Lasers Med Sci (2008) 23:11–18
DOI 10.1007/s10103-007-0445-y

ORIGINAL ARTICLE

Laser light influences cellular viability and proliferation in diabetic-wounded fibroblast cells in a dose- and wavelength-dependent manner

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Received: 21 August 2006 / Accepted: 1 December 2006 / Published online: 15 March 2007

Abstract Phototherapy stimulates metabolic processes in healing wounds. Despite worldwide interest, phototherapy is not firmly established or practiced in South Africa. This study aimed to determine which dose and wavelength would better induce healing in vitro. Diabetic-induced wounded fibroblasts were irradiated with 5 or 16 J/cm^2 at 632.8, 830, or 1,064 nm. Cellular morphology, viability (Trypan blue and apoptosis), and proliferation (basic fibroblast growth factor) were then determined. Cells irradiated with 5 J/cm^2 at 632.8 nm showed complete wound closure and an increase in viability and basic fibroblast growth factor (bFGF) expression. Cells irradiated at 830 nm showed incomplete wound closure and an increase in bFGF expression. Cells irradiated at 1,064 nm showed incomplete closure and increased apoptosis. All cells irradiated with 16 J/cm^2 at all three wavelengths showed incomplete wound closure, increased apoptosis, and decreased bFGF expression. This study showed that diabetic-wounded cells respond in a dose- and a wavelength-dependent manner to laser light. Cells responded the best when irradiated with a fluence of 5 J/cm^2 at a wavelength of 632.8 nm.

Keywords Apoptosis · bFGF · Laser · Photostimulation · Proliferation · Viability

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Introduction

Low level laser therapy (LLLT) has been investigated and used clinically for over 30 years, and the worldwide interest in LLLT is illustrated by its use in more than 85 institutions in over 37 countries [1]. LLLT includes wavelengths of between 500 and 1,100 nm and typically involves a fluence of 1 to 4 J/cm^2 using lasers with output powers of 10 to 90 mW [2]. The visible red (e.g., He–Ne, 632.8 nm) and infrared portions of the spectrum (e.g., GaAIAs 800 to 900 nm) have been shown to have highly absorbent and unique therapeutic effects in living tissues [3] and seem to provide the best results [4]. Reports of LLLT applied to soft tissues in vitro and in vivo suggest stimulation of specific metabolic processes in healing wounds, although low doses of LLLT are stimulatory, but high doses are suppressive [5]. For stimulative effects, there is a narrow and well-defined range of parameters of light, while the inhibitive fluence and intensity ranges are not so exactly determined and are much broader [6]. Parameters such as wavelength, fluence, and intensity play the most important roles in both the stimulation and inhibition of cellular metabolism.

Fibroblasts are cells of paramount importance in the process of wound healing, and the stimulatory effects of LLLT on fibroblast proliferation in vitro are well established. Some of the literature also alludes to the fact that LLLT may have no effect or an inhibitory effect on fibroblast proliferation and activity. Almeida-Lopez et al. [7] suggest that LLLT acts by improving in vitro fibroblast proliferation, and a smaller laser exposure time results in higher proliferation. Wavelength also seems to play a role in the stimulation or inhibition of fibroblasts. Abergel et al. [8] showed that a Nd:YAG laser suppressed collagen synthesis, while the He–Ne and Ga–As (gallium–arsenide) laser stimulated collagen production. Thus, the Nd:YAG

laser may be useful in the treatment of keloids and hypertrophic scars, while low-energy lasers may be useful in enhanced wound healing. Studying the mitotic rate in human fibroblast cells, Lubart et al. [9] found a nonlinear dose and intensity dependence after irradiating with two different wavelength ranges.

Similar results were obtained by Bolton et al. [10] who used an 860-nm diode laser. Their results suggested a relationship between fibroblast proliferation and succinic dehydrogenase (enzyme involved in the respiratory chain). At 2 J/cm^2, both cellular proliferation and enzyme activity were significantly increased, whereas at a fluence of 16 J/cm^2, inhibition of both parameters was noted. Kreisler et al. [11] irradiated periodontal ligament fibroblasts twice and three times at 24-h intervals in vitro to a GaAIAs diode laser (wavelength, 809 nm; power output, 10 mW; and fluence of 1.96, 3.92, or 7.84 J/cm^2). Proliferation of cells was assessed by the Almar blue assay, where they found an increase in activity in all irradiated cell cultures as compared to un-irradiated controls. The difference was highly significant ($P<0.001$) on days 1 and 2 after irradiation and decreased slightly but still remained significantly increased on day 3. Khadra et al. [12] irradiated human oral fibroblast cells in vitro to a GaAIAs diode laser and found that fluences of 1.5 and 3 J/cm^2 increased cellular proliferation.

Various cytokines and growth factors are important at each stage of wound healing. LLLT has been shown to stimulate the production of basic fibroblast growth factor (bFGF), a multifunctional polypeptide that supports fibroblast proliferation and differentiation [13, 14]. Yu et al. [15] studied the effect of cellular proliferation and the release of bFGF after irradiating fibroblast and endothelial cells with a 660-nm laser and found that the maximal release of bFGF was at a fluence of 2.2 J/cm^2. A further effect of bFGF is the transformation of fibroblasts into myofibroblasts, which are responsible for wound contraction. Pourreau-Schneider et al. [16] showed a direct and large transformation of cultured fibroblasts into myofibroblasts as early as 24 h after laser irradiation (He–Ne). Medrado et al. [17] also found a positive effect of LLLT on myofibroblasts.

It is estimated that between 2 and 5% of the population is affected by diabetes, and approximately one half of all diabetics will require surgery [18]. There are "mechanical" impairments and metabolic defects, which result in delayed wounded healing in diabetes. The effects of diabetes on wound healing include retardation of closure, delayed contraction, effects on granulocytes, defects in chemotaxis, interference with collagen synthesis, and effects on red blood cells [18]. Various authors have found a positive effect of LLLT on diabetic wound healing in rodents [19–25] and humans [26–31].

This study aimed to establish which wavelength (632.8, 830, or 1,064 nm) and fluence (5 or 16 J/cm^2) would better induce wound healing in a diabetic fibroblast wounded in vitro model.

Materials and methods

Cell culture

Human skin fibroblast cells (WS1; Adcock S.A., ATCC CRL 1502) were grown as described by Hawkins and Abrahamse [32, 33]. An in vitro diabetic wound model was based on Rigau et al. [34], Hamuro et al. [35], and Vinck et al. [36]. Cells were cultured in complete media containing an additional 17 mMol/l D-glucose. To determine the effects of the lasers, cells were detached by trypsinization (1 ml/25 cm^2, 0.25% trypsin–0.03% ethylenediamine tetraacetic acid), and 6×10^5 cells in 3 ml complete culture media 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 induced 30 min before laser irradiation [34].

Laser set-up and irradiation

Wounded diabetic-induced WS1 cells were irradiated in the dark with three different lasers, namely, the He–Ne (632.8 nm), diode (830 nm), and Nd:YAG (1,064 nm) lasers. Laser parameters are shown in Table 1. Cells were irradiated with a fluence of either 5 or 16 J/cm^2 on days 1 and 4 (72 h between irradiations). Post-laser irradiation on day 4, cells were incubated for an hour (37°C) before tests, were performed. Normal unwounded un-irradiated cells and diabetic-wounded un-irradiated cells were used as controls. After irradiation and incubation for an hour, cells were detached by trypsinization and re-suspended to a final concentration of approximately 1×10^5 cells/100 μl.

Morphology

Cellular morphology was determined by inverted microscopy (Olympus CKX41); the formation of spindle-shaped cells in wounded cultures was evaluated, characterizing normal processes involved in wound healing [34]. Colony formation, haptotaxis (orientation of cells at the wound margin), chemotaxis (migration), and the number of cells in the central scratch were also evaluated.

Cellular viability

Cellular viability was determined by Trypan blue exclusion test and apoptosis (caspase 3/7). Trypan blue is a vital stain

Table 1 Laser parameters

Laser	Wavelength	Wave emission	Power output	Power density	Spot size	Duration of irradiation
He–Ne[a]	632.8 nm	CW	23 mW	2.206 mW/cm^2	9.1 cm^2	37 min (5 J/cm^2) 2 h (16 J/cm^2)
Diode	830 nm	CW	55 mW	6 mW/cm^2	9.1 cm^2	13 min 45 s (5 J/cm^2) 44 min (16 J/cm^2)
Nd:YAG	1,064 nm	CW	1 W	12.7 mW/cm^2	78.5 cm^2	6 min 34 s (5 J/cm^2) 21 min (16 J/cm^2)

[a] Due to expanding, 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.
CW Continuous wave emission

used in estimating the proportion of viable cells in a population. An equal volume of 0.4% Trypan blue (Sigma Aldrich, S.A. T8154) in hanks-balanced salt solution (Adcock S.A., 10-543F) was added to re-suspended cells and allowed to incubate at room temperature for 5 to 15 min. The number of viable and non-viable cells was counted and the percentage viability calculated. The Caspase-Glo™ 3/7 assay (Whitehead Scientific S.A., Promega, TB323) was used to measure the activity of caspase-3 and 7. The addition of reagent results in cellular lysis followed by substrate cleavage by caspase, and as a result, a luminescent signal is generated by luciferase. Negative controls consisted of reagent and culture media without cells. A positive control was included by inducing apoptosis in 1×10^6 cells/ml using 0.5 μg/ml Actinomycin D (Sigma Aldrich, S.A. A5156-1VL). An equal volume of reagent was added (25 μl), contents mixed and incubated at room temperature for 3 h. Luminescence was read using the Junior EG and G Berthold luminometer and reported in reading light units (RLU). Luminescence is proportional to the amount of caspase activity present.

Cellular proliferation

The bFGF released into culture media after irradiation was determined using the indirect enzyme-linked immunosorbent assay (ELISA). Human bFGF (Sigma Aldrich S.A., F0291) in PBS-T (10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween 20) was used as a positive control and to establish a standard curve. A serial dilution of 25 μg/ml bFGF was made in 100 μl carbonate–bicarbonate buffer (Sigma Aldrich S.A., C-3041), ranging from 25 μg/ml to 6.1 ng/ml. The standard was used to determine the concentration of the unknown samples. Isotype-matched, nonspecific mouse immunoglobulin (Mouse IgG1 Kappa, Sigma Aldrich S.A. M90935) in PBS-T was used as a negative control.

Two hundred microliters of sample (100 μl culture media and 100 μl carbonate–bicarbonate buffer) was incubated overnight at 4°C in Nunc Maxisorp 96-well microplate (Adcock S.A., 3894). Plates were washed three times with PBS-T using a microplate washer (BioRad PW40). Two hundred microliters monoclonal anti-fibroblast growth factor primary antibody (Sigma Aldrich S.A., F6162; diluted 1:10000 in PBS-T) was added to each well and incubated at room temperature for 2 h. The coating solution was removed and washed as before. Two hundred microliters of goat anti-mouse IgG (Fab specific), horseradish peroxidase-conjugated secondary antibody (Whitehead Scientific S.A., D0705; diluted 1:4,000 in PBS-T) was added to each well and incubated at room temperature for 2 h. The coating solution was removed and washed as before. One hundred microliters of freshly prepared 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Scientific Group, BD 555214) was added. After 30-min incubation at room temperature, color development was stopped by adding 100 μl 1 mol/ml H_2SO_4 and read spectrophotometrically at 450 nm (BioRad Plus microplate spectrophotometer).

Statistical analysis

Each experiment was performed four times, and each test was done in duplicate, the average of which was used. Statistical analysis was done using the one-tailed Student's *t* test, using SigmaPlot version 8.0, and was considered statistically significant when $P<0.05$ and plotted using positive and negative standard error bars.

Results

Morphology

Figure 1 shows the typical morphology of diabetic WS1 cells. Cells were long and slender, and when a wound was inflicted into the monolayer, cells began to change their direction of growth and migrate into the central scratch in an attempt to close the 'wound'. Wounded diabetic-induced WS1 cells were irradiated at a wavelength of 632.8, 830, or 1,064 nm with a fluence of 5 or 16 J/cm^2 on days 1 and 4 and incubated for an hour post-irradiation on day 4. When irradiated at a wavelength of 632.8 nm, the rate of cellular

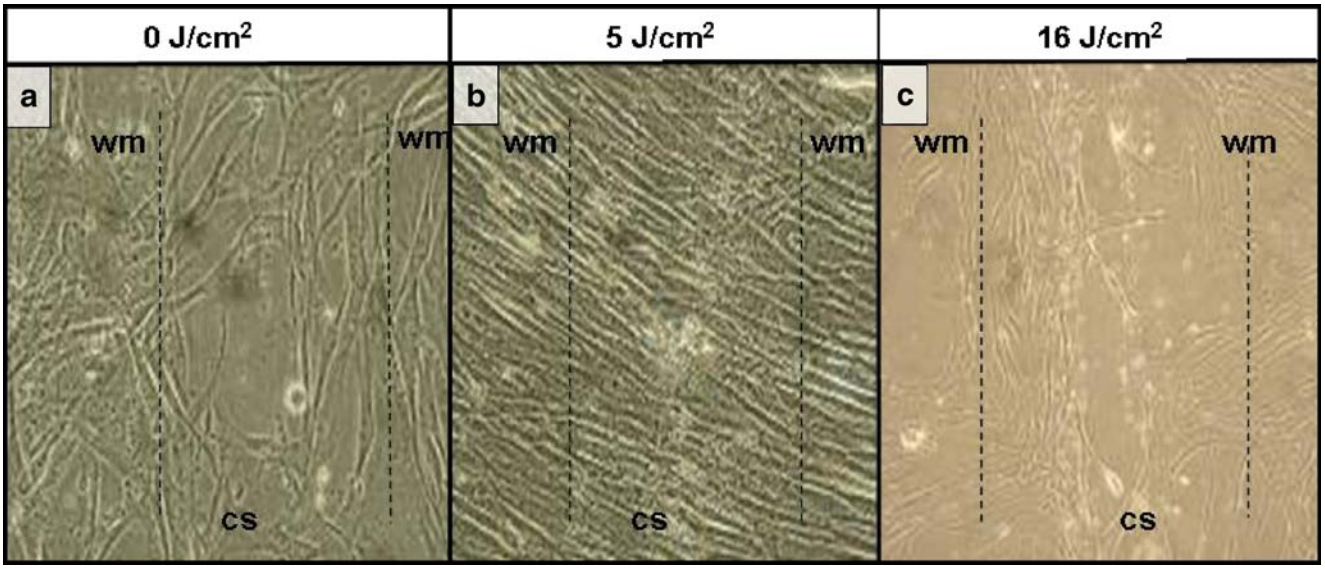

Fig. 1 Micrograph (400×) of diabetic-wounded WS1 cells irradiated on days 1 and 4 at a wavelength of 632.8 nm with a fluence of either 5 J/cm^2 (**b**) or 16 J/cm^2 (**c**) and incubated for an hour at 37°C post-irradiation on day 4. A wound margin (*wm*) can clearly be seen both sides of the central scratch (*cs*), and cells have migrated into the cs. Cells irradiated with a fluence of 5 J/cm^2 (**b**) showed complete wound closure compared to un-irradiated cells (**a**), indicating a stimulatory effect. Cells irradiated with a fluence of 16 J/cm^2 (**c**) showed incomplete closure, indicating an inhibitory effect

migration of cells irradiated with 5 J/cm^2 into the central scratch was significantly higher than the diabetic-wounded control cells, as well as cells irradiated with 16 J/cm^2. There was complete wound closure by day 4, whereas controls and cells irradiated with 16 J/cm^2 showed incomplete wound closure (Fig. 1a–c). Cells irradiated at a wavelength of 830 nm with a fluence of 5 J/cm^2 showed more migration into the central scratch compared to diabetic-wounded un-irradiated cells and cells irradiated with 16 J/cm^2; there was a higher number of cells in the central scratch, however, there was still areas of incomplete wound closure. Diabetic-wounded cells irradiated at 1,064 nm with a fluence of 5 J/cm^2 did not show an increased rate of cellular migration as compared to cells irradiated with 16 J/cm^2. There was incomplete wound closure in both cases, as well as in diabetic-wounded un-irradiated cells.

Cellular viability

Cellular viability was determined by the Trypan blue exclusion test (% viability; Fig. 2) and apoptosis (caspase 3/7; Fig. 3). In all three wavelengths, diabetic-wounded un-irradiated cells showed slightly decreased % viability compared to normal un-irradiated cells, whereas the increase in apoptosis was significant ($P<0.000$ for 632.8 nm, $P=0.008$ for 830 nm, and $P=0.002$ for 1,064 nm).

Diabetic-wounded cells irradiated at a wavelength of 632.8 nm with a fluence of 5 J/cm^2 showed a significant increase in % viability compared to diabetic-wounded un-irradiated cells ($P=0.001$) and cells irradiated with 16 J/cm^2 ($P=0.047$). Compared to normal un-irradiated cells, there was no significant change. There was a significant increase in caspase-3 and 7 compared to normal un-irradiated cells ($P<0.000$), however when compared to diabetic wounded un-irradiated cells there was no significant change ($P=0.063$). Cells irradiated with a fluence of 16 J/cm^2 showed a decrease in % viability, but was not significant when compared to normal un-irradiated cells ($P=0.052$). The increase in apoptosis compared to normal and diabetic wounded un-irradiated cells

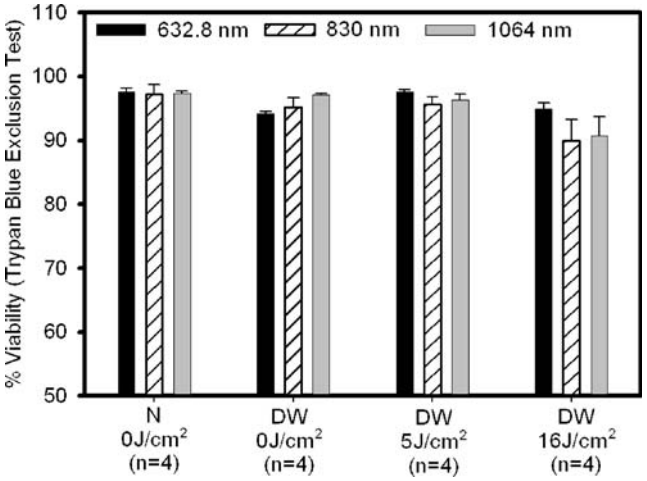

Fig. 2 Cellular viability was determined by the Trypan blue exclusion test (% viability) in diabetic-wounded (*DW*) WS1 cells irradiated with either 5 or 16 J/cm^2. Unwounded normal (*N*) and DW un-irradiated WS1 cells were used as controls. DW cells irradiated at a wavelength of 632.8 nm with a fluence of 5 J/cm^2 showed a significant increase in % viability compared to DW un-irradiated cells ($P=0.001$), as well as cells irradiated with 16 J/cm^2 ($P=0.047$). Cells irradiated with 16 J/cm^2 to all three wavelengths showed a decrease in % viability

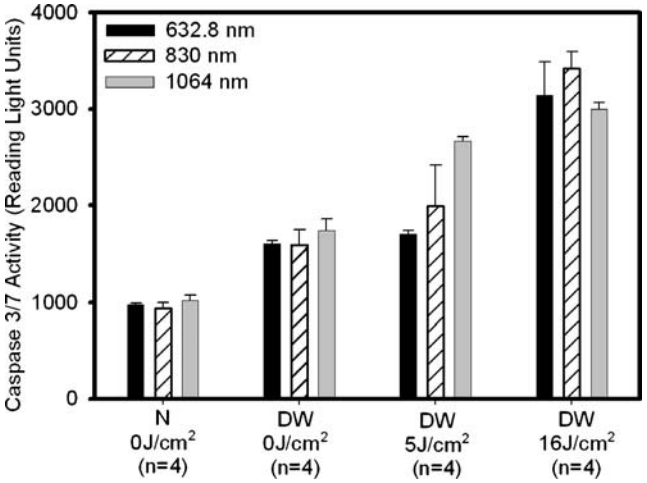

Fig. 3 Caspase 3/7 activity was determined as a function of apoptosis in diabetic-wounded (*DW*) WS1 cells irradiated with 5 or 16 J/cm^2. Unwounded normal (*N*) and DW un-irradiated WS1 cells were used as controls. There was a significant increase in DW cells (5 J/cm^2) irradiated at 632.8 and 1,064 nm compared to N ($P<0.000$). The same cells irradiated at 1,064 nm also showed a significant increase compared to DW un-irradiated cells ($P<0.000$). Cells irradiated with 16 J/cm^2 at 632.8, 830, and 1,064 nm showed a significant increase compared to N ($P=0.001$, $P<0.000$, and $P<0.000$, respectively) and DW un-irradiated cells ($P=0.004$, $P<0.000$, and $P<0.000$, respectively), as well as DW cells irradiated with 5 J/cm^2 ($P=0.006$, $P=0.021$, and $P=0.008$, respectively). DW un-irradiated cells showed a significant increase compared to N un-irradiated cells ($P=0.006$ for 632.8 nm, $P=0.021$ for 830 nm, and $P=0.008$ for 1,064 nm)

was significant ($P=0.001$ and $P=0.004$ respectively), and cells irradiated with 5 J/cm^2 ($P=0.006$).

Cells irradiated at a wavelength of 830 nm with a fluence of either 5 or 16 J/cm^2 and incubated for an hour showed no significant change in % viability. Cells irradiated with 5 J/cm^2 showed an increase in caspase-3 and 7 compared to normal un-irradiated cells; however, this increase was not significant ($P=0.05$). On the other hand, cells irradiated with 16 J/cm^2 showed a significant increase in caspase-3 and 7 compared to normal and diabetic wounded un-irradiated cells ($P<0.000$) and cells irradiated with 5 J/cm^2 ($P=0.021$). Cells irradiated with 16 J/cm^2 showed an increase in caspase-3 and 7 compared to the same cells irradiated at a wavelength of 1,064 nm ($P<0.000$).

When irradiated at a wavelength of 1,064 nm with either 5 or 16 J/cm^2, there was no significant change in % viability, although cells irradiated with 16 J/cm^2 showed a decrease compared to normal and diabetic wounded un-irradiated cells ($P=0.066$ and $P=0.076$, respectively). There was, however, a significant increase in caspase-3 and 7 in cells irradiated with either 5 or 16 J/cm^2 compared to normal and diabetic wounded un-irradiated cells ($P< 0.000$). Cells irradiated with 16 J/cm^2 showed higher caspase-3 and 7 activity than cells irradiated with 5 J/cm^2 ($P=0.008$). There was a significant increase in caspase-3 and 7 in diabetic-wounded cells irradiated at 1,064 nm with 5 J/cm^2 compared to the same cells irradiated to a wavelength of 632.8 nm ($P<0.000$).

Cellular proliferation

Cellular proliferation was determined by measuring bFGF expression by indirect ELISA. Human bFGF was used to establish a standard curve from which sample concentration was determined (Fig. 4). Diabetic-wounded cells irradiated at 632.8 nm with 5 J/cm^2 showed a significant increase in bFGF expression compared to normal ($P=0.006$) and diabetic wounded un-irradiated cells ($P=0.002$), as well as cells irradiated with a fluence of 16 J/cm^2 ($P=0.004$). Compared to cells irradiated at a wavelength of 1,064 nm, there was a significant increase in cells irradiated with 5 J/cm^2 ($P=0.028$). There was no significant change in cells irradiated with a fluence of 16 J/cm^2 compared to un-irradiated cells. Just as cells irradiated at 632.8 nm with 5 J/cm^2, there was a significant increase in cellular proliferation in diabetic-wounded cells irradiated at 830 nm with a fluence of 5 J/cm^2 compared to normal ($P=0.002$) and diabetic wounded un-irradiated cells ($P<0.000$). Cells irradiated with a fluence of 16 J/cm^2 showed a significant decrease compared to normal un-irradiated cells ($P=0.015$) and cells irradiated with 5 J/cm^2 ($P<0.000$). Cellular proliferation as determined by bFGF expressed into the culture media showed no significant change in cells

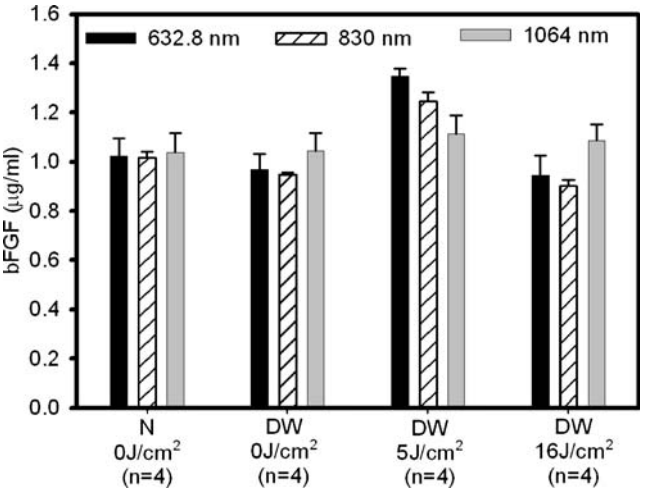

Fig. 4 Cellular proliferation was determined by measuring basic fibroblast growth factor (*bFGF*) expression by indirect ELISA in diabetic-wounded (*DW*) WS1 cells irradiated with either 5 or 16 J/cm^2. Unwounded normal (*N*) and DW un-irradiated WS1 cells were used as controls. bFGF concentration was determined from the standard run with each plate. DW cells irradiated with 5 J/cm^2 at a wavelength of 632.8 nm or 830 nm showed a significant increase in bFGF expression compared to N ($P=0.006$ and $P=0.002$, respectively) and DW un-irradiated cells ($P=0.002$ and $P<0.000$, respectively), as well as DW cells irradiated with 16 J/cm^2 ($P=0.004$ and $P<0.000$, respectively). Cells irradiated at 1,064 nm showed no significant change

irradiated with either 5 or 16 J/cm^2 at a wavelength of 1,064 nm. Diabetic-wounded cells irradiated with 16 J/cm^2 showed a significantly higher bFGF concentration than the same cells irradiated at a wavelength of 830 nm (P=0.038).

Discussion

Overall, this study proved that the model chosen, namely diabetic-induced wounded fibroblast cells, was suitable for basic research investigations concerning the biological mechanisms of LLLT on human cells. When WS1 cells were grown in additional glucose (17 mMol/l) to simulate a diabetic in vitro model, cellular migration was not inhibited, corresponding to the work of Abrahamse et al. [37]. Diabetic-wounded irradiated and un-irradiated cells were still able to change their direction of growth (haptotaxis) and migrate (chemotaxis) into the central scratch. This is in contrast with the work conducted by Hamuro et al. [35], where they found that culturing human aortic endothelial cells (HAEC) in 10 or 30 mM glucose inhibited cellular migration, while McDermott [38] found an increase in cellular migration when human corneal epithelial (HCE) cells were grown in 17.5 mM glucose. Diabetic wounded un-irradiated cells showed no significant difference in proportion viability compared to normal un-irradiated cells; however, there was a significant increase in apoptosis (caspase 3/7). A number of studies have found that there is a significant increase in apoptosis [39–43] when cells are grown in conditions of hyperglycemia. If cells were left to incubate for longer, there may have be a significant decrease in proportion viability in diabetic wounded un-irradiated cells, as there is an increase in apoptosis (caspase 3/7).

Irradiation of diabetic wounded cells at a wavelength of 632.8 nm (He–Ne laser with output power of 23 mW) with a fluence of 5 J/cm^2 on days 1 and 4 increased cellular migration and wound closure. There was complete wound closure compared to un-irradiated cells. In a similar study conducted by Hawkins and Abrahamse [33] using WS1 cells, there was a rapid migration of cells when irradiated to a He–Ne laser with a fluence of 5 J/cm^2 on 2 consecutive days. When diabetic-wounded cells were irradiated at a wavelength of 830 or 1,064 nm with a fluence of 5 J/cm^2, the rate of cellular migration was slower than cells irradiated to 632 nm, with more areas of incomplete wound closure. Cells irradiated at 1,064 nm showed the least amount of wound closure. All cells irradiated with a fluence of 16 J/cm^2 to all three wavelengths showed a slower rate of cellular migration with incomplete wound closure, indicating that phototherapy can stimulate cellular migration in a dose and, more significantly, wavelength-dependent manner.

Diabetic-wounded cells irradiated with a fluence of 5 J/cm^2 at a wavelength of 632.8 or 830 nm showed a significant increase in apoptosis (caspase 3/7 activity) when compared to normal un-irradiated cells. However, when compared to diabetic-wounded un-irradiated cells, there was no significant difference. The increase in caspase 3/7 activity is likely due to growing cells in a hyperglycemic environment and not laser-induced. Carnevalli et al. [44] found that irradiation of CHO K-1 cells to a semiconductor laser (830 nm; 10 mW; 2 J/cm^2) prevents apoptosis, as well as re-establishing cellular homeostasis, and Wang et al. [45] found that irradiation of lung adenocarcinoma cells (He–Ne laser) with a fluence exceeding 60 J/cm^2 induces apoptosis in a fluence-dependent manner. When diabetic-wounded WS1 cells were irradiated with a fluence of 16 J/cm^2 to either 632.8 or 830 nm, the increase in caspase activity was significant when compared to both normal and diabetic-wounded un-irradiated cells. Diabetic-wounded cells irradiated with either 5 or 16 J/cm^2 at a wavelength of 1,064 nm had significantly increased caspase 3/7 activity. Laser irradiation can stimulate wound healing and protect cells from apoptosis at a fluence of 5 J/cm^2 or inhibit wound healing and activate caspase 3/7 at 16 J/cm^2, and the effects are, thus, dose dependent.

This study showed that diabetic-wounded WS1 cells responded in a dose and, more significantly, a wavelength-dependent manner to laser light. At the correct wavelength (632.8 or 830 nm), a fluence of 5 J/cm^2 was stimulatory; there was complete wound closure by day 4 and cells were protected from apoptosis, while cells irradiated with 16 J/cm^2 showed incomplete wound closure and increased apoptosis. Cells have adapted and responded to the laser light, and cellular repair mechanisms have been triggered despite being subjected to three stressors, namely, growth in a hyperglycemic environment, wound induction, and laser irradiation. A wavelength of 1,064 nm showed the least amount of wound closure and cellular proliferation (bFGF), particularly at a fluence of 16 J/cm^2. This study showed that not only is it important to choose the correct fluence, but it is also important in choosing the correct wavelength. Although studies have shown 1,064 nm to be stimulative in phototherapy, this study found the opposite. This difference in wavelength is likely due to the higher output power (W) of the Nd:YAG laser (1 W), as lower irradiances have been confirmed to be more effective than higher irradiances [46–48].

Acknowledgments The National Laser Center (NLC; contract grant sponsor, research grant), South Africa; National Research Foundation (NRF), South Africa; Medical Research Council (MRC), South Africa and the University of Johannesburg Research Council (URC; contract grant sponsor, research grant) supported this research. Lasers were supplied by the NLC.

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