Lasers in Medical Science (2019) 34:421–425
https://doi.org/10.1007/s10103-018-2559-9

BRIEF REPORT

Promotion of wound healing through low-fluence ablative fractional laser treatment in diabetic mice

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Received: 20 February 2018 / Accepted: 6 June 2018 / Published online: 14 June 2018
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Abstract

Chronic ulcers are a significant cause of morbidity in diabetic patients, which can greatly affect a patient's quality of life. While numerous methods have been developed to promote and enhance wound healing in diabetic patients, a convenient, effective treatment for diabetic ulcers has yet to be established. Here, we demonstrate the promotion of wound healing using a low-fluence (2 mJ/spot) ablative fractional laser (AFL) treatment in diabetic mice. Treatment was shown to confer increases in mRNA expression and in protein abundance of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), along with decreases in mRNA expression and protein abundance of transforming growth factor beta (TGF-β). Taken together, these results suggest that low-fluence AFL treatment can be used to promote healing in chronic diabetic wounds.

Keywords Diabetics · Fractional laser · Wound healing · Wound repair · Regeneration

Introduction

Diabetes is one of the most common metabolic diseases worldwide, affecting ~8.8% of all individuals in 2015 [1], resulting in significant long-term complications in patients with uncontrolled disease [2]. Chronic ulcers are among the most common of these complications, resulting in significant morbidity, which can greatly affect a patient's quality of life [3].

Various approaches have been used to promote wound healing in diabetic patients, including skin grafts; use of growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF); and even stem cell therapy; however, none of these approaches has been widely adopted. Skin grafts are frequently associated with scarring and contraction, while application of growth factors can be inconvenient and is typically not cost-effective. Application of stem cells is also inconvenient, requiring special equipment and facilities, combined with an uncertain safety profile due to the possibility of malignant transformation [4–6].

Recently, ablative fractional laser (AFL) treatment has been shown to improve wound healing in patients with chronic ulcers [7, 8]. AFL accelerates re-epithelialization, inducing rapid wound healing from the normal skin surrounding the microthermal zone [9]. This approach has been successfully used to treat a variety of skin diseases including acne scars and photo-damaged skin [10]. It is now performed to various skin conditions such as alopecia and vitiligo beyond the scars, although the exact mechanism has not been elucidated fully [11, 12]. In the present study, we examined the therapeutic effects of AFL treatment on impaired wound healing in a diabetic animal model and provide insights into the mechanism of action underlying these results.

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Materials and methods

Animals

Seven-week-old male db/db mice, spontaneous type 2 diabetic animal model, were purchased from Samtako Bio Korea Co. (Osan, Korea). All mice were maintained under standard laboratory conditions (25 °C, 60% relative humidity, and a 12-h

light/dark cycle) and acclimated for 2 weeks prior to experimentation. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at St. Vincent's Hospital, The Catholic University of Korea (Animal IRB 16-2).

Pilot study for determining appropriate energy level

Before collecting data, we performed a round of preliminary investigations to determine an appropriate energy level for AFL treatment. First, the back hair of all mice was shaved with a hair clipper, followed by a hair removal cream 1 day prior to the experiment. Four 15 mm × 15 mm wounds were formed on each of our test mice by removing full-thickness skin with scissors ($n = 3$). Each mouse was treated with an ablative CO_2 fractional laser (eCO2, Lutronic, Goyang, Korea) with different pulse energy of 2, 6, and 12 mJ/spot (spot size of 120 μm, and 300 spots/cm^2) across each wound edge and the surrounding normal skin. One wound was used for each energy setting, with the remaining wound used as the untreated control. Following treatment, wounds were covered with Tegaderm (3M, Maplewood, MN). Treatment was performed once per week (a total of 2 sessions for 16 days) beginning on the fourth day after wounding and observed for 16 days.

Determination of the number of laser treatments at the same level

Mice ($n = 8$) were divided into two groups and treated with ablative CO_2 fractional laser therapy either once or twice per week. Two wounds per mouse were made. Ablative CO_2 fractional laser therapy was performed using the same method described in the pilot study, using an energy setting of 2 mJ/spot. All mice were treated beginning on the fourth day and observed for 14 days.

Measurement of wound closure

Photographs were taken on the day of surgery and postoperative days 4, 7, 11, and 14. Wound area was analyzed by tracing the wound margin using Image J software (NIH, Bethesda, MD).

RT-PCR analysis

Total RNA was isolated from skin samples collected from the wound margin using Trizol Reagent (Life Technologies, Carlsbad, CA). cDNA was generated using the Reverse Transcription Master Premix (Elpis Biotech, Daejeon, Korea) according to the manufacturer's instructions. cDNA was then analyzed by real-time PCR using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Mannheim, Germany), and run on a LightCycler 2.0 (Roche Diagnostics). All primer sequences are listed in Table 1.

Western blot

Skin samples were collected from the wound margin and were homogenized in lysis buffer. After quantification, 20 μg of total protein per sample was run on an SDS polyacrylamide gel and transferred to a 0.45-μm PVDF membrane (Thermo Fisher Scientific, Waltham, MA). Samples were then immunoblotted using the following antibodies: GAPDH (Cell Signaling Technology, Beverly, MA), VEGF-A (Abcam, Cambridge, MA), bFGF (Cusabio Biotech, Wuhan, China), and TGF-β (Abcam). The membrane was probed with anti-rabbit IgG-horseradish peroxidase conjugates at room temperature for 1 h, and the bands were visualized using an enhanced chemiluminescence (ECL) substrate (Thermo Fisher Scientific).

Statistical analyses

The degree of wound closure was determined by comparing treated and untreated control based using an independent samples t test after Bonferroni's correction. Treatment responses were assessed based on energy level and the treatment frequency across each time point tested. Differences in mRNA expression of growth factors in skin samples between the laser treatment and control groups at each time point were compared by independent t test. p values ≤ 0.05 were considered significant.

Results

Effective wound healing in low-fluence AFL treatment

The percentage of the remaining wound area was significantly smaller in the 2 mJ/spot (11.4 ± 0.15%), 6 mJ/spot (17.2 ± 1.7%), and 12 mJ/spot (12.8 ± 2.8%) groups, relative to untreated controls (28.6 ± 3.4%) on day 16 ($p < 0.05$ for all). The most effective energy level was the 2 mJ/spot setting, although the statistical comparison was not performed (Fig. 1a, b).

Minimal differences based on treatment frequency

The percentage of the remaining wound area on day 14 was 55.4 ± 7.9% in the control group, compared with 31.3 ± 9.4% and 32.1 ± 9.2% in the once and twice weekly treatment groups, respectively (Fig. 1d). No significant differences were observed between the once and twice weekly treatment groups at the fluence of 2 mJ/spot (Fig. 1c, d).

Table 1 Primer sequences for real-time polymerase chain reaction

Gene	Forward	Reverse
VEGF	CAACATCACCATGCAGAT	TCACCGCCTTGGCTTGTCAC
bFGF	GGACGGCTGCTGGC TTCTAA	CCAGTTCGTTTCAGTGCCAC ATAC
TGF-β	AACAATTCCTGGCGTTACCTT	CTGCCGTACAACTCCAGTGA
GAPDH	TGCACCACCAACTGCTTAGC	TCTTCTGGGTGGCAGTGATG

Increase in mRNA expression of VEGF and bFGF, combined with decreases in TGF-β after treatment

VEGF mRNA expression in the treatment group (2.38 ± 0.03-fold) was significantly higher than that of untreated controls (0.79 ± 0.25-fold) on day 4, followed by gradual decreases thereafter, with no significant differences evident by day 11 ($p < 0.05$; Fig. 2a). bFGF mRNA expression was also elevated in the treatment group (1.67 ± 0.30-fold), relative to controls (0.97 ± 0.15-fold), though this increase was sustained until at least day 11 ($p < 0.05$; Fig. 2b). In contrast, TGF-β mRNA expression in the treatment group (5.33 ± 0.47-fold) was significantly lower than that of untreated controls (8.73 ± 1.73-fold) on day 14 ($p < 0.05$; Fig. 2c).

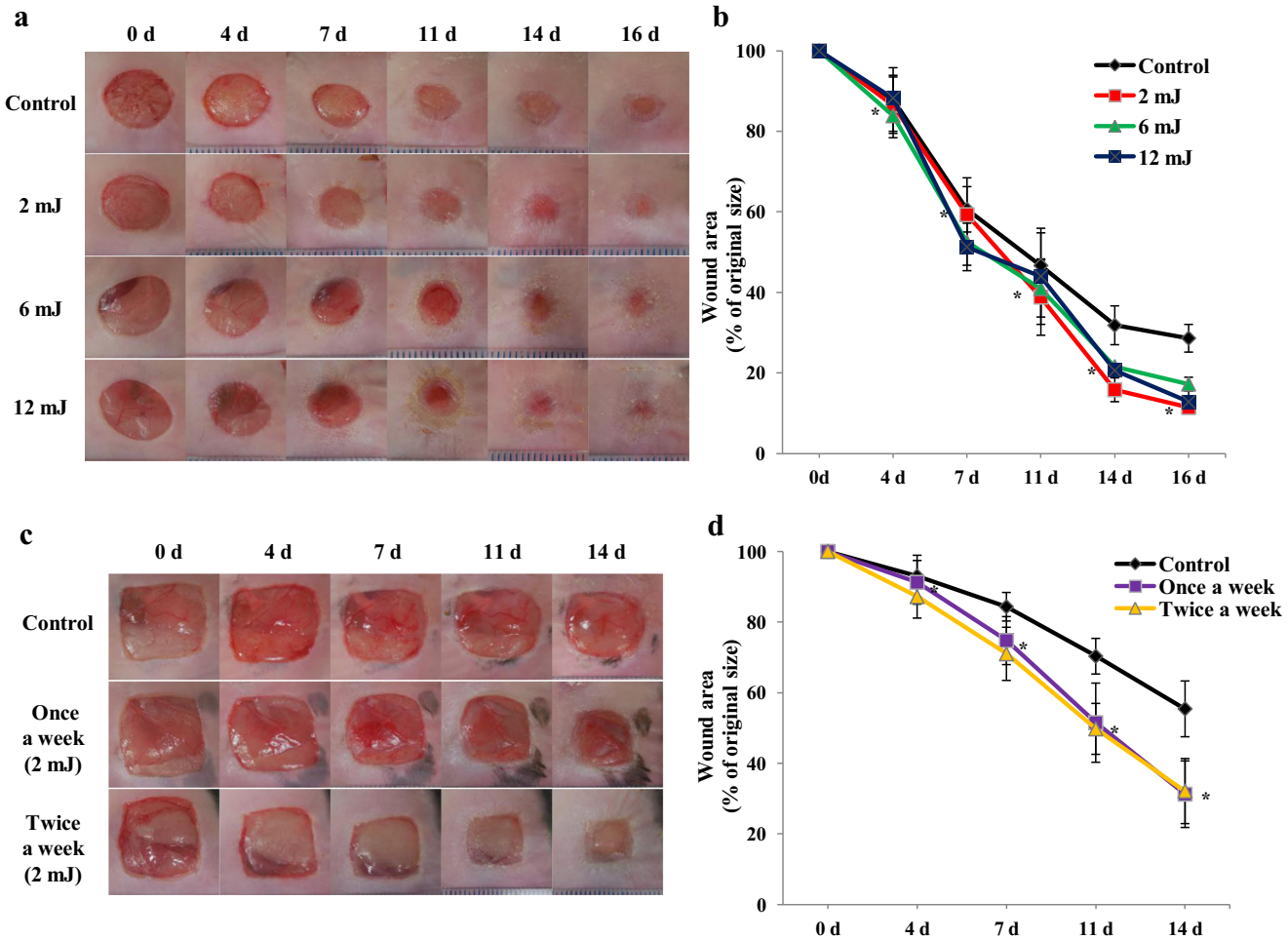

Fig. 1 Wound area closure after ablative fractional laser treatment. **a** The pictures at day 0 were taken immediately after injury. Control wounds received no treatment; each laser treatment group was treated once per week, beginning on day 4 post-injury. **b** The percentage of wound area closure at each time point (days 0, 4, 7, 11, 14, and 16) was measured to compare wound size relative to the original wound area (day 0). Data are presented as mean ± SEM; *$p < 0.05$. **c** Each group was treated using an energy setting of 2 mJ/spot, either once or twice per week. **d** The percentage of wound area closure at each time point was compared against the original wound area on day 0. The data are presented as mean ± SEM; *$p < 0.05$

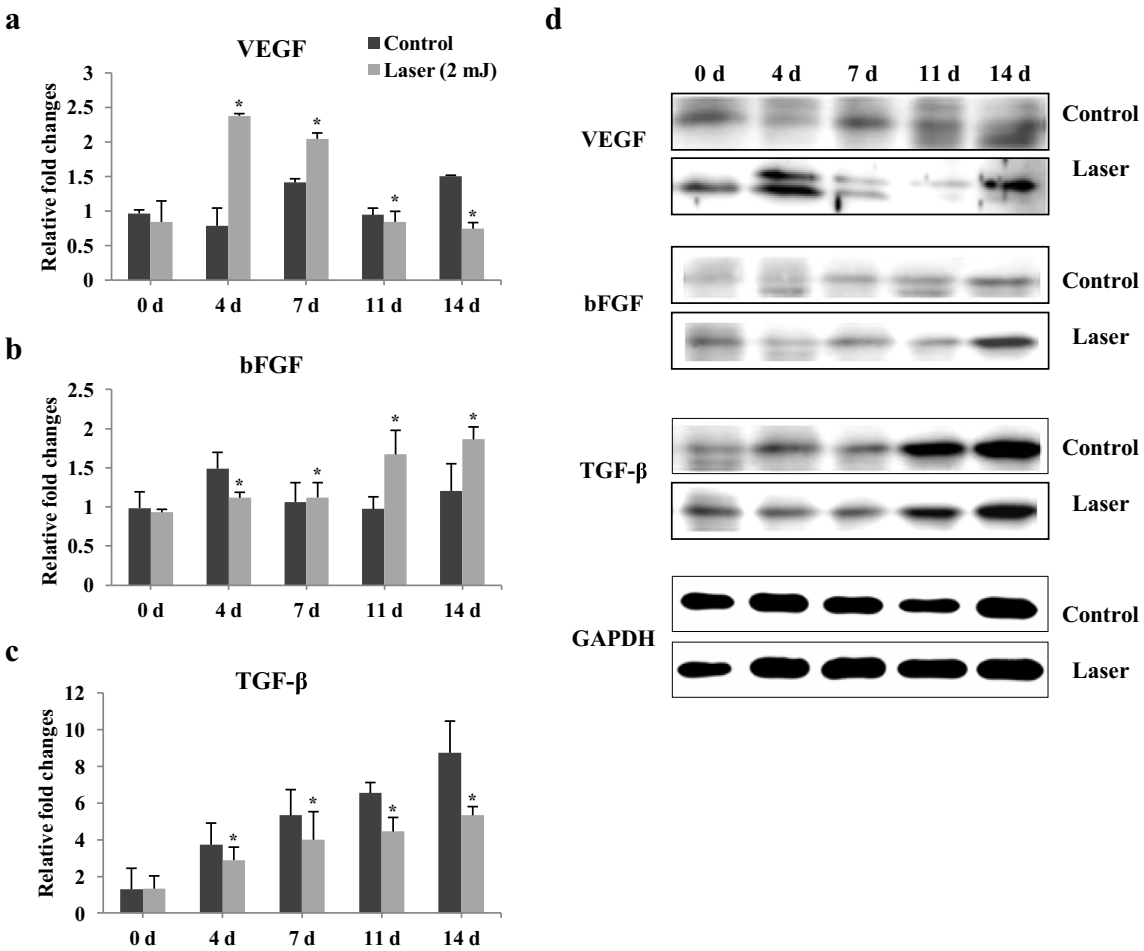

Fig. 2 Changes in the expression of the growth factors associated with wound healing. The mRNA expression levels of **a** VEGF, **b** TGF-β, and **c** bFGF at each time point (days 0, 4, 7, 11, 14, 16) were measured by real-time PCR. Results are presented as the relative fold change of each growth factor compared with normal skin at each time point. The data are presented as mean ± SEM; $*p < 0.05$, laser (2 mJ) vs. control. **d** Expression of VEGF, TGF-β, and bFGF proteins at each time point was examined by western blot

Increases in VEGF and bFGF and decreases in TGF-β protein level after AFL treatment

AFL-treated wounds exhibited a 3.7-fold increase in VEGF protein levels relative to untreated controls on day 4. Similarly, bFGF protein levels were increased up to 1.5-fold in treated wounds compared with controls, with the greatest differences seen on day 14. As with mRNA expression, the treatment group showed a marked decrease in TGF-β protein levels compared with untreated controls on day 11 (Fig. 2d).

Discussion

We demonstrated that AFL treatment (2, 6, and 12 mJ/spot) was effective to promote wound healing in a diabetic mouse model. The low-fluence AFL treatment (2 mJ/spot) was as effective as high-fluence treatment (6 and 12 mJ/spot), and little difference was shown between once- and twice-weekly treatments. The effect of AFL treatment does not seem to be related to column depth, since the column becomes deeper with higher fluence.

The low-fluence AFL treatment promotes wound healing via stimulation of various growth factors rather than simple skin contraction. The low-fluence AFL treatment was shown to increase mRNA and protein levels for both VEGF and bFGF, while decreasing levels of TGF-β. These changes are consistent with that of enhanced wound healing, as VEGF and bFGF stimulate a number of important wound healing processes, including cell proliferation, vasculogenesis, and collagen synthesis, all of which are impaired in chronic diabetic wounds [13]. Furthermore, overexpression of TGF-β has been reported in the chronic wounds of diabetes patients and is considered a symptom of prolonged wound healing [14]. In the present study, the laser group after AFL treatment showed increases in VEGF and bFGF expression, similar to the normal wound healing process, combined with the resolution of TGF-β overexpression.

This study had some limitations. First, mouse skin does not perfectly recreate the condition seen in human skin; however, our results were comparable with previous reports of laser treatment in humans, suggesting that mice are a suitable model for this condition. In addition, we investigated the expression of various growth factors at various time points within our treatment protocol. Expansion of this analysis could help to determine the optimal protocol for use in animal models. Finally, the role of individual growth factors was not examined in this study.

Taken together, the data presented here show that AFL treatment promotes wound healing in chronic wounds via stimulation of VEGF and bFGF expression in a mouse model of diabetes. This approach represents a promising treatment option for chronic impaired wounds, offering both convenience and efficacy. Further clinical studies will be needed to confirm these results.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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