Photomedicine and Laser Surgery
Volume 24, Number 6, 2006

Pp. 705–714
DOI: 10.1089/PHO.2006.1076

Effect of Multiple Exposures of Low-Level Laser Therapy on the Cellular Responses of Wounded Human Skin Fibroblasts

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ABSTRACT

Objective: **This study aimed to establish the behavior of wounded human skin fibroblasts (HSF) after helium-neon (HeNe) (632.8 nm) laser irradiation using one, two, or three exposures of different doses, namely, 2.5, 5.0, or 16.0 J/cm^2 on each day for 2 consecutive days.** ***Background Data:*** **Low-level laser therapy (LLLT) is a form of phototherapy used to promote wound healing in different clinical conditions. LLLT at than adequate wavelength, intensity, and dose can accelerate tissue repair. However, there is still conflicting information about the effect of multiple irradiations on the cellular responses of wounded cells.** ***Methods:*** **Cellular responses to HeNe laser irradiation were evaluated by measuring changes in cell morphology, cell viability, cell proliferation, and damage caused by multiple irradiations.** ***Results:*** **A single dose of 5.0 J/cm^2, and two or three doses of 2.5 J/cm^2 had a stimulatory or positive effect on wounded fibroblasts with an increase in cell migration and cell proliferation while maintaining cell viability, but without causing additional stress or damage to the cells. Multiple exposures at higher doses (16 J/cm^2) caused additional stress, which reduces cell migration, cell viability, and ATP activity, and inhibits cell proliferation.** ***Conclusion:*** **The results show that the correct energy density or fluence (J/cm^2) and number of exposures can stimulate cellular responses of wounded fibroblasts and promote cell migration and cell proliferation by stimulating mitochondrial activity and maintaining viability without causing additional stress or damage to the wounded cells. Results indicate that the cumulative effect of lower doses (2.5 or 5 J/cm^2) determines the stimulatory effect, while multiple exposures at higher doses (16 J/cm^2) result in an inhibitory effect with more damage.**

INTRODUCTION

LOW-LEVEL LASER THERAPY (LLLT) is a type of photomodulation—the use of photons to modulate biological activity.[1–6] The effects are biochemical and not thermal, and therefore cannot cause damage to living tissues at a cellular level.[5] While selection of the correct wavelength and power density (W/cm^2) is important, consistent application of the necessary dose (energy density or fluence: in joules per cm^2 [J/cm^2]) is required if the best therapeutic effects are to be achieved.

Photons produced by lasers can stimulate healing in acute and chronic wounds that are healing slowly.[7] If a rough surface is illuminated by visible laser light, the laser light that is coherent shows a speckled structure due to constructive interference.[1,8] This interference creates gradient forces in the cell, inducing a change in the distribution of organelles. Although absorption of the photon is the key mechanism in photobiomodulation, the photon can, in principle, pass through the cell without being absorbed and still initiate changes.[8]

Different substances absorb light of different wavelengths for example; the cells of injured skin are more sensitive than those of intact tissue. Once the target cells have absorbed the photons a cascade of biochemical events occur with the ultimate result of accelerated wound healing.[6] Laser therapy is thought to work through a variety of mechanisms:

- Photons from a laser probe are absorbed into the mitochondria and cell membranes of the target cells.[5]

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- After a cell absorbs photons the energy is incorporated into a molecule to increase kinetic energy, activate or deactivate enzymes or alter physical or chemical properties of main macromolecules.[5]
- Growth factor response within cells and tissue as a result of increased ATP and protein synthesis, improved cell proliferation and change in cell membrane permeability to calcium up-take.
- A cascade of metabolic effects results in various physiological changes which results in improved tissue repair, faster resolution of the inflammatory response, and a reduction in pain.[5]

In human fibroblasts, there are several molecules that serve as photoreceptors. In the visible range absorption peaks are found around 420, 445, 470, 560, 630, 690, and 730 nm. At longer wavelengths, a general decrease in absorption is observed.[8] Tunér and Hode[1] recommend treating open wounds with a dosage of 0.5 J/cm^2 and the skin next to the wound with 3–4 J/cm^2. Mester et al. surveyed the LLLT treatment of over 1000 patients with chronic ulcers; they showed 50–100% healing, variation being related to the type of lesion and clinical condition.[7] Skin ulcers with compromised healing remain a major problem for plastic and dermatological surgeons. Low levels of laser energy have been shown to increase the blood flow rate and volume and to accelerate the wound healing process,[9] thus raising the possibility in augmenting treatment for skin ulcers. Woodruff et al. conducted a study from 24 original research papers to determine the efficacy of laser therapy in wound repair and their results revealed significant positive effects on specific indices of healing, for example, acceleration of inflammation, augmentation of collagen synthesis, reduced healing time and reduction in wound size.[10] The studies concluded that laser therapy is a highly effective tool for promoting wound repair and pain relief.[10]

The total dose of light energy delivered is the product of the power density (or irradiance), irradiated area, and the treatment time. In most studies this total dose is considered the main variable with respect to the effects of laser irradiation. Until now, only a few studies have evaluated the importance of the laser power density or light intensity (W/cm^2).[11] Photo-biomodulation of human fibroblasts by LLLT has been described in morphological studies and in studies on cell functions such as proliferation and collagen production.[11] Power densities in these experiments varied from 0.1 to 24.7 mW/cm^2, while the total energy dose varied between 0.02 and 12 J/cm^2. Depending on the irradiation conditions, effects were found on collagen production—stimulatory as well as inhibitory—and on cell proliferation—also positive and negative.[11] Belotsky et al. studied the effect of 5 and 12 J/cm^2 and concluded that an inhibitory effect of light intensity dependence was more pronounced at 12 J/cm^2. Al-Watban and Zhang concluded that LLLT using a pulsed or continuous wavelength (CW) laser at the appropriate dosimetry and frequency provides acceleration in wound healing in rats and that the effects of treatment using CW (635 nm) laser are higher than pulse frequency.[13] Kreisler et al. investigated the effects of low-level laser irradiation on the proliferation of human gingival fibroblasts.[14] Cells were irradiated at energy fluences of 1.96–7.84 J/cm^2 using an 809 nm semiconductor laser operated at an output of 10 mW in the continuous wave (CW) mode. Laser treatment was performed alternatively once, twice, and three times in a 24 h interval. After lasing, incubation was continued for 24 h. The study concluded that irradiated cells revealed a considerably higher proliferation activity 24 h after irradiation but decreased in an energy-dependent manner after 48 and 72 h. The results indicated that repeated treatments might be necessary to achieve a positive laser effect in clinical applications.[14]

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.[15–17] These processes reflect the behaviour of individual cells as well as the properties of the cell sheet as a surrogate tissue. To perform a wound healing assay, a wound is typically introduced in a cell monolayer according to Cha et al. using an object such as a pipette tip or syringe needle to create a cell-free zone.[18] The monolayers recover and heal the wound in a process that can be observed over a time course of 3–24 h. The wound heals in a stereotyped fashion—cells polarize toward the wound, initiate protrusion, migrate, and close the wound.[18]

Currently, no universally accepted theory can explain the mechanism of laser biomodulation. Although a theoretical understanding is not necessary to establish effects, the lack of knowledge complicates the evaluation of conflicting reports found in literature. The main parameters that should be described when discussing continuous wave LLLT are laser type and wavelength, laser power (mW), power density (W/cm^2) collimation or divergence of the laser beam (intensity), laser exposure time and treatment schedule, method of application (contact or non-contact method), and total laser energy or fluence (J/cm^2) delivered to the treatment area.[19]

This study aimed to establish cellular responses of wounded[2–4] human skin fibroblasts to helium-neon (632.8 nm) laser irradiation using multiple exposures of different doses, namely, 2.5, 5.0, or 16.0 J/cm^2. This study aimed to establish if multiple or cumulative exposures could alter cellular responses in a dose-dependent manner.

METHODS

Cell culture

Human skin fibroblast monolayer cultures (ATCC CRL1502 WS1) were grown in Eagle's minimal essential medium with Earle's balanced salt solution and 2 mM L-glutamine that was modified to contain 1.0 mM sodium pyruvate, 0.1 mM non-essential amino acids, 1% fungizone, 1% penicillin-streptomycin, and supplemented with 10% V/V fetal bovine serum. The cultures were incubated at 37°C with 5% CO_2 and 85% humidity.[18] Cells were trypsinized using a 0.25% (w/v) trypsin/0.03% EDTA solution in Hanks balanced salt solution (HBSS), and approximately 6.5×10^5 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.[20]

Laser irradiations

Irradiations were performed with a helium-neon (Spectra-physics Model 127) laser, at a wavelength of 632.8 nm, 33 mW (output power), 3.03 mW/cm^2 (power density), and 3.4 cm

diameter spot size using one, two, or three doses of either 2.5, 5.0, or 16.0 J/cm^2 on each day for 2 consecutive days with incubation at 37°C between exposures. Since the laser has a power density of 3 mW/cm^2 and spot size of 3.4 cm, the light is divergent and not as harmful as a narrow parallel beam, which allows the entire volume of intense laser light to be focused or concentrated on one small area.[21] Using an average laser power density of 3 mW/cm^2, the duration of each exposure was calculated at 13 min 45 sec for the 2.5 J/cm^2, 27 min 30 sec for the 5 J/cm^2, and 88m 00s for the 16 J/cm^2 dose. 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.[3,4] Each scratch was irregular and the size of the wounds ranged from 1–2 mm in diameter.[3,4] Each experiment was repeated on different populations (n = 4) of fibroblast cells between passage 13 and 31 (normal and wounded) for each dose, and each biochemical assay was performed in duplicate.

Cellular responses

Changes in wounded fibroblast cell morphology were evaluated by light microscopy. Following laser irradiation, the fibroblasts were trypsinized from the 3.4 cm culture dishes, and the cell suspension (1×10^5 cells/100 μL) was used to assess changes in viability (trypan blue exclusion test and ATP luminescence), cell proliferation (neutral red assay), and damage or additional stress caused by the irradiation (caspase 3/7 assay). The culture medium was used to assess basic fibroblast growth factor (bFGF) expression and alkaline phosphatase (ALP) enzyme activity.

Changes in cell morphology. The control (un-irradiated or 0 J/cm^2) and wounded fibroblast behaviour was observed using an inverted microscope. The number and intensity of colony formation, the haptotaxis (direction or orientation) of the edge fibroblasts, the number of fibroblasts present in the center of the scratch, and chemotaxis-chemokinesis (movement or migration of cells across the central scratch) were evaluated to determine the activity of fibroblasts.[2,4]

Trypan blue exclusion test. A vital dye such as Trypan blue is usually excluded from viable cells, but can enter through "holes" in damaged cell membranes and stain the cytoplasm, thus acting as an indicator of nonviable cells. For the Trypan exclusion test, 20 μL of the cell suspension was mixed with 20 μL of 0.4% Trypan blue (Sigma T8154) in HBSS. The solution was incubated for 5 min, loaded into a hemocytometer-counting chamber, and the total number of cells per milliliter, the total number of viable (unstained) cells, and total number of nonviable (stained) cells were counted to determine the % viability.[20] The % viability can be defined as the number of viable cells (translucent) ÷ total cell number × 100.

ATP cell viability assay. The CellTiter-Glo luminescent cell viability assay (Promega G7570) is based on the quantitation of ATP present, which signals the presence of metabolically active cells or viable cells.[22] An equal volume of reconstituted CellTiter-Glo reagent was added to 50 μL of cell suspension (1×10^5 cells/100 μL). The contents were mixed by an orbital shaker for 2 min to induce cell lysis and were then incubated at room temperature for 10 min to stabilize the luminescent signal, which was recorded.[22,23]

Neutral red assay. The proliferating activity after irradiation was determined by the neutral red assay (Sigma N2889) based on the ability of living cells to take up the neutral red dye from the medium and retain it in their lysosomes. 5×10^4 cells in complete EMEM were incubated with 10% neutral red (33 μg/mL) for 1 h at 37°C, fixed with 1% formaldehyde for 30 min, and solubilized with 1% acetic acid in 50% ethanol for 30 min. Absorbance was read at 550 nm.[24]

Basic-fibroblast growth factor. 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 enzyme-linked immunosorbent assay (ELISA)[25,26] was identified to evaluate the expression of bFGF using a monoclonal anti-human basic fibroblast growth factor (Sigma F6162) primary antibody diluted to a minimum titer of 5 μg/mL in PBS-T (10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween 20). A pure protein of human bFGF (Sigma F0291) was used as a positive control to establish a standard curve, while isotype matched, non-specific mouse immunoglobulin (Sigma, M9035, Mouse IgG Kappa) was used as a negative control. The Nunc Maxisorp 96-well microplate was washed after each incubation step with three washes of PBS-T. An anti-mouse IgG (Fab specific) peroxidase-conjugated antibody (Sigma A9917) diluted in PBS-T was used as the secondary antibody, while Sigma Fast™ OPD tablets (Sigma P9187) were used as the substrate for the colorimetric detection. The orange-yellow colour development was stopped after 30 min with 3M HCl, and the positive wells were read at 450 nm.[26]

ALP enzyme assay. Alkaline phosphatase (ALP) is a membrane-bound enzyme released in inflammation, remodeling, and cell proliferation, and has been used as a marker for wound healing.[24,27] ALP enzyme activity was measured by the colorimetric assay using *p*-nitrophenyl phosphate as a substrate. Culture medium was removed from each plate after laser 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, 0.5 mM magnesium acetate, 110 mM NaCl, and 0.22% Triton X-100 for 30 min at 37°C. 20 mM *p*-nitrophenyl phosphate (ρ-NPP Sigma N7653) was added and the reaction was incubated at 37°C for 30 min.[24,27] The amount of *p*-nitrophenol liberated was measured at 405 nm.

Apoptosis. The caspase-Glo 3/7 (Promega G8090) assay is a luminescent assay that measures caspase-3 and -7 activity. Apoptosis can be induced by stress from growth factor withdrawal or irradiation, and is mediated by a cascade of highly specific proteases known as caspases.[28] Caspases 3, 6, and 7 are effector caspases that cleave cellular substrates and precipitate apoptotic death.[28] The addition of caspase-Glo 3/7 reagent results in cell lysis, followed by caspase cleavage of the substrate and generation of a luminescent signal produced by luciferase. 25 μL of the cell suspension (1×10^5 cells/100 μL) was added to 25 μL of caspase-Glo 3/7 reagent, and the tube was mixed and incubated at room temperature for 3 h. The lu-

minescent signal was recorded, which is directly proportional to the amount of caspase activity present. A positive control was included by inducing apoptosis using Actinomycin D. Apoptosis was induced in a 1×10^6 cells/mL suspension of human skin fibroblast cells by the addition of 0.5 µg/mL Actinomycin D (5 mg/mL). The cell culture was incubated for 19 h in a 37°C, 5% CO_2 incubator.

Statistical analysis

The results were recorded for statistical analysis using SigmaPlot 8.0, and the significant change between the un-irradiated control (0 J/cm^2) and the irradiated wounded cells was calculated and graphically represented with statistical analysis ($p < 0.05$; $n = 4$). The p value was calculated using the Student t-test to determine significant differences ($p < 0.05$) between the un-irradiated wounded control and the irradiated wounded cells.

RESULTS

Cell morphology

The cell morphology of the wounded un-irradiated control cells showed a clear wound margin where the central scratch had been performed. On day 1, there was little or no migration of the cells and a clear wound margin was maintained, whereas after the overnight incubation, there was evidence of migration with movement of the cells across the central scratch (Fig. 1).[2,3,4] Wounded fibroblasts exposed to one, two, or three doses of either 2.5 or 5.0 J/cm^2 on two consecutive days responded with morphological changes that indicated a stimulatory effect. Wounded cells exposed to one exposure of 5.0 J/cm^2 (Fig. 2), two exposures of 2.5 J/cm^2 (Fig. 3), and three exposures of 2.5 J/cm^2 (Fig. 4) showed an increase in the rate of chemotaxis-chemokinesis as the cells migrated across the central scratch in an attempt the close the wound. There was also an increase in haptotaxis or change in the orientation of the edge fibroblasts as cells were stimulated to migrate in the direction across the central scratch increasing the number of fibroblasts present in the central scratch. The morphological changes indicate that the laser irradiation has a positive effect on the migration of wounded cells across the central scratch. A high dose of 16 J/cm^2 (Fig. 5) resulted in morphological changes that indicated an in-

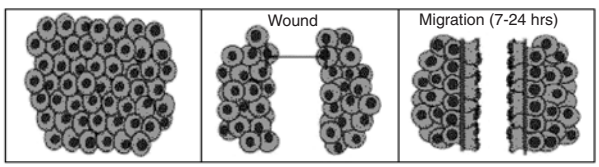

FIG. 1. A central scratch was performed across a confluent monolayer according to the method described by Rigau et al. to simulate a wound condition.[3,4] The effect of the wounding and laser irradiation was studied using colony formation (formation of colonies in the central zone of the wound or on the edge of the scratch), haptotaxis (change of orientation of the edge cells), chemotaxis-chemokinesis (movement or migration of cells across the wound) and the number of fibroblasts present in the central scratch to indicate the rate of cell migration.[4]

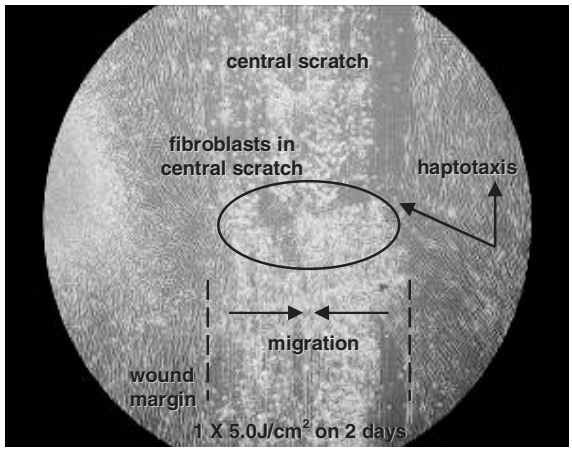

FIG. 2. Wounded fibroblasts exposed to a single dose of 5.0 J/cm^2 showed an increase in the number of fibroblasts present in the central scratch with an increase in the rate of chemotaxis and haptotaxis accelerating wound closure.

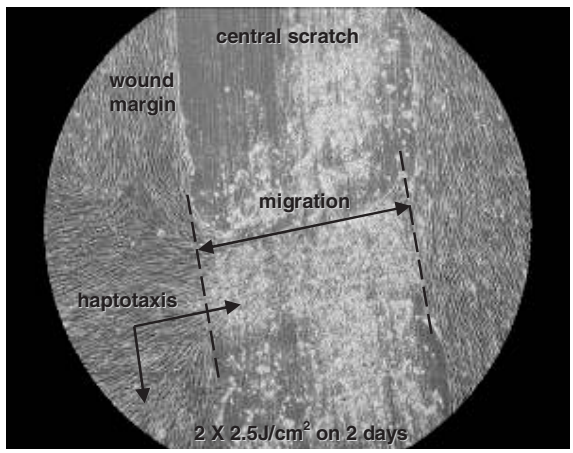

FIG. 3. The number of fibroblasts increased dramatically in the centre of the scratch indicating migration of fibroblasts across the central scratch with an increase in the rate of haptotaxis also indicating a stimulatory effect.

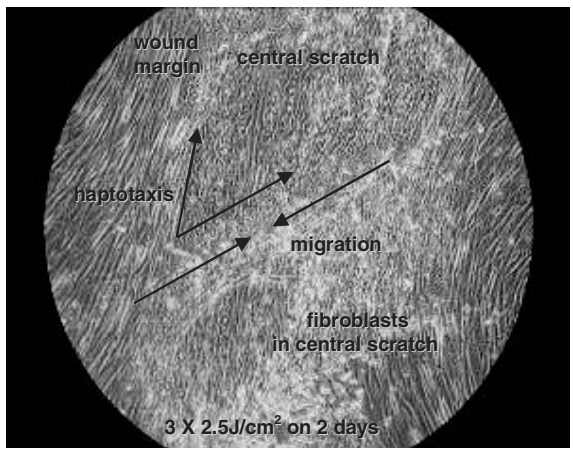

FIG. 4. The stimulatory effect of laser irradiation was evident since wounded fibroblasts exposed to three doses of 2.5 and 5.0 J/cm^2 showed good migration and haptotaxis with almost complete wound closure in areas.

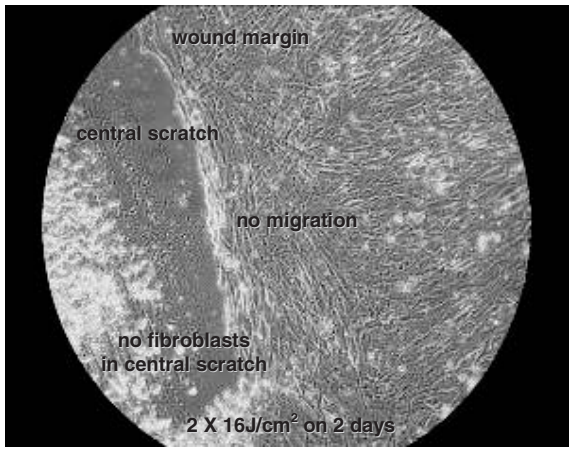

FIG. 5. A high dose of 16.0 J/cm^2 appeared to inhibit fibroblast migration and haptotaxis and there was little evidence of migration across the central scratch supporting previous evidence that higher doses inhibit cell responses.

hibitory effect. There was little or no chemotaxis-chemokinesis of the wounded cells across the central scratch. There was also little evidence of haptotaxis and no evidence of fibroblasts present in the central scratch. The morphological changes indicate that higher doses inhibit cell migration, which prevents fibroblasts from closing the central scratch. In the clinical situation, this delay in wound closure may lead to other complications such as wound infections and unsightly scar tissue formation.

Trypan blue exclusion test

Wounded fibroblasts exposed to laser irradiation maintained the cell viability after one exposure of either 2.5 or 5.0 J/cm^2, two exposures of 2.5 J/cm^2, and three exposures of either 2.5 or 5.0 J/cm^2 when compared to the un-irradiated control, indicating that lower doses do not cause additional damage or stress to the wounded cells. Wounded cells exposed to one exposure of 16 J/cm^2 ($p = 0.023$), two exposures of 16 J/cm^2 ($p = 0.011$), and three exposures of 16 J/cm^2 ($p = 0.013$) on 2 consecutive days showed a decrease in cell viability, indicating that multiple exposures at a high dose has an inhibitory effect since the cumulative doses may cause additional stress or damage to the wounded cells, reducing the cell viability (Fig. 6).

ATP cell viability assay

Wounded fibroblasts exposed to laser irradiation maintained cell viability after one exposure of either 2.5 J/cm^2 ($p = 0.729$) or 5.0 J/cm^2 ($p = 0.423$), two exposures of 2.5 J/cm^2 ($p = 0.799$), and three exposures of 5.0 J/cm^2 ($p = 0.160$) when compared to the un-irradiated control. Chemical energy within the cell in the form of ATP promotes normalization of cell function and healing, so an increase in ATP after one exposure of either 2.5 or 5.0 J/cm^2 and two exposures of 2.5 J/cm^2 may stimulate wounded cells and promote healing. The results show that there was a decrease in the cell viability or mitochondrial activity after one exposure of 16 J/cm^2 ($p = 0.052$), two exposures of either 5 J/cm^2 ($p = 0.173$) or 16 J/cm^2 ($p = 0.001$), and three exposures of either 2.5 J/cm^2 ($p = 0.021$), 5.0 J/cm^2, or 16 J/cm^2 ($p = 0.001$). Both the trypan blue and ATP assay showed that wounded cells exposed to three exposures of 5 J/cm^2 on 2 consecutive days responded with a higher cell viability than 2.5 or 16 J/cm^2, but still less than the un-irradiated control, indicating a biostimulatory response at 5 J/cm^2, which is dependent on a combination of the dose (J/cm^2) and number of exposures. The cell viability results show that one exposure of 5.0 J/cm^2 on 2 consecutive days maintains the cell viability, but two or three exposures of 5.0 J/cm^2 reduce viability, indicating that the cumulative dose administered determines the total biomodulating effect on the wounded cells (Fig. 6).

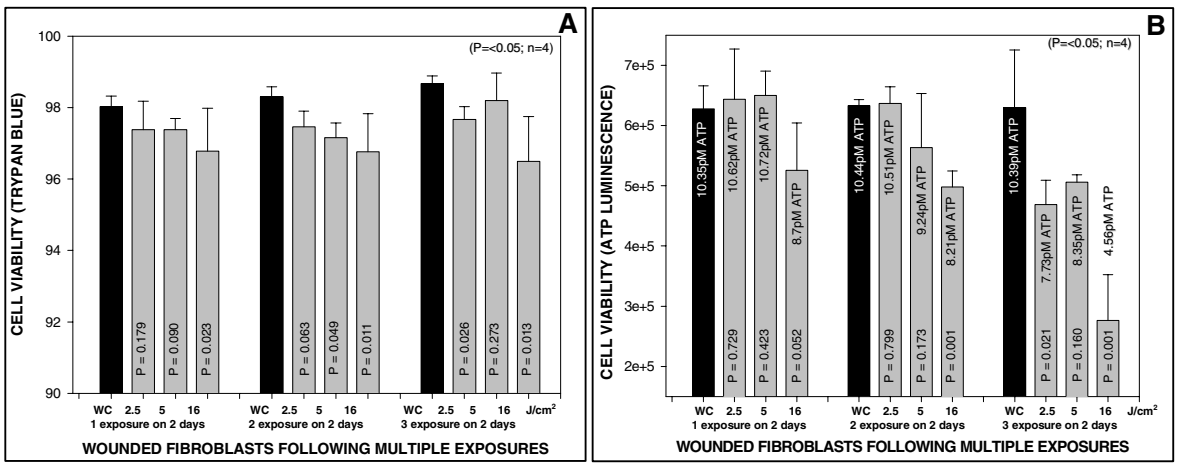

FIG. 6. The effect of multiple exposures of low-level laser therapy on wounded fibroblasts was evaluated by changes in cell viability using the trypan blue exclusion assay and ATP luminescence. Changes in the % viability **(A)** showed a decrease in the cell viability for all the exposures after a dose of 16 J/cm^2, while the % viability was maintained for the doses of 2.5 and 5.0 J/cm^2. The ATP luminescent assay was used to measure changes in cell viability **(B)**. A single exposure of 2.5 J/cm^2 (2.6%) or 5 J/cm^2 (3.6%) and two exposures of 2.5 J/cm^2 (0.6%) stimulated ATP activity, while a dose of 16 J/cm^2 was inhibitory with a decrease in ATP luminescence by 16.72% after one exposure, 21.35% after two exposures, and 56.07% after three exposures.

Neutral red assay

The neutral red assay was used to measure changes in cell proliferation. Wounded fibroblasts responded with an increase in cell proliferation after one exposure of 5.0 J/cm^2 on 2 consecutive days, two exposures of 2.5 J/cm^2, and three exposures of 2.5 J/cm^2. These results indicate that wounded cells respond better to a single exposure of 5 J/cm^2 or multiple exposures of 2.5 J/cm^2—supporting published findings[1] that the cumulative dose administered determines the biostimulatory effect. Wounded fibroblasts responded with a decrease in cell proliferation after one exposure of either 2.5 or 16 J/cm^2, two exposures of either 5.0 or 16 J/cm^2 ($p = 0.034$), and three exposures of 16 J/cm^2 ($p = 0.069$), indicating a bioinhibitory effect dependent on the dose (5 or 16 J/cm^2) and number of exposures.

Basic fibroblast growth factor

Fibroblast growth factor–basic (bFGF) is a potent mitogenic agent for fibroblasts and may play an important role *in vivo* in cell proliferation and differentiation associated with tissue regeneration and wound healing.[25,26] Wounded fibroblasts responded with an increase in cell proliferation after two exposures of 2.5 J/cm^2 on two consecutive days ($p = 0.012$), while the release of bFGF was similar to the un-irradiated control after one exposure of 5.0 J/cm^2, two exposures of 5.0 J/cm^2, and three exposures of either 2.5 or 5.0 J/cm^2. These results show that there was a significant release of bFGF with the multiple exposure of 2.5 J/cm^2, which stimulates and regulates cell proliferation and wound healing. Wounded fibroblasts responded with a decrease in cell proliferation after one exposure of either 2.5 J/cm^2 ($p = 0.0156$) or 16 J/cm^2 ($p = 0.041$), two exposures of 16 J/cm^2 ($p = 0.003$), and three exposures of 16.0 J/cm^2 ($p = 0.035$), indicating a bioinhibitory effect dependent on the dose (5 or 16 J/cm^2) and number of exposures. The cell proliferation results show that one exposure of 2.5 J/cm^2, two exposures of 5.0 J/cm^2, and three exposures of either 2.5 or 5.0 J/cm^2 does not stimulate or inhibit cell proliferation, since the values are similar to the un-irradiated wounded controls (Fig. 7). The results also suggest that a single dose of 2.5 J/cm^2 may not be sufficient to stimulate cell proliferation, but that the cumulative effect of two exposures of 2.5 J/cm^2 may provide an effect equivalent to that of a single dose of 5 J/cm^2.

ALP enzyme activity

During wound healing and inflammation, fibroblasts express elevated ALP.[24,27] Wounded fibroblasts showed an increase in ALP enzyme activity after one exposure of 5.0 J/cm^2 ($p = 0.268$) on 2 consecutive days, which may indicate a phenotypic response related to cell proliferation during wound healing. Wounded fibroblasts showed an increase in the ALP activity after two exposures of 16 J/cm^2 ($p = 0.006$) and three exposures of 16 J/cm^2 ($p = 0.001$), indicating that multiple exposures of a high dose (16 J/cm^2) may cause additional stress or damage to the cell membrane of the wounded fibroblasts to cause an increase in the release of the ectoenzyme ALP anchored in the plasma membrane. The increase in the ALP activity may indicate that a high dose of 16 J/cm^2 results in the up-regulation of ALP expression, which requires the cessation of proliferation.[24] One exposure of either 2.5 or 16.0 J/cm^2, two exposures of either 2.5 or 5.0 J/cm^2, and three exposures of either 2.5 or 5.0 J/cm^2 had an ALP enzyme activity similar to that of the un-irradiated control, indicating that the dose and number of exposures did not have a biostimulatory or bioinhibitory effect on the proliferation of wounded fibroblasts (Fig. 8).

Apoptosis

The caspase 3/7 luminescence assay was used to identify if the irradiation caused additional stress, which induces higher levels of apoptosis. The caspase 3/7 activity for 5.0 J/cm^2 increased by 3.91% after one exposure, 6.87% after two exposures, and 13.88% after three exposures when compared to the

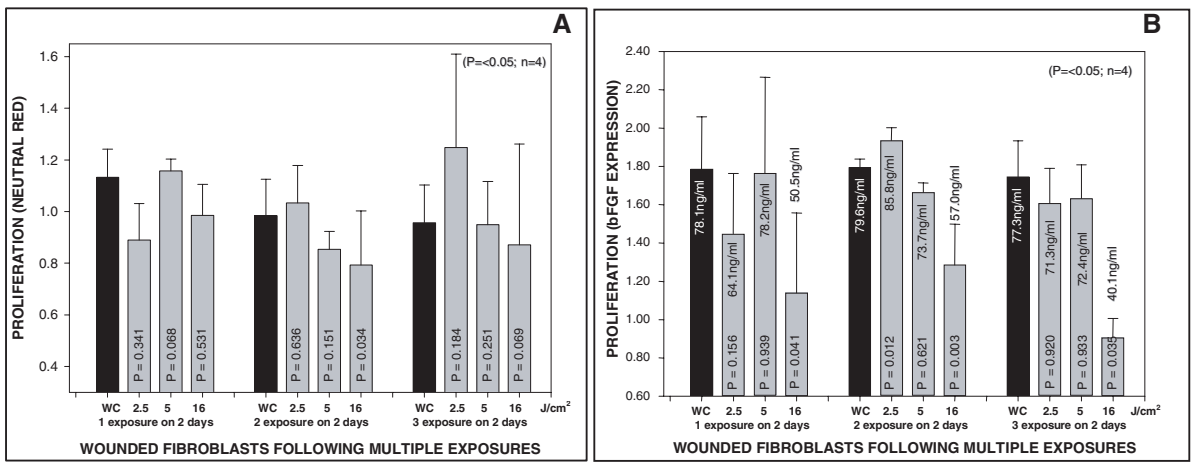

FIG. 7 The neutral red assay was used to measure changes in cell proliferation **(A)**. Wounded fibroblasts showed an increase in cell proliferation after one exposure of 5 J/cm^2, two exposures of 2.5 J/cm^2, and three exposures of 2.5 J/cm^2 while there was a decrease in cell proliferation after one exposure of either 2.5 or 16 J/cm^2, two exposures of either 5 or 16 J/cm^2 and three exposures of either 5 or 16 J/cm^2. Wounded fibroblasts responded with an increase in bFGF expression **(B)** after two exposures of 2.5 J/cm^2, while 5 J/cm^2 maintained bFGF within control limits and expression decreased after one exposure of either 2.5 or 16 J/cm^2, two exposures of 16 J/cm^2, and three exposures of 16 J/cm^2.

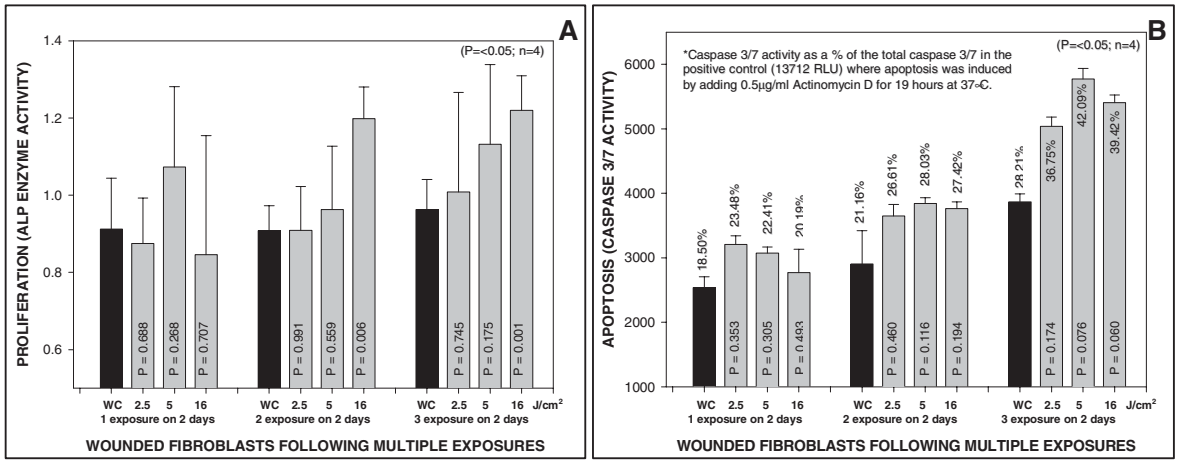

FIG. 8. The ALP enzyme activity assay was used as a marker for wound healing **(A)**. Wounded fibroblasts exposed to a single dose of 5.0 J/cm² showed an increase in ALP activity, which may be related to changes during wound healing. High doses like 16 J/cm² showed an increase in the ALP activity with multiple exposures indicating additional damage or stress resulting in the release of the ALP anchored in the plasma membrane. The caspase 3/7 luminescent assay was used to identify if the irradiation caused additional stress, which induces higher levels of apoptosis (**B**). The caspase 3/7 activity for 16 J/cm² increased by 1.69% after one exposure, 6.26% after two exposures, and 11.21% after three exposures when compared to the normal un-irradiated wounded control. Results indicate that one or two exposures do not cause additional stress; however, three exposures may cause additional stress, which may induce apoptosis.

un-irradiated wounded control, while the activity for 16 J/cm² increased by 1.69% after one exposure, 6.26% after two exposures, and 11.21% after three exposures. Results indicate that one or two exposures do not cause additional stress; however, three exposures may cause additional stress, which may result in higher levels of apoptosis or cell-programmed death. The results show that there was no significant increase in the caspase 3/7 activity, and that although higher doses (16 J/cm²) may have a bioinhibitory effect, the laser irradiation does not cause an excessive increase in cell stress or damage and there is no resultant increase in the levels of apoptosis. Three exposures of either 5 J/cm² (p = 0.076) or 16 J/cm² (p = 0.060) show the highest level of caspase 3/7 activity providing further evidence that the cumulative dose may cause additional stress, which ultimately has a bioinhibitory effect.

These results were supported by preliminary findings using flow cytometry with Annexin V-FITC to measure early apoptosis and propidium iodide (PI) to measure late apoptosis or necrosis. The results show that the number of apoptotic cells increased as the dose increased, with 16 J/cm² having the highest number of early apoptotic (55.05%) and late apoptotic or necrotic cells (31.20%). The results also show that the number of apoptotic cells increased from 42.30% for one exposure of 16 J/cm² to 55.05% for two exposures, indicating that multiple irradiations may cause additional stress, which induces apoptosis. The PI results showed that cells exposed to a single dose of 2.5 J/cm² (12.65%) had a similar value to a single dose of 16 J/cm² (11.10%), indicating that a single irradiation did not result in gross injury to cells. The number of late apoptotic or necrotic cells increased from 11.10% for one exposure of 16 J/cm² on 2 consecutive days to 31.20% for two exposures of 16 J/cm², indicating that multiple irradiations with a high dose results in injury to the cells and release of cytoplasmic components into the surrounding environment.

DISCUSSION

LLLT from the red and near-infrared region corresponds well to the characteristic energy and absorption levels of the relevant components of the respiratory chain. Photons enter the tissue and are absorbed in the mitochondria and at the cell membrane. The photonic energy is converted to chemical energy within the cell, in the form of ATP, which leads to normalization of cell function, pain relief and healing. Cell membrane permeability alters, and then physiological changes occur. These physiological changes affect macrophages, fibroblasts, endothelial cells, mast cells, bradykinin, and nerve conduction rates.[29] It is difficult to understand how a laser of a specific wavelength should be able to induce stimulatory and inhibitory effects on the same type of cell or tissue. Low doses of light intensify the formation of a transmembrane electrochemical proton gradient in the mitochondria, which is followed by a calcium release from the mitochondria into the cytoplasm by an antiport process, which, in turn, subsequently triggers or stimulates various biological processes such as RNA and DNA synthesis, cell mitosis and protein secretion mitosis and cell proliferation. At higher doses, however, too much calcium is released, which causes hyperactivity of calcium-adenosine triphosphatase (ATPase) calcium pumps and exhausts the ATP pool of the cell, thereby inhibiting cell metabolism.[29,30]

Karu stated that 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[31] however if the cells are wounded then laser biostimulation aims to normalize cell function to stimulate healing and repair. Abe et al. reported that under serum deprivation, a fraction of fibroblasts undergo cell death, while others survive and express alkaline

phosphatase (ALP) for at least several weeks.[32] Serum or growth factor deprivation induces ALP expression which is inversely correlated with cell growth.[32]

Results indicate that one exposure of 5.0 J/cm^2 or two exposures of 2.5 J/cm^2 stimulate cell proliferation and maintain cell viability and mitochondrial activity without causing additional stress to the cells—indicating that the cumulative dose administered on one day determines the stimulatory or inhibitory effect of the laser irradiation on cellular responses. Mester et al. demonstrated that smaller doses with appropriate periods of time in-between are more effective than treatments that are administered close together.[7] Abergel et al. used fibroblast cultures and demonstrated that smaller doses with longer intervals between exposures were more effective.[33] Laser therapy has been shown to be cumulative (the dose from one treatment lasts some time, and what "remains" of the dose is added to the dose at the next treatment), it is vital that treatments are not too close together, so as to avoid a situation where the accumulated dose eventually ends up above the biostimulating range or even in the bioinhibitory range, with consequently poorer results[1] (Fig. 9).

The He-Ne laser beam has a Gaussian beam which is far from homogenous and the power density (3.03 mW/cm^2) over the irradiated area (3.4 cm) may vary considerably. 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.[1] In the clinic, there is no single cell effect instead a very complex and multipath cascade of processes that influences every cell in the body at each particular moment. In wound healing, it has been shown to be beneficial to treat with closer intervals in the beginning (every other day or every third day for 2 weeks) and then with longer intervals (once a week for a few weeks) until the wound has healed.[1]

This study investigated the effect of low-power He-Ne laser irradiation with a laser output of 33 mW corresponding to the energy density of either 2.5, 5, or 16 J/cm^2, where treatment was performed one, two, or three times on each day for 2 consecutive days. Morphologically, wounded cells responded to one, two, or three exposures of 2.5 and 5.0 J/cm^2 with an increase in chemotaxis (migration), haptotaxis (change in orientation), and fibroblasts present in the central scratch indicating a biostimulatory effect on the morphological changes of wounded fibroblasts in an attempt to close the wound or central scratch. A low dose of either 2.5 or 5.0 J/cm^2 and fewer exposures (one or two) maintained the cell viability, whereas higher doses (16 J/cm^2) and more exposures (three) significantly reduced the cell viability indicating that the dose and cumulative effect from the accumulated dose determines the biomodulating effect of the laser irradiation. The ATP results support published findings that suggest that the cumulative dose administered determines the biostimulatory or bioinhibitory effect of the laser irradiation.[1] Cell proliferation studies indicate that one exposure of 5.0 J/cm^2 and two exposures of 2.5 J/cm^2 stimulate cell proliferation, while three exposures of 2.5 or 5.0 J/cm^2 maintain proliferation relative to the control and one, two, or three exposures of 16.0 J/cm^2 show a significant decrease in cell proliferation, indicating a bioinhibitory effect that is dose dependent.

During wound healing and inflammation, fibroblasts express elevated ALP.[24] Fibroblasts undergo phenotypic modulation as their microenvironment changes. The increased expression of ALP activity is one of the phenotypic characteristics of fibroblasts during wound healing and chronic inflammation. The up-regulation of ALP expression requires the cessation of pro-

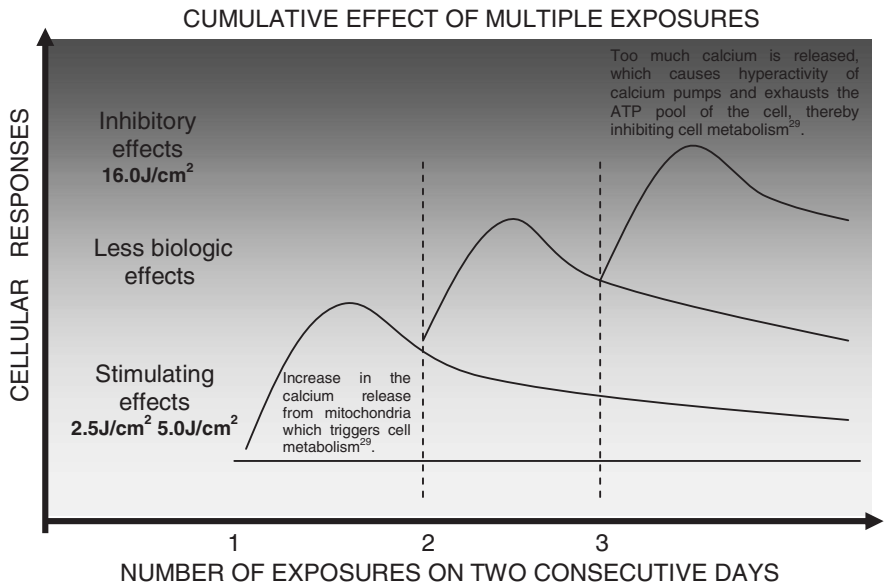

FIG. 9. Laser therapy has been shown to be cumulative. The dose from one treatment lasts some time and what "remains" of the dose is added to the dose at the next treatment. Adequate time between doses is essential to allow the cells time to respond to the initial dose and will also avoid a situation where the accumulated dose eventually ends up above the bio-stimulating range or even in the bioinhibitory range, with consequently poorer results.[1]

liferation. However, growth arrest alone is unlikely to be sufficient for the elevated ALP expression in wounds and inflammation because fibroblasts are inactive under normal conditions *in vivo*. Results show that 16 J/cm^2 and multiple exposures of 16 J/cm^2 causes an increase in the release of ALP and caspase 3/7 activity from additional stress and damage to the cells whereas lower doses (2.5 or 5.0 J/cm^2) and fewer exposures do not induce higher levels of apoptosis or cellular damage.

Results show that multiple exposures at higher doses (16 J/cm^2) cause additional stress, which reduces the cell viability and ATP activity and inhibits cell proliferation. A specific combination of energy density or fluence (J/cm^2) and number of exposures are necessary for stimulatory effects of low-level laser irradiation. Laser radiation has the capability to inhibit (16 J/cm^2) or stimulate (5.0 J/cm^2) cellular activity in a dose dependent manner with the absence of significant heating. Effects of LLLT on the wound healing process is one of the most fully studied aspects of this type of therapy and influences all phases of this very complex process.[5]

CONCLUSION

The Arndt-Schultz Law[34] states that there is a threshold amount of energy (laser light) that is required to effect a change in cellular activity. When the dosage is increased above threshold, the degree of cellular biological activity also increases. When the dosage increases further, above a certain level a plateau effect occurs with no increase in cellular activity. When the dosage is increased above the plateau level, there is an inhibitory effect upon the cells.[34] The results show that the correct energy density or fluence (J/cm^2) and number of exposures can stimulate cellular responses of wounded fibroblasts and promote cell migration and cell proliferation by stimulating mitochondrial activity and maintaining viability without causing additional stress or damage to the wounded cells. Results indicate that the cumulative dose administered on 1 day determines the stimulatory or inhibitory effect of the laser irradiation on cellular responses. This study has identified that a single dose of 5 J/cm^2 or multiple exposures of 2.5 J/cm^2 with adequate time between exposures may be effective in the treatment of wounds in the clinical situation by accelerating wound closure. A single dose of 5 J/cm^2 or multiple exposures of 2.5 J/cm^2 promotes wound healing by stimulating migration, mitochondrial activity, and proliferation of wounded fibroblasts, while maintaining viability and without causing additional stress or damage to the cells.

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