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Efficacy of three different laser wavelengths for *in vitro* wound healing

Denise Hawkins-Evans & Heidi Abrahamse

Laser Research Group, Faculty of Health Sciences, University of Johannesburg, Johannesburg, South Africa

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Correspondence:
Prof. Heidi Abrahamse, Laser Research Group, Faculty of Health Sciences, University of Johannesburg, P.O. Box 17011, Doornfontein, Johannesburg 2028, South Africa.
Tel: +27 11 559 6406
Fax: +27 11 559 6558
e-mail: habrahamse@uj.ac.za

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None declared.

Summary

Background and objective: Despite contradictory reports on the effect of laser light on cell proliferation, studies have shown that appropriate doses and wavelengths of laser light are therapeutically beneficial in tissue repair and pain control. This study aimed to establish if the dose and/or wavelength influenced the biological responses of irradiated *in vitro* fibroblasts – 1 h after laser irradiation.

Materials and methods: This study aimed to establish cellular responses of normal and wounded human skin fibroblasts to helium-neon (632.8 nm), diode (830 nm) and Nd:YAG (1064 nm) laser irradiation using one exposure of 5 or 16 J/cm^2 on day 1 and again on day 4.

Results: Wounded cells exposed to 5 J/cm^2 using 632.8 nm showed an increase in cell migration and haptotaxis, a stable increase in the release of interleukin-6 (IL-6), a decrease in caspase 3/7 activity, an increase in ATP viability and an increase in cell proliferation – 1 h after the final exposure. The results confirm that changes in parameters such as ATP viability, cytokine expression (IL-6), cell proliferation (alkaline phosphatase enzyme activity) and DNA damage can be observed directly after the laser irradiation. The amount of DNA damage and cytotoxicity may be related to duration of the laser irradiation, which is dependent on the power density (mW/cm^2) of each laser.

Conclusion: The results indicate that 5 J/cm^2 using 632.8 nm results in a stimulatory effect that is more effective than 830 and 1064 nm. The results suggest possible mechanisms by which the wavelength may potentially influence the cellular responses of wounded cells.

Low-level lasers improve wound healing (1, 2). Cell proliferation and protein expression are important steps in this process. Laser irradiation at certain fluences and wavelengths can enhance the release of growth factors from fibroblasts and stimulate cell proliferation *in vitro* (1). As a treatment modality low-level laser therapy (LLLT) remains controversial because the exact mechanism by which laser light causes photochemical reactions is still unknown and studies have argued that it simply does not work (3, 4). However, positive studies on cultured human fibroblasts have shown that helium-neon (HeNe, 632.8 nm) or near infrared low-level laser irradiation increases collagen production and cell number (4). Lack of quality control and poor experimental design has produced negative results in some studies and criticism of many of the positive studies (3). The most striking general feature in the negative studies is the use of very low doses, ineffective treatment techniques or inadequate conclusions on the observations made (5). Another important consideration is that successful *in vitro* results do not always directly translate to positive results in the *in vivo* application (6). Negative as well as positive studies are equally important in the search for optimal treatment parameters.

Karu (7) stated that the laser effect depends on the radiation wavelength, dose, and intensity as well as on the cell culture conditions. Many studies have examined the effect of LLLT on fibroblasts in culture. Almeida-Lopes *et al.* (2001), Pereira *et al.* (2002) and Azevedo *et al.* (2006) reported that laser irradiation stimulates fibroblast proliferation (8–10), while Colver and Priestley concluded that HeNe (633 nm, 5 mW, 1 mm diameter beam) laser irradiation (three times a day for 3 days) did not have a significant effect on cell proliferation or cellular migration (11).

The authors have published a series of papers confirming the positive effect of LLLT on human skin fibroblasts and have concluded the following:

(i) Studies have confirmed that the central scratch is sufficient to successfully induce a reproducible wound environment because there are specific cellular responses such as cell viability and cytotoxicity that distinguish wounded from normal un-irradiated cells (12–14).

(ii) Studies have shown that 5 J/cm^2 using a HeNe laser (632.8 nm, 3 mW/cm^2) stimulates migration, proliferation and mitochondrial activity of wounded fibroblasts to accelerate wound closure (14).

(iii) Higher doses (10 and 16 J/cm^2) of LLLT are characterized by a decrease in cell viability and cell proliferation with a

significant amount of damage to the cell membrane and DNA (12).

(iv) 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) results in an inhibitory effect (15) and

(v) A duration of between 1 and 3 h post-irradiation is sufficient to measure the direct effect of laser radiation on cells (16).

Abrahamse *et al.* reported on the effect of wavelength and fluence on the cellular responses of diabetic wounded human skin fibroblasts, 24 h post-irradiation (17, 18). Therefore, it became necessary to report the cellular responses 1 h post-irradiation to establish the effect shortly after exposure while excluding other effects (cell proliferation and protein expression) that are only demonstrated after some time (16). This study presents the cellular responses directly after laser irradiation and excludes any time-dependent modifications. Initial *in vitro* studies established the effect of one, two or three exposures (14, 16) on 1 or on 2 consecutive days (12–16) while this study establishes the effect after a single exposure on day 1 and day 4. The main reason was to establish *in vitro* results that may be more practical and applicable to the clinical environment.

Diabetic wounded irradiated cells (5 J/cm^2) have shown an increase in IL-6 cytokine expression, proliferation and migration compared with diabetic wounded un-irradiated cells (0 J/cm^2), indicating a stimulatory effect of the laser light (19). This study investigates the role of IL-6 cytokine expression in cell–cell communication and the effect on cell migration and proliferation. The effect of laser irradiation on IL-6 is important because a direct association between IL-6, migration and proliferation may accelerate the inflammatory phase and reduce the time for complete wound healing.

This study aimed to establish cellular responses of normal and wounded human skin fibroblasts to helium-neon (632.8 nm), diode (830 nm) and Nd:YAG (1064 nm) laser irradiation using one exposure of 5 or 16 J/cm^2 on day 1 and day 4. This study showed that the biological effects of laser therapy are dependent on the dose and wavelength. Results suggest that IL-6 may be directly associated with cell proliferation and cell migration. The results suggest possible mechanisms by which the wavelength may potentially influence the cellular responses of wounded cells and implicates hypoxic cell injury as a cause of wavelength-dependent reduced cell function.

Materials and methods

Cell culture procedure

Human skin fibroblast (WS1) monolayer cultures (ATCC CRL1502) were grown to confluence in Eagle's minimal essential medium (EMEM) with Earle's balanced salt solution according to previously published methods (12–18). Cells were trypsinized using a 0.25% (w/v) trypsin, 0.03% EDTA solution in Hanks balanced salt solution and approximately 6.5×10^5 cells (in 3 ml culture medium containing phenol red) were seeded in 3.4 cm diameter culture plates and incubated overnight to allow the cells to attach (20). The culture plates were randomly assigned to each treatment group of normal, wounded, irradiated and un-irradiated for the specific dose (5 and 16 J/cm^2) and wavelength (632.8, 830 and 1064 nm).

Once the fibroblasts had attached, 2 ml of culture medium was removed, a wound was induced and the plates were incubated at 37 °C for 30 min before the cells were irradiated (21–23). According to Rigau *et al.* (1995), a central scratch should induce a wound that ranges between 1 and 2 mm therefore a 2 mm sterile plastic pipette was used to create reproducible wounds with the same diameter, length and depth through the cell monolayer (22). Many studies have used this central scratch method to successfully reproduce a wound environment for *in vitro* studies on wound healing (24–27).

Laser specifications and exposure regime

Normal and wounded fibroblasts were exposed to either 5 or 16 J/cm^2 on day 1 and day 4 while control cells received 0 J/cm^2. Cell culture dishes containing 6.5×10^5 cells were placed under the laser beam and irradiated with the culture dish lid off at room temperature in the dark on a dark surface. Irradiations were performed with a HeNe laser, a 830 nm diode laser and a 1064 nm Nd:YAG laser (Table 1).

A fiber optic was used to transmit the laser light from the laser tip ($\pm$ 5 mm) to the culture dish after the beam was expanded to 3.4 and 10 cm for the 830 and the 1064 nm laser, respectively.

Table 1. Summary of laser parameters used

	Helium-neon	Diode laser	Nd:YAG laser
Wavelength	632.8 nm	830 nm	1064 nm
Spectrum	Visible	Near-infrared	Near-infrared
Irradiation mode	Continuous wave (CW)	Continuous wave (CW)	Continuous wave (CW)
Power output	18.8 mW	54 mW	1 W
Power density	2.07 mW/cm^2	5.95 mW/cm^2	12.73 mW/cm^2
Energy density	5 or 16 J/cm^2	5 or 16 J/cm^2	5 or 16 J/cm^2
Spot size/area	3.4 cm/9.1 cm^2	3.4 cm/9.1 cm^2	10 cm/78.5 cm^2
Duration: 5 J/cm^2	40 min 15 s	14 min 33 s	6 min 32 s
16 J/cm^2	128 min 49 s	44 min 49 s	20 min 57 s
Exposures	Days 1 and 4	Days 1 and 4	Days 1 and 4

The fiber optic for the 830 nm diode was < 6 cm above the culture dish while this distance increased to 17 cm for the 1064 nm Nd:YAG laser. As the loss in power output was negligible, the power output recorded was used to calculate the duration of each exposure. The HeNe laser beam was transmitted through a lens and then a scanning mirror to change the direction of the beam by 90° and direct it onto the culture dish. The beam was expanded and clipped to obtain a 3.4 cm diameter spot size (area 9.1 cm^2), which was equal to the area of the cell culture dish used. As the culture dish was located some distance from the laser, the power output was reduced by approximately 21.3% and the duration of laser irradiation was compensated accordingly. As the laser tip was expanded so that the spot size area was the same area as the culture dish bottom, the entire dish was irradiated for a specific duration to deliver 5 or 16 J/cm^2 accordingly (Table 1). The HeNe (632.8 nm) beam was clipped so that a homogenous beam profile was obtained while the 830 and 1064 nm have flat beam profiles, which results in a homogenous beam distribution across the entire culture dish.

Because 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), the response observed may reflect the cumulative dose from day 1 and day 4. This response would represent the one observed in the clinical situation after the successful completion of a treatment regimen on day 1 and day 4. Because small doses with appropriate periods of time in between may be more effective than treatments that are very close, it is important to measure the biological effect in response to the cumulative dose rather than after a single dose of a treatment regimen. The cellular responses to the cumulative dose were measured 1 h (day 4) after the final laser irradiation to measure the effect of the laser irradiation shortly after exposure and to exclude time-dependent responses such as cell proliferation, protein expression and cell repair mechanisms that may only be observed after some time.

Preliminary 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 (13, 15). Temperature fluctuations were monitored using a digital thermometer and a maximum temperature change of 2.0 ± 0.6 °C was recorded during therapy. The EMEM (Invitrogen, Carlsbad, CA, USA) medium has a pH indicator (6.8–8.4), which utilizes a sodium bicarbonate buffer system (2.5 g/l), and therefore requires 5% CO_2 to maintain the required pH. When exposed to ambient levels of CO_2, the sodium bicarbonate in the medium causes MEM to become basic very rapidly and the medium changes from red to purple, indicating a rise in pH. The medium was checked regularly for changes in the pH indicator to ensure that the pH was maintained between 7.0 and 7.4 throughout the experiments.

Biological assays

Changes in cell morphology

The control (un-irradiated or 0 J/cm^2) and wounded fibroblast behavior was observed using an inverted microscope (Olympus S.A. CKX41) and changes were recorded daily using an Olympus Camedia C3030 digital camera (Olympus, Center Valley, PA, USA). Cell morphology was assessed using colony formation, haptotaxis, chemotaxis and the number of cells present in the central scratch (28). The morphology results presented in this study reflect changes observed 1 h post-irradiation (day 4).

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 cell viability (ATP luminescence and caspase 3/7 activity) and DNA damage (Comet assay). The culture medium was used to assess cell proliferation markers [alkaline phosphatase (ALP) enzyme activity and basic fibroblast growth factor], the effect on cell migration [interleukin-6 (IL-6)] and damage or additional stress caused by the irradiation (LDH membrane integrity). The culture medium (containing fetal bovine serum) contains all the small metabolites released by the cells. Because control cells are incubated in the same media but are not irradiated, the markers found in the conditioned medium are released from the cells in response to the laser irradiation and are not present due to serum supplementation.

Cell–cell communication

Fibroblasts are important sources of inflammatory cytokines early in wound healing. The IL-6 enzyme-linked immunosorbent assay (ELISA) assay (BD OptEIA 550799; Becton Dickinson and Company, San Jose, CA, USA) was used for the quantitative *in vitro* determination of human IL-6 (hIL-6) in cell culture supernatants (29). Approximately 100 μl of each sample (stored at − 20 °C) was added to each well with 50 μl of ELISA diluent (0.09% sodium azide) and then mixed, covered and incubated under constant shaking at 250 rpm ($6.98 \times g$) at 15–25 °C for 2 h. The solution was aspirated and each well was washed five times with 300 μl/well washing buffer. After washing, 100 μl of biotinylated anti-human IL-6 monoclonal antibody was added, mixed, covered and incubated under constant shaking at 250 rpm ($6.98 \times g$) at 15–25 °C for 1 h. The solution was aspirated and each well was washed seven times with 300 μl/well washing buffer. One-hundred microliters of 3,3′,5,5′-tetramehylbenzidine (TMB) substrate solution was added and the plate was covered and incubated at 15–25 °C for 30 min in the dark. Fifty microliters 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 on a BioRad Benchmark Plus Microplate Spectrophotometer (Bio-Rad Laboratories, Hertfordshire, UK) (29, 30).

Early marker of apoptosis

The caspase-Glo 3/7 (Promega G8090, Madison, WI, USA) assay was performed according to previously published methods (15, 19). Briefly, 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

luminescent signal was recorded, which is directly proportional to the amount of caspase activity present (31).

ATP cell viability assay

The CellTiter-Glo luminescent cell viability assay (Promega G7570) was performed according to previously published methods (15, 18). Briefly, 50 µl of CellTiter-Glo reagent was added to 50 µl of cell suspension (1×10^5 cells/100 µl) and mixed for 2 min to induce cell lysis. After a 10 min incubation at room temperature the luminescent signal was recorded using a Berthold EG & G Junior luminometer (Berthold Technologies GmbH & Co KG, Bad Wildbad, Germany) (32, 33).

Basic-fibroblast growth factor (bFGF)

The indirect ELISA (34, 35) was performed according to previously published methods (15, 18). Briefly, 100 µl of culture medium was diluted in 100 µl carbonate–bicarbonate buffer (Sigma-Aldrich C3041, St. Louis, MO, USA) and incubated overnight at 4 °C. The following day the coating solution was removed and 200 µl of anti-human bFGF (Sigma-Aldrich F6162, St. Louis, MO, USA) primary antibody (1 : 6500 or 5 µg/ml) in PBS-T (10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween 20) was added and the plate incubated at room temperature for 2 h. Each incubation step was followed with three washes of PBS-T. Two hundred microliters of anti-mouse IgG (Fab specific) peroxidase-conjugated antibody (Santa Cruz Biotechnology sc-2005 200 µg/0.5 ml, Santa Cruz, CA, USA) diluted (1 : 4000) in PBS-T was used as the secondary antibody while 100 µl of TMB substrate reagent (BD Biosciences #555214, Rockville, MD, USA) was added for colorimetric detection. The orange-yellow color development was stopped after 30 min with 1 mol/L H_2SO_4 and the positive wells were read at 450 nm (34, 35).

ALP enzyme assay

ALP enzyme activity was performed according to previously published methods (12, 14, 15, 19). Briefly, 50 µl of the culture medium was incubated with 50 µl of 0.5 M *N*-methyl-D-glucamine buffer (pH 10.5) at 37°C for 30 min. Twenty millimolar ρ-nitrophenyl phosphate (ρ-NPP; Sigma-Aldrich N7653, St. Louis, MO, USA) was added and the reaction was incubated at 37 °C for 30 min (36, 37). The amount of ρ-nitrophenol liberated was measured at 405 nm.

LDH membrane integrity

The CytoTox 96® non-radioactive cytotoxicity assay (Promega G1780, Madison, WI, USA) was performed according to previously published methods (12, 16–18). Briefly, 50 µl of culture medium was mixed with 50 µl of reconstituted substrate, covered with foil and incubated at room temperature for 30 min. Fifty microliters of stop solution (1 M acetic acid) was added and the absorbance read at 490 nm (38).

DNA damage

The Comet assay was performed according to Collins (39) and previously published methods (12, 16, 18). The Comet assay consists of four basic steps to measure DNA damage namely cell lysis, alkaline unwinding, electrophoresis where relaxed coils are pulled out of the nucleiod 'head' forming the 'tail' of a comet-like image and neutralization (39). One hundred comets per gel were visually analyzed at random and the cells 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).

Statistical analysis

Each biological assay was performed in duplicate and the average was used to obtain a final sample number of $n = 4$. The Student *t*-test was used to analyze the difference between the un-irradiated control (0 J/cm^2) and the irradiated cells and the difference between the different data groups (wavelengths and dose). Because the Comet assay gives data in scores or arbitrary units, the Wilcoxon rank sum (Mann–Whitney) for non-parametric data was used to confirm the results obtained from the Student *t*-test. Statistical significance was accepted at the 0.05 level (95% confidence interval; $n = 4$). The ± value or error bars in the figures indicate standard error of the mean.

Results

Effect on cell migration

Cell morphology

Normal human skin fibroblasts are long slender cells that grow in monolayer sheets (Fig. 1A, × 400 magnification). Cells irradiated with higher doses of 16 J/cm^2 showed changes indicating cell stress or damage with some fragmented cells (cell lysis) and debris (Fig. 1B, × 200 magnification). Large vacuoles were observed in the cytoplasm of some cells irradiated with 5 and 16 J/cm^2 using 830 nm. Large clear cytoplasmic vacuoles that displace the nucleus may indicate cellular injury or may be associated with thermal damage (Fig. 1C, × 400 magnification) (37).

Wounded un-irradiated cells demonstrated a clear wound margin (wm) on either side of the central scratch with some evidence of haptotaxis (h) but with very little migration (m) and few fibroblasts present in the central scratch (Fig. 1D, G and J). Wounded fibroblasts exposed to 5 J/cm^2 using 632.8 nm showed a higher rate of cell migration and haptotaxis with an increase in the number of fibroblasts present in the central scratch. The result suggests that a dose of 5 J/cm^2 stimulates cell migration of fibroblasts (Fig. 1E). Wounded cells exposed to 16 J/cm^2 using 632.8 nm showed a less migration of cells across the central scratch, however, there was evidence of haptotaxis. The result suggests that a dose of 16 J/cm^2 stimulates haptotaxis; however, there was a delay in cell migration (Fig. 1F).

Wounded fibroblasts exposed to 5 J/cm^2 using 830 nm showed a higher rate of cell migration, haptotaxis and number of fibroblasts

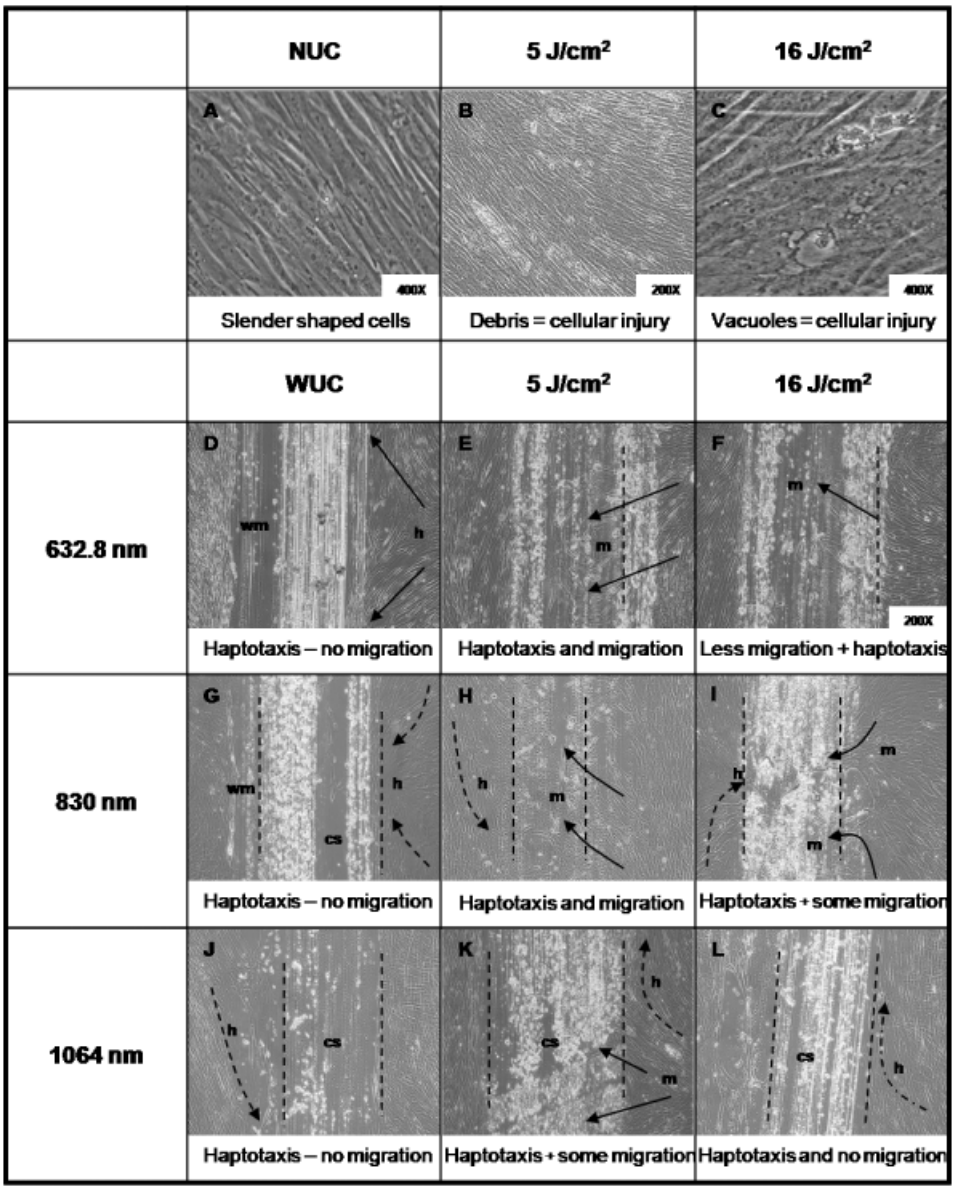

Fig. 1. Fibroblast behavior was observed using an inverted microscope. Changes in cell morphology were digitally recorded daily. Morphology results presented in this figure reflect changes observed 1 h after the final laser irradiation (day 4). Normal (NUC) and wounded (WUC) un-irradiated cells were used as control cells. Morphological changes that were assessed included haptotaxis (h), chemotaxis–chemokinesis or migration (m), colony formation and number of fibroblasts present in the central scratch (cs) as the cells migrate across the wound margin (wm) (× 200 magnification).

present in the central scratch compared with the wounded un-irradiated control on day 4 (Fig. 1H). The result shows that some of the fibroblasts have migrated completely across the central scratch to the other wound margin suggesting that the laser irradiation stimulates wound closure. Wounded cells exposed to 16 J/cm^2 using 830 nm showed very little migration of cells across the central scratch; however, there was evidence of haptotaxis. The results showed that 16 J/cm^2 stimulated haptotaxis; however, there was a delay in cell migration (Fig. 1I). Wounded cells exposed to 5 and 16 J/cm^2 using 1064 nm showed very little migration of cells across the central scratch, however, there was evidence of haptotaxis (Fig. 1K and L).

IL-6 expression

The hIL-6 assay was used to elucidate the role of the cytokine in cell migration and cell proliferation. Wounded cells exposed to 5 or 16 J/cm^2 using 632.8, 830 and 1064 nm showed an increase in the release of IL-6 when compared with the normal cells irradiated with the same dose and wavelength (Fig. 2). Wounded cells exposed to 16 J/cm^2 using 632.8 nm showed an increase in the release of IL-6 when compared with the wounded un-irradiated control ($P = 0.044$), normal cells exposed to 16 J/cm^2 ($P = 0.023$) and wounded cells exposed to 5 J/cm^2 ($P = 0.037$).

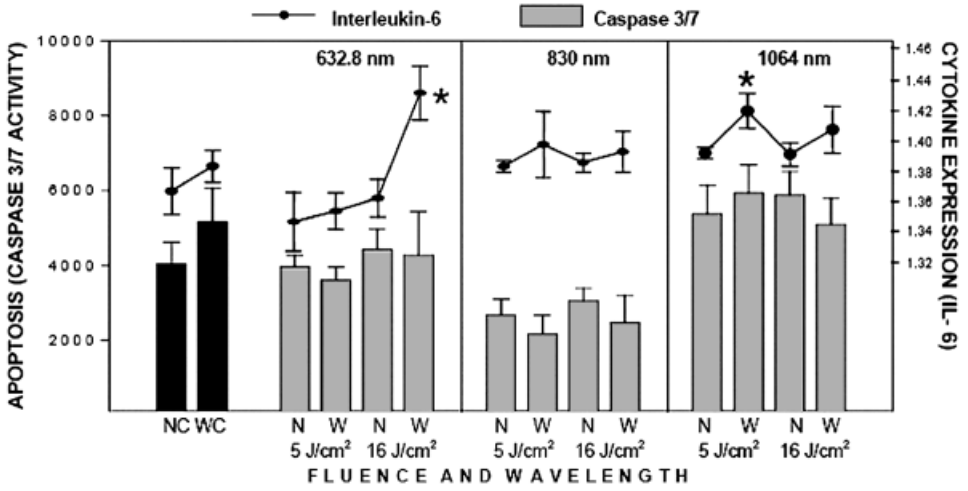

Fig. 2. hIL-6 assay was used to identify changes in the release of cytokines following irradiation while caspase 3/7 assay was used to identify an increase in cell stress that may ultimately induce apoptosis. Normal (N) and wounded (W) cells exposed to 16 J/cm^2 using 632.8 nm while normal and wounded cells exposed to 5 and 16 J/cm^2 using 1064 nm showed an increase in caspase 3/7 activity when compared with 632.8 and 830 nm. Wounded cells exposed to 16 J/cm^2 using 632.8 nm and wounded cells exposed to 5 J/cm^2 using 1064 nm showed an increase in the release of IL-6 ($n = 4$ *$P \leq 0.05$).

Wounded cells exposed to 5 or 16 J/cm^2 using 830 nm have an increase in the release of IL-6 when compared with the un-irradiated control, however, this difference did not prove to be statistically significant. The result corresponds to a decrease in caspase 3/7 activity indicating that the increase in cytokines not only assists wound healing but may also have a protective role. Wounded cells exposed to 5 J/cm^2 using 1064 nm showed an increase in IL-6 when compared with the wounded un-irradiated control ($P = 0.05$) and when compared with normal cells exposed to 5 J/cm^2 using 1064 nm ($P = 0.036$). There were no significant differences between the different wavelengths indicating that the release of IL-6 is dependent on the dose administered and not on the wavelength.

Table 2. Percentage change for cell viability using ATP luminescence

	632.8 nm	830 nm	1064 nm
Normal 5 J/cm^2	− 11.15%	− 5.77%	− 5.47%
Wounded 5 J/cm^2	7.11%	− 15.70%	5.51%
Normal 16 J/cm^2	− 13.69%	− 0.34%	− 5.38%
Wounded 16 J/cm^2	− 12.88%	− 8.16%	3.77%

Effect on cell viability

Early marker of apoptosis

The caspase 3/7 luminescent assay was used to identify if laser irradiation caused additional stress, which induces higher levels of apoptosis. Significant differences identified between the different wavelengths specific for each biological assay are summarized and presented in Table 4. The results show that there was a statistical difference ($P < 0.05$) between wounded cells exposed to 5 J/cm^2 using 632.8 nm and between wounded cells exposed to 5 J/cm^2 using 830 nm indicating that the wavelength can influence cell stress.

ATP cell viability

The ATP luminescent assay was used to determine the percentage change between the un-irradiated control cells and the irradiated normal and wounded fibroblasts (Table 2). Wounded cells exposed to 5 J/cm^2 using 830 nm showed a statistical difference ($P < 0.05$) when compared with the cells exposed to the same dose using 1064 nm, whereas wounded cells exposed to 5 J/cm^2 using 632.8 nm showed a statistical difference when compared with a wavelength of 830 nm (Table 4). The results confirm that wavelength can influence the cell viability of irradiated cells.

Effect on cell proliferation

ALP activity and bFGF

The ALP enzyme activity assay was used as a marker of wound healing while the expression of bFGF was used to assess the release of growth factor, which stimulates the proliferation of fibroblasts and is important for normal wound repair. Wounded un-irradiated cells had an increase in the release of bFGF when compared with normal un-irradiated cells indicating the mechanical injury or central scratch stimulates the release of growth factor to promote cell proliferation in the natural healing process of wounds (Fig. 3). An increase in ALP activity implies no cell growth while a decrease in ALP activity implies that the cells have enough nutrients, cytokines and growth factors to support cell proliferation. Normal cells exposed to 5 J/cm^2 using 632.8 nm showed an increase in the concentration of bFGF when compared with normal un-irradiated cells ($P = 0.05$) suggesting that there are sufficient growth factors to support proliferation (Table 3).

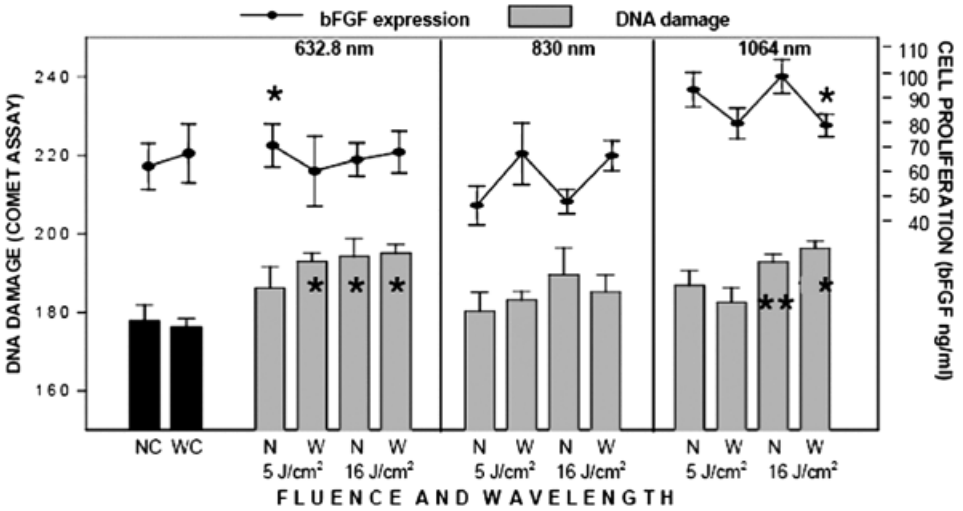

Fig. 3. Basic-fibroblast growth factor (bFGF) determined the rate at which growth factors were released while the Comet assay was used determine if irradiation caused an increase in DNA damage. Wounded cells exposed to 5 J/cm^2 using 1064 nm showed a decrease in DNA damage after 1 h. Normal (N) and wounded (W) cells exposed to 16 J/cm^2 using 632.8, 830 and 1064 nm showed an increase in LDH cytotoxicity after 1 h when compared with the normal (NC) and wounded (WC) un-irradiated control. Wounded cells exposed to 16 J/cm^2 using 1064 nm showed a decrease in bFGF ($n = 4$ $^*P \leq 0.05$, $^{**}P \leq 0.001$).

Table 3. Percentage change for ALP enzyme activity and LDH cytotoxicity

	ALP enzyme activity			LDH cytotoxicity		
	632.8 nm	830 nm	1064 nm	632.8 nm	830 nm	1064 nm
Normal 5 J/cm^2	4.60%	− 1.23%	5.23%	13.08%	− 0.76%	12.91%
Wounded 5 J/cm^2	− 11.45%	2.21%	11.11%	− 4.27%	− 0.35%	6.39%
Normal 16 J/cm^2	− 10.53%	− 4.72%	2.89%	6.078%	− 0.57%	14.63%
Wounded 16 J/cm^2	− 20.61%	4.79%	16.83%	− 4.70%	− 0.71%	11.65%

ALP, alkaline phosphatase; LDH, lactate dehydrogenase.

Wounded cells exposed to 16 J/cm^2 using 1064 nm showed a decrease in the bFGF concentration when compared with the wounded un-irradiated control cells ($P = 0.05$). Wounded cells exposed to 5 J/cm^2 ($P = 0.07$) or 16 J/cm^2 ($P = 0.059$) using 1064 nm showed an increase in ALP activity when compared with the normal cells exposed to the same dose. Significant differences for the ALP enzyme activity were identified for 5 and 16 J/cm^2 between the different wavelengths, supporting evidence that the dose and wavelength influences the stimulatory or inhibitory effect of laser irradiation (Table 4).

Effect on membrane integrity and DNA damage

LDH membrane integrity assay

CytoTox 96® non-radioactive cytotoxicity assay was used to measure membrane integrity (Table 3). The results show that normal and wounded cells exposed to 5 and 16 J/cm^2 using 1064 nm show more damage than the same cells exposed to the same dose using 830 nm. Wounded cells exposed to 5 J/cm^2 using 632.8 nm showed less damage than wounded cells to 5 J/cm^2 using 1064 nm. The results clearly show that 1064 nm laser irradiation may have an undesirable thermal effect that results in cellular damage. The results also suggest that damage may be influenced by different wavelengths.

DNA damage using the Comet assay

The Comet assay was used to determine the extent of DNA damage or single strand breaks following laser irradiation (Fig. 3). Wounded cells exposed to 5 or 16 J/cm^2 using 632.8 nm showed an increase in the DNA damage after 1 h when compared with the normal cells irradiated with the same dose indicating that the damage observed may not be due to the irradiation dose administered but rather that inducing a wound or central scratch induces damage. Wounded cells exposed to 5 J/cm^2 ($P = 0.02$) and wounded cells exposed to 16 J/cm^2 ($P = 0.015$) using 632.8 nm both showed an increase in DNA damage when compared with the wounded un-irradiated control cells. This result indicates that the dose (5 or 16 J/cm^2) and the wavelength are important factors that determine the extent of cellular and molecular damage on irradiated cells. This was supported by significant differences identified between the different wavelengths for doses of 5 and 16 J/cm^2 (Table 4).

Normal cells exposed to 16 J/cm^2 using 1064 nm showed an increase in DNA damage ($P = 0.0008$) when compared with the

Table 4. Summary of statistical differences between the different wavelengths for each specific biological assay

*Significant differences between the wavelengths of 632.8, 830 and 1064 nm.			
Caspase 3/7	Wounded exposed to 5 J/cm^2 using 632.8 nm	Versus	Wounded exposed to 5 J/cm^2 using 830 nm*
ATP cell viability	Wounded exposed to 5 J/cm^2 using 632.8 nm	Versus	Wounded exposed to 5 J/cm^2 using 830 nm*
	Wounded exposed to 5 J/cm^2 using 830 nm	Versus	Wounded exposed to 5 J/cm^2 using 1064 nm*
ALP enzyme activity	Wounded exposed to 5 J/cm^2 using 632.8 nm	Versus	Wounded exposed to 5 J/cm^2 using 830 nm*
	Wounded exposed to 16 J/cm^2 using 632.8 nm	Versus	Wounded exposed to 16 J/cm^2 using 830 nm*
	Wounded exposed to 5 J/cm^2 using 632.8 nm	Versus	Wounded exposed to 5 J/cm^2 using 1064 nm*
	Wounded exposed to 5 J/cm^2 using 830 nm	Versus	wounded exposed to 5 J/cm^2 using 1064 nm*
	Wounded exposed to 16 J/cm^2 using 830 nm	Versus	Wounded exposed to 16 J/cm^2 using 1064 nm*
Membrane integrity LDH	Wounded exposed to 5 J/cm^2 using 632.8 nm	Versus	Wounded exposed to 5 J/cm^2 using 1064 nm*
Comet assay DNA damage	Wounded exposed to 5 J/cm^2 using 632.8 nm	Versus	Wounded exposed to 5 J/cm^2 using 1064 nm*
	Wounded exposed to 16 J/cm^2 using 632.8 nm	Versus	Wounded exposed to 16 J/cm^2 using 830 nm*
	Wounded exposed to 16 J/cm^2 using 830 nm	Versus	Wounded exposed to 5 J/cm^2 using 1064 nm*

*$P \leq 0.05$.

ALP, alkaline phosphatase; ATP, adenosine triphosphate; LDH, lactate dehydrogenase.

normal un-irradiated control, while wounded cells exposed to 16 J/cm^2 using 1064 nm also showed an increase ($P = 0.029$) when compared with the wounded un-irradiated control indicating that 16 J/cm^2 using 1064 nm causes additional damage to the cells. Wounded cells exposed to 5 J/cm^2 using 1064 nm show less damage than wounded cells exposed to 16 J/cm^2 using 1064 nm ($P = 0.03$).

Discussion

The purpose of phototherapy is to stimulate or activate target cells to improve wound healing in soft tissue and to induce healing in slow-to-heal or non-healing wounds (2). Laser therapy assists the natural healing processes of the body: if there is a need for these processes, such as in the relief of a painful condition, or repair of damaged tissues, then the normal healing mechanisms occur more efficiently (2). Normalization is the keystone of laser therapy and phototherapy aims to restore homeostasis. The resultant clinical benefits include pain relief, tissue repair and increase blood circulation.

Cell morphology results show that a dose of 5 J/cm^2 using 632.8 and 830 nm appeared to stimulate cell migration in wounded cells at a higher rate than wounded un-irradiated cells or cells exposed to 16 J/cm^2. Wounded cells exposed to 5 or 16 J/cm^2 using 1064 nm did not respond with an increase in cell migration; however, there was evidence of haptotaxis indicating that the irradiation did not damage cell function but did delay cell migration. Cell morphology results suggest that migration of fibroblasts may be dependent on the dose and wavelength; however, haptotaxis appears to occur regardless of the dose or wavelength.

IL-6 has a systemic effect which indicates that any increase in IL-6 may not only promote migration and proliferation to accelerate wound healing but may also assist in the immune responses of lymphocytes preventing infection and the spread of pathogens. The results indicate that wounded irradiated cells show an increase in the release of IL-6 1 h after irradiation. This result indicates that the release of cytokines is not only dependent on the physiological status of the cells (wounded) but also on the dose and wavelength. Results suggest that laser irradiation has an additional therapeutic benefit by stimulating the release of cytokines to promote cell–cell communication, migration and proliferation to assist the natural healing process. The results appear to suggest that laser irradiation may stimulate the release of IL-6, which may be directly related to cell proliferation rather than cell migration, because wounded cells exposed to 16 J/cm^2 using 632.8 nm showed an increase in IL-6, decrease in ALP and increase in bFGF but a decrease in cell migration. However, wounded cells exposed to 5 J/cm^2 using 632.8 and 830 nm showed a direct association between an increase in IL-6, increase in cell proliferation and increase in cell migration. Future studies with hydroxyurea (Sigma H8627) will confirm if IL-6 has a direct effect on cell proliferation or cell migration because hydroxyurea is known to have an anti-proliferative effect and inhibits DNA synthesis but allows protein synthesis and cell migration (40).

Caspase results show that wounded cells exposed to 5 and 16 J/cm^2 using 632.8 nm show less cell stress than normal cells exposed to the same dose. The result suggests that the physiological status of the cells at the moment of irradiation is important when measuring the laser effect. Karu (1998) stated that light stimulates cells that are growing poorly at the moment of irradiation. Thus, if a cell is fully functional at the moment of irradiation, there is nothing for laser irradiation to stimulate, and no therapeutic benefit will be observed (41); however, if the cells are wounded then laser biostimulation aims to normalize cell function, restore homeostasis and stimulate healing and repair. The results show that wounded cells exposed to 5 J/cm^2 using 632.8 and 830 nm show less cell stress when compared with the normal irradiated cells or the un-irradiated controls, which indicates that laser irradiation stimulates the cells to normalize cell function. A wavelength of 830 nm resulted in statistically less cell stress than 632.8 nm indicating that both the dose and wavelength are important factors that influence the stimulatory effect.

The ALP enzyme activity and bFGF results suggest that the regulation of cell proliferation is dependent on the release of growth factors and the stage of wound healing. Wounded cells exposed to 16 J/cm^2 using 1064 nm showed an increase in ALP

activity and decrease in the release of bFGF after 1 h indicating a cessation of proliferation. The results suggest that either a dose of 16 J/cm^2 using 1064 nm stimulates wounded cells to enter the fibroplastic phase earlier with the cessation of cell proliferation or that that the dose could cause cellular injury, which may have an inhibitory effect like reducing cell proliferation prematurely.

The results for wounded cells exposed to 5 J/cm^2 using 632.8 nm may suggest that 1 h after irradiation there is an initial delay in the release of bFGF while the cells were still actively proliferating. However, 24 h after laser irradiation the release of bFGF increased (19) which may ultimately stimulate the wound to enter the fibroplastic phase earlier. At this stage the bFGF remains high but there is also an increase in ALP activity, which implies the cessation of proliferation (36). This suggests that once wound healing has been completed the ALP activity increases, signaling wound healing, which will be followed by a decrease in bFGF to regulate proliferation and to avoid overgrowth or overactive proliferation and excessive scar tissue formation. The relationship between bFGF and ALP enzyme activity may indicate that possibly the parameters are at their optimal activity at different times following irradiation. The result suggests that laser irradiation stimulates the release of bFGF and the increased level of bFGF may ultimately stimulate the wound to enter the fibroplastic phase earlier. The increase in ALP activity may indicate successful wound healing and the cessation of proliferation (granulation tissue formation).

Both DNA damage and cellular damage were low for wounded cells exposed to 5 J/cm^2 using 1064 nm, which may suggest a therapeutic benefit for wound healing since the laser irradiation does not cause additional stress or damage to the wounded cells. This result is supported since a wavelength of 1064 nm showed statistical less cytotoxicity than 632.8 nm. Normal and wounded cells exposed to 16 J/cm^2 using 1064 nm show more DNA damage than 5 J/cm^2 indicating that a higher dose (16 J/cm^2) results in more cellular and molecular damage. A dose of 5 J/cm^2 using 1064 nm appears to be favorable to achieve a beneficial response that may improve that rate of wound healing.

Free radicals may be formed within cells by the action of UV light or X-rays, by intracellular oxidative reactions or by the metabolism of chemicals of drugs. Free radicals may be a direct cause of cell damage in hypoxia, chemical injury or radiation damage. From the cell morphology results large cytoplasmic vacuoles were observed in normal fibroblasts exposed to 830 nm laser irradiation indicating the accumulation of water in the cell. Cloudy swelling is an almost universal morphological expression of reversible injury. The cell swells because of the accumulation of water within them (42). Hypoxic cell injury results in failure of the cell's aerobic respiratory function, that is oxidative phosphorylation with the formation of energy-rich ATP, which takes place in the mitochondria. The cell morphology results indicate that higher doses (16 J/cm^2) cause reversible damage to the cells. A proposed mechanism may include the formation of free radicals from irradiation damage where the dose administered is above the survival threshold of the target cells resulting in an inhibitory effect. The results suggest that normal cells exposed to 5 and 16 J/cm^2 using 830 nm may show signs of cloudy swelling or hypoxic cell injury with a decrease in ATP activity (failure of oxidative phosphorylation), a decrease in the release of bFGF (diminished protein synthesis), an increase in ALP activity, a decrease in cell proliferation and an increase in cytotoxicity (increased membrane permeability).

The results from this study show that wounded un-irradiated cells show differences in cell morphology, ATP viability, caspase 3/7 activity, IL-6, bFGF concentration and LDH cytotoxicity when compared with normal un-irradiated providing evidence that a central scratch successfully damages the cells and induces a wound environment. Successful *in vitro* results do not always directly translate to positive results in the *in vivo* application (9); therefore, the laser parameters identified in this *in vitro* study will be used to investigate the effect of laser irradiation on a wounded artificial skin model before being transferred to an *in vivo* model. The model consists of human keratinocytes in the epidermis and human fibroblasts and rat-tail collagen in the dermis using a floating collagen gel method otherwise known as the RAFT method (43, 44). The model is a suitable alternative method to animal testing and cell culture since its natural three-dimensional structure closely reproduces an *in vivo* situation (45). This alternative will provide an effective method to bridge the gap between *in vitro* and *in vivo* experiments.

It is well known that LLLT is dependent on the combination of laser parameters such as wavelength, power, energy, and secondarily, irradiated area, time and number of irradiations (10). The fact that irradiation of cells with visible light of the same wavelength and absorption of this light by the same molecules has a positive effect (increased proliferation) and a negative one (damage to intracellular systems) may be explained by different reactions prevailing in cases of lower and higher doses (41). This study confirmed the above statement that the dose determines the stimulatory or inhibitory effect but also clearly demonstrates that the wavelength can influence the cellular response. The results obtained support findings by Almeida-Lopes *et al.* (2001) (8) where laser irradiation improved the proliferation of fibroblasts and where smaller exposure times resulted in higher proliferation rates. Almeida-Lopes *et al.* (2001) also investigated the effect of different wavelengths (670, 780, 692 and 786 nm) using the same fluence (2 J/cm^2) and found that the infrared laser induced higher cell proliferation than the visible laser when the power outputs were different. However, lasers of equal power output presented similar effects on cell growth independently of their wavelengths (8). The results from this study indicate that the dose, wavelength and physiological status of the cells (normal or wounded) should be included as parameters when reporting the effects of low-energy laser irradiation. Results from this study confirm previous reports that have shown that the increase or decrease of cell growth depends on the applied wavelength, on the irradiance, on the pulse sequence modulated to laser beams (constant, periodic or random pulses), on the type of cells (fibroblasts) and on the density of the cells in tissue cultures.

A recent paper from Karu (46) gave the following wavelength ranges for the LLLT action spectrum: (i) 613.5–623.5 nm, (ii)

667.5–683.7 nm, (iii) 750.7–772.3 nm, (iv) 812.5–846.0 nm. In the clinical environment the different wavelengths are known for their specific penetration depths. Wavelengths in the 600–700 nm range are chosen for treating superficial tissue and wavelengths between 780 and 950 nm are chosen for deeper-seated tissues (46). However, in a single monolayer of fibroblasts the penetration depth has a limited effect on the cellular responses so other mechanisms have to be responsible for the different effects that are observed. Mognato *et al*. reported that the combination of 808 nm (continuous wave diode) and 905 nm (pulsed wave diode) in the range of 1–60 J/cm^2 was superior to the effect achieved when a single wavelength of 808 or 905 nm was used. The results also confirmed previous observations where only the proliferation of slow growing cells appeared to be stimulated by laser light (47).

Each interaction between molecules, macromolecules or living cells is basically electromagnetic and governed by photons. For this reason, electromagnetic influences like laser light of the correct wavelength will have a remarkable impact on the regulation of living processes. Different wavelengths have different energy levels and can have various effects on cells and tissue. The results from this study suggest possible mechanisms by which the wavelength may potentially influence the cellular responses of wounded cells:

(i) different *optical penetration depths* through tissue or how much light is lost as it travels through tissue or cells which depends on the ratio between absorption and scattering,

(ii) *the absorption*, as visible and near-infrared light may be absorbed by different components of the cellular respiratory chain such as chromophores in cytochrome *c* oxidase or porphyrins, which results in the production of reactive singlet oxygen species or superoxide anions O_2^- (48). Non-specialized photoacceptors absorb light of a specific wavelength (different photoacceptors absorb different wavelengths) (41),

(iii) *activating different pathways*, visible light induces photochemical changes in the mitochondria which activates a chain of biological events whereas infrared light directly affects membrane channels resulting in changes in membrane permeability, temperature and pressure gradients (8),

(iv) *cell signalling*, the correct wavelength and power density can increase ATP production and cell membrane perturbation which could lead to permeability changes and second messenger activity resulting in functional changes. Visible laser light results in an increase in oxidative metabolism, an increase in growth factor and increase in the release of cytokines,

(v) *power density*, DNA damage and cytotoxicity may be related to duration of the laser irradiation (which is dependent on the power density). For example the duration ranges from 6 min 32 s for 1064 nm to 40 min 15 s for 632.8 nm to administer a dose of 5 J/cm^2, when a power density of 12.73 and 2.07 mW/cm^2 is used,

(vi) *thermal damage*, when less laser light is absorbed by target cells more energy is transmitted leading to inadvertent heating and significant thermal damage and,

(vii) *type of laser*, diode lasers (830 nm) emit light with a shorter coherence length than gas lasers and the biological effects of light from a 633 nm diode laser are less obvious than those from a HeNe laser.

Conclusion

The results from this study show that wounded cells exposed to 5 J/cm^2 using 632.8 and 830 nm show an increase in cell migration and haptotaxis; a stable increase in the release of IL-6 and an increase in cell proliferation indicating that the dose and wavelength are important laser parameters that can influence the biological response of cells. Results suggest that IL-6 may be directly associated with cell proliferation and cell migration. Laser irradiation using 5 J/cm^2 using 1064 nm appears to have a favorable stimulatory effect on wounded cells, which may be directly related to the duration or the laser exposure, the dose and the wavelength. The results indicate that 5 J/cm^2 using 632.8 nm is more effective than 830 and 1064 nm. The amount of DNA damage and cytotoxicity may be related to duration of the laser irradiation, which is dependent on the power density (mW/cm^2) of each laser. This factor may be important when selecting a laser for a specific application where a minimum amount of damage or cytotoxicity is required.

The results confirm that changes in parameters such as ATP viability, cytokine expression (IL-6), cell proliferation (ALP enzyme activity) and DNA damage can be observed directly after the laser irradiation. This is important in determining the biostimulatory mechanism because the direct or immediate effect of the laser irradiation is observed and any time-dependent modifications are excluded. The *in vitro* results from this study identified optimal laser parameters that will be used for the initiation of *in vitro* studies using the wounded skin equivalent model and moreover for *in vivo* wound healing studies.

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