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The effect of 880 nm low level laser energy on human fibroblast cell numbers: a possible role in hypertrophic wound healing

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Abstract

Low level lasers (LLLs) have been shown to induce therapeutic effects in wound healing. However, there have been few LLL studies on burn wounds which may become unsightly, hypertrophic and impair function. Inhibitory effects on the healing of fibrotic wounds, prone to hypertrophy may be expected to reasonably reduce the problems accompanying hypertrophic scarring. The effects of LLL wavelengths and treatment parameters on wound healing cells in vitro often demonstrate meaningful results and without concurrent ethical difficulties of clinical trials. This experiment investigated the effect of an 880 nm, 16 mW GaAlAs diode at 2.4 and 4 J/cm^2 on cell numbers of two human fibroblast cell lines, derived from hypertrophic scar (HF) and normal dermal explants (NF), respectively. After irradiation by 880 nm LLL, cell numbers were measured utilising methylene blue bioassay and read by the spectrophotometer in the same microculture plates. HF and NF exhibited decreased cell numbers as compared to sham-irradiated controls. HF cell number, after 2.4 J/cm^2, was significantly lower on day 5 ($P<0.05$). The NF cell numbers were significantly lower on day 4 and/or day 5 ($P<0.05$). The results have implications on hypertrophic wound healing and further studies are required.
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Keywords: In vitro fibroblasts; 880 nm wavelength; Wound healing; Hypertrophic scar

1. Introduction

Low level laser (LLL) energy/therapy was initially employed over 30 years ago for its photobiostimulatory effect on sluggish-healing ulcers and wounds such as burn wounds [1,2]. From the limited clinical studies that have been carried out fairly recently, some of them have demonstrated positive outcomes [3–5]. The small numbers may be because randomized, controlled clinical trials in wound healing involving LLLs are difficult to conduct partly owing to ethical reasons. Another reason is because of the postulated systemic effect of LLL therapy which would also apply, in animal studies. Thus, it is very difficult to study the effect of LLLs on the healing of burn wounds or developing hypertrophic scars. Apart from the occasional study such as one using an argon laser on hypertrophic and keloid scars [6], there is a dearth of investigation into this area. Furthermore, the lack of a readily available animal model, able to exhibit human-like hypertrophic scars poses further problems on evaluating the effect of LLL energy on burn wounds [7]. Investigators may therefore, preferably undertake in vitro studies to first of all, determine the effects of specific wavelengths and other treatment parameters on different types of human wound healing cells. There have been many studies on the varying effects by different LLL wavelengths and other treatment parameters on an important wound healing cell, the fibroblast [8–14].

It has been strongly suggested that the biomodulatory effect of LLL energy is wavelength dependent [15,16]. We have reported the stimulatory effect of LLL energy on hypertrophic scar-derived fibroblasts cell numbers after irradiation with the 660 nm red light wavelength on fibroblasts [13]. However, a photobiostimulatory effect on hypertrophic scar healing is not required since excessive fibroplasia [13,17,18] is counterproductive in wounds that are susceptible to hypertrophic scarring. Apart from the wavelengths that have been shown to induce biostimulatory effects such as increasing cell proliferation, others should be tested for their effects which may be different. The literature, however, includes a considerable number of

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LLL studies which exhibited biostimulatory or 'no effects' on irradiated cells but very few studies reported inhibitory effects [18,19]. One of them [18] demonstrated there was an inhibitory effect on fibroblast proliferation mediated via the supernatant collected from a suspension of macrophage-like cells that had been irradiated by 880 nm GaAlAs diode. It was suggested by the investigators [18] that this could have been due to stimulation of the secretion of an inhibitory factor or factors into the supernatant by LLL. As an inhibitory effect on fibroblast cell numbers in abnormal fibrotic wounds may be expected to reasonably reduce the problems of developing hypertrophic scars [13] which are marked by abnormal and exuberant collagen deposition secondary to an increased vascularization response in the scar and an increased fibroblast cell density [20], the 880 nm wavelength was employed in this study. Unlike the demonstrated stimulatory effects of red light wavelengths on fibroblasts cell numbers, investigations are lacking on the inhibitory effect of LLL energy on hypertrophic scar fibroblasts which affect fibroplasia in the wound healing process [21]. Although other co-existing factors such as excessive collagen production would affect fibroplasia but wound fibroblast cell numbers also could be expected to contribute to the exuberant deposition of collagen.

In this study, the two cell-lines were originally derived from 'discarded' biopsy explants from consenting donors, matched for race, age, gender and the region of the donor site [13]. The dosages of 2.4 and 4 J/cm^2 were selected as employed in a previous study [13] and as supported by the literature. These energy fluences are within the 'therapeutic window' of up to 4 J/cm^2 that are frequently employed clinically in the treatment of wounds and ulcers [22–25]. The dosage or energy fluence/density in laser irradiation is the intensity of energy, i.e., J/cm^2 or the product of the power density (W/cm^2) and the duration of irradiation in seconds, being delivered to the receiving tissue(s) which in this study is a cell-suspension. The details of the parameters used in this study are provided in Section 2.3 below.

2. Materials and methods

2.1. Cell cultures

Hypertrophic scar derived fibroblasts and normal dermal fibroblasts were grown as described [13]. In summary, both primary cell lines were obtained as follows. The tissue biopsies were removed from DMEM (Gibco) that had penicillin and streptomycin incorporated into it. They were washed with phosphate-buffered saline (PBS) (Oxoid), cut into fragments of less than 1 mm in any dimension, cultivated in 50-ml culture flasks and incubated at 37 °C in humidified atmosphere with 5% CO_2. The primary culture medium, DMEM with 10% foetal bovine serum, L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml) was added to the flask after 24 h. The explanted cells were then fed every 3 days.

When nearing confluency, short trypsinization selected the fibroblasts from other cell types. After the suspension was centrifuged, the collected fibroblasts were subcultured in culture medium in a fresh flask. Subculturing the cells in this manner but with longer trypsinization was continued until they were harvested at passage 4 for storage in liquid nitrogen. For this study, the cells were retrieved from cryopreservation, thawed and cultured for two more passages before they were irradiated with 880 nm LLL energy. The fibroblasts were characterised immunohistochemically (Dako Products, Japan) to exclude that they were not other cell types as had been described [13].

2.2. Instrumentation

The laser power output emitted from the 16 mW laser diode equipment (3ML, Omega Universal Technologies) was new and used solely for the study. However, it was tested with an optical power meter (OLS, OPM93, Omega Universal Technologies) just before irradiation of the cell-suspensions during each experiment. The equipment was thoroughly checked by the manufacturer's agent for accuracy at regular intervals.

2.3. 880 nm LLL irradiation

Preparation of both primary cell lines, hypertrophic scar (HF) and normal dermal explants (NF) for irradiation was similar. The procedures were carried out under a vertical laminar airflow tissue culture hood (Class II, Type A/B3, Nuaire). Each specified well in alternate columns of the microculture plates (Linbro) had inoculated into it, 100 μl of cell suspension consisting of approximately 1000 cells in primary culture medium. Half of the inoculated wells received 880 nm irradiation of 2.4 J/cm^2 from a laser diode. The tip of the diode was positioned perpendicular, 0.4 cm distance from the surface of the culture medium. The other half of the cells-containing wells were sham-irradiated as the diode was similarly positioned but not switched on. The parameters of the laser irradiation were:

(a) Source: Noncoherent Omega superluminous diode.
(b) Average power output: 16 mW.
(c) Spot size at 0.4 cm distance: 0.296 cm^2.
(d) Source spectral bandwidth at 95% intensity: 6 nm.
(e) Total angle of divergence: 30°.
(f) Pulsing frequency: 5,000 Hz.
(g) Pulse duration: 160 μs.
(h) Power density at 0.4 cm distance: 0.054 W/cm^2.
(i) Duration of exposure: 44.5 s for energy density of 2.4 J/cm^2 in experiments A and B.

(j) Duration of exposure: 74 s for energy density of 4 J/cm^2 in experiments C and D.

Immediately following irradiation at 2.4 J/cm^2, all the empty wells were filled with culture medium. The culture plates were marked and sealed, positioned next to each other on the same shelf of an incubator, with humidified atmosphere, 5% CO_2 and 37 °C. For interpretation of results optimally, for each cell line (HF and NF), five sets of culture plates were prepared for the aim of stopping one plate from each set per day for 5 days. They were called experiments A and B, respectively.

The above procedure was replicated but the cells were irradiated with energy density of 4 J/cm^2 (experiments C and D, respectively).

2.4. Post-irradiation conditions for methylene blue bioassay

On day 1, the first plate was removed from the incubator for processing, 18 h after the irradiation. Then at each subsequent 24 h, another plate was taken out of the incubator and processed for spectrophotometry after day 5. A modified methylene blue method as described previously [26] was employed when all the microplates were bioassayed and optically read at 650 nm using a spectrophotometer (Anthos Labtec Instruments) for absorbances. Prior to the bioassay, the cells were stained with methylene blue for 30 min, then gently rinsed with 0.01 M borate buffer three times, and elution of the dye with a 1:1 ratio of acid–alcohol.

Conversion of the absorbency readings of the cell-containing wells into cell numbers has been described [13]. It involved seeding determined numbers of serially diluted cells into specific wells of identical microculture plates (Linbro), incubation under the conditions stated earlier, careful removal of the culture medium and rinsing the monolayer of cells with PBS and then fixing with 100% methanol. The specific cell numbers were highly correlated with their absorbency values. Thus, by linear regression, cell numbers were estimated by the absorbances of the cell-containing wells.

For consistency, all the processed microculture plates were read on the same day, under identical conditions.

3. Results

The results of the experiments revealed that laser energy of 880 nm wavelength at 16 mW and 2.4 J/cm^2 had a very mild inhibitory effect on both the primary cell lines, HF derived from hypertrophic scar explants and NF from normal dermal biopsy explants. As shown in Fig. 1, in experiment A, on days 1 to 4, the HF cell numbers as compared to controls were not significantly decreased except for day 5, when it was statistically significantly lower ($P<0.05$, Mann–Whitney, M–W, U-test) and in experiment B, the NF cell numbers were also lower and reached significance on days 4 and 5 ($P<0.05$, Mann–Whitney U-test).

Fig. 2 illustrates the results of experiments C and D after the cell lines were exposed to similar laser parameters but of an energy density of 4 J/cm^2. The results of experiment C showed that HF cell numbers were lower but not significant on any of the 5 days. In experiment D, the NF cells were significantly lower on just day 5 ($P=0.05$, Mann–Whitney U-test). The percentage differences in cell numbers on each of the 5 days for all the experiments are presented in the legends of the charts.

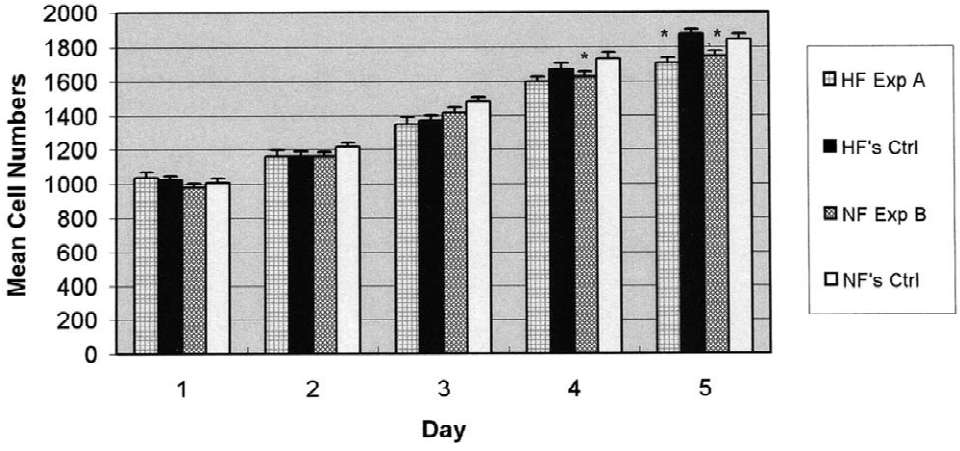

Fig. 1. Effect of 880 nm wavelength at 2.4 J/cm^2 on HF (experiment A) and NF (experiment B) cell lines where in each, $N=32$. * Denotes that cell number was significantly lower than control ($P<0.05$, M–W U-test). Error bars are standard errors of the means. The percentage decrease in HF cell numbers following irradiation at 2.4 J/cm^2 as compared to the respective control are: day 1: 1.4%; day 2: 0%; day 3: 1.3%; day 4: 4.2%; day 5: 8.8%*. The percentage decrease in NF cell numbers following irradiation at 2.4 J/cm^2 as compared to the respective control are: day 1: 2.8%; day 2: 4.8%; day 3: 4.6%; day 4: 6%*; day 5: 5.9%*.

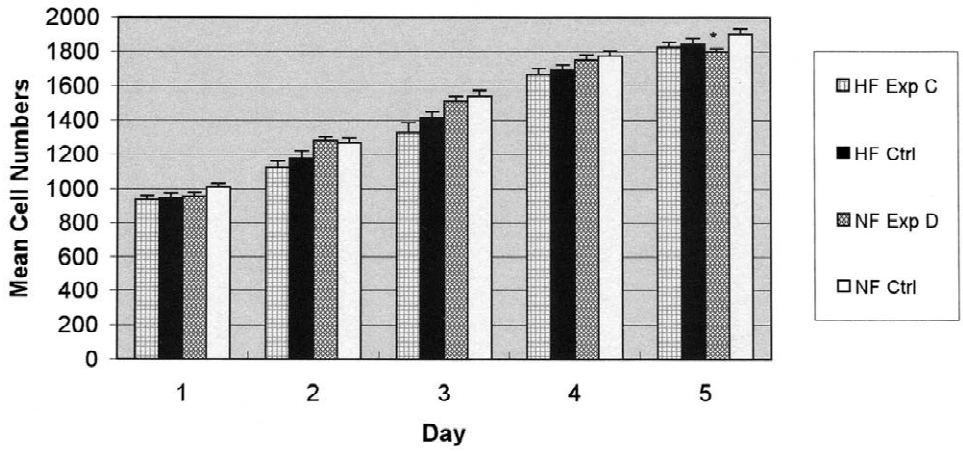

Fig. 2. Effect of 880 nm wavelength at 4 J/cm^2 on HF (experiment C) and NF (experiment D) cell lines where in each, $N=32$. * Denotes that cell number was significantly lower as compared to control ($P=0.05$, M–W U-test). Error bars are standard errors of the means. The percentage decrease in HF cell counts following irradiation at 4 J/cm^2 as compared to the respective control are: day 1: 1.2%; day 2: 5%; day 3: 5.1%; day 4: 1.5%; day 5: 0.7%. Percentage decrease in NF cell counts following irradiation at 4 J/cm^2 as compared to control are: day 1: 5.2%; day 2: 1.3%; day 3: 1.9%; day 4: 1.4%; day 5: 5.3%*.

4. Discussion

In the treatment of ulcers or wounds that do not heal normally, some clinicians have employed LLL irradiation or therapy of different wavelengths [2–5,27–29]. As hypertrophic scars can present many debilitating, long-term problems [19,20,30], both physical and psychological, the findings of this in vitro investigation are discussed in such a context even though there are some limitations in attempting to extrapolate the results to the in vivo state. Nevertheless, by knowing the effect of the 880 nm infrared wavelength laser energy on in vitro fibroblasts under highly controlled conditions would answer the basic question if further research with this wavelength is recommended in the quest for a modality to treat this debilitating complication as currently, there is no routinely effective form of therapy for hypertrophic wounds [20]. Otherwise, the search may be directed at other LLL wavelengths that can therapeutically counteract or reduce the abnormal fibroplasia or the development of hypertrophic scars.

The normal wound healing process is described as an orderly concert of repair activities work, mainly controlled by numerous and significant levels of growth factors [31,32]. The clinical manifestation of hypertrophic scars indicate that this orderly control may be lacking in hypertrophic wounds. It may be due to an abundance of growth factors, fibroblast growth factors (FGFs) and others [17] along with increased angiogenesis [19,32]. In a study on the behaviour of tissue repair cells including fibroblasts and their cytokines in foetal wound healing, it was suggested that excessive transforming growth factor β caused scarring [17]. It could be speculated that if appropriate laser therapy can biomodulate the abnormal hypertrophic wound healing process and bring the cytokines to more orderly levels, the fibroblast cell counts may be reduced and could potentially limit the excessive deposition of collagen in the healing scar tissue. The mild inhibitory effect found in this study may be possibly the result of the 880 nm irradiation inducing the release of inhibitory factors in the cells.

It also has been suggested that stimulatory, no response or inhibitory effects result from the different wavelengths and parameters including dosages, affect different photoacceptors of the respiratory chain [15,33]. The higher energy density of 4 J/cm^2 did not appear to have a greater inhibitory effect on the fibroblasts in this study. In the HF cell line, the lower cell numbers did not even reach significance on any of the 5 days suggesting that there was hardly any cumulative effect over the successive days.

Contrary to the stimulatory effect of selected red light wavelengths, the 880 nm wavelength at 2.4 and 4 J/cm^2 employed in this study only decreased slightly the fibroblast cell numbers of both cell lines in all the 5 days following irradiation. The lower NF cell numbers reached significance on days 4 and 5 after irradiation at 2.4 J/cm^2 could indicate that this lower energy density level was possibly more suitable to induce an inhibitory effect than 4 J/cm^2. This outcome could be relevant in that low energy levels of laser irradiation may induce mild oxidative modifications [15] and reduce cell functions [33]. Unlike the structural changes caused by substantial doses of ultraviolet irradiation [34] or high level lasers, it is likely that the mild reactive oxidative modifications brought about by LLL will not amount to cell death. If this is indeed the case and since the collagenase level in postburn hypertrophic scar fibroblasts was found to be lower than in normal fibroblasts which implied reduced activity in the modulation of collagen [32,35], the abnormal process of excessive collagen deposition during healing may be

modulated after appropriate LLL irradiation. Also, as hypertrophic scars are abundant in mRNAs for procollagen, in particular types I and III [21], it can be speculated that if suitable laser irradiation parameters could cause a dampening effect on these activities, the effect would be therapeutic.

Traditionally, inhibitory effects on in vitro cells are associated with higher than optimum LLL dosages [10,15,24,25,33], but this was not likely the case in this study. The two energy densities employed were within the 'therapeutic safety window' [24,33,36] and are not likely to cause irreversible damage to the cells. It has been suggested that higher than optimum laser dosages can cause excessive superoxide, O_2 to form and without the production of superoxide dismutase to counter the harm these free radicals have on DNA [11,15].

Although we studied the effect of a single exposure of LLL energy on the cell numbers of two fibroblast cell lines, the 880 nm wavelength may have induced some sustained inhibition on the factors influencing the production of FGFs. The FGFs, especially FGF-1 and FGF-2 mediate the soft tissue repair process and they promote chemotaxis, differentiation and proliferation of wound healing cells [31,32] especially fibroblasts [34]. Thus, we speculate that the 880 nm wavelength employed in our study may have inhibited the effects of these growth factors or on the growth factors themselves.

It has been shown that the response of LLL on cells may be dose-dependent as well as wavelength-dependent [10,16,18,19,37–39]. Even with an appropriate wavelength that can be absorbed by the targeted tissues, employing the correct power density, exposure time and energy density are important parameters which require further evaluation [15,16,23,24]. As depicted in the Arndt–Schultz curve, described by Ohshiro and Calderhead [24], the optimum LLL dose should induce the required best effect without causing cell mortality through excessive dosage. However, small amounts of laser-induced singlet oxygen photoproduced by natural porphyrins may help normalize DNA synthesis and cytoplasmic activities [38] such as modulating Ca^{2+} influx into the cytoplasm for promoting the normal cell mitotic process [11] which is aimed for during fibrotic wound healing.

It also has been postulated that the 880 nm infrared LLL wavelength's interactions occur at the irradiated cell membranes by cell chromophores, e.g., endogenous porphyrins [11] which considerably alters the transmembrane transport of various cations, notably Ca^{2+}. LLL-mediated increased cell membrane permeability to Ca^{2+} may trigger the chain of events related to more orderly release of cytokines [33,37,38] towards better or normal wound healing. Furthermore, it was reported that increased calcium uptake rates in macrophages, which are also important cells in wound healing, following LLL, were wavelength, dose and pulse frequency-dependent [39].

Although LLL therapy is frequently applied to dermal conditions, uncertainties remain regarding the best and most appropriate wavelength and treatment parameters [36,40,41] for the treatment of hypertrophic wounds or recent hypertrophic scars. The mild inhibitory effects by the 880 nm wavelength in this study should be investigated further for greater effectiveness including that on the role of FGFs. The results also prompt the need for more work on the effect of 880 nm and other LLL parameters such as other wavelengths and various other parameters [24,33,38,41] on these essentially intradermal nodules of collagen bundles that although they lie parallel and close to the skin surface, could cause deep, crippling effects on the patient's functions.

5. Conclusion

This study found that 880 nm LLL from a 16 mW laser diode, pulsed at 5 KHz, at energy densities of 2.4 and 4 J/cm^2 mildly decreased HF cell counts but significance was reached on day 5 only, after the 2.4 J/cm^2 irradiation. The higher dosage of 4 J/cm^2 failed to demonstrate any greater effect than the 2.4 J/cm^2 irradiation and its effect was even milder. Since the NF cell numbers were also inhibited, the results suggest that this wavelength at the selected parameters may not be as suitable for enhancing the closure of sluggish-healing ulcers.

Future studies should measure other cell functions to help relate them to LLL-induced inhibitory effects and in turn, be related to dermal burn wound healing conditions where hypertrophic scars present problems to patients.

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