MEDICAL SCIENCE MONITOR

LAB/IN VITRO RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2019; 25: 9446-9457
DOI: 10.12659/MSM.918268

Received: 2019.06.23
Accepted: 2019.08.01
Published: 2019.12.11

Shixiang Plaster, a Traditional Chinese Medicine, Promotes Healing in a Rat Model of Diabetic Ulcer Through the receptor for Advanced Glycation End Products (RAGE)/Nuclear Factor kappa B (NF-κB) and Vascular Endothelial Growth Factor (VEGF)/Vascular Cell Adhesion Molecule-1 (VCAM-1)/Endothelial Nitric Oxide Synthase (eNOS) Signaling Pathways

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF 1 Ji Fei*
BCDF 2 Yi-Ming Ling*
BCF 1 Man-Jie Zeng
ACDEG 1 Kai-Wei Zhang

1 Department of Orthopedics, The First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine, Guiyang, Guizhou, P.R. China
2 Department of Orthopedics, Shaoxing Hospital of Traditional Chinese Medicine, Shaoxing, Zhejiang, P.R. China

* Ji Fei and Yi-Ming Ling contributed equally to this study

Corresponding Author: Kai-Wei Zhang, e-mail: zkw1973@aliyun.com

Source of support: This study was funded by Guizhou Province Science and Technology Cooperation Program and Youth Science and Technology Talents Growth Project of Guizhou Provincial Department of Education

Background: Shixiang plaster is a traditional Chinese medicine has been used to treat chronic ulcers, including diabetic ulcers. Aminoguanidine is a hydrazine derivative that inhibits the formation of advanced glycosylation end products (AGEs). This study aimed to investigate the effects of shixiang plaster and aminoguanidine on wound healing in the streptozotocin-induced rat model of diabetes and the molecular mechanisms involved.

Material/Methods: Sprague-Dawley rats treated with intraperitoneal streptozotocin and given surgical wounds were divided into the untreated chronic ulcer group (n=10), the aminoguanidine group (n=10), the shixiang plaster group (n=10), and the control group with sham surgery (n=10). Granulation tissue samples underwent light microscopy to evaluate angiogenesis and immunohistochemistry to identify AGE, vascular endothelial growth factor (VEGF), and CD34 expression. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot measured mRNA and protein expression of receptor for advanced glycation end products (RAGE), vascular cell adhesion molecule-1 (VCAM-1), nuclear factor kappa B (NF-κB) and endothelial nitric oxide synthase (eNOS).

Results: The shixiang plaster group showed a significant increase in angiogenesis in ulcer granulation tissue, significantly reduced expression of AGEs and increased expression of VEGF and CD34 expression in granulation tissue compared with the untreated chronic ulcer group ($p<0.05$). The shixiang plaster group showed significantly down-regulated expression of RAGE and VCAM-1 compared with the untreated chronic ulcer group ($p<0.05$). Shixiang plaster promoted angiogenesis by activating the NF-κB p65 associated pathway and eNOS activation.

Conclusions: Shixiang plaster promoted healing in a rat model of diabetic ulcer through the RAGE/NF-κB and VEGF/VCAM-1/eNOS signaling pathways.

MeSH Keywords: Diabetic Angiopathies • Neovascularization, Physiologic • Wound Healing • Wounds and Injuries

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/918268

3686 1 6 49

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

Background

Worldwide, type 1 and type 2 diabetes mellitus (DM) is an increasingly prevalent public health problem that results in patient morbidity and mortality [1,2]. In 2014, it was estimated that there were approximately 425 million people aged between 20–79 years who suffered from diabetes mellitus, and the global prevalence was estimated to reach 592 million by the year 2035 [3]. DM is the tenth most common causes of mortality, which is designated by the World Health Organisation (WHO) to be a chronic metabolic disorder caused by an acquired or genetic deficiency in insulin [4]. DM results in damage to all major organ systems, particularly the nervous system and vascular system and results in microvascular diseases that include retinopathy, neuropathy, and nephropathy [5].

Delayed healing of skin ulcers is a complication of DM that is caused by diabetic microvascular disease and results in the failure of complete healing of skin wounds resulting in chronic ulcers, particularly in the lower limb [6]. Wound healing, or tissue repair, is a complex physiological process and involves several phases that include inflammation, cell proliferation, fibrosis, and remodeling that all depend on angiogenesis, which is the hallmark of granulation tissue [7]. The inflammatory phase and the proliferation phase form granulation tissue and trigger re-epithelialization, and the remodeling phase involves cells and non-cellular components of connective tissue to support newly synthesized epithelium. These phases of tissue repair have been studied and targeted to accelerate the healing of chronic ulcers.

Schafer et al. [8] reported that increased levels of reactive oxygen species (ROS) were associated with delayed wound healing. Upregulation of Toll-like receptor 4 (TLR4) also contributes to the healing of chronic wounds in hyperglycemic animal models [9]. Adjunctive treatments for skin ulcers include extracellular matrix (ECM) proteins, the use of negative-pressure wound therapy (NPWT), bio-engineered skin substitutes, growth factors, the use of stem cells and gene therapy, angiotensin receptor analogs, and inhibitors of inflammatory cytokines [10]. However, chronic ulcers develop from a lack of blood supply, and healing can be difficult when tissue ischemia is present.

Studies have shown that wound healing is dependent on the degree of tissue hydration, which is reduced with increasing degrees of fibrosis found in chronic wounds [11]. Advanced glycation end products (AGEs) have been shown to have a role in the occurrence and development of diabetic ulcers [12]. Aminoguanidine is a hydrazine derivative that inhibits the formation of advanced glycosylation end products (AGEs) and suppresses inflammation in diabetic skin wounds [13].

Although treatments used in Western medicine are used to control blood glucose levels in patients with diabetes mellitus, treatments for non-healing chronic diabetic ulcers remain a challenge [14]. In traditional Chinese medicine theory, the drainage of wounds and removal of necrotic tissue are recommended. Shixiang plaster is a traditional Chinese medicine that contains calamine, frankincense, *Halloysitum rubrum*, calcined bone, and borneol [15]. In a previously reported clinical study, we found that shixiang plaster could significantly improve wound healing in patients suffering from chronic ulcers, but the specific mechanisms involved were not studied [16].

Therefore, this study aimed to investigate the effects of shixiang plaster and aminoguanidine on wound healing in the streptozotocin-induced rat model of diabetes and the molecular mechanisms involved.

Material and Methods

Animals

Specific pathogen-free (SPF) male Sprague-Dawley rats weighing 300–350 were obtained from the Experimental Animal Center of Guizhou Medical University, Guiyang, China (License No. SCXK 2012-0001). The rats had free access to food and water. The rats were housed at a temperature of 25±5°C, with a humidity of 50±5% and a 12-hourly light and dark cycle. The animal experiments were conducted and approved by the Ethical Committee of The First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine, Guiyang, China.

The rat model of diabetic ulcer

Before the rat model of diabetes was prepared, the rats fasted for 18 h and had free access to water. Rats (n=30) were in the diabetes model were given a single intraperitoneal injection of streptozotocin, at a dose of 65 mg/kg body weight in 0.1 mmol/L sodium citrate pH 4.0 (Sigma-Aldrich, St. Louis, MO, USA). Rats that achieved random blood glucose levels of ≥16.7 mmol/L or fasting blood glucose levels of ≥11.1 mmol/L, were included in the diabetes model. Rats in the normal group were injected intraperitoneally with 0.1 mmol/L of sodium citrate (n=10). In this study, the rat model of diabetes was achieved and maintained during the study period.

Rats were anesthetized by intraperitoneal injection with ketamine hydrochloride (100 mg/kg) and an area of hair measuring 3×3 cm on the back of the rats was removed using a depilatory cream before surgery. Following depilation, the hairs surrounding the skin wounds were dried using 75% alcohol to prevent infection. In the center of the exposed skin, a round surgical wound measuring 5×5 mm was created using a skin

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

biopsy punch, and the full skin layers were removed using ophthalmic scissors to establish the chronic ulcers in the rat model.

Drug preparation, study groups, and treatment

Preparation of shixiang plaster included calamine (500 g), frankincense (150 g), myrrh (150 g), *Halloysitum rubrum* (150 g), *Fructus gardeniae* (150 g), calcined bone (150 g), and borneol (150 g), which were dissolved and mixed in 2 L of heated sesame oil to form a pastes. The paste was mixed with 200 g of beeswax to synthesize the shixiang plaster.

Preparation of aminoguanidine included stearic acid, liquid paraffin, vaseline, isopropyl ester, glycerol, nipagin oil, and aminoguanidine, which were mixed to form a cream. Following surgery to create the skin wound, the diabetic rats in the chronic ulcer model were divided into three groups, that included the chronic ulcer group (n=10), the aminoguanidine group (n=10), and the shixiang plaster group (n=10). The rats in the chronic ulcer group and the control group were treated with topical application of stearic acid, liquid paraffin, vaseline, isopropyl ester, glycerol, and nipagin oil, without aminoguanidine. The rats in the shixiang plaster group were treated by topical application of shixiang plaster at a thickness of 2 mm over the wound. The rats in the aminoguanidine group were treated by topical application of aminoguanidine cream at a thickness of 2 mm.

Sample preparation

At day 7 and day 14 following topical treatment of the skin wounds, the rats in each group were anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg). At the end of the study, the granulation tissues from the skin ulcers were removed and fixed in 10% formaldehyde solution (Sigma-Aldrich, St. Louis, MO, USA) and paraffin-embedded for sectioning for light microscopy. Fresh tissues were also sampled and stored at –70°C for molecular analysis.

Immunohistochemistry

The paraffin-embedded rat skin granulation tissues were sectioned at 4 µm onto glass slides. The tissue sections were dewaxed and rehydrated in graded ethanol. Endogenous peroxidase was blocked in 3% hydrogen peroxide (Beyotime Biotech., Shanghai, China) for 10 min at 37°C. Non-specific antibody binding was blocked with normal goat serum (Hyclone, Logan, UT, USA) at room temperature for 15 min. The tissue sections were incubated at 4°C overnight with the primary antibodies. The primary antibodies were incubated on the tissue sections overnight at 4°C and included rabbit anti-rat advanced glycosylation end products (AGEs) polyclonal antibody (1: 2000) (Cat. No. ab23722) (Abcam, Cambridge, MA, USA), rabbit anti-rat vascular endothelial growth factor (VEGF) polyclonal antibody (1: 2000) (Cat. No. ab53465) (Abcam, Cambridge, MA, USA), rabbit anti-rat CD34 monoclonal antibody (1: 3000) (Cat. No. ab185732) (Abcam, Cambridge, MA, USA). The tissue sections were washed with PBS and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1: 1000) (Cat. No. ab6721) (Abcam, Cambridge, MA, USA) at 37°C for 1 h. The tissues were then stained with DAB and hematoxylin for 3 min, differentiated with 0.1% alcohol hydrochloride for 3 min, and dehydrated with graded alcohols for 3 min. Tissue sections were counterstained with hematoxylin, mounted, and coverslipped. The immunostained tissue sections were evaluated by light microscopy.

Histology

The tissue sections were stained histochemically using hematoxylin and eosin (H&E) (Beyotime Biotech., Shanghai, China) and were examined by light microscopy, as previously described [17]. Photomicrographs of the tissue sections were taken using a light microscope (Olympus, Tokyo, Japan) at a magnification of ×400.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs of granulation tissues that had been stored fresh at –70°C were extracted using TRIzol reagent (Beyotime Biotech., Shanghai, China), according to the manufacturer's instructions. Complementary DNAs (cDNAs) were synthesized using an RNA transcription kit (Western Biotech., Chongqing, China). The qRT-PCR assay was performed using a SYBR Green I PCR amplification kit (Western Biotech., Chongqing, China), based on the synthesized cDNAs. The primers used for receptor for advanced glycation end products (RAGE), vascular cell adhesion molecule-1 (VCAM-1), nuclear factor kappa B (NF-κB) and endothelial nitric oxide synthase (eNOS) are shown in Table 1. The qRT-PCR assay was performed using the FTC-3000P Real-Time Fluorescence PCR system (Funglyn BioTech. Inc., Toronto, Canada). Gene expression was calculated using the $2^{-\Delta Ct\ (2\text{-}[(Ct\ of\ gene)\ -\ (Ct\ of\ U6)])}$ method [18].

Western blot

Granulation tissues were lysed using RIPA buffer, and the harvested lysates were centrifuged at 10000×g for 10 min to obtain the protein-containing supernatants. The proteins were then separated using 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Amresco Inc., Solon, OH, USA) and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA) with the Trans-Blot SD cell instrument (Bio-Rad Laboratories). PVDF membranes were treated with rabbit anti-rat RAGE monoclonal antibody

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

Table 1. Primers used in the polymerase chain reaction (PCR) assay.

Gene		Sequences	Length (base pairs)
RAGE	Forward	5'-GGGACTCTTCACGCTTCGG-3'	187
	Reverse	5'-ACCTTCAGGCTCAACCAACAG-3'	
VCAM-1	Forward	5'-GAACCCAAACAAAGGCAGAG-3	136
	Reverse	5'-GAAAACCATCACTTGAGCAGG-3	
NF-κBp65	Forward	5'-TCTGTTTCCCCTCATCTTTCC-3	168
	Reverse	5'-GGGTGCGTCTTAGTGGTATCTG-3	
eNOS	Forward	5'-GCAACAAACCGAGGCAATC-3	189
	Reverse	5'-GGTCCAGCCATGTTGAATACAG-3	
β-actin	Forward	5'-CCCATCTATGAGGGTTACGC-3	150
	Reverse	5'-TTTAATGTCACGCACGATTTC-3	

(1: 3000) (Cat. No. ab228861) (Abcam, Cambridge, MA, USA), rabbit anti-rat VCAM-1 monoclonal antibody (1: 3000) (Cat. No. ab134047) (Abcam, Cambridge, MA, USA), rabbit anti-rat NF-κB p65 polyclonal antibody (1: 2000) (Cat. No. ab16502) (Abcam, Cambridge, MA, USA), rabbit anti-rat phosphorylated NF-κB p65 (p-NF-κB p65) polyclonal antibody (1: 2000) (Cat. No. ab86299) (Abcam, Cambridge, MA, USA), rabbit anti-rat eNOS (1: 2000) (Cat. No. ab199956) (Abcam, Cambridge, MA, USA), and rabbit anti-rat β-actin monoclonal antibody (1: 3000) (Cat. No. ab179467) (Abcam, Cambridge, MA, USA) at 4°C overnight. PVDF membranes were then continuously incubated using HRP-labeled goat anti-rabbit IgG (1: 2000) (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 1 h. Western blot bands were imaged using an ECL kit (Thermo Scientific Pierce, Rockford, IL, USA) for 2 min in the dark. The relative grey density for bands was calculated using the LabworksTM Analysis Software (LabWorks, Upland, CA, USA).

Statistical analysis

Data were presented as the mean±standard deviation (SD) and analyzed with the SPSS version 19.0 software (SPSS Inc., Chicago, IL, USA). Tukey's post hoc analysis of variance (ANOVA) test was used to compare differences between multiple groups. Student's t-test was used to compare differences between two groups. Statistical significance was represented by $p<0.05$.

Results

Shixiang plaster promoted angiogenesis in granulation tissue of ulcers in the rat model

Angiogenesis in the granulation tissue of healing skin ulcers was evaluated by light microscopy (Figure 1A). The degree of angiogenesis in the granulation tissue of the normal ulcer group was significantly less than in the chronic ulcer group (Figure 1B; $p<0.05$) at day 7 and day 14 post-surgery. Treatment with aminoguanidine and shixiang plaster significantly increased angiogenesis compared with the chronic ulcer group at day 7 and day 14 post-surgery (Figure 1B, $p<0.05$). However, at day 7 post-surgery, shixiang plaster demonstrated relative more angiogenesis compared with the aminoguanidine group (Figure 1B; $p<0.05$).

Shixiang plaster reduced the levels of advanced glycosylation end products (AGEs) in granulation tissue of ulcers in the rat model

In this study, immunohistochemistry was used to examine the expression of AGE2 in granulation tissue of the rat model with chronic skin ulcers (Figure 2A). The results showed that AGE levels in the chronic ulcer group were significantly increased compared with the normal ulcer group (Figure 2B; $p<0.05$) at day 7 and day 14. However, treatment with both aminoguanidine and shixiang plaster significantly reduced the levels of AGE compared with the chronic ulcer group (Figure 2B; $p<0.05$) at day 7 and day 14. Treatment with shixiang plaster resulted in significantly lower levels of AGE compared with the aminoguanidine group (Figure 2B; $p<0.05$), which suggested that shixiang plaster had a greater effect on the expression of AGE.

Treatment with shixiang plaster and vascular endothelial growth factor (VEGF) and CD34 expression in granulation tissue of ulcers in the rat model

Previous studies have shown that VEGF and CD34 participate in new vessel formation in tissue repair [19,20]. In this study, VEGF and CD34 were detected in granulation tissue of chronic ulcers using immunohistochemistry (Figure 3). The results showed that expression levels of both VEGF (Figure 3A) and CD34 (Figure 3B) in the chronic ulcer group were significantly

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

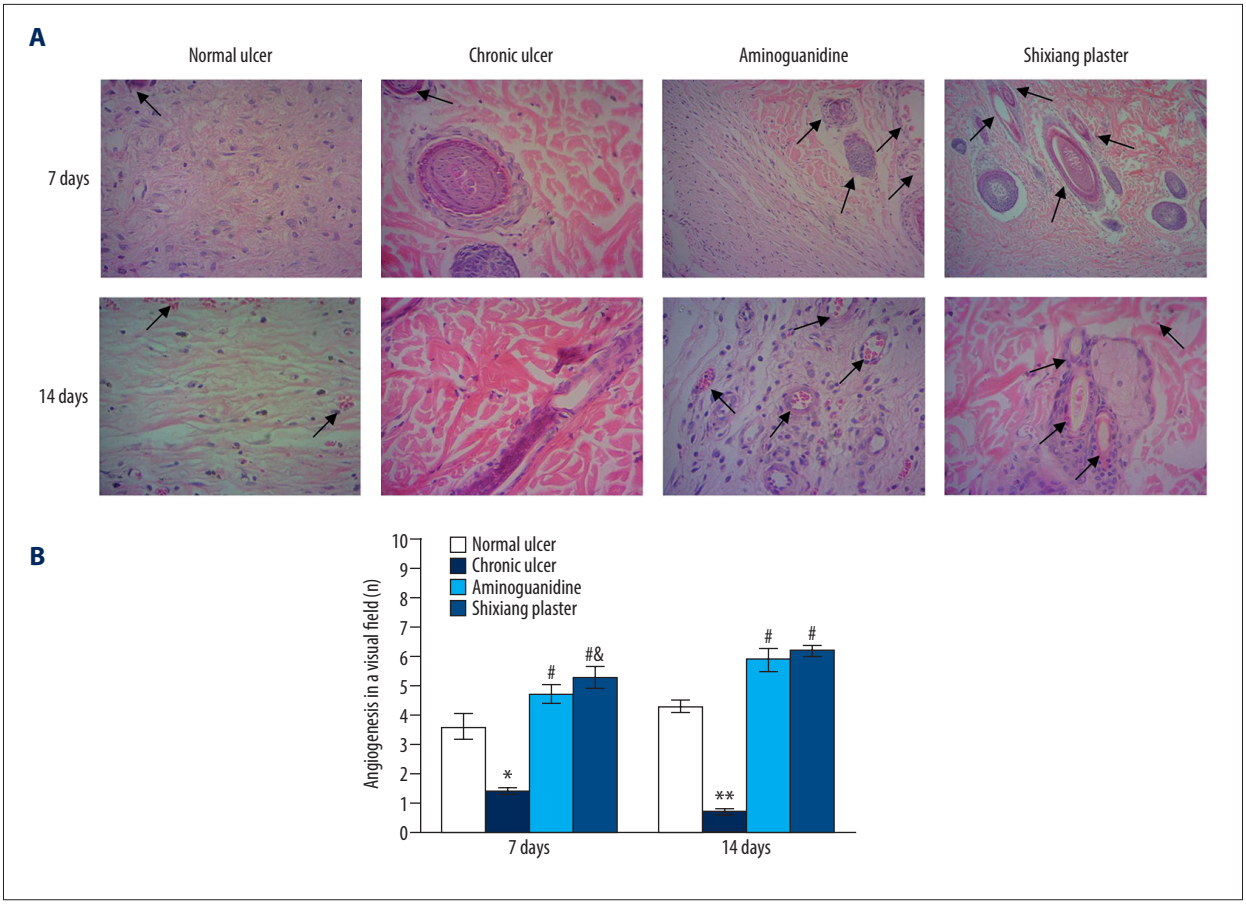

Figure 1. Evaluation of angiogenesis in skin ulcers in the diabetic rat model study groups and the effects of shixiang plaster. (**A**) Photomicrographs of the light microscopy of the skin wounds from rats in the different treatment groups show the degree of angiogenesis in the granulation tissue during healing. Hematoxylin and eosin (H&E). (**B**) Statistical analysis of angiogenesis in a visual field. The black arrows represent the formed angiogenesis. * p<0.05, ** p<0.01 *vs.* the normal ulcer group. # p<0.05 *vs.* the chronic ulcer group. & p<0.05 *vs.* the aminoguanidine group.

reduced compared with the normal ulcer group (p<0.05) at day 7 and day 14 post-surgery. Treatment with both aminoguanidine and shixiang plaster significantly increased the expression of VEGF (Figure 3A) and CD34 (Figure 3B) compared with the chronic ulcer group (p<0.05). However, aminoguanidine treatment showed a greater effect on VEGF expression, and shixiang plaster treatment showed a greater effect on CD34 expression (Figure 3A, 3B).

Shixiang plaster down-regulated the expression of angiogenesis-associated molecules in granulation tissue of ulcers in the rat model

The expression of the angiogenesis-associated molecules, receptor for advanced glycation end products (RAGE) [21] and vascular cell adhesion molecule-1 (VCAM-1) [22], were also determined using Western blot (Figure 4A). The results showed that expression of RAGE (Figure 4B) and VCAM-1 (Figure 4C) molecules were significantly lower in the chronic ulcer group compared with the normal ulcer group (p<0.05) at day 7 post-surgery. Although both aminoguanidine and shixiang plaster could significantly suppress RAGE expression (Figure 4B) and VCAM-1 expression (Figure 4C) (p<0.05), shixiang plaster had a greater effect.

Shixiang plaster stimulated angiogenesis by activating the nuclear factor kappa B (NF-κB) p65 pathway

The NF-κB molecule also has a role in angiogenesis. NF-κB expression was detected using Western blot (Figure 4A). NF-κB p65 expression was significantly higher (Figure 4D) and p-NF-κB p65 expression was significantly lower (Figure 4E) in the chronic ulcer group compared with the normal ulcer group (p<0.05) at day 7 post-surgery. However, both aminoguanidine and shixiang plaster significantly reduced NF-κB p65 expression (Figure 4D) and significantly increased p-NF-κB p65 expression compared with the chronic ulcer group (p<0.05) (Figure 4E).

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

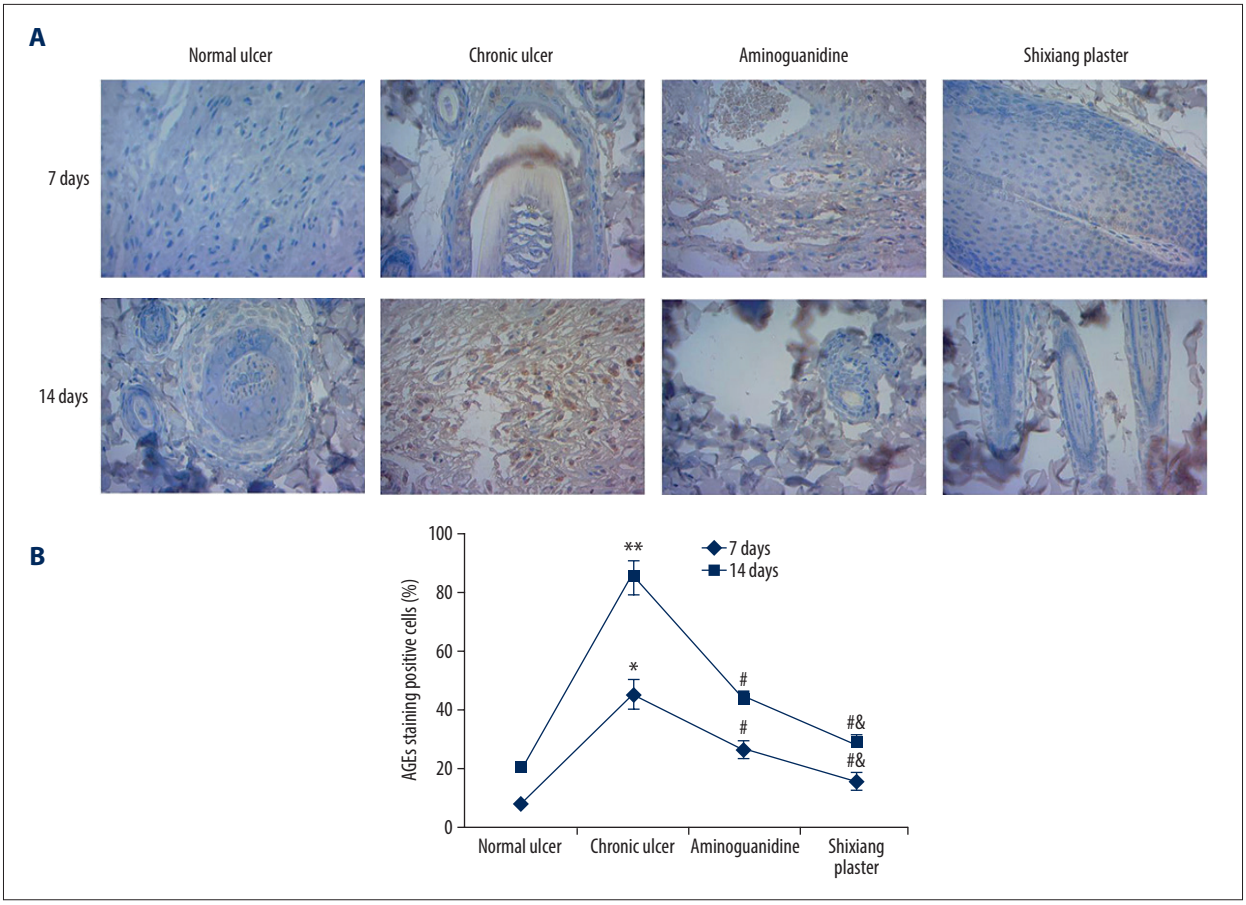

Figure 2. Immunohistochemistry for the expression of advanced glycosylation end products (AGEs) in granulation tissue in skin ulcers in the diabetic rat model study groups and the effects of shixiang plaster. (**A**) Photomicrographs of the light microscopy of the skin wounds from rats in the different treatment groups show the immunohistochemistry staining for the expression of AGEs (brown). (**B**) Statistical analysis for cells positively stained for AGEs. * $p<0.05$, ** $p<0.01$ *vs.* the normal ulcer group. # $p<0.05$ *vs.* the chronic ulcer group. & $p<0.05$ *vs.* the aminoguanidine group.

Shixiang plaster promoted angiogenesis by activating endothelial nitric oxide synthase (eNOS)

In this study, the angiogenesis associated molecule, eNOS, was also investigated with Western blot (Figure 4A). The results showed that the expression of eNOS in the chronic group was significantly lower than the normal ulcer group (Figure 4F; $p<0.05$) at day 7 post-surgery. However, treatment with aminoguanidine and shixiang plaster significantly increased the expression of eNOS compared with the chronic ulcer group (Figure 4F; $p<0.05$). Shixiang plaster also decreased the expression of RAGE and VCAM-1, modulated the expression of NF-κB p65 and increased the expression of eNOS compared with the chronic ulcer group at day 14 post-surgery (Figure 5; $p<0.05$).

Shixiang plaster modified angiogenesis by modulating mRNA expression of RAGE, VCAM-1, NF-κB p65, and eNOS

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) also showed that the mRNA expressions of RAGE (Figure 6A), VCAM-1 (Figure 6B), and NF-κB (Figure 6C) were significantly increased, and eNOS (Figure 6.D) was significantly reduced in the chronic ulcer group compared with the normal ulcer group ($p<0.05$). Treatment with both aminoguanidine and shixiang plaster significantly reduced mRNA expression of RAGE (Figure 6A), VCAM-1 (Figure 6B), and NF-κB (Figure 6C), and significantly increased mRNA expression of eNOS (Figure 6D) compared with the chronic ulcer group ($p<0.05$).

Discussion

The mechanisms involved in healing in patients with diabetes who have chronic skin ulcers are complex. Recently, studies

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

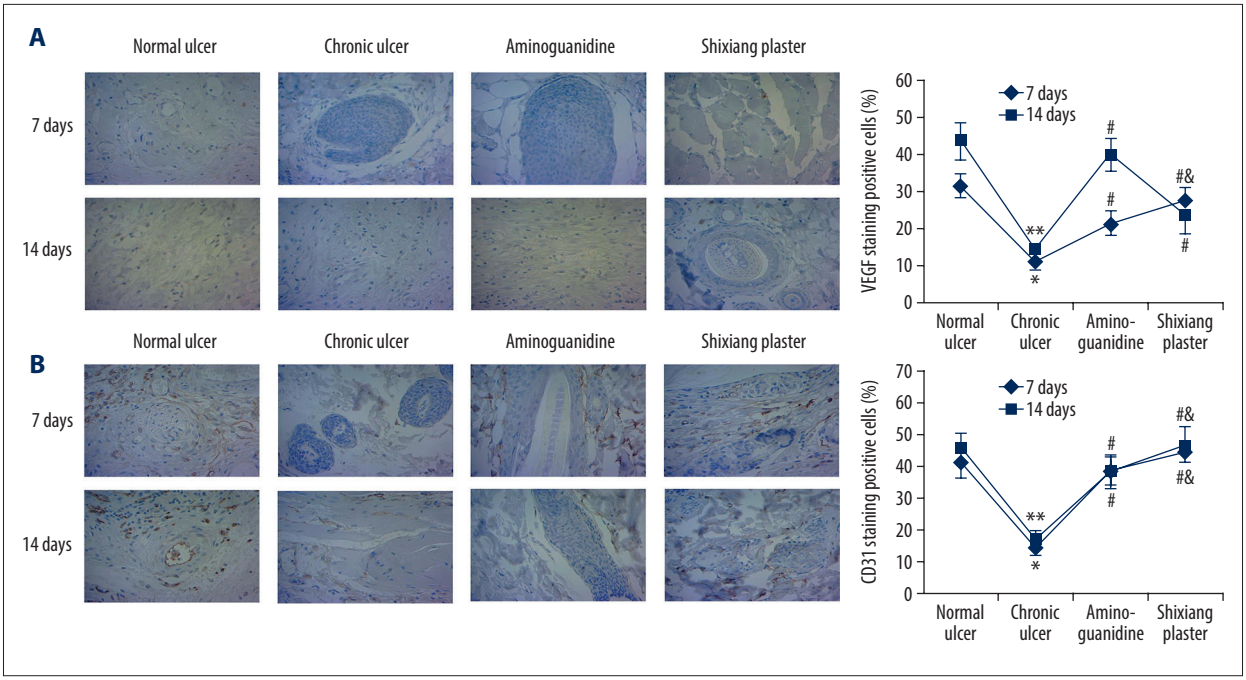

Figure 3. Immunohistochemistry for the expression of vascular endothelial growth factor (VEGF) and CD34 in skin ulcers in the diabetic rat model study groups and the effects of shixiang plaster. (**A**) Photomicrographs of the light microscopy of the skin wounds from rats in the different treatment groups show the immunohistochemistry staining for the expression of VEGF (brown) and statistical analysis. (**B**) Photomicrographs of the light microscopy of the skin wounds from rats in the different treatment groups show the immunohistochemistry staining for the expression of CD34 (brown) and statistical analysis. * p<0.05, ** p<0.01 *vs.* the normal ulcer group. # p<0.05 *vs.* the chronic ulcer group. & p<0.05 *vs.* the aminoguanidine group.

have shown that histological changes are associated with chronic ulcers that are resistant to healing [23,24]. The process of angiogenesis that occurs in granulation tissue is sensitive to the oxidative stress, and inhibition of oxidative stress may be a potential target to promote healing of ulcers in patients with diabetes [25,26]. Studies are ongoing to identify more effective drugs to promote healing of in chronic ulcers. In the present study, the traditional Chinese medicine, shixiang plaster, showed promising results in the promotion of healing of chronic skin ulcers in the streptozotocin-induced rat model of diabetes.

In traditional Chinese medicine theory, the drainage of wounds and removal of necrotic tissue are recommended. Shixiang plaster is a traditional Chinese medicine that contains calamine, frankincense, *Halloysitum rubrum*, calcined bone, and borneol, which could have beneficial effects on healing and tissue repair [15]. Previous studies have shown that angiogenesis is the most direct effector for the healing of wounds or ulcers [27,28]. The findings from the present study showed that treatment with both aminoguanidine, a hydrazine derivative that inhibits the formation of advanced glycosylation end products (AGEs), and shixiang plaster treatment significantly increased angiogenesis at day 7 and day 14 post-surgery, which was a finding supported by previous studies [13,29].

In diabetes, persistent hyperglycemia results in protein glycosylation and the accumulation of AGEs in the skin, which is a risk factor for the injury and chronic ulcers [30]. Studies have shown that AGEs increase the chronicity of diabetic ulcers, which are a serious complication of diabetes [31]. In this study, treatment with aminoguanidine and shixiang plaster significantly reduced the levels of AGEs in granulation tissue in the rat model of diabetic ulcer. However, treatment with shixiang plaster had a greater inhibitory effect on the expression of AGEs, which indicated that shixiang plaster suppressed AGEs in granulation tissue to promote healing, which is consistent with effects of other drugs used to treat diabetic ulcers [32,33].

Reduced angiogenesis during healing and the formation of granulation tissue is a reason for the reduced healing of chronic diabetic ulcers [34,35]. Although previous studies have identified growth factors and associated signaling pathways that participate in angiogenesis, vascular endothelial growth factor (VEGF) has an important role [36]. Also, cells that express CD34 trigger the angiogenesis and mediate the acceleration of wound healing [37]. Therefore, in the present study, the expression of VEGF and CD34 were investigated in the granulation tissue of chronic ulcers in the rat model of diabetes. The findings showed that treatment with aminoguanidine and shixiang plaster significantly increased the expression of VEGF

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

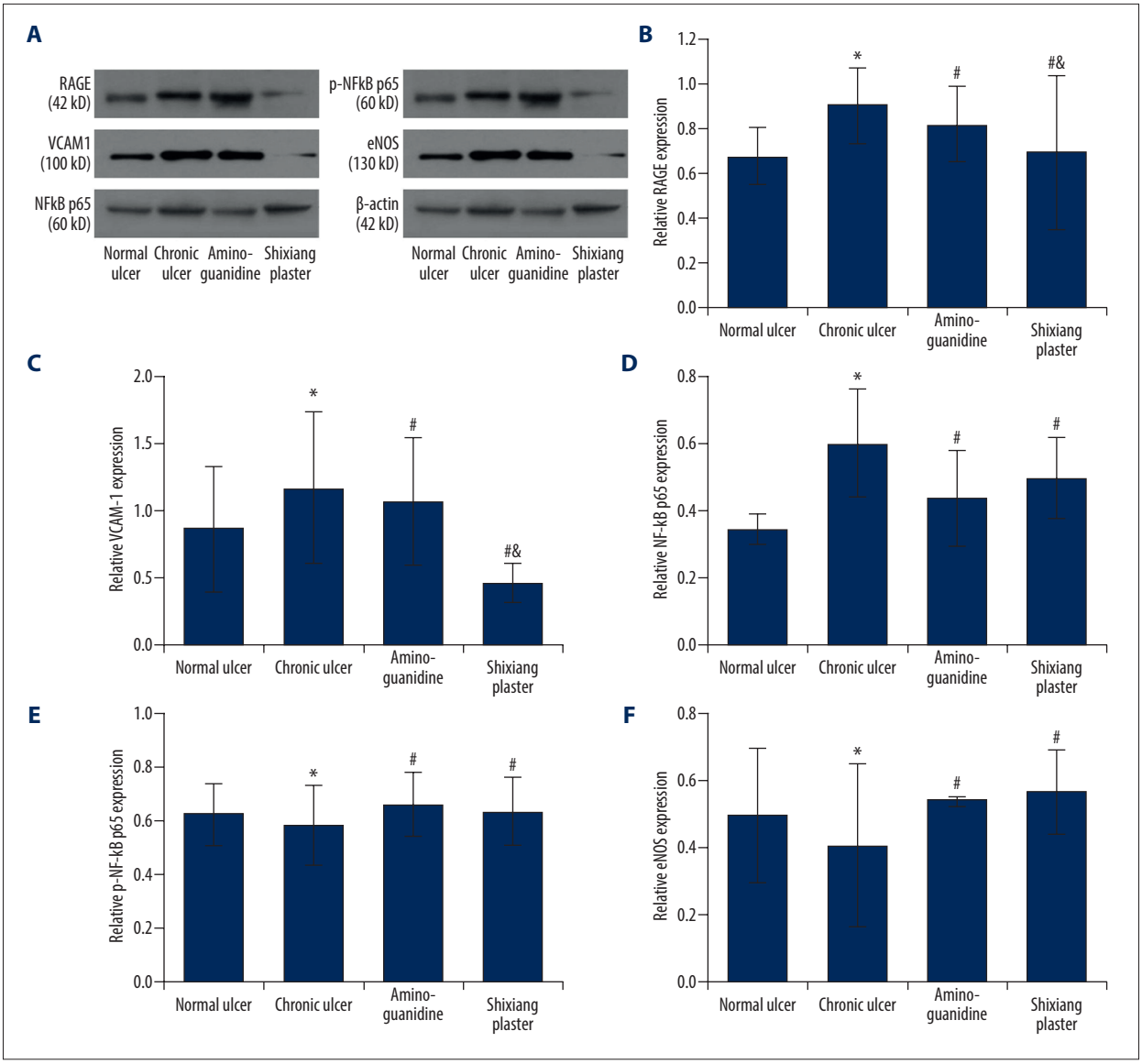

Figure 4. Expression of angiogenesis factors in granulation tissue of chronic ulcers at 7 days post-surgery in skin ulcers in the diabetic rat model study groups and the effects of shixiang plaster. (**A**) Western blot images. (**B**) Statistical analysis for the expression of receptor for advanced glycation end products (RAGE). (**C**) Statistical analysis for the expression of vascular cell adhesion molecule-1 (VCAM-1). (**D**) Statistical analysis for the expression of nuclear factor kappa B (NF-κB). (**E**) Statistical analysis for the expression of p-NF-κB. (**F**) Statistical analysis for the expression of endothelial nitric oxide synthase (eNOS). * p<0.05 *vs.* the normal ulcer group. # p<0.05 *vs.* the chronic ulcer group. & p<0.05 *vs.* the aminoguanidine group.

and CD34 in the rats in the chronic ulcer group, but aminoguanidine had a greater effect on VEGF expression, and shixiang plaster had a greater effect on CD34 expression, which is consistent with the findings from a previous study on the effects of aminoguanidine on wounds [13,38]. However, this study was the first to report the effects of treatment with shixiang plaster on the expression of VEGF and CD34 expression in a model of chronic diabetic ulcer.

Long-term hyperglycemia induces the expression of receptor for advanced glycation end products (RAGE) in tissues [39]. The direct interaction between AGEs and RAGE forms an integrated signaling pathway, induces dysfunction of granulation tissue and cells, and promotes chronicity in diabetic ulcers [40]. The results from the present study showed that shixiang plaster down-regulated the expression of RAGE to promote healing of chronic ulcers by suppressing the AGEs/RAGE signaling pathway, which is similar to the effects other drugs to treat chronic wounds [40,41]. Previous studies have also shown

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

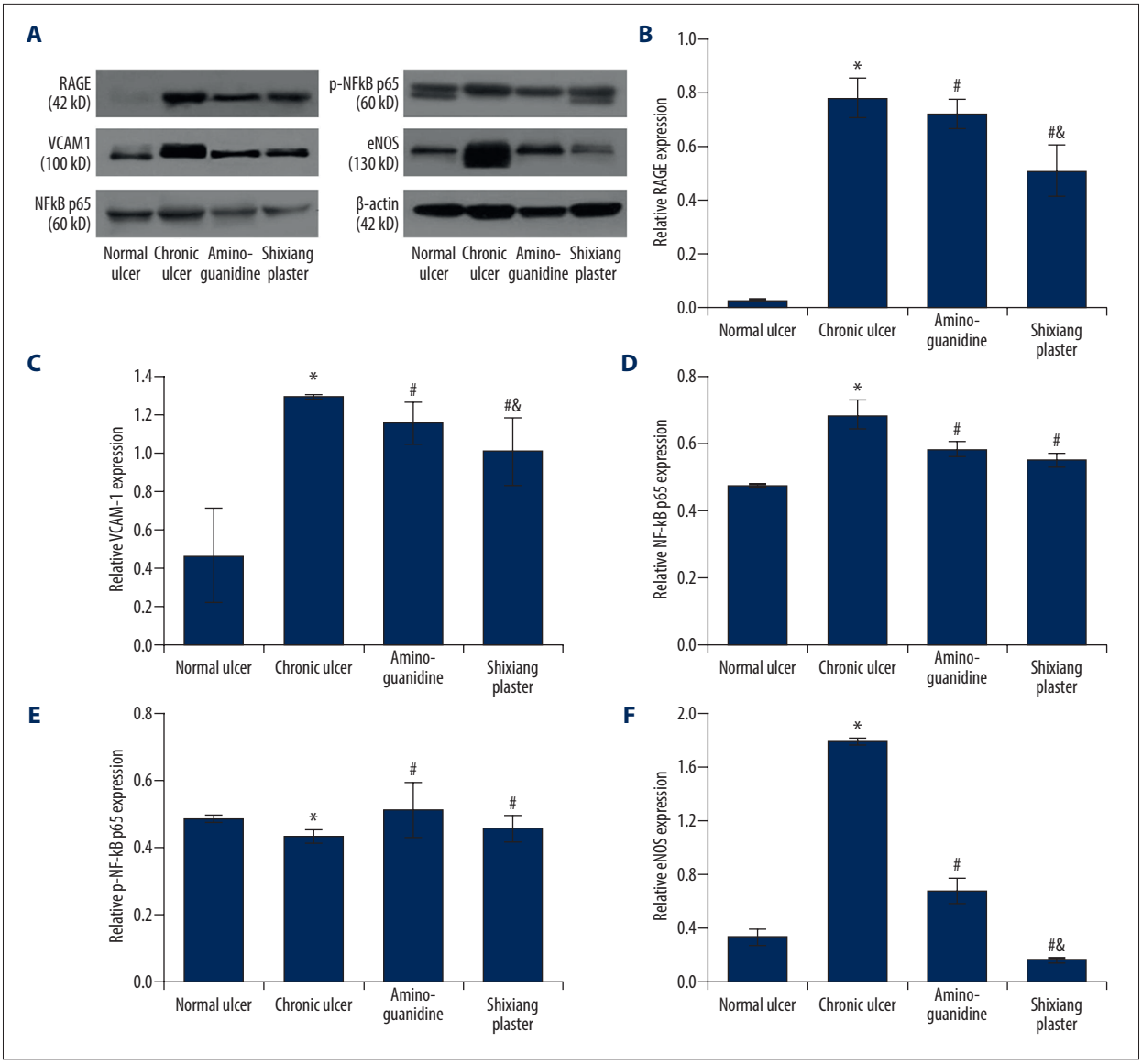

Figure 5. Expression of angiogenesis factors in granulation tissue of chronic ulcers at 14 days post-surgery in skin ulcers in the diabetic rat model study groups and the effects of shixiang plaster. (**A**) Western blot images. (**B**) Statistical analysis for the expression of receptor for advanced glycation end products (RAGE). (**C**) Statistical analysis for the expression of vascular cell adhesion molecule-1 (VCAM-1). (**D**) Statistical analysis for the expression of nuclear factor kappa B (NF-κB). (**E**) Statistical analysis for the expression of p-NF-κB. (**F**) Statistical analysis for the expression of endothelial nitric oxide synthase (eNOS). * $p<0.05$ *vs.* the normal ulcer group. # $p<0.05$ *vs.* the chronic ulcer group. & $p<0.05$ *vs.* the aminoguanidine group.

that inhibition of vascular cell adhesion molecule-1 (VCAM-1) could enhance angiogenesis, which was also demonstrated in this study as treatment with shixiang plaster significantly inhibited VCAM-1 expression in granulation tissues of ulcers in the rat model [22,42]. Therefore, shixiang plaster could directly promote healing by suppressing the AGEs/RAGE signaling pathway to indirectly heal the ulcer by enhancing angiogenesis and inhibit VCAM-1 expression.

Nitric oxide (NO) is a key regulator of physiological and pathological angiogenesis [43]. Endothelial nitric oxide synthase (eNOS) can modulate VEGF-mediated angiogenesis while blocking eNOS inhibits the inducible effect of VEGF on angiogenesis [44]. Silencing the expression of the eNOS gene was shown to impair angiogenesis in an animal model of ischemia [45]. Therefore, eNOS is involved in angiogenesis-associated signaling pathways in healing and tissue repair. This study showed that treatment with shixiang plaster significantly increased the expression of eNOS expression in the chronic ulcer group.

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

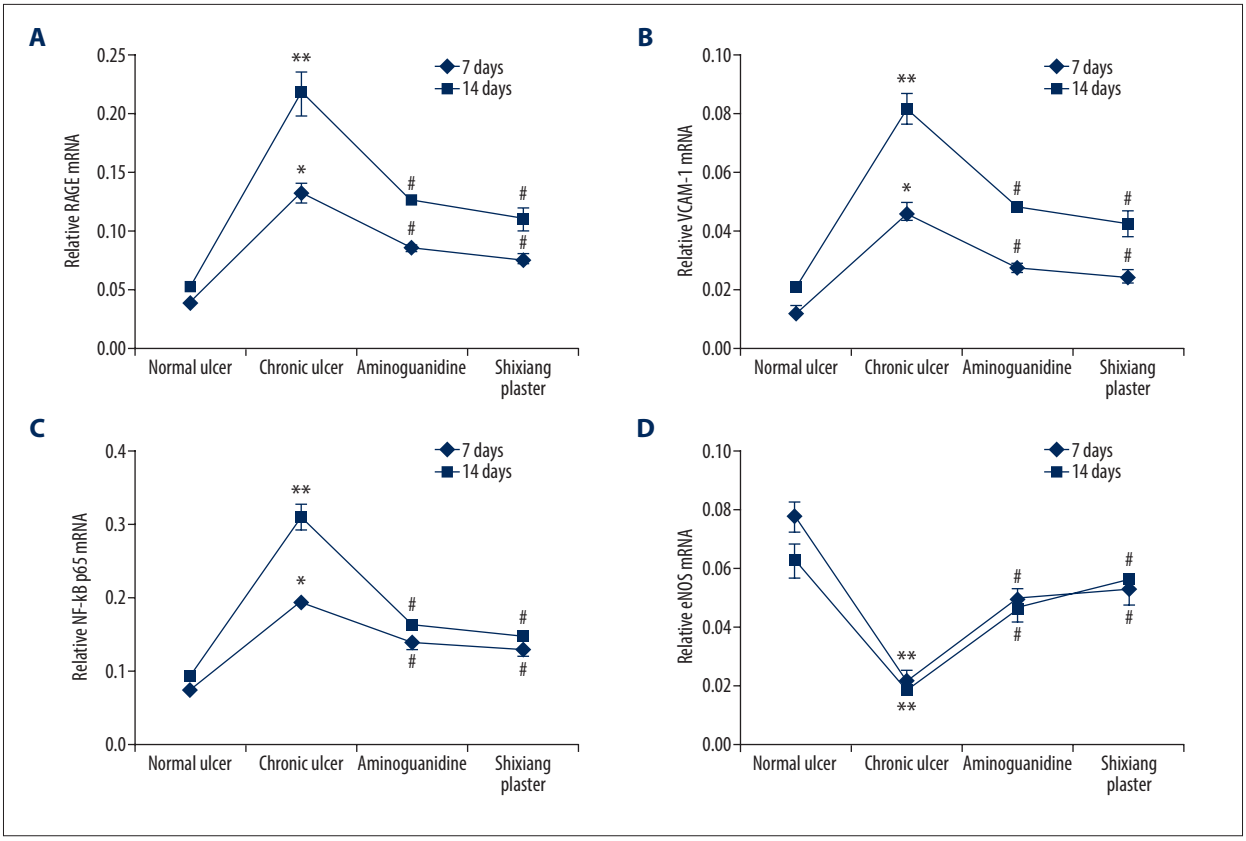

Figure 6. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) for mRNA expression of factors associated with angiogenesis in skin ulcers in the diabetic rat model study groups and the effects of shixiang plaster. (**A**) Statistical analysis for RAGE mRNA expression. (**B**) Statistical analysis for VCAM-1 mRNA expression. (**C**) Statistical analysis for NF-κB mRNA expression. (**D**) Statistical analysis for eNOS mRNA expression. * p<0.05, ** p<0.01 *vs.* the normal ulcer group. # p<0.05 *vs.* the chronic ulcer group.

A previous study also reported that the combination of AGEs and RAGE resulted in intracellular oxidative stress and activation of NF-κB [46]. The activation and accumulation of NF-κB in the form of phosphorylated NF-κB (p-NF-κB) occurs during inflammations and tissue damage [47]. The findings from the present study showed that the expression of NF-κB was reduced and the expression of p-NF-κB was increased in granulation tissues in the rat model following treatment with shixiang plaster. Also, in this study, aminoguanidine, an inhibitor of AGEs, was a positive control for healing of chronic ulcers [48]. However, although aminoguanidine showed greater efficacy in the promotion of healing, the neurotoxicity associated with the use of this compound limits its clinical use [49]. However, shixiang plaster is simple to use, is associated with less tissue damage, and is based on herbal compounds that have less toxicity than many drug treatments. Although the findings from this small and short-term study in an animal model have demonstrated beneficial effects for the use of shixiang plaster, future prospective clinical studies are needed to examine its efficacy and mechanisms of action in the treatment of chronic diabetic ulcers and in tissue repair.

Conclusions

This study aimed to investigate the effects of shixiang plaster and aminoguanidine on wound healing in the streptozotocin-induced rat model of diabetes and the molecular mechanisms involved. Topical treatment of healing skin ulcers in the rat model with shixiang plaster reduced the expression of advanced glycation end products (AGEs) and receptor for advanced glycation end products (RAGE) in granulation tissue of chronic ulcers. Blocking AGEs activated nuclear factor kappa B (NF-κB) and upregulated p-NF-κB expression, which further caused cell injury and promoted expression of vascular endothelial growth factor (VEGF), CD34, and endothelial nitric oxide synthase (eNOS). Treatment with shixiang plaster increased the expression of vascular cell adhesion molecule-1 (VCAM-1) in granulation tissues of the chronic ulcer. These findings, in skin ulcers in the streptozotocin-induced rat model of diabetes, showed that topical treatment with shixiang plaster promoted angiogenesis and accelerated healing through the RAGE/NF-κB and VEGF/VCAM-1/eNOS signaling pathways.

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

Acknowledgments

This study was funded by Guizhou Province Science and Technology Cooperation Program and Youth Science and Technology Talents Growth Project of Guizhou Provincial Department of Education.

Conflict of interest

None.

References:

1. Jaworski M, Panczyk M, Sliwczyriski A et al: Severe depressive episode with psychotic symptoms and type 2 diabetes: A 2010–2017 longitudinal study. Med Sci Monit, 2019; 25: 1760–68
2. Adu MD, Malabu UH, Malau-Aduli AEO et al: Enablers and barriers to effective diabetes self-management: A multi-national investigation. PLoS One, 2019; 14: e0217771
3. Forouhi NG, Wareham NJ: Epidemiology of diabetes. Medicine, 2014; 42: 698–702
4. Infante-Garcia C, Garcia-Alloza M: Review of the effect of natural compounds and extracts on neurodegeration in Animal models of diabetes mellitus. Int J Mol Sci, 2019; 20: E2533
5. Rosenson RS, Fioretto P, Dodson PM: Does microvascular disease predict macrovascular events in type 2 diabetes? Atherosclerosis, 2011; 218: 13–18
6. Fan Y, Wu W, Lei Y, et al. Edaravone-loaded alginate-based nanocomposite hydrogel accelerated chronic wound healing in diabetic mice. Mar Drug, 2019; 17: E285
7. Braiman-Wiksman L, Solomonik I: Novel insights into wound healing sequence of events. Toxicol Pathol, 2007; 35: 767–79
8. Schafer M, Werner S: Oxidative stress in normal and impaired wound repair. Pharmacol Res, 2008; 58: 165–71
9. Peng J, Zheng H, Wang X et al: Upregulation of TLR4 via PKC activation contributes to impaired wound healing in high-glucose treated kidney proximal tubular cells. PLoS One, 2017; 12: e0178147
10. Baltzis D, Eleftheriadou I, Veves A: Pathogenesis and treatment of impaired wound healing in diabetes mellitus: New insights. Adv Ther, 2014; 31: 817–36
11. Atiyeh BS, Hayek SN: An update on management of acute and chronic open wounds: The importance of moist environment in optimal wound healing. Med Chem Rev, 2014; 1: 111–21
12. Han Y, Sun T, Tao R et al: Clinical application prospect of umbilical cord-derived mesenchymal stem cells on clearance of advanced glycation end products through autophagy on diabetic wound. Eur J Med Res, 2017; 22: 11
13. Tian M, Qing C, Niu Y et al: Effect of aminoguanidine intervention on neutrophils in diabetes inflammatory cells wound healing. Exp Clin Endocrinol Diabetes, 2013; 121: 635–42
14. Moulik PK, Mtonga R, Gill GV: Amputation and mortality in new-on-set diabetic foot ulcers stratified by etiology. Diabetes Care, 2003; 26: 491–94
15. Fei J, Chen JY, Zhang KW et al: Treating chronic ulcer with shixiang plaster: A randomized controlled trial. Liaoning Chin Med J, 2015; 42: 236–38
16. Fei J, Chen JY, Zhang KW et al: Multicenter study of treating chronic ulcer with shixiang plaster. Chin J Exp Trad Med Form, 2015; 21: 179–81
17. Luan Q, Pan L, He D et al: SC79, the AKT activator protects cerebral ischemia in a rat model of ischemia/reperfusion injury. Med Sci Monit, 2018; 24: 5391–97
18. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔct method. Methods, 2001; 25: 402–8
19. Moreira IS, Fernandes PA, Ramos MJ: Vascular endothelial growth factor (VEGF) inhibition, a critical review. Anticancer Agents Med Chem, 2007; 7: 223–45
20. das Neves LM, Leite GP, Marcolino AM et al: Laser photobiomodulation (830 and 660 nm) in mast cells, VEGF, FGF, and CD34 of the musculocutaneous flap in rats submitted to nicotine. Lasers Med Sci, 2017; 32: 335–41
21. Lei C, Zhang S, Cao T et al: HMGB1 may act via RAGE to promote angiogenesis in the later phase after intracerebral hemorrhage. Neuroscience, 2015; 295: 39–47
22. Fukushi J, Ono M, Morikawa W et al: The activity of soluble VCAM-1 in angiogenesis stimulated by IL-4 and IL-13. J Immunol, 2000; 165: 2818–23
23. Peppa M, Uribarri J, Vlassara H: The role of advanced glycation end products in development of atherosclerosis. Curr Biab Res, 2004; 4: 31–36
24. Andrade TAM, Masson-Meyers DS, Caetano GF et al: Skin changes in streptozotocin-induced diabetic rats. Biochem Biophys Res Commun, 2017; 490: 1154–61
25. Okonkwo UA, DiPietro LA: Diabetes and wound angiogenesis. Int J Mol Sci, 2017; 18: E1419
26. Ren C, Li N, Li S et al: Limb ischemic conditioning improved cognitive deficits via eNOS dependent augmentation of angiogenesis after chronic cerebral hypoperfusion in rats. Aging Dis, 2018; 9: 869–79
27. Zhang Y, Li L, Liu Y et al: PKM2 released by neutrophils at wound site facilitates early wound healing by promoting angiogenesis. Wound Repair Regen, 2016; 24: 328–36
28. Saad I, Fournier CT, Wilson RL et al: Thioredoxin-1 augments wound healing and promote angiogenesis in a murine ischemic full-thickness wound model. Surgery, 2018; 164: 1077–86
29. Chang PC, Tsai SC, Jheng YH et al: Soft-tissue wound healing by anti-advanced glycation end-products agents. J Dent Res, 2014; 93: 388–93
30. Richards JE, Hutchinson J, Mukherjee K et al: Stress hyperglycemia and surgical site infection in stable nodaibetic adults with orthopedic injuries. J Trauma Acute Care Surg, 2014; 76: 1070–75
31. Driver VR, Reyzelman A, Kawalec J et al: A prospective, randomized, blinded, controlled trial comparing transdermal continuous oxygen delivery to moist wound therapy for the treatment of diabetic foot ulcers. Ostomy Wound Manage, 2017; 63: 12–18
32. Shim HS, Choi JS, Kim SW: A role for postoperative negative pressure wound therapy in multitissue hand injuries. Biomed Res Int, 2018; 2018: 3629643
33. Aunin E, Broadley D, Ahmed MI et al: Exploring a role for regulatory miRNAs in wound healing during ageing: Involvement of miR-200c in wound repair. Sci Rep, 2017; 7: 3257
34. Singh R, Garcia-Gomez I, Gudehithlu KP et al: Bitter melon extract promotes granulation tissue growth and angiogenesis in the diabetic wound. Adv Skin Wound Care, 2017; 30: 16–26
35. Weinherimer-Haus EM, Judex S, Ennis WJ et al: Low-intensity vibration improves angiogenesis and wound healing in diabetic mice. PLoS One, 2014; 9: e91355
36. Moreira IS, Femandes PA, Ramos MJ: Vascular endothelial growth factor (VEGF) inhibition, a critical review. Anticancer Agents Med Chem, 2007; 7: 233–45
37. Kanji S, Das M, Aggarwal R et al: Nanofiber-expanded human umbilical blood-derived CD34+ cell therapy accelerates murine cutaneous wound closure by attenuating pro-inflammatory factors and secreting IL-10. Stem Cell Res, 2014; 12: 275–88
38. Ge K, Lu SL, Qing C: [Aminoguanidine and impaired wound healing in diabetes.] Zhonghua Yi Xue Za Zhi, 2004; 84: 966–68 [in Chinese]
39. Kay AM, Simpson CL, Stewart JA: The role of AGE/RAGE signaling in diabetes-mediated vascular calcification. J Diabetes Res, 2016; 2016: 6809703
40. Wang Q, Zhu G, Cao X et al: Blocking AGE-RAGE signaling improved functional disorders of macrophages in diabetic wound. J Diabetes Res, 2017; 2017: 1428537
41. Serban AI, Stanca L, Geicu OI et al: RAGE and TGF-beta 1 cross-talk regulate extracellular matrix turnover and cytokine synthesis in AGEs exposed fibroblast cells. PLoS One, 2016; 11: e0152376
42. Kim TK, Park CS, Na HJ et al: Ig-like domain 6 of VCAM-1 is a potential therapeutic target in TNF-alpha induced angiogenesis. Exp Mol Med, 2017; 49: e249

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

43. Yoshida D, Akahoshi T, Kawanaka H et al: Roles of vascular endothelial growth factor and endothelial nitric oxide synthase during revascularization and regeneration after partial hepatectomy in a rat model. Surg Today, 2011; 41: 1622–29
44. Papapetropoulos A, Carcina-Cardena G, Madri JA: Nitric oxide production contributes to the antiogenic properties of vascular endothelial growth factor in human endothelial cells. Clin Invest, 1997; 100: 3131–39
45. Chin LC, Kumar P, Palmer JA et al: The influence of nitric oxide synthase 2 on cutaneous wound angiogenesis. Br J Dermatol, 2011; 165: 1223–35
46. Schiekofer S, Andrassy M, Chen J et al: Acute hyperglycemia causes intracellular formation of CML, and activation of rats, p42/p44 MAPK, and nuclear factor-κB in PBMCs. Diabetes, 2003; 52: 621–33
47. Bierhaus A, Humpert PM, Moreos M et al: Understanding RAGE, the receptor for advanced glycation end products. J Mol Med (Berl), 2005; 83: 876–86
48. Xiong DD, Zhang M, Li N et al: Mediation of inflammation, obesity and fatty liver disease by advanced glycation endoproducts. Eur Rev Med Pharmacol Sci, 2017; 21: 5172–78
49. Soro-Paavonen A, Forbes M: Novel therapeutics for diabetic micro- and macrovascular complications. Curr Med Chem, 2006; 13: 1777–88

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]