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Traditional Chinese medicine Zizhu ointment alleviates inflammatory activity during wound healing in diabetic rats

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Diabetic ulcers are a severe complication in patients with diabetes that often lead to amputation. The etiology of diabetes-induced wound healing impairment is multi-faceted. Persistent inflammation and deficient tissue regeneration have been suggested to impair wound healing in diabetes patients. Although new therapeutic approaches have been developed, many chronic wound treatments remain unsatisfactory, and more promising therapeutics are urgently needed. We thus developed diabetic wounding models with or without bacterial infection in rats that are optimized for testing a traditional Chinese medicine-Zizhu ointment. We observed increased proinflammatory markers and decreased angiogenesis in both non-infected and infected diabetic wounded rats. These changes were normalized by Zizhu ointment. Additionally, activation of Phosphatidylinositol-3-kinase (PI3K), c-Jun N-terminal kinase (JNK), and nuclear factor-kappa B (NF-κB) were observed in diabetic wounded rats, whereas Zizhu ointment significantly inhibited activation of these proteins. Our results suggest that Zizhu ointment promotes wound healing by inhibiting the inflammatory response.

Key words: Zizhu ointment, diabetic wounding, inflammation, angiogenesis, nuclear factor-kappa B (NF‑κB).

INTRODUCTION

Diabetes is a complex, chronic illness and has become a global public health problem associated with multifactorial risk-reduction strategies beyond glycemic control (Munhoz and Frode, 2018). Diabetes affects approximately 350 million people worldwide, with predictions that this number will triple by 2050 (Kuo et al., 2016). Diabetic ulcers are a major, persistent complication of diabetes, often leading to pain, suffering, and a poor quality of life for patients.

About 58% of ulcers become clinically infected (Prompers et al., 2007), often leading to amputation. A series of factors, including decreased cell and growth factor response as well as sustained chronic inflammation, lead to diminished peripheral blood flow and decreased local angiogenesis, all of which can contribute to lack of healing in persons with diabetic ulcers (Hardwicke et al., 2011; Han and Ceilley, 2017).

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Wound healing requires a well-orchestrated integration of molecular and cellular processes that recover damaged wound tissue into its normal state after injury. The molecular mechanism of wound healing is associated with inflammation, cell division, vascularization and tissue formation (Tan et al., 2019). Diabetic patients experience deficits in the precisely orchestrated course of events observed in normal healing, and bacterial colonization or infection further disrupts this process (Falanga, 2005). The mechanism underlying this impaired wound healing is complicated. One of the important causes is a sustained inflammatory response as well as postponed proliferation and remodeling periods (Landén et al., 2016). It is well documented that diabetes-associated impaired wound healing is partially due to increased proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α (Witte and Barbul, 1997; Barrientos et al., 2008; DeClue and Shornick, 2015). Some clinical evidence also shows that diabetic patients exhibit compromised cell proliferation, reduced angiogenesis, and reduced growth factors (Steed, 1995; Falanga, 2005).

Although the field of wound healing management is well established, the current treatment of chronic diabetic foot ulcers remains unsatisfactory. Therefore, more effective therapeutic approaches need to be established. Due to increasing multi-resistant bacteria and a slow development of new antibiotics, wound care professionals have turned to traditional medicine alternatives for wound healing treatments (Dorai, 2012). Traditional Chinese medicine has been developed more than 2000 years, including various forms of Chinese herbal medicine, diet therapy, mind/body exercises (Qigong and Tai Chi), and Tui Na (Farquhar, 2018). It has long been used in the Chinese culture to treat complex diseases such as diabetes mellitus. Many Chinese herbs have been used to treat diabetes mellitus and its complications and significantly promote diabetic wound healing (Lau et al., 2008, 2009; Hsiao et al., 2012; Wu et al., 2012).

It has been demonstrated that Zizhu ointment improves the cure rate of chronic skin ulcers of lower limbs in 72 medical cases (Lu et al., 2015). In our current study, we evaluate whether Zizhu ointment improves wound healing in diabetic rats with or without bacterial infection.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley (SD) rats were purchased from Charles River laboratory (Wilmington, MA) and kept in an animal colony with a 12 h light/12 h dark cycle with food as well as water *ad libitum*. 8-week-old male rats, weighing 150 to 250 g, were used in this study. The animal experiments followed the Guiding Principles for the Care and Use of Animals at the Shanghai University of Traditional Chinese Medicine.

Induction of diabetic rats

Diabetic rats were generated by 8-weeks high fat diet. Rats with plasma glucose >16.7 mmol/L were classified as diabetic models (group B). Water intake and weight were monitored throughout the study. To confirm the diabetic state, a fasting blood glucose measurement was repeated on the day of euthanasia.

Induction of wound animal

Rats were anesthetized by Isoflurane in biological hood (Southern Anesthesia and Surgical). The dorsal fur of the rat was shaved off and sprayed with 70% ethanol before wound formation. Non-diabetic or diabetic rats were burned with a hot metal plate (90 degree, 10 s/kg).

Diabetic wounding rats

Non-diabetic or diabetic rats were burned with a hot metal plate, named as wounded rats (group A) or diabetic wounded rats (group D). Different dosage of Zizhu ointment was applied daily on the wounded area for 2 weeks, beginning 1 day after the rats were wounded. D group rats were treated with vehicle, low, medium, high dose Zizhu ointment, and b-FGF Spray named as Z0, Z1, Z2, Z3 and C, respectively.

Diabetic wounding infectious rats

Diabetic rats were exposed to the same treatment as the previous diabetic wounded rats. Then, the diabetic wounded rats were inoculated with combined S. aureus or P. aeruginosa as previously described (Mendes et al., 2012) and named diabetic wounded infectious rats (group F). Group F rats were subgroup to four groups: GZ0, GZ1, GZ2, GZ3, and E treated with vehicle, low, medium, high dose Zizhu ointment and b-FGF Spray, respectively.

Zizhu ointment preparation

The main components of the Zizhu ointment consist of cinnabar, *Arnebiae radix*, *Sanguis draconis*, *Astragali radix*, *Colla corii asini*, and borneol. The solvent is made from white vaseline, lanolin, poloxamers, polyethylene glycol, propylene, glyceryl monostearate, ethyl 4-hydroxybenzoate, and water. All the raw herbs were obtained from Shanghai University of Traditional Chinese Medicine. All the herbs in the Zizhu ointment have been authenticated by the Shanghai University of Traditional Chinese Medicine (Shanghai, China). The preparation of Zizhu ointment followed the preparation technique of traditional Chinese ointment medicine. The entire process is carried out under bacteria-free environment. The mixed herbs were boiled into 75% ethanol then filtered all insoluble substance. The solvent components were boiled into water and cool down to room temperature. 50/25/12.5% of Zizhu solution (w/w) mixed with solvent as high/medium/low dose Zizhu ointment, respectively.

Immunochemistry

Wound tissue was collected after animals were euthanized with isoflurane exposure. Then, wound tissue samples were fixed in 4% paraformaldehyde (in PBS) at 4°C overnight. The tissue was then processed with a standard dehydration process in a battery of increasing ethanol concentrations for 1 day. The tissue was then embedded in paraffin in a container, then sliced with a histological microtome (Leica). Slices of 8 μm thick tissues were mounted onto slides and dried out on a heated plate overnight. The slides were then washed in deionized water, followed by PBS. To perform heat-

induced antigen retrieval, the slides were placed in Citrate 6 buffer (Sigma) at 94°C for 20 min. The slides were then washed in PBS and permeabilized in 0.2% Triton-X-100 in PBS for 10 min at room temperature. Subsequently, samples were blocked in 5% goat serum (Millipore) for 1 h at room temperature. Primary and secondary antibodies were diluted in blocking solution. Incubation in primary antibody CD34 (Abcam, ab81289) or VEGF (Santa Cruz, sc-7269) was performed overnight at 4°C, and in secondary antibodies (1:200) for 1 h at room temperature. Then samples were performed in ABC kit (Vectorlabs) and DAB (Vectorlabs) solution according to manufactory instruction.

Elisa

The concentration of inflammatory cytokines, including IL6, C - reactive protein (CRP) and NO in the wound tissue, was determined using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturers' protocols. Protein concentrations were measured and calculated with the help of the standard curve. The values were normalized to the total protein content determined by the Pierce BCA assay.

Western blot

Wound tissue samples were lysed in RIPA buffer (Sigma) including a complete/phosphatase stop cocktail (Millipore). Protein concentration was measured using the Pierce BCA assay (Thermo). The lysates (20 μg/lane) were then separated through 6 to 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The transferred membrane was incubated with blocking solution [5% milk (BD) in TBST] for 1 h at room temperature, followed by overnight incubation with a primary antibody at 4°C. The membrane was incubated with the HRP-conjugated secondary antibody (Millipore) for 1 h at room temperature. Immunoreactivity was detected with an ECL kit (Millipore). The optical density of the immunoreactivity bands was analyzed using ImageJ (NIH). Antibodies: p-JNK (Cell signaling, 4671S), JNK (Cell signaling, 9252S), p-P65 (Cell signaling, 3033S), P65 (Cell signaling, 6956S), p-PI3K (Cell signaling, 4228S), GAPDH (Cell signaling, 5174S), PI3K (Abcam, AB40776), and IL-6 (Abcam, AB9324).

RNA isolation and real-time PCR

Total RNA was isolated from wound tissue using PureLink RNA Mini Kit (Thermo fisher) according to the manufacturer's protocol. First-strand cDNA was synthesized using SuperScript® III First-Strand Synthesis System from Life Technologies. Real-time PCR was performed, using SYBR Green as an indicator, employing an ABI StepOne Plus Real-Time PCR system. PCR was carried out for 40 cycles, each of which consisted of a 15 s incubation at 95 and 60°C for 1 min. Fluorescence was read during the reaction, allowing a continuous monitoring of the amount of PCR product. The data were normalized using GAPDH mRNA. Primers were as follows: VEGF, forward 5'-CTGCTGCAATGATGAAGCCC-3, reverse 5'-TCCCGAAACCCTGAGGGAG-3'; E-cadherin, forward 5'-CGAAGGCCTAAGCACAACAGC-3', reverse 5'-CGGTGTACACAGCATTCCACG-3; GAPDH, forward 5'-TGCTGAGTATGTCGTGGAGT-3', reverse 5-GTCTTCTGAGTGGCAGTGAT-3'.

Statistical analysis

Data were expressed as mean ± Standard Error Mean (SEM). Results with $P < 0.05$ were considered statistically significant (*$P <$ 0.05, **$P < 0.01$, ***$P < 0.001$). All results were analyzed and graphed by using GraphPad Prism. Between-groups, differences were measured using the Student's t test. For multiple group comparisons, we used one-way analysis of variance, followed by Bonferroni's post hoc test.

RESULTS

Effect of Zizhu ointment on inflammatory responses

To determine inflammatory responses in diabetic non-infectious and infectious wounding rat models, we treated 3-month-old diabetic wounding rats with varying doses of Zizhu ointment or b-FGF Spray for 2 weeks (Figure 1). Wounding tissues were collected for various assays. Previous studies have shown that proinflammatory markers-C-reactive protein (CRP), interleukin 6 (IL-6), and proinflammatory mediator nitric oxide (NO)-were increased in diabetic wounding rats (Tripathi et al., 2007). Then, we examined the effects of Zizhu ointment on inflammatory markers by Elisa assays in diabetic wounding rat. We found that the levels of CRP, IL6, and NO were significantly increased in diabetic wounding rats (group D) compared to wounding rats (group A) or diabetic rats (group B) (Figure 2A). However, a significant reduction in CRP, IL6, and NO levels were observed at different doses of Zizhu ointment treatment as well as in the positive control treatment (b-FGF Spray, group C) compared to vehicle-treated diabetic wounding rats (group Z0) (Figure 2A).

Consistent with diabetic wounding rats, different doses of Zizhu ointment (group GZ1, GZ2, GZ3) and b-FGF Spray treatment (group E) significantly decreased the levels of CRP, IL6, and NO compared to vehicle treated diabetic infectious wounding rats (group GZ0) (Figure 2B).

Effect of Zizhu ointment on angiogenesis of wounding tissue

Angiogenesis plays an important role in wound healing and is associated with the expression of angiogenic factors, such as CD34 and VEGF (Li et al., 2005; Bitto et al., 2013). The rate of angiogenesis and proliferation of wounding tissues was measured by CD34, VEGF, and E-cadherin.

In diabetic non-infectious wounding rats, we found that different doses of Zizhu ointment treatment remarkably increased CD34 and VEGF expression in comparison to Z0 rats (Figure 3A and B). In addition, Z2 group had a higher CD34 and VEGF than Z1 and Z3 group (Figure 3A and B). We also measured the VEGF and E-Cadherin gene expression by RT-PCR. The expression of VEGF and E-Cadherin genes were decreased in D group more than groups A and B (Figure 3C). Zizhu ointment treated groups Z1, Z2 and b-FGF spray treated C group showed
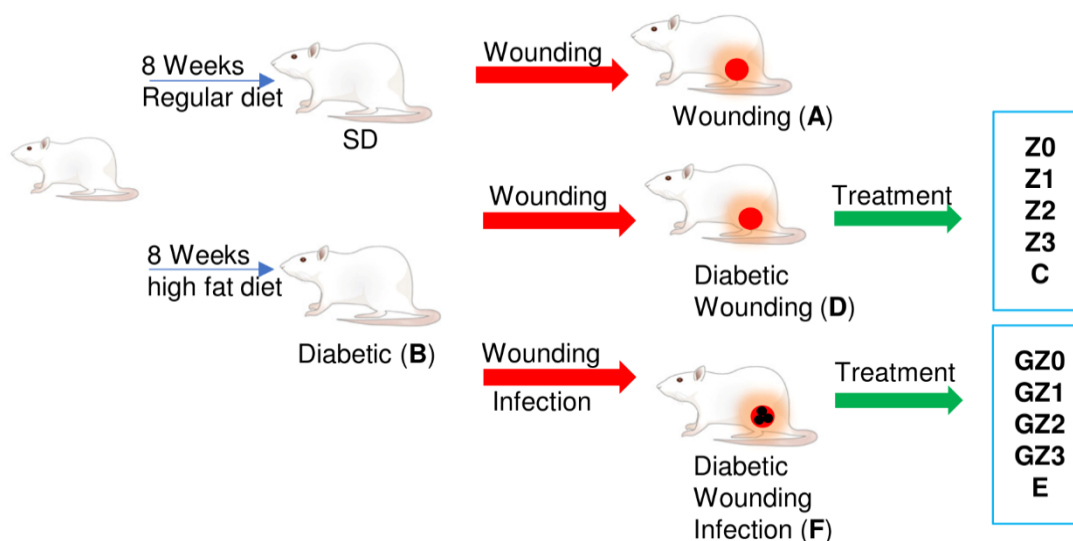
Figure 1. Experimental design for high-fat diet induces diabetic wounding rats with Zizhu ointment treatment. A, wounding rats; B, diabetic rats; D, diabetic wounding rats; Z0, Z1 Z2, Z3 and C as vehicle, low dose, medium dose, high dose Zizhu ointment or b-FGF spray treated wounding diabetic rats, respectively; F, diabetic infected wounding rats; GZ0, GZ1, GZ2, GZ3 and E as vehicle, low dose, medium dose, high dose Zizhu ointment or b-FGF spray treated diabetic infected, wounding rats.
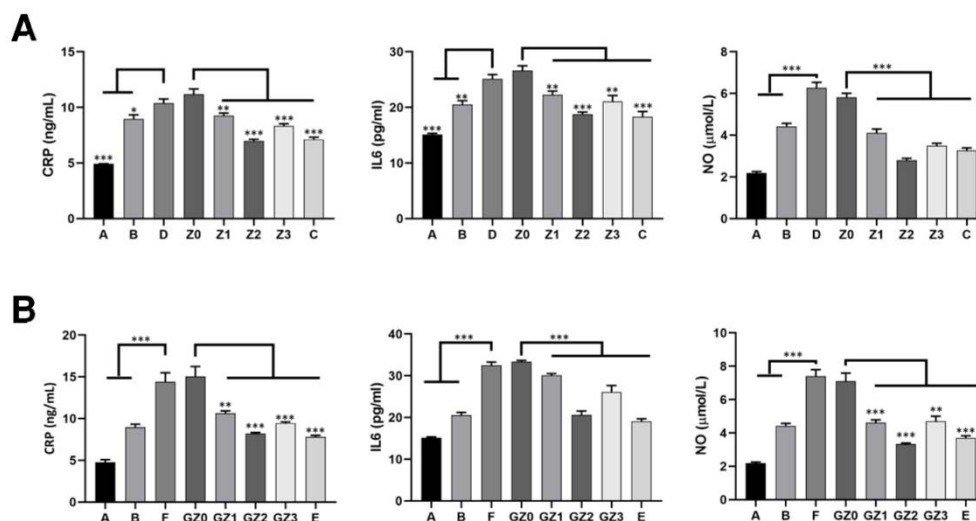

Figure 2. Effect of Zizhu ointment on proinflammatory responses on diabetic wounding rats. (A) Production of pro-inflammatory cytokines such as CRP, IL6 and NO in Zizhu oinitment treated diabetic wounding rats. (B) Production of pro-inflammatory cytokines such as CRP, IL6 and NO in Zizhu ointment treated, diabetic wounding, infected rats.

increased VEGF mRNA expression compared to group Z0. In contrast, only groups Z1 and C showed upregulated E-cadherin mRNA level compared to Z0 (Figure 3C).

In diabetic infectious wounding rats, medium dose Zizhu ointment treated group GZ2 exhibited significantly increased CD34 and VEGF expression in comparison to vehicle treated group GZ0 (Figure 3D and E). Diabetic

infectious wounding group F also showed decreased VEGF and E-cadherin gene expression when compared with groups A and B (Figure 3F). Different dose Zizhu ointment treated GZ1, GZ2, and GZ3 groups and b-FGF Spray treated group F showed increased VEGF and E-cadherin gene expression than vehicle treated GZ0 group (Figure 3F).
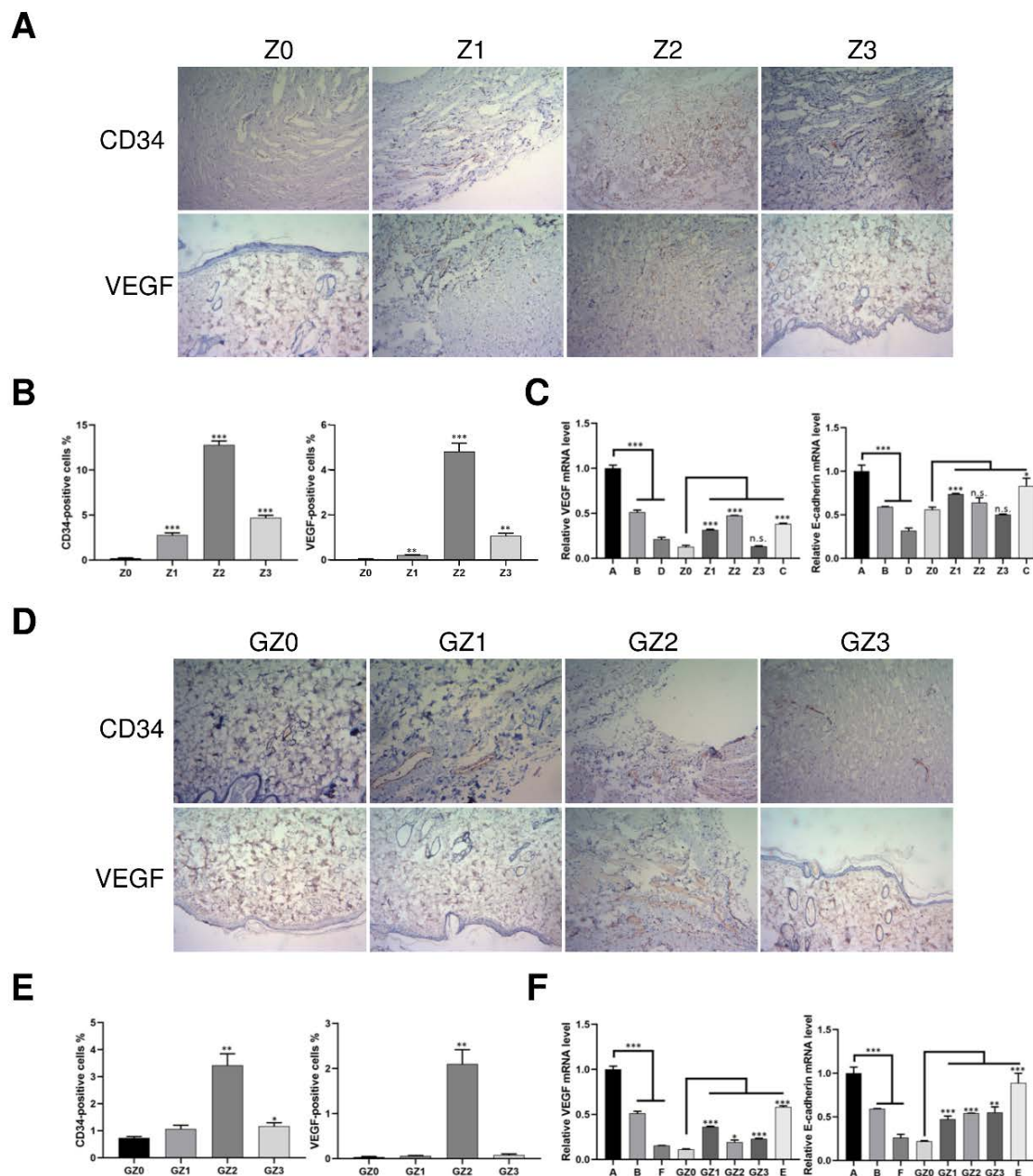
Figure 3. Evaluation of angiogenesis with the expression of VEGF and CD34 in wound tissue. (A-C) In diabetic wounding rats, CD34 (upper) and VEGF (lower) expression in wound tissue (A). Quantification of CD34 (left) and VEGF (right) expression levels (B). The relative mRNA levels of E-cadherin and VEGF genes were determined by real-time PCR (C). (D-F) In diabetic wounding infectious rats, CD34 (upper) and VEGF (lower) expression in wound tissue (D). Quantification of CD34 (left) and VEGF (right) expression levels (E). The relative mRNA levels of E-cadherin and VEGF genes were determined by real-time PCR (F).

Effect of Zizhu ointment on inflammatory pathways

Previous studies reported that NF-κB, JNK and PI3K proteins were increased in wound tissues from diabetic individuals (Zhu et al., 2012; Sakamoto et al., 2016; Wang et al., 2016). Therefore, we next investigated whether NF-κB, JNK, and PI3K pathways were involved in the effects of Zizhu ointment regulating diabetic wound healing.

As expected, increased p-PI3K/PI3K, p-P65/P65, and p-JNK/JNK were observed in diabetic wounding group D more than in diabetic group A or wounding group B (Figure 4A and B). Intriguingly, different doses of the Zizhu ointment significantly inhibited activation of PI3K,
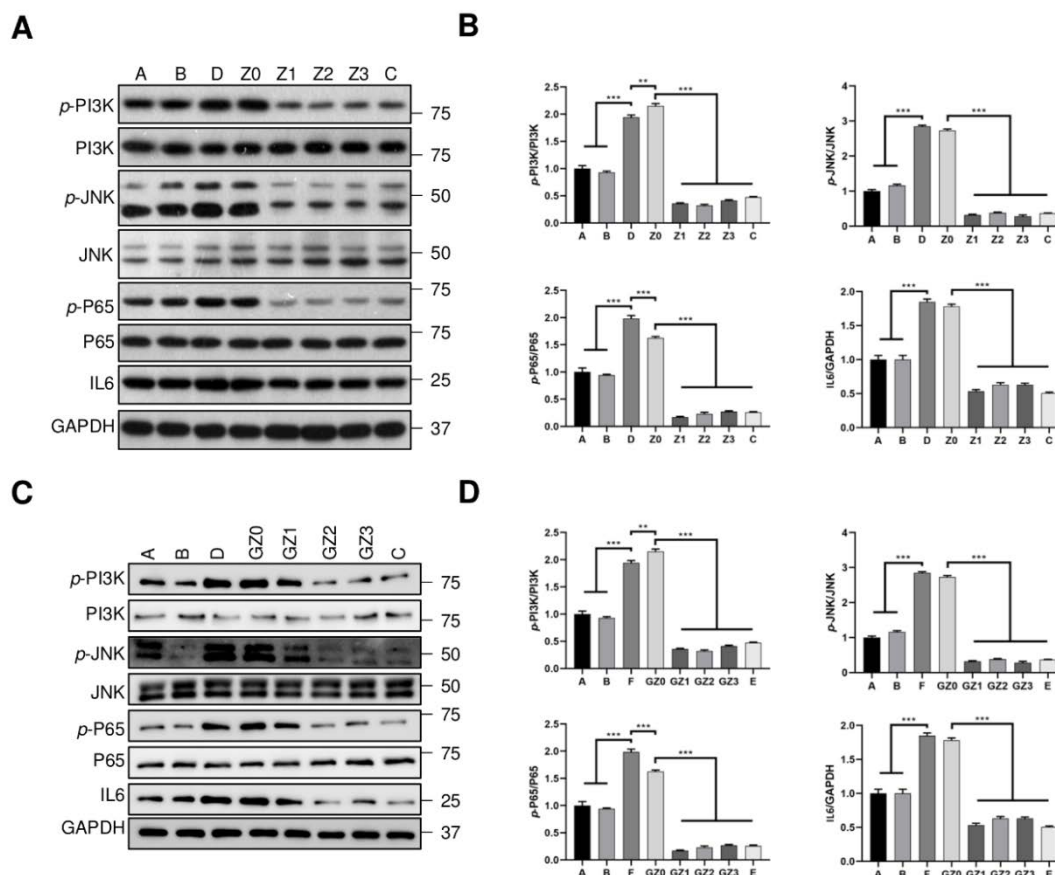
Figure 4. Effect of Zizhu ointment on inflammatory pathways in diabetic wounding rats. (A-B) Wound tissue lysates of diabetic wounding rats were subjected to immunoblot analysis using specific antibodies (A). Quantification of PI3K, JNK, p65 and IL6 proteins in wound tissue (B). (C-D) Wound tissue lysates of diabetic wounding infectious rats were subjected to immunoblot analysis using specific antibodies (C). Quantification of PI3K, JNK, p65 and IL6 proteins in wound tissue (D).

P65, and JNK in diabetic wounding tissues (Figure 4A and B). We also found that IL6 protein level was decreased in different doses of Zizhu ointment treated rats than vehicle treated group Z0 (Figure 4A and B).

In diabetic infectious wounded rats, p-PI3K/PI3K, p-P65/P65, and p-JNK/JNK levels were increased in group F more than in diabetic A group or wounded B group (Figure 4C and D). Different dose Zizhu ointment treated GZ1, GZ2, and GZ3 groups showed significantly decreased activation of PI3K, P65, and JNK in diabetic infectious wounded tissues (Figure 4C and D). IL6 protein level was also decreased in different dose Zizhu ointment treated rats than vehicle treated rats (Figure 4C and D).

DISCUSSION

In this study, it was found that Zizhu ointment increases angiogenesis and decreases the expression of proinflammatory genes in wounding tissues. We further found that Zizhu ointment improves wound healing through inhibiting inflammation-related PI3K, JNK and NF-κB activation. These data suggest a potential role for Zizhu ointment as a new wound dressing for treating the wounds of diabetic patients.

Diabetic foot ulcers affect 15% of patients with diabetes and are a leading cause of amputation (Peppa et al., 2009). Diabetic foot ulcer is a complicated clinical problem which cannot be rescued by a single method or standard drug. Currently, the FDA has only approved growth factor and cell therapies for diabetic foot ulcer which are not routinely used in clinical treatment. Topical application of growth factors or cytokines directly to stimulate diabetic wound healing has been explored to no avail as a result of the fast diffusion and drying of the drug spray in the open wound. Evidently, efforts to explore new methods and strategies are urgently needed in dealing with diabetic wound healing. Traditional Chinese medicine has been utilized clinically to treat various forms of trauma and to help wound healing.

Traditional Chinese medicine has been reported to have multiple functions, including the regulation of wound healing (Ye et al., 2003), regulation of the immune system (Zhao et al., 2003), antioxidant effects (Kuang et al., 2006), and anti-inflammatory effects (Yang et al., 2007). According to this evidence, we developed a new compound formula, consisting of cinnabar, *Arnebiae radix*, *Sanguis draconis*, *Astragali radix*, *Colla corii asini*, and borneol. Furthermore, we previously showed that Zizhu ointment promotes wound healing in 72 clinical cases (Lu et al., 2015). To explore the cellular mechanisms for the promotion of wound healing by Zizhu ointment, we developed diabetic wounded rats and diabetic, infected and wounded rats and examined the related pathways.

Wound healing is a complex biophysiological process that involves cell proliferation, angiogenesis, inflammation, and wound closure and remodeling (Peppa et al., 2009; Lau et al., 2012). Wounds trigger the acute inflammatory response, in which neutrophils, monocytes and mast cells infiltrate to the site of injury and produce cytokines (Trautmann et al., 2000). They are responsible for removing non-functional host cells and bacteria. Further, they coordinate repair through production of a broad spectrum of factors that influence angiogenesis, fibroplasias and extracellular matrix synthesis (DiPietro, 1995). In the final stage of wound healing, extracellular matrix remodeling, fibroblast proliferation, angiogenesis and re-epithelialization are responsible for wound closure and scar formation (Clark, 2001). Impaired wound healing arises from abnormal inflammatory response (Acosta et al., 2008), decreased angiogenesis (Duraisamy et al., 2001), and insufficient fibroblast proliferation (Hehenberger et al., 1998). In the present study, therefore, we explore the effects of Zizhu ointment on inflammatory response, angiogenesis and cell proliferation.

Appropriate production of proinflammatory cytokines is important for recruiting neutrophils and removing bacteria and other infections from the wound area (Hantash et al., 2008). On the other hand, sustained expression of cytokines is harmful since it may result in the progression of chronic wounds. The levels of CRP, IL6, and NO were measured in the wounds of diabetic or diabetic and infected rats in this study. It was found that all three markers were significantly increased in the wound tissue of diabetic and diabetic infected animals than in the wound tissue of normal rats. Limited production of CRP, IL6 and NO can improve wound healing but excessive production will result in the formation of ulcers (Pasche et al., 2008; McFarland-Mancini et al., 2010; Tan et al., 2019). Here, Zizhu ointment can significantly reduce the concentration of all three markers in a dose-dependent manner. These markers not only act as a mediator in the inflammatory cell, but they also play an important role in modulating cell proliferation, angiogenesis, and wound healing.

Angiogenesis is an essential process in the progression of wound healing and the most potent angiogenic factor is the vascular endothelial growth factor (VEGF) (Lee et al., 2009). On the other hand, the CD34 is an endothelial antigen that has been used to highlight the microvasculature vessel density as a direct marker for the degree of angiogenesis (Caiado et al., 2011). Our data indicates that upregulation of angiogenic factors such as VEGF and CD34 were also observed in the wounded tissue lysate from diabetic rats and diabetic infected rats following treatment with Zizhu ointment. In this study, Zizhu ointment accelerated diabetic or diabetic infectious wound healing through the VEGF signaling pathways. During wound-induced inflammation, NF-κB nuclear translocation and JNK phosphorylation are considered to play a critical role (Rämet et al., 2002; Heng, 2011). To investigate the mechanism of the inhibitory effect of Zizhu ointment on the inflammatory response further, we analyzed changes in the activation of the NF-κB and JNK pathways. In the present study, diabetic or diabetic wound-inflicted rats displayed activated JNK phosphorylation and NF-κB pathway. Zizhu ointment significantly inhibited this activation of JNK and NF-κB pathway in diabetic or diabetic infected models. These findings suggest that Zizhu ointment administration, in particular, inhibits the JNK and NF-κB dependent inflammatory response, and represents a promising approach for protecting wounds against excessive inflammation.

This study demonstrates that traditional Chinese medicine-Zizhu ointment plays an important role in wound healing in diabetic rats with or without bacterial infection. Further, our data also indicates that the wound healing effects of Zizhu ointment might be due to the regulation and coordination of inflammation, angiogenesis and tissue regeneration. This study provided us with scientific evidence that the traditional Chinese medicine-Zizhu ointment is a promising complementary supplement for diabetic patients with wound healing defects.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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