Comparison of the Low Level Laser Therapy Effects on Cultured Human Gingival Fibroblasts Proliferation Using Different Irradiance and Same Fluence

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Background and Objective: The low level laser therapy (LLLT) has been used in Dentistry to improve wound healing. In order to analyse the effect of LLLT on the in vitro proliferation of gingival fibroblasts we developed a primary culture of human gingival fibroblasts.

Study Design/Materials and Methods: The cell line named LMF was grown in Dulbecco’s Modified Eagle’s medium (DME) with either 5% (nutritional deficit) or 10% fetal bovine serum (fbs). Laser irradiation was carried out with diode lasers with the following wavelengths: 670 nm (L1), 780 nm (L2), 692 nm (L3), and 786 nm (L4). The fluence was fixed in 2 J/cm². For growth analysis, control (not irradiated) and treated cultures (irradiated) were plated in 60 mm diameter culture dishes for 12 h before the irradiation.

Results: We found that cells cultured in nutritional deficit condition grown in medium supplemented by only 5% fbs presented a cell proliferation rate significantly smaller that cell grown in ideal culture conditions (10% fbs). However, when irradiated, cells in nutritional deficit presented cell growth similar or higher than that of control cells grown in ideal culture conditions. Using the same fluence, the infrared laser induced a higher cell proliferation than visible laser when the power outputs were different. However, lasers of equal power output presented similar effect on cell growth independently of their wavelengths.


Key words: cell culture; diode laser; human fibroblast; wound healing

INTRODUCTION

The low level laser therapy (LLLT) has been used in Dentistry to improve wound healing. However, most of the studies on the effects of LLLT on wound healing were undertaken in skin [1–11]. These studies showed the laser effects on wound such as increasing cellular metabolic processes, increasing the regenerative potential of the biological tissues, increasing neovascularization and formation of regenerative tissue [1–11]. However, there are a lesser number in mucous membranes [11–16]. Additionally, these former studies were generally carried out in animal [15].

More recently, in vitro studies of laser effects have been performed [17–29]. The greater advantage of this kind of study is that one can isolate a specific part of a determinate process [30,31]. The vast majority of in vivo studies were carried out either on established cell lines or on primary-cultured skin fibroblasts [27,28,32–40]. However, only a small number of studies were done with cell lines derived from human mucous membranes [11,17,18,41–43].

It is known that lasers with different wavelengths produce different effects on fibroblasts. In the literature, the studies comparing the effects of laser using visible and near-infrared wavelengths showed differences most of them indicating that the best results were obtained when visible wavelength was used [27–29,33,34,41,43,44].

Two other aspects of LLLT on fibroblasts are related to the dosage and culture conditions. Lower fluence rates present no significant effect, whereas high fluence rates present inhibitory effect. An ideal fluence to obtain response using fibroblasts in vitro is around 2 J/cm² [5,11,24,25,40,43,45–49]. Additionally, the response of the tissue in vivo to the LLLT is directly correlated to stress conditions, thus it is lacking when applied to healthy tissue [32,43,50,51].

The aim of this work was to study the effect of LLLT on fibroblasts primary cultured from human oral mucosa. We compared the effect on cell proliferation of visible lasers vs infrared lasers, keeping the fluence constant at 2 J/cm² and using different irradiance. We believe that the results of this study will be of clinical relevance helping the
clinicians in the choice of the more adequate parameters for the LLLT to be used in each clinical condition.

**MATERIALS AND METHODS**

**Cell Culture**

In order to obtain a cell line of human gingival fibroblasts we used a primary explant technique [30,52]. A fragment of human oral mucosa was obtained through a biopsy of gingival tissue. The consent of the patient was given and the Ethical Committee of the University of São Paulo approved the project. The tissue was finely chopped, rinsed, and the pieces seeded onto the culture surface in a small volume of medium. The explants were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented by 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil) and 1% antibiotic–antimycotic solution (Sigma) in a humidified air–5% CO₂ atmosphere. We observed an adherence of the explants to the culture surface followed by an outgrowth of cells. After the first passage, the cell line presenting fibroblast-like phenotype was named LMF (Fig. 1). The cell line was maintained in the same culture conditions as explants.

Looking for a situation in vitro that would simulate cell stress in vivo we grew the LMF cells in different fetal bovine serum concentrations (serum-free, 5 and 10%). We observed cell growth only when LMF cells were grown with DME medium containing 5 or 10% fetal bovine serum (Fig. 2). Then, all experiments were done in cell grown with serum concentrations higher than 5%.

**Laser Irradiation**

Laser irradiation was carried out with diode lasers with the following wavelengths: 670 nm (L1), 780 nm (L2), 692 nm (L3), and 786 nm (L4). The power output and the focal spot were fixed: 10 mW and 0.01 cm² (L1), 50 mW and 0.01 cm² (L2), 30 mW and 0.01 cm² (L3 and L4), respectively. By setting the fluence (2 J/cm²) at the handpiece, the apparatus automatically determined the time of exposure in order to obtain a homogeneous fluence applied to the full extension of the dish.

In order 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 to the light, during the experiment, all dishes were covered by black box.

**Experiments**

For growth analysis, control (non-irradiated) and treated cultures (irradiated) were plated on 60 mm diameter culture dishes (1.8 × 10⁴ cells per dish). The cultures were incubated in humidified air–5% CO₂ atmosphere for 12 h before the irradiation. Four irradiations were applied 12 h apart from each other. Three identical samples from each group were taken at random for cell counting 2, 4 and 6 days after the first irradiation. The study was done in two parts. Firstly, we compared the effect of lasers with different power outputs on cell proliferation: visible laser (L1) vs. infrared laser (L2), using same fluence and different irradiance for both. Secondly, we used lasers of equal power output: visible laser (L3) vs. infrared laser (L4), using same fluence and same irradiance for both.

**Growth Curves**

Growth curves were carried out as previously described [30,53]. Briefly, the cell number was determined by counting the viable cells in a hemocytometer using the Trypan blue dye exclusion assay. For each time three dishes of each group were counted.

**Statistical Analysis**

Each data point corresponded to mean ± standard error (SEM) of cell numbers from three dishes. The data were compared by either ANOVA complemented by Tukey's test.

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Fig. 1. Photomicrograph of LMF fibroblasts grown on DME medium supplemented by 10% fetal bovine serum (× 100).

Fig. 2. Growth curves of LMF cells growing on DME medium supplemented by different fetal, bovine serum concentrations. Note that growing is only observed in cultures treated with more than 5% fetal bovine serum. Additionally, with 5% serum concentration the cell growth was significantly smaller than that of cultures grown on ideal serum concentration (10%).
RESULTS

In order to determine the best fetal bovine serum (FBS) concentration that would reasonably decrease the LMF cell growth rate, we grew the cells in medium supplemented by different serum concentrations. The growth curves of LMF cells growing on DMEM medium supplemented by 0, 5, and 10% serum concentration is represented in Figure 2. Growing was only observed in cultures treated with more than 5% FBS. Additionally, with 5% serum concentration the cell growth was significantly smaller than that of cultures grown on ideal serum concentration (10%).

Firstly, we compared the effect of cell proliferation of lasers with different power outputs: visible laser (L1) vs. infrared laser (L2). Growth curves of LMF cells irradiated with same fluence (2 J/cm²), but different diode lasers (L1 and L2) are represented in Figure 3. The groups grown on 5% serum that were irradiated, independently of the laser used showed growth rates higher than those of LMF control cells grown on the same conditions. Cells irradiated with infrared (L2) presented cell growth significantly higher than cells irradiated with visible light (L1).

Secondly, we used lasers of equal power output: visible laser (L3) vs. infrared laser (L4). Growth curves of LMF cells irradiated with same fluence (2 J/cm²), but different diode lasers (L3 and L4) are expressed in Figure 4. All groups that were irradiated, independently of either serum concentration or kind of laser used showed growth similar or higher than that of LMF control cells grown on ideal serum concentration (10% FBS). Cells grown on DMEM medium supplemented by 10% FBS and irradiated presented growth similar to that of control cells grown on ideal conditions. LMF cells grown on medium containing 5% serum and irradiated showed growth significantly higher than that of control cells grown on the same condition. There were no differences in growth between cultures treated with the different lasers.

DISCUSSION

We developed a fibroblastic cell line from human gingival tissue. The fibroblasts, named LMF, were used for testing the effects of LLLT on in vitro cell proliferation. We found that cells cultured in nutritional deficit condition grown in medium supplemented by only 5% FBS presented a cell proliferation rate significantly smaller that cell grown in ideal culture conditions (10% FBS). However, when irradiated, the cells in nutritional deficit presented cell growth similar or higher than that of control cells grown in ideal culture condition. Using the same fluence, the infrared laser induced a higher cell proliferation than visible laser when the power outputs were different. However, lasers of equal power output presented similar effect on cell growth independently of their wavelengths.
It is known that the LLLT has its better application in tissues under stress [32,43,50,51]. Thus, our first objective was to determine the best culture conditions for the LMF fibroblasts in order to simulate in vitro a stress situation of the tissue in vivo. For this reason, we cultured the cells in medium supplemented by different fbs concentrations. Cells cultured in serum-free medium presented no growth, while cells grown in medium containing only 5% fbs presented a growth rate significantly smaller than that of cells grown in ideal serum concentration (10%). Then, for our study we used cells grown in 5% fbs (nutritional deficit), which would represent cells in stress, and cells grown in ideal culture condition (10% fbs), representing normal cells.

Firstly, we compared the effect of irradiation using the same fluence (2 J/cm²) but obtained from lasers of different wavelengths and different power outputs. This fluence was chosen because it has been reported as the best fluence for stimulating fibroblast growth in vitro and in vivo [5,24,25,40,43,45]. Cells in nutritional deficit and irradiated presented higher growth rates than that of control group (non-irradiated). It ratifies the fact that the LLLT intensifies the cellular functions when it is in a stress condition [43,50,51]. This increase could be induced by growth autocrine factors, which are synthesised by the cell itself [56].

Moreover, cells treated with infrared radiation presented growth rates higher than those of cells treated with visible light. This result is not in accordance with the literature where the visible light is pointed as more active in cell proliferation induction [27,41,43,44]. The power output of the visible light used (10 mW) was five times lower than that of infrared (50 mW). Therefore, the period of time of laser exposure for reaching the same fluence was more than five times higher for visible light (200 s) than for infrared light (40 s). Hence, we would suggest that shorter radiation exposure is better in order to increase cell growth in vitro. However, at this point this suggestion may not be valid as different light and wavelengths are being discussed.

Based on the results with lasers of different power outputs, we decided to extend our study using radiation of same fluence and same radiance. We used fixed time of laser exposure and the only variable would be the laser wavelength. In this condition we did not observe difference in cell proliferation between cultures treated with visible light and cultures treated with infrared laser. Thus, with equal exposure time and equal fluence there were similar response to the laser therapy. This finding supports our suggestion that differences in the period of laser exposure can cause differences in cell growth.

Confirming reports in the literature [41,43,50,58,59], cells grown in ideal culture conditions (10% fbs) do not respond to the LLLT as cells in nutritional deficit. There was no significant growth improvement after the irradiation. However, the overall arrangement of cells grown in ideal conditions and irradiated was different to that of non-irradiated cells. When irradiated, fibroblasts assume a polarised pattern, forming bundles in different directions. Some authors [8,57] have already reported this finding. If it also happens in vivo it could explain both the better aesthetic healing and the less wound contracture obtained after surgeries using laser than conventional surgeries.

We have shown interesting effects of LLLT on proliferation of fibroblasts cultured from oral mucosa. The cell growth of stressed cells was similar to that of control cells grown in ideal conditions. Moreover, the best results were obtained with shorter radiation exposure, when using infrared light. We believe that our results will be of clinical relevance helping the clinicians in the choice of the more adequate parameters for the LLLT to be used in each clinical conditions. However, more studies must be done to elucidate the effects of LLLT in oral mucous and other human tissues, not only in cell growth but also in the morphological and physiological aspects of cells.

**CONCLUSIONS**

In human gingival fibroblasts we concluded the following:

1. The primary culture with fibroblasts can be obtained from gingival normal human explants, resulting in a stable cell line.
2. The use of medium with 5% fbs showed to be an ideal model to analyse the effect of laser therapy, as the cellular growth decreased proportionally to the fbs concentration.
3. The administrated fluence of 2 J/cm² stimulates the human gingival fibroblasts when they are in a nutritional deficit (stress condition).
4. Increased proliferation rate was the effect of LLLT on fibroblasts in vitro in cells grown in nutritional deficit condition (5% fbs), when using a fluence of 2 J/cm². This effect on fibroblasts grown under ideal growth conditions (10% fbs) was low and no significant.
5. The infrared laser (780 nm) induced significantly higher cell growth in cells grown in nutritional deficit than the visible laser (670 nm). However, lasers of equal power outputs (red—692 nm and...
REFERENCES


