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ORIGINAL ARTICLE

Luciane Hiramatsu Azevedo · Fernanda de Paula Eduardo · Maria Stella Moreira · Carlos de Paula Eduardo · Márcia Martins Marques

Influence of different power densities of LILT on cultured human fibroblast growth

A pilot study

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Abstract The aim of this in vitro study was to analyze the influence of different power densities of low-intensity laser therapy (LILT) on the biomodulation of human gingival fibroblasts. The cells were cultured in nutritional deficit. Laser irradiation was carried out with a GaAlAs diode laser (λ-660 nm, 2 J/cm^2). The irradiation was done twice with 12-h interval using the punctual technique, at continuous mode and in contact. The experimental groups were: I, control, nonirradiated; II, power of 10 mW and intensity≅142.85 mW/cm^2; III, 29 mW and 428.57 mW/cm^2. Growth curves were obtained by using the trypan blue dye exclusion assay. The cell growth of the irradiated groups was significantly higher than control group ($P \leq 0.05$). The cultures of Group II presented cell growth superior to that of Group III. Based on the conditions of this study, we concluded that the power density influences cell growth in an inversely proportional manner.

Keywords Biomodulation · Cell culture · Cell proliferation · 660-nm diode laser · Low-power laser

L. H. Azevedo · F. de Paula Eduardo
Department of Stomatology, School of Dentistry,
University of Sao Paulo,
Sao Paulo, Brazil

M. S. Moreira · C. de Paula Eduardo · M. M. Marques
Department of Restorative Dentistry, School of Dentistry,
University of Sao Paulo,
Sao Paulo, Brazil

M. M. Marques (✉)
Faculdade de Odontologia, Departamento de Dentística, Av.
Prof. Lineu Prestes, Universidade de São Paulo,
2227, Cidade Universitária,
São Paulo 05508 900, Brazil
e-mail: mmmarques@usp.br
Tel.: +55-11-30917839
Fax: +55-11-30917839

Introduction

The low-intensity laser therapy (LILT) has biostimulatory effect on cell proliferation in vitro [1–3]. Additionally, LILT improves wound healing in vivo [4, 5].

The in vitro biostimulation by LILT is dependent on laser irradiation parameters such as wavelength, laser output power [1], and energy density [2, 6]. Similar parameters can have different effects on cultured cells. Parameters that effectively improve cell growth can impair protein synthesis [2, 3]. For this reason, it is crucial to know the correct combination of parameters (e.g., wavelength, power density, and energy density) to ultimately reach the desirable effects on patients.

Several parameters have been used to induce cell proliferation in vitro. Most studies have tested different energy densities [6–8]. In our experience, the energy density of 2–4 J/cm^2 has shown to be mostly effective on improving cell growth [1–3]. Power density could also be of importance in reaching cell proliferation improvement. However, the effect of changes in this parameter is still unclear because, until now, the authors have reported only the laser output power. Most studies report neither the power nor the irradiation area impairing calculation of the final power density.

A particular laser output power, when applied to different irradiation areas, culminates in different power densities. The aim of this paper is to analyze the effect of different power densities (mW/cm^2) of a low-power diode laser on the biomodulation of human gingival fibroblasts.

Materials and methods

This project has been approved by the Ethical Committee of the School of Dentistry of the University of São Paulo.

Cell culture

Human gingival fibroblasts (LMF cell line) [1] were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical, St. Lois, MO), supplemented by 10% fetal bovine serum (FBS) (Cultilab, Campinas, SP, Brazil) and 1% antibiotic–antimycotic solution (Sigma) in a humidified air–5% CO_2 atmosphere. Cells were used at the tenth to 14th passages.

Experiments

For growth analysis, control and treated cultures were plated into 35-mm diameter culture dishes (1×10^3 cells per dish). To simulate a situation of cell stress, the cultures were then grown on DMEM supplemented by only 5% of FBS. In this situation, the growth rate of fibroblasts is diminished [1, 3, 9], allowing the observation of possible differences in cell growth between treated and control cultures. The cultures were incubated in a humidified air–5% CO_2 atmosphere for 2 days before the irradiation. For the maintenance of cell viability, the culture medium was exchanged by fresh medium every other day.

Laser irradiation

Laser irradiation was carried out with a 660-nm low intensity gallium–aluminun–arsenate (GaAlAs) diode laser (model Kondortech, Brazil). The parameter settings are described at Table 1.

The LaserCheck power meter (Coherent, Santa Clara, CA, USA) verified the output of our equipment. Laser application was done in contact through the bottom of the culture dish. The laser beam did not transpose, the culture medium being applied straight to the cell monolayer instead.

To avoid influence of second-order variables, the cells of all experimental groups, including the control group, were exposed to the same environmental and stress conditions such as temperature, humidity, and light. Regarding the light, all dishes were covered by a black box during the experiment.

Experimental groups

I. Control, no laser application
II. Irradiated with 10 mW
III. Irradiated with 29 mW

Cell growth

Growth curves were carried out as described elsewhere. Briefly, the cell numbers were determined by counting the viable cells in a hemocytometer using the trypan blue dye exclusion assay. The cells were counted 2, 6, and 9 days after the irradiation. The number of viable cells harvested from each Petri dish was obtained by following the mathematical sentence:

$$\frac{UC \times D \times 10^4}{\#SQ}$$

where, UC is the unstained cell count (viable cells), D is the dilution of the cell suspension and #SQ is the number of counted squares of the hemocytometer.

Statistical analysis

Each data point corresponded to mean±standard error (SEM) of cell numbers from three dishes. The data were compared by ANOVA complemented by Tukey's test. The level of significance was 5% ($P\leq0.05$).

Results

Cell growth

The growth curves are shown in Fig. 1. The number of cells of control cultures (G I) was maintained stable. These cultures did not reach confluence. The cell numbers of control cultures were significantly smaller than those of the other two lased groups at all experimental times ($p<0.01$).

Cultures of Group II (142.85 mW/cm^2) presented a significantly higher number of cells when compared to the

Table 1 Laser parameters settings

Group	Irradiation mode	Number of irradiated points	Output power (mW)	Beam spot (cm^2)	Irradiation time (s)	Number of irradiations/12-h interval	Power density (mW/cm^2)	Energy density (J/cm^2)
II	Contact and punctual	60	10	0.07	14	2	142.85	2
III	Contact and punctual	60	29	0.07	≅4.8	2	428.57	2

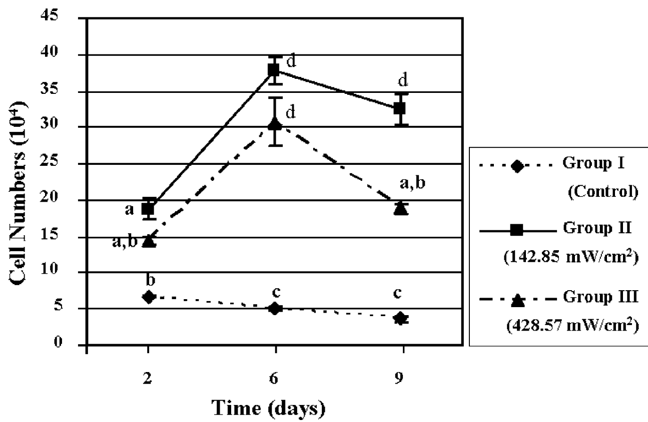

Fig. 1 Graphic representation of growth curves of control LMF cultures (*GI*), cultures irradiated with a total of 2 J/cm^2 and 142.84 mW/cm^2 (*GII*), and 428.57 mW/cm^2 (*GIII*). Different *letters* indicate statistical differences ($p<0.01$)

control cultures through the whole experiment ($p<0.01$). The cultures presented increased growth until the sixth day after irradiation. After that time, these cultures reached confluence, and the cell number was maintained ($p>0.05$). These cultures reached cell number values threefold to tenfold higher at 2 and 9 days after irradiation, respectively, than those of control cultures. At the end of the experiment (ninth day), these cultures presented cell numbers significantly higher than those of cultures of group III ($p<0.01$).

Cultures of Group III (428.57 mW/cm^2) presented significantly higher number of cells when compared to the control cultures from the sixth day after irradiation until the end of the experiment ($p<0.01$). The cultures presented increased growth until the sixth day after irradiation. After that time the cultures presented a significant decrease in cell number ($p<0.01$). These cultures did not reach confluence. These cultures reached cell number values twofold to sixfold higher at 2 and 9 days after irradiation, respectively, than those of the control cultures.

Discussion

According to the study by van Breughel and Dop Bärr [10], power density appears to be more important than total dose in wound healing. Wound healing is dependent on cell proliferation. It would then be important to test different power densities in the studies of cell proliferation in vitro.

This in vitro study demonstrated that LILT at the same energy density can influence differently the cultured human fibroblast growth in function of power density. The irradiated cultures presented growth rates significantly higher than those of control cultures. Moreover, there was an inverse relationship between power density and cell growth. Therefore, the smallest power density tested (142.85 mW/cm^2) elicited the highest cell growth. Additionally, only cultures in this group reached confluence. This could be observed at the phase microscope and was confirmed by the cell numbers that where similar at sixth and ninth days after irradiation. The nonirradiated control group showed no growth during the experimental time, and group III showed significant decrease in the cell numbers on sixth day after irradiation.

The control cultures maintained stable cell numbers. This means that they did not grow. It was expected once they were grown in nutritional deficit, and fibroblasts need at least 10% of serum supplementation in the culture medium for presenting their characteristic cell growth. On the other hand, the lased groups, although also growing in nutritional deficit, showed significant growth. It occurred because the low-power laser irradiation can stimulate stressed cells to grow [1–3]. This biostimulation for the group II (142.85 mW/cm^2) was enough to make the cultures confluent in 6 days after irradiation. It is known that fibroblasts present contact inhibition that occurs when cells are in complete contact with adjacent cells, as in a confluent culture, when the cell proliferation stops. For this reason, the number of cells after 6 days was maintained in group II.

Previous studies indicated that LILT at energy densities up to 4 J/cm^2 had stimulating effects, whereas higher energy fluences had rather inhibitory characteristics [2, 11]. For this reason, we used 2 J/cm^2 and obtained an increase in cell growth. However, it is known that LILT is dependent on the combination of laser parameters such as wavelength, power, energy, and secondarily, irradiated area, time, and number of irradiations. In this way, it is possible to obtain an energy density from different outputs of power laser, irradiation times, and irradiation areas. Trying to determine the best laser irradiation parameters for reaching improvement of cell growth, we studied the effect of power density variations using the same energy density (2 J/cm^2).

On the basis of the literature [6, 12] and on the conditions of the laser equipment used, we have chosen to work with 142.85 mW/cm^2 as the best power density and 428.57 mW/cm^{2}as the highest power density, and these powers were checked using a power meter. Using the laser tip in contact with the bottom of the culture dishes, we knew that the irradiated spot size was 0.07 cm^2, and the times of irradiations were determined to reach a power density of 2 J/cm^2. Finally, we were able to determine the power densities used (142.85 and 428.57 mW/cm^2).

The highest power density used led to the small growth rate, and the cultures were not able to maintain confluence, as group II did. Probably this result was due to some temperature change. In fact, Kert and Rose [13], showed in 1989 that above 500 mW /cm^2, there is an increase in temperature that would be able to damage cells in culture. On the other hand, we chose to work with a power density of 142.85 mW/cm^2 as the smallest parameter. By working with smaller powers, the time of irradiation to reach 2 J/cm^2 would be extremely high and the cultures would stay out of the incubator for long times, which could cause damage in the cells related to changes in temperature and culture medium pH.

It is difficult to compare our results with the observations of wound healing in the literature because most studies report only the power and wavelength of the laser

irradiation and the power density is not mentioned. However, the increase in power density has shown impairment of in vivo wound healing [12]. On the other hand, most studies report only changes in energy density, which has been well-established in the in vitro proliferation effects [14].

We believe that our results will be of clinical relevance These results would help the clinicians in the choosing the power density, and, consequently the intensity power. However, more studies must be done to elucidate the effects of different power densities on cultured human fibroblast growth.

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