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Low Level Laser (Biophotomodulation) Therapy for the Treatment of Diabetic Foot Ulcers with 532 nm KTP Laser Induces Wound Healing, Fibroblast Proliferation and Over-expression of TGF-β

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ABSTRACT
Development of diabetic foot ulcer can be considered as the most apprehensive complication, exhausting both patients and the health care system. Due to treatment high failure rate, different modalities of treatment have been developed. Recently, low-level-laser-therapy (LLLT) was investigated on cell culture and on few clinical trials, utilizing infrared/near-infrared machines. The clinical and histological benefit of KTP-532nm LLLT on ulcers and the tissue expression of TGF-β before/after LLLT investigated in this study including eleven patients with empirical LLLT. Tissue samples were submitted for histopathological assessment from four patients before and after LLLT. Immunohistochemical expression of TGF-β was evaluated, using Wilcoxon-Signed statistical test.
All patients responded dramatically, 3 (27.27%) completely healed, 7 (63.36%) were poorly compliant despite their obvious response. One-patient (9.09%) ended with toe amputation because of underlying osteomyelitis. All histological sections showed dramatic increase of fibroblast TGF-β after receiving LLLT.
KTP-53nm LLLT enhances ulcer healing and TGF-β expression.
Keywords: Diabetic ulcer, foot ulcer, low-level biophotomodulation therapy, KTP 532 nm
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INTRODUCTION
Diabetes Mellitus is the most common disease process associated with lower limb amputation, accounting for approximately half of non-traumatic amputations in North America and Europe [1,2]. Up to 85% of lower-limb, amputations in diabetic patients are preceded by foot ulcers that fail to heal [3]. About 2% to 3% of all diabetic patients will develop a foot ulcer every year, and many of these will require prolonged hospitalization for treatment of complications of ensuing infection or gangrene [4,5].
The main underlying cause for the development of diabetic foot ulcers is peripheral neuropathy and microangiopathy.
- Neuropathy: 30-50% of diabetic patients can be affected by distal neuropathy of lower limbs. Both types of diabetes [I and II) are similarly at risk. More than 60% of diabetic patients' foot ulcers are primarily due to underlying neuropathy. Loss of nerve function correlates with chronic hyperglycemia, as reflected in the mean level of glycosylated hemoglobin over time [6]. Ischemia of the endoneurialmicrovascular circulation induced by metabolic abnormalities from prolonged hyperglycemia is believed to be the underlying mechanism for nerve deterioration [7,8].
- Ischemia: Primarily ischemic ulcers without substantial accompanying neuropathy accounts for approximately 15-20% of foot ulcers, and another 15-20% have a mixed neuropathic-vascular etiology [9]. Besides the well-known microangiopathic effect of longstanding diabetes mellitus, there is an increased risk of developing atherosclerotic changes in the intimal layer of lower limb arteries. Interestingly, in diabetic patients, smoking does not seem to be associated with recurrent foot ulcers or risk of amputation from ischemia [3,10,11]. These data conflict with information about patients without diabetes, which clearly indicates that smoking is a risk factor for claudication and amputation [12].
Grading Diabetic Foot Ulcers:
Different grading systems have been suggested for proper classification of the severity of diabetic foot ulcers, some are designed for clinical practice and others are more suitable for research purposes. The first published classification system of the diabetes ulcers, Meggitt-Wagner system, is a linear system consisting only six grades (0-5, where 0 is intact skin), the first three grades being related to depth. The limitation of this system is that it not includes peripheral arterial disease and infection[49].
The most suitable grading system for clinical practice is the University of Texas (UT) grading system, which relies on grading ulcers according to depth of involvement (grade) and further staging ulcers into stage A (presence of infection), B (presence of ischemia) and C (both infection and ischemia)[49]. In this study, we adopted UT classification system for proper description and follow up of results (table 1).

Table 1: University of Texas (UT) Classification/Scoring system of diabetic foot ulcers.

Stage	Grade 0	Grade I	Grade II	Grade III
A	Pre- or post-ulcerative lesion completely	Superficial ulcer, not involving tendon capsule or	Ulcer penetrating to tendon or capsule	Ulcer penetrating to bone or joint

	epithelialized	bone		
B	Infection	Infection	Infection	Infection
C	Ischemia	Ischemia	Ischemia	Ischemia
D	Infection & Ischemia	Infection & Ischemia	Infection & Ischemia	Infection & Ischemia
Score: Grade_____ Stage_____				

The SAD grading system relies on three parameters; size of the lesion (area and depth), presence and absence of ischemia (arteriopathy), and presence or absence of neuropathy (denervation). This system is superior to the abovementioned schemes by adding neuropathy as an accessory parameter, which is useful in clinical audit.
Other systems like SINBAD, PEDIS, Van Acker/Peters and DUSS classification systems rely on similar parameters only adding ulcer site [49].

Wound Healing and Growth Factors

Failure of wound healing is another manifestation of macro and microangiopathic effects of diabetes resulting in ischemia. Once the protective barrier is broken, the normal (physiologic) process of wound healing is immediately set in motion. The classic model of wound healing is divided into three or four sequential, yet overlapping, phases: (1) hemostasis (not considered a phase by some authors), (2) inflammation, (3) proliferation and (4) remodeling.
Many growth factors are released immediately after wound ensues and during the process of wound healing. Platelet-derived growth factor (PDGF) released from platelet alpha-granules immediately after injury, attracts neutrophils, macrophages, and fibroblasts to the wound and serves as a powerful mitogen. Macrophages, endothelial cells, and fibroblasts also synthesize and secrete PDGF. Platelet-derived growth factor stimulates fibroblasts to synthesize new extracellular matrix, predominantly non-collagenous components such as glycosaminoglycans (GAGs) and adhesion proteins. PDGF also increases the amount of fibroblast-secreted collagenase, indicating a role for this cytokine in tissue remodeling [13,14].
Transforming growth factor-beta (TGF-β) directly stimulates collagen synthesis and decreases extracellular matrix degradation by fibroblasts [15,16,40]. It is released from platelets and macrophages at the wound. In addition, TGF-β is released from fibroblasts and keratinocytes. It acts in an autocrine fashion to further stimulate its own synthesis and secretion. TGF-β also chemoattracts fibroblasts and macrophages to the wound. It is regarded as a profibrotic growth factor, which accelerates wound repair at the expense of increased fibrosis. It has been implicated in pathological fibrosis in multiple different organ systems and is thought to be a factor in the formation of intestinal adhesions. TGF-β stimulates extracellular matrix (ECM) synthesis and accumulation, increases integrin expression and therefore enhances cell-matrix interactions[15,16,40].
The exogenous application of several growth factors has been shown to accelerate normal healing as well as improve healing rates and efficacy in impaired models of healing.The best-studied growth factors with the most promise to improve healing are PDGF, TFG-β, and members of the FGF family. Several obstacles must be overcome before widespread use, including efficacious application, vehicle development, and cost [17,18-27].
Cell studies with cultured human keratinocytes, endothelial cells, and fibroblasts indicated potential effects of near infrared light in the treatment of chronic skin ulcers [28,29].
Few experimental studies of biophotomodulation irradiation of human and animal cells in culture media document positive biophotomodulatory effects of biophotomodulation irradiation. Various types of cells involved in wound or soft-tissue repair or cell lines relating to soft tissues (human and animal stem cells, endothelial cells, smooth muscle cells, keratinocytes, fibroblasts, and others) respond differently to irradiation, also depending on irradiation parameter [28,29,30-34]. LLL irradiation resulted in an increased fibroblast proliferation in-vitro [34]. Biophotomodulation irradiation can promote cell migration and cell proliferation by stimulating mitochondrial activity and maintaining viability without causing damage to the wounded cells [30].

Biophotomodulatory Low Level Laser Therapy (LLLT) of Skin Wounds

Few clinical trials on using LLLT for the treatment of non-healing wounds has been reported, however; the use of laser beam for removal of skin pigments is well established. Different types of laser machines are available for this purpose, most utilize Nd:YAG 1064 nm, and Ruby 694 nm for their depth of penetration reaching the hypodermis, Fig. 1. KTP 532 nm is of less value in this regard because of its lowest depth of penetration affecting no deeper than the dermo-epidermal junction[50].

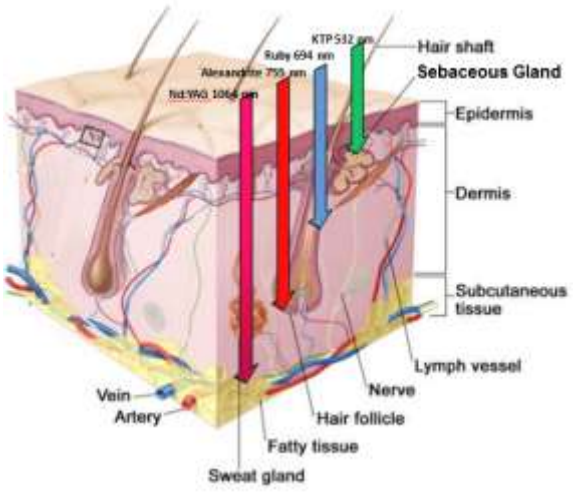

Fig. 1: Schematic representation of penetration of different laser types on skin tissue.

Objectives

1. Investigating the clinical and histological benefit of low-level biophotomodulation therapy (LLLT) on diabetic foot ulcers, using KTP 532 nm laser beam.
2. Investigating the tissue expression of TGF-β before and after LLLT.

PATIENTS, MATERIALS AND METHODS

Patients

During the period extending from October 20, 2018 to Apr. 23, 2019, eleven volunteer patients, accepted to participate in this clinical trial of using of LLLT in the treatment of diabetic foot ulcer, the age range was 25-76 years old, four of them also accepted to take pretreatment and post-treatment biopsy specimens one week apart. A written consent was obtained from all patients, with approval of the local Ethical Committee of the health directorate in Babylon, and in accordance with Declaration of Helsinki-Ethical Principles for medical research involving human subjects.

All patients were complaining of high-grade (I, II and III) diabetic foot ulcers depending on the University of Texas staging system of diabetic foot ulcers, with variable control of blood glucose level, and were subjected to different modalities of surgical and medical treatment but none of them showed accepted response, especially with antibiotics. As shown in the table-2:

Table 2: Grades & stages of the studied subjects

Grade	Stage	No. of cases
I	A	7
I	C	1
II	A	2
III	D	1
Total		11

Treatment scheme

Local and systemic antibiotic therapy was withdrawn from all patients, and local debridement under local or general anesthesia was done. Pre-biophotomodulation therapy biopsy samples were submitted to the pathology department. All patients of this study were subjected to LLLT according to the specifications listed below. Biopsy samples were taken exactly one week later. During this interval, strict control of blood glucose levels was done by timed bedside monitoring, adjusted exceeding 200 mg/dl. Simple oral antibiotic cover was reintroduced in some patients according to their clinical situation. Frequency of sessions and number of pulses were empirical and was influenced by patient attendance which was subjective due to unsettled Iraqi conditions.

Laser parameters

The machine used was diode laser KTP 532 nm purchased from ARC Laser company, GmbH. Power: 8 W, pulse length: 100 msec. (0.1 sec), spot size: 0.9 cm2, energy: 0.8 J, power density: 8.8 w/cm2, energy density: 0.8 J/cm2 and frequency: 6 pulses/sec. Specifications of handpiece: Large Spot-HS11020 10x5 mm spot (at 15 mm distance).

Empirical biophotomodulation therapy was done in a circular manner at the ulcer edge between the skin and the ulcer bed, with horizontal scanning manner to the bed, repeating the procedure 10 times, the speed of movement was slow with a range of 2-3 pulses per area, the total time for the whole biophotomodulation session was 5-10 min, according to the ulcer size. The aforementioned LLLT regimen was repeated three times for each patent, one week apart.

Histological Examination

Histological sections were prepared with H&E stains and immunohistochemical staining for TGF-β purchased from ABCam corporation. Statistical analysis was done using the Wilcoxon-signed test. A p value less than 0.05 was regarded significant.

RESULTS

Clinical Response

All patients participated in this study showed a dramatic response after the first week of commencing LLLT. Three patients out of eleven (27.27%) were totally cured. Seven patients (63.36%) did not continue treatment, despite the dramatic improvement and the nice-looking granulation tissue formation. One patient (9.09%) ended with toe amputation because of underlying osteomyelitis (figure-2).

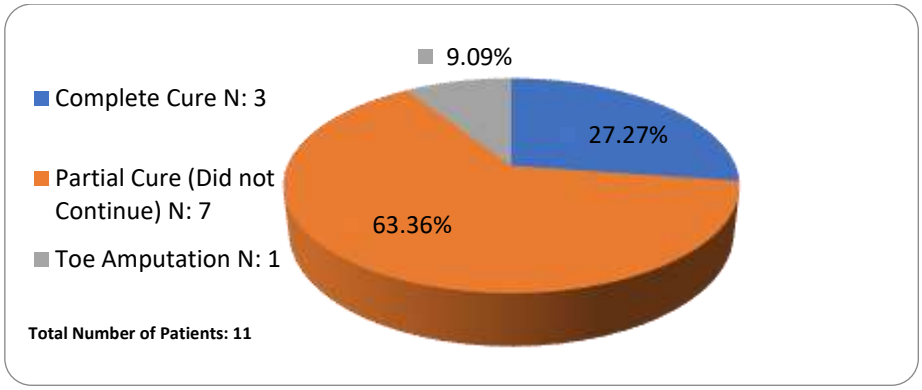

Fig. 2: Pie chart illustrating the clinical cure rates of diabetic foot in eleven patients participated in this clinical trial.

Figure 3: pre, during, and post 3 months of biophotomodulation sessions and Ray amputation to 5th metatarsal.

Histopathological Changes

Biopsy samples taken from diabetic foot ulcer bases before commencing LLLT showed four indistinguishable layers of necrotic tissue material, under which lies a layer of inflammatory infiltrate with variable degrees of granulation tissue formation and an inner layer of fibrosis. The degree and extent of fibrous tissue formation differ in all cases with no distinct correlation with ulcer size and duration of ulceration. A repeated biopsy after 10 days of biophotomodulatory laser therapy revealed an increased thickness of the fibrous tissue layer with the appearance of activated fibroblasts in a large number (Fig. 4 and 5). The overlying granulation tissue layer showed a brisk vascular activity with fibrous tissue deposition in all of the four cases in this study.

The degree of fibrous tissue response on H&E histopathological sections was graded into (0; when the fibrous tissue shows no signs of active fibroblasts, +; when foci of active fibroblasts were identified in less than 20% granulation tissue areas, ++; active fibroblasts identified within 20-50% of granulation tissue and underlying fibrous tissue layer, +++; active fibroblasts seen in more than 50% of granulation tissue). The p value was 0.024.

The degree of fibroblast TGF-β expression was graded in the same manner into 0, +, ++, +++ depending on the number of cells with positive staining result (0: negative, +; less than 20% of cells, ++; 20-50% of cells and +++; more than 50% of cells). There was a good correlation between LLLT and clinical response with a p value of 0.038.

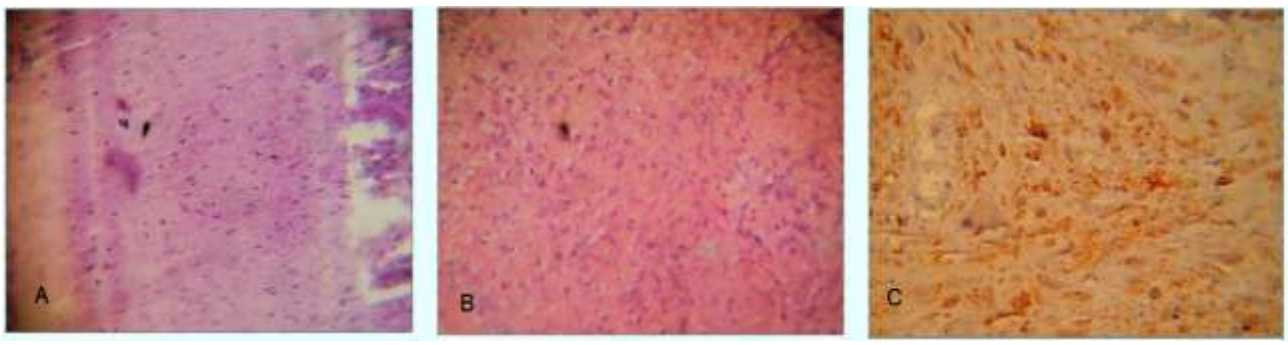

Fig. 4: A. Histological appearance of fibrous tissue region in diabetic foot ulcers before LLLT. B. Histological appearance of thesame case after LLLT, showing brisk fibrous tissue with activated fibroblasts. C. Immunohistochemical staining for TGF-β, for the same case after LLLT, showing moderately increased expression pattern.

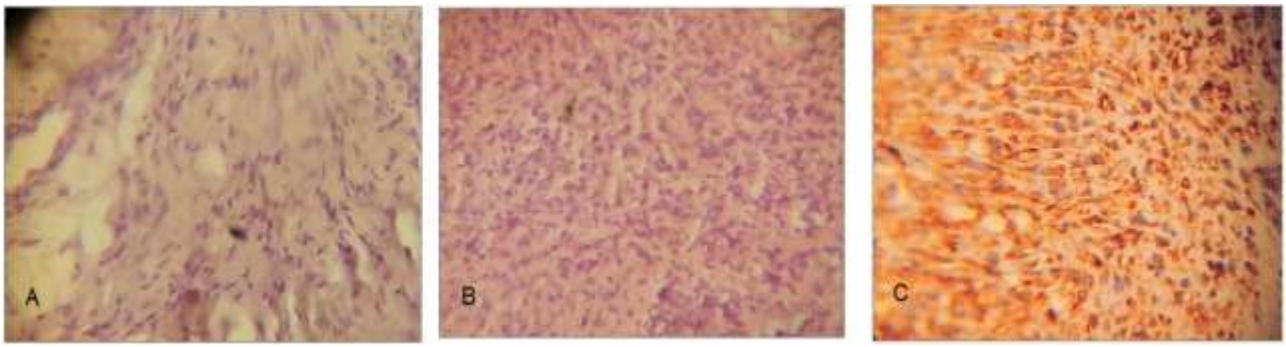

Fig. 5: A. Histological appearance of fibrous tissue region in diabetic foot ulcers before LLLT. B. Histological appearance of the same case after LLLT, showing brisk fibrous tissue with activated fibroblasts. C. Immunohistochemical staining for TGF-β, for the same case after LLLT, showing markedly increased expression pattern.

DISCUSSION

Skin wounds in nondiabetic subjects heal by the formation of granulation tissue and contraction by fibrosis, whereas in diabetics, the closure of the wounds is primarily resulted by both formation of granulation tissue and re-epithelialization[35]. Though epithelial repair in simple superficial wounds is not delayed in diabetes, but the repair of deeper wounds by the ability to form collagen in is severely hindered[35]. In diabetes wound healing can further be delayed by inadequate granulation tissue formation, probably explained by a defect in fibroblast activity [36]. In chronic diabetic foot ulcers, the fibroblasts have impaired proliferative capability compared with those in intact skin[37]. According to our clinical and immunohistochemical study, fibroblasts are well proliferated and well activated with very active granulation tissue formation (Figures 4 and 5].

TGF-β In Wound Healing

The TGF-β superfamily comprises TGF-β types 1, 2, and 3; activin A, B, and C; bone morphogenic proteins; anti-Müllerian hormone and growth differential factors. The primary sources of synthesis and production of these factors include fibroblasts, macrophages, platelets and keratinocytes [38]. Ligands of TGF-β superfamily bind to type II TGF-β receptor, a serine/threonine receptor kinase, which intern phosphorylates TGF-β type I receptor[39]. This interaction leads to the activation of the SMAD pathway through which the R-SMADs are phosphorylated, and through binding to a common SMAD mediator (SMAD4), they form R-SMAD/co-SMAD complexes. Thereafter, these complexes are transported into the nucleus, where it regulates the expression of a different of genes[40].

One of the pathways through which TGF-β is activated is integrin αvβ6 that interacts with inactive complexes of TGF-β1with its latency-associated protein (LAP) causing the activation of TGF-β1. This integrin will activate TGF-β1 by binding to the RGD motif present in LAP [41,42].

Clinical Response

In this study, the clinical response to 532 nm KTP LLLT was dramatic, with complete or incomplete healing of diabetic foot ulcers in 91% of patients. Only one patient out of 11 ended with metatarsal amputation due to underlying osteomyelitis at the time of presentation, but the ulcer eventually completely cured with complete epithelialization (Fig. 2). This study also used the lowest energy limit (0.8 J/cm2) in each session with dramatic results, compared to other studies illustrated below.

The healing effect of combined 660 and 890 nm LED biophotomodulation treatment on diabetic leg ulcers was tested by Minatel et al in a double-blind randomized placebo-controlled study including 23 patients. Mean ulcer healinnd formation of granulation tissue rates were significantly higher in the treatment group than in the placebo group at each of 15, 30, 45, 60, 75, and 90 days of treatment. While placebo-treated ulcers were cleaned, dressed with 1% silver sulfadiazine cream, and treated with placebo biophotomodulation radiation (< 1.0 J/cm2). Ulcers in the treatment group got the same treatment but a 3 J/cm2 dose. After one month, ulcers in the treatment group expressed 56% more granulation and achieved 79.2% faster healing compared to placebo group. After 90 days, 58.3% of ulcers had healed fully and 75% had achieved 90–100% healing. In contrast, in the placebo group, only one ulcer healed fully and no ulcer attained more than 90% healing[43].

In the same context, a clinical trial by Zhou et al. investigated the healing effect of irradiated (633 nm) chronic foot ulcers in sixty diabetics and non-diabetics. They reported the remarkable clinical response confirmed by immunohistochemical increase of heat-shock protein 70 expression [44].

Schindl et al. noticed in his study, an increment of temperature after a single treatment with low-intensity biophotomodulation irradiation (632.8 nm) as a marker of circulatory augmentation in the skin of subjects with diabetic microangiopathic ulcers [45].

Kaviani et al. investigated twenty-three subjects with diabetic foot ulcers, using 685 nm laser machine and energy density of 10 J/cm2. He observed a significant clinical improvement in wound healing of biophotomodulation-treated patients compared with the placebo[46].

Saltmarche et al. examined the value of low level biophotomodulation therapy of 785 nm for wound healing. They utilized infrared biophotomodulation clusters of 16×5 mW and a 50 mW source of both 785 nm and applied 2-4 Joules on each site, depending on the intensity of skin pigmentation. The empirical therapy was applied daily for five days in the 1st week, and three times weekly from the

second to the ninth week or until complete ulcer healing. Around 62% of the open wounds attained significant clinical improvement, measured by reduction of the wound size. While 43% had complete closure, 14.3% had mild improvement; 23.8% exhibited any change[47].

In comparison to laser therapy, Landau et al. studied the influence of 400–800 nm broadband visible light on sixteen patients with diabetic or venous foot ulcers. The treated group, their wounds illuminated twice daily with 43.2 J/cm2 while the placebo group their wounds were illuminated with only 2.4 J/cm2 which was considered as non-therapeutic. Conventional wound care was continued on all patients. At the end of the clinical trial, 90% of wounds of the treatment group were healed, while only 33% of patients in the placebo group had wound closure[48].

To our best knowledge, this is the first study conducted with 532 nm LLLT on diabetic foot ulcers, evaluated clinically and histologically and supported by immunohistochemical staining to compare the grade of expression of TGF-β before and after commencing therapy. Low energy biophotomodulation beams of 532 nm wavelengths and 0.8 J/cm2 has even proved more effective than biophotomodulation therapies with other wavelength devices emitting at wavelengths of infrared or near infrared. Studies illustrated above with the latter type of devices only achieved clinical and histological response on high energy density.

CONCLUSIONS

1. Even at low energy density, 532 nm KTP LLLT can be very beneficial, with histological and immunohistochemical proof, in the treatment of diabetic foot ulcer.
2. Due to the relative safety of KTP 532 nm on the skin, it can be applied to open wounds without drastic effects.
3. Biophotomodulation therapy of diabetic foot ulcers has resulted in increased expression of TGF-β in fibroblasts, which may play a role in induction of fibroblast growth and differentiation, and hence wound healing.

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