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Effect of laser therapy on attachment, proliferation and differentiation of human osteoblast-like cells cultured on titanium implant material

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Abstract

The aim of this in vitro study was to investigate the effect of low-level laser therapy (LLLT) on the attachment, proliferation, differentiation and production of transforming growth factor-ß$_1$ (TGF-β_1) by human osteoblast-like cells (HOB). Cells derived from human mandibular bone were exposed to GaAlAs diode laser at dosages of 1.5 or 3 J/cm^2 and then seeded onto titanium discs. Non-irradiated cultures served as controls. After 1, 3 and 24 h, cells were stained and the attached cells were counted under a light microscope. In order to investigate the effect of LLLT on cell proliferation after 48, 72 and 96 h, cells were cultured on titanium specimens for 24 h and then exposed to laser irradiation for three consecutive days. Specific alkaline phosphatase activity and the ability of the cells to synthesize osteocalcin after 10 days were investigated using p-nitrophenylphosphate as a substrate and the ELSA-OST-NAT immunoradiometric kit, respectively. Cellular production of TGF-β_1 was measured by an enzyme-linked immunosorbent assay (ELISA), using commercially available kits. LLLT significantly enhanced cellular attachment ($P<0.05$). Greater cell proliferation in the irradiated groups was observed first after 96 h. Osteocalcin synthesis and TGF-β_1 production were significantly greater ($P<0.05$) on the samples exposed to 3 J/cm^2. However, alkaline phosphatase activity did not differ significantly among the three groups. These results showed that in response to LLLT, HOB cultured on titanium implant material had a tendency towards increased cellular attachment, proliferation, differentiation and production of TGF-β_1, indicating that in vitro LLLT can modulate the activity of cells and tissues surrounding implant material.

Keywords: Low level laser therapy; Titaninum implant; Human osteoblasts; Cell attachment; Differentiation; Proliferation; TGF-β_1

1. Introduction

The effect of low level laser therapy (LLLT) on bone regeneration has become a focus of recent research. LLLT is based on biostimulation of the tissues with monochromatic light. Various biostimulatory effects have been reported on wound healing [1,2] and collagen synthesis [3,4] in vivo and in vitro. With respect to bone, LLLT has been shown to modulate inflammation [5], accelerate cell proliferation [6] and enhance healing [7–10]. Ozawa and co-workers reported that LLLT resulted in significant increases in cellular proliferation, bone nodule formation and alkaline phosphatase (ALP) activity [11]. Our recent experimental animal studies showed that LLLT enhances the functional attachment of titanium implants to bone and promotes bone healing and mineralization [7,12].

The successful outcome of endosseous implant treatment is dependent on the wound-healing process and on the potential of osteogenic cells to induce new bone formation around the implant.

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Bone formation at the implant–bone interface is a complex physiological process, regulated by systemic hormones and local factors produced by the skeletal cells [13]. It involves sequential events such as cellular attachment, proliferation, differentiation and deposition of bone matrix.

Transforming growth factor β_1 (TGF-β_1) has potent effects on wound-healing and regulation of bone formation and resorption. TGF-β_1 stimulates the replication of precursor cells of the osteoblast lineage and has a direct stimulatory effect on bone–collagen synthesis [14]. Exposing the cells at the implant–tissue interface to laser treatment may influence their endogenous expression of TGF-β_1, thereby modulating the healing process and enhancing the quality of bone formation. Therefore, optimal laser therapy should promote the production and release of local factors at levels that enhance bone formation.

A recent in vitro study [15] showed that LLLT enhances attachment and proliferation of human gingival fibroblasts on titanium implant material. However, of great clinical relevance for bone regeneration around the implant is the response of the osteoblasts, the bone-forming cells. When in contact with implant surfaces, osteoblast-like cells behave differently from oral fibroblasts [16,17]. Little is known about the effect of LLLT on the attachment and proliferation of osteoblasts on titanium implant material.

The purpose of the present study was therefore to investigate the response to LLLT, using a GaAlAs diode laser, of osteoblast-like cells derived from human mandibular alveolar bone, cultured on titanium implant material, with special reference to initial attachment, proliferation, differentiation and production of TGF-β_1.

2. Materials and methods

2.1. Implants

Test implant discs measuring 10 mm in diameter and 1 mm in thickness were made from sheets of commercially pure titanium grade 4. The surfaces of all titanium 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 mirrorpolished for 1 h.

Prior to use, the discs were treated with trichlorethylene in an ultrasonic bath for 15 min, rinsed with 100% ethanol and then treated with 100% ethanol in an ultrasonic bath for 3×10 min. The samples were thereafter autoclaved.

2.2. Surface characterization

The surface topography of five titanium samples was characterized qualitatively and quantitatively using a scanning electron microscope (SEM, 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 (S_a), the maximum peak-to-valley roughness (S_t) and the developed surface area ratio (S_{dr}). Each sample was measured at three sites. The field area measured was 1000×1000 μm. Before use, the titanium surfaces were also examined by energy-dispersive X-ray microanalysis (EDX) to look for metal contaminants.

2.3. 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 anodised metal. Each titanium disc was placed in the centre of the culture dish and laser irradiated at dosages of 1.5 or 3 J/cm^2. 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.

2.4. Cell culture

Human mandibular bone specimens without any clinical or radiographic evidence of pathology were obtained from several patients undergoing oral surgery at the Department of Maxillofacial Surgery, Karolinska Hospital, Stockholm, Sweden. The specimens were taken from the molar region, harvested and maintained by a modification of the method described by Beresford et al. [18] as reported elsewhere [19,20]. After culture of the bone fragments for 3–4 weeks in a humidified atmosphere of 95% air, cells that had migrated from the bone fragments were harvested and characterized using three different assays [17,20]. In brief, these cells were stained for alkaline phosphatase activity (ALP) and their responsiveness to 1.25-$(OH)_2D_3$ and ability to synthesize osteocalcin were investigated in serum-free culture medium. The specific activity of ALP was also assayed at the basal level as well as in response to 1.25-dihydroxyvitamin, using p-nitrophenylphosphate as the substrate (Jassen Chimica, Geel, Belgium). Although most of the cells were ALP positive, only those

expressing high levels of 1.25-$(OH)_2D_3$-responsive alkaline phosphatase (ALP) activity were selected for the study. These cells have the capacity to synthesize osteocalcin, which is a characteristic of bone-forming cells (data not shown).

The culture medium was the minimum essential medium, alpha modification (α-MEM; Gibco, Grand Island, NY, USA), supplemented with penicillin/streptomycin solution and L-glutamine. During the experiments 10% fetal calf serum (FCS) was added to the medium. Exposure to ambient light was avoided as much as possible. The study protocol was approved by the Ethics Committee at Karolinska Institute, Stockholm. In all experiments, cells were used at first passage.

2.5. Cellular attachment

Osteoblast-like cells were released from the culture dishes using 0.025 trypsin in PBS containing 0.02% EDTA. The cells were washed three times in PBS, centrifuged, resuspended, and then plated in three 35 mm diameter Petri dishes. Two dishes were subjected to laser exposure with 1.5 and 3 J/cm^2, respectively, the third served as a control. Following irradiation, the cells were resuspended in growth medium and plated at a density of 2×10^4 cells/cm^2 on forty-five titanium discs placed on the bottom of 24-multiwell dishes. Thereafter, the titanium discs were separated into three groups: Group I (untreated control) served as a reference for in vitro growth of HOB and Groups II and III received laser doses of 1.5 and 3 J/cm^2, respectively. Cell attachment was observed after 1, 3 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 HOB 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, Steinheim, Germany) for another 15 min. The samples were then allowed to dry in the dark [21].

The number of attached cells was counted in each of 10 randomly selected fields from each titanium disc using a microscope (Nikon, Eclipse E600, Japan) connected to an image analyser (Soft Imaging System GmbH, Germany). All measurements were calculated using a (x10) magnification objective. Five samples from each group were used in the experiment, giving a total of 450 micrographs.

2.6. Cellular proliferation

Forty-five titanium discs were placed on the bottom of 24-multiwell dishes and plated with HOB at a density of 2×10^4 cells/cm^2. The titanium discs were randomly allotted to one of three groups as described above. After 24 h, the experimental groups were exposed to laser irradiation using a GaAlAs diode laser for three consecutive days, at dosages of 1.5 or 3 J/cm^2. Cell proliferation was observed after 48, 72 and 96 h. The titanium discs were then gently washed twice with PBS. Attached HOB were stained and counted using a microscope as described above.

2.7. 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.

2.8. Cell differentiation

2.8.1. Alkaline phosphatase activity

Eighteen titanium discs were placed on the bottom of 24-multiwell dishes and plated with HOB at a density of 2×10^4 cells/cm^2. The titanium discs were randomly allotted to one of three groups as described above. After 24 h culture, the experimental groups were exposed to laser irradiation using a GaAlAs diode laser for three consecutive days, at dosages of 1.5 or 3 J/cm^2. Culture media were collected and frozen after 10 days. 40 μl of each sample and standard were pipetted into a 96-well plate and *p*-nitrophenyl phosphate substrate was added. The samples were incubated at room temperature for 30 min in the dark. The reaction was stopped with 50 μl 3 N NaOH, and the absorbance was read at 405 nm using a microplate reader. The Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA) protein assay was used to measure the concentration of total protein in the same culture medium collected. 10 μl of each sample and standard were pipetted into a 96-well plate and 200 μl of diluted dye reagent was added to each well. The samples were incubated at room temperature for at least 5 min. The absorbance was read at 595 nm.

2.8.2. Osteocalcin production

Cells were seeded onto six specimens of each group at a density of 2×10^4 cells/cm^2 and incubated until the cells reached confluence. At confluence (after 10 days), the specimens were moved to a new culture plate and the medium was replaced with serum-free medium containing 0.1% bovine serum albumin (BSA) supplemented with 10^{-8} M 1.25$(OH)_2D_3$. The cultures were harvested 96 h later and the conditioned medium was collected and frozen at 20 °C until assayed. Osteocalcin production by the cultures was determined using an ELSA-OST-NAT immunoradiometric kit [22,23] and a gamma counter.

2.9. Transforming growth factor β_1

Eighteen titanium discs were placed on the bottom of 24-multiwell dishes and plated with HOB at a density of 2×10^4 cells/cm^2. The titanium discs were randomly allotted to one of three groups as described above. After 24 h, the experimental groups were exposed to laser irradiation using a GaAlAs diode laser for three consecutive days, at dosages of 1.5 or 3 J/cm^2. The level of total TGF-β_1 in the conditioned media was assessed using a commercially available ELISA kit (Amersham Pharmacia Biotech, Sweden) specific for human TGF-β_1. Immediately prior to the assay, the media were acidified by the addition of 1 M HCl for 10 min, followed by neutralization with 1 M NaOH. The treated samples (conditioned media) were placed in 96-well plates coated with monoclonal antibody to TGF-β_1 for 1 h. After the unbound proteins were removed, the wells were incubated with a polyclonal antibody against TGF-β_1 (detection antibody) for 1 h, washed, incubated with the biotinylated second antibody (anti-rabbit Ig, conjugated to biotin) for another 1 h, washed, and then incubated with a horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody for 30 min. This was followed by a wash and color development. Color development was stopped by the addition of 0.19 M sulfuric acid. Absorbance at 450 nm was measured with a microplate reader and sample concentrations were determined by comparing the absorbance value to a known standard curve.

2.10. Statistical analysis

Statistical analysis was performed using SPSS 11 for Windows 98. Descriptive statistics were used to describe mean and standard deviations. The significance of difference between means of the irradiated and control groups in HOB cultured on titanium was tested by independent *t*-test. The level of significance was set at ($P<0.05$). The data presented are from one of two identical experiments performed in parallel using biopsies from two different donors. Moreover, the experiments were repeated three times.

3. Results

3.1. Surface analyses

SEM examination confirmed that the surfaces of the implant discs were smooth, with no irregularities. Surface analysis by EDX showed that all surfaces were clean and consisted of titanium only. Quantitative analysis by Leica TCS 4D confocal laser microscopy yielded a mean deflection value of (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 (Fig. 1).

3.2. Cellular attachment

As shown in Fig. 2, after incubation for 1, 3 and 24 h, the number of cells initially attaching to the implant surfaces was significantly greater in the irradiated groups than in the control group ($P<0.05$). However, there were no significant differences between the two irradiated groups.

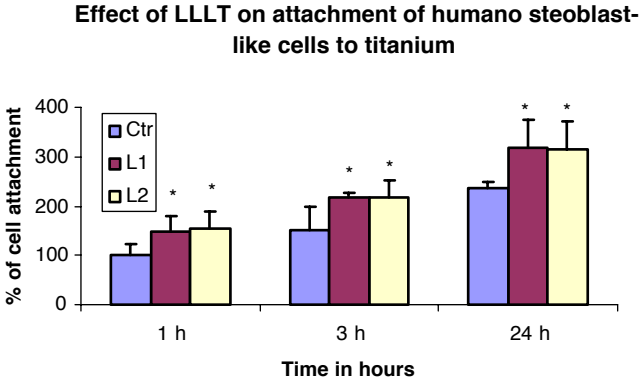

Fig. 2. Initial attachment of human osteoblasts to titanium surfaces. The percentage of attached osteoblasts on all specimens was measured after 1, 3 and 24 h of culture. Each bar represents the mean of percentage of attached osteoblasts ±SD of irradiated ($L1=1.5$ J/cm^2, $L2=3$ J/cm^2) and control groups ($n=5$ in each group) in one of two identical experiments. The difference between the experimental groups and the control group was significant *($P<0.05$).

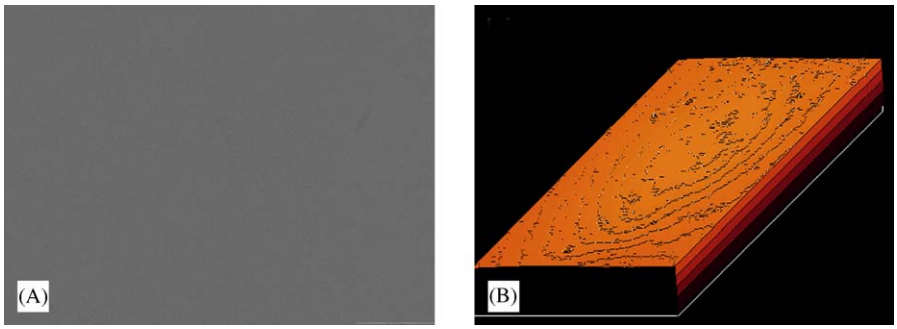

Fig. 1. (a) Scanning electron micrograph of the prepared titanium surface. Scale bar = 10 μm (b) Computer-generated image of the surface.

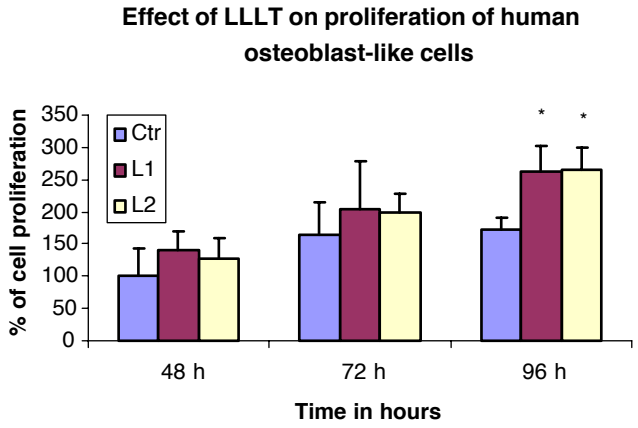

Fig. 3. Proliferation of human osteoblasts on titanium surfaces. Each bar represents the mean of percentage of cell proliferation ±SD of irradiated ($L1 = 1.5\,J/cm^2$, $L2 = 3\,J/cm^2$) and control groups ($n = 5$ in each group) in one of two identical experiments. The difference between the experimental groups and the control group at 96 h was significant *($P < 0.05$).

3.3. Cellular proliferation

The proliferation of human osteoblasts cultured on titanium after 48, 72, and 96 h is presented in Fig. 3. The number of osteoblasts in each sample increased with the incubation time and after 96 h was significantly higher in the irradiated groups than in the control group ($P < 0.05$). However, there was no significant difference in the number of osteoblasts between irradiated and control groups at 48 and 72 h. There were also no significant differences between the two irradiated groups.

3.4. Cell viability

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

3.5. Alkaline phosphatase activity

The specific alkaline phosphatase activity was expressed in relation to the total protein. The differences in ALP activity between irradiated and control groups were not statistically significant (Table 1).

3.6. Osteocalcin production

Osteocalcin synthesis by human osteoblast-like cells in response to stimulation by $1.25(OH)_2D_3$ was significantly greater ($P < 0.05$) in the group exposed to $3\,J/cm^2$. Osteocalcin production in the group exposed to $1.5\,J/cm^2$ was not statistically different from that of the control group (Table 1).

Table 1
Data of ALP activity and production of osteocalcin and TGF-β_1 by human osteoblast-like cells cultured on titanium implant

Variables	ALP/total protein (%)	Osteocalcin (ng/ml)	TGF-β_1 (ng/ml)
Control	100 ± 35.79	4.72 ± 0.35	1.69 ± 0.16
$1.5\,J/cm^2$	124.46 ± 29.28	5.22 ± 0.55	2.03 ± 0.67
$3\,J/cm^2$	164.07 ± 94.09	6.26 ± 1.26*	2.32 ± 0.54*

Results are mean ± SD.
*$P < 0.05$ level of statistical significance.

3.7. Transforming growth factor-β_1

The production of TGF-β_1 in conditioned media from the cultures exposed to $3\,J/cm^2$ was significantly higher than in the non-irradiated cultures (Table 1). The level of TGF-β_1 produced by cells irradiated with $1.5\,J/cm^2$ was not statistically significant.

4. Discussion

The growth and differentiation of osteoblasts, the bone forming cells, is central to the regeneration of bone around dental implants. It is proposed that biostimulation by LLLT may enhance the osteogenic potential of these cells. Although there are positive reports from both in vitro and in vivo experiments, the results of some studies of LLLT effects on bone healing are contradictory, e.g. laser biostimulation had no relevant effect [24] while in other studies it promoted significant stimulation of bone matrix synthesis [25]. Such discrepancies might be attributable to variations in the irradiation protocols and experimental models used.

From a biological standpoint, the use of human oral diploid cells as a test system for LLLT studies is more reliable than aneuploid cell lines derived from other tissues and species in culture. One of the principal differences between primary cell cultures and established cell lines is the capacity of the latter for unlimited growth. The vigorous nature of some of cell lines may mask laser-induced effects. Boulton [26] has demonstrated experimentally that the effect of LLLT is more pronounced in slow growing cultures. However, several studies utilizing established cell lines have demonstrated a beneficial effect of LLLT [27].

In the present study, the bone cells were derived directly from the target tissue, human mandibular alveolar bone. The use of this known primary culture model may limit the potential for phenotypic changes. Following incubation with $1.25(OH)_2D_3$, there was a significant increase in osteocalcin production compared with the basal level, confirming the osteogenic potential of these cells.

In order to investigate the response of human osteoblast-like cells to LLLT, the cells were cultured on titanium implant material and irradiated with a GaAlAs diode laser. The selected parameters were initial attachment and proliferation, differentiation, and production of TGF-β_1.

Initial attachment after 1, 3 and 24 h was significantly higher for the laser-exposed cells than for the non-exposed (control) cells. After 48 and 72 h of culture, the differences in cell proliferation were not statistically significant. However, at 96 h, proliferation was significantly higher in the irradiated cells. These results are in agreement with those of Ozawa et al. [11] and Ueda et al. [6]. However, the present study disclosed no significant differences between the two laser doses, indicating that increasing the dose to the range of 3 J/cm^2 does not further enhance the initial attachment and proliferation of osteoblast-like cells.

Alkaline phosphatase activity is regarded as a marker of osteoblast differentiation [28]. Early progenitor cells do not express ALP activity, but differentiate through a defined number of cell divisions, ultimately expressing a mature osteoblast phenotype: a postmitotic, osteogenic cell with ALP activity [29]. Therefore the effect of laser therapy on ALP activity may reflect the effect of laser irradiation on bone formation. The present data disclosed no significant increase in ALP activity with both laser doses used in the study. In agreement with these results, Coombe et al. [24], in an experimental study using cells isolated from human osteosarcoma, reported that ALP activity was not significantly affected by laser irradiation.

In contrast, other studies have reported a significant increase in ALP activity after LLLT [27,30]. In a recent study by Ueda et al. application of Ga–Al–As diode laser (wavelength 830 nm, power output 500 mW, energy density 0.48 to 3.84 J/cm^2) to osteoblast-like cells isolated from fetal rat calvariae significantly stimulated cellular proliferation, bone nodule formation, ALP activity, and ALP gene expression [6].

Osteocalcin synthesis and production of TGF-β_1 by the cells derived from human mandibular bone were significantly greater in the samples exposed to 3 J/cm^2. This may account for an acceleration of osteoblast differentiation due to increases in the irradiation dose, suggesting that this stimulatory effect is dose dependent. The significant increases in TGF-β_1 and osteocalcin production by cells exposed to LLLT is probably attributable not only to such factors as the selection of wavelength and doses, but also on the ability of laser irradiation to accelerate cellular activity such as ATP synthesis [31], early osteoblastic differentiation [32], and release of growth factors [33].

During the early stages of wound healing, the energy requirements of the cell are increased. For this purpose, the appropriate dose for application of LLLT would be the one which enhances TGF-β_1 production by osteoblasts.

The doses used in this study are similar to those in several previous studies which indicated that 1–5 J/cm^2 is effective for inducing positive effects [34,35]. Our observation showed that the irradiation on three consecutive days with a dose of 3 J/cm^2 enhanced production of osteocalcin and TGF-β_1. Ozawa et al. and Saito et al. reported that the stimulatory effects of bone formation are achieved by repeated irradiation on three consecutive days, rather than one application using the same LLLT parameters [11,36].

Several laser systems are currently being applied for stimulation of tissue regeneration. In selecting optimal laser therapy for experimental or clinical application, it is important that the properties of each device are carefully evaluated [37]. The present results have demonstrated that LLLT using a GAAlAs diode laser has a positive biostimulatory effect on osteoblast-like cells and does not cause cell damage. Cell viability was greater than 90% for all experimental groups and it should be noted that the application of laser irradiation did not cause any apparent damage to the cells.

This in vitro study and our recently published animal experiments [7,12] indicate that LLLT enhances hard tissue-implant integration by stimulating the production of factors involved in inflammation and wound-healing and in bone cell metabolism. These results may help to define optimal laser light doses to the tissue-implant area and contribute to the development of a clinical model of laser regimes for implant therapy.

5. Conclusion

In this cellular model, LLLT enhanced the attachment and proliferation of cells derived from human mandibular bone, cultured on titanium implant material. Exposure to laser light of 3 J/cm^2 significantly increased osteocalcin and TGF-β_1 production, suggesting that LLLT stimulates differentiation of osteoblast-like cells in a dose-dependent manner. The results indicate that LLLT modulates the activity of cells and tissues surrounding an implant. The introduction of laser therapy for implant treatment seems feasible and may be of therapeutic benefit in accelerating healing.

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