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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/cm², 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/cm² inducing a biostimulatory response and 20 to 50 J/cm² determining a bioinhibitory and cytotoxic effect. Accordingly, DNA content was generally increased for the 10 J/cm² dose and decreased for the 50 J/cm² 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/cm² was found. Further investigations using the biostimulatory dose of 10 J/cm² emerged from this study are needed to establish the ideal treatment regimens in the laboratory as well as in clinical practice. © 2014 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.19.10.108002]

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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/cm² 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 $\mu\text{g}/\text{ml}$ 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 $6 \text{ W} \pm 20\%$. 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 Kruskal–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/cm²) 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/cm² versus CTR, $p < 0.005$; 3 days: **, 10, 20, and 50 J/cm² 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/cm² laser irradiated groups exhibited a significantly decreased viability, whereas the 10 J/cm² 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/cm² irradiated groups and decreased DNA content for the 50 J/cm² 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/cm²) 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/cm²) 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/cm²) or nonirradiated (control, CTR). Data are means; bars are SDs. No statistically significant differences were found.

50 J/cm² 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/cm². The other doses (1, 5, and 10 J/cm²) 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/cm²) 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 $\times$ magnification): (a) nonirradiated control group; (b) cells irradiated at 1 J/cm²; (c) cells irradiated at 5 J/cm²; (d) cells irradiated at 10 J/cm²; (e) cells irradiated at 20 J/cm²; and (f) cells irradiated at 50 J/cm².

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/cm² 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/cm² inducing a slight increase and doses of 20 and 50 J/cm² 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/cm² were almost viable and comparable with untreated cells and had a status near to confluence. On the opposite, cells treated with 50 J/cm² dose appeared almost red stained, indicating an ongoing apoptotic status and those treated with 20 J/cm² 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/cm² 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/cm² as well as for the 20 J/cm² laser irradiated groups compared with the nonirradiated controls, whereas the 10 J/cm² 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/cm² 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/cm² irradiated groups and decreased DNA content for the 50 J/cm² 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/cm² 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/cm² 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/cm² 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/cm² 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/cm² 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/cm² irradiated groups as well as a trend toward decreased VEGF release for the 20 and 50 J/cm² 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/cm² induces a biostimulatory cell response whereas doses of 20 or 50 J/cm² 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 practice¹¹ 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/cm² 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.

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References

1. I. Marini, M. R. Gatto, and G. A. Bonetti, "Effects of superpulsed low-level laser therapy on temporomandibular joint pain," *Clin. J. Pain* **26**(7), 611–616 (2010).
2. M. Scoletta et al., "Effect of low-level laser irradiation on bisphosphonate-induced osteonecrosis of the jaws: preliminary results of a prospective study," *Photomed. Laser Surg.* **28**(2), 179–184 (2010).
3. K. M. Alghamdi, A. Kumar, and N. A. Moussa, "Low-level laser therapy: a useful technique for enhancing the proliferation of various cultured cells," *Lasers Med. Sci.* **27**(1), 237–249 (2012).
4. J. D. Carroll et al., "Developments in low level light therapy (LLLT) for dentistry," *Dent. Mater.* **30**(5), 465–475 (2014).
5. M. Mozzati et al., "Influence of superpulsed laser therapy on healing processes following tooth extraction," *Photomed. Laser Surg.* **29**(8), 565–571 (2011).
6. P. Angeletti et al., "Effect of low-level laser therapy (GaAlAs) on bone regeneration in midpalatal anterior suture after surgically assisted rapid maxillary expansion," *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **109**(3), e38–e46 (2010).
7. H. Long et al., "The effectiveness of low-level laser therapy in accelerating orthodontic tooth movement: a meta-analysis," *Lasers Med. Sci.* (2013) [Epub ahead of print].
8. S. Incerti Parenti et al., "Effect of low-level laser irradiation on osteoblast-like cells cultured on porous hydroxyapatite scaffolds," *Ann. Ist. Super. Sanita* **49**(3), 255–260 (2013).
9. G. A. Guzzardella et al., "Laser stimulation on bone defect healing: an *in vitro* study," *Lasers Med. Sci.* **17**(3), 216–220 (2002).
10. N. Bloise et al., "Investigation of low-level laser therapy potentiality on proliferation and differentiation of human osteoblast-like cells in the absence/presence of osteogenic factors," *J. Biomed. Opt.* **18**(12), 128006 (2013).
11. R. M. Huertas et al., "Effect and clinical implications of the low-energy diode laser on bone cell proliferation," *Biol. Res. Nurs.* **16**(2), 191–196 (2014).
12. H. D. Arisu, E. Türköz, and O. Bala, "Effects of Nd:Yag laser irradiation on osteoblast cell cultures," *Lasers Med. Sci.* **21**(3), 175–180 (2006).
13. F. Chellini et al., "Low pulse energy Nd:YAG laser irradiation exerts a biostimulative effect on different cells of the oral microenvironment: an *in vitro* study," *Lasers Surg. Med.* **42**(6), 527–539 (2010).
14. E. Stein et al., "Phenothiazine chloride and soft laser light have a biostimulatory effect on human osteoblastic cells," *Photomed. Laser Surg.* **27**(1), 71–77 (2009).
15. H. Bayram et al., "Effect of low level laser therapy and zoledronate on the viability and ALP activity of Saos-2 cells," *Int. J. Oral Maxillofac. Surg.* **42**(1), 140–146 (2013).
16. A. R. Coombe et al., "The effects of low level laser irradiation on osteoblastic cells," *Clin. Orthod. Res.* **4**(1), 3–14 (2001).
17. M. Fini et al., "Bone remodeling, humoral networks and smart biomaterial technology for osteoporosis," *Front. Biosci.* **S2**(2), 468–482 (2010).
18. A. D. Petri et al., "Effects of low-level laser therapy on human osteoblastic cells grown on titanium," *Braz. Dent. J.* **21**(6), 491–498 (2010).
19. M. Xu et al., "Low-intensity pulsed laser irradiation affects RANKL and OPG mRNA expression in rat calvarial cells," *Photomed. Laser Surg.* **27**(2), 309–315 (2009).

20. S. Fujita et al., "Low-energy laser stimulates tooth movement velocity via expression of RANK and RANKL," *Orthod. Craniofacial Res.* **11**(3), 143–155 (2008).
21. B. A. Altan et al., "Metrical and histological investigation of the effects of low-level laser therapy on orthodontic tooth movement," *Lasers Med. Sci.* **27**(1), 131–140 (2012).
22. A. Domínguez, C. Gómez, and J. C. Palma, "Effects of low-level laser therapy on orthodontics: rate of tooth movement, pain, and release of RANKL and OPG in GCF," *Lasers Med. Sci.* (2013) [Epub ahead of print].
23. S. M. Chim et al., "Angiogenic factors in bone local environment," *Cytokine Growth Factor Rev.* **24**(3), 297–310 (2013).
24. J. Hou et al., "In vitro effects of low-level laser irradiation for bone marrow mesenchymal stem cells: proliferation, growth factors secretion and myogenic differentiation," *Lasers Surg. Med.* **40**(10), 726–733 (2008).
25. J. Szymanska et al., "Phototherapy with low-level laser influences the proliferation of endothelial cells and vascular endothelial growth factor and transforming growth factor-beta secretion," *J. Physiol. Pharmacol.* **64**(3), 387–391 (2013).
26. N. Kipshidze et al., "Low-power helium: neon laser irradiation enhances production of vascular endothelial growth factor and promotes growth of endothelial cells in vitro," *Lasers Surg. Med.* **28**(4), 355–364 (2001).
27. S. Hakki and S. B. Bozkurt, "Effects of different setting of diode laser on the mRNA expression of growth factors and type I collagen of human gingival fibroblasts," *Lasers Med. Sci.* **27**(2), 325–331 (2012).
28. K. R. Byrnes et al., "Low power laser irradiation alters gene expression of olfactory ensheathing cells in vitro," *Lasers Surg. Med.* **37**(2), 161–171 (2005).
29. J. Feng, Y. Zhang, and D. Xing, "Low-power laser irradiation (LPLI) promotes VEGF expression and vascular endothelial cell proliferation through the activation of ERK/Sp1 pathway," *Cell. Signal.* **24**(6), 1116–1125 (2012).
30. A. Khanna et al., "Augmentation of the expression of proangiogenic genes in cardiomyocytes with low dose laser irradiation in vitro," *Cardiovasc. Radiat. Med.* **1**(3), 265–269 (1999).
31. M. M. Jawad et al., "Effect of 940 nm low-level laser therapy on osteogenesis in vitro," *J. Biomed. Opt.* **18**(12), 128001 (2013).
32. S. Bouvet-Gerbetaz et al., "Effects of low-level laser therapy on proliferation and differentiation of murine bone marrow cells into osteoblasts and osteoclasts," *Lasers Surg. Med.* **41**(4), 291–297 (2009).
33. M. Soleimani et al., "The effects of low-level laser irradiation on differentiation and proliferation of human bone marrow mesenchymal stem cells into neurons and osteoblasts-an in vitro study," *Lasers Med. Sci.* **27**(2), 423–430 (2012).
34. E. M. Czekanska et al., "A phenotypic comparison of osteoblast cell lines versus human primary osteoblasts for biomaterials testing," *J. Biomed. Mater. Res. A* **102**(8), 2636–2643 (2014).
35. J. Tuner and L. Hode, *The Laser Therapy Handbook*, 4th ed., Prima Books AB, Grangesberg, Sweden (2004).

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