44 pg/ml) was significantly increased compared with the content in the Con group (122.04 ± 0.55 pg/ml). On the 14th day, compared to that in the positive Con group (111.59 ± 0.26 pg/ml), VEGF in the BO group (116.08 ± 1.07 pg/ml) increased significantly (Fig. 6B). These results showed that BO could increase the blood VEGF content after scalding.

TGF-β1 is closely related to wound healing. On the 7th day, compared with the concentration in the Con group (129.24 ± 5.81 ng/ml), the concentration of TGF-β1 in the BO group was increased (305.98 ± 6.17 ng/ml). On the 14th day, compared to that in the Con group (209.82 ± 6.10 ng/ml), the concentration of TGF-β1 in the BO group significantly increased (356.87 ± 5.24 ng/ml). On the 28th day, the concentration in the treatment group (79.96 ± 3.74 ng/ml) was significantly increased compared with that in the Con group (127.07 ± 4.75 ng/ml) (Fig. 6C). These results showed that BO could increase the content of TGF-β1 in blood with time after scalding; at a later time, BO could decrease its content as well.

BO activates the PI3K-AKT-mTOR pathway

The PI3K-AKT-mTOR signaling pathway plays an important role in wound healing and it can be activated by many cellular factors. To examine the effect of BO on the PI3K-AKT-mTOR signaling pathway, the results of relevant proteins in this pathway were evaluated with Western blot analysis. The results showed that 1) from the 14th to 28th days, the expression of the p-PI3K/p-AKT/p-mTOR proteins was increased by external wound stimulation; 2) on the 14th and 28th days, the expression of the p-PI3K/p-AKT/p-mTOR proteins in tissues was increased, and the protein concentrations in the BO and BMS groups were significantly increased compared with those in the Con group ($P < 0.05$) (Fig. 7B-D). The phosphorylated protein content in the BO group increased more than that in the BMS group on the 28th day. These results suggest that BO could upregulate the expression of the PI3K/AKT/mTOR pathway.

Discussion

Burn and scald stress trauma not only causes local damage to the body but also induces systemic inflammatory responses mediated by neuroendocrine[20], cytokine and inflammatory mediators. Those factors not only delay the healing of local burn and scald wounds but also cause serious complications, including pain, making treatment difficult[21]. The healing of burn and scald wounds involves three complex processes, which include inflammation, tissue hyperplasia and regeneration. These three processes are not independent but overlap and develop in a certain order[22]. Exogenous damage will stimulate the body to secrete a variety of growth factors, such as VEGF, TGF-β1, and TNF-α, that promote cell migration, proliferation and differentiation to help wound constriction and recovery. This damage and

these factors can also activate signaling pathways associated with wound repair, such as the PI3K-AKT-mTOR pathway[11].

Inflammation is the first step after tissue damage, and it is very important for skin repair[11, 23]. However, prolonged inflammatory reactions will be harmful to the body, producing some complex complications. Therefore, inhibiting inflammation and pain during tissue recovery is also important. To study the anti-inflammatory and analgesic effects of BO, we used a variety of classical inflammatory animal models, including auricle swelling in mice and toe swelling in rats, and hot-plate tests. The results showed that BO could inhibit ear swelling in mice and toe swelling in rats (* $p < 0.05$, ** $p < 0.01$), and it showed strong anti-inflammatory activity. Moreover, the analgesia results demonstrated that the pain threshold was increased in mice treated with BO (** $p < 0.01$) compared with that in control mice, illustrating that BO could relieve pain.

When the wound presents with early inflammation, the body's defense system is activated and forms a protective barrier, stimulating growth factor secretion[24]. TNF-α and VEGF are two of those cell factors and are the key factors in the wound healing process. As one of the most important proinflammatory cytokines, TNF-α participates in vasodilatation, edema formation and leukocyte adhesion to the epithelium through the expression of adhesion molecules; it regulates blood coagulation and contributes to oxidative stress in sites of inflammation[7]. Studies had shown that drugs could decrease the release of inflammatory factors, such as TNF-α, to reduce the inflammatory reaction and promoted wound healing[25, 26]. We found that BO could inhibit the release of TNF-α, which might be related to the anti-inflammatory mechanism of BO. However, to determine whether other factors can be linked to the anti-inflammatory effects of BO, further experiments are needed. For example, Chen[27] found that inhibiting the expression of IL-6 and IL-10 could reduce the inflammatory response to promote wound healing. The secretion of VEGF in the healing process also exerts and important effect, and VEGF will promote the proliferation of vascular endothelial cells and their survival, accelerating the formation of new blood vessels[28–31]. The blood vessels are the channels of nutrient delivery in the body[32], and ensure that the tissues and organs are provided with sufficient requirements for new skin formation[33]. Our results indicated that when skin was scalded by high temperature, BO could promote the release of VEGF to facilitate wound healing. In addition, collagen is necessary for repairing wounds. Studies had illustrated that TGF-β1 could regulate type I collagen gene expression and translation[34, 35]. However, collagen I continuously increases in tissue will cause pathological scars to develop. Our results showed that the TGF-β1 content of the BO group tended to increase early but decreased later. Based on these results, we speculated that under the stimulation of BO, TGF-β1 stimulated the release of type I collagen early to promote skin repair. Then, to avoid collagen over-proliferation and scar formation, TGF-β1 expression is downregulated to inhibit the increase in collagen I.

The PI3K/AKT/mTOR signaling pathway is found in various cells and participates in cell growth, proliferation, apoptosis, and differentiation[31, 36, 37]. PI3K can be activated by growth factors, such as VEGF and TGF-β1[11, 38]. The activated state of growth factor receptors quickly phosphorylates phosphatidylinositol(4,5) P2 (PIP2) to obtain phosphatidylinositol(3,4,5)P3 (PI3P)[39]. Protein kinase B

(AKT) is one of the proteins downstream of PI3K; it is recruited by PI3P and phosphorylated by 3-phosphoinositide-dependent kinases (PDK-1)[40]. Then, p-AKT can regulate the protein level of phosphorylated mTOR to promote cellular growth and reproduction[41]. Fibroblasts are a major force involved in wound healing. When tissue injury occurs, this signaling pathway is activated by increased VEGF and TGF-β1, which then promotes the migration of fibroblasts to the wound site, and the fibroblasts begin to proliferate and differentiate, producing myofibroblasts[42, 43]. These cells can help contract the wound border and are helpful for producing collagen-like proteins, which are necessary for healing wounds and forming new skin tissue, accelerating the repair of burn wounds[44–46]. Our Western blotting analysis showed that compared with the control group, the BO-treated group had significantly increased activation of the PI3K/AKT/mTOR signaling pathway, and the phosphorylated proteins of this pathway were gradually upregulated. This finding suggested that the preliminary mechanism by which BO promoted burn repair might be related to the activation of the PI3K/AKT/mTOR signaling pathway

Conclusion

In this study, we find that BO has obvious analgesic and anti-inflammatory effects, and a scald test in rats and mice confirmed that BO could significantly promote the healing of scalded wounds. Importantly, we find that BO can accelerate wound healing via the PI3K/AKT/mTOR signaling pathway and increase the crucial cellular factors VEGF and TGF-β1 to help further tissue recovery. The study has preliminarily explained its pharmacological basis for the treatment of burns and scalds, which is of certain significance for the clinical application of BO. Our study also provides new treatment methods for burns. However, there are more questions we do not have the answers to, such as the specific mechanism of the anti-inflammatory and analgesic effects of BO, the other signaling pathways that can promote wound healing, or whether BO can help to heal the skin festering induced by diabetes. These results are preliminary and further studies are required to evaluate the specific mechanism of BO.

Abbreviations

AKT
protein kinase B; AST:aspartate transaminase; BMS:Burns Moisturizing Scald; BO:burn ointment; BSA:bovine serum albumin; Cr:creatinine; DAB:diaminobenzidine; ELISA:enzyme-linked immunosorbent assay; H&E:hematoxylin-eosin; GAPDH:glyceraldehyde phosphate dehydrogenase; mTOR:mammalian target of rapamycin; PAGE:SDS-polyacrylamide gel electrophoresis; PBS:phosphate buffered saline; PDK-1:3-phosphoinositide-dependent kinases; PI3K:phosphatidylinositol 3-kinase; PIP3:phosphatidylinositol (3,4,5) P3; PVDF:polyvinylidene difluoride; SDS:sodium dodecyl sulfate; TCM:Traditional Chinese medicine; TGF-β1:transforming growth factor-beta1; TNF-α:Tumor Necrosis Factor-alpha; VEGF:vascular endothelial growth factor; PIP2:phosphorylates phosphatidylinositol (4,5) P2.

Declarations

Acknowledgments

We are very grateful that all funding agencies for supporting this research.

Authors' contributions

Meixian Xiang and Hanwen Su designed the study. Dali Gan performed the animal and cell culture experiments and Qiyuan Su wrote and modified the manuscript. Yan Yao and evaluated and quantitated the histopathological and immunohistochemical staining. SuqinYang analyzed and interpreted the data. All authors reviewed the manuscript.

Funding

This work was supported by the Natural Science Foundation of China (31200264), and the Fundamental Research Funds for Central Universities South-Central University for Nationalities (CZY19028, CZY20048).

Availability of data and materials

The datasets used during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The experiments involving animals were approved by the Institutional Animal Care and Use Committee of South-Central University for Nationalities (Wuhan, China).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interest.

References

1. Shan Y, Peng L, Liu X, Chen X, Xiong J, Gao J. Silk fibroin/gelatin electrospun nanofibrous dressing functionalized with astragaloside IV induces healing and anti-scar effects on burn wound. Int J Pharmaceut. 2015;479(2):291–301.
2. Kaddoura I, Abu-Sittah G, Ibrahim A, Karamanoukian R, Papazian N. Burn injury: review of pathophysiology and therapeutic modalities in major burns. Ann Burns Fire Disasters. 2017;30(2):95–102.
3. Allgower M, Schoenenberger GA, Sparkes BG. Burning the largest immune organ. Burns. 1995;21(Suppl 1):7–47.
4. Wang Y, Beekman J, Hew J, Jackson S, Issler-Fisher AC, Parungao R, et al. Burn injury: Challenges and advances in burn wound healing, infection, pain and scarring. Adv Drug Deliv Rev. 2018;123:3–

17.

5. Li HL, Chen LP, Hu YH, Qin Y, Liang G, Xiong YX, et al. Crocodile oil enhances cutaneous burn wound healing and reduces scar formation in rats. Acad Emerg Med. 2012;19(3):265–73.
6. Sumiyoshi M, Kimura Y. Enhancing effects of a chromone glycoside, eucryphin, isolated from Astilbe rhizomes on burn wound repair and its mechanism. Phytomedicine. 2010;17(10):820–9.
7. Zelova H, Hosek J. TNF-alpha signalling and inflammation: interactions between old acquaintances. Inflamm Res. 2013;62(7):641–51.
8. Matsuda N, Hattori Y. Systemic inflammatory response syndrome (SIRS): molecular pathophysiology and gene therapy. J Pharmacol Sci. 2006;101(3):189–98.
9. Roskoski RJ. Vascular endothelial growth factor (VEGF) and VEGF receptor inhibitors in the treatment of renal cell carcinomas. Pharmacol Res. 2017;120:116–32.
10. Eming SA, Martin P, Tomic-Canic M. Wound repair and regeneration: mechanisms, signaling, and translation. Sci Transl Med. 2014;6(265):265 sr6.
11. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. Wound Repair Regen. 2008;16(5):585–601.
12. Gonzalez AC, Costa TF, Andrade ZA, Medrado AR. Wound healing - A literature review. An Bras Dermatol. 2016;91(5):614–20.
13. Lv C, Wu X, Wang X, Su J, Zeng H, Zhao J, et al. The gene expression profiles in response to 102 traditional Chinese medicine (TCM) components: a general template for research on TCMs. Sci Rep. 2017;7(1):352.
14. Shen C, Zhang Z, Xie T, Ji J, Xu J, Lin L, et al. Rhein suppresses lung inflammatory injury induced by human respiratory syncytial virus through inhibiting NLRP3 inflammasome activation via NF-kappaB pathway in mice. Front Pharmacol. 2019;10:1600.
15. Kim YJ, Lee JY, Kim HJ, Kim DH, Lee TH, Kang MS, et al. Anti-inflammatory effects of Angelica Sinensis (Oliv.) Diels water extract on RAW 264.7 induced with lipopolysaccharide. Nutrients. 2018;10(5):647.
16. Wei WL, Zeng R, Gu CM, Qu Y, Huang LF. Angelica sinensis in China-A review of botanical profile, ethnopharmacology, phytochemistry and chemical analysis. J Ethnopharmacol. 2016;190:116–41.
17. Gundogdu G, Gundogdu K, Nalci KA, Demirkaya AK, Yilmaz TS, Demirkaya MF, et al. The effect of parietin isolated *From Rheum Ribes L* on in vitro wound model using human dermal fibroblast cells. Int J Low Extrem Wounds. 2019;18(1):56–64.
18. Zhang Q, Hu F, Guo F, Zhou Q, Xiang H, Shang D. Emodin attenuates adenosine triphosphateinduced pancreatic ductal cell injury in vitro via the inhibition of the P2 × 7/NLRP3 signaling pathway. Oncol Rep. 2019. doi:10.3892/or.2019.7270.
19. Mesquita RL, Silva PI, Melo ESS, Oliveira KO, Fontes-Pereira AJ, Freitas JJ, et al. Effect of low-intensity therapeutic ultrasound on wound healing in rats subjected to third-degree burns. Acta Cir Bras. 2016;31(1):36–43.

20. Gokakin AK, Deveci K, Kurt A, Karakus BC, Duger C, Tuzcu M, et al. The protective effects of sildenafil in acute lung injury in a rat model of severe scald burn: A biochemical and histopathological study. Burns. 2013;39(6):1193–9.
21. Naseri N, Kalantar K, Amirghofran Z. Anti-inflammatory activity of Echium amoenum extract on macrophages mediated by inhibition of inflammatory mediators and cytokines expression. Res Pharm Sci. 2018;13(1):73–81.
22. Adam J. Singer. RAFC. Cutaneous wound healing. N Engl J Med. 1999(341):738–746.
23. Glim JE, Beelen RH, Niessen FB, Everts V, Ulrich MM. The number of immune cells is lower in healthy oral mucosa compared to skin and does not increase after scarring. Arch Oral Biol. 2015;60(2):272–81.
24. Eming SA, Krieg T, Davidson JM. Inflammation in wound repair: molecular and cellular mechanisms. J Invest Dermatol. 2007;127(3):514–25.
25. Weimann E, Silva M, Murata GM, Bortolon JR, Dermargos A, Curi R, et al. Topical anti-inflammatory activity of palmitoleic acid improves wound healing. Plos One. 2018;13(10):e205338.
26. Coppe JP, Kauser K, Campisi J, Beausejour CM. Secretion of vascular endothelial growth factor by primary human fibroblasts at senescence. J Biol Chem. 2006;281(40):29568–74.
27. Chen Y, Tian L, Yang F, Tong W, Jia R, Zou Y, et al. Tannic acid accelerates cutaneous wound healing in rats via activation of the ERK 1/2 signaling pathways. Adv Wound Care (New Rochelle). 2019;8(7):341–54.
28. Yen YH, Pu CM, Liu CW, Chen YC, Chen YC, Liang CJ, et al. Curcumin accelerates cutaneous wound healing via multiple biological actions: The involvement of TNF-alpha, MMP-9, alpha-SMA, and collagen. Int Wound J. 2018;15(4):605–17.
29. Yan D, He Y, Dai J, Yang L, Wang X, Ruan Q. Vascular endothelial growth factor modified macrophages transdifferentiate into endothelial-like cells and decrease foam cell formation. Biosci Rep. 2017;37(3):BSR20170002.
30. Wagner VP, Curra M, Webber LP, Nor C, Matte U, Meurer L, et al. Photobiomodulation regulates cytokine release and new blood vessel formation during oral wound healing in rats. Lasers Med Sci. 2016;31(4):665–71.
31. Peng N, Gao S, Guo X, Wang G, Cheng C, Li M, et al. Silencing of VEGF inhibits human osteosarcoma angiogenesis and promotes cell apoptosis via VEGF/PI3K/AKT signaling pathway. Am J Transl Res. 2016;8(2):1005–15.
32. Johnson KE, Wilgus TA. Vascular endothelial growth factor and angiogenesis in the regulation of cutaneous wound repair. Adv Wound Care (New Rochelle). 2014;3(10):647–61.
33. Lohman AW, Billaud M, Isakson BE. Mechanisms of ATP release and signalling in the blood vessel wall. Cardiovasc Res. 2012;95(3):269–80.
34. Kubota K, Okazaki J, Louie O, Kent KC, Liu B. TGF-beta stimulates collagen (I) in vascular smooth muscle cells via a short element in the proximal collagen promoter. J Surg Res. 2003;109(1):43–50.

35. Chen SJ, Yuan W, Mori Y, Levenson A, Trojanowska M, Varga J. Stimulation of type I collagen transcription in human skin fibroblasts by TGF-beta: involvement of Smad 3. J Invest Dermatol. 1999;112(1):49–57.
36. Polivka JJ, Janku F. Molecular targets for cancer therapy in the PI3K/AKT/mTOR pathway. Pharmacol Ther. 2014;142(2):164–75.
37. Karar J, Maity A. PI3K/AKT/mTOR Pathway in Angiogenesis. Front Mol Neurosci. 2011;4:51.
38. Sheppard K, Kinross KM, Solomon B, Pearson RB, Phillips WA. Targeting PI3 kinase/AKT/mTOR signaling in cancer. Crit Rev Oncog. 2012;17(1):69–95.
39. Morotti M, Becker CM, Menada MV, Ferrero S. Targeting tyrosine-kinases in ovarian cancer. Expert Opin Investig Drugs. 2013;22(10):1265–79.
40. Shimamura H, Terada Y, Okado T, Tanaka H, Inoshita S, Sasaki S. The PI3-kinase-Akt pathway promotes mesangial cell survival and inhibits apoptosis in vitro via NF-kappa B and Bad. J Am Soc Nephrol. 2003;14(6):1427–34.
41. Xu J, Wang S, Feng T, Chen Y, Yang G. Hypoglycemic and hypolipidemic effects of total saponins from Stauntonia chinensis in diabetic db/db mice. J Cell Mol Med. 2018;22(12):6026–38.
42. Klass BR, Grobbelaar AO, Rolfe KJ. Transforming growth factor beta1 signalling, wound healing and repair: a multifunctional cytokine with clinical implications for wound repair, a delicate balance. Postgrad Med J. 2009;85(999):9–14.
43. Verrecchia F, Mauviel A. Transforming growth factor-beta signaling through the Smad pathway: role in extracellular matrix gene expression and regulation. J Invest Dermatol. 2002;118(2):211–5.
44. Xiao W, Tang H, Wu M, Liao Y, Li K, Li L, et al. Ozone oil promotes wound healing by increasing the migration of fibroblasts via PI3K/Akt/mTOR signaling pathway. Biosci Rep. 2017;37(6):BSR20170658.
45. Brenner AK, Andersson TT, Bruserud O. The complexity of targeting pi3k-akt-mtor signalling in human acute myeloid leukaemia: the importance of leukemic cell heterogeneity, neighbouring mesenchymal stem cells and immunocompetent cells. Molecules. 2016;21(11):1512.
46. Ohlund D, Elyada E, Tuveson D. Fibroblast heterogeneity in the cancer wound. J Exp Med. 2014;211(8):1503–23.

Figures

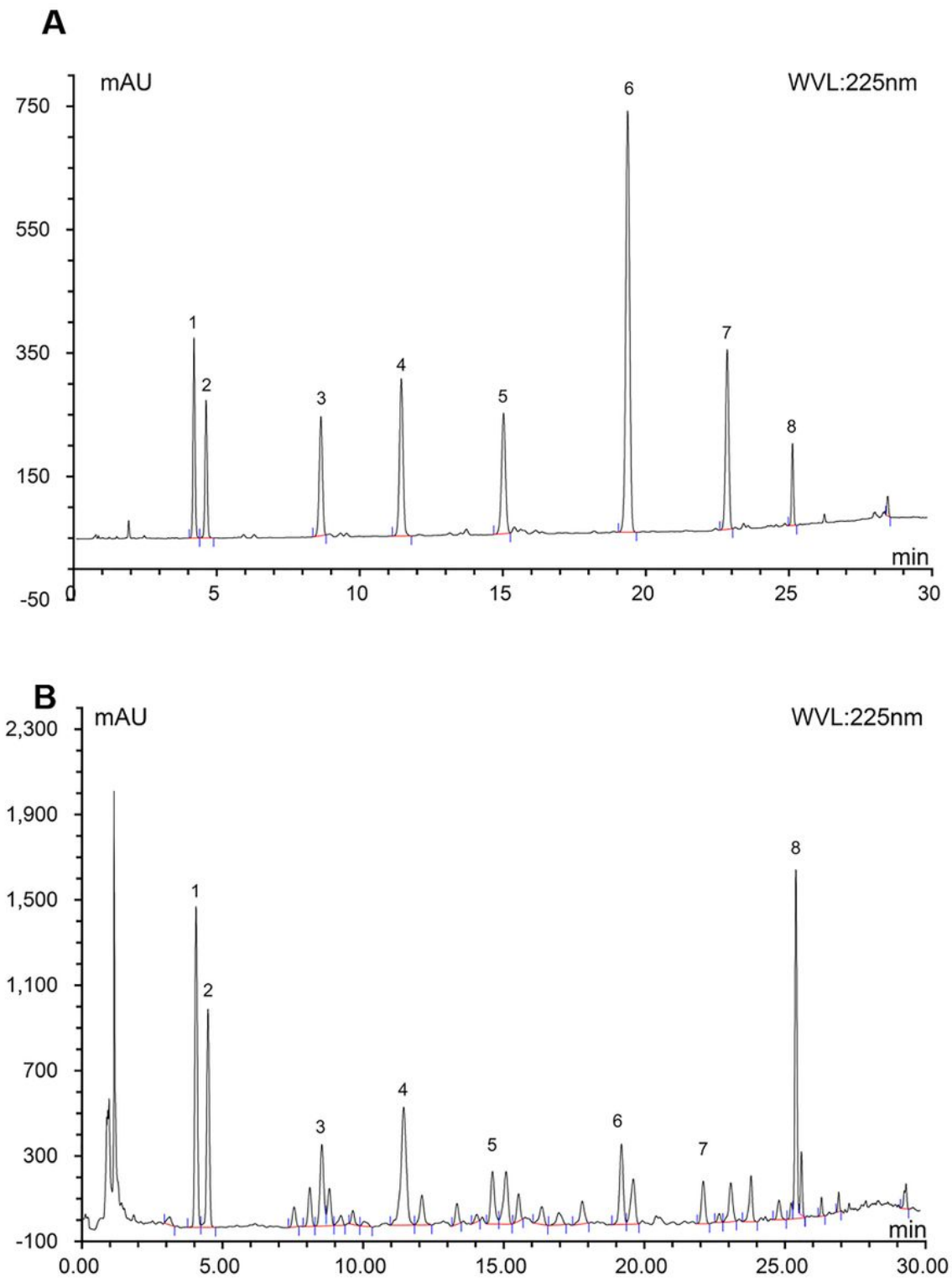

Figure 1

The result of HPLC analysis. A. The content determination of standards; B The content determination of sample. 1.Aloe-emodin; 2. Lobetyolin; 3. Rhein; 4. ferulic acid; 5. Asarinin; 6. Chrysophanol; 7 .emodin; 8. Borneol

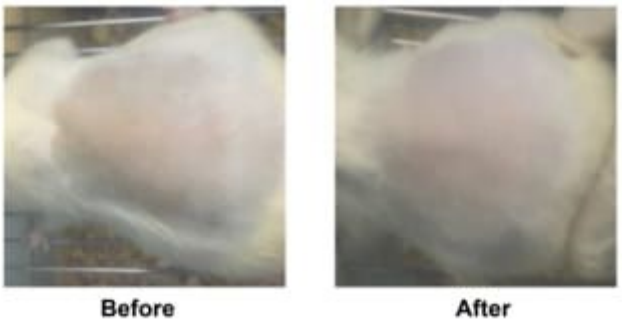

Figure 2

The result of irritating experiment. Rat skin condition before administration, the skin condition of rats after 72 hours of treatment with BO (n=30).

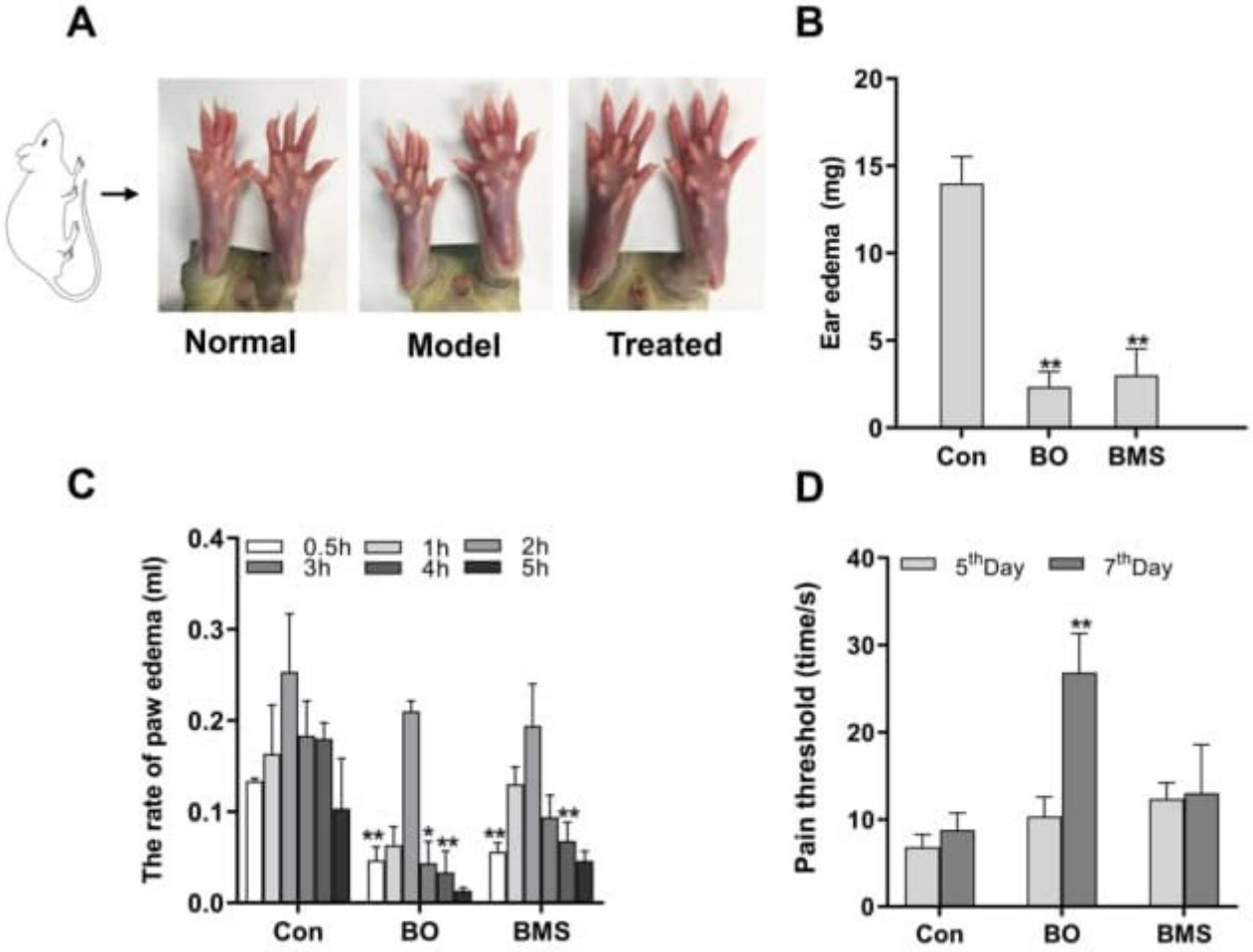

Figure 3

The anti-inflammatory and analgesic effects of BO. The negative control group (Con), the treatment group (BO) and the positive group burns moisturizing scald (BMS). (A) Picture of rat toe before making model and after, before treatment and after. (B) The result of mouse ear edema, the data were expressed as the mean ± SD (n=30), compared with the control group, *P<0.05□**P<0.01. (C) The results of the rat's toe

swelling, the data are expressed as the mean ± SD (n=10), compared with the control group, *P<0.05, **P<0.01. (D) The results of the pain threshold, the data are expressed as the mean ± SD (n=27), compared with the control group, *P<0.05, **P<0.01.

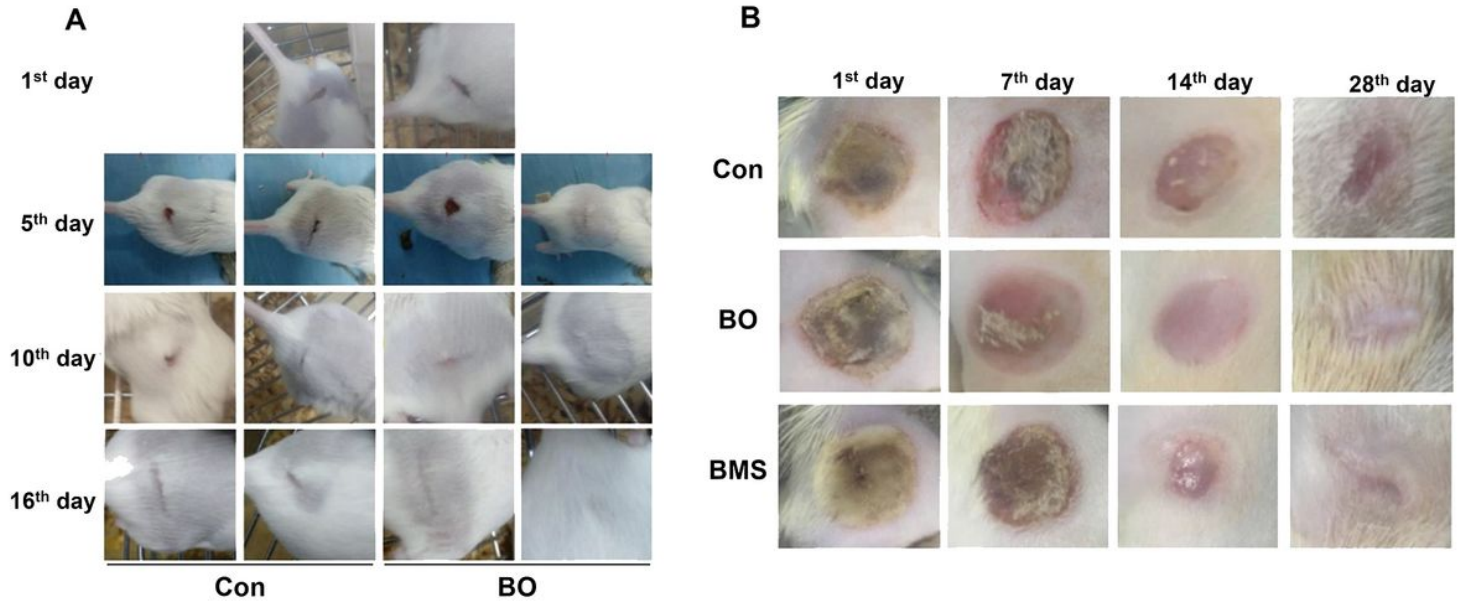

Figure 4

Pictures of wounds healing. (A)The pictures of wound healing in mice' knife wound, the healing of process was observed and photographed at 1st, 5th, 10th and 16th day. Control group (Con) and the treatment group (BO), which were coated 3 times a day for 16 days(n=30). (B)The pictures of the scald wound, the process of healing was observed and photographed at intervals of 7th, 14th and 28th day, and the negative control group (Con), the treatment group (BO) and the positive group burns moisturizing scald (BMS), who were coated 3 times a day for 28 days(n=35).

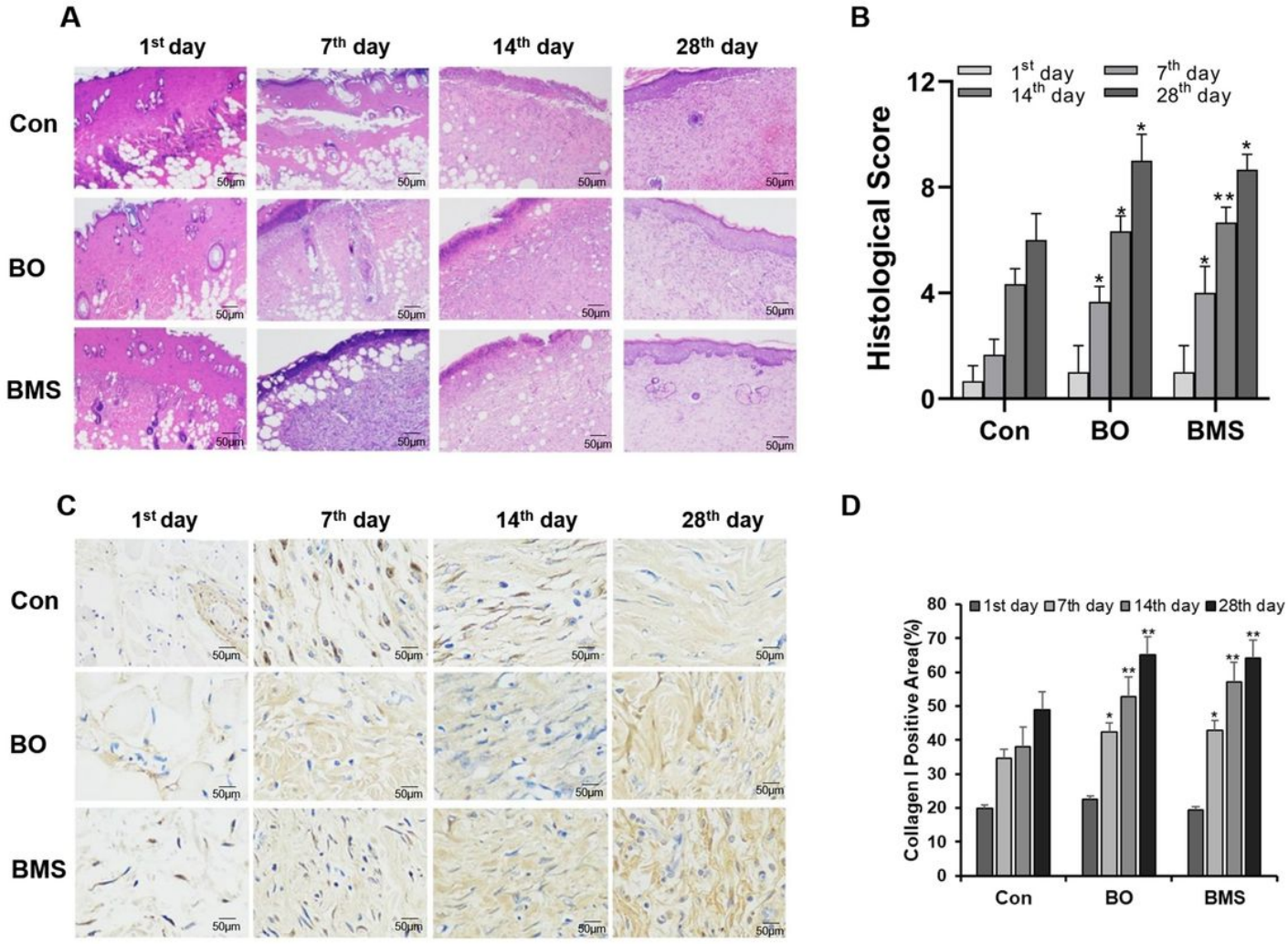

Figure 5

(A) H&E staining of scald wound in rats. Specimens were taken from the 1st, 7th, 14th and 28th day after modeling. The negative control group (Con), the treatment group (BO) and the positive group burns moisturizing scald (BMS), (n=35). (B) The results of histological score. Compare with Con group, BO group's wound was significantly healing on 28th day (*P<0.05), BMS group's wound was prominently healing on 14th day (*P<0.05, **P<0.01). (C) Immunohistochemistry of scald wound in rats. Specimens were taken from the 1st, 7th, 14th and 28th day after modeling. The negative control group (Con), the treatment group (BO) and the positive group burns moisturizing scald (BMS), (n=35). (D)The results of collagen I expression semi-quantitation, compared with the control group, BO could upregulate the collagen I of wound area markedly from the 7th day; *P<0.05, **P<0.01.

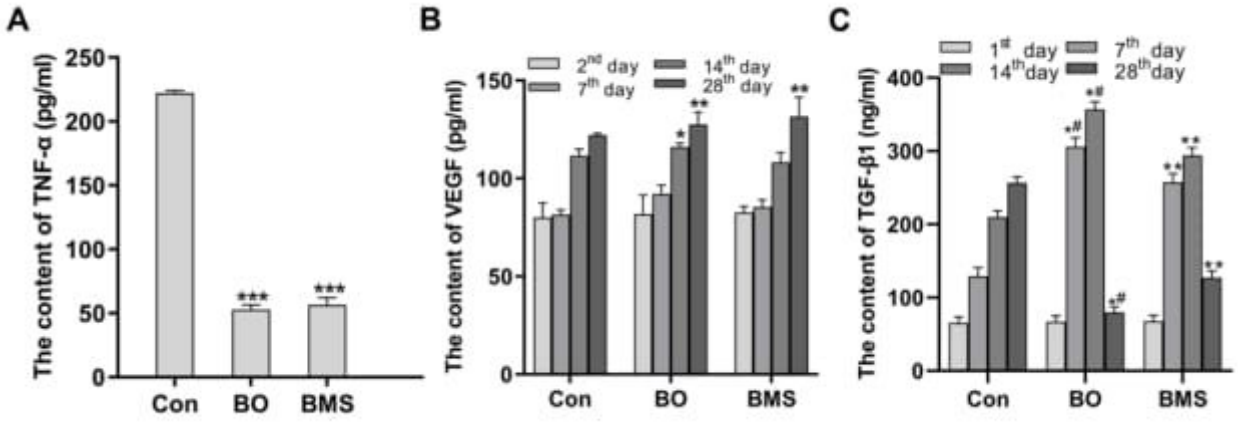

Figure 6

The content of Cytokines. (A) The content of TNF-α, the data are expressed as the mean ± SD (n=35), compared with the control group, *P<0.05, **P<0.01, ***P<0.001. (B) The content of VEGF, which was tested at 2nd, 7th, 14th, 28th days. The data were expressed as the mean ± SD (n=35), compared with the control group, *P<0.05, **P<0.01, ***P<0.001. (C) The content of TGF-β1, which was tested at 1st, 7th, 14th, 28th days. The data were expressed as the mean ± SD (n=35), compared with the control group, *P<0.05, **P<0.01; compared with the BMS group, #P<0.05, *#P<0.01.

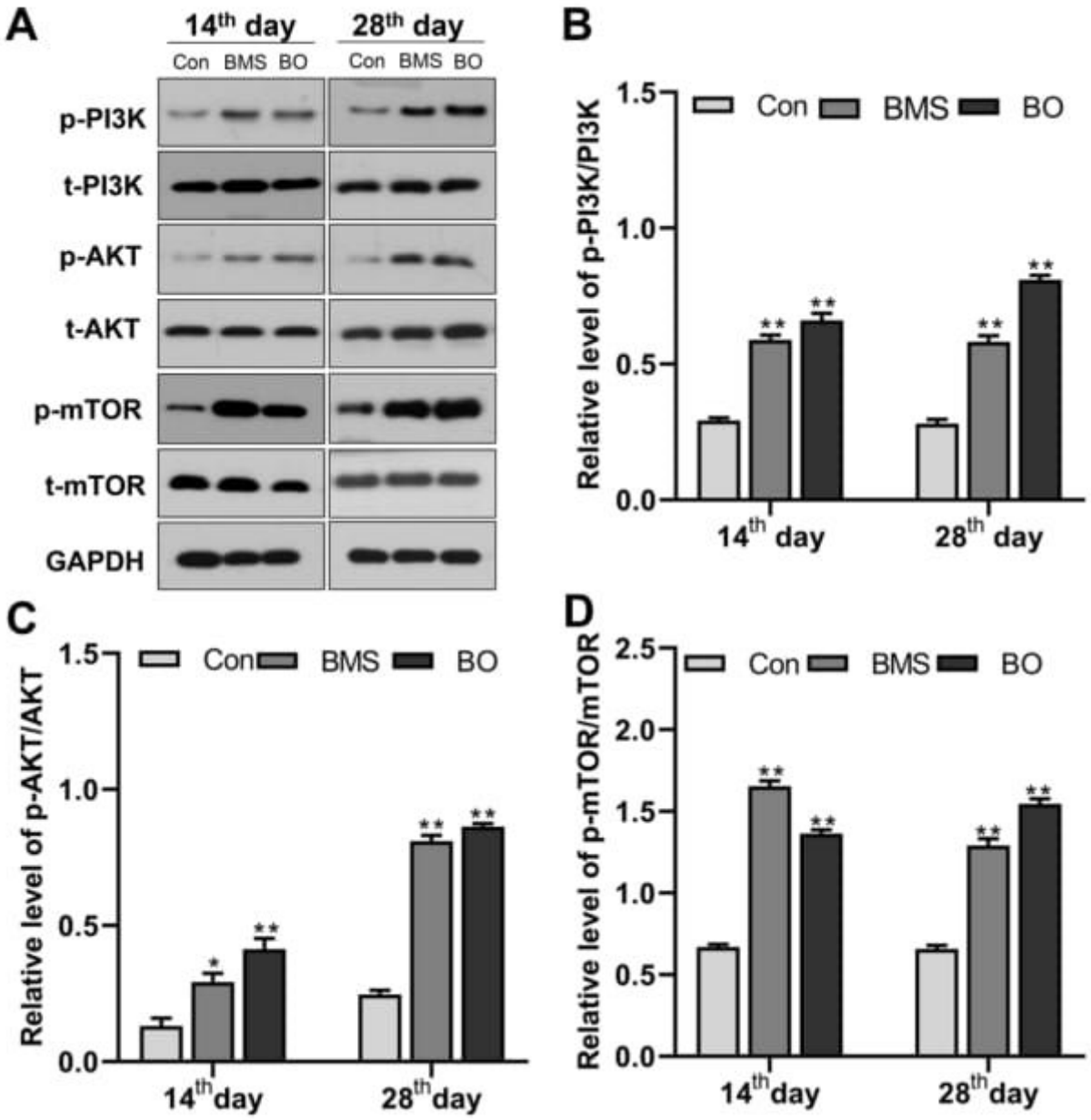

Figure 7

The PI3K/AKT/mTOR signaling pathway protein expression levels are shown as the mean grayscale value of the matching bands in the bar graph. (A) Representative bands of relevant proteins. Protein levels of (B) p-PI3K/t-PI3K, (C) p-AKT/t-AKT, (D)p-mTOR/t-mTOR. Data were presented as the mean ± SD of three experiments. Compared with the control group, *P<0.05, **P<0.01.