Influence of Broad-Spectrum and Infrared Light in Combination with Laser Irradiation on the Proliferation of Wounded Skin Fibroblasts

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ABSTRACT

Objective: This study aimed to establish if broad-spectrum or infrared (IR) light in combination with laser therapy can assist phototherapy and accelerate cell proliferation to improve the rate of wound healing. Background Data: The effect of laser light may be partly or completely reduced by broad-spectrum light. There are few studies that investigate the benefit or detriment of combining laser irradiation with broad-spectrum or IR light. Methods: Wounded human skin fibroblasts were irradiated with a dose of 5 J/cm² using a helium-neon laser, a diode laser, or a Nd:YAG laser in the dark, in the light, or in IR. Changes in cell proliferation were evaluated using optical density at 540 nm, alkaline phosphatase (ALP) enzyme activity, cytokine expression, and basic fibroblast growth factor (bFGF) expression. Results: The optical density and ALP enzyme activity indicate that 5 J/cm² using 1064 nm in the light is more effective in increasing cell proliferation or cell growth than 830 nm in the light, but not as effective as 632.8 nm in the light. bFGF expression shows that the response of wounded cells exposed to 5 J/cm² in IR light is far less than the biological response of wounded cells exposed to 5 J/cm² in the dark or light. The results indicate that wounded cells exposed to 5 J/cm² using 632.8 nm in the dark results in a greater increase in IL-6 when compared to cells exposed to 5 J/cm² in the light or in IR. Conclusion: Results indicate that 5 J/cm² (using 632.8 nm in the dark or 830 nm in the light) is the most effective dose to stimulate cell proliferation, which may ultimately accelerate or improve the rate of wound healing.

INTRODUCTION

Light is considered part of the spectrum of electromagnetic energy. This spectrum includes wavelengths of energy that vary considerably in length, from the very short waves in the ultraviolet end of the spectrum (gamma and x-rays) to the very long waves at the infrared (IR) extreme of the spectrum (Fig. 1). The visible portion of the spectrum is composed of wavelengths that range from 400 to 800 nm; however, visibility depends on the intensity and the background, since 500-mW 810-nm light is easily seen in semi-darkness. Tissue is generally more transparent to near-IR wavelengths (800–1200 nm) than to visible light. Photons in the optical field (either as visible, ultraviolet, or IR light) can be transmitted or absorbed when they come in contact with matter.

Several studies have investigated the effect of different wavelengths on cell proliferation. Lubart et al. (1992) examined the effect of 360-nm, 632.8-nm, and 780-nm light on fibroblast cells and suggested that low-level laser therapy (LLLT) in the visible and the near-IR region is due to cell respiration stimulation by either the endogenous porphyrins in the cell or by the cytochromes. Loevschall and Arenholt-Bindslev (1994) investigated the effect of 812-nm irradiation on the proliferation of fibroblasts and demonstrated an increased incorporation of tritiated thymidine, which suggests that LLLT can increase DNA synthesis.

Studies have documented the positive effect of laser irradiation on cell proliferation. Almeida-Lopes et al. (2001) studied the effect of LLLT on cultured fibroblasts using diode lasers at 670 nm, 780 nm, 692 nm, and 786 nm, and the fluence was...
fixed at 2 J/cm². IR laser induced a higher rate of cell proliferation than visible laser when the power outputs were different. However, lasers of equal power output presented similar effect on cell growth independently of their wavelengths. LLLT acts by improving the in vitro fibroblast proliferation, and a smaller exposure time results in higher proliferation rates. Karu (1982) demonstrated stimulating biological effects in cell cultures from monochromatic incoherent light. Cell cultures that are first irradiated with laser light and have consequently exhibited biological effects, which are then irradiated with broad-spectrum (non-monochromatic and incoherent) light, subsequently have their laser-produced biological effects reduced to almost nothing. This indicates that there are more mechanisms at work than simply the excitation of polarization-sensitive chromophores. The influence of ambient light may influence the outcome of both clinical and laboratory studies, and may well explain some of the variability in the results/effects reported in the literature.
Moore et al. (2005) studied the effect of wavelength on proliferation and found that maximum proliferation occurred with 665-nm and 675-nm light, whereas 810-nm light was inhibitory to the proliferation of cultured fibroblasts. The observations suggest that both wavelength and cell type influence the cell proliferation response to LLLT.

LLLT at wavelengths of 950, 660, and 570 nm results in a higher rate of proliferation in cultured fibroblasts in vitro, which indicates a possible stimulatory effect on wound healing in vivo at specific dosimetric parameters. Mendez et al. (2004) studied the effect of GaAlAs laser (830 nm, 35 mW) irradiation with doses of 20 or 50 J/cm² on cutaneous wounds in the dorsum of Wistar rats. The results showed that irradiated subjects showed increased collagen production and organization when compared to non-irradiated controls. The study concluded that LLLT could have a positive biomodulatory effect on the repair of cutaneous wounds.

The induced activation of proliferative processes in the wound occurs due to development of not only local, but also systemic processes, whose nature remains largely unexplored. Samoilova et al. (2003) reported evidence that, 30 min after irradiation of a small area of the volunteer’s body surface with polychromatic visible light together with IR polarized light (400–3400 nm, 95% of polarization) at a therapeutic dose (12 J/cm²), soluble factors appeared in circulating blood, which were able to stimulate proliferation of human keratinocytes in primary culture. Samoilova et al. (2003) suggested that a rapid rise of growth-promoting activity of the entire circulating blood (systemically) may be a consequence of transcutaneous photo-modification of the small amount of light-modified blood in superficial blood vessels (locally) and of the effect of such blood on its entire circulating volume. Zhevago and Samoilova (2004) further suggest that therapy employing polychromatic visible and IR light would promote an increase in the number of lymphocytes in peripheral blood and enhance their response to antigenic stimuli.

Visible and IR irradiation of laser and non-laser sources has a pronounced wound healing effect that develops as a consequence of local and systemic light effects, but many aspects of their mechanisms are still unclear. Lagan et al. (2002) investigated the efficacy of combined phototherapy and LLLT (660–960 nm, diode, 5 kHz pulsed, 12 J/cm²) in the management of chronic venous ulceration when used in conjunction with standardized nursing intervention. From the wound and pain assessment, there was an apparent clinical difference in the rate of wound healing, and a continued reduction in wound size was evident in the treatment group.

There are few studies that investigate the benefit or detriment of combining laser irradiation with broad-spectrum (visible white light) or IR light (ExoTerra Heat Glo; 240 V, 100 W). This study aimed to establish if broad-spectrum or IR light has any benefit when irradiating wounded human skin fibroblasts with a dose of 5 J/cm² using a HeNe (632.8 nm) laser, a diode (830 nm) laser, or a Nd:YAG (1064 nm) laser. Results showed that only the decrease in IL-6 expression and increase in cell growth or proliferation differentiated wounded cells exposed to 5 J/cm² using 1064 nm in IR light from cells exposed to 1064 nm in the dark, indicating that IR light can support laser irradiation with 1064 nm providing that the duration of exposure is minimal to limit the undesirable thermal effects. This study determined that broad-spectrum white light or IR light in combination with laser therapy could assist phototherapy to accelerate cell proliferation, which may ultimately improve the rate of wound healing.

**METHODS**

**Cell culture procedure**

Human skin fibroblast monolayer cultures (American Type Culture Collection [ATCC], Manassas, VA; CRL1502 WS1) were grown in Eagle’s minimal essential medium (EMEM) with Earle’s balanced salt solution that was modified to contain 2 mM l-glutamine, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1% fungizone, and 1% penicillin-streptomycin, and supplemented with 10% V/V fetal bovine serum (serum-rich medium). The cultures were incubated at 37°C with 5% CO₂ and 85% humidity. Cells were trypsinized using a 0.25% (W/V) trypsin 0.03% EDTA solution in Hanks balanced salt solution (HBSS). Approximately 6.5 × 10⁵ cells (in 3 mL of culture medium) were seeded in 3.4-cm-diameter culture plates and incubated overnight to allow the cells to attach.

**Laser specifications and exposure regime**

Once the fibroblasts had attached, 2 mL of culture medium was removed, and a wound was induced before the cells were irradiated (Table 1). For the simulated wound environment, confluent monolayers were first scratched with a sterile pipette of 2 mm in diameter, and the plates were incubated at 37°C for 30 min before they were irradiated. Each scratch was irregular, and the size of the wounds ranged from 1 to 2 mm in diameter. Wounded cells were exposed to 5 J/cm² either in the dark or in broad-spectrum white light (Philips fluorescent lights). A Nd:YAG laser was used to perform the irradiation in the dark, but the Nd:YAG laser was placed out of the field of view. A toothbrush was used to prevent any reflection of the laser beam on the wound site or any unintended areas.

**Table 1. Summary of Laser Parameters Used**

<table>
<thead>
<tr>
<th>Power density</th>
<th>Wavelength</th>
<th>Power output</th>
<th>Energy density</th>
<th>Spot size/area</th>
<th>Duration</th>
<th>Exposures</th>
</tr>
</thead>
<tbody>
<tr>
<td>632.8 nm</td>
<td>18.8 mW</td>
<td>2.07 mW/cm²</td>
<td>5 J/cm²</td>
<td>3.4 cm/9.1 cm²</td>
<td>41 min 40 sec</td>
<td>Days 1 and 4</td>
</tr>
<tr>
<td>830 nm</td>
<td>54 mW</td>
<td>5.95 mW/cm²</td>
<td>5 J/cm²</td>
<td>3.4 cm/9.1 cm²</td>
<td>12 min 49 sec</td>
<td>Days 1 and 4</td>
</tr>
<tr>
<td>1064 nm</td>
<td>1 W</td>
<td>12.73 mW/cm²</td>
<td>5 J/cm²</td>
<td>10 cm/78.5 cm²</td>
<td>6 min 34 sec</td>
<td>Days 1 and 4</td>
</tr>
</tbody>
</table>

**TABLE 1. SUMMARY OF LASER PARAMETERS USED**
cent tube; 36 W, 25 mW/cm²) or in IR light (ExoTerra Heat Glo; 240 V, 100 W, 103.9 mW/cm², 17.5 cm above cells) on days 1 and 4.

Scanning mirrors (HeNe) or fiber optics (diode and Nd:YAG) were used to direct the laser light to the culture dish located on the bench top. Cell culture dishes were positioned under the laser beam on a black surface and irradiated at room temperature (with the culture dish lid off). Control experiments determined that fibroblasts could be irradiated at room temperature for approximately 2.5 h without adversely influencing the cellular responses of the cells. The cellular response measurements were made 24 h (day 5) after laser irradiation, to observe the modifications or responses that occur with time.19

Since the HeNe laser has a power density of 2.07 mW/cm² and a spot size of 3.4 cm, the light is divergent and is not as harmful as a narrow parallel beam, which allows the entire volume of intense light to be focused or concentrated on one small area.15 A penetration depth of even some microns can be regarded as sufficient for in vitro and in vivo studies, since most of the relevant target cells required for wound healing are located within the epidermis and upper dermis. However, the Nd:YAG laser, with a wavelength of 1064 nm (near-IR), has a greater-than-desired penetration depth, so the laser energy is less absorbed by target tissue, and more energy is transmitted beyond the target (Fig. 1). This unintended effect can lead to inadvertent heating of deeper tissues and clinically significant thermal damage.1

**Biological assays**

Following laser irradiation, the fibroblasts were trypsinized from the 3.4-cm culture dishes, and the cell suspension (1 × 10⁶ cells/100 μL) was used to assess the effect of laser irradiation on cell proliferation using optical density and basic fibroblast growth factor (bFGF) expression. The culture medium was used to assess alkaline phosphatase (ALP) enzyme activity and cytokine expression (interleukin-6 [IL-6]).

**Optical density.** Spectroscopy at 540 nm was used to measure cell proliferation or cell density of both irradiated and control samples.20 The optical density of 4 × 10⁴ cells in 100 μL of complete EMEM was measured at 540 nm.

**Alkaline phosphatase (ALP) enzyme activity.** ALP is a membrane-bound enzyme released in inflammation, remodeling, and cell proliferation and has been used as a marker for wound healing.21,22 ALP enzyme activity was measured by the colorimetric assay using p-nitrophenyl phosphate as a substrate. A total of 500 μL of culture medium was removed from each plate after each irradiation. 50 μL of the culture medium was pre-incubated with 50 μL of 0.5 M N-methyl-D-glucamine buffer, pH 10.5, 0.25 mM magnesium acetate, 110 mM NaCl, and 0.2% Triton X-100 for 30 min at 37°C. 20 mM p-nitrophenyl phosphate (p-NPP; Sigma N7653) was added and the reaction was incubated at 37°C for 30 min.21,22 The amount of p-nitrophenol liberated was measured at 405 nm.

**Cytokine expression.** The IL-6 enzyme-linked immunosorbent assay (ELISA) assay (BD OptEIA 550799) was used for the quantitative in vitro determination of human IL-6 from cell culture supernatants.23 Fibroblasts are important sources of inflammatory cytokines early in wound healing. Approximately 100 μL of each sample (stored at −20°C), or 100 μL of the standard and 50 μL of ELISA diluent (30 mL of animal serum with 0.09% sodium azide) was incubated under constant shaking at 250 rpm at 15–25°C for 2 h. The solution was removed, and each well was washed five times before 100 μL of working detector (biotinylated anti-human IL-6 monoclonal antibody containing fetal bovine serum and ProClin™-150 as preservative) was added and the plate incubated under constant shaking at 250 rpm at 15–25°C for 1 h. The solution was removed, and each well was washed seven times before 100 μL of 3,3’,5,5’-tetramethylbenzidine (TMB) substrate solution was added, and the plate incubated at 15–25°C for 30 min in the dark. 50 μL of stop solution (1 M phosphoric acid) was added, and the plate was incubated for 1 min. The absorbance was read within 5 min of adding the stop solution at 450 nm with a wavelength correction at 570 nm.23,24

**Basic fibroblast growth factor (bFGF).** Fibroblast growth factors are potent regulators of cell proliferation and differentiation, and are important in normal development, tissue maintenance, and wound repair.25 The indirect ELISA26 was used to evaluate the expression of bFGF. A pure protein of human bFGF (Sigma F0291) was used as a positive control to establish a standard curve. A total of 100 μL of culture medium (antigen) was diluted in 100 μL of carbonate-bicarbonate buffer (Sigma C3041) and incubated overnight at 4°C. On the following day, the coating solution was removed and 200 μL of diluted bFGF (5 μg/mL) monoclonal primary antibody (Sigma F6162) in PBS-T (10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween 20) was added; then, the plate was incubated at room temperature for 2 h. Each incubation step was followed with three waxes of PBS-T. 200 μL of anti-mouse IgG (Fab-specific) peroxidase-conjugated antibody (Santa Cruz sc-2005 200 μg/0.5 mL) diluted (1:4000) in PBS-T was used as the secondary antibody, while 100 μL of TMB substrate reagent (BD Biosciences 555214) was added for colorimetric detection. The orange-yellow color development was stopped after 30 min with 1 mol/L H₂SO₄, and the positive wells were read at 450 nm.26

**Statistical analysis**

Each experiment was repeated on different populations of fibroblast cells between passages 13 and 31. Each biological assay was performed in duplicate, and the average of the two results was used to obtain a final sample number of n = 4. The results were recorded for statistical analysis using SigmaPlot 8.0, and the mean, standard deviation, standard error, and percentage change between the un-irradiated control (0 J/cm²) and the irradiated cells was calculated for each data group and graphically represented. The one-way Student t-test was used to analyze the difference firstly between the un-irradiated control (0 J/cm²) and the irradiated cells, and secondly between the different data groups. Statistical significance was accepted at the 0.05 level (95% confidence interval).

**RESULTS**

The effect of laser irradiation on wounded fibroblasts was evaluated by a number of biological assays. The cellular response mea-
measurements were made 24 h (day 5) after laser irradiation and assessed cell proliferation, cytokine, and growth factor expression.

**Optical density**

The optical density at $A_{540}$ nm was used to measure changes in cell density that may result from proliferation or cell growth (Fig. 2). Wounded cells exposed to 5 J/cm$^2$ using 830 nm in the IR showed a decrease in cell density when compared to cells exposed to 5 J/cm$^2$ using 830 nm in the light ($p = 0.054$). Wounded cells exposed to 5 J/cm$^2$ using 830 nm in the dark showed an increase in cell density when compared to cells exposed in the light; however, the difference did not prove to be statistical. The result corresponds to the ALP enzyme activity assay, indicating that irradiation in the light or dark did not influence cell growth (Table 2). Wounded cells exposed to 5 J/cm$^2$ using 1064 nm in the light showed an increase in cell density when compared to cells using 5 J/cm$^2$ using 830 nm in the light ($p = 0.027$). The results indicate that cell proliferation or cell growth can be influenced by the wavelength or treatment condition (Table 3).

**ALP enzyme activity**

The ALP enzyme activity assay was used to determine changes in cell growth or proliferation following laser irradiation (Fig. 2). Wounded cells exposed to 5 J/cm$^2$ using 632.8 nm in the light showed a decrease in ALP enzyme activity when compared to cells exposed to 5 J/cm$^2$ using 632.8 nm in IR ($p = 0.025$).

The ALP results for wounded cells exposed to 5 J/cm$^2$ using 632.8 nm in the light corresponds with an increase in bFGF, which indicates that when ALP enzyme activity is normal or decreased the cells are growing with sufficient nutrients and growth factors to support proliferation and cell growth.

Wounded cells exposed to 5 J/cm$^2$ using 830 nm in the light showed a decrease in ALP enzyme activity when compared to cells exposed to 5 J/cm$^2$ using 830 nm in IR ($p = 0.0006$). The ALP results for wounded cells exposed to 5 J/cm$^2$ using 830 nm in the light corresponds with an increase in bFGF and optical density. Wounded cells exposed to 5 J/cm$^2$ using 830 nm in the dark showed an increase in ALP enzyme activity when compared to wounded cells exposed to 5 J/cm$^2$ using 830 nm in IR ($n = 4$).
in IR (p = 0.019). However, there was no statistical difference in ALP activity for wounded cells exposed to 5 J/cm² using 830 nm in the dark or in the light, suggesting that 830 nm in the light is more effective than 830 nm in the dark, but that 830 nm in IR is more effective than 830 nm in the dark (Table 2).

Wounded cells exposed to 5 J/cm² using 1064 nm in the dark showed an increase in ALP enzyme activity when compared to wounded cells exposed to 5 J/cm² using 830 nm in the light (p = 0.031). The results indicate that wounded cells exposed to 5 J/cm² using 1064 nm in the dark respond with an increase in ALP enzyme activity or decrease in cell proliferation or cell growth when compared to cells exposed to 1064 nm in IR (p = 0.0007). Wounded cells exposed to 5 J/cm² using 1064 nm in the dark showed a decrease in ALP enzyme activity when compared to wounded cells exposed to 5 J/cm² using 632.8 nm in the dark (p = 0.0008), indicating a higher rate of cell growth or proliferation following exposure to 1064 nm in the dark. The results indicate that cell proliferation or cell growth can be influenced by the wavelength or treatment condition. Wounded cells exposed to 5 J/cm² using 1064 nm in IR showed a decrease in ALP enzyme activity when compared to wounded cells exposed to 5 J/cm² using 632.8 nm in IR (p = 0.037) or 830 nm in IR (p = 0.023), indicating that IR light may support irradiation using 1064 nm, providing that the duration of exposure is minimal to limit the undesirable thermal effects (Table 2).

### Cytokine expression

The IL-6 ELISA assay was used to identify changes in cytokine expression that may influence migration, proliferation, or differentiation of wounded cells following laser irradiation. Wounded cells exposed to 5 J/cm² using 632.8 nm in the dark showed an increase in the IL-6 concentration when compared to wounded cells exposed to 5 J/cm² using 632.8 nm in the light (p = 0.029) or in IR (p = 0.005). The results indicate that 632.8 nm in the dark results in a greater increase in IL-6 when compared to cells exposed to 5 J/cm² in the light or in IR (Fig. 3).

Wounded cells exposed to 5 J/cm² using 830 nm in IR showed a decrease in the IL-6 concentration when compared to wounded cells exposed to 5 J/cm² using 830 nm in the dark (p = 0.004). The results indicate that 830 nm in the light results in a greater increase in IL-6 when compared to cells exposed to 5 J/cm² in the dark or in IR. The results show that cells exposed to 5 J/cm² using 830 nm in IR results in a decrease in IL-6, supporting evidence that IR light may cause undesirable thermal damage that may inhibit biological responses (Table 2).

Wounded cells exposed to 5 J/cm² using 1064 nm in the dark showed an increase in the IL-6 concentration when compared to wounded cells exposed to 5 J/cm² using 1064 nm in IR (p = 0.044). The results indicate that wounded cells respond equally to 5 J/cm² using 1064 nm in the dark or in IR. The results suggest that IR may support LLLT, providing that the duration of exposure is minimal to limit the undesirable thermal effects (Table 2).
Wounded cells exposed to 5 J/cm² using 1064 nm in the dark showed an increase in the IL-6 concentration when compared to the wounded cells exposed to 5 J/cm² using 830 nm in the dark (p<0.038). However, wounded cells exposed to 5 J/cm² using 632.8 nm in the dark showed an increase in the IL-6 concentration when compared to the wounded cells exposed to 5 J/cm² using 1064 nm in the dark (p<0.026) and 830 nm in the dark (statistically non-significant [NS]). The results suggest that 632.8 nm in the dark is more effective in stimulating the release of IL-6, than 830 nm or 1064 nm in the dark.

Wounded cells exposed to 5 J/cm² using 830 nm in IR showed a decrease in the IL-6 concentration when compared to wounded cells exposed to 5 J/cm² using 1064 nm in IR (p<0.047). Wounded cells exposed to 5 J/cm² using 632.8 nm in the light showed an increase in the IL-6 concentration when compared to the wounded cells exposed to 5 J/cm² using 830 nm in the dark (p<0.026) and 1064 nm in the dark (statistically non-significant [NS]). The results suggest that 632.8 nm in the dark is more effective in stimulating the release of IL-6, than 830 nm or 1064 nm in the dark.

bFGF

The bFGF ELISA assay was performed to determine changes in the concentration of bFGF following wounding and laser irradiation (Fig. 4). bFGF was used as an indicator of cell proliferation since these growth factors are potent regulators of cell proliferation during wound repair. The effect of broad-spectrum and infrared light in combination with laser irradiation also influenced cell viability which was measured using ATP viability, caspase 3/7 activity as an early marker of apoptosis and changes in membrane integrity or LDH cytotoxicity. Wounded cells exposed to 5 J/cm² using 632.8 nm in light, dark, or infrared (IR). Wounded cells exposed to 5 J/cm² using 632.8 nm in the dark, 830 nm in the light, and 1064 nm in the light showed an increase in IL-6 that could exert a growth inducing effect on target cells. Wounded cells exposed to 5 J/cm² using 830 nm in IR showed a decrease (p = 0.006) in the IL-6 concentration and demonstrates the thermal effect of IR light that could exert a growth inhibitory effect (n = 4).

Wounded cells exposed to 5 J/cm² using 1064 nm in the dark showed an increase in the IL-6 concentration when compared to the wounded cells exposed to 5 J/cm² using 830 nm in the dark (p = 0.038). However, wounded cells exposed to 5 J/cm² using 632.8 nm in the dark showed an increase in the IL-6 concentration when compared to the wounded cells exposed to 5 J/cm² using 1064 nm in the dark (p = 0.026) and 830 nm in the dark (statistically non-significant [NS]). The results suggest that 632.8 nm in the dark is more effective in stimulating the release of IL-6, than 830 nm or 1064 nm in the dark.

Wounded cells exposed to 5 J/cm² using 830 nm in IR showed a decrease in the IL-6 concentration when compared to the wounded cells exposed to 5 J/cm² using 1064 nm in IR (p = 0.047). Wounded cells exposed to 5 J/cm² using 632.8 nm in the light showed an increase in the IL-6 concentration when compared to the wounded cells exposed to 5 J/cm² using 830 nm in IR (p = 0.019) and 1064 nm in IR (NS). The results indicate the biological response of cells irradiated in IR is dependent on the duration of the exposure, since a longer exposure (12 min 45 sec) results an inhibitory effect while a shorter exposure (6 min 30 sec) results in a more positive response (Table 3).

FIG. 3. The interleukin-6 (IL-6) enzyme-linked immunosorbent assay (ELISA) assay was used for the quantitative in vitro determination of human IL-6 from cell culture supernatants. Wounded fibroblasts were exposed to 5 J/cm² using 632.8, 830, or 1064 nm in light, dark, or infrared (IR). Wounded cells exposed to 5 J/cm² using 632.8 nm in the dark, 830 nm in the light, and 1064 nm in the light showed an increase in IL-6 that could exert a growth inducing effect on target cells. Wounded cells exposed to 5 J/cm² using 830 nm in IR showed a decrease (p = 0.006) in the IL-6 concentration and demonstrates the thermal effect of IR light that could exert a growth inhibitory effect (n = 4).
pase 3/7 activity, which indicates that the bFGF concentration may be influenced by cell viability and the extent of cell damage or stress (wounded and irradiated). Wounded cells exposed to 5 J/cm² using 632.8 nm in IR show an increase in ATP viability, a decrease in caspase 3/7 activity, and an increase in bFGF concentration when compared to cells exposed to 5 J/cm² using 1064 nm in the light. Wounded cells exposed to 5 J/cm² using 632.8 nm in IR show an increase in ATP viability, an increase in LDH cytotoxicity, a decrease in caspase 3/7 activity, and a decrease in bFGF concentration when compared to cells exposed to 5 J/cm² using 1064 nm in the dark. The results indicate that IR light can support LLLT since it has a similar effect to using LLLT in the light. The effect may not be optimal when compared to using LLLT in the dark; however, it may still have some alternative applications (Table 2).

Wounded cells exposed to 5 J/cm² using 632.8 nm in the dark showed an increase in bFGF concentration when compared to wounded cells exposed to 5 J/cm² using 830 nm in the dark (p = 0.0003) and 1064 nm in the dark (p = 0.018). The increase in bFGF concentration corresponds to an increase in ATP viability, an increase in IL-6, and a decrease in caspase 3/7 activity, indicating that exposure to 632.8 nm in the dark is more effective than 830 nm in the dark and 1064 nm in the dark. Wounded cells exposed to 5 J/cm² using 830 nm in the dark showed an increase in bFGF concentration when compared to wounded cells exposed to 5 J/cm² using 1064 nm in the dark. The increase in bFGF concentration with 830 nm corresponds to an increase in ATP viability, an increase in IL-6, and an increase in caspase 3/7 activity; these were not statistically significant, but the LDH cytotoxicity results indicate that exposure to 1064 nm in the dark is more effective than 830 nm in the dark. Cells exposed to 5 J/cm² using 632.8 nm in the light showed an increase in bFGF when compared to cells exposed to 5 J/cm² using 830 nm in the light. The decrease in bFGF concentration with 830 nm corresponds with an increase in caspase 3/7 activity, a slight decrease in IL-6, and an increase in ATP activity for 830 nm, which indicates that exposure to 830 nm in the light is more effective than 632.8 nm in the light but not as effective as 632.8 nm in the dark (Table 3).

Wounded cells exposed to 5 J/cm² using 632.8 nm in the light show an increase in bFGF concentration when compared to wounded cells exposed to 5 J/cm² using 1064 nm in the light (p = 0.035). The increase in bFGF concentration with 632.8 nm corresponds with an increase in IL-6, which indicates that exposure to 632.8 nm in the light is more effective than 1064 nm in the light but not as effective as 632.8 nm in the dark. Wounded cells exposed to 5 J/cm² using 830 nm in the light show an increase in bFGF concentration when compared to wounded cells exposed to 5 J/cm² using 1064 nm in the light. The increase in bFGF concentration with 830 nm corresponds with an increase in ATP viability, an increase in IL-6, a decrease in LDH cytotoxicity, and a decrease in caspase 3/7 activity, which indicates that exposure to 830 nm in the light is more effective than 1064 nm in the light (Table 3).

**DISCUSSION**

Karu²⁸ stated that the laser effect depends on the radiation wavelength, dose, and intensity as well as on the cell culture
Cell Proliferation of Irradiated Fibroblasts

conditions. There are biological limits to the effects of LLLT: the proliferation of fast growing cells cannot be stimulated, nor can all cellular functions be activated. Not all cells in tissues or cellular cultures will respond to irradiation in exactly the same way. The total response from the cells may represent the average rather than the true value, which has little meaning in the clinic but is important in a cell study. Many experiments have been performed in vitro, and the reaction seen or not seen in an in vitro experiment reflects the effect of laser therapy on a single isolated cell.29

Laser light stimulates cells that are growing poorly at the moment of irradiation. Thus, if cells are fully functional at the moment of irradiation or are growing in a serum-rich environment (10% FBS), there is nothing for laser irradiation to stimulate, and no therapeutic benefit will be observed. Karu has shown that the effect of laser light may be partly or completely reduced by broad-spectrum light. When wounded or scratched, cell monolayers respond to the disruption of cell-cell contacts with an increased concentration of growth factors at the wound margin and by healing the wound through a combination of proliferation and migration.30–32 These processes reflect the behavior of individual cells as well as the properties of the cell sheet as a surrogate tissue. The monolayers recover and heal the wound in a process that can be observed over a time course of 3–24 h.18

ALP expression is negatively correlated with cell growth and accompanied by a change into serum-growth-factor-independen
t survival.33 Under pathological conditions such as wound healing and inflammation, morphological and functional features of fibroblasts are modulated by factors such as growth factors and cytokines.34 Growth factor antagonists induce ALP expression when added to cells cultured in serum, while the addition of serum suppresses the ALP induction.33 Serum or growth factor deprivation induces ALP expression, which is inversely correlated with cell growth. The expression of ALP is upregulated by DNA synthesis inhibition and is inversely related with proliferation.35,36 Abe et al. (1998) also reported a negative correlation between ALP expression and cell growth.33

The optical density and ALP enzyme activity results indicate that 5 J/cm² using 1064 nm in the light is more effective for increasing cell proliferation or cell growth than 830 nm in the light, but not as effective as 632.8 nm in the light. The ALP enzyme activity results show that wounded cells exposed to 5 J/cm² using 632.8 nm in the light exhibit a statistical increase in cell proliferation or cell growth when compared to wounded cells exposed to 632.8 nm in IR light but not to wounded cells exposed to 830 nm in the light. The results show that wounded cells exposed to 5 J/cm² using 830 nm in IR light show a statistical decrease in optical density and statistical increase in ALP enzyme activity when compared to wounded cells exposed to 5 J/cm² using 830 nm in the light indicating an increase in cell proliferation or cell growth for cells exposed in the light. Results show that wounded cells exposed to 5 J/cm² using 632.8, 830, and 1064 nm in the dark respond with an increase in ALP enzyme activity, which indicates a decrease in cell growth or cell proliferation while wounded cells exposed to 5 J/cm² using 632.8 nm or 1064 nm in the light respond with an increase in cell density. Wounded cells exposed to 5 J/cm² using 1064 nm in IR light show an increase in cell proliferation or cell growth when compared to cells exposed to 632.8 or 830 nm in IR light, indicating that IR light supports 1064 nm.

IL-6 is a pleiotropic cytokine produced by many cell types 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. The results indicate that wounded cells exposed to 5 J/cm² using 632.8 nm in the dark results in a greater increase in IL-6 when compared to cells exposed to 5 J/cm² in the light or in IR. The results indicate that wounded cells exposed to 5 J/cm² using 830 nm in the light results in a greater increase in IL-6 when compared to cells exposed to 5 J/cm² in the dark or in IR. Wounded cells exposed to 5 J/cm² using 1064 nm in the light showed a decrease in the IL-6 when compared to cells exposed to 5 J/cm² in the dark or in IR, indicating that wounded cells respond equally to 1064 nm in the dark or in IR.

Results show that wounded cells exposed to 5 J/cm² using 632.8 or 830 nm respond in a similar way. Both wavelengths show the highest concentration of bFGF with laser irradiation in the light and the lowest concentrations with laser irradiation in IR. The results for wounded cells irradiated in IR light correspond to the decrease in cell viability, increase in LDH cytotoxicity and increase in caspase 3/7 activity indicating the laser irradiation in IR causes additional stress and damage that inhibits biological responses of the cells.

The results measuring changes in cell viability, IL-6, and bFGF concentration show the following: 5 J/cm² using 632.8 nm in the dark is more effective than 830 nm in the dark; 632.8 nm in the dark is more effective than 1064 nm in the dark and 1064 nm in the dark is more effective than 830 nm. Results also show that 5 J/cm² using 830 nm in the light is more effective than 632.8 nm in the light but not as effective as 632.8 nm in the dark; 632.8 nm in the light is more effective than 1064 nm in the light but not as effective as 632.8 nm in the dark and exposure to 830 nm in the light is more effective than 1064 nm in the light. Therefore the results indicate that 632.8 nm in the dark is the most effective. The bFGF results show that the response of wounded cells exposed to 5 J/cm² in IR is far less than the biological response of wounded cells exposed to 5 J/cm² in the dark or light.

CONCLUSION

Wounded cells respond with an increase in cytokine expression when exposed to 5 J/cm² using 632.8 nm in the dark or 830 nm in the light. Results showed that only the decrease in IL-6 expression, increase in cell growth or proliferation and increase in LDH cytotoxicity differentiated wounded cells exposed to 5 J/cm² using 1064 nm in IR from cells exposed to 1064 nm in the dark indicating that IR light can support laser irradiation with 1064 nm providing that the duration of exposure is minimal to limit the undesirable thermal effects.

Wounded cells exposed to 5 J/cm² using 632.8 nm in the light results in an increase in cell growth or proliferation, increase in bFGF concentration and decrease in caspase 3/7 activity when compared to cells exposed in the dark. The results support findings from Hawkins and Abrahamse, which reported that 5 J/cm² stimulates mitochondrial activity, cell proliferation, and migration to accelerate wound closure. The results also support findings by Enwemeka et al., which
demonstrated that phototherapy is a highly effective therapeutic armamentarium for tissue repair and pain relief.

Cell proliferation studies showed an increase in cell growth using 632.8, 830, and 1064 nm in the light. Wounded cells respond to 5 J/cm² using 632.8 or 830 nm in IR with decrease in cell proliferation indicating an undesirable thermal effect of IR light that may inhibit other biological responses such as the expression of bFGF. The results show that wounded cells exposed to 5 J/cm² using 632.8 nm in the light respond with an increase in bFGF expression whereas wounded cells exposed to 1064 nm in the light respond with a decrease in bFGF expression. The results indicate that the effect of laser radiation is dependent on the wavelength and irradiation conditions (light, dark, or IR) since broad-spectrum light may affect the biological response of cells to laser light. The results indicate that 5 J/cm² using 632.8 nm results in an increase in cell density, cell proliferation, bFGF expression, and cytokine expression, indicating a stimulatory effect that was less significant in cells exposed to 830 and 1064 nm. The use of broad-spectrum light in combination with 632.8-nm laser therapy results in an increase in cell proliferation, cell density, and bFGF expression, indicating that broad-spectrum light may have a potential therapeutic benefit despite reports which suggest that the effect of laser light may be partly or completely reduced by broad-spectrum light. The combination of broad-spectrum light and IR light appears to be dependant on the wavelength and duration of the exposure or power density (mW/cm²). The results from this study suggest that the combination of broad-spectrum light and IR light may benefit wound healing providing the combination is matched with a specific dose, wavelength and duration of exposure.

To conclude, our results show that wounded cells responded optimally to 5 J/cm² using 632.8 nm in the dark, 830 nm in the light, and 1064 nm in the dark by maintaining cell viability, reducing cytotoxicity, and stimulating the release of cytokines and growth factors, thus ultimately stimulating cell proliferation and cell growth. The results indicate that the exposures identified may ultimately stimulate wounded cells to accelerate wound healing.

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