Determining optimal dose of laser therapy for attachment and proliferation of human oral fibroblasts cultured on titanium implant material

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Abstract: The purpose of this study was to investigate the influence of single or multiple doses of low-level laser therapy (LLLT) on attachment and proliferation of human gingival fibroblasts in a standardized, reproducible in vitro model. Titanium discs were randomly allotted to one of three groups: group I served as a control, group II was exposed to a single laser dose of 3 J/cm², and the three subgroups in group III were exposed to laser doses of 0.75, 1.5, and 3 J/cm². To examine the possible thermal effects of laser exposure on the cell culture, the temperature in the Petri dish was measured for every dose used, before and during irradiation. For attachment assays, groups II and III were exposed to laser irradiation and then seeded onto titanium discs. In group III, the exposures were repeated after 3 and 6 h. Cells were cultured for 6 and 24 h and stained with Hoechst and Propidium. Attached cells were counted under a light microscope. To investigate the effect of LLLT on cell proliferation after 48 h, 72 h, and 7 days, cells were cultured on titanium discs for 24 h and then exposed to laser irradiation for 1 day and 3 consecutive days, respectively. Cell proliferation was determined by counting cells under the microscope and by a cell proliferation enzyme-linked immunosorbent assay system. No increase of temperature of the cell cultures occurred before or during laser exposure at any of the doses tested. Both single and multiple doses of LLLT significantly enhanced cellular attachment (p < 0.05). The proliferation assays showed higher cell proliferation (p < 0.05) in group III at doses of 1.5 and 3 J/cm² after 72 h and 7 days, with agreement between staining and enzyme-linked immunosorbent assay. It is concluded that, in this cellular model, the attachment and proliferation of human gingival fibroblasts are enhanced by LLLT in a dose-dependent manner. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 73A: 55–62, 2005

Key words: low-level laser; titanium implant; human gingival fibroblasts; attachment; proliferation

INTRODUCTION

Clinical and experimental studies have shown that the long-term stability of dental implants requires sustained osseointegration and a properly functioning barrier at the transmucosal passage of the implant. Although many problems associated with osseous healing of dental implants have been addressed, the sealing of the implant surface through soft tissue remains to be a crucial determinant of the therapeutic outcome.1,2 Recently, soft tissue stability has attracted increasing attention in implant research. In this context, it is claimed that laser therapy offers major benefits in enhancing regeneration of the oral mucosa and establishment of an intact functional barrier at the transmucosal passage of the abutment.

Low-level laser therapy (LLLT) has been used for more than 20 years in clinical practice and is known to modulate various biological processes.3–6 LLLT is a nonthermal modality: usually the temperature changes associated with treatment are negligible.7 A number of different laser light sources, including helium-neon, ruby, and gallium arsenide have been used to deliver LLLT in different treatments and schedules. However, the use of LLLT is still not widely accepted by the medical community.8

Several in vitro studies on cultured human fibro-
blasts have demonstrated a variety of LLLT effects, including increased cell numbers, increased DNA synthesis, and increased collagen production. Another interesting finding is that laser irradiation also stimulates cell attachment to a plastic substrate. The effects of LLLT on cell culture have been studied most extensively by Karu. It was also reported that the stimulation of cellular proliferation is possible only at certain doses of laser irradiation.

Despite extensive research to validate many claims, there are few scientifically controlled, randomized, double-blind clinical trials. The importance of choosing the adequate level of energy is emphasized by many authors but the recommended energy for obtaining optimal “biostimulation” varies markedly.

In the present study, human gingival fibroblasts were cultured on titanium implant material to investigate the influence of single or multiple doses of LLLT on cellular attachment and proliferation in a standardized, reproducible in vitro model.

**MATERIALS AND METHODS**

**Implants**

Test implant discs measuring 10 mm in diameter and 1 mm in thickness were made from sheets of commercially pure, grade 4 titanium. The surfaces of all the discs were standardized by polishing with Chem cloth and colloidal silica paste with a grain size of 0.04 μm (OP-S suspension) according to Struers Metalog Guide. The implants were mirror-polished for 1 h.

Before use, the discs were treated with trichloroethylene in an ultrasonic bath for 15 min, rinsed with 100% ethanol, and then treated with 100% ethanol in an ultrasonic bath for 3 min. The samples were then autoclaved.

**Surface characterization**

The surface topography of five titanium samples was characterized qualitatively and quantitatively using a scanning electron microscope (Philips XL30 ESEM, Holland) and a confocal laser scanning microscope (Leica TCS 4D, Germany). For numerical characterization, the parameters evaluated were the average height deviation value (S₀), the maximum peak-to-valley roughness (Sₚ), and the developed surface area ratio (Sdr). Each sample was measured at three sites. The field area measured was 1000 × 1000 μm. Before use, the titanium surfaces were also examined using energy-dispersive X-ray microanalysis to look for metal contaminants.

**Laser treatment**

A photon-plus, gallium-aluminum-arsenide (GaAlAs) diode laser device (Rønvig Dental AS, Denmark) was used in this study. This system operates in the near-infrared spectrum at a continuous wavelength of 830 nm and a power output of 84 mW. To ensure uniform exposure of the whole culture dish (35-mm-diameter Petri dish, NUNC), the beam was specially expanded. The distance from the probe to the cell layer was 9 cm. The culture dish was placed in a standardized position in a milled hollow in anodized metal. Each titanium disc was placed in the center of the culture dish and laser irradiated. The laser doses used in this experiment have been reported to stimulate fibroblast numbers in vitro. The control dishes were maintained under a laminar flow hood for a period of time corresponding to those of experimental groups, but were not irradiated.

**Exposure regimen**

The titanium discs were randomly separated into three groups: group I (untreated control) served as a reference for in vitro growth of human gingival fibroblasts (HGF). Group II was exposed to a single dose of 3 J/cm² of LLLT corresponding to an exposure time of 360 s and the three subgroups in group III were exposed to multiple doses of 0.75, 1.5, and 3 J/cm² corresponding to exposure times of 90, 180, and 360 s, respectively.

For the attachment assay, group II was exposed to a single laser dose of 3 J/cm², at 0 h and the cells were allowed to incubate for 6 or 24 h. The samples in group III were irradiated at 0 and 3 h and allowed to incubate for 6 h, or irradiated at 0, 3, and 6 h and allowed to incubate for 24 h [Fig. 1(a)].

For the proliferation assays, after 24 h culture, the samples in group II were irradiated with a single dose of 3 J/cm² at
24 h whereas the samples in group III were irradiated daily for 3 consecutive days with three different doses of 0.75, 1.5, and 3 J/cm² [Fig. 1(b)].

Cell culture

HGF were grown from biopsies obtained during oral surgery, cultured, and maintained in plastic culture flasks (NUNC, Denmark) according to the technique described by Liu et al. The cells were cultured in Dulbecco’s modified Eagle medium (Sigma), supplemented with 5% fetal calf serum and penicillin (50 IU/mL)/streptomycin (50 μg/mL) solution. The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells for the experiments were harvested between the fourth and eighth passages. Exposure to ambient light was avoided as much as possible. The study protocol was approved by the Ethics Committee at Karolinska Institutet, Stockholm.

Temperature measurement

To exclude the thermal effect of laser on cell culture, for every dose used, the temperature in the Petri dish was measured before and during laser exposure. For this purpose, a commercial infrared temperature probe (80T-IR; FLUKE, Germany) was used. The temperature probe is sensitive to within ±0.1°C.

Cell attachment

Fibroblasts were released from the culture dishes using 0.025 trypsin in phosphate-buffered saline (PBS) containing 0.02% ethylenediaminetetraacetic acid. The cells were washed three times in PBS; centrifuged, resuspended, and then plated in three 35-mm-diameter Petri dishes. After irradiation, the cells were resuspended in growth medium and plated at a density of 2 × 10⁴ cells/cm² on 60 titanium discs which were placed on the bottom of 24-multwell dishes. Cellular attachment was observed after 6 h and 24 h. Unattached cells were then pipetted from the surfaces, and washed twice with PBS to collect any unattached cells remaining on the surfaces. Attached HGF were stained with (0.5 mg/mL) Hoechst (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 15 min followed by (0.5 mg/mL) Propidium iodide (Sigma-Aldrich Chemie GmbH) for another 15 min. The samples were then allowed to dry in the dark.

After drying, the titanium discs were mounted to slides. Ten randomly selected fields from each titanium disc were captured using a microscope (Eclipse E600; Nikon, Japan) with a (×10) magnification objective. The first two points were set at a central position. The following two points were then taken at the right and left of the central positions with equal distance. Three points at the top and three at the bottom of the surfaces were also analyzed. Thirty titanium discs were observed for each time point, giving a total of 600 micrographs. Images were imported into an image analyzer (Soft Imaging System GmbH, Germany). The number of attached cells was then counted and the mean of cell number for each titanium disc was calculated for statistical analysis.

Cell proliferation

Proliferation assays (staining)

Ninety titanium discs were placed on the bottom of 24-multwell dishes and plated with HGF at a density of 2 × 10⁴ cells/cm² for the 48- and 72-h exposures and a density of 5 × 10³ cells/cm² for the 7-day exposure. After 24 h culture, the experimental groups were irradiated as described above. Cell proliferation was observed after 48 h, 72 h, and 7 days, respectively, with two changes of medium. Thirty titanium discs were observed from each time point. At the termination of each experiment, the discs were gently washed twice with PBS. Attached HGF were stained and counted using a microscope as described above.

Proliferation assays (pyrimidine incorporation)

The cellular proliferation rate was also estimated using a Biotrak cell proliferation enzyme-linked immunosorbent assay (ELISA) system, version 2 from Amersham Pharmacia Biotech (Uppsala, Sweden). This technique is based on the incorporation of the pyrimidine analog BrdU into the DNA of proliferating cells. Ninety titanium discs were placed on the bottom of 24-multwell dishes and plated with HGF at a density of 2 × 10⁴ cells/cm² for the 48- and 72-h time points and a density of 5 × 10³ cells/cm² for the 7-day time point. Thirty titanium discs were observed for each time point. After 24 h of culture, the experimental groups were exposed to laser irradiation as described above. BrdU was added to the cells 24 h before the proliferation assay, after changing media. At harvest, the culture medium was removed, the cells were fixed, and the DNA was denatured by addition of a fixative. The peroxidase-labeled anti-BrdU binds to the BrdU incorporated in newly synthesized, cellular DNA. The immune complexes were detected by the subsequent substrate reaction, and the resultant color was read at 450 nm in a microtiter plate spectrophotometer. The absorbance values correlated directly to the amount of DNA synthesis and thereby to the number of proliferating cells in culture.

Cell viability

Cell viability

At the termination of the experiment, viability was assessed by applying the staining technique described above. The uptake of propidium iodide indicates damage to the cell membrane: necrotic cells thus identified were excluded from the cell count.

Statistical analysis

Analyses for determination of differences between groups were accomplished using a one-way analysis of variance with a multiple comparison test (Bonferroni). The data pre-
sented are from one of two identical experiments performed in parallel using biopsies from two different donors. Moreover, the experiments were repeated three times.

**RESULTS**

**Surface analyses**

Scanning electron microscopy examination confirmed that the surfaces of the implant discs were smooth, with no irregularities. Surface analysis by energy-dispersive X-ray showed that all surfaces were clean and consisted of pure titanium. Quantitative analysis by Leica TCS 4D confocal laser microscopy yielded a mean deflection value ($S_a$) of 0.477 μm, a maximum peak-to-valley roughness ($S_t$) of 14.25 μm, and a developed surface area ratio ($S_{dr}$) of 1.01.

**Temperature measurement**

The temperature in the Petri dishes before and during laser exposure showed no increase of temperature of the cell cultures within the limits of our equipment at any of the doses tested.

**Cell attachment**

The number of attached cells on the implant surfaces of the irradiated groups with single (3 J/cm$^2$) and multiple (only 1.5 and 3 J/cm$^2$) exposures was significantly ($p < 0.05$) larger than that on the control after incubation for 6 and 24 h (Fig. 2). However, there were no significant differences between single and multiple doses. Exposing the cells to 0.75 J/cm$^2$ did not enhance cellular attachment compared with the control.

**Cell proliferation**

Proliferation assays (staining)

Figure 3 summarizes the proliferation of human fibroblasts cultured on titanium after 48 h, 72 h, and 7 days. The analyses showed that cell proliferation was significantly higher on the samples exposed to multiple doses of 1.5 and 3 J/cm$^2$ than the control group after 72 h and 7 days ($p < 0.05$). There were no significant differences between the control group and the samples exposed to a single dose of 3 J/cm$^2$ (group II).

**Cell viability**

Cell viability, assessed at the end of the experiments, showed no differences between experimental and control groups, exceeding 90% in all cases.

**DISCUSSION**

The quality of the seal between an implant and oral mucosa is a determinant of successful implant ther-
apy. In the search to promote the implant–soft tissue interaction, the effect of laser therapy on gingival fibroblasts is an important field of investigation. The

Figure 3. Cell proliferation of HGF on titanium surfaces (staining) was measured after 48 h (a), 72 h (b), and 7 days (c). Each bar represents the mean of cell numbers ± SD of multiple doses group III (0.75, 1.5, 3 J/cm²), single dose group II (3 J/cm²), and control group I (n = 5 each dose) in one of two identical experiments. The differences between the irradiated (multiple doses 1.5 and 3 J/cm²) and control groups were significant after 72 h and 7 days (*p < 0.05).

Figure 4. Cell proliferation of HGF on titanium surfaces (ELISA) was measured after 48 h (a), 72 h (b), and 7 days (c). Each bar represents the mean of pyrimidine incorporation ± SD of multiple doses group III (0.75, 1.5, 3 J/cm²), single dose group II (3 J/cm²), and control group (n = 5 each dose) in one of two identical experiments. The differences between the irradiated (multiple doses 1.5 and 3 J/cm²) and control groups were significant after 72 h and 7 days (*p < 0.05).
ability of LLLT to promote a favorable soft tissue healing response has been described in several experimental and a few clinical studies. Positive findings from animal studies encouraged us to conduct the present study using a simple and reliable in vitro model. The purpose was to investigate in vitro the influence of single or multiple GaAlAs laser exposures on attachment and proliferation of HGF, as a step toward determining the optimal laser dose for enhancing implant–tissue interaction.

Attachment, a vital cell function essential for maintaining the integrity of tissues, involves protein synthesis and cell division. Because there is neither cementum nor fiber insertion on the surface of an implant, lack of attachment around implants may lead to peri-implantitis and eventual failure. Boulton and Marshall demonstrated that laser-irradiated cultures exhibited a significant increase in the number of human skin fibroblasts grown on plastic substrate in comparison with their respective nonirradiated controls after 24 and 48 h. In another study, Bosatra et al. demonstrated that laser irradiation with 2 J/cm² could produce a remarkable accumulation of fibrillar material in the outer part of the fibroblast cytoplasm. These findings are also supported by Karu et al. who evaluated the adhesion of HeLa cells to glass specimens after laser irradiation. They showed increased cell–cell and cell–glass adhesion after laser irradiation. In the present study, the number of attached HGF on the titanium surfaces of the irradiated groups with a single dose of 3 J/cm² and multiple doses of 1.5 and 3 J/cm² were significantly higher than that on the control after incubation for 6 and 24 h. However, our findings showed that compared with a single dose, multiple exposures did not enhance initial cellular attachment.

The proliferation assays after culturing cells on titanium specimens for 48 h determined by both cell counting and ELISA showed that the irradiated cells had slightly higher proliferation than the nonirradiated cells, but the difference was not statistically significant. Interestingly, cell proliferation was significantly stimulated by multiple doses of 1.5 and 3 J/cm² as the experiment was continued to 72 h and 7 days. These findings are in agreement with the results reported by Almeida-Lopes et al. However, the proliferation assay suggests that the single irradiation did not attain an effect equivalent to the multiple doses. Kreisler et al. clearly revealed considerably higher proliferation activity 24 h after laser irradiation, decreasing in an energy-dependent manner over 48 and 72 h, indicating that repeated laser applications are necessary to achieve a positive effect on proliferation. On this basis, in the present study, HGF grown on the titanium specimens were lazed for 3 consecutive days (24, 48, and 72 h).

The results are consistent with those of earlier reports that multiple exposures are more effective than a single dose for acceleration of bone formation or fibroblast growth, although conflicting results have been reported. Low-level laser within the visible and invisible wavelengths as well as continuous and pulsed lasers have been used. Because of the numerous variable parameters such as the wavelength of the laser system, power output, time of irradiation, and distance of the fiber from the specimen, it is very difficult to compare results from different investigations. This may explain the conflicting results on the effect of LLLT on wound and osseous healing as well as cell culture.

Several other studies have reported satisfactory tissue healing rates at helium-neon laser exposure levels between 1 and 4 J/cm². Laser-enhanced biostimulation has been reported to induce intracellular metabolic changes, resulting in faster cell division, rapid matrix production (increased collagen, myofibrolasts, etc.) and cell movement. However, higher laser energy doses were reported to inhibit cell proliferation.

In previous studies, cells cultured under conditions of nutritional deficiency were shown to be more susceptible to laser irradiation. For this reason, we used cells grown in a low serum concentration which would represent cells under stress. It has been reported that under conditions of stress, laser irradiation has an increased effect on DNA synthesis and proliferation.

The temperature measurements confirmed that the laser irradiation did not produce any significant temperature increase in the Petri dishes. This indicates that, in this cellular model, a thermal component in the laser effect seems unlikely. The result seems to be in agreement with studies by Boulton and Marshall and Quickenden and Daniels in which it was reported that the temperature remained unchanged in fibroblast suspension during irradiation. Schneede et al. using a microthermo probe in a monolayer of cells, reported a temperature increase of less than 0.065°C at a laser irradiation of 40 mW/cm². On the contrary, the temperature of tissues treated with laser irradiation has changed as much as 0.3°–0.62°C or 0.1°C. Furthermore, a study by Mochizuki-Oda et al. using laser irradiation with 830- and 652-nm wavelengths on rat brain showed an increase of 4.4°–4.7°C for both wavelengths. Stimulation of cellular adenosine triphosphate production was, however, observed only after irradiation with 830 nm. The results suggest that the increase in tissue adenosine triphosphate content did not result from the thermal effect, but from a specific effect of the laser operated at the 830-nm wavelength.

The parameters frequently used for evaluating the wound healing in animal models may not be relevant for describing the clinical settings. This may be attrib-
ulated to the differences in soft and hard tissue anatomy and physiology. Major interspecies differences in hormonal responsiveness were reported, even between species as closely; related as the rat and the mouse. Furthermore, the mechanisms underlying the process of wound healing in response to laser treatment involve a sequence of events that includes cell adhesion, migration, growth, and differentiation, as well as secretion of extracellular matrix. In vitro techniques provide unique potential for analyzing and understanding these sequences, although the optimal dose cannot be assessed by these approaches. Therefore, combining the results from our previous animal studies and a direct comparison of cellular responses to LLLT in the present study may provide information that would aid the selection of an optimal dose of LLLT, if one could take in consideration the reflection and the scattering of the tissues.

**CONCLUSION**

In the present in vitro study, the attachment and proliferation of HGF cultured on titanium implant material were enhanced by LLLT, with no differences in initial cellular attachment between single and multiple doses. The proliferation assays suggest that the single dose did not achieve effects comparable to multiple doses. Exposing the cells to 0.75 J/cm² did not promote cellular attachment and proliferation compared with nonirradiated cells. However, these findings do not answer the frequent questions by both physicians and laser manufacturers about optimal laser light parameters and whether these parameters would also be optimal under in vivo conditions. Further studies are needed to clarify these issues.

**References**

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