# Moist exposed burn ointment promotes cutaneous excisional wound healing in rats involving VEGF and bFGF

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**Abstract.** Cutaneous delayed wounds are a challenging clinical problem, and vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) exhibit key roles in wound healing. Moist exposed burn ointment (MEBO), a Chinese burn ointment with a USA patented formulation, has been reported to promote chronic ischemic and neurogenic ulcer healing in patients; however, the underlying mechanisms remain unclear. In the present study, MEBO significantly promoted the formation of granulation tissue in cutaneous excisional wounds, shortened the time of wound healing, and increased neovascularization and the number of fibroblasts. Furthermore, as well as enhancing the protein expression, MEBO application also increased the gene expression of VEGF and bFGF. The results indicate that MEBO promotes cutaneous excisional wound healing by at least partially enhancing VEGF and bFGF production, implicating the potential uses of MEBO for delayed cutaneous wound healing.

## Introduction

Cutaneous wounds, known as ulcers, are an extremely common clinical problem and often arise following acute or chronic mechanical causes, physical or chemical burns, frostbite, infections, and disorders, including rheumatism, diabetes, peripheral vascular disease, lipodermatosclerosis and malignant tumors (1,2). Furthermore, cutaneous wound healing may be significantly delayed due to the aforementioned factors. At present, delayed cutaneous wounds are one of major burdens for health care, and lead to a reduced quality of life in patients who suffer from this type of wound (3,4).

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Wound healing is a complicated biological process involving a series of dynamic events, including hemostasia, inflammation, cell proliferation and differentiation, neovascularization, granulation tissue formation, collagen synthesis, epithelialization, and wound contraction. Increasing evidence has established the hypothesis that growth factors, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), are key regulators of normal and abnormal angiogenesis, and tissue repair in animals and humans (5-8). VEGF promotes angiogenesis/vasculogenesis and vascular permeability, and enhances endothelial cell proliferation and migration as well as the adhesion of leukocytes (5,9). Further research revealed that VEGF stimulates hydrogen sulfide synthesis and release from endothelial cells, thus leading to subsequent endothelial cell growth, migration and permeability, microvessel formation, and wound healing (10). In addition, VEGF promotes epithelialization and collagen deposition in the wound (11). FGF-2, known as bFGF, is a member of a large FGF family and induces angiogenesis, endothelial cell and fibroblast proliferation, and wound healing (12-14). Recent data indicated that bFGF-mediated angiogenesis refers to endothelial cell proliferation, migration and tube formation by activating c-Jun N-terminal kinase/stress-activated protein kinase signaling (15). Notably, the expression of VEGF and bFGF was increased following skin injury, particularly in the early stages of healing, showing greatest intensity at the center of the wound, with progressive decline in intensity towards the periphery and almost no VEGF or bFGF in uninjured skin (16,17). VEGF and bFGF, however, are decreased in delayed cutaneous wounds, including diabetic wounds and chronic ulcers (17-19). Inhibition of VEGF and bFGF by neutralizing antibodies results in a decrease in the migration of fibroblasts and a delay in wound healing (20), while treatment with recombinant bFGF and VEGF, or overexpression of VEGF accelerates wound healing (14,21,22). Therefore, it is a potentially clinically beneficial to increase the levels of VEGF and bFGF in cutaneous wounds, particularly in delayed wound healing.

In the past few decades, moist exposed burn ointment (MEBO), a Chinese burn ointment with a USA patented formulation since 1995, which was developed at the China National Science and Technology Centre in Beijing in 1989 (23,24), has been used to treat thickness burns in clinical practice and has achieved beneficial efficacy (25-28). MEBO contains

sesame oil, β-sitosterol, berberine, and other small quantities of plant ingredients from Chinese herbal remedies, including *Coptis chinensis* Franch., *Scutellaria baicalensis* Georgi, *Phellodendron Chinese* Schneid., *Pheretima aspergillum* (E Perrier) and *Papaver somniferum* L. (29). Further research indicated that β-sitosterol exhibits anti-inflammatory effects (30) and that berberine exhibits antimicrobial effects (31). Clinical and experimental studies have shown that MEBO exhibits analgesic and antimicrobial effects, and reduces the treatment time of burns (25-28,32,33). Furthermore, MEBO induces debridement and epithelial repair, associated with improved scar quality and reduced costs of treatment for patients with burns (25,27,28,33). MEBO has been observed to promote chronic ischemic and neurogenic ulcer healing, and reduces the time of wound healing in patients (34,35); however, the underlying mechanisms remain unclear.

The aim of the present study was to investigate the effects of MEBO on cutaneous excisional wounds in experimental rats and to explore the underlying mechanisms.

## Materials and methods

*Reagents*. MEBO was purchased from Shantou MEBO Pharmaceuticals Co., Ltd. (Shantou, China). Recombinant bovine bFGF (rb-bFGF) was purchased from Zhuhai Yisheng Biological Pharmaceutical Co. (Zhuhai, China). Rabbit polyclonal antibodies against VEGF and bFGF were purchased from Abcam (Cambridge, MA, USA), and secondary mouse anti-rabbit peroxidase-conjugated monoclonal antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA). TRIzol reagent was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). A bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

*Wound preparation and experimental design*. Sixty male Sprague-Dawley rats (age, 8-weeks; weight, 220-250 g) were purchased from the Animal Experimental Center of Guangxi Medical University (Nanning, China; SCXKGui2009-0002). The study was approved by the Ethics Committees of Youjiang Medical University for Nationalities (Baise, China) and Guangxi University of Chinese Medicine. Rats were housed in a temperature- and humidity-controlled room with a 12-h ligh/dark cycle and had free access to food and water. Following 1-week acclimatization, cutaneous full-thickness excisional wounds were prepared as previously described with some modifications (36). Briefly, the rat hair was shaved on the dorsal side following anesthesia with pentobarbital sodium (39 mg/kg), and the skin was cleaned with 70% ethanol. A 2.4-cm diameter full-thickness skin defect was created on the back by skin punch biopsy under aseptic conditions. Two wounds were created in each rat. Next, the rats were randomly divided into three groups based on different treatments: Model (n=20), MEBO (n=20) and rb-bFGF (n=20). The wounds in the model group were covered with a single dressing soaked with physiological saline and double dry sterile dressings; wounds in the MEBO group were covered with dry sterile dressings following direct coating of the wound with 1 mm thick MEBO; and wounds in the rb-bFGF group were covered with dry sterile dressings following by spraying of the wound with rb-bFGF. The sterile dressings were changed daily following clearing of wound liquefaction with sterile dressings. All procedures were in accordance with internationally accepted principles for laboratory animal use and care, as found in the European Community Guidelines (EEC Directive of 1986; 86/609/EEC) and the US guidelines (NIH publication #85-23, revised in 1985).

*Histopathological observation*. Rats were euthanized using pentobarbital sodium. Granulation tissue was collected from eight-day-old wounds not containing healthy skin margin. Each tissue was fixed using 10% formalin, processed for paraffin embedding and stored at 4˚C. The following parameters were evaluated with hematoxylin and eosin (H&E) staining on multiple serial sections (4-5 $\mu$m): Neovascularization, fibroblast proliferation and inflammatory cell infiltration. All analysis was performed at x200 magnification.

*Western blot analysis*. The protein expression of VEGF and bFGF in granulation tissue from eight-day-old wounds was determined by western blotting, as previously described with certain modifications (37). Briefly, tissue homogenates from wound granulation were prepared using RIPA lysis buffer. Insoluble material was removed by centrifugation (12,000 x g for 20 min; 4˚C; Thermo Forma, Osteroden, Germany), and the protein concentrations of the supernatants were determined using a BCA protein assay kit. Equal quantities of protein (50 $\mu$g) were separated via SDS-PAGE. The protein was electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The blots were incubated with the primary antibody overnight at 4˚C and then with the secondary antibody for 1-2 h at room temperature. The protein bands were detected by an Enhanced Cheminluminescent Plus detection reagent kit (Amersham Pharmacia Biotech, Amersham, UK).

*Quantification of mRNA levels by reverse transcription-polymerase chain reaction (RT-PCR)*. Total RNA was extracted from eight-day-old wound granulation tissue using TRIzol reagent according to the manufacturer's instructions, and subsequently transcribed into cDNA. cDNA was then transcribed into mRNA by RT-PCR. The product was separated via agarose gel electrophoresis. The sequences of primers used were as follows: Forward: 5'-CGGAAGATTAGGGAGTTTTG-3' and reverse: 5'-AGGGATGGGTTTGTCGTGT-3' for VEGF; forward: 5'-GCGTCCGGGAGAAGAGCGAC-3' and reverse: 5'-GCCAGGTACCGGTTCGCACA-3' for bFGF; forward: 5'-CAGTGCCAGCCTCGTCTCAT-3' and reverse: 5'-AGGGGCCATCCACAGTCTTC-3' for GAPDH; and forward: 5'-CACCCGCGAGTACAACCTTC-3' and reverse: 5'-CCCATACCCACCATCACACC-3' for β-actin.

*Statistical analysis*. The data are presented as the mean ± standard deviation. Statistical analysis was performed using the SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Significant differences among groups were analyzed by one-way analysis of variance, and differences between means were determined by Fisher's least significant difference post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*MEBO promotes the formation of granulation tissue and wound healing*. MEBO was initially successfully designed to treat burns. Previously, MEBO was observed to promote chronic ulcer healing (34,35); however, the underlying mechanisms remain to be fully elucidated. In the current study, a full-thickness skin defect was induced by skin punch biopsy, and treatment with MEBO for eight days significantly promoted the formation of granulation tissue when compared with the model group. Furthermore, the time of wound healing in the MEBO group was shorter compared with that of the model group (P<0.01; Fig. 1). In addition, rb-bFGF was designed as a positive control medicine for MEBO. Consistent with MEBO, rb-bFGF also caused the formation of granulation tissue and shortened the time of wound healing when compared with the model group (P<0.01); however, the time of wound healing in the MEBO group was also shorter than that of the rb-bFGF group (P<0.05). The data indicate that MEBO promotes cutaneous excisional wound healing, similar to that of rb-bFGF, indicating that there is a similarity in the effects of MEBO and rb-bFGF in the treatment of wounds.

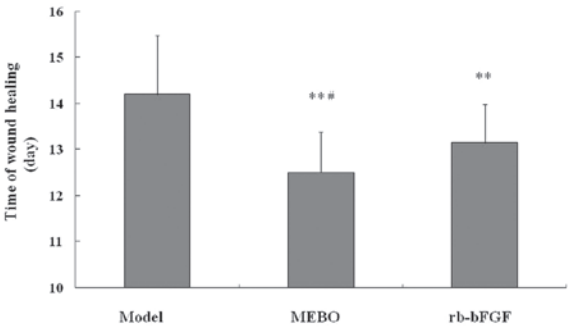


Figure 1. MEBO reduced the time of wound healing. Following treatment, the time of wound healing of the rats in each group was recorded. **P<0.01, vs. the model group; #P<0.05, vs. the recombinant bovine-basic fibroblast growth factor (rb-bFGF) group, n=20. MEBO, moist exposed burn ointment.

*MEBO increases neovascularization and fibroblasts in granulation tissue*. To determine the mechanisms underlying the effects of MEBO, the histology of granulation tissue in three groups was observed following wound treatment for eight days. As shown in Fig. 2, there were numerous novel capillaries and fibroblasts in granulation tissue in the MEBO group, which were more abundant compared with the model group and rb-bFGF group, when observed at x200 magnification. In addition, rb-bFGF treatment for eight days resulted in marked increases in the numbers of capillaries and fibroblasts in granulation tissue when compared with the model group. These data suggest that MEBO promotes neovascularization and enhances fibroblast proliferation and/or migration.

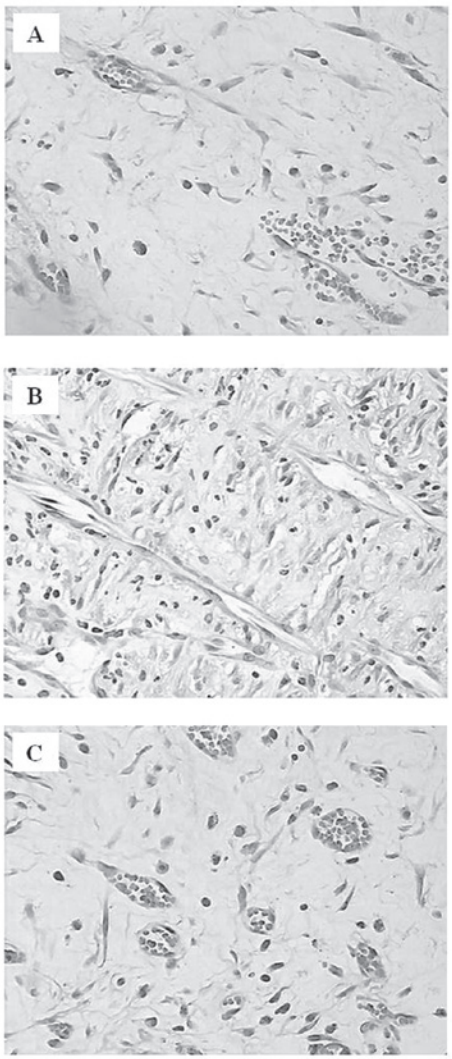


Figure 2. Effects of MEBO on the histopathology of the wounds. Granulation tissue was collected from eight-day-old wounds and then analyzed histopathology using hematoxylin and eosin staining (magnification, x200). (A) Model group, (B) MEBO group and (C) recombinant bovine-basic fibroblast growth factor group. MEBO, moist exposed burn ointment.

*Effects of MEBO on the protein expression of VEGF and bFGF*. MEBO promoted neovascularization and increased fibroblasts in granulation tissue. As previously described, VEGF and bFGF levels are closely correlated with neovascularization and fibroblast proliferation (5-8). To further determine the association between MEBO and VEGF/bFGF, western blotting was used to determine the growth factor levels. Local administration of MEBO for eight days markedly increased the levels of VEGF and bFGF by ~77.5 and 90.8%, respectively (all P<0.01; Fig. 3), when compared with the model group. VEGF and bFGF protein expression in the rb-bFGF group were higher compared with the model group (all P<0.01). Notably, the levels of VEGF and bFGF in the MEBO group were higher compared with the rb-bFGF group (P<0.05 and P<0.01, respectively). The results indicate that MEBO at least partially increases the protein expression levels of VEGF and bFGF to promote wound healing.

*Effects of MEBO on the gene expression of VEGF and bFGF*. qPCR analysis (Fig. 4) indicated that MEBO treatment for eight days led to increases in the mRNA expression of VEGF and bFGF by 40.9 and 97.1%, respectively, when compared with the model group (all P<0.01). The rb-bFGF treatment also increased the gene expression of VEGF and bFGF (all P<0.01). Furthermore, the mRNA levels of VEGF and bFGF genes in

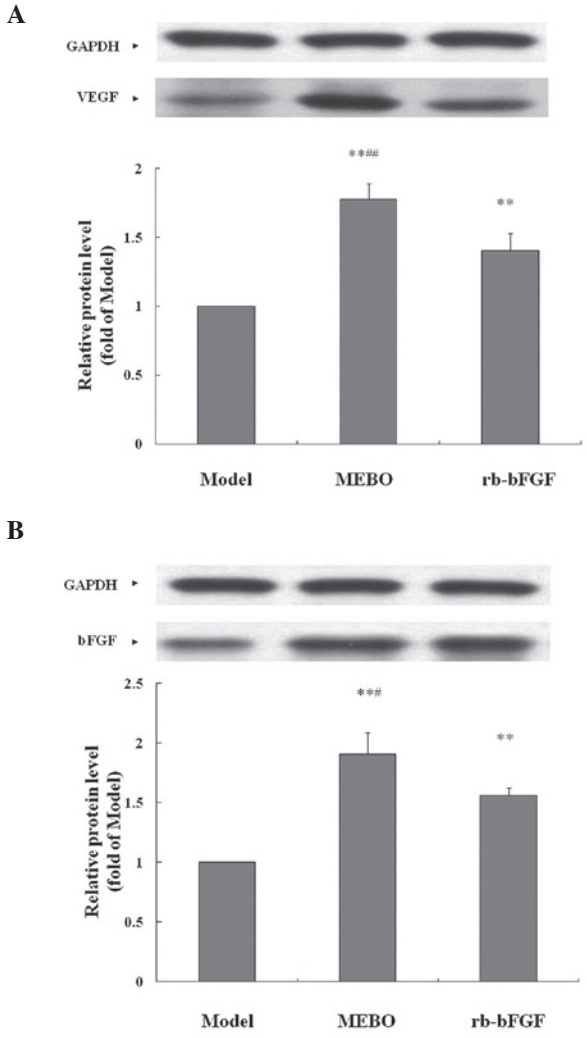


Figure 3. MEBO enhanced the protein expression of VEGF and bFGF. Following treatment for eight days, granulation tissue was collected, and the protein expression in the granulation tissue was determined by western blot analysis. (A) Relative protein level of VEGF. (B) Relative protein level of bFGF. $^{**}$P<0.01, vs. the model group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01, vs. the rb-bFGF group. MEBO, moist exposed burn ointment; VEGF, vascular endothelial growth factor; rb-bFBF, recombinant bovine-basic fibroblast growth factor.

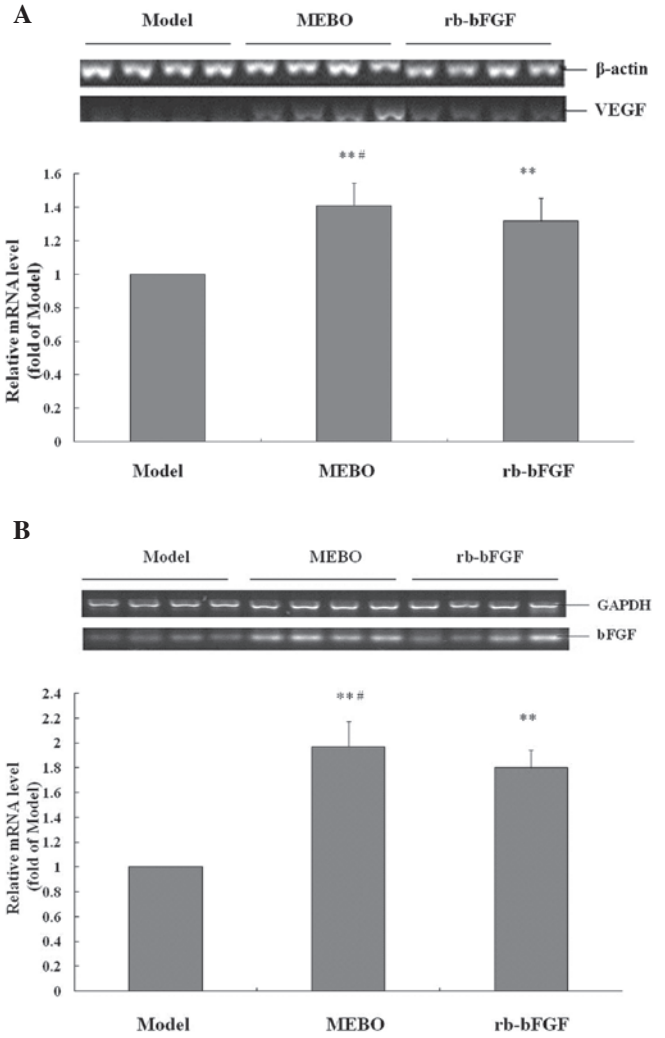


Figure 4. MEBO increased the gene expression of VEGF and bFGF. Total RNA was extracted from eight-day-old wound granulation, and mRNA level was analyzed by quantitative polymerase chain reaction. (A) Relative mRNA level of VEGF. (B) Relative mRNA level of bFGF. $^{**}$P<0.01, vs. the model group; $^{\#}$P<0.05, vs. the rb-bFGF group. MEBO, moist exposed burn ointment; VEGF, vascular endothelial growth factor; rb-bFBF, recombinant bovine-basic fibroblast growth factor.

the MEBO group were higher compared with the rb-bFGF group (all P<0.05).

## Discussion

In China and other Asian countries, traditional Chinese medicine has been widely used to treat wounds in clinical practice due to its beneficial effects; however, convincing evidence is lacking, and the mechanisms remain unclear.

In the present study, MEBO, a Chinese herbal ointment, promotes granulation tissue formation and reduces the time of wound healing in cutaneous excisional wounds, suggesting that it is effective for wound healing, which is consistent with the results of previous studies (34,35,38). It is well established that excisional wounds invariably destroy tissue integrity, and lead to vascular injury and fibrin-fibronectin clot formation, thus leading to platelet recruitment, and subsequently upregulation of growth factors and cytokines, including VEGF and bFGF (5,39), which triggers the formation of granulation tissue and wound healing. The wound healing process involves migration and proliferation of cells, including vascular endothelial cells and fibroblasts. Further investigation has demonstrated that MEBO increases neovascularization in the granulation tissue, implicating the beneficial effects of MEBO on vascular endothelial cell proliferation. Furthermore, MEBO also increased fibroblasts in the granulation tissue. Increases in the number of fibroblasts primarily arise due to the proliferation of resident fibroblasts in response to growth factors, including bFGF, and fibroblasts migrating from the surrounding connective tissue into the wound site (40). It was observed that recombinant bFGF accelerates the wound healing process (14,41), thus, rb-bFGF was used as a positive control for MEBO. In the current study, rb-bFGF promoted the formation of granulation tissue, increased neovascularization and the number of fibroblasts in the granulation tissue, and reduced the time of wound healing, which were all consistent with the effects of MEBO.

It is generally accepted that VEGF induces endothelial cell proliferation and migration, promotes vascular permeability and angiogenesis, increases collagen deposition (5,9-11), and that bFGF mediates angiogenesis, promotes the proliferation and migration of endothelial cells and fibroblasts proliferate and migration (12-15,42). Therefore, it was inferred that MEBO promotion of wound healing in rats involves growth factors, including VEGF and bFGF. In order to confirm this hypothesis, western blot analysis and RT-PCR were respectively used to analyze the protein and mRNA expression of VEGF and bFGF. Notably, MEBO enhanced the protein expression of VEGF and bFGF and also elevated their mRNA expression. A previous study reported that MEBO enhanced α-smooth muscle actin in fibroblasts, indicating that MEBO activates fibroblasts (43). It is hypothesized that leukocytes, including fibroblasts and endothelial cells are the primary origin of VEGF and bFGF (41). Furthermore, bFGF upregulates the expression of VEGF (9,41). Decreased levels of VEGF and/or bFGF, or inhibition of their activity leads to the limited migration of fibroblasts and the delay of wound healing (20,44). In the present study, rb-bFGF enhanced the protein and gene expression of VEGF and bFGF, which is similar to the results of a previous study (45). It is well known that the expression of VEGF and bFGF is increased following skin injury, particularly in the early stage of healing. A previous report showed that the acute wound fluid, which is rich in VEGF and bFGF, increases the mRNA levels of VEGF and bFGF (45). Since a similar effect between MEBO and rb-bFGF in treating cutaneous excisional wounds was observed, it was concluded that MEBO promotes wound healing by increasing VEGF and bFGF production.

Furthermore, compared with rb-bFGF, MEBO took less time to promote wound healing, and led to a higher production of VEGF and bFGF. In addition, increasing evidence indicates that MEBO promotes wound contraction and improves scar quality of wounds (43,46). Therefore, MEBO is hypothesized to be an ideal therapy for cutaneous wounds.

In conclusion, MEBO promotes cutaneous excisional wound healing by at least partially enhancing the production of VEGF and bFGF, which reveals part of the underlying mechanism and suggests the use of MEBO for the treatment of delayed healing cutaneous wounds.

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