

Therapeutic effect of the epidermal growth factor on diabetic foot ulcer and the underlying mechanisms

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Abstract. The present study aimed to explore the therapeutic effect and underlying mechanism of epidermal growth factor (EGF) on the wound healing of diabetic foot ulcers (DFU). A total of 48 rabbits with DFU were randomly divided into 2 groups, comprising the treatment and control groups. Full-thickness skin (10x10 mm) was excised from the thigh of each rabbit. The wounds in the treatment group were treated with 100 mg/l EGF once a day for 1 month. The control group received no treatment. At 20 days following treatment, new granulation tissues that formed beyond the edge of the wound were collected for subsequent analysis. Tissues from rabbits in the treatment group produced a greater number of fibroblasts, which exhibited a fibroblastic morphology when compared with those in the control group. In the treatment group, a larger number of these fibroblasts were observed as clusters, and there were numerous blood vessels when compared with the control group. The fibroblasts in the control group exhibited an irregular morphology, contained fewer organelles and the surrounding collagenous fibers were sparse. These fibroblasts also demonstrated a disordered arrangement and it was revealed that the wound healed at a slower rate compared with the treatment group. Endogenous EGF mRNA detection revealed that there was a significant difference ($P<0.05$) in the relative gray value of EGF mRNA between the treatment (103.27 ± 4.27) and control (63.88 ± 4.36) groups. In conclusion, EGF may accelerate the healing of DFU, and exogenous EGF treatment may upregulate the expression of EGF mRNA in newly generated tissues.

Introduction

Diabetic foot ulcer (DFU) is a disorder observed primarily in developing countries. The blood supply in the extremities of patients with diabetes is poor, thus leading to the development of DFUs (1). The wounds require a long time to heal and may be incurable; dry gangrene can also develop as a result of DFU, which may lead to amputation in severe cases (1). Diabetes associated with acquired coagulopathies, particularly those caused by vitamin K deficiency or liver disease, may lead to excessive bleeding from the DFU and delay the healing process (2). In these cases, the use of fibrin adhesives and collagen matrices to the affected area are required (3). Such wounds can be infected by various pathogens that are difficult to treat, including fungi from the genera *Fusarium* or *Aspergillus* (4,5). The study of the pathogenesis of DFU has focused on poor wound healing and delayed wound healing due to dry gangrene (6,7). Numerous studies have attempted to elucidate the mechanisms underlying refractory DFU and signal transduction pathways in DFU lesions; however these studies have not been successful (8,9). A study suggested that offloading, debridement and moist dressing are currently used to treat DFUs with little success (9). Unsuccessful DFU treatment is caused by a range of factors, including the varying comorbidities observed for each diabetic patient (10). Poor wound healing is considered to be associated with poor glycemic control, refractory infection, anti-inflammatory cytokines and a relative or absolute deficiency of corresponding cytokine receptors, and vascular lesion (11). Refractory DFU lesions primarily form due to decreased expression levels of epidermal growth factor (EGF) and its receptor in DFU lesions (12). It has been demonstrated that applying active EGF to the DFU lesion may promote wound healing (13,14). EGF may also promote the proliferation of gliocytes and fibroblasts, neo-epidermal thickening, peripheral nerve regeneration, as well as mediate the proliferation, migration and differentiation of gliocytes and fibroblasts (15). Previous studies have demonstrated that EGF stimulates protein synthesis by modulating the replication and signal transduction of epidermal cell DNA and RNA (16,17). Previous studies investigated the repair function of the EGF receptor in the colonic mucosa of patients with ulcerative colitis (18-21). The authors demonstrated that EGF was involved in repairing inflamed mucous membranes

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in these patients. On the basis of these previous studies, and in order to aid the development of novel pharmaceutical devices based on nanotechnology for the treatment of refractory or non-healing DFU, the present study attempted to elucidate the mechanism underlying the therapeutic effect of EGF on DFU.

Materials and methods

Animals. A total of 65 healthy New Zealand white rabbits (age, 2 years old; male to female, 1:1; weight, 2.5–3.0 kg) were provided by the Jiexiang Xian Yongwang Rabbit Sales Center (Jining, China). All the rabbits were conventionally raised with natural light, laminar circulation filter air, an indoor temperature of $25 \pm 1^\circ\text{C}$ and the humidity at $40 \pm 5\%$ (22–24). All animals had free access to food and water. The present study was approved by the Ethics Committee of The Affiliated Qingdao Hiser Hospital of Qingdao University (Qingdao, China).

Experimental reagents. The following materials and reagents were used in the present study: EGF solution (Wuhan Healthgen Biotechnology Corp., Wuhan, China); Sumianxin (Jilin Huamu Animal Health Product Co., Ltd., Changchun, China); alloxan monohydrate (Hefei Bomei Biotechnology Co., Ltd., Hefei, China); glucometer and blood glucose kit (Hua Xia Kangning Xuzhou Medical Technology Co., Ltd., Xuzhou, China); RNA PCR kit (AMV) Ver.3.0 (Takara Biotechnology Co., Ltd., Dalian, China); and the RNApur Pure Tissue kit [cat. no. DP431; Tiangen Biotech (Beijing) Co., Ltd., Beijing, China].

Experimental equipment. The following equipment were used in the present study: NanoDrop ND-1000 Ultraviolet Spectrometry Photometer (Shanghai Spectrum Instruments Co., Ltd., Shanghai, China); CO_2 incubator (Shanghai SANTN Instruments Co., Ltd., Shanghai, China); a gel imaging system (UVP, Inc., Upland, CA, USA); a -80°C freezer (Wuxi Guanya Refrigeration Technology Co., Ltd., Wuxi, China); an light microscope; a transmission electron microscope; Nucleic Acid and Protein Analyzer (Suzhou Qile Electron Technology Co., Ltd., Kunshan, China); and a gradient PCR instrument (Beijing Bohui Innovation Technology Co., Ltd., Beijing, China).

Induction of type II diabetes. New Zealand rabbits were fed with a high-fat/high-sucrose diet for 2 months prior to the induction of type II diabetes, as previously described (25,26). Following this 2-month period, diabetes was induced by administering rabbits with 50 mg/kg body weight alloxan monohydrate (2.5%) via the ear of each rabbit every 3 days following 6 h of fasting. On day 10, the rabbits were fasted overnight (8–10 h with water) prior to the collection of blood samples. The rabbits that exhibited a fasting blood glucose level >11.1 mmol/l were admitted to the type II diabetes model. A total of 48 rabbits were included in the present study as 17 rabbits did not meet the experimental standard.

Experimental groups and therapeutic measures. The remaining 48 type II diabetes rabbits were randomly and equally divided into treatment and control groups. The legs of all rabbits were disinfected with 100 ml iodophor solution (Shandong Lierkang Medical Technology Co., Ltd., Dezhou, China) and animals

were anesthetized using 200 mg/kg Sumianxin administered to the hind leg, where muscle was abundant. A 10×10 mm section of full-thickness skin was excised, followed by a radial debridement of the wound. For the treatment group, 100 mg/l EGF solution was applied to the wound every day for 1 month; continuous contact between the EGF solution and wound was maintained as described previously (27). No treatment was administered to the control group. During the experiment, the feeding and sanitary conditions of the 2 groups were the same. A digital camera was used to capture images of the DFU wound, and record the wound healing rate and healing area in real time once every five days, using Masson and hematoxylin and eosin (HE) staining. The wound healing rate calculation formula was as follows: Wound healing rate = (original wound area - non healing area)/original wound area.

Histological analysis. Following 20 days of treatment, a 2-mm region of newly produced granulation tissue from the DFU site was collected from all rabbits in both groups. Prior to tissue collection, the rabbits were injected with 200 mg/kg body weight Sumianxin intramuscularly (22,23). The collected samples were irrigated with distilled water and divided into four sections. These tissue sections were then used for Masson staining, HE staining, electron microscopy observation and reverse transcription-polymerase chain reaction (RT-PCR) analysis of EGF mRNA levels.

Masson staining. Healing and granulation tissue specimens were removed from the surface of the diabetic foot ulcer. The samples were fixed in 10% formaldehyde solution, embedding in paraffin and dewaxed (thickness, 6 μm). Staining was performed according to the manufacturer's protocol connective tissue Masson staining kit (Abcam, Cambridge, UK). Samples were incubated with Masson staining solution at 37°C for 5 min. Following two washes with 0.2% acetic acid for 5 min, the sections were stained with 1% aniline for 5 min at room temperature. Following two more washing steps with 0.2% acetic acid for 5 min, sections were dehydrated 10 sec at room temperature with absolute alcohol and rendered transparent 5 min at room temperature using xylene. Sections were observed using a light microscope (magnification, $\times 400$).

HE staining. After 15 days, healing specimens were removed from the surface of the diabetic foot ulcer of the experimental group and fixed (10 min; 20°C) in 40 g/l paraformaldehyde solution. Gradient ethanol dehydration (35°C , 1.5 h) and xylene (30 min; 35°C) was used in the sample preparation prior to embedding the specimens in paraffin (60 min, 56°C). The specimens were sliced into 5- μm -thick sections and xylene was used to dewax the samples (37°C , 10 min). They were stained with HE for 5 min 35°C . The growth of fibroblasts and blood vessels was observed using a light microscope (magnification, $\times 1,600$).

Molecular level detection. Total RNA was extracted using TRIzol reagents (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) from the granulation tissue samples. This was performed according to the manufacturer's instructions. The synthesis of cDNA was carried out using a reverse transcription kit (RevertAid First Strand cDNA Synthesis kit;

Table I. Primer sequences used for reverse transcription-polymerase chain reaction analysis.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Size (bp)
Epidermal growth factor	TTGCTGCTCTACCTCCACCAT	CTGCATTACATTTGTTGTGC	354
β -actin	CAACACGCCGGCCATGTA	TCCATGCCCAGGAAGGAG	429

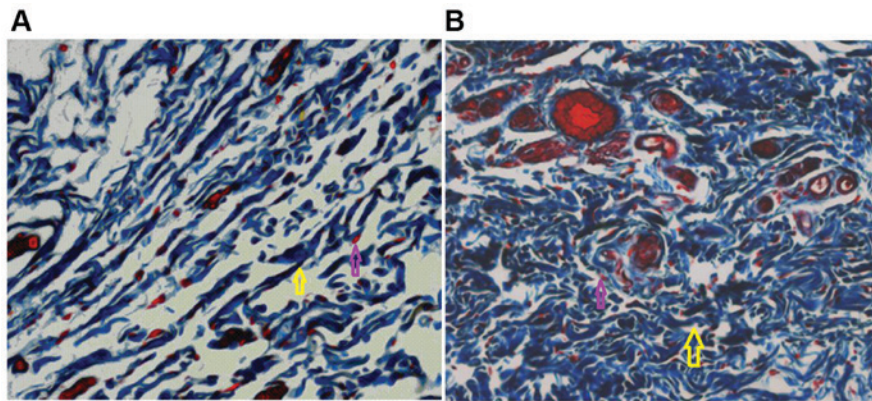


Figure 1. EGF increases granulation tissue production and the number of clustered fibroblasts. Masson staining was performed at 20 days following wound generation and treatment (A) with or (B) without EGF (magnification, x40). Yellow arrows highlight areas of clustered fibroblasts and the purple arrows highlight neutrophils. EGF, epidermal growth factor.

Fermentas; ThermoFisher Scientific, Inc.). The reaction mixture was incubated at 37°C for 30 min followed by inactivated at 85°C for 5 min. Polymerase chain reaction was performed with cDNA as template and the reaction volume was 20 μ l. EGF mRNA expression was analyzed by semi-quantitative RT-PCR using the Qiaquick PCR kit (Qiagen, Inc., Valencia, CA, USA). The thermocycling conditions were as follows: 42°C for 30 min, 94°C for 5 min, and 40 cycles of 94°C for 45 sec and 60°C for 80 sec. β -actin was used as internal reference gene. Amplification products were analyzed using the following protocol: A total of 5 μ l/lane RT-PCR reaction mixture was separated by 2% agarose gel electrophoresis at a voltage of 80 V. Samples were visualized using ethidium bromide. A gel imaging system was used to observe the gel and capture images, and the gray values were calculated using software (Shanghai Peiqing Science and Technology Co., Ltd., Shanghai, China). Densitometry analysis was performed using BioID (Vilber Lourmat, Marne la-Vallée, France). The primer sequences for EGF and β -actin are shown in Table I.

Capillary number. Fresh granulation tissue samples (1.0x1.0 mm) were obtained from the wound surfaces of animals of the control and treatment groups. Samples were fixed for 10 min at 20°C with 20% formaldehyde, dehydrated at 35°C for 1.5 h and embedded in paraffin for 2 min 22°C. Continuous sections (thickness, 5 μ m) were stained with HE solution as described. A light microscope was used to analyze the sections (magnification, x400). The number of capillaries with a complete lumen in each visual field was calculated and three visual fields per section were observed.

Statistical analysis. The data is presented as mean \pm standard deviation. Experiments were performed in duplicate. SPSS

statistics software (version 19.0; IBM Corp., Armonk, NY, USA) was used for data analysis. A normal distribution detection and homogeneity of variance test were conducted prior to comparison tests among groups. Non-normal data were analyzed using Kruskal-Wallis test. $P < 0.05$ was used to indicate a statistically significant difference.

Results

EGF increases wound healing rates. The wound healing of rabbits in the EGF treatment group was faster than that of rabbits in the control group (Fig. 1). The mean time for the wound to become fully epithelized. In the experimental group, 78% of the animals were healed following 12 days of treatment, while in the control group 25% of animals exhibited wound closure following 25 days observation. The rabbits in the control group healed by day 25 and the healing rate was 35%.

EGF increases granulation tissue production, neutrophil numbers, collagen uniformity and the extracellular matrix. The number of neutrophils in the experimental group was lower than that in the control group. Histological sections from the treatment and control groups were collected at 20 days following treatment. Granulation tissue production and the number of clustered fibroblasts were increased in the treatment group when compared with the control group (Fig. 1). The collagen bundles in the treatment group were dense and ordered, and the extracellular matrix was more abundant when compared with that of the control group (Fig. 2). In the control group, wound healing was relatively slow, histological structures were not evident and the collagen fibers were relatively sparse (Fig. 2).

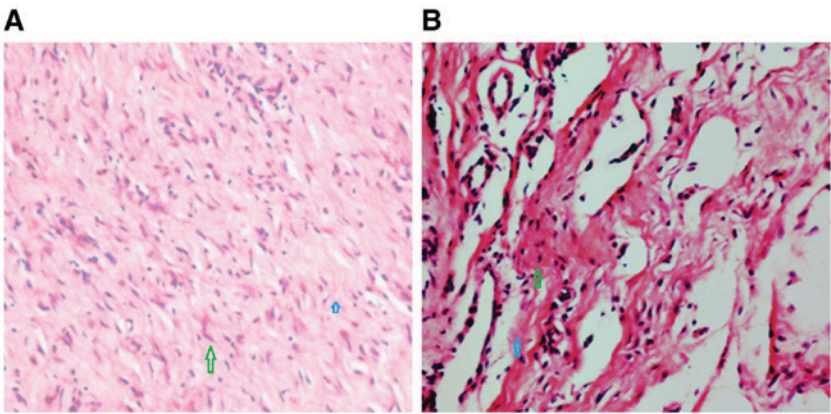


Figure 2. EGF increases the production of extracellular matrix and causes collagen structures to become dense and ordered. Hematoxylin and eosin staining was performed at 20 days following generation of the wound and treatment (A) with or (B) without EGF (magnification, x40). Blue arrows highlight the extracellular matrix and green arrows highlight collagen structures EGF, epidermal growth factor.

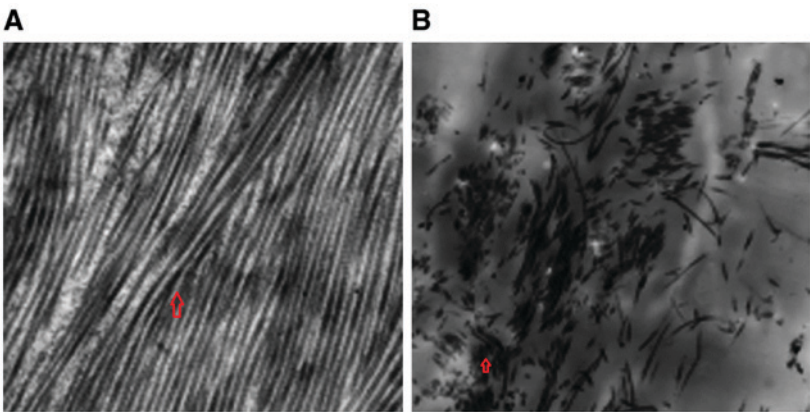


Figure 3. EGF increases the number of capillaries and fibroblasts, and improves the morphology of fibroblasts. Transmission electron microscopy was performed at 20 days following generation of the wound and treatment (A) with or (B) without EGF (magnification, x1,600). Red arrows highlight fibroblasts. EGF, epidermal growth factor.

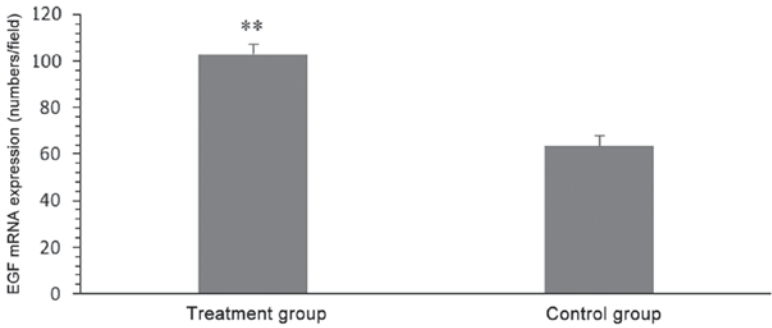


Figure 4. Epidermal growth factor mRNA expression in the treatment and control groups. **P<0.01

EGF increases the number of capillaries and fibroblasts, and improves fibroblast morphology. The number of fibroblasts and capillaries were increased in the treatment group compared with the control group (Fig. 3). The fibroblasts in the treatment group exhibited a uniform morphology and were abundant in organelles when compared with the fibroblasts in the control group.

Exogenous EGF treatment increases EGF mRNA expression. The relative gray value of EGF mRNA in the treatment group

was higher than that of the control group was (103.27±4.27 vs. 63.88±4.36; P<0.05; Fig. 4).

Discussion

When normal tissue is acutely injured, epithelial cells, fibrocytes and fibroblasts are damaged and the normal physiological process of wound healing is initiated (28). In DFU, the wound healing process is hindered by the accumulation of advanced glycation end products (AGEs), which is due to

the high blood glucose levels of patients with diabetes (29,30). AGEs competitively bind to the EGF receptor, thus preventing the binding of EGF and perpetuating the initial injury to the vascular endothelial cells and fibroblasts (31,32). This leads to an increase in apoptosis of injured vascular endothelial cells and fibroblasts, and a decrease in cytokines and collagen production; collectively these phenomena decrease angiogenesis (33). The levels of inflammatory mediators also increase in the extracellular matrix of DFU lesions (34,35). The wound healing process is inhibited by decreased neutrophil granulocyte numbers, inflammation, inflammatory cell chemotaxis and granulation tissue production (36). This type of wound takes a long time to heal and may never heal completely. However, topical treatments consisting of antibacterial nanoparticles (such as silver nanoparticle between 1 and 100 nm) have been used against different pathogens, particularly *Staphylococcus aureus* and it was observed that these treatments can encourage wound healing (37,38).

Previous studies have demonstrated that the degradation of growth factors, such as EGF, and their cell surface receptors are the main cause of refractory DFU wounds (39,40). EGF was the first growth factor used to study wound healing (41,42). A previous *in vitro* study demonstrated that EGF may promote mitosis and glycolysis (43). EGF receptors exist on the majority of cell surface membranes (44,45). EGF induces the migration of inflammatory cells away from the wound, leading to an improvement of the wound microenvironment and the nutritional status of the tissue (46). Also, EGF can promote epidermal proliferation increasing fibroblast numbers in partial thickness wounds (47-49). Cellulose promotes cell proliferation and wound repair (50). The aforementioned studies support the results of the present study, which indicates that EGF may serve a positive role in the DFU wound healing process.

EGF is secreted by circulating platelets, macrophages and mononuclear cells (51). The results of the present study demonstrated that EGF mRNA expression in the group treated with exogenous EGF was higher than that of the control group. This may demonstrate that exogenous EGF promotes the expression of endogenous EGF, which upregulates partial EGF gene expression (52,53). Experiments with single and multiple linear regression models should be designed and regression analyses performed to support the notion that exogenous EGF promotes endogenous EGF protein expression.

In conclusion, the results of the present study suggest that EGF binds to its corresponding receptor on epidermal cell and fibroblast cell surface membranes in order to build collagenous tissue, and accelerate the generation of wound granulation and epithelial tissues, which accelerate the wound healing process. The treatment of diabetic foot ulcer is long and expensive, and causes great pain to patients and families. Therefore, it is of great importance to find a novel treatment method DFUs. EGF demonstrated positive effects in promoting wound healing.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JZ and ZX designed the study. WH, QD and ZW were responsible for the collection and analysis of the data. JM and XC performed data analysis and interpretation. JZ and ZX prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The Affiliated Qingdao Hiser Hospital of Qingdao University (Qingdao, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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