Open access • Journal Article • DOI:10.1007/S10103-003-0262-X

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Published on: 01 Jan 2003 - Lasers in Medical Science (Springer-Verlag)

Lasers Med Sci (2003) 18: 95–99
DOI 10.1007/s10103-003-0262-x

ORIGINAL ARTICLE

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Received: 15 October 2002 / Accepted: 7 May 2003

Abstract *Background and Objective*: As Light Emitting Diode (LED) devices are commercially introduced as an alternative for Low Level Laser (LLL) Therapy, the ability of LED in influencing wound healing processes at cellular level was examined. *Study Design/Materials and Methods*: Cultured fibroblasts were treated in a controlled, randomized manner, during three consecutive days, either with an infrared LLL or with a LED light source emitting several wavelengths (950 nm, 660 nm and 570 nm) and respective power outputs. Treatment duration varied in relation to varying surface energy densities (radiant exposures). *Results*: Statistical analysis revealed a higher rate of proliferation ($p < 0.001$) in all irradiated cultures in comparison with the controls. Green light yielded a significantly higher number of cells, than red ($p < 0.001$) and infrared LED light ($p < 0.001$) and than the cultures irradiated with the LLL ($p < 0.001$); the red probe provided a higher increase ($p < 0.001$) than the infrared LED probe and than the LLL source. *Conclusion*: LED and LLL irradiation resulted in an increased fibroblast proliferation *in vitro*. This study therefore postulates possible stimulatory effects on wound healing *in vivo* at the applied dosimetric parameters.

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Keywords Biostimulation · Fibroblast proliferation · Light Emitting Diodes · Low Level Laser · Tetrazolium salt

Introduction

Since the introduction of photobiostimulation into medicine, the effectiveness and applicability of a variety of light sources, in the treatment of a wide range of medical conditions [1–5] has thoroughly been investigated, *in vitro* as well as *in vivo*. The results of several investigations are remarkably contradictory. This is at least in part a consequence of the wide range of indications, as well as the wide range of suitable parameters for irradiation and even the inability to measure the possible effects after irradiation with the necessary objectivity [4,6,7]. A lack of theoretical understanding can also be responsible for the existing controversies. In fact, theoretical understanding of the mechanisms is not necessary to establish effects, though it is necessary to simplify the evaluation and interpretation of the obtained results. As a consequence, the widespread acceptance of especially Low Level Laser (LLL) therapy, in the early seventies is faded nowadays and biostimulation by light is often viewed with scepticism [8]. According to Baxter [4,9], contemporary research and consumption in physiotherapy is in particular focused on the stimulation of wound healing. Tissue repair and healing of injured skin are complex processes that involve a dynamic series of events including coagulation, inflammation, granulation tissue formation, wound contraction and tissue remodelling [10]. This complexity aggravates research within this cardinal indication.

Research in this domain mostly covers LLL studies, but the current commercial availability of other light sources, appeals research to investigate as well the effects of those alternative light sources, e.g. Light Emitting Diode (LED) apparatus.

The scarcity of literature on LED is responsible for consultation of literature originating from LLL studies

[11] but it may be wondered if this literature is representative for that purpose. As in the early days of LLL therapy, the stimulating effects upon biological objects were explained by its coherence [12,13], while the beam emitted by LED's on the contrary produces incoherent light. Though the findings of some scientists [9,14,15,16,17] pose nowadays that the coherence of the light beam is not responsible for the effects of LLL therapy. Given that the cardinal difference between LED and LLL therapy, coherence, is not of remarkable importance in providing biological response in cellular monolayers [5], one may consult literature from LLL studies to refer to in this LED studies.

The purpose of this preliminary study is to examine the hypothesis that LED irradiation at specific output parameters can influence fibroblast proliferation. Therefore, irradiated fibroblasts cultures were compared with controls. The article reports the findings of this study in an attempt to promote further discussion and establish the use of LED.

Materials and methods

Cell isolation and culture procedures

Fibroblasts were obtained from 8-days old chicken embryos. Isolation and disaggregation of the cells was performed with warm trypsin according the protocol described by Ian Freshney (1994) [18]. The primary explants were cultivated at 37 °C in Hanks' culture Medium supplemented with 10% Fetal Calf Serum, 1% Fungizone, 1% L-Glutamine and 0.5% Penicillin-Streptomycin. When cell growth from the explants reached confluence, cells were detached with trypsine and subcultured during 24 hours in 80-cm^2 culture flasks (Nunc™) in 12 ml of primary culture medium. After 72 hours the cells were removed from the culture flasks by trypsinization and counted by Bürker hemocytometry. For the experiment, cells from the third passage were plated in 96-well plates (Nunc™) with a corresponding area of 0.33 cm^2, they were subcultured at a density of 70.000 cell/cm^2. Cultures were maintained in a humid atmosphere at 37 °C during 24 hours.

All supplies for cell culture were delivered by N.V. Life Technologies, Belgium, except for Fetal Calf Serum (Invitrogen Corporation, UK)

Irradiation sources

In this study two light sources, a Light Emitting Diode (LED) device and a Low Level Laser (LLL) device, were used in comparison to control cultures.

The used LLL was an infrared, GaAlAs Laser (Unilaser 301P, MDB-Laser, Belgium) with an area of 0.196 cm^2, a wavelength of 830 nm, a power output ranging from 1–400 mW and a frequency range from 0–1500 Hz.

The Light Emitting Diode device (BIO-DIO preprototype, MDB-Laser, Belgium), consisted of three wavelengths emitted by separate probes. A first probe, emitting green light, had a wavelength of 570 nm (power-range, 10–0.2 mW), the probe in the red spectrum, had a wavelength of 660 nm (power-range, 80–15 mW) and the third probe had a wavelength of 950 nm (power-range, 160–80 mW) and emitted infrared light. The area of all three probes was 18 cm^2 and their frequency was variable within the range of 0–1500 Hz.

Exposure regime

Prior to irradiation, the 96-well plates were microscopically verified, to guarantee that the cells were adherent, and to assure that there was no confluence, nor contamination. Following aspiration of 75% Hanks' culture Medium irradiation started. The remaining 25% (50 µl) medium avoided dehydration of the fibroblasts throughout irradiation.

The 96-well plates were randomly assigned in the treated (LLL or green, red or infrared LED's) or the control group.

For the treatments in this study, the continuous mode was applied as well for the LLL as for the three LED-probes. The distance from light source to fibroblasts was 0.6 cm. LLL therapy consisted of 5 seconds irradiation at a power output of 40 mW resulting in a radiant exposure of 1 J/cm^2. The infrared and the red beam delivered radiant exposures of 0.53 J/cm^2 and the green beam emitted 0.1 J/cm^2, corresponding to exposure-times of respectively 1 minute, 2 minutes or 3 minutes and a respective power output of 160 mW, 80 mW or 10 mW.

After these handlings, the remaining medium was removed and new Hanks'culture medium was added, followed by 24 hours of incubation.

One irradiation (LLL or LED) was performed daily, during three consecutive days according to the aforementioned procedure. Control cultures underwent the same handling, but were sham-irradiated.

Determination of cell proliferation

The number of cells within the 96-well plates, as a measure for repair [19], was quantified by a sensitive and reproducible colorimetric proliferation assay [20, 21]. The colorimetric assay was performed at two different points of time to determine the duration of the effect of the used light sources.

This assay exists of a replacement of Hanks'culture medium by fresh medium containing tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) 24 or 72 hours after the third irradiation, for MTT analysis as described by Mosmann (1983) [22]. Following a 4 hour incubation at 37 °C, the MTT solution was substituted by lysing buffer, isopropyl alcohol. The plates were temporarily shaken to allow dissolution of the produced formazan crystals. After 30 minutes of exposure to the lysing buffer, absorbance was measured. The absorbance at 400 to 750 nm, which was proportional to fibroblast proliferation, was determined using an ELx800 counter (Universal Microplate Reader, Bio-Tek Instruments INC).

The complete procedure from isolation to MTT assay was executed six times (Trial A, B, C, D, E and F) while it was impossible to irradiate all the investigated number of wells with the same LED apparatus on one day. All the trials included as much control as irradiated wells, but the number of control and irradiated wells in each trial varied, depending on the number of available cells after the second subculturing. A further consequence of the available number of cells is the number of probes examined per trial. Varying from 4 probes in trial A and F to 1 probe in trial B, C, D and E.

Incubation period before proliferation analyses numbered 24 hours. To investigate if the stimulatory effect tends to occur immediately after irradiation or after a longer period of time, incubation in trial F lasted 72 hours.

An overview of the followed procedures regarding incubation time before proliferation analysis, number of analysed wells for each trial and the number of probes examined per trial is given in Table 1. As a consequence of the differences in procedures followed and because each trial started from a new cell line, the results of the five trials must be discussed separately.

Statistical analysis

Depending on the amount of groups to be compared within each trial and depending on the p-value of the Kolmogorov-Smirnov

Table 1 Fibroblast proliferation after LED and LLL irradiation

	Groups	Mean number of fibroblasts[a]
Trial A		
n = 64	Control	0.595 ± 0.056
TP = 24 h	Irradiated (LLL)	0.675 ± 0.050*
	Irradiated (LED-infrared)	0.676 ± 0.049*
	Irradiated (LED-red)	0.741 ± 0.059*
	Irradiated (LED-green)	0.775 ± 0.043*
Trial B		
n = 368	Control	0.810 ± 0.173
TP = 24 h	Irradiated (LLL)	0.881 ± 0.176*
Trial C		
n = 368	Control	0.810 ± 0.173
TP = 24 h	Irradiated (LED-infrared)	0.870 ± 0.178*
Trial D		
n = 192	Control	0.886 ± 0.084
TP = 24 h	Irradiated (LED-red)	0.917 ± 0.066*
Trial E		
n = 192	Control	0.818 ± 0.075
TP = 24 h	Irradiated (LED-green)	0.891 ± 0.068*
Trial F		
n = 64	Control	0.482 ± 0.049
TP = 72 h	Irradiated (LLL)	0.454 ± 0.065*
	Irradiated (LED-infrared)	0.487 ± 0.044
	Irradiated (LED-red)	0.446 ± 0.044*
	Irradiated (LED-green)	0.442 ± 0.035*

[a] Mean number of fibroblasts as determined by MTT analysis ± SD and significances (*$p < 0.001$) in comparison to the control group
n = number of analysed wells for each group within a trial
TP = Time Pre-analysis, incubation time before proliferation analysis was performed

test of normality, a T-test or one-way ANOVA was used for parametrical analyses and a Kruskal-Wallis or Mann-Whitney-U test was used for nonparametrical comparisons. Statistical significance for all tests was accepted at the 0.05 level. For this analysis Statistical Package for Social Sciences 10.0 (SPSS 10.0) was used.

Results

The results, presented in Table 1, show that cell counts by means of MTT assay revealed a significant ($p < 0.001$) increase in the number of cells in comparison to their respective sham-irradiated controls, for all the irradiated cultures of trial A, B, C, D, and E, except the irradiated groups in trial F.

Moreover, the results of trial A showed that the effect of the green and red LED probe was significantly ($p < 0.001$) higher than the effect of the LLL probe. With regard to the amount of proliferation the green probe yielded a significantly higher number of cells, than the red ($p < 0.001$) and the infrared probe ($p < 0.001$). Furthermore, the red probe provided a higher increase in cells ($p < 0.001$) than the infrared probe.

The infrared LED source and the LLL provided a significant ($p < 0.001$) higher number of cells than the control cultures but no statistical significant difference was recorded between both light sources.

The trials A, B, C, D, and E, regardless of the number of probes used in each trial, were analysed after 24 hours of incubation after the last irradiation. The incubation period of trial F lasted 72 hours.

The means of trial F illustrated that the effect was opposite after such a long incubation. The control cultures had significantly ($p < 0.001$) more fibroblasts than the irradiated cultures, with the exception of the LED-infrared group that showed a not significant increase of cells. Further analysis, revealed that the green probe yielded a significantly lower number of cells, than the red ($p < 0.001$) and the infrared probe ($p < 0.001$) and that the red probe provided a higher decrease ($p < 0.001$) than the infrared probe. Laser irradiation induced a significant decrease of fibroblasts in comparison to the infrared irradiated cultures ($p < 0.001$) and the control cultures ($p = 0.001$). LED irradiation with the green and the red probe revealed no statistical significant differences.

Discussion

Despite the failure of some studies [2,23] to demonstrate beneficial effects of laser and photodiode irradiation at relatively low power levels (< 500 mW) on fibroblast proliferation, this study provides experimental support for a significant increased cell proliferation. Therefore these results confirm previous studies that yielded beneficial stimulating effect [1,15,24,25]. Remarkably though is the higher increase, noted after irradiation at lower wavelengths (570 nm). Van Breughel et al. [26] observed a general decrease in absorption at longer wavelengths and concluded that several molecules in fibroblasts serve as photoacceptors, resulting in a range of absorption peaks (420, 445, 470, 560, 630, 690 and 730 nm). The wavelength of the used 'green' LED probe is the closest to one of these peaks.

Karu [5] also emphasises that the use of the appropriate wavelength, namely within the bandwidth of the absorption spectra of photoacceptor molecules, is an important factor to consider.

In this particular context, penetration depth can almost be ignored as virtually all wavelengths in the visible and infrared spectrum will pass through a monolayer cell culture [12]. The irradiance (W/cm^2) on the contrary, could have had an important influence on the outcome of this study. The higher increased proliferation by the lower wavelengths is possibly a result of the lower irradiance of these wavelengths. Lower irradiances are confirmed by other experiments to be more effective than higher irradiances [11,16,26].

The used radiant exposures reached the tissue interaction threshold of 0.01 J/cm^2 as described by Pöntinen [17], but in the scope of these results it also needs to be noticed that there is a substantial difference in radiant exposure between the LLL (1 J/cm^2), the green LED probe (0.1 J/cm^2) and the remaining LED probes (0.53 J/cm^2). Consequently, the results of especially trial

A and F must be interpreted with the necessary caution. It is possible that the determined distinction between the used light sources and the used probes is a result from the various radiant exposures applied during the treatments of the cultures.

Notwithstanding the increased proliferation revealed with MTT analysis 24 hours after the last irradiation, this study was unable to demonstrate a stimulating effect when analysis was performed 72 hours after the last irradiation. Moreover, this longer incubation period even yielded an adverse effect. Although a weakening of the photostimulating influence over time is acceptable, it can not explain a complete inversion. Especially in the knowledge that a considerable amount of authors still ascertain an effect after a longer incubation period [24,27]. In an attempt to illuminate this finding, one can suppose that the circadian response of the cells triggered by the LED and the LLL [12,28] forfeited after a prolonged period (72 hours) in the dark. The most obvious explanation is even though a decreased vitality and untimely cell death in the irradiated cell cultures as a result of reaching confluence at an earlier point of time than the control cultures. The cells of a confluent monolayer have the tendency to inhibit growth and finally die when they are not subcultured in time. No other reasonable explanations could be found for this discrepancy.

Photo-modulated stimulation of wound healing is often viewed with scepticism. The real benefits of Light Emitting Diodes, if any, can only be established by histological and clinical investigations performed under well controlled protocols. Despite these remarks, this study suggests beneficial effects of LED and LLL irradiation at the cellular level, assuming potential beneficial clinical results. LED application on cutaneous wounds of human skin may be assumed useful at the applied dosimetric parameters, but future investigation is necessary to explain the mechanisms of LED biomodulation and to provide sufficient guidelines in the use of the most effective parameters for LED treatment. Subsequently resolving the lack of scientific evidence and nullifying the controversial acknowledgements of the effect of LED can bring about a widespread acceptance for the use of LED in clinical settings.

Persons in good health rarely require treatment for wound healing, as posed by Reddy et al. [1,3] light has a possible optimal effect under conditions of impaired healing. Postponed wound healing is a time-consuming and often expensive complication. Thus, future prospects must remind to examine the therapeutic efficacy of LED on healing-resistant wounds.

Acknowledgements The authors are grateful to Prof. Deridder for supplying the laboratory as well as the material necessary for this investigation, and to Ms. François, laboratory worker, for providing the culture medium and for the technical support.

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