Irradiation with a 632.8 nm Helium-Neon Laser with 5 J/cm² Stimulates Proliferation and Expression of Interleukin-6 in Diabetic Wounded Fibroblast Cells

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ABSTRACT

Background: The use of lasers has been shown to stimulate wound healing in vivo and in vitro. There is an increase in wound closure, cell viability, proliferation, and cytokine expression. If laser parameters can be optimized and standardized, and the underlying mechanisms better understood, this phototherapy can become an alternative safe treatment to slow-to-heal wounds, such as in patients with diabetes. This study aimed to determine the effect on cellular proliferation, migration, and cytokine [interleukin-6 (IL-6)] expression in diabetic and diabetic wounded fibroblast cells (WS1) post-laser irradiation.

Methods: Diabetic and diabetic wounded WS1 cells were irradiated at 632.8 nm (23 mW) with 5 J/cm² or 16 J/cm². IL-6 level, cellular proliferation (neutral red assay), and morphology were then determined.

Results: Diabetic cells irradiated with 5 J/cm² showed no significant change, while diabetic wounded cells showed an increase in IL-6 level, proliferation, and migration. On the other hand, diabetic and diabetic wounded cells irradiated with 16 J/cm² showed a significant decrease in proliferation and evidence of cellular damage, and wounded cells showed no migration.

Conclusion: This study showed that phototherapy at the correct fluence stimulates IL-6 expression, proliferation, and cellular migration in diabetic wounded cells. A fluence of 5 J/cm² stimulates diabetic wound healing in vitro, while 16 J/cm² is inhibitive.

INTRODUCTION

A WOUND IS A PATHOLOGICAL STATE in which tissues become separated or destroyed, while wound healing is the complex sequence of events directed toward closure of the defect, usually by replacement with scar-forming connective tissue. Wound healing is aimed at reversing the loss of structural integrity caused by injury to the tissue.¹ Normal wound healing requires both destructive and reparative processes in controlled balance. Proteases and growth factors play an important role in regulating this balance, and if it is disrupted in favor of degradation then delayed healing ensues, which is a trait of chronic wounds.² Cytokines are involved in all phases of wound healing and regulate migration, proliferation, differentiation, and metabolism of mammalian cells. It appears that it is the balance of these cytokines and other mediators rather than the mere presence or absence of one or more cyto-

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kines that plays a decisive role in regulating the initiation, progression, and resolution of wounds.\textsuperscript{3}

A wide range of cytokines involved in wound healing have been identified. Interleukin (IL)-6 is a pleiotropic cytokine (21–28-kDa glycoprotein) produced by lymphoid and nonlymphoid cells that acts on a wide range of tissues and cells, exerting growth-inducing, growth-inhibitory, and differentiation-induction effects, depending on the nature of the target cells.\textsuperscript{34} IL-6 also regulates immune activity, the acute-phase response to injury and infection, inflammation, oncogenesis, and hematopoiesis.\textsuperscript{4–6} IL-6 is produced by normal constituents of the skin, including epidermal cells, fibroblasts, and endothelial cells,\textsuperscript{7} and plays a crucial role in the pathophysiology of wound healing. Local wound levels of various cytokines, including IL-6, correlate with the stage of wound healing. Baker et al.\textsuperscript{8} found that IL-6 levels peaked 1 day after surgery and decreased by day 7. This initial increase following injury or wounding and decrease over time are also found in other studies.\textsuperscript{9–11}

The literature shows that cytokines are important mediators in the pathogenesis of autoimmune insulin-dependent diabetes mellitus.\textsuperscript{12} IL-6 is expressed by adipose tissue and centrally in hypothalamic nuclei with its receptor that involves regulation of body fat. Serum levels of IL-6 are known to correlate with body mass index, and similarly correlate with metabolic parameters such as insulin sensitivity. Additionally, IL-6 can enhance glucose serum levels and may be involved in type 1 diabetes.\textsuperscript{4} It has been observed that anti–IL-6 inhibits the development of insulin-dependent diabetes in NOD/Wehi mice, and this supports the concept that IL-6 can play a role in autoimmune disease.\textsuperscript{12} It has been found that plasma IL-6 levels are elevated in patients with poorly controlled diabetes and in diabetes-induced monocytes in vitro.\textsuperscript{13} Fu et al.\textsuperscript{14} studied mRNA expression of IL-6 in diabetic ulcers and the surrounding normal skin from the same patients. It was found that the mRNA expression levels of IL-6 were increased in both conditions. Fahey et al.\textsuperscript{15} analyzed wound fluid for total number of leukocytes, tumor necrosis factor-\textalpha, and IL-6 aspirated from wound chambers implanted in normal mice and those with diabetes at different time intervals. On days 1 and 3, IL-6 levels were similar between the two groups; however, on day 7 mice without diabetes showed significantly higher IL-6 levels. It is the opinion of these authors that delayed wound healing in diabetes is associated with altered IL-6 levels in wound fluid during the late inflammatory phase of wound healing.\textsuperscript{15}

Phototherapy, or low-level laser therapy, is used in many medical fields and other professions for wound healing; however, this therapy is still not established, and controversy surrounds it. This is due to the lack of an understanding of the underlying mechanisms of action and the fact that there is a threshold limit: below the limit no effects will be seen; above the limit inhibitive or negative effects will be seen. As a result of this threshold limit, it is important in choosing the correct parameters such as fluence/dose (radiant energy per unit area, measured in J/cm\textsuperscript{2}), wavelength (distance from crest to crest of an electromagnetic wave, measured in nm), power density (amount of light energy incident per unit area, measured in W/cm\textsuperscript{2}), and irradiation time. Irradiation with low-level lasers has been shown to elicit a wide variety of effects, including accelerated wound healing, increased microcirculation, and stimulation of the immune system. All of these effects can be useful in diabetic wound healing, as it is well known that patients with diabetes suffer from delayed healing and are more susceptible to infection than patients without diabetes. Phototherapy has been shown to stimulate wound healing in murine and in vitro models, as well as in patients with diabetes.\textsuperscript{16,17} Laser therapy is an alternative, safe, and side effect–free treatment that is appealing to patients.\textsuperscript{17} Despite the advocacy of this treatment, there is a reluctance to accept it due to the lack of understanding,\textsuperscript{18} and further research to assess the effectiveness of biostimulation for diabetic wound healing is needed.\textsuperscript{17}

It has been shown that laser irradiation at certain fluences and wavelengths can enhance the release of growth factors and stimulate cell proliferation.\textsuperscript{19} In in vitro studies, Gavish et al.,\textsuperscript{20} Hawkins and Abrahame,\textsuperscript{3} and Novoselova et al.\textsuperscript{21} found an increase in IL-6 ex-
pression after laser irradiation. In another similar study, Hawkins and Abrahamse also found an increase in cellular proliferation after irradiation of normal and wounded human fibroblast cells (WS1) to a He-Ne laser (632.8 nm) with a fluence of 5 J/cm². In an in vivo study, Zhevago and Samoilova found that laser irradiation (480–3,400 nm, 12 J/cm²) of a small area of the body (sacral area) resulted in a decrease in pro-inflammatory cytokines (tumor necrosis factor-α, IL-6, and interferon-γ) 0.5 h post-irradiation. Similar results were seen after in vitro irradiation of blood.

It was the aim of this study to determine the effect of irradiating diabetic wounded and unwounded human fibroblast cells at 632.8 nm with 5 or 16 J/cm² on cellular proliferation, migration, and IL-6 expression.

**MATERIALS AND METHODS**

**Cell culture**

Human skin fibroblast cells (WS1) (ATCC CRL 1502, Adcock Ingram S.A., Bryanston, SA) were grown as described previously. An in vitro diabetic model with a simulated wound was set up according to Hamuro et al. and McDermott et al. WS1 cells were continuously cultured for several passages in complete medium containing additional glucose (17 mM) in 75-cm² culture flasks until required. The medium had a basal glucose concentration of 5.6 mM; thus diabetic induced cells were grown in a total glucose concentration of 22.6 mM. To determine the effects of the laser, cells were detached by trypsinization (1 mL/25 cm², 0.25% trypsin–0.03% EDTA), and 6 x 10⁵ cells in 3 mL of complete culture medium (with added glucose) were seeded into 3.3-cm-diameter culture plates as determined by the Trypan blue exclusion test. Plates were incubated overnight to allow the cells to attach. A wound was made in the confluent monolayer of WS1 cells by pressing a sterile pipette down onto the culture dish to scrape the cell sheet, thereby exposing a cell-free zone in the center of the culture dish. The medium was replaced, and the cells were left at 37°C for 30 min before irradiation.

**Laser setup and irradiation**

Diabetic and diabetic wounded WS1 cells were irradiated in the dark with a He-Ne laser at a wavelength of 632.8 nm. Laser parameters are shown in Table 1. Cells were irradiated once with a fluence of either 5 J/cm² or 16 J/cm². Normal unwounded unirradiated cells and diabetic wounded unirradiated cells were used as controls. After irradiation, cells were detached by trypsinization and resuspended to a final concentration of approximately 1 x 10⁵ cells/100 µL.

**Cellular morphology**

Cellular morphology was determined by inverted microscopy (Olympus model CKX41, Wirsam, Richmond, Johannesburg, SA). The formation of spindle-shaped cells in wounded cultures was evaluated, characterizing normal processes involved in wound healing. Colony formation, haptotaxis (change in direction of growth), cellular migration, and the number of cells in the central scratch were also evaluated.

**Expression of human IL-6 (hIL-6)**

The photometric enzyme-linked immunosorbent assay (ELISA) was used for the quantitative determination of hIL-6 in the culture medium of laser-irradiated and unirradiated control cells. The assay is based on the quantitative sandwich ELISA principle using the Human IL-6 ELISA Kit (catalog number 1534475, Roche Applied Science, Randburg, SA), which uses two monoclonal mouse antibodies directed against two different epitopes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tbody>
<tr>
<td>Laser</td>
<td>He-Ne³</td>
</tr>
<tr>
<td>Wavelength</td>
<td>632.8 nm</td>
</tr>
<tr>
<td>Wave emission</td>
<td>Continuous wave</td>
</tr>
<tr>
<td>Power output</td>
<td>23 mW</td>
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<tr>
<td>Power density</td>
<td>2.206 mW/cm²</td>
</tr>
<tr>
<td>Spot size</td>
<td>9.1 cm²</td>
</tr>
<tr>
<td>Duration of irradiation</td>
<td>37 min (5 J/cm²)</td>
</tr>
<tr>
<td></td>
<td>2 h (16 J/cm²)</td>
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³Because of expansion, clipping, and reflecting of the laser beam to the cells, power was lost, and as a result output power readings were multiplied by 0.871 to convert to the value at the cells.
of IL-6. Both of these antibodies recognize epitopes that are essential for receptor binding, which enables the specific determination of biologically active hIL-6.

The assay was performed in streptavidin-precoated plates. A six-point standard was run from which the concentration of the samples were determined. Twenty microliters of sample or standard and 200 μL of immunoreagent (incubation buffer, anti–IL-6-biotin, and peroxidase-conjugated anti–IL-6 detection antibody) were put in the appropriate wells and incubated at room temperature for 2 h while shaking at 250 rpm. The plate was then washed four times with washing buffer using a plate washer (model PW40, Bio-Rad, Rosebank, Johannesburg). Two hundred microliters of 3,3’,5,5’-tetramethylbenzidine substrate was then added and incubated in the dark at room temperature while shaking at 250 rpm. After 30 min, 50 μL of stop solution (1 M H2SO4) was added and mixed for 1 min. Absorbance at 450 nm (reference wavelength 690 nm) was then read (Benchmark Plus microplate spectrophotometer, Bio-Rad).

Neutral red (NR) assay

NR is a cationic, nontoxic vital dye employed as a histologic stain for proliferating cells. NR is selectively taken up and concentrated by mitotic cells. The NR assay was used to evaluate cellular proliferation after laser irradiation. Approximately $5 \times 10^4$ cells/mL were incubated at 37°C for 1 h in complete medium with 10% NR (33 μg/mL) (catalog number N2889, Sigma Aldrich, Kempton Park, Johannesburg). Cells were then fixed with 1% formaldehyde for 30 min and solubilized with 1% acetic acid in 50% ethanol (50 μL) for 30 min. Absorbance was read at 550 nm (Benchmark Plus microplate spectrophotometer, Bio-Rad).

Statistical analysis

Each experiment was performed four times ($n = 4$), and each sample was done in duplicate, the average of which was used. Statistical analysis was done using the one-tailed Student’s $t$ test and one-way analysis of variance using SigmaPlot version 8.0 (Systat Software, San Jose, CA), and differences were considered statistically significant when $P < 0.05$. Data were plotted using positive and negative standard error bars. Statistical probability is represented as $P < 0.05$, $P < 0.01$, and $P < 0.001$.

RESULTS

Cellular migration and change in direction of growth were assessed by inverted microscopy in wounded cultures. In wounded cultures, the central scratch and wound margin could be clearly seen (Fig. 1). There was no structural difference between normal wounded and diabetic wounded WS1 cells, as well as between normal and diabetic unwounded cells. Diabetic cells irradiated with 5 J/cm² showed no structural difference compared to normal and diabetic unirradiated cells, while cells irradiated with 16 J/cm² began to show signs of stress with some open spaces where cells began to shed from the culture plate. Wounded unirradiated cells showed no signs of cellular migration (Fig. 1a). On the other hand, diabetic wounded cells irradiated with 5 J/cm² showed colony formation, where the cells began to group at the wound margin, as well as a change in the direction of growth and cellular migration (Fig. 1b). Diabetic wounded cells irradiated with 16 J/cm² showed no signs of cellular migration and began to show signs of stress with cells appearing shrunken and cellular lysis (Fig. 1c).

Following laser irradiation of diabetic and diabetic wounded WS1 cells (632.8 nm; 2.206 mW/cm²) with 5 or 16 J/cm², hIL-6 expression was determined by ELISA (Fig. 2). Normal wounded and diabetic wounded unirradiated control cells (0 J/cm²) showed an insignificant increase in hIL-6 compared to normal and diabetic unirradiated cells, respectively. Diabetic induced WS1 cells showed higher levels of hIL-6 compared to normal and diabetic unirradiated cells, respectively. Diabetic induced WS1 cells showed higher levels of hIL-6 compared to nondiabetic cells; however, this increase was not significant. Compared to normal and normal wounded unirradiated control cells, both diabetic ($P < 0.05$) and diabetic wounded ($P < 0.01$) cells irradiated with 5 J/cm² showed a significant increase. Diabetic wounded cells irradiated with 5 J/cm² showed a significant increase compared to diabetic and diabetic wounded unirradiated control cells.
FIG. 1. A diabetic wound model was induced in human skin fibroblast cells (WS1). Thirty minutes post-wound induction, diabetic wounded cells were irradiated once with a He-Ne laser (632.8 nm) with a fluence of 5 or 16 J/cm². Control cells were not irradiated (0 J/cm²). Post-laser irradiation, cellular morphology was evaluated for colony formation, haptotaxis, and cellular migration. (a) Unirradiated diabetic wounded cells showed no evidence of colony formation or cellular migration; cells remained long and slender. (b) Diabetic wounded cells irradiated with 5 J/cm² began to show signs of haptotaxis and migration, as well as colony formation. (c) Cells irradiated with 16 J/cm² began to show signs of stress, and cells appeared shrunken with some cellular lysis. There was no evidence of colony formation, haptotaxis, or migration.

FIG. 2. Diabetic (D) and diabetic wounded (DW) WS1 cells were irradiated once with a He-Ne laser (632.8 nm) with a fluence of 5 J/cm² or 16 J/cm² 30 min post-wound induction. Normal (N), normal wounded (NW), D, and DW control cells were not irradiated (0 J/cm²). Following laser irradiation, the expression of IL-6 was assessed by ELISA. DW cells irradiated with 5 J/cm² showed a significant increase in IL-6 expression compared to N and NW unirradiated cells (P < 0.01), D and DW unirradiated cells (P < 0.01 and P < 0.05, respectively), and D cells irradiated with 5 J/cm² (P < 0.05). D cells irradiated with 5 J/cm² only showed an increase compared to N and NW unirradiated cells (P < 0.05). Cells irradiated with 16 J/cm² showed a significant decrease compared to cells irradiated with 5 J/cm² (P < 0.01).
(P < 0.01 and P < 0.05, respectively), while diabetic cells (5 J/cm²) showed an insignificant increase. Diabetic wounded cells irradiated with 5 J/cm² also showed a significant increase in hIL-6 expression compared to diabetic cells irradiated with 5 J/cm² (P < 0.05). Cells irradiated with 16 J/cm² showed no significant change compared to all unirradiated control cells. However, compared to cells irradiated with 5 J/cm², diabetic and diabetic wounded cells irradiated with 16 J/cm² showed a significant decrease in hIL-6 (P < 0.01).

The ability of cells to retain NR in their lysosomes was used as a measure of cellular proliferation following laser irradiation (Fig. 3). There was no significant change between wounded and unwounded unirradiated control cells, as well as between diabetic induced and nondiabetic WS1 cells. Diabetic wounded cells irradiated with 5 J/cm² showed an increase in NR that approached statistical significance compared to normal, normal wounded, and diabetic wounded unirradiated cells (P = 0.05, P = 0.057, and P = 0.057, respectively). The decrease seen in diabetic cells (5 J/cm²) was insignificant compared to all the controls. Diabetic and diabetic wounded cells irradiated with 16 J/cm² showed a significant decrease compared to normal, normal wounded, diabetic, and diabetic wounded unirradiated control cells (P < 0.05). Diabetic wounded cells irradiated with 5 J/cm² showed a significant increase in NR retention compared to normal, normal wounded, diabetic, and diabetic wounded unirradiated control cells (P < 0.01) and diabetic wounded cells irradiated with 16 J/cm² (P < 0.001).

DISCUSSION

Among the clinical symptoms caused by diabetes mellitus, delayed wound healing poses a potential risk for patients. Approximately 15% of individuals with diabetes mellitus will develop foot ulcers, and 6% of these will require hospitalization for the treatment of such
When applied correctly, and the correct laser parameters are chosen, laser therapy can facilitate wound healing in slow-to-heal wounds, even in diseased conditions such as diabetes.\textsuperscript{16,17}

Laser therapy has been shown to enhance tissue repair by releasing growth factors from fibroblasts.\textsuperscript{3,30} IL-6 is an important cytokine involved in wound healing, stimulating both proliferation and differentiation. Laser irradiation has been found to increase cytokine (IL-6) expression in aortic smooth muscle cells\textsuperscript{20} and normal wounded and unwounded WS1 fibroblast cells,\textsuperscript{3} as well as immune cells.\textsuperscript{21} This study showed that diabetic wounded cells irradiated with 5 J/cm\textsuperscript{2} at 632.8 nm (2.206 mW/cm\textsuperscript{2}) showed a significant increase in cellular proliferation as shown by the increase in hIL-6 expression and NR retention compared to diabetic cells irradiated with 5 J/cm\textsuperscript{2}. It has been shown that the effect of phototherapy on normal regenerating tissue may be insignificant. Thus if a cell is growing normally and fully functional and irradiated with laser light, there is nothing to be stimulated, and thus no effect will be observed. This explains the increase in proliferation in irradiated (5 J/cm\textsuperscript{2}) diabetic wounded cells compared to diabetic cells. There was no significant difference in IL-6 or proliferation between wounded and unwounded unirradiated control cells; thus the increase observed in irradiated diabetic wounded cells was a direct result of the laser irradiation. Diabetic wounded cells (5 J/cm\textsuperscript{2}) also showed an increase in proliferation (hIL-6 and NR) and migration compared to diabetic wounded unirradiated cells (0 J/cm\textsuperscript{2}), indicating a stimulatory effect of the laser light.

At a higher fluence, irradiation can induce the opposite effect. Diabetic wounded cells irradiated with 16 J/cm\textsuperscript{2} showed no significant change in hIL-6 expression and no signs of cellular migration, cells began to show signs of cellular damage, and there was a significant decrease in NR retention. These results correlate with those of Hawkins and Abrahamse.\textsuperscript{3} Similar studies\textsuperscript{22} have found an increase in cellular and genetic damage when cells were irradiated with 16 J/cm\textsuperscript{2}. This study further confirms that a fluence of 16 J/cm\textsuperscript{2} inhibits wound healing in diabetic cells in vitro. It is thus important when performing studies using low-level lasers that the correct parameters, particularly fluence, are chosen. A study using this therapy may be interpreted as negative or having no effect due to incorrect parameters.

Morohoshi et al.\textsuperscript{13,31} found an increase in IL-6 expression in monocytes grown in 22 and 33 mM glucose. Although this study found an increase in hIL-6 expression in diabetic induced unirradiated WS1 cells compared to nondiabetic unirradiated cells, this increase was not significant. Cells were continuously cultured for several passages in 22.6 mM glucose; thus this insignificance could not be attributed to an insufficient incubation time in a hyperglycemic environment. IL-6 has been linked to the pathogenesis of type 1 diabetes,\textsuperscript{4,12,13} and altered IL-6 levels have also been associated with delayed wound healing in diabetes.\textsuperscript{15} The increase in IL-6 in irradiated diabetic WS1 cells stimulated proliferation and migration, important events in wound healing. In a similar study, diabetic wounded WS1 cells were irradiated on days 1 and 4, and hIL-6 was determined. There was no longer a significant increase in hIL-6 (data not shown); levels had returned to those found in nondiabetic WS1 cells, and there was complete wound closure. This correlates with the literature, which states that IL-6 levels peak and decrease over time.\textsuperscript{5–11}

It is evident from this study that low-level He-Ne laser irradiation (632.8 nm, 2.206 mW/cm\textsuperscript{2}) at the correct fluence (5 J/cm\textsuperscript{2}) can stimulate the expression of cytokines (more specifically IL-6), increase proliferation, and stimulate cellular migration in diabetic wounded fibroblast cells. Phototherapy appears to be beneficial in diabetic wound healing and should be further optimized in vivo so that this therapy can become routinely used as an alternative treatment for slow-to-heal wounds, not only in patients with diabetes, but in all wound cases.

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