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ORIGINAL ARTICLE

The combination effect of ultrasound and laser therapy on wound healing in diabetic rat model: histological and biomechanical evaluations

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Abstract

Background Defect in wound healing is a serious problem in diabetes. There are several physical treatment approaches such as ultrasound and laser therapy. This study was designed to determine the combination effect of ultrasound and laser on diabetic wound healing in a rat model.

Methods Thirty Wistar rats were divided into five groups; healthy control, diabetic control, laser (wavelength of 905 nm), ultrasound (frequency of 3 MHz), and laser plus ultrasound for 5 days a week for 2 weeks. Diabetes was induced by injection of streptozotocin (55 mg/kg). Afterward, a 3-cm wound was made with an incision on the back of the animals. On the 14th day of the treatments, blood fasting glucose was determined by glucometer. Also, biomechanical and histological assessments of the tissues were performed by Van Gieson, trichrome stains, and tensiometry methods, respectively.

Results The collagen and elastin amounts were significantly decreased in diabetic control group in comparison to healthy group. The tensile parameters, collagen, and elastin amounts in diabetic rats under different treatments were remarkably increased than in diabetic control rats. Also, the combination of ultrasound and laser caused more increase in collagen and elastin levels as well as tissue tensile parameters than ultrasound or laser.

Conclusion It appears that ultrasound or laser intervention is effective in the wound healing of diabetes condition partly through increasing the amount of collagen and elastin and also in improving the biomechanical properties. In addition, the combination therapy of ultrasound and laser might be more effective than each one alone.

Keywords Diabetes · Wound · Ultrasound · Laser · Physical therapy

Introduction

Diabetes is among the most common endocrine diseases with several complications, including impaired wound healing [1–3]. Due to reduced angiogenesis, poor blood flow, and decreased pain and heat, the risk of inducing and developing wounds in diabetic patients is high. So that, small wounds gradually grow in deep wounds, and finally, along with the delay in the wound healing process, the possibility of amputation in diabetics increases by causing infection and its spread to the underlying tissues and bones [4].

Since the high cost of treatment, long-term hospitalization, and the high percentage of amputations, various methods have been used to treat diabetic wounds. In many cases, these methods have not been satisfactory for the complete treatment of diabetic wound [1, 5]. One of the physical methods of wound healing is the use of ultrasound (US), which has been considered a non-invasive

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and cost-effective method. In general, US waves play an effective role in wound healing by affecting the inflammatory phase and the proliferation phase [6]. Some studies have shown that in the treatment of diabetic and venous foot ulcers, US waves with a frequency of 0.5 to 3 MHz have accelerated the healing process to some extent due to increased blood flow, angiogenesis, and increased protein synthesis such as collagen and growth factors [7]. Also, it has been indicated that US waves by stimulating the cell proliferation phase through increased inflammatory mediators and migration of fibroblasts to the wound bed, could play an effective role in healing [8].

Various studies show that laser treatment can be considered as an adjunct treatment of wounds and cutaneous lesions by reducing pain, infection, and shortening the recovery period [9, 10]. Based on the useful features and high efficiency of laser therapy, it is even considered as a suitable alternative approach for the common treatment of diabetic wounds [11]. In some other animal studies, low-power infrared laser irradiation on diabetic wounds increased granulation, tissue formation and tissue stiffness [12]. In human studies, the use of low-power laser for diabetic foot wounds led to an increase in the quality of life and a decrease in the wound healing time [13]. Thus, laser therapy has been described as an effective method for the treatment of diabetic wounds.

Today, the use of combination therapy for wound healing is developing [14–16]. It was previously reported that the combination of US and electric field stimulation techniques can overcome their limitations and greatly increase the probability of chronic skin ulcer eradication [17]. It has been suggested that using US and laser treatment mechanisms simultaneously can lead to more favorable results in the treatment of pressure ulcers [16]. According to the above, photobiomodulation with low-level laser therapy accelerates biochemical reactions. Absorption of laser photon energy by cells can be converted into chemical kinetic energy. On the other hand, low-intensity US therapy has biomechanical and non-thermal effects on tissue. Micromassage of cells following US radiation causes collagen synthesis and improves tissue tensile strength. Therefore, it seems that the simultaneous use of laser photobiomodulation effects and biomechanical effects caused by US can more effectively accelerate the wound healing process through biochemical-biophysical mechanisms. With regard to having fewer side effects and a better safety, together with certain controversies surrounding each one of these two approaches, prompted us to clarify the combined effectiveness of these two methods on the diabetic wound healing in terms of biomechanical properties and histopathology.

Materials and methods

Study design

Thirty Wistar rats (220–250 g) were kept at 22 ± 2 °C with about 60% humidity. All animals were housed in the separate cages with a 12-h light/dark cycle and had free access to their related diet and water. The rats were divided into five groups: healthy control (labeled as control), diabetic control (labeled as diabetic), diabetic exposed to laser, diabetic exposed to US, diabetic exposed to laser and US (labeled as combined) for 5 days a week for 2 weeks. First, following overnight fasting, diabetes was induced by injection of streptozotocin (STZ) at a dose of 55 mg/kg body weight intraperitoneally (i.p.). The fasting blood glucose (FBG) level of animals before STZ injection was 92 ± 12 mg/dl which was within the normal range. On the 3rd day after STZ injection, FBG level was 558 ± 55 mg/dl which confirmed diabetic rats. The level of FBG was determined by a glucometer (Accu-Chek, Roche, Germany). Then, the rats were anesthetized by i.p. injection of ketamine-xylazine (90 and 10 mg/kg respectively). In the next stage, a 3-cm wound including dermis and epidermis was induced with an incision on the back of the animals. The day of surgery was considered as a start day of follow-up. It is mentionable, one of the techniques for mimicking a wound model with impaired healing process is the diabetic model [18]. So, the wound model in diabetic rats was selected according to similar previous studies [19, 20].

The effect of US and laser radiation on the wound healing process was evaluated. For this purpose, the animals were fixed and irradiated. As mentioned earlier, the treatments were performed 5 days a week for 2 weeks. The laser therapy was performed by using a laser system (Laser med, model 4098) with 905-nm wavelength, power of 50 mW, in 18-s period at 10 points in wound area (totally 180 s). US therapy with the frequency of 3 MHz and duty factor of 20% was performed by using the US system (ITO, US-750). The pulsed US with 0.5 W/cm^2 intensity and 5-min irradiation time was used. Low-level laser treatment (less than 100–200 mW) and low-intensity pulsed US waves (less than 1 W/cm^2) have only non-thermal effects. The non-thermal effects of US waves are in the form of micro-massage and do not have destructive effects. The energy density of the laser in this study was about (5 J/cm^2), which was in the range of non-ablative laser, and this energy density was lower than its value in the treatment of skin lesions and resurfacing. The treatment protocol in this study was similar to most experimental and clinical studies [7]. The use of low-level 50 mW laser with 180-s irradiation time as well as pulsed US waves with low intensity of 0.5 W/cm^2 and 5-min irradiation time in this study had no thermal or destructive effects, and no skin side effects were observed in the treatment groups.

On the 14th day, FBG in the healthy control group was 87 ± 9 mg/dl. FBG in different diabetic groups was 535 ± 13, 462 ± 16, 492 ± 54, and 475 ± 24 mg/dl, respectively, which indicated diabetes condition at the end of the treatment period. Then, the animals were sacrificed, and the wound tissue was evaluated as follows.

Histopathological assessment

The tissue samples were isolated from the wound site at the dimensional of 1 cm^2. Then, they were placed in 10% formalin and sent to the laboratory. After preparing pathological slides from the tissue samples, Van Gieson and trichrome stains were performed to evaluate the amount of elastin and collagen in the tissues [21]. The images were then examined by a pathologist, and the amount of collagen and elastin in each image was scored between 1 and 5.

Biomechanical evaluation

Tensiometry was used to determine the wound tensile strength [22]. In this phase, a skin strip containing a healed wound (perpendicular to the incision) in 50 × 5 mm was removed and placed into normal saline. The tissue was positioned in the tensiometer clamps (fiber tester micro 50). The load was applied to the skin, and the force–elongation curve was plotted by a computer automatically. The parameters of load at the peak, energy at the break, and strain at the break were examined.

Statistical analysis

The normality of the data was assessed by the Shapiro–Wilk test. The Kruskal–Wallis test followed by the Mann–Whitney *U* test was performed to compare the values of collagen and elastin in the different experimental groups. Also, a one-way analysis of variance (ANOVA) with LSD post hoc test was used to compare the biomechanical properties. *p*-values < 0.05 for ANOVA and *p*-value < 0.01 for the Mann–Whitney test were considered statistically significant. Statistical analysis was performed by SPSS V.22 software.

Results

Collagen content

The amount of collagen for the study groups is shown in Fig. 1. The amount of collagen was significantly decreased in diabetic control (diabetic) group in comparison to healthy control (control) group ($p = 0.003$). The collagen amount in diabetic rats treated with laser, US, and the combination of laser and US was remarkably increased relative to diabetic control rats ($p = 0.002$, $p = 0.003$, $p = 0.001$, respectively). Also, the combination of laser and US caused more increase in the level of collagen as compared to laser or US alone ($p = 0.002$, $p = 0.004$, respectively).

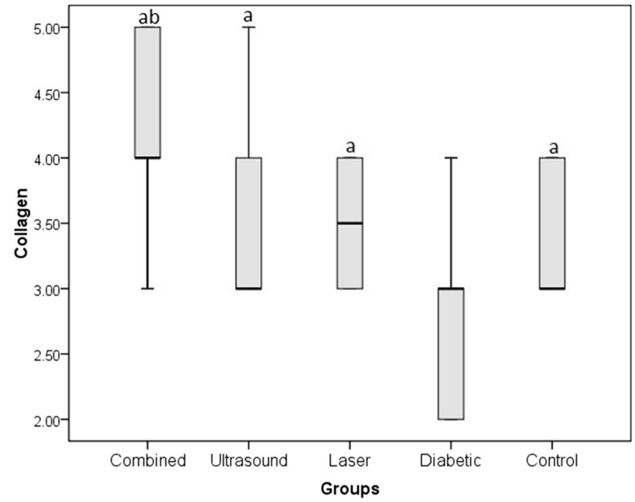

Fig. 1 Box plots of collagen content (scores between 1 and 5) in the different experimental groups. Data are median (horizontal lines), interquartile range (boxes), and min–max values (error bars). **a** Significant data in comparison to the diabetic group. **b** Significant data in comparison to the single modality treated and control groups

Elastin content

Elastin amount of diabetic control rats was considerably lower than that of healthy control rats ($p = 0.001$). The amount of elastin in diabetic groups exposed to laser, US, and the combination of laser and US was significantly increased in comparison to diabetic control group ($p = 0.001$, $p = 0.003$, $p = 0.001$, respectively). The increasing trend in the elastin level was similar to the collagen level (Fig. 2). Also, the combination of laser and US resulted in more increase in the elastin amount relative to laser or US alone ($p = 0.002$, $p = 0.003$, respectively). Photographic images of wound appearance in the different experimental groups are shown in Fig. 3.

Biomechanical parameters (energy at break, load at peak, and strain break)

In Table 1, the different biomechanical parameters on 14th day of different treatments are presented. Also, the curve of force versus elongation is shown in Fig. 4.

Energy at the break was significantly higher in the US, laser, and the combination therapy groups compared with

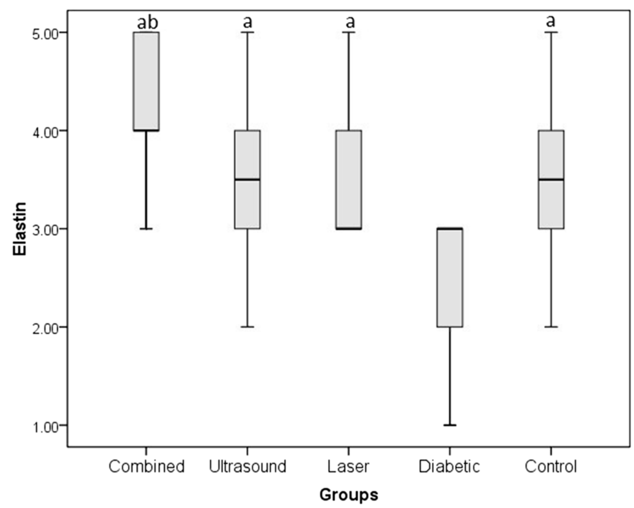

Fig. 2 Box plots of elastin content (scores between 1 and 5) in the different experimental groups. Data are median (horizontal lines), interquartile range (boxes), and min–max values (error bars). **a** significant data in comparison to the diabetic group. **b** Significant data in comparison to the single modality treated and control groups

diabetic group ($p = 0.01$, $p = 0.02$, $p = 0.001$, respectively). Also, energy at the break showed a significant increase in the combination treatment group relative to US or laser group alone ($p = 0.028$, $p = 0.027$, respectively).

The Load at Peak was significantly higher in the combination therapy, US, and laser groups than diabetic groups ($p = 0.001$; for all groups). The combination therapy group led to more increase in this parameter as compared to US or laser groups alone ($p = 0.003$, $p = 0.013$, respectively).

Strain at break in diabetic groups exposed to laser, US, and the combination of laser and US was considerably increased in comparison to diabetic control group (($p = 0.02$, $p = 0.04$, $p = 0.001$, respectively). The combination of laser and US showed more increase in this parameter in comparison to US or laser group alone ($p = 0.01$, $p = 0.02$, respectively). As shown in Fig. 4, US and laser treatment improved the biomechanical properties of the wound, and the combination therapy (US + laser) was more effective in increasing wound tissue tensile parameter. The improvement in wound tensile strength may be due to collagen and elastin formation in the treatment groups.

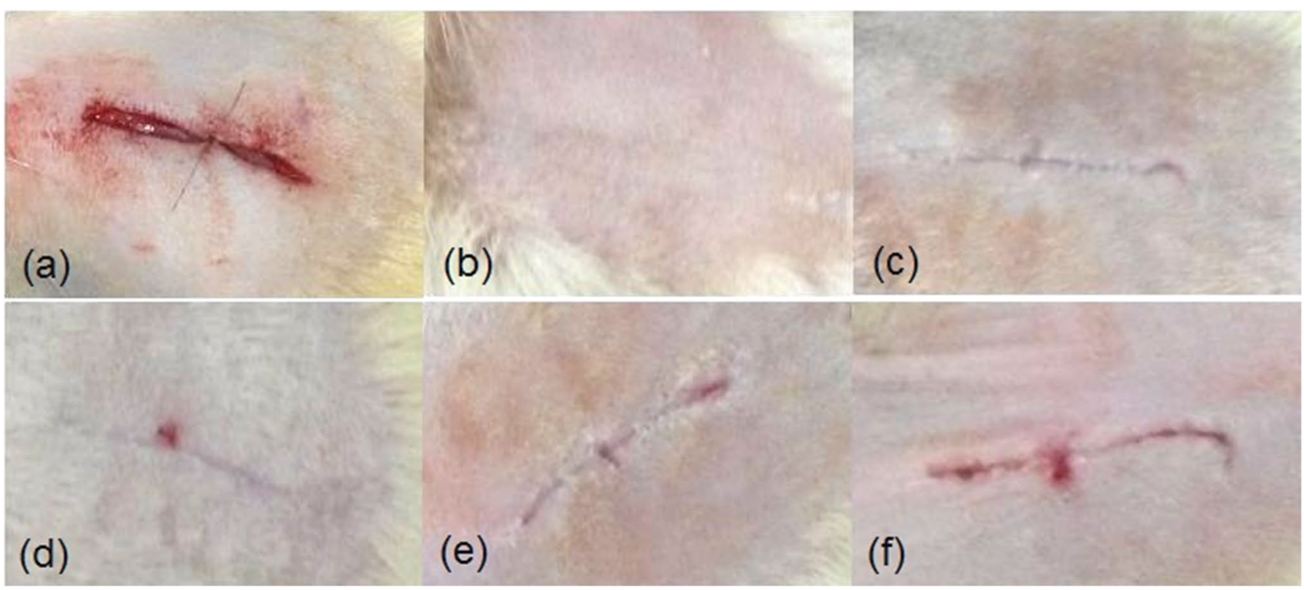

Fig. 3 Wound appearance in the different experimental groups on the day of surgery (0th day) and 14th day of treatments. **a** 0th day, **b** combined, **c** ultrasound, **d** laser, **e** control, **f** diabetic

Table 1 Biomechanical properties of skin on the 14th day of different groups (mean ± SD)

Parameter	Ultrasound	Laser	Combined	Control	Diabetic
Load at peak (cN)	3804.7 ± 167.6	3997.1 ± 212.4	4745.3 ± 222.8	3134.3 ± 406. 1	2213.3 ± 413.6
Energy at break (N m)	0.291 ± 0.02	0.289 ± 0.03	0.412 ± 0.06	0.283 ± 0.08	0.16 ± 0.08
Strain at break (%)	46.3 ± 10.9	48.1 ± 3.9	66.4 ± 5.7	50.4 ± 11.6	29.934 ± 7.551

cN centinewton, *N m* Newton meter

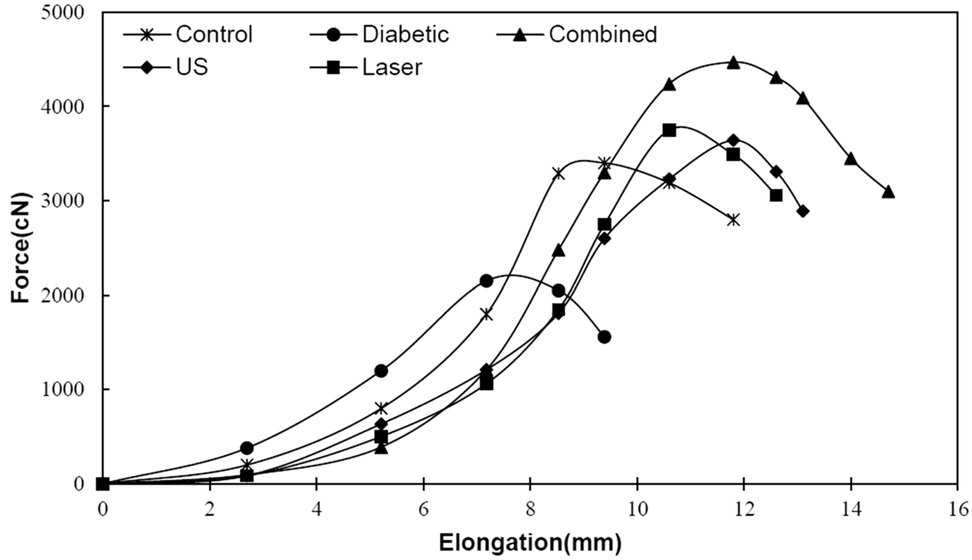

Fig. 4 Force–elongation curves of the skin in the control, diabetic, and different treatment groups (cN: centinewton)

Discussion

In recent decades, electric fields, laser, and US have been developed as an alternative methods for the treatment of acute and chronic wounds. The latest researches have focused on changing the forms of procedures to reduce the wound healing time. The findings of these experiments have demonstrated the potential benefits of these methods on wound healing [9, 23, 24]. This study was designed to evaluate the combined effects of US and laser therapy on the wound healing in induced diabetic rat. Our results showed that using laser and US increased collagen and elastin levels significantly. Also, the combination of US and laser had more effect on the amount of collagen and elastin.

It was shown that biological stimulation might promote wound mending by increasing collagen synthesis, cell proliferation, and by reducing inflammatory processes [25]. Also, photochemical and photomechanical stimulation of the skin by low-power laser could produce energy at the surface of mitochondria and could accelerate the conversion of myofibroblasts to fibroblasts, collagen synthesis, and ultimately wound healing [26]. It was also revealed that laser radiation at specific frequencies may control the cellular proliferation and promote the secretion of cytokines from fibroblasts resulting in enhanced pro-collagen synthesis, greater connection of existing collagen, and faster epithelial healing [9, 27]. It was indicated that US could influence the synthesis of collagen, increase the tensile strength of collagen, decrease the inflammatory stage of healing, and promote the proliferation phase of healing [28]. The present results showed that the use of laser and US therapy caused an increase in the amount of collagen and elastin and were therefore effective in healing of diabetic wounds. In an experimental study, the impact of US and laser therapies on the wound healing in rats was compared [29]. Both therapies led to the increased level of hydroxyproline as a key factor for assessment of collagen metabolism and stimulation of the synthesis and composition of collagen. In this study, healthy animals were used, and laser showed more effective results as compared with US [29]. In a comparative research on wound healing in mice, after 14 days of treatments with laser and US alone, US showed more positive effects due to increased level of collagen and anti-inflammatory function. Regardless of the fact that healthy mice with a small sample size were used, it was concluded that laser and US could be used during the early days and the end of healing course, respectively [30]. In a study conducted by Shalaby et al., the effect of US and laser on the wound healing in diabetic mice was compared. Laser showed more efficiency on the skin injury healing in comparison with US. It was determined that both physical treatments could accelerate the cutaneous wound healing in diabetic-induced animal model [31]. In a comparative clinical study, the therapeutic effectiveness of US and laser on ulcer healing of venous leg was evaluated. It was concluded that US and laser could be as conservative therapies for small-size ulcers of venous leg [7]. Although some limitations were observed such as small sample size, the simultaneous effect of these modalities was suggested. In a study about tendon healing in healthy rats, the level of tissue hydroxyproline among different groups including laser, US, and combined laser plus US was not significant. It was found that the combination of laser and US might not be always required [32]. In a case report, using the combination of US and laser led to complete healing of pressure ulcer after 12 weeks [16].

In the present study, US, laser, and the combination of US and laser increased the elasticity of the diabetic skin as indicated by energy at break, load at peak, and strain at break.

Based on the previous studies, if the collagen is restored because of the diminished skin laxity and improved elasticity, dermal wellbeing increases [33]. So, the increased elasticity of the diabetic skin under US and laser treatments might be in part attributed to the increased amount of collagen. The present study had some limitations such as the level of skin hydroxyproline was not determined. Although the current study is of smaller size, the results are promising, and it is recommended the testing of low-frequency US therapy and laser therapy on wound healing in clinical practice on a larger scale.

Conclusion

It seems that laser or US therapy is effective in the wound healing of diabetes disease in part through increasing the amount of collagen and elastin and also in improving the biomechanical properties. In addition, the combination therapy of laser and US could be more effective than each one alone. Further studies in this area would be welcomed.

Author contribution All the authors contributed to the study conception and design. Study design, material preparation, data collection, and analysis were performed by Ali Ebrahiminia, Korosh Khanaki, Kamran Ezzati, and Rouhollah Gazor. The first draft of the manuscript was written by Shahram Taeb, and all the authors commented on previous versions of the manuscript. All the authors read and approved the final manuscript.

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Data Availability The data are available upon request to the first author or the corresponding author of this study and with the permission of the journal and compliance with ethical standards.

Declarations

Ethical statements This experimental study was approved by the Ethics Committee of Guilan University of Medical Sciences, Rasht, Iran [IR.GUMS.REC.1396.404]. All the institutional and national guidelines for the care and use of laboratory animals were followed.

Conflict of interest The authors declare no competing interests.

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