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Different doses of low-level laser irradiation modulate the *in vitro* response of osteoblast-like cells

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Abstract. Because osteoblasts play a key role in bone remodeling and the influence of low-level laser therapy on this process is not clear, Saos-2 human osteoblast-like cells were irradiated by a gallium–aluminum–arsenide diode laser (915 nm) for 10, 48, 96, 193, and 482 s using doses 1, 5, 10, 20, and 50 J/cm^2, respectively. A control group was not irradiated. Morphology, viability, and cytotoxicity analyses were carried out after 1 hr, 1 day, and 3 days. Deoxyribose nucleic acid (DNA) content and release of vascular endothelial growth factor (VEGF), receptor activator of nuclear factor kappa B ligand (RANKL), and osteoprotegerin (OPG) were evaluated. Viability was modulated by laser irradiation in a dose-dependent manner, with 10 J/cm^2 inducing a biostimulatory response and 20 to 50 J/cm^2 determining a bioinhibitory and cytotoxic effect. Accordingly, DNA content was generally increased for the 10 J/cm^2 dose and decreased for the 50 J/cm^2 dose. A rapid and transitory trend toward increased RANKL/OPG ratio and a tendency toward a delayed increase in VEGF release for doses of 1 to 10 J/cm^2 was found. Further investigations using the biostimulatory dose of 10 J/cm^2 emerged from this study are needed to establish the ideal treatment regimens in the laboratory as well as in clinical practice. [DOI: 10.1117/1.JBO.19.10.108002]

Keywords: biophysical stimulation; bone remodeling; diode laser; *in vitro* study; osteoblast-like cells.

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1 Introduction

Low-level laser therapy (LLLT) is an ever-growing clinical tool for a range of medical applications, including reduction of inflammatory processes, pain relief, and acceleration of wound healing.[1,2] The mechanisms behind LLLT are based on photochemical and photobiological reactions on cells and tissues that are due to stimulation by light alone without any thermal increase, which is different from other ablating or heating-mediated laser treatments.[3] The best documented clinical applications for LLLT are in the musculoskeletal system for the treatment of pain, osteoarthritis, and tendinitis.[4] Although LLLT applications for dentistry are not as well evidenced as in the musculoskeletal system, many experimental studies investigate LLLT effects to accelerate alveolar bone healing after tooth extraction,[5] and to promote bone regeneration in the midpalatal suture after maxillary expansion.[6] Besides the positive role of LLLT for pain relief in orthodontics, it is still controversial its influence on the rate of orthodontic movement of teeth.[7]

The modulation of the biological processes underlying bone remodeling by LLLT is a highly promising strategy because it is easy to administrate, noninvasive, painless, and no adverse effect has been detected so far, thus potentially having a great beneficial impact on patients' quality of life. It has attracted interest among researchers because it may hold potential clinical advantages in orthopedics and dentistry as an adjuvant therapy.[4,8,9] However, to improve its clinical use, the key to success with LLLT has yet to be searched in the identification of the proper technique and in the understanding of the optimal parameters to achieve the desired effect. Several wavelengths, exposure times, doses, pulsing modes, and powers of laser irradiation have been proposed to influence the proliferation of various cultured cells using different laser equipment, treatment protocols, and experimental conditions.[3] This makes it difficult to obtain unambiguous and comparable results, clearly indicating the need for LLLT parameters to be examined more rigorously at a cellular level prior to widespread incorporation into clinical practice.

The aim of the present study was to *in vitro* apply a wide range of doses of 1 to 50 J/cm^2 by a gallium–aluminum–arsenide (GaAlAs) diode laser with a wavelength of 915 nm on osteoblast-like cells, which resemble human mature osteoblast phenotype and have a key role in bone remodeling, in order to identify the LLLT settings responsible for biostimulatory or bioinhibitory effects by means of morphology, viability, deoxyribose nucleic acid (DNA) synthesis, and cytotoxicity analyses. The release of receptor activator of nuclear factor kappa B ligand (RANKL), osteoprotegerin (OPG), and vascular endothelial growth factor (VEGF) was evaluated after laser irradiation because they are relevant key molecules affecting bone remodeling and neoangiogenesis.

2 Materials and Methods

2.1 *Cell Culture*

Saos-2 human osteoblast-like cells (ATCC® HTB-85™, Manassas) were cultured in Dulbecco modified Eagle's medium

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(DMEM; Sigma-Aldrich, St. Louis, Missouri) with 10% fetal calf serum (Lonza Walkersville Inc., Walkersville), 100 IU/ml penicillin and 100 μg/ml streptomycin solution (Gibco Invitrogen SRL, San Giuliano Milanese, MI, Italy) at 37°C in a controlled humidified atmosphere (95% air/5% CO_2). When confluent, cells were detached with 0.05% (w/v) trypsin and 0.02% (w/v) ethylenediamine tetra-acetic acid (EDTA) and counted. A cell suspension of 3×10^4 cells/ml was seeded into black 24-multiwell tissue culture plates. Plates were returned in the controlled humidified incubator for 24 h to allow cell adhesion.

2.2 *Laser Irradiation*

After a 24-h incubation, cells were irradiated with a GaAlAs diode laser (Pocket Laser, Orotig s.r.l., Verona, Italy). The laser equipment had a wavelength of 915 ± 10 nm and a maximum power output of 6 W $\pm$ 20%. The laser beam was delivered by an optical fiber 0.6 mm in diameter that was defocused at the tip by a concave lens to cover the growth area of each well (1.91 cm^2) at a distance of 19 mm. A 100 Hz pulse irradiation mode was used with a duty cycle of 50% and a set power of 1 W (corresponding to an output power of 0.575 W, as measured at hand piece aperture). A single session of laser irradiation was carried out; the doses corresponding to the pulsed irradiation for 10, 48, 96, 193, and 482 s were, respectively, of 1, 5, 10, 20, and 50 J/cm^2. Laser irradiation was carried out in wells individually and perpendicularly with lids off and in the absence of the culture medium because serum could interfere with the reaction during irradiation. Immediately before irradiation, DMEM was removed, phosphate-buffered solution (PBS, Sigma-Aldrich) was added and then, immediately after irradiation, fresh culture medium was replaced. The same amount of cells was seeded in control wells (CTR), cultivated for the same experimental times and treated under identical conditions except for laser irradiation. Black multiwell plates were used in order to avoid cross-irradiation among wells and to minimize light reflections. Cultures were kept at 37°C in a humidified atmosphere of 95% air/5% CO_2 for 1 h, 1 day, and 3 days. At each experimental time, cultures were processed for the evaluation of viability (AlamarBlue, AbD Serotec, Oxford, UK) and DNA quantification (Quant-iT™ PicoGreen® dsDNA, Life Technologies, Carlsbad, California). Cell culture supernatants of laser exposed and not exposed wells were collected and analyzed to evaluate the cell damage (cytotoxicity Detection Kit, lactate dehydrogenase (LDH), Roche Diagnostics GmbH, Mannheim, Germany), the release of VEGF (Boster Immunoleader ELISA kit, Fremont, California), OPG and RANKL (Cloud-Clone Corp., Houston, Texas).

2.3 *Viability Assay*

The AlamarBlue assay was used to quantitatively measure the viability of Saos-2 cells. At each experimental time, culture medium was removed from wells, cells were extensively washed with PBS and then 100 μl of diluted AlamarBlue solution (final concentration 1:10) was added to each well. Plates were incubated at 37°C for further 4 h. The colorimetric reaction was measured spectrophotometrically at 570- and 625-nm wavelengths with a microplate absorbance reader (iMark, Biorad-Laboratories Inc., Hercules, California).

2.4 *DNA Quantification*

Laser irradiated and control wells were repeatedly washed with PBS, the plates were then frozen at −80°C and thawed at room temperature for three times. For the complete lysis and elution of the cells and their cellular content, 100 μl of Tris-EDTA buffer with sodium dodecyl sulphate 0.01% solution were added to each well. A working solution of the PicoGreen® (Life Technologies, Carlsbad, California) reagent was added and incubated with experimental samples in the dark for 3 min at room temperature. The fluorescence was read at 490ex- to 520em wavelengths, the readings expressed as relative fluorescence units and the DNA amount of each sample calculated above a standard curve.

2.5 *Supernatant Measurements*

The degree of cell damage was evaluated by measuring the LDH activity released from the cytosol of damaged cells into the supernatant of irradiated and control cells by using the LDH cytotoxicity detection kit (Roche Diagnostics GmbH). Briefly, 100 μl of laser exposed- and not exposed-cell supernatant was mixed with 100 μl of kit reaction mixture. After a 30-min incubation at room temperature in the dark, the optical absorbance was measured at 490 nm with a reference correction at 625 nm with a microplate reader (iMark, Biorad-Laboratories Inc.).

At each experimental time, the release of OPG, RANKL (Cloud-Clone Corp.), and VEGF (Boster Immunoleader ELISA kit) in culture medium was analyzed by enzyme-linked immunosorbent assay kits following manufacturer's instructions.

2.6 *Cell Morphology*

Cell morphology at 1 and 3 days following laser exposure was assessed by the LIVE/DEAD® Viability/Cytotoxicity Kit. Briefly, cells were rinsed in PBS and incubated with 150 μl of the reaction mixture (2 μM Calcein AM and 4 μM EthD-1) for 45 min in the dark at room temperature. Then, stained cells were observed by an inverted light/fluorescence microscopy (IX71, Olympus Italia s.r.l, Milano, Italy) equipped with a long-pass filter for the simultaneous viewing of both probes. Images were grabbed with a digital camera (XC30, Olympus Italia s.r.l).

2.7 *Statistical Analysis*

Data were shown as mean $\pm$ standard deviation (SD). The differences between the laser irradiation protocols were analyzed using a Kruskall–Wallis H test and *post hoc* analysis with a Bonferroni-corrected Mann–Whitney U test for each experimental time. The differences between the three experimental times were evaluated with Friedman test followed by a Bonferroni-corrected Wilcoxon paired sign-rank test for each laser irradiation protocol. Statistical analyses were performed using the statistical software SPSS for Windows (version 18.0; 2009; SPSS Inc., Chicago, Illinois). The limit for statistical significance was set at $p < 0.05$.

3 Results

3.1 *Viability Assay*

A single application of laser affected cells already after 1 h from irradiation, with the 50 J/cm^2 treated group having a

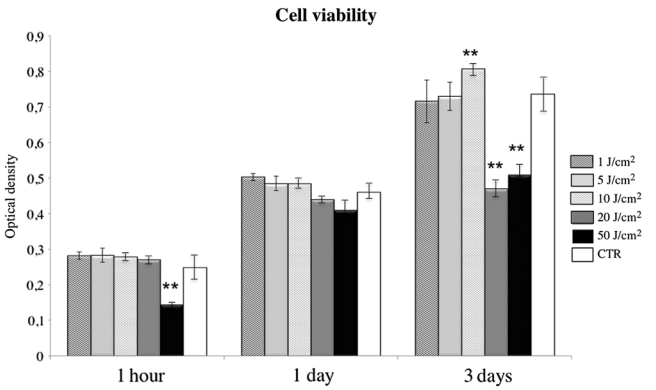

Fig. 1 Viability results of Saos-2 cells irradiated with a single laser session of different doses (1, 5, 10, 20, and 50 J/cm^2) or nonirradiated (control, CTR). Data are means; bars are standard deviations (SDs). Kruskal Wallis test followed by a Bonferroni-corrected Mann–Whitney *U* test: 1 h: **, 50 J/cm^2 versus CTR, $p < 0.005$; 3 days: **, 10, 20, and 50 J/cm^2 versus CTR, $p < 0.005$. No statistically significant differences were found at 1 day.

significantly lower viability compared with the nonirradiated controls ($p < 0.005$, Fig. 1). After 3 days, the 20 and the 50 J/cm^2 laser irradiated groups exhibited a significantly decreased viability, whereas the 10 J/cm^2 laser irradiated group showed a significantly higher viability compared with the nonirradiated controls ($p < 0.005$, Fig. 1). No statistically significant difference was found between the three experimental times for each laser irradiation protocol.

3.2 *DNA Quantification*

A generally increased DNA content for the 10 J/cm^2 irradiated groups and decreased DNA content for the 50 J/cm^2 irradiated groups compared with nonirradiated controls were found at each experimental time; although the differences between the groups were not statistically significant (Fig. 2). In agreement with the viability results, this trend was more evident after 3 days from irradiation. No statistically significant difference was found between the three experimental times for each laser irradiation protocol.

3.3 *Supernatant Measurements*

Although the differences between the groups were not statistically significant, the single laser irradiation with a dose of

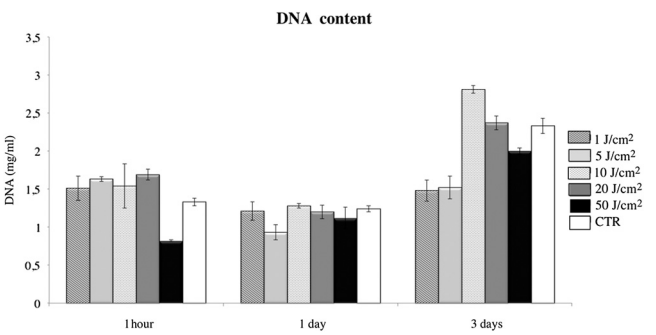

Fig. 2 Deoxyribose nucleic acid quantification measurements of Saos-2 cells irradiated with a single laser session of different doses (1, 5, 10, 20, and 50 J/cm^2) or nonirradiated (control, CTR). Data are means; bars are SDs. No statistically significant differences were found.

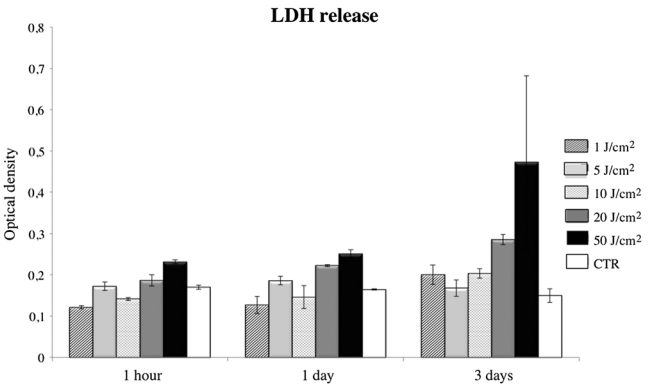

Fig. 3 Lactate dehydrogenase release of Saos-2 cells irradiated with a single laser session of different doses (1, 5, 10, 20, and 50 J/cm^2) or nonirradiated (control, CTR). Data are means; bars are SDs. No statistically significant differences were found.

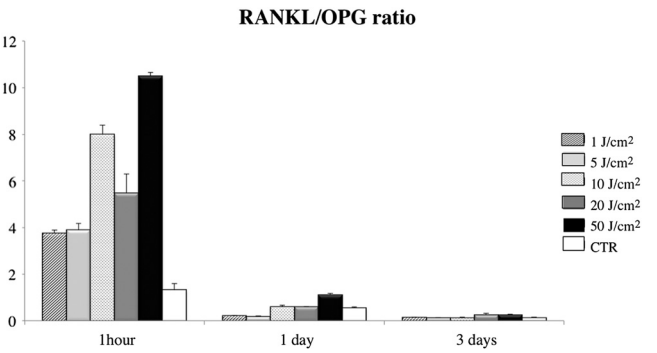

Fig. 4 Receptor activator of nuclear factor kappa B ligand/osteoprotegerin ratio of Saos-2 cells irradiated with a single laser session of different doses (1, 5, 10, 20, and 50 J/cm^2) or nonirradiated (control, CTR). Data are means; bars are SDs. No statistically significant differences were found.

50 J/cm^2 exhibited a tendency toward an increased release of LDH, which is the indicator of cell damage, at all experimental times compared with nonirradiated controls (Fig. 3). The same trend was evident also for the dose of 20 J/cm^2. The other doses (1, 5, and 10 J/cm^2) got LDH levels comparable or lower than controls. No statistically significant difference was found

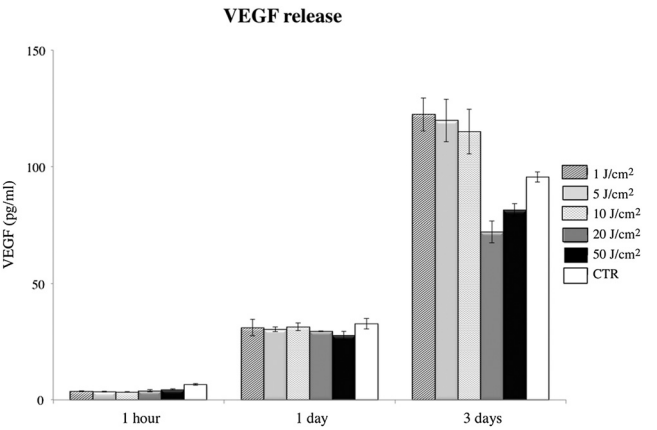

Fig. 5 Vascular endothelial growth factor release of Saos-2 cells irradiated with a single laser session of different doses (1, 5, 10, 20, and 50 J/cm^2) or nonirradiated (control, CTR). Data are means; bars are SDs. No statistically significant differences were found.

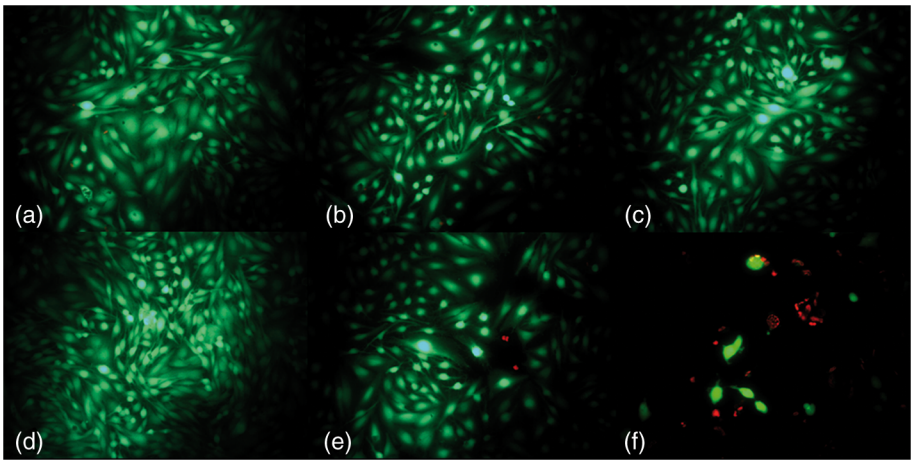

Fig. 6 Morphological characterization with fluorescent probes (live/dead staining) marking viable (green stained) and dead (red stained) cells (10× magnification): (a) nonirradiated control group; (b) cells irradiated at 1 J/cm^2; (c) cells irradiated at 5 J/cm^2; (d) cells irradiated at 10 J/cm^2; (e) cells irradiated at 20 J/cm^2; and (f) cells irradiated at 50 J/cm^2.

between the three experimental times for each laser irradiation protocol.

Generally, at 1 h, all laser irradiated cells promptly responded to the biophysical stimuli with an increase of the RANKL/OPG ratio and then this effect, in the absence of repeated stimuli, was lost at longer times (Fig. 4). Although the differences between the groups were not statistically significant, the RANKL/OPG ratio was generally increased for the 50 J/cm^2 laser irradiated groups compared with nonirradiated controls. No statistically significant difference was found between the three experimental times for each laser irradiation protocol.

VEGF release showed a tendency toward a dose-dependent and delayed response after a single laser irradiation with doses of 1, 5, and 10 J/cm^2 inducing a slight increase and doses of 20 and 50 J/cm^2 inducing a slight decrease after 3 days in comparison with nonirradiated controls, although without any statistical significance (Fig. 5).

3.4 *Cell Morphology*

Figure 6 showed the morphological appearance of laser treated and untreated cells after 3 days from irradiation. Cells treated with laser doses of 1, 5, and 10 J/cm^2 were almost viable and comparable with untreated cells and had a status near to confluence. On the opposite, cells treated with 50 J/cm^2 dose appeared almost red stained, indicating an ongoing apoptotic status and those treated with 20 J/cm^2 seemed viable but at a lower stage of confluence and with several apoptotic cells.

4 Discussion and Conclusions

In this study, an early effect on cell proliferation was found after 1 h from a single laser irradiation, with the 50 J/cm^2 treated group having a significantly lower viability compared with the nonirradiated controls. This result was more evident after 3 days from laser application: a decreased viability was detected for the 50 J/cm^2 as well as for the 20 J/cm^2 laser irradiated groups compared with the nonirradiated controls, whereas the 10 J/cm^2 laser irradiated group showed a significantly higher viability compared with the controls. Our results supported the idea that laser irradiation administered at low doses between 1 and 10 J/cm^2 induces a biostimulatory cell response whereas higher doses determine a bioinhibitory effect.[3] Reports on the effects of laser irradiation on osteoblastic cells are controversial: some authors find a significant increase in cell proliferation,[10–12] whereas others do not.[13–16] However, direct comparison with the current study is inappropriate because of different laser equipment, treatment protocols, *in vitro* models, and conditions used. None of the previous works irradiating Saos-2 with laser confirm the viability results by quantifying the DNA content. In our study, although the differences between the groups were not statistically significant, a tendency toward increased DNA content for the 10 J/cm^2 irradiated groups and decreased DNA content for the 50 J/cm^2 irradiated groups compared with nonirradiated controls was found at each experimental time. This general trend was consistent with that of the viability assays and, similarly, was more evident at 3 days. The hypothesis of a bioinhibitory effect for the dose of 50 J/cm^2 was further confirmed by morphological images showing a vast majority of dead cells and by the increment in LDH release compared with the nonirradiated controls that is indicative of cytotoxicity, especially at 3 days. Also the dose of 20 J/cm^2 exhibited a tendency toward cytotoxicity because of increased LDH release compared with the nonirradiated controls at each experimental time, whereas the doses of 1, 5, and 10 J/cm^2 showed levels of LDH more comparable with those of controls, and therefore, did not evoke cytotoxic effects.

The RANKL/OPG ratio was also investigated into the present study because it determines whether bone is formed or removed during remodeling. Activation of the remodeling cycle starts with osteoblasts that increase the expression of the RANKL; by coupling via binding with its RANK, which is highly expressed on the osteoclast membrane, this lead to expansion of the osteoclast progenitor pool, increased survival of these cells, differentiation into mononucleated progenitor cells, fusion into multinucleated osteoclasts, and then activation. Simultaneously, osteoblasts can balance this process by modulating the expression of OPG, a secretory soluble receptor. OPG is an inhibitor of RANK receptor. This reduces the effect of RANKL on osteoclastogenesis and in effect constitutes an efficient negative layer of control.[17] Although the differences between the groups were not statistically significant, a tendency toward an increased RANKL/OPG ratio for the 50 J/cm^2 laser irradiated groups compared with nonirradiated controls was found at each experimental time. As a general trend, our data

showed a rapid and transitory increase in the RANKL/OPG ratio for all the doses after a single laser irradiation. Only 2 *in vitro* studies have analyzed the expression of the RANKL/OPG ratio after laser irradiation with contrasting results: the first study found an increase in the RANKL/OPG ratio in human alveolar bone-derived cells seeded onto titanium disks and exposed to irradiation with a dose of 3 J/cm^2 using a GaAlAs diode laser with a wavelength of 780 nm,[18] whereas the other study detected a decrease in the RANKL/OPG ratio in rat calvarial cells irradiated with a diode laser with a wavelength of 650 nm.[19] However, our *in vitro* findings seem to confirm data from preclinical and clinical studies in which an increase of the RANKL/OPG ratio was observed in laser-treated rats and patients leading to an acceleration of bone remodeling.[20–22]

An estimation of VEGF release was carried out into the present study because it is a powerful growth factor and promotes vascularization that is an integral part of bone remodeling.[23] VEGF production by osteoblasts induces endothelial cell proliferation, angiogenesis, and capillary permeability, acts as a potent attractant for osteoblasts and osteoclasts, and can directly enhance bone resorption and osteoclast survival.[23] In the present study, a trend toward increased VEGF release for the 1, 5, 10 J/cm^2 irradiated groups as well as a trend toward decreased VEGF release for the 20 and 50 J/cm^2 irradiated groups was found compared with nonirradiated controls after 3 days from a single laser irradiation. Few *in vitro* studies have so far analyzed VEGF release after laser irradiation on several cell phenotypes, such as human endothelial cells, smooth muscle cells, cardiomyocytes, dermal or gingival fibroblasts, bone marrow derived mesenchymal stem cells, or olfactory glial cells.[24–30] No study has investigated laser effects on VEGF expression using osteoblast-like cells. From our findings, it can be hypothesized that a single laser irradiation modulates VEGF release in a dose-dependent and time-delayed manner.

Our results show that the viability of Saos-2 cells is modulated by a single laser irradiation in a dose-dependent manner. A dose of 10 J/cm^2 induces a biostimulatory cell response whereas doses of 20 or 50 J/cm^2 determine a bioinhibitory effect. Further studies on multiple laser exposures and cell observation at increased experimental times are needed to strengthen cell responses with more evident and significant results than that of a single irradiation owing to a cumulative laser effect, similarly to previous *in vitro* studies.[10,16,31–33] This could lead to establish the ideal treatment regimens in the laboratory as well as in clinical practice. A Saos-2 cell line was chosen in this study because it accurately resembles the behavior of human primary mature osteoblast phenotype in terms of matrix mineralization, cytokine, and growth factor patterns,[34] and in order to obviate to the variability of primary cells. A GaAlAs diode laser was employed because it is one of the most popular into clinical practice[11] and, at a wavelength of 915 nm, it is known to have a higher penetration depth compared with other laser types.[3] Our study protocol was planned to determine the optimal dose of laser irradiation to get a biostimulatory effect onto osteoblasts for a useful and safe clinical application. It was, therefore, decided to keep constant the output power, the pulsing of the radiation, and the treated area while varying the dose as the main study variable because it has been recognized as the most important laser parameter responsible of the biologic response.[35] This study is a first step toward further investigations on LLLT potentiality on bone cells in order to bridge the gap between *in vitro* research and biomedical applications. The maximally stimulating dose of 10 J/cm^2 emerged from our data could be used for coculture studies using laser irradiation on both osteoblasts and osteoclasts in order to enhance our knowledge on laser effects on bone remodeling.

Acknowledgments

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Biographies of all the authors are not available.