Low-frequency ultrasound enhances vascular endothelial growth factor expression, thereby promoting the wound healing in diabetic rats

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Received September 28, 2018; Accepted August 16, 2019

DOI: 10.3892/etm.2019.8051

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Key words: low-frequency ultrasound, vascular endothelial growth factor, wound healing, diabetic rats

Abstract. Diabetes is a chronic metabolic disease with a high prevalence worldwide, which typically delays or impairs wound healing, potentially causing death. Low-frequency ultrasound treatment promotes the repair of various injuries and may promote wound healing. The aim of the present study was to determine whether low-frequency ultrasound can accelerate wound healing, as well as investigate its effects on the expression of vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β1, interleukin (IL)-6 and tumor necrosis factor (TNF)-α in diabetic rats. A total of 45 Wistar rats were intraperitoneally injected with 1% streptozocin following intraperitoneal injection of pentobarbital sodium anesthesia. Subsequently an incision wound was created in the skin of back. The area of the wound was recorded to calculate the rate of wound healing. The expression of VEGF and TGF-β1 was determined via immunohistochemical analysis and their mRNA and protein levels were measured via reverse transcription-quantitative PCR analysis. The results revealed that when compared with the control group, low‑frequency ultrasound treatment significantly increased wound healing rate in diabetic rats and markedly increased the mRNA and protein levels of VEGF and TGF-β1. US treatment also reduced the mRNA and protein levels of TNF-α and IL-6. In conclusion, the results of the present study indicated that low‑frequency ultrasound promotes the expression of VEGF and TGF-β1, and inhibits the expression of IL-6 and TNF-α, thereby promoting wound healing in diabetic rats.

Introduction

Diabetes is a highly prevalent chronic metabolic disease, affecting >400 million individuals worldwide, which often causes delayed or impaired wound healing (1), representing a major health concern and a heavy socioeconomic burden. According to incomplete statistics, 20% of patients with diabetes suffer from foot ulcers and >20% of patients require amputations (2-4). The rate of amputation is markedly higher among diabetic patients compared with the general population (2,5). Wound healing in diabetes is delayed and several therapeutic approaches are ineffective (6,7). The etiology of diabetic foot ulcers is complex. Age, sex, vascular disease, infection, blood pressure and smoking may affect the progression of diabetic foot ulcers, and the majority of studies have reported that the pathogenesis of diabetic foot ulcers is closely associated with ischemia, neuropathy and infection (8,9). Angiopathy (9), particularly in vascular diseases of the lower extremities, is the earliest and most common complication leading to the development of diabetic foot ulcers. Additionally, diabetic microangiopathy is a risk factor for diabetic foot. Due to long-term hyperglycemia, diabetic patients accumulate a large number of advanced glycation end-products in vivo, resulting in endothelial cell damage and apoptosis, thickening of the intimal vascular wall, luminal stenosis or obstruction (10). Furthermore, endothelial cell damage promotes platelet adhesion, erythrocyte aggregation and microthrombosis, leading to insufficient irrigation of the affected limb, with ensuing ischemia, hypoxia and eventually diabetic ulcer formation or aggravation of diabetic foot ulcers (10-12).

Wound healing is a complex biological process that may be divided into three stages: Inflammatory response, cell differentiation and proliferation, and tissue repair (13). Numerous factors may delay the wound healing process, including the inhibition of cytokine production by fibroblasts and inflammatory cells. During the early stages of wound healing, the overexpression of inflammatory factors, such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α (14,15), severely impairs the formation of granulation tissue and further delays wound healing. However, a number of studies have revealed that growth factors, including vascular endothelial growth factor (VEGF) (2,4,16), epidermal growth factor (17,18),...
and transforming growth factor-β (TGF-β) (19,20), serve an important role in the promotion of wound healing.

A number of treatment methods have been developed to promote wound healing in diabetic foot ulcers, including vascular reconstruction, negative pressure treatment, stem cell transplantation therapy, hyperbaric oxygen therapy and tissue engineering technology, among others (21-25). Although these treatment methods have improved the wound healing of diabetic foot ulcers, their efficacy is unsatisfactory. Hence, an increasing number of topical treatments have been developed, particularly involving debridement methods including surgical, biological, and dressing debridement, and have been widely applied for the treatment of diabetic patients in the clinical setting (26). Debridement eliminates necrotic tissue, decreases chronic inflammatory factor levels, increases cytokine secretion, promotes the growth of granulation tissue and reduces the absorption of toxins during necrosis tissue decomposition and degradation (27-29). Therefore, debridement is widely used in the clinical setting to promote wound healing in patients with diabetes (30). In addition, ultrasonic debridement may promote the repair of various injuries, including those of the bone, tendon, muscle, cartilage and ligament (31-33).

The aim of the present study was to determine whether low-frequency ultrasound accelerates wound healing and tissue regeneration in diabetic rats, and to investigate its effects on the expression of VEGF, TGF-β1, IL-6 and TNF-α.

Materials and methods

Animals. A total of 45 female Wistar rats, weighing 250-300 g, were purchased from the Laboratory Animal Center of North Sichuan Medical College. They were fed for 1 week at 18-2˚C in 12 h light/dark cycle with access to food and water ad libitum, and a humidity of 50-60%. The animals were handled humanely according to the guidelines provided in the Guide for the Care and Use of Laboratory Animals, published by the National Institute of Health (34). The rats were anesthetized by intraperitoneal administration of 2.25% pentobarbital sodium (45 mg/kg). All animal procedures were approved by the Ethics Committee of Affiliated Hospital of North Sichuan Medical College [approval no. 2016ER(A)022].

Streptozotocin (STZ)-induced diabetic rat model and experimental groups. A diabetic rat model was established in all Wistar rats using STZ, as described previously (35-37). Rats were intraperitoneally injected with 1% STZ (60 mg/kg) following anesthesia. A total of 1 week after STZ injection, the blood glucose levels of all rats were >16.7 mmol/l. The rats were subsequently placed in a prone position on a fixed plate, where a circular area (3.0 cm in diameter) was marked on the skin of the back. A skin incision was created and cleaned with iodine. The diabetic animals were then randomly divided into three groups (n=15) according to the different treatments administered: the untreated control group; the ultrasound (US) treatment group and the common treatment group. In rats of the US group, the wound was cleaned with normal saline and treated with low-frequency US (frequency, 1 MHz; sound intensity, 1.0 W/cm²) for 15 min. The working sound intensity range of low-frequency US is adjustable from 0.1-1.1 W/cm², with a frequency of 1 MHz and a repetition frequency of 1 KHz with continuous waves, accounting for 20% of the air ratio. The skin wound was then irradiated with an US sound intensity of 1.0 W/cm² once per day for 21 days. The sterile head of the ultrasound machine was connected to the ultrasonic debridement machine, with a saline bag used as the washing solution. When atomized water drops appeared on the machine head subsequent to first use, the front of the machine head was tilted to contact the wound surface at a 45° angle. The irradiated wound surface was then moved at a constant and slow speed. After horizontal scanning, the irradiated wound surface was vertically scanned to ensure irradiation of all wound surfaces. In the common treatment group, the wound was cleaned with normal saline alone once per day (35-37).

Estimation of the wound healing rate. All rats were observed on day 7, 14 and 21 after wound formation. The area of the wound was recorded to calculate the rate of wound healing as follows: Wound healing rate=(1-remaining-wound area/initial wound area) x100% (38).

Histological analysis. A biopsy sample was obtained from the wound edge on day 7, 14 and 21 to determine the pathological changes occurring within the wound. Part of the biopsy specimens was fixed in 4% paraformaldehyde at 4˚C for 48 h, embedded in paraffin sectioned (4 µm) and stained with hematoxylin for 3-8 min and eosin for 1-3 min (H&E) at room temperature to examine the pathological changes. The remaining part of the tissue was frozen at -70˚C to extract total RNA from rat skin.

Immunohistochemical (IHC) analysis. IHC semi-quantification analysis was performed as described previously (39), using a horseradish peroxidase-3,3′-diaminobenzidine (HRP-DAB) staining kit (Beyotime Institute of Biotechnology). Tissue sections from the different groups were blocked by 3% H2O2 at room temperature for 15 min, and then incubated with the following primary antibodies for 1 h: anti-rabbit VEGF (Abcam; cat. no. ab19393; 1:100) and anti-rabbit TGF-β1 (Abcam; cat. no. ab92486; 1:100) at 4˚C. Each antibody was diluted in PBS. Subsequently, samples were incubated with biotinylated goat anti-rabbit antibodies (Abcam; cat. no. ab6721; 1:100) for 30 min at room temperature. The specific binding of the secondary to primary antibodies was visualized using HRP for the enzymatic conversion of the chromogenic substrate DAB into a brown precipitate. The sections were mounted, cleared, cover-slipped, and examined under a fluorescence microscope (magnification, x100). The scale bar was 100 µm.

Reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from rat skin using the TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. Samples were then reverse transcribed using the Bestar™ qPCR RT kit (Takara Bio, Inc.). The RT conditions were 37˚C for 15 min and 98˚C for 5 min. qPCR was performed using the ABI Prism 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the SYBR® Premix Ex Taq™ kit (Takara Bio, Inc.). PCR amplification conditions were as follows: denaturing at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 1 min. GAPDH was
used for normalization. Data are presented as fold difference and were calculated using the $2^{\Delta\Delta Cq}$ relative expression method (40). The primers used were as follows: VEGF forward 5'-TCCAGGAGTACCCCGATGA-3' and reverse, 5'-CTCCGC TCTGAACAGGCT-3'; TGF-β1 forward 5'-TAAGGCTCG CCAGTCCCC-3' and reverse, 5'-GGTTTTGTCAAGAT TCGGTGTT-3'; TNF-α forward 5'-CTCTCAATTCCGTCT CGTGG-3' and reverse, 5'-TCCGCTTGGGTGTTTGC-3'; IL-6 forward, 5'-GCCCTTCTTGCGGACTGATGTT-3' and reverse, 5'-GCTCTGAATGACTCTGCTT-3'; GAPDH forward, 5'-TGAACGGGAAGCTCATTGG-3' and reverse, 5'-TCCACCACCTGTITGCTGTA-3'.

Western blotting. Total protein was extracted from cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) and determined using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology) in accordance with the manufacturer's protocol. Subsequently, 20 mg of the cell lysate was separated via 10% SDS-PAGE and transferred to PVDF membranes (EMD Millipore). The membranes were
then blocked with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 2 h at room temperature and incubated with the following primary antibodies obtained from Abcam overnight at 4˚C: Anti-rabbit VEGF (Abcam; cat. no. ab11939; 1:500), anti-rabbit TGF-β1 (Abcam; cat. no. ab92486; 1:1,000), anti-mouse TNF-α (Abcam; cat. no. ab9739; 1:1,000), anti-rabbit IL-6 (Abcam; cat. no. ab208113; 1:500) and anti-rabbit GAPDH (Abcam; cat. no. ab9385; 1:1,000). The immune complexes were then immunoblotted with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (Beijing ComWin Biotech Co., Ltd; 1:2,000). Immunodetection was performed using enhanced chemiluminescence reagents (Fdbio Science) by Image J 1.8.0 (National Institutes of Health).

Statistical analysis. Data were analyzed using SPSS 23.0 software (IBM Corp.), and were presented as the mean ± standard deviation. Data were analyzed using an unpaired Student’s t-test and one-way ANOVA with Bonferroni’s post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Low-frequency US treatment accelerates wound healing in diabetic rats. After each post-operative treatment, the width of the wound was measured every 7 days, and the rate of wound healing was calculated with the aforementioned formula. The results demonstrated that, compared with the control group, the size of the wound area was significantly decreased in the US treatment group (Fig. 1A). As presented in Fig. 1B, the wound healing rate in the US treatment group was higher compared with the control (25.12±2.06 vs. 14.89±2.53%; P<0.05) on the 7th day. However, as the treatment time was prolonged, the difference in wound healing rate became markedly higher between the US treatment group and the control group (14th day, 56.98±3.76 vs. 19.91±2.72%; P<0.01; 21st day, 85.62±4.16 vs. 26.89±2.64%; P<0.001). In addition, the wound healing rate in the US treatment group was higher compared with the common treatment group at day 7, 14 and 21 post-treatment (14th day, 56.98±3.76 vs. 28.86±2.54%; P<0.05, 21st day, 85.62±4.16 vs. 39.63±2.54%; P<0.01). Furthermore, the wound healing rate of diabetic rats treated with normal saline for 21 days was significantly higher compared with the control group (39.63±2.54 vs. 26.89±2.64%; P<0.05). The results indicated that low-frequency US treatment accelerates wound healing in diabetic rats.

Histological and IHC analysis. To determine the effects of low frequency US treatment on diabetic rat wound healing, H&E staining was performed to investigate the pathological
changes that occurred in all treatment groups. The results obtained from the biopsy specimens collected from the wound margin revealed a marked inflammatory response occurring on the 7th day. As the treatment time was prolonged, the inflammatory response decreased, and when compared with the control, more fibroblasts, collagen fibers and neovascularization were observed in the rats of the US treatment group on days 14 and 21 (Fig. 2A). IHC analysis was performed to determine the role of VEGF (Fig. 2B) and TGF-β1 (Fig. 2C) in the wound healing process of diabetic rats. Following treatment at all times, the results indicated that the expression of TGF-β1 and VEGF was significantly increased in the US treatment group compared with the control group. Expressions were also significantly increased in the US group compared with the common treatment group, and TGF-β1 and VEGF levels in the common treatment group were higher compared with the control group. Furthermore, as the treatment time prolonged, the expression of TGF-β1 and VEGF in the wound of US treated rats decreased on the 14th day, and the expression of TGF-β1 and VEGF then increased on the 21st day (Fig. 2B and C).

Low frequency US treatment upregulates the expression of TGF-β1 and VEGF in diabetic rats. To further investigate the difference in expression of TGF-β1 and VEGF in diabetic rats after receiving treatment, the mRNA and protein expression of these molecules was assessed in the wounds of diabetic rats via RT-qPCR and western blotting, respectively. The results revealed that on the 7th day, mRNA expression did not exhibit any marked difference among the three groups, except for the TGF-β1. TGF-β1 mRNA levels did not exhibit any marked differences among the three groups and TGF-β1 protein expression in the US group was significantly increased when compared with the control group (P<0.05). However, the mRNA (Fig. 3) and protein (Fig. 4) expression of VEGF in the US group were significantly increased on the 14th day (P<0.01 in both mRNA and protein) and 21st day (P<0.05 in the mRNA and P<0.01 in the protein) compared with the control group (Figs. 3A

Figure 3. Reverse transcription-quantitative PCR results of VEGF, TGF-β1, TNF-α and IL-6 in each group following treatment. The expression of (A) VEGF and (B) TGF-β1 in the US group was significantly increased on the 14th day and 21st day compared with the control. Furthermore, as treatment time prolonged, the expression of (C) TNF-α and (D) IL-6 were gradually reduced in the US group, with the expression being lowest on the 21st day. *P<0.05, **P<0.01 and ***P<0.001 as indicated. VEGF, vascular endothelial growth factor; TGF-β1, transforming growth factor-β1; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; US, ultrasound.
Figure 4. VEGF, TGF-β1, TNF-α and IL-6 in each group were determined after treatment via western blotting. (A) The expression of the four proteins were determined using western blot analysis. (B) The expression of VEGF in the US treatment group was significantly increased on 14th and 21st day compared with the control group. (C) The expression of TGF-β1 in the US treatment group was significantly increased on 14th and 21st day compared with the control group. (D) The expression of TNF-α in the US treatment group were gradually reduced, with the lowest expression level being exhibited on the 21st day as treatment time was prolonged. (E) The expression of IL-6 in the US treatment group were gradually reduced, with the lowest expression level being exhibited on the 21st day. *P<0.05 and **P<0.01 as indicated; #P<0.05 as indicated. VEGF, vascular endothelial growth factor; TGF-β1, transforming growth factor-β1; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; US, ultrasound.
and 4A and B); the mRNA (Fig. 3) and protein (Fig. 4) expression of TGF-β1 in the US group were significantly increased on the 14th day (P<0.01 in both mRNA and protein) and 21st day (P<0.05 in the mRNA and P<0.01 in the protein) compared with the control group (Fig. 3B and Fig. 4A-C), and the common treatment group (P<0.05; Fig. 3A and B and Fig. 4B and C). In addition, the mRNA and protein levels of TGF-β1 and VEGF in the common treatment group were increased when compared with the control group (P<0.05). Furthermore, as treatment time prolonged, the mRNA and protein levels of TGF-β1 and VEGF were markedly increased on the 14th day compared with the 7th day. Levels then decreased on the 21st day compared with the 14th day.

Low-frequency US treatment suppresses the inflammatory response of diabetic rats. To confirm the effect of low-frequency US treatment on the inflammatory response of rats receiving treatment during the wound healing process, levels of IL-6 and TNF-α were determined via RT-qPCR and western blotting. As the treatment time was prolonged, the mRNA (Fig. 3) and protein (Fig. 4) levels of IL-6 and TNF-α in the US treatment group were reduced. The results were similar for the common treatment group. However, the mRNA and protein expression levels of IL-6 and TNF-α in the control group were increased (Figs. 3C and D and 4D and E). Additionally, the mRNA and protein levels of IL-6 and TNF-α in the US treatment group were lower compared with those in the control group on days 7 and 14 (7th day, P<0.05 in both TNF-α and IL-6; 14th day, P<0.01 in both TNF-α and IL-6). The mRNA and protein levels of IL-6 and TNF-α were the lowest compared with the control group on the 21st day (P<0.001, Figs. 3C and D and 4D and E). Furthermore, the mRNA expression of IL-6 and TNF-α in the US treatment group were significantly reduced compared with the common treatment group at day 14 (P<0.05 in both TNF-α and IL-6) and 21 (P<0.01 in the TNF-α, and P<0.05 in the IL-6; Fig. 3C and D); the protein expression of IL-6 and TNF-α in the US treatment group were significantly reduced compared with the common treatment group at day 14 and 21 (P<0.05 in both TNF-α and IL-6; Fig. 4D and E).

Discussion

Diabetic patients often suffer from diabetic foot ulcers, occasionally requiring amputation, which severely affects patient health and poses a major socioeconomic burden to patients' families and society. Despite several interesting and promising experimental results, their application in the clinical setting has not been satisfactory to date. Hence, a novel and effective therapeutic method is urgently required. With advances in medical technology, previous studies have indicated that US treatment may promote the repair of various injuries, including bone, tendon, muscle, cartilage and ligament injuries (31-33). The results of the current study demonstrated that the wound healing rate of diabetic rats in the US treatment group (85.62±4.16%) was higher compared with that in the other groups at 21 days after treatment, indicating that low-frequency US may enhance epithelialization and granulation tissue formation, thereby accelerating wound healing in diabetic rats. These effects may be mediated by decreasing the inflammatory response and promoting the production of growth factors.

The process of wound healing may be divided into inflammatory response, cell differentiation, cell proliferation and tissue repair stages (13). TNF-α and IL-6 serve a dual role by promoting as well as hindering wound healing (41). During the early stages of the inflammatory response, TNF-α and IL-6 promote the chemotaxis of inflammatory cells to remove necrotic tissues and pathogens, and promote the production and secretion of various cell growth factors including VEGF, basic fibroblast growth factor and IL-8 (14,15), further accelerating the differentiation and proliferation of various repair cells, extracellular matrix formation and neovascularization, ultimately promoting wound healing (42). However, the continuation of the inflammatory response and overexpression of TNF-α and IL-6 may cause the accumulation of harmful substances and severely impair granulation tissue formation and wound healing (43). The results of the present study demonstrated that the expression of IL-6 and TNF-α in US treatment group on the 7th, 14th and 21st day decreased with the time. However, the expression levels of IL-6 and TNF-α in the control group were markedly increased compared with those determined on the first day. Additionally, as the treatment time was prolonged, the expression of IL-6 and TNF-α in US treatment group was gradually downregulated. These results indicated that low-frequency US markedly inhibited the expression of IL-6 and TNF-α in diabetic rats after treatment for 7 days and that their expression was gradually downregulated with increased treatment duration.

A number of studies have demonstrated that angiogenesis is the physiological basis of wound repair, which is regulated by cytokines via different signaling pathways that promote or inhibit wound healing (37,38). VEGF is a soluble factor that is one of the key regulators of angiogenesis, which binds to the VEGF receptor to induce the proliferation and migration of vascular endothelial cells via autocrine and paracrine pathways, ultimately regulating neovascularization (2,4,16,44). Additionally, TGF-β1 serves a key role in wound healing by mediating the chemotaxis of inflammatory cells, the differentiation and proliferation of fibroblasts, and the production and degradation of collagen and extracellular matrix (19,20,45). Decreased expression and dysfunction of TGF-β1 and its receptors may hamper wound healing. In the present study, the results of IHC examination indicated that the expression of TGF-β1 and VEGF was significantly increased in the US group compared with the control group. The highest result was observed on day 14 of US treatment, which subsequently decreased by day 21. To further investigate whether low-frequency US promoted the expression of TGF-β1 and VEGF, RT-qPCR and western blotting were performed. The results demonstrated that the mRNA expression of TGF-β1 and the mRNA and protein expressions VEGF did not differ significantly among the three groups on day 7. However, compared with the control group, the expression of TGF-β1 and VEGF in the US treatment group were significantly increased on days 14 and 21. The results confirmed that low-frequency US increased the expression of TGF-β1 and VEGF after treatment for 14 days, further promoting wound healing.

In conclusion, the present study indicated that low-frequency US increased the expression of TGF-β1 and VEGF, induced the proliferation and differentiation of vascular endothelial cells and fibroblasts, and regulated the process of neovascularization in a diabetic rat model. Furthermore, it
also reduced the expression of IL-6 and TNF-α after treatment for 7 days and regulated and suppressed the abnormal inflammatory response, thereby accelerating wound healing in diabetic rats. Although the different effects of radiation on diabetic wound healing have been verified, the underlying mechanism of low frequency US in wound healing should be elucidated in following pharmacodynamic experiments.

Acknowledgements

Not applicable.

Funding

The present study was funded by the Scientific Research Foundation of the Education Department of Sichuan Province, China (grant no. 150ZB0186) and the Research and Development Program of North Sichuan Medical College (grant no. CBY13-A-ZP01).

Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors’ contributions

LC conceived the current study, analyzed the majority of the data and wrote the initial draft of the manuscript. QZ, XC, JW and LW refined the study design, performed additional analyses and finalized the manuscript.

Ethics approval and consent to participate

The present study was approved by IRB of the Affiliated Hospital of North Sichuan Medical College Approval Notice (file no. 2016 ER(A)022).

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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