The Role of Laser Fluence in Cell Viability, Proliferation, and Membrane Integrity of Wounded Human Skin Fibroblasts Following Helium-Neon Laser Irradiation†

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Background: In medicine, lasers have been used predominantly for applications, which are broadly termed low level laser therapy (LLLT), phototherapy or photobiomodulation. This study aimed to establish cellular responses to Helium-Neon (632.8 nm) laser irradiation using different laser fluences (0.5, 2.5, 5, 10, and 16 J/cm^2) with a single exposure on 2 consecutive days on normal and wounded human skin fibroblasts.

Materials and Methods: Changes in normal and wounded fibroblast cell morphology were evaluated by light microscopy. Changes following laser irradiation were evaluated by assessing the mitochondrial activity using adenosine triphosphate (ATP) luminescence, cell proliferation using neutral red and an alkaline phosphatase (ALP) activity assay, membrane integrity using lactate dehydrogenase (LDH), and percentage cytotoxicity and DNA damage using the Comet assay.

Results: Morphologically, wounded cells exposed to 5 J/cm^2 migrate rapidly across the wound margin indicating a stimulatory or positive influence of phototherapy. A dose of 5 J/cm^2 has a stimulatory influence on wounded fibroblasts with an increase in cell proliferation and cell viability without adversely increasing the amount of cellular and molecular damage. Higher doses (10 and 16 J/cm^2) were characterized by a decrease in cell viability and cell proliferation with a significant amount of damage to the cell membrane and DNA.

Conclusions: Results show that 5 J/cm^2 stimulates mitochondrial activity, which leads to normalization of cell function and ultimately stimulates cell proliferation and migration of wounded fibroblasts to accelerate wound closure. Laser irradiation can modify cellular processes in a dose or fluence (J/cm^2) dependent manner. Lasers Surg. Med. 38:74–83, 2006. © 2006 Wiley-Liss, Inc.

Key words: laser therapy; cellular effect; phototherapy; photobiomodulation; inhibitory; stimulatory

INTRODUCTION

Phototherapy has been used for many years and is used in medical and dental professions; however, it is still not an established therapeutic modality [1]. In addition to accelerated wound healing, the main advantages of phototherapy include prevention of side effects of drugs and significantly accelerated functional recovery [3]. Low energy laser irradiation produces significant bioeffects, which are manifested in biochemical, physiological, and proliferative phenomena in various enzymes, cells, tissues, organs, and organisms [3]. Laser radiation has a wavelength dependent capability to alter cellular behavior in the absence of significant heating [4]. Phototherapy includes wavelengths of between 500 and 1,100 nm and typically involves the delivery of 1–4 J/cm^2 to treatment sites. Red laser light (632.8 nm) appears to be the most effective frequency of laser at a cellular level. There is controversy about the results observed previously as visible laser light can cause stimulatory or inhibitory depending on factors such as the energy, wavelength, and irradiation time [5].

Beginning from the late 1960s, Endre Mester, a Hungarian physician, began a series of experiments with monochromatic light. Mester observed that in many cases the skin incisions made to implant recalcitrant cells appeared to heal faster in treated animals compared to incisions of control animals that were not treated with light [6–8].

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 [9–11]. These processes reflect the behavior 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. (1996) [12] using an object such as a pipette tip or syringe needle to create a cell-free zone. The monolayers recover and heal the wound in a process that can be observed over a time course of 3–24 hours. The wound heals in a stereotyped fashion—cells polarize toward the wound, initiate protrusion, migrate, and close the wound. In vitro wound closure can be monitored by manually imaging samples [13] or by the repopulation of

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the cleared area with cells over time. The residual gap between the migrating fibroblasts is measured and can be expressed as a percentage of the initial total scratch area. The central scratch has been used with multiple cell types and, as the monolayers heal the wound in a characteristic manner, they have been used to study cell polarization, matrix remodeling, cell migration, and numerous other processes [12].

Kornyei et al. (2000) [14] used an in vitro scratch-wound model to investigate astroglial responses to mechanical injury while Saga et al. (2003) [15], Liang et al. (2004) [16], Farooqui et al. (2004) [17], and Walker et al. (2004) [18] used the cell scratch-wound model to study the proliferative and migratory responses of different cells. Lau et al. (2001) [19] focused on the production of four inflammatory cytokines in primary culture using an injury model which simulated in vivo mechanical trauma and reported that all four cytokines began to increase 1 hour post-scratch and remained at high levels throughout the experiment [19].

Helium-Neon laser light (1–10 mW) can penetrate as far as 0.5 mm into freshly excised human skin and delivers the highest relative percentage of incident energy to a certain volume of tissue [20]. With longer wavelengths emitted by infrared (IR) lasers, the depth of penetration has been shown to be even greater, reaching several millimeters [21]. A penetration depth of even some microns can be regarded as sufficient because most of the relevant target cells of low level laser irradiation namely fibroblasts, keratinocytes, macrophages, and endothelial cells for the induction of wound healing, are located within the epidermis and upper dermis [20].

Each cell contains a number of power plants, called mitochondria. The function of these power plants is to produce adenosine triphosphate (ATP), the form of energy, which can be used by the cell to function properly [20]. Low level laser light reaches the mitochondria of low lying cells where the photonic energy is absorbed by the collector surfaces and is converted to chemical energy [3] within the cell in the form of ATP as an additional source of energy. Mitochondria produce more ATP, which leads to normalization of cell function, pain relief, and healing [3,4]. A sufficiently high supply of cellular energy enables cells to work under optimum conditions and is the essential prerequisite to ensure successful self-healing process [20].

The complex physiological process of wound healing commences at the time of injury. The immune and circulatory systems are stimulated while cell migration, cell division, and several chemical and cellular responses occur. The three overlapping phases of healing are the inflammatory phase, followed by the proliferative phase and matrix remodeling [22]. Any device that can accelerate any of these processes could accelerate the healing process of wounds [22]. Literature indicates that laser photobioactivation accelerates inflammation, modulates the level of prostaglandin, enhances the action of macrophages, promotes fibroblast proliferation, facilitates collagen synthesis, fosters immunity, and even accelerates the healing process [22]. Fibroblasts are cells of paramount importance in the process of wound healing. At low doses (2 J/cm^2) phototherapy stimulates fibroblast proliferation while higher doses (16 J/cm^2) are suppressive, pointing to the dose dependence of biological responses after light exposure. Low energy laser irradiation alters the cellular function by influencing protein synthesis, cell growth and differentiation, cell motility, membrane potential and binding affinities, neurotransmitter release, ATP synthesis, and prostaglandin synthesis [23,24].

The unique properties of lasers create an enormous potential for specific therapy of skin diseases and require an understanding of the mechanisms of light interaction with tissue as well as the properties of the laser itself. Modification of current lasers and innovative advances with biomedical laser instrumentation may eventually allow the physician to match optimally the laser and the treatment procedure with the lesion [25]. Currently, the wavelengths, dosage schedules, and appropriate conditions to be treated are not established. Because of the large number of positive reports and the innocuous nature of the treatment, further clinical evaluation of laser therapy is warranted.

This study aimed to establish cellular responses of normal and wounded [2,12] human skin fibroblasts to Helium-Neon (632.8 nm) laser irradiation using different laser fluences or doses (0.5, 2.5, 5, 10, and 16 J/cm^2) with a single exposure on 2 consecutive days. This study aimed to identify the laser fluence (J/cm^2) that would improve cell viability and cell proliferation with minimal damage that would ultimately improve or accelerate wound healing in vivo.

METHODOLOGY

Cell Culture

Human skin fibroblast monolayer cultures (ATCC CRL1502 WS1) were grown in Eagle's minimal essential medium with Earle's BSS and 2 mM L-glutamine that was modified to contain 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. The cultures were incubated at 37°C with 5% CO_2 and 85% humidity [26]. Cells were trypsinized using a 0.25% (w/v) trypsin –0.03% EDTA solution in HBSS and approximately 6.5×10^5 cells (in 3 ml culture medium) were seeded in 3.3 cm diameter culture plates and incubated overnight to allow the cells to attach [27].

Laser Irradiations

Irradiations were performed with a Helium-Neon (Spectraphysics Model 127) laser, at a wavelength of 632.8 nm, 3 mW/cm^2 output power, and 3.3 cm diameter spot size with a single dose of 0.5, 2.5, 5, 10, or 16 J/cm^2 on 2 consecutive days with an incubation at 37°C between the two exposures. Since the laser has an output power of 3 mW/cm^2 and spot size of 3.3 cm, the light is divergent and is not as harmful as a narrow parallel beam that allows the entire volume of intense laser light to be focused or concentrated on one small area [28]. The intensity of laser light at the target depends primarily on the parallelity and diameter of the

laser beam [28]. Using an average laser power density of 3 mW/cm^2, the duration of each exposure was calculated at 2 minutes 45 seconds for the 0.5 J/cm^2 dose, 13 minutes 45 seconds for the 2.5 J/cm^2, 27 minutes 30 seconds for the 5 J/cm^2, 55 minutes 00 seconds for the 10 J/cm^2, and 88 minutes 00 seconds for the 16 J/cm^2 dose. To simulate a wound environment, confluent monolayers were first scratched with a sterile pipette of 2 mm diameter and the plates were incubated at 37°C for 30 minutes before they were irradiated [2]. Each scratch was irregular and the size of the wounds ranged from 1 to 2 mm in diameter [2,12]. All tests were performed on different populations ($n = 6$) of cells for each sample group (normal and wounded) for each dose and each biochemical assay was performed in duplicate.

Changes in normal and wounded fibroblast cell morphology were evaluated by light microscopy. Changes following laser irradiation were evaluated by assessing the mitochondrial activity (ATP luminescence), cell proliferation (neutral red and ALP enzyme assay), membrane integrity (LDH and percentage cytotoxicity), and DNA damage (Comet assay). The results were recorded for statistical analysis and the significant change between the unirradiated control (0 J/cm^2) and the irradiated normal or wounded cells was calculated and graphically represented with statistical analysis ($P =< 0.05$; $n = 6$).

Changes in Cell Morphology

The control, normal fibroblasts, and wounded fibroblast behavior were 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].

ATP Cell Viability Assay

The CellTiter-Glo luminescent cell viability assay is based on the quantitation of ATP present, which signals the presence of metabolically active cells or viable cells [29]. An equal volume of reconstituted CellTiter-Glo reagent was added to 50 μl of cell suspension. The contents were mixed on an orbital shaker for 2 minutes to induce cell lysis. The contents were incubated at room temperature for 10 minutes to stabilize the luminescent signal and the luminescence was recorded [29].

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. Cells (5×10^4) in complete EMEM were incubated with 10% neutral red (33 μg/ml) for 1 hour at 37°C, fixed with 1% formaldehyde for 30 minutes, and solubilized with 1% acetic acid in 50% ethanol for 30 minutes. Absorbance was read at 550 nm [30].

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 [30]. ALP enzyme activity was measured by the colorimetric assay using ρ-nitrophenyl phosphate as a substrate. 500 μl of culture medium was removed from each plate after each irradiation. 100 μl of the culture medium was pre-incubated with 100 μl of 0.5M N-methyl-D-glucamine buffer, pH 10.5, 0.5 mM magnesium acetate, 110 mM NaCl, and 0.22% Triton X-100 for 30 minutes at 37°C. 20 mM ρ-nitrophenyl phosphate (ρ-NPP; Sigma N7653) was added and the reaction was incubated at 37°C for 30 minutes [30]. The amount of ρ-nitrophenol liberated was measured at 405 nm.

LDH Membrane Integrity Assay

The CytoTox 96® non-radioactive cytotoxicity assay measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. 500 μl of culture medium was removed from each plate after each irradiation. 100 μl of the culture medium was mixed with an equal volume of reconstituted substrate mix. The plate was covered with foil and incubated at room temperature for 30 minutes, protected from light. 100 μl of stop solution was added and the absorbance read at 490 nm [31]. Maximum LDH release with complete cell lysis was induced by incubating the plate at −80°C for approximately 30 minutes followed by thawing at 37°C for 15 minutes [31]. The percentage cytotoxicity was calculated by dividing the experimental LDH release OD_{490} by the maximum LDH release OD_{490}.

The Comet Assay for DNA Damage

The Comet assay was performed according to Collins (2000) [32]. After laser irradiation, the cells were harvested by trypsinization and resuspended to approximately 1×10^5 cells. The Comet assay protocol has four steps namely: lysis, DNA unwinding in electrophoresis solution, electrophoresis, and finally neutralization. Gels were stained with 20 μl of a 1 μg/ml 4′6-diamidine-2-phenylindol dihydrochloride (DAPI) and viewed on an Olympus BH2-RFCA Epifluorescent Microscope. One hundred comets per gel were visually analyzed at random. Cells were scored according to the five recognizable classes of comets, ranging from class 0, (undamaged, no discernible tail), to class 4, (almost all DNA in tail, insignificant head). Each comet was allocated a value depending on its classification to obtain an overall score ranging from 0 to 400 arbitrary units for each gel. The average arbitrary unit for each slide was calculated with a higher number of arbitrary units indicating more DNA damage.

RESULTS

Changes in Cell Morphology

The wound margin remained clearly defined in the unirradiated control while irradiated (2.5 and 5 J/cm^2) cultures showed a greater rate of migration of the

fibroblasts across the wound in an attempt to close the central scratch (wound). The occurrence of haptotaxis or change in the orientation of the edge cells was more evident and occurred sooner in irradiated cultures than in un-irradiated controls. Irradiated (2.5 and 5 J/cm^2) cultures had more fibroblasts present in the central scratch indicating an increase in the rate of chemotaxis or migration of cells across the central scratch. Morphological changes were mainly observed in irradiated wounded fibroblasts, indicating a stimulatory effect of phototherapy especially at 2.5 and 5 J/cm^2 (Fig. 1), while cells exposed to 16 J/cm^2 showed little evidence of migration indicating an inhibitory effect. The wounded cells exposed to 16 J/cm^2 were morphologically different from wounded un-irradiated cells and showed characteristics of cell stress and damage with debris, fragmented cells, and shedding. The higher doses (10 and 16 J/cm^2) had a decrease in the number of fibroblasts present in the central scratch indicating a decrease in the rate of chemotaxis or migration of cells and there was little evidence of haptotaxis indicating a slower response of the cells. A dose of 16 J/cm^2 had an inhibitory effect on wounded fibroblasts when compared to 5 J/cm^2, which showed the highest rate of migration and haptotaxis with the highest number of fibroblasts present in the central scratch.

ATP Cell Viability

The results from the ATP cell viability assay showed that normal cells exposed to a single dose on 2 consecutive days responded with an increase in the cell viability after 0.5 J/cm^2 ($P = 0.033$) and 5 J/cm^2 ($P = 0.046$), while at higher doses of 10 and 16 J/cm^2 there was a decrease in the cell viability. A dose of 2.5 J/cm^2 did not increase or decrease the cell viability when compared to the normal un-irradiated control and the viability was maintained (Fig. 2).

The ATP results for wounded cells showed a significant decrease in the cell viability at higher doses of 10 J/cm^2 ($P = 0.009$) and 16 J/cm^2 ($P = 0.017$), while the intermediate doses of 2.5 J/cm^2 ($P = 0.587$) and 5 J/cm^2 ($P = 0.721$) maintained the cell viability and did not show a significant change from the un-irradiated control (Fig. 2). At a low dose of 0.5 J/cm^2 wounded cells appeared to show a decrease in the cell viability, however the change did not prove to be significant ($P = 0.0121$). The results indicate the higher doses may be harmful to the cells resulting in a decrease in the cell viability while intermediate doses may not adversely affect the cells and the viability is maintained within normal control limits.

From the results there was a significant decrease in the cell viability of normal cells between the intermediate dose

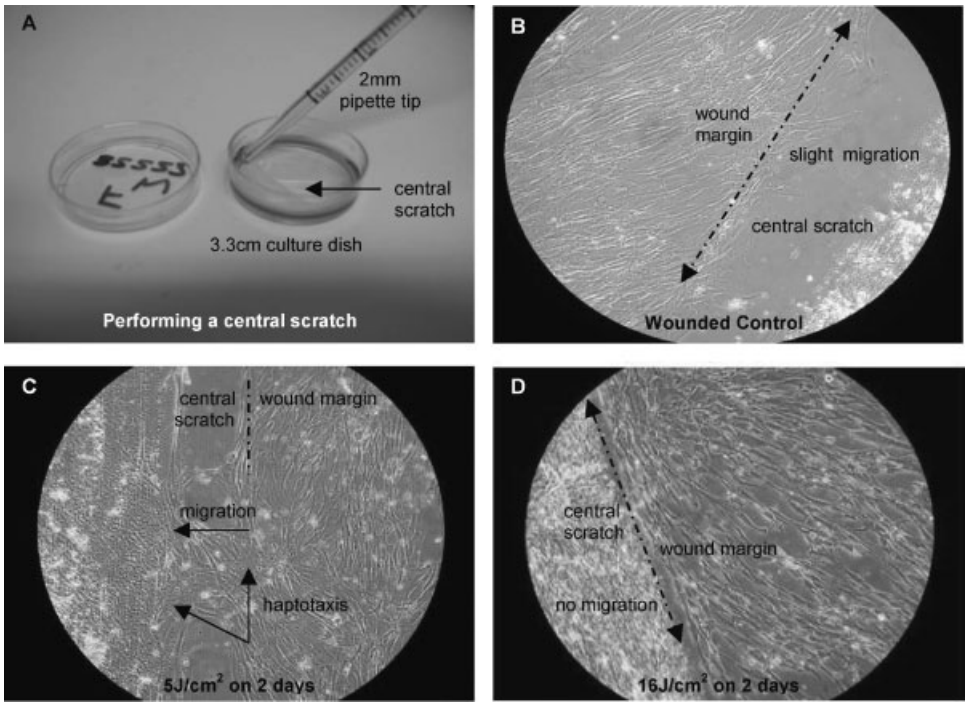

Fig. 1. **A**: A wound is typically introduced in a cell monolayer using a 2 mm sterile pipette tip to create a cell-free zone [2,16]. Criteria for assessing the wounded un-irradiated control (**B**) and the wounded irradiated cell morphology (**C** and **D**) were: (i) rate of chemotaxis-chemokinesis or migration of cells, (ii) haptotaxis or change in orientation of edge fibroblasts, (iii) colony formation of cell along the wound margin, and (iv) the number of fibroblasts present in the central scratch. C: Wounded fibroblasts following a single exposure of 5 J/cm^2 on 2 consecutive days showed evidence of fibroblasts in the central scratch, haptotaxis, and chemotaxis indicating that a dose of 5 J/cm^2 has a stimulatory effect. D: Cells exposed to 16 J/cm^2 on 2 consecutive days showed little evidence of migration with evidence of lysis, debris, and fragmented cells and some cells detached from the culture vessel indicating that a dose of 16 J/cm^2 has an inhibitory effect.

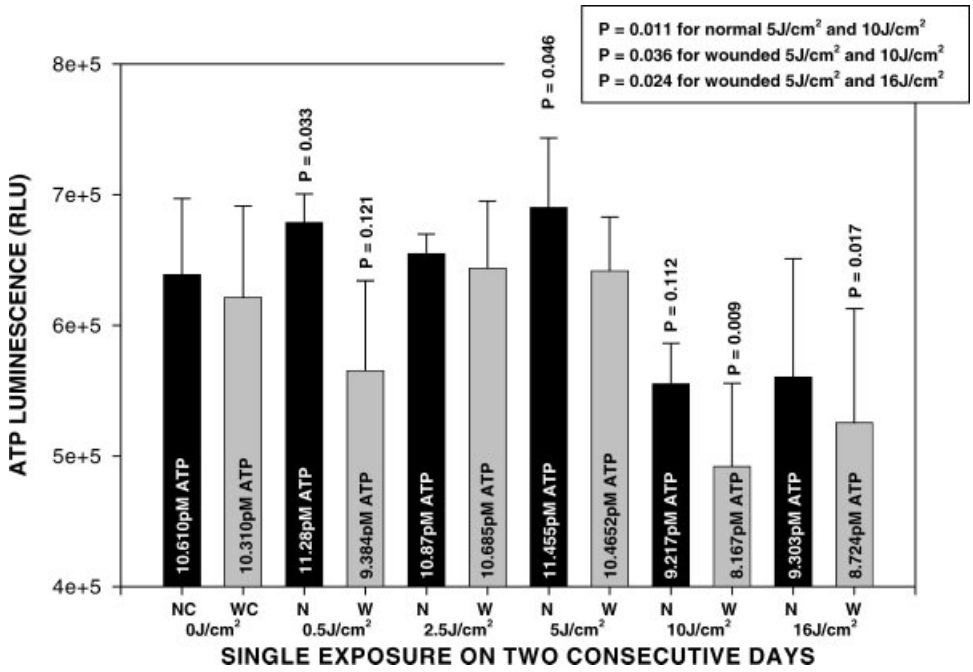

Fig. 2. The ATP luminescent assay was used to assess the mitochondrial activity as an indicator of cell viability following laser irradiation. Normal and wounded cells were irradiated with a single dose ranging from 0.0 to 16 J/cm^2 on 2 consecutive days. At higher doses of 10 and 16 J/cm^2 there was a remarkable decrease in the cell viability while a dose of 5 J/cm^2 stimulates mitochondrial activity, which leads to normalization of cell function, pain relief, and healing. The *P*-value was calculated using the *t*-test to determine significant differences ($P = <0.05$) between the un-irradiated normal or wounded control and the irradiated normal or wounded cells. The graph represents a normal (non-wounded) un-irradiated control (NC), a wounded un-irradiated control (WC), normal (non-wounded) irradiated cells (N), and wounded irradiated cells (W).

of 5 J/cm^2 and the high dose of 10 J/cm^2 ($P = 0.011$), while there was also a significant decrease in the cell viability of wounded cells between the intermediate dose of 5 J/cm^2 and the high dose of 10 J/cm^2 ($P = 0.036$) and 16 J/cm^2 ($P = 0.024$) supporting previous evidence that higher doses may negatively affect the viability of cells.

Neutral Red Assay

The results from the neutral red assay indicate for a single dose on 2 consecutive days showed that normal fibroblasts responded with an increase in cell proliferation after a dose of 0.5 J/cm^2 ($P = 0.153$), 5 J/cm^2 ($P = 0.131$), and 10 J/cm^2 ($P = 0.162$) and a decrease in cell proliferation after 16 J/cm^2 ($P = 0.149$), however none of the doses showed a significant change in cell proliferation when compared to normal un-irradiated control. At higher doses of 10 and 16 J/cm^2, the normal fibroblasts showed a decrease in cell viability but no significant decrease in cell proliferation indicating that the cells are fully functional and there is nothing for the laser to stimulate but that higher doses may be detrimental to some cells reducing the viability. The results indicate that where maximum regeneration is occurring naturally, the laser irradiation does not appear to enhance cell proliferation [4]. The wounded fibroblasts responded with a decrease in the proliferation rate after 0.5 J/cm^2 ($P = 0.212$), 2.5 J/cm^2 ($P = 0.163$), 10 J/cm^2 ($P = 0.093$), and 16 J/cm^2 ($P = 0.504$), while the proliferation rate increased after a single dose of 5 J/cm^2. The neutral red assay may be more sensitive than the ALP enzyme assay since it showed that there was a statistical increase in the proliferation between wounded fibroblasts exposed to a single dose of 2.5 J/cm^2 ($P = 0.002$) and 5 J/cm^2 and between 5 and 10 J/cm^2 ($P = 0.064$) indicating that the correct dose and physiological state of the cells at the moment of irradiation is an important factor influencing the biostimulatory effect and that phototherapy at 5 J/cm^2 normalizes or stimulates the proliferation rate of wounded cells that are growing poorly at the time or irradiation. The higher doses of 10 and 16 J/cm^2 have an inhibitory effect with a decrease in both cell proliferation and cell viability (Fig. 3).

ALP Enzyme Activity

The results for a single dose on 2 consecutive days showed that normal fibroblasts responded with an increase in ALP enzyme activity after a dose of 5 J/cm^2 ($P = 0.165$) and a decrease after 10 J/cm^2 ($P = 0.764$), however none of the doses showed a significant change in cell proliferation when compared to normal un-irradiated control. The wounded fibroblasts responded with an increase in the ALP enzyme activity after 0.5 J/cm^2 ($P = 0.189$) and 5 J/cm^2 ($P = 0.171$), while there was a decrease in the enzyme activity after 2.5 J/cm^2 ($P = 0.401$), 10 J/cm^2 ($P = 0.193$), and 16 J/cm^2 ($P = 0.325$), however none of the doses showed a significant

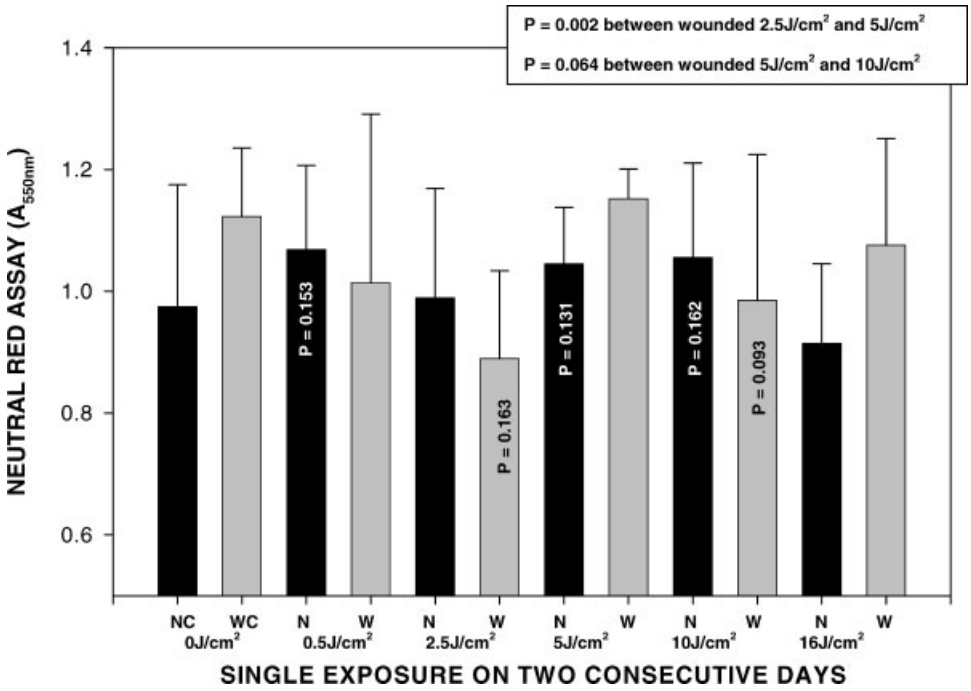

Fig. 3. The neutral red assay was used to assess proliferating activity after irradiation based on the ability of living cells to take up the neutral red dye from the medium and retain it in their lysosomes. Normal and wounded cells were irradiated with a single dose ranging from 0.0 to 16 J/cm^2 on 2 consecutive days. Phototherapy had a positive influence on wounded cells irradiated at 5 J/cm^2 with a slight increase in the proliferation rate while all the other exposures of 0.5, 2.5, and 10 J/cm^2 showed a decrease in the proliferation rate. Normal fibroblasts did not show a significant difference from the un-irradiated control indicating that the laser irradiation did not stimulate cell proliferation since the cells were fully functional at the time of irradiation.

change in cell proliferation when compared to wounded un-irradiated control (Table 1). The increased expression of ALP activity is one of the phenotypic characteristics of fibroblasts during wound healing and chronic inflammation. The upregulation of ALP expression requires the cessation of proliferation. 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. The wounded fibroblasts have an ALP enzyme activity less than normal cells, which may indicate a phenotypic response [30] related to an increase in proliferation initiated by the simulated wound environment. During wound healing, the proliferative or migratory phase begins a few hours after injury and continues for a few days to weeks. However, fibroblasts only reach the maximum relative number of cells after 5 days post-wounding so any increase in the rate of proliferation as seen with a single dose of 5 J/cm^2 on 2 consecutive days would ultimately accelerate the natural healing process of wounds.

LDH Membrane Integrity Assay

The results for the LDH membrane integrity or cellular damage and percentage cytotoxicity showed that normal cells exposed to a single dose on 2 consecutive days did not display a significant increase in the cellular damage even at

TABLE 1. ALP Enzyme Activity After a Single Exposure on 2 Consecutive Days

	Normal HSF			Wounded HSF		
Dose (J/cm^2)	ALP $A_{405\ nm}$	% Change[a]	*P*-value	ALP $A_{405\ nm}$	% Change	*P*-value
0.0	1.127			0.953		
0.5	1.157	2.66	0.499	1.187	24.55	0.189
2.5	1.131	0.35	0.618	0.876	−8.08	0.401
5	1.249	10.83	0.165	1.161	21.83	0.171
10	1.033	−8.34	0.764	0.811	−14.90	0.193
16	1.120	−0.62	0.689	0.846	−11.23	0.325

[a]Percentage change between the normal or wounded un-irradiated control and the irradiated normal or wounded fibroblasts.

the higher doses of 10 J/cm^2 ($P = 0.099$) and 16 J/cm^2 ($P = 0.192$). The percentage cytotoxicity ranged from 31.6% for the un-irradiated normal control to 37.09% for 10 J/cm^2 and 35.68% for 16 J/cm^2 (Fig. 4). Results from a separate study for a single exposure on 1 day showed a significant increase in cellular damage for 10 J/cm^2 ($P = 0.051$) indicating that the overnight incubation at 37°C may give the cells enough time to recover, normalize cell function and repair any damage. The percentage cytotoxicity results for normal fibroblasts exposed to 10 J/cm^2 support evidence that low levels of laser irradiation administered over 2 days (37.09%) results in less cellular damage than a single exposure on 1 day (44.95%). The results suggest that the fibroblasts may become sensitized or conditioned to the low dose of irradiation and that the dose on the second day may not have the same effect as a single dose on one day. The fibroblast cells may initiate a protective mechanism after the first exposure that allows the cells to remember an insult and adapt or possible become resistant to the same stimulus or insult.

Results from a separate study for a single exposure on 1 day showed a significant increase in cellular damage for wounded cells exposed to 10 J/cm^2 ($P = 0.048$) while cells exposed to a single dose on 2 consecutive days failed to show a significant increase after 10 J/cm^2 ($P = 0.403$). The percentage cytotoxicity results for wounded fibroblasts exposed to 10 J/cm^2 support evidence that low levels of laser irradiation administered over 2 days (34.15%) results in less cellular damage than a single exposure on 1 day (47.44%). The LDH and percentage cytotoxicity results failed to show a significant increase in the cellular damage for any of the doses, however the percentage cytotoxicity did increase from 33.7% for the wounded un-irradiated control to 36.53% for 0.5 J/cm^2 and decreased to 32.52% for 5 J/cm^2 indicating a stimulatory effect where the cell function has normalized stimulating healing and repair. The results showed that wounded cells exposed to 5 J/cm^2 had significantly less cellular damage after 2 days when compared to 0.5 J/cm^2 ($P = 0.008$), 2.5 J/cm^2 ($P = 0.023$), and 16 J/cm^2 ($P = 0.032$).

The Comet Assay for DNA Damage

The results for normal fibroblasts showed that only the higher doses of 10 J/cm^2 ($P = 0.012$) and 16 J/cm^2 ($P = 0.011$) had a significant increase in the amount of DNA damage. The results indicate that as the dose increases the amount of DNA damage also increases. The results suggest an initial repair mechanism is functional after the second day since normal cells exposed to a single dose of 10 J/cm^2 on 1 day had 256 arbitrary units compared to 166 arbitrary units for a single exposure on 2 consecutive days. The results show a significant increase in the extent of DNA damage between 0.5 and 10 J/cm^2 ($P = 0.048$) and between 0.5 and 16 J/cm^2 ($P = 0.047$).

The results for wounded fibroblasts showed a significant increase in DNA damage after 0.5 J/cm^2 ($P = 0.045$)

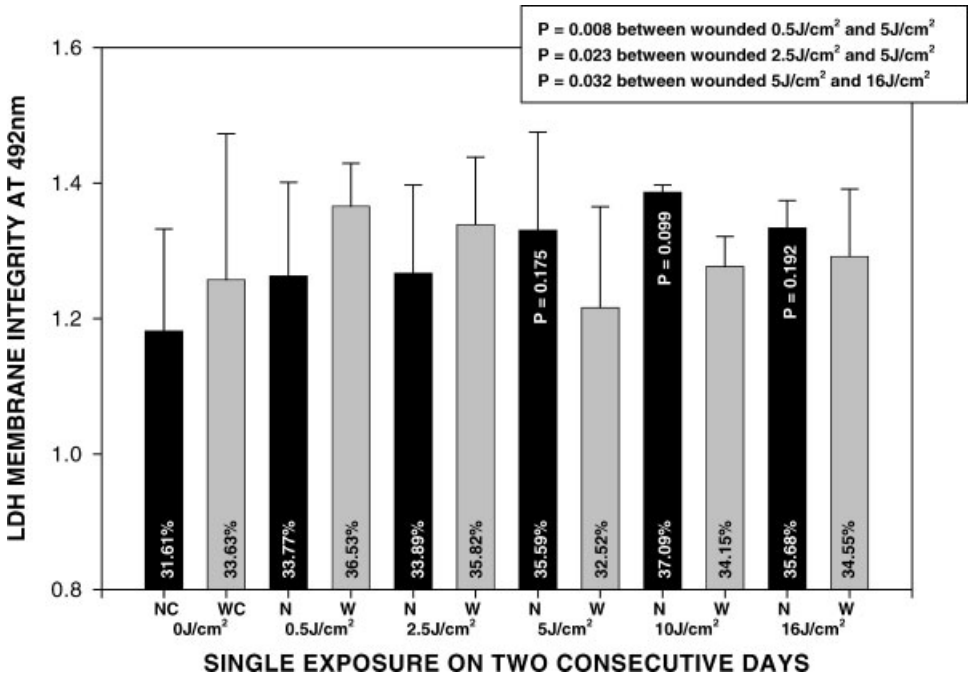

Fig. 4. The LDH membrane integrity assay was used to assess the degree of cellular damage following laser irradiation. Normal and wounded cells were irradiated with a single dose ranging from 0.0 to 16 J/cm^2 on 2 consecutive days. Wounded cells showed an initial increase in the percentage cytotoxicity at doses of 0.5 and 2.5 J/cm^2 possibly indicating that the dose was not sufficient to overcome the wounding while the percentage cytotoxicity decreased after 5 J/cm^2 possibly indicating a sufficient dose adequate to stimulate the cells to recover and repair.

possibly indicating that the dose was too low to stimulate the cells to normalize cell function and initiate healing and repair (Fig. 5). The results also showed a significant increase after 16 J/cm^2 ($P = 0.008$) indicating that the dose was too high and caused additional damage which inhibited the cellular response to wounding and slowed down the repair process. The other doses of 2.5, 5, and 10 J/cm^2 did not show a significant increase in DNA however, just like the percentage cytotoxicity and LDH results 5 J/cm^2 did show a decrease in DNA damage indicating a repair process. The results show that a high dose of 16 J/cm^2 causes the highest amount of DNA damage when compared to other doses like 10 J/cm^2 ($P = 0.029$), 5 J/cm^2 ($P = 0.008$), 2.5 J/cm^2 ($P = 0.022$), and 0.5 J/cm^2 ($P = 0.050$).

DISCUSSION

Phototherapy, when used in an appropriate manner, can stimulate the healing of injured tissues such as those of the dermis [5]. Investigations into the mechanisms involved have shown that many of the types of cells whose interaction results in dermal repair can be affected in a therapeutically advantageous manner by treatment with phototherapy both in vitro and in vivo. Proliferation of fibroblasts, endothelial cells, and keratinocytes maintained in adverse conditions can also be stimulated [22]. Karu (1987) found that IR laser (620 nm) stimulated DNA and RNA synthesis rates, enzyme activity, and cAMP levels. It is postulated that the respiratory chain is stimulated, activating ATP turnover, increasing H+, and ultimately triggering an increase in cell proliferation. The stimulating effects of light appear to occur in "sluggish" cell cultures or in circumstances of decreased activity such as trophic ulcers and indolent wounds, where low tissue oxygen concentration and pH inhibit cell growth. Conversely, where maximum regeneration is occurring naturally, laser did not appear to enhance the process [33]. The magnitude of the laser biostimulation effect depends on the physiological state of the cell at the moment of irradiation. This explains why the effect is not always detectable, as well as the variability of the results reported in the literature. Karu (1987) stated that light stimulates cell proliferation if the cells are growing poorly at the time or irradiation. Thus, if a cell is fully functional, there is nothing for laser irradiation to stimulate, and no therapeutic benefit will be observed [33].

Major morphological changes characterized the effect of incubating irradiated wounded fibroblasts overnight since they demonstrated a much higher rate of chemotaxis (migration), haptotaxis (change in orientation at the wound edge and colony formation). A dose of 16 J/cm^2 had an inhibitory effect on wounded fibroblasts when compared to 5 J/cm^2, which showed the highest rate of migration and haptotaxis with the highest number of fibroblasts present in the central scratch.

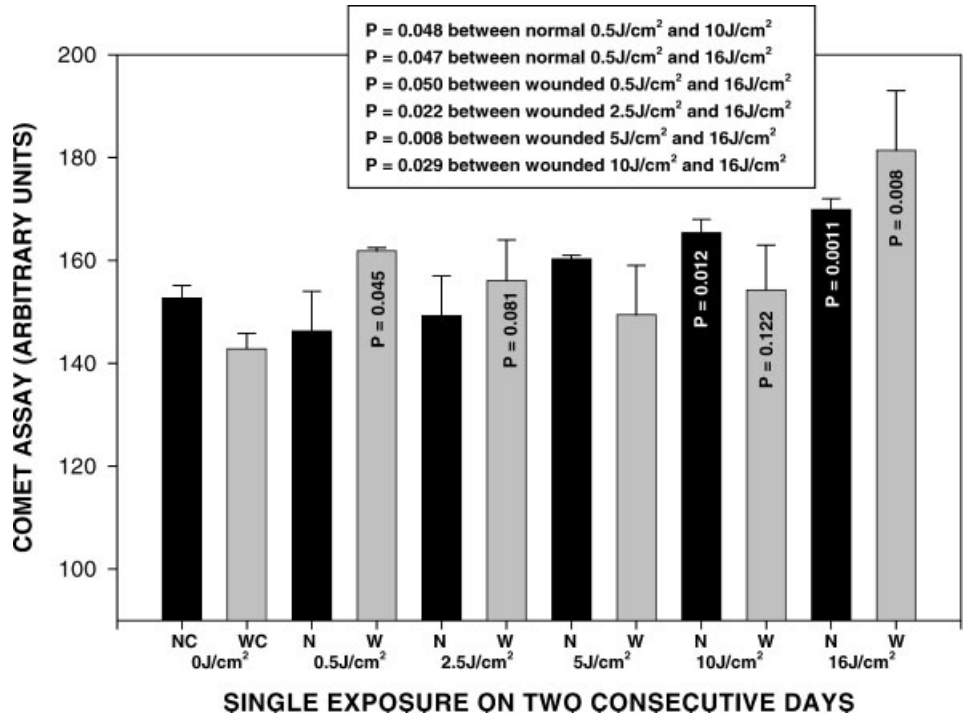

Fig. 5. The Comet assay was used to asses the degree of DNA damage following laser irradiation. Normal and wounded cells were irradiated with a single dose ranging from 0.0 to 16 J/cm^2 on 2 consecutive days. Wounded cells showed an initial increase in the DNA damage at doses of 0.5 and 2.5 J/cm^2 possibly indicating that the dose was not sufficient to overcome the wounding while the DNA damage decreased after 5 J/cm^2 possibly indicating a sufficient dose adequate to stimulate the cells to recover and repair. Wounded cells showed an increase in the DNA damage at doses of 10 and 16 J/cm^2.

The wounded fibroblasts exposed to a single dose of 0.5 J/cm^2 on 2 days showed a decrease in the cell viability, an increase in cellular damage and an increase in DNA damage indicating that the dose was too small to reverse the damage caused from the wounding. Higher doses of 10 and 16 J/cm^2 showed a decrease in cell viability, decrease in cell proliferation with an increase in DNA damage after 16 J/cm^2 but no increase in cellular damage indicating an early repair process triggered by wounding, irradiation, and overnight incubation at 37°C. Wounded cells responded to the intermediate dose of 2.5 J/cm^2 with maintained cell viability, a decrease in cell proliferation, slight increase in both cellular and DNA damage indicating that the dose was not enough to stimulate or harm wounded fibroblasts. A single dose of 2.5 J/cm^2 may be effective on 1 day since the results indicate that cells adapt or become sensitized so that the effect is not as great on the second day. Wounded cells responded to a single dose of 5 J/cm^2 with maintained cell viability, an increase in cell proliferation, slight decrease in both cellular, and DNA damage. The results indicate that 5 J/cm^2 stimulates the wounded cells to normalize cell function, increase cell proliferation, and promote healing and repair.

The results support Karu (1987) [33] where wounded cells appeared to respond better to laser irradiation since the physiological state of the cells at the moment of irradiation was compromised and the cells responded better when they were "stressed" or damaged resulting in a higher rate of cell proliferation, chemotaxis, and haptotaxis. The results also suggest that the irradiation of fibroblasts is not detrimental to the cells since they have the ability to recover, adapt, proliferate, and respond to changes in their environment. If the irradiation doses were too harmful to the cells they would loose their ability to recover and adapt and would not be able to overcome the insult of wounding or irradiation and would not be able to survive in cell culture.

CONCLUSION

From the cell morphology results, wounded cells responded to a dose of 2.5 and 5 J/cm^2 with an increase inchemotaxis-chemokinesis and haptotaxis indicating a stimulatory effect. The results indicate that a fluence of 5 J/cm^2 on 2 days stimulates mitochondrial activity (ATP activity) and cell proliferation without adversely affecting the cell viability or damaging membrane integrity or causing DNA damage. Higher doses (10 and 16 J/cm^2) result in a decrease in cell viability and mitochondrial activity with an increase in percentage cytotoxicity and DNA damage. The results showed that the percentage cytotoxicity was less for wounded fibroblasts exposed to a single dose of 5 J/cm^2 on 2 consecutive days when compared to the other doses and to a single dose of 5 J/cm^2 on 1 day supporting the benefits of irradiating a wound on 2 consecutive days. Results show that wounded cells exposed to a single exposure of 5 J/cm^2 on 2 consecutive days respond better than normal cells, un-irradiated wounded cells or wounded cells irradiated with 0.5, 2.5, 10, or 16 J/cm^2 indicating that a laser fluence of 5 J/cm^2 on 2 consecutive days may ultimately accelerate wound healing in vivo. Results support the concept that laser irradiation can modify cellular processes in a dose or fluence (J/cm^2) dependent manner.

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