Ann Ist Super Sanità 2013 | Vol. 49, No. 3: 255-260
DOI 10.4415/ANN_13_03_04

Effect of low-level laser irradiation on osteoblast-like cells cultured on porous hydroxyapatite scaffolds

Serena Incerti Parenti[a], Silvia Panseri[b], Antonio Gracco[c], Monica Sandri[b], Anna Tampieri[b] and Giulio Alessandri Bonetti[a]

[a] Reparto di Ortognatodonzia, Dipartimento di Scienze Biomediche e Neuromotorie (DIBINEM), Università di Bologna, Bologna, Italy
[b] Istituto di Scienza e Tecnologia dei Materiali Ceramici, Consiglio Nazionale delle Ricerche (ISTEC-CNR), Faenza (RA), Italy
[c] Reparto di Ortognatodonzia, Dipartimento di Neuroscienze, Università di Padova, Padova, Italy

Abstract

Objective. To determine the effect of laser irradiation at a low dose on human osteoblast-like cells.

Materials and methods. 32 porous hydroxyapatite scaffolds currently used for bone tissue engineering were seeded with MG63 cells and irradiated or not with a GaAlAs diode laser (wavelength 915 nm, dose 2 J/cm^2) using different power density and exposure duration.

Results. After 72-h incubation, cells showed well spread morphology and good adhesion on both laser-treated and untreated scaffolds. Laser irradiation did not interfere in cell viability and proliferation as compared with the non-irradiated controls.

Conclusion. This study suggests that there is no effect of 915 nm laser irradiation at a dose of 2 J/cm^2 on the proliferation rate of MG63 cells. Future investigations are needed to compare different dose and wavelength regimens in order to determine the optimal set of laser parameters for maximum cell yield and safe clinical application.

Key words

- biomaterials
- human osteoblast-like cells
- low-level laser therapy
- porous hydroxyapatite scaffolds
- three-dimensional cultures

INTRODUCTION

In the last decades a new paradigm of worldwide biomedical research has been represented by tissue engineering, a multidisciplinary approach aiming at replacing, repairing or even regenerating diseased or injured tissues to their original state and function [1]. Rather than the employment of prostheses or organ transplantation proposed by traditional regenerative medicine [1], one of the most promising innovative strategies underlying tissue engineering depends on employing scaffolds that serve as a three-dimensional (3D) extracellular matrix (ECM) on which cells can migrate, proliferate and form the desired tissue. The ideal biomaterial for a scaffold would provide the physical and chemical stimulus to selectively promote attachment, growth and differentiation of the cells and their assembly into 3D tissues [2].

The traditional approach to repair large bone defects included the use of autografts (with the disadvantages of donor site morbidity, limited donor bone supply, as well as different anatomic and structural problems) or allografts (with the risk of eliciting an immunological response as well as inducing transmissible diseases) [3]. The increasing demand for the development of synthetic materials that mimic natural bone tissue has the potential to overcome these limitations. This has led researchers to investigate new technologies in order to enhance the recruitment, attachment and growth of bone cells with the biomaterials. Likewise in the process of discovery of drugs and, in general, of medical products the mission should be to favour the translation of new treatments which may potentially have a great beneficial impact on patients' quality of life as well as on public health [4-6].

Recently, scientific literature has shown that the use of low-level laser therapy (LLLT) may be beneficial in accelerating bone tissue healing [7-10]. LLLT refers to irradiation with red-beam or near-infrared lasers (*e.g.* helium-neon and gallium-aluminum-arsenide) characterized by a wavelength of 600-1100 nm, an output power of 1-500 mW, and an energy density of 0.04-50 J/cm^2 [11]. The effect is not thermal, but rather related to photochemical reactions in the cells, which generate a series of modifications in tissue metabolism [12]. This is in contrast to high-energy lasers (*e.g.* carbon dioxide and neodymium-YAG), which can raise tissue temperatures high enough to cut and vaporize them [11, 13].

Address for correspondence: Serena Incerti Parenti, Reparto di Ortognatodonzia, Dipartimento di Scienze Biomediche e Neuromotorie, Università di Bologna, Via San Vitale 59, 40125 Bologna, Italy. E-mail: serena.incerti2@unibo.it.

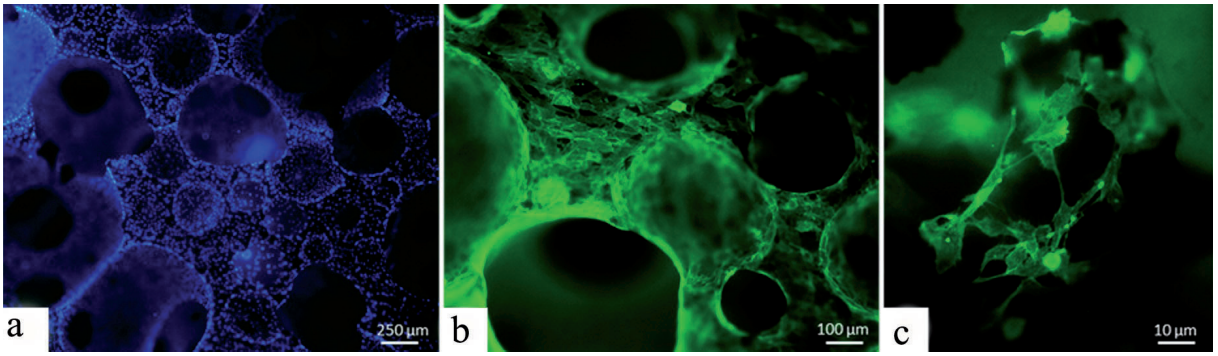

Figure 1
Immunofluorescence images of cells seeded on HA scaffold. Cell nuclei labeled with DAPI showed a well colonization of the scaffold (a). Cells exhibited a good morphology with no differences between each group both on the external surface (b) and in the inner section (c) of the scaffold. Scale bars: (a) 250 µm, (b) 100 µm, (c) 10 µm.

In particular, LLLT has recently attracted major interest in the field of tissue engineering of bone tissue because it can facilitate fracture consolidation by means of increased angiogenesis, collagen fiber deposition, bone cell proliferation and differentiation [8, 14-17]. This may lead to potential benefits in orthopaedics for bone fracture healing, in maxillofacial surgery for bone healing after fracture or surgical assisted rapid maxillary expansion (SARME) [18, 19] and, also, in dentistry to assist osseointegration of implants [20], to promote alveolar bone repair after tooth extraction [21], bone regeneration in midpalatal suture after rapid maxillary expansion (RME) [22] and bone remodelling during orthodontic movement of teeth [23, 24].

Nevertheless, the biological mechanisms that mediate these effects are not fully understood and need to be examined more rigorously at a cellular level. To date, *in vitro* studies have been carried out under non-standardized experimental conditions using two-dimensional (2D) cell cultures, while only a few of them did use 3D cell cultures which more accurately resemble the *in vivo* situation [25, 26]. It is well known that the flat surface of a conventional 2D culture is not representative of the physiological cell-cell and cell-ECM interactions found in real tissues [27]. Biomimetic scaffolds have shown potential in cell culturing and in investigating different aspects of the cell-matrix interaction in 3D like cell proliferation, migration and apoptosis or such physiological cell-cell and cell-ECM interactions. For all these reasons it was decided to use for this study a biomimetic porous hydroxyapatite (HA) scaffold, already used in clinic as a bone substitute, to investigate the effects of laser irradiation at a low dose on 3D human osteoblast-like culture.

MATERIALS AND METHODS

MG63 human osteoblast-like cells (Lonza, Italy) were cultured in Dulbecco Modified Eagle's (DMEM, PAA, Austria), containing 1% penicillin/streptomycin (100 U/mL-100 µg/ml) supplemented with 10% fetal bovine serum (FBS) and kept at 37 °C in an atmosphere of 5% CO_2. Cells were detached from culture flasks by trypsinization and centrifuged; cell number and viability were checked with trypan-blue dye exclusion test.

Scaffolds

The porous HA scaffolds is already commercially available as a biomimetic bone graft (Engipore, Finceramica S.p.A., Italy). Briefly, the samples (diameter 10.00 mm; height 4.00 mm) were prepared with a technology based on slurry expansion. As bioceramic powder was used commercial HA (Finceramica S.p.A., Italy) nanopowder calcined at 1000 °C for 5 h in a conventional muffle furnace. An aqueous slurry with high ceramic powder concentration (60 wt%) and opportune amounts of Dolapix CA (Zschimmer & Schwarz, Lahnstein, Germany) as dispersing agent was prepared. After 6 h of ball-milling with zirconia balls, Dermocin BS Conc (Fratelli Ricci, Italia) as foaming agent was added to the suspension and expanded in a known volume (40-60 vol% of the total) to achieve a controlled morphology and an inner porosity close to 80 vol%. The expanded suspension was poured into a mould and dried at room temperature. To consolidate and stabilize the structure of the "green" samples, a heat treatment was performed in an air furnace at 1250 °C for 2 h.

Cell seeding

Prior to seeding with cells, 32 scaffolds were subjected to a 1 h preconditioning soak in phosphate-buffered saline (PBS) at 37 °C and, then, placed into 24 multiwell tissue culture plates, with one scaffold per well. Several plates were used to increase the distance between the wells and avoid cross-laser-irradiation.

Cell seeding on top of the scaffolds was performed by dropping 100 µl of cell suspension (1 x 10^5 cells) onto the scaffold surface, and allowing cells attach for 1 h (37 °C, 5% CO_2), before addition of 1.5 mL medium in 24 multiwell plates. After a 6 h incubation step, each scaffold was carefully placed in a new 24 multiwell plate to eliminate any contribution of remnant cells from the cell suspension that might grow into the scaffold from its bottom surface. The medium was change every 2 days.

Laser irradiation

Three days after cells were seeded on scaffolds, laser irradiation was carried out using a gallium-aluminum-arsenide (GaAlAs) diode laser (Pocket Laser, Orotig

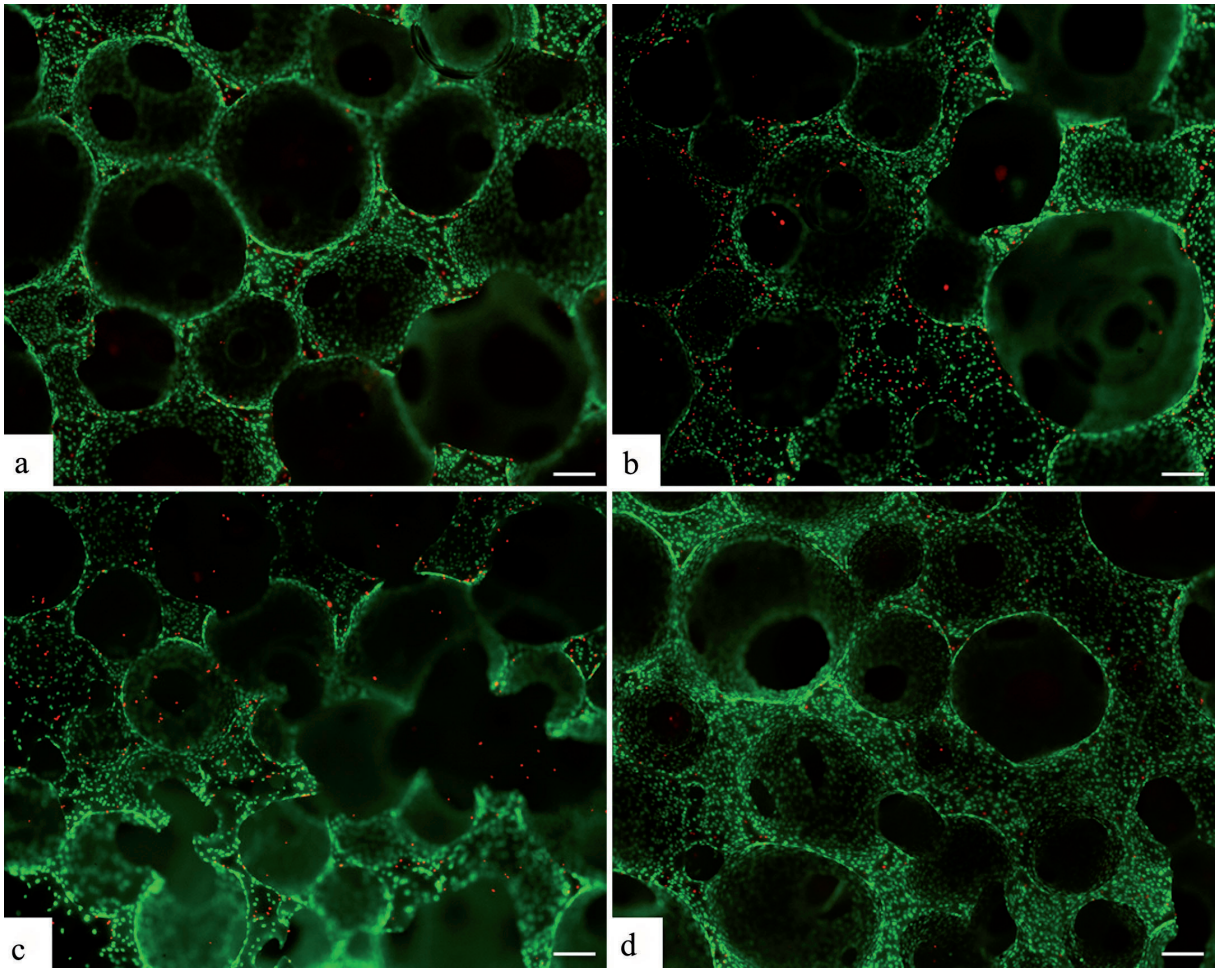

Figure 2
Cell viability analysis showed a very high ratio of live cells (in green) compared with the dead cells (in red) in all the groups. Group 1 (a), Group 2 (b), Group 3 (c), Group 4 (d). Scale bars: 200 µm.

s.r.l., Verona, Italy), which has a wavelength of 915 nm ± 10 nm and a maximum power output of 6 W ± 20% (sourced from the equipment manufacturer's specifications).

The laser beam was delivered in a continuous operating mode by an optical fiber 0.6 mm in diameter that was defocused at the tip by a concave lens to cover the whole top surface of each scaffold at a fixed distance of 14 mm (beam spot size at culture surface 1.13 cm^2; area irradiated 0.78 cm^2).

A single treatment session was performed at a dose of 2 J/cm^2. While keeping the dose constant, various power and exposure duration were used. Scaffolds were, thus, equally assigned to the following experimental conditions:

- Group 1 (8 scaffolds): underwent laser irradiation at a 10 mW power output for 157 seconds;
- Group 2 (8 scaffolds): underwent laser irradiation at a 50 mW power output for 31 seconds;
- Group 3 (8 scaffolds): underwent laser irradiation at a 90 mW power output for 17 seconds;
- Group 4 (8 scaffolds): did not undergo laser irradiation: served as control.

The laser equipment was calibrated by the manufacturer just prior to the study. Irradiation was carried out in a partial darkness, without other light influences except for the laser and in the absence of culture medium, which was removed immediately before and added immediately after irradiation.

All the groups were kept at 37 °C in an atmosphere of 5% CO_2 for 72 h and then morphological analysis and viability assays were performed.

Morphological analysis

Phalloidin staining was performed to assess cell adhesion and morphology of 2 samples for each group. After permeabilized with 0.5% Triton X-100 for 15 minutes, FITC-conjugate phalloidin solution was added for 30 minutes at 37 °C. After washing, 300 nM 4'-6-diamidino-2-phenylindole (DAPI) solution was added for 5 minutes in order to stain cell nuclei (Molecular Probes). One sample was finely cut with a scalpel in order to examine also the internal surface. The analysis was performed by an inverted fluorescence microscope (Nikon Ti-E, Nikon Corporation, Tokyo, Japan).

Cell viability and proliferation assays

For cell viability assay, samples were stained with a Live/Dead viability kit as according to the manufacturer's instructions (BioVision Research

Products, Mountain View, CA, USA). Briefly, the Live/Dead stain was prepared by adding equal amounts of 1mM Live-Dye and 2.5 mg/mL Propidium Iodide to the provided staining buffer. Samples were incubated with the stain for 15 minutes in the dark at 37 °C. Then the samples were rinsed 3 times in PBS before viewing using an inverted fluorescence microscope. One scaffold for each group was finely cut to examine the infiltration and viability of cells in the center region.

Cell proliferation was assessed using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. MTT was added to cell culture (0.5 mg/ml medium) and incubated at 37 °C for 3 h in darkness. Afterwards, the unreacted dye was removed and dimethylsulfoxide was added to dissolve the intracellular insoluble purple formazan product into a colored solution. The absorbance of the resulting solution was measured at 570 nm on a UV-visible spectrophotometric reader (Lambda 35 UV/VIS Spectrometer; Perkin Elmer Instrument, USA).

Statistical analysis

Data from *in vitro* cellular proliferation assay were presented as means ± standard deviations (SD). Between groups comparison was carried out with the One-Way ANOVA test ($p < 0.05$).

RESULTS

Cells grew into the porous scaffold structure and they entered into the scaffolds without any difference between the groups *(Figure 1a)*. Phalloidin staining did not show differences in cell morphology compared between each group. Attached cells exhibited their characteristic shape both on the external surface and into porous *(Figure 1b)*. Inner section of each scaffolds showed that, after laser irradiation, cells that had already entered the porous structures and attached the inner surface were not negatively affected by the treatment *(Figure 1c)*.

A very high ratio of live cells was well visible on each scaffold surface *(Figure 2a-d)*. The very small ratio of dead cells was comparable between laser-treated *(Figure 2a-c)* and untreated scaffolds *(Figure 2d)*.

Quantitative analysis with MTT test showed no difference in terms of cell viability between the groups (Group 1: 2.3708 ± 0.2051; Group 2: 2.3281 ± 0.3051; Group 3: 2.3781 ± 0.0889). Laser irradiation did not decrease cell growth in a statistically significant manner compared with non-irradiated controls (Group 4: 2.3221 ± 0.1687).

DISCUSSION

Nowadays, the focus of bone tissue engineering has moved towards the synthesis of 3D scaffolds with suitable mechanical properties, having the ability to promote cell function, in particular enhancing osteoblast adhesion, growth, and differentiation, as well as inducing the differentiation of mesenchymal cells into osteoblasts [28-30]. Increasing interest may be brought about the use of LLLT, since it has the potential to increase cell proliferation or to induce differentiation into specific cell types or both.

Porous HA scaffolds have been well described physically and chemically, including their macro- and micro-structure, mechanical properties [31, 32] but, so far, no studies have been carried out concerning the possible effects of LLLT on such cell-seeded scaffolds.

In the present study, focusing on growth behavior of human MG63 human osteoblast-like cells, a single 915 nm laser irradiation at a low dose (2 J/cm^2) did not interfere in cell adhesion and proliferation on porous HA scaffolds after an incubation period of 72 h. Cells showed well spread morphology and good adhesion on both the laser-treated and untreated scaffold trabeculae, but no statistically significant difference was observed in the proliferation rate when comparing laser-irradiated and non-irradiated samples. These finding suggests the lack of effect of 915 nm laser irradiation at a dose of 2 J/cm^2 on MG63 cell growth, thus being in contrast with previous studies demonstrating an increased proliferation of laser-irradiated osteoblastic cells grown on 2D cultures [8, 14, 16, 17, 33, 34]. This may be due to a range of factors, including laser dose, wavelength and different experimental conditions. Laser irradiation administered at a low dose should be more likely to produce a biostimulatory cell response when compared to the same wavelength at high dose [11]. Saracino *et al.* [35] had previously found that 910 nm laser irradiation inhibited proliferation in MG63 cells at a dose that was 3 times higher (6.7 J/cm^2), but stimulated the expression of proteins essential for bone formation due to an inverse correlation between cell growth and tissue specific gene expression. It is, therefore, possible to assume that, under the conditions of this *in vitro* study, a dose of 2 J/cm^2 was too high to excite physiological cell proliferation. However, possible laser effects on tissue specific gene expression should be taken into proper account in future investigations.

LLLT has been reported in the literature with wavelengths of 600-1100 nm, but the most effective cellular response might result from using laser wavelengths of 600-700 nm [11]. In this study a single treatment session was carried out using a diode laser with a wavelength of 915 nm because the equipment was already available in our Department [8, 36]. A dose of 2 J/cm^2 was chosen because it has already been reported as the most effective to stimulate cell proliferation under *in vitro* conditions [11]. While keeping constant this value, 3 different power outputs of 10 mW [37], 50 mW [38], and 90 mW [39] were used for respectively 157 seconds, 31 seconds, and 17 seconds. Power and exposure duration were chosen as the treatment variables between the laser-irradiated groups based in previous studies demonstrating that power density and exposure duration can significantly influence cell growth *in vitro* [40, 41]. The lack of significant difference in the proliferation rate between the laser-irradiated groups indicates that future investigations comparing the effects of laser irradiation on MG63 cells at different dose and wavelength regimens are needed in order to determine the optimal set of laser parameters for maximum cell yield and the safe combination for clinical application.

So far, there have been few attempts to evaluate the effects of LLLT on human osteoblast-like cells [8, 34-36] and, particularly, no data are yet available on 3D cell cultures. Moving from 2D to 3D cultures is motivated by the need to work with cellular models that mimic the functions of living tissues, leading to more useful data and more relevant research [27]. Renno *et al.* [26] observed that a single 830 nm laser irradiation at a dose that was 5 times higher compared with the one used in the present study (10 J/cm^2) had an inhibitory effect on proliferation of osteoblastic cells seeded on 3D glass-ceramic scaffolds and, conversely, a stimulatory effect if cells were seeded on 2D standard monolayers [8], hypothesizing that interactions of laser with matrix compounds and structure should be taken into proper account. It is, therefore, reasonable to assume that, under the conditions of this *in vitro* study, laser irradiation failed to determine any change in cell growth also due to reflection, refraction or absorption in the material of the scaffold.

CONCLUSIONS

Under the experimental conditions of this 3D *in vitro* study, a single 915 nm laser application at a dose of 2 J/cm^2 did not interfere in adhesion and morphology of MG63 human osteoblast-like cells seeded on porous HA scaffolds currently used for bone tissue engineering after an incubation period of 72 h. No statistically significant difference was observed in the proliferation rate when comparing laser-irradiated and non-irradiated samples, suggesting the lack of effect of laser irradiation on cell growth. Future investigations are needed to compare the effect of laser irradiation on MG63 human osteoblast-like cells at different dose and wavelength regimens in order to determine the optimal set of laser parameters for maximum cell yield and for safe clinical application.

Acknowledgments

The authors wish to express gratefulness to Orotig s.r.l., Verona, Italy for providing the laser therapy apparatus.

Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

Received on 31 December 2012.
Accepted on 8 May 2013.

REFERENCES

1. Barbetta A, Bedini R, Pecci R, Dentini M. Role of X-ray microtomography in tissue engineering. *Ann Ist Super Sanità* 2012;48(1):10-8. DOI: 10.4415/ANN_12_01_03.
2. Trubiani O, Fulle S, Traini T, Paludi M, la Rovere R, Orciani M, Caputi S, Piattelli A. Functional assay, expression of growth factors and proteins modulating bone-arrangement in human osteoblasts seeded on an anorganic bovine bone biomaterial. *Eur Cell Mater* 2010;20:72-83.
3. Hattar S, Berdal A, Asselin A, Loty S, Greenspan DC, Sautier JM. Behaviour of moderately differentiated osteoblast-like cells cultured in contact with bioactive glasses. *Eur Cell Mater* 2002;4:61-9.
4. Garaci E, Rasi G. A critical evaluation of the process of drug discovery and evaluation: is the current approach the best possible one? *Ann Ist Super Sanità* 2011;47(1):1. DOI: 10.4415/ANN_11_01_01.
5. Garaci E, Popoli P. New challenges in translational medicine: transforming the advancement of science into cures. Preface. *Ann Ist Super Sanità* 2011;47(1):5-7. DOI: 10.4415/ANN_11_01_02.
6. Belardelli F, Rizza P, Moretti F, Carella C, Galli MC, Migliaccio G. Translational research on advanced therapies. *Ann Ist Super Sanità* 2011;47(1):72-8. DOI: 10.4415/ANN_11_01_15.
7. Mizokami T, Aoki K, Iwabuchi S, Kasai K, Yamazaki Y, Sakurai T, Samejima K, Yoshii N. Low reactive level laser therapy - A clinical study: Relationship between pain attenuation and the serotonergic mechanism. *Laser Ther* 1993;5(4):165-8.
8. Renno ACM, McDonnell PA, Parizotto NA, Laakso EL. The effects of laser irradiation on osteoblast and osteosarcoma cell proliferation and differentiation *in vitro*. *Photomed Laser Surg* 2007;25(4):275-80. DOI: 10.1089/pho.2007.2055.
9. Hawkins D, Abrahamse H. Effect of multiple exposures of low-level laser therapy on the cellular responses of wounded human skin fibroblasts. *Photomed Laser Surg* 2006;24(6):705-14. DOI: 10.1089/pho.2006.24.705.
10. Tracey S, Gracco A. Lasers in Orthodontics. In: Graber LW, Vanarsdall RL, Vig KWL (Eds). *Orthodontics. 5th Edition current principles and techniques*. St. Louis, Mo: Elsevier Mosby; 2012. p 1051-73.
11. Alghamdi KM, Kumar A, Moussa NA. Low-level laser therapy: a useful technique for enhancing the proliferation of various cultured cells. *Lasers Med Sci* 2012;27(1):237-49. DOI: 10.1007/s10103-011-0885-2.
12. Brosseau L, Robinson V, Wells G, Debie R, Gam A, Harman K, Morin M, Shea B, Tugwell P. Low-level laser therapy (Classes I, II and III) for treating rheumatoid arthritis. *Cochrane Database Syst Rev* 2005;19(4):CD002049.
13. Pogrel MA, Chen JW, Zhang K. Effects of low-energy gallium-aluminum-arsenide laser irradiation on cultured fibroblasts and keratinocytes. *Lasers Surg Med* 1997;20(4):426-32. DOI: 10.1002/(SICI)1096-9101(1997)20:4<426::AID-LSM8>3.0.CO;2-S.
14. Ozawa Y, Shimizo N, Kariya G. Low-energy laser irradiation stimulates bone nodule formation at early stages of cell culture in rat calvarial cells. *Bone* 1998;22(4):347-54. DOI: 10.1016/S8756-3282(97)00294-9.
15. Pinheiro ALB, Limeira F, Gerbi M. Biomodulatory effects of LLLT on bone regeneration. *Laser Ther* 2001;13:73-9.
16. Ueda Y, Shimizu N. Effects of pulse frequency of low-level laser therapy (LLLT) on bone nodule formation in rat calvarial cells. *J Clin Laser Med Surg* 2003;21(5):271-7.
17. Fujimoto K, Kiyosaki T, Mitsui N, Mayahara K, Omasa S, Suzuki N, Shimizu N. Low-intensity laser irradiation stimulates mineralization via increased BMPs in MC3T3-E1 cells. *Lasers Surg Med* 2010;42(6):519-26. DOI: 10.1002/lsm.20880.
18. Pogrel MA, Kavan LB. Surgically assisted rapid maxillary expansion in adults. *Int J Adult Orthod Orthog Surg* 1992;7(1):37-41.

19. Angeletti P, Pereira MD, Gomes HC, Hino CT, Ferreira LM. 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* 2010;109(3):e38-46. DOI: 10.1016/j.tripleo.2009.10.043.
20. Petri AD, Teixeira LN, Crippa GE, Beloti MM, De Oliveira PT, Rosa AL. Effects of low-level laser therapy on human osteoblastic cells grown on titanium. *Braz Dent J* 2010;21(6):491-8. DOI: 10.1590/S0103-64402010000600003.
21. Takeda Y. Irradiation effect of low laser on alveolar bone after tooth extraction. Experimental study in rats. *Int J Oral Maxillofac Surg* 1998;17(6):388-91.
22. Saito S, Shimizu N. Stimulatory effects of low-power laser irradiation on bone regeneration in midpalatal suture during expansion in the rat. *Am J Orthod Dentofac Orthop* 1997;11(5):525-32.
23. Cruz DR, Kohara EK, Ribeiro MS, Wetter NU. Effects of low-intensity laser therapy on the orthodontic movement velocity of human teeth: a preliminary study. *Lasers Surg Med* 2004;35(2):117-20. DOI: 10.1002/lsm.20076.
24. Kawasaki K, Shimizu N. Effects of low-energy laser irradiation on bone remodeling during experimental tooth movement in rats. *Lasers Surg Med* 2000;26(3):282-91. DOI: 10.1002/(SICI)1096-9101(2000)26:3<282::AID-LSM6>3.0.CO;2-X.
25. Abramovitch-Gottlib L, Gross T, Naveh D, Geresh S, Rosenwaks S, Bar I, Vago R. Low-level laser irradiation stimulates osteogenic phenotype of mesenchymal stem cells seeded on a three-dimensional biomatrix. *Lasers Med Sci* 2005;20(3-4):138-46. DOI: 10.1007/s10103-005-0355-9.
26. Renno AC, McDonnell PA, Crovace MC, Zanotto ED, Laakso L. Effect of 830 nm laser phototherapy on osteoblasts grown in vitro on Biosilicate scaffolds. *Photomed Laser Surg* 2010;28(1):131-3.
27. Pampaloni F, Reynaud EG, Stelzer EH. The third dimension bridges the gap between cell culture and live tissue. *Nat Rev Mol Cell Biol* 2007;8(10):839-45. DOI: 10.1038/nrm2236.
28. Drury JL, Mooney DJ. Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials* 2003;24(24):4337-51. DOI: 10.1016/S0142-9612(03)00340-5.
29. Chen QZ, Efthymiou A, Salih V, Boccaccini AR. Bioglass-derived glass-ceramic scaffolds: study of cell proliferation and scaffold degradation *in vitro*. *J Biomed Mater Res A* 2008;84(4):1049-60. DOI: 10.1002/jbm.a.31512.
30. Meleo D, Bedini R, Pecci R, Mangione F, Pacifici L. Microtomographic and morphometric characterization of a bioceramic bone substitute in dental implantology. *Ann Ist Super Sanità* 2012;48(1):59-64. DOI: 10.4415/Ann_12_01_10.
31. Martinetti R, Dolcini L, Belpassi A, Quarto R, Mastrogiacomo M, Cancedda R, Labanti M. Inspired Porosity for Cells and Tissues. *Key Engineering Materials* 2004;254-256:1095-8.
32. Martinetti R, Dolcini L, Mangano C. Physical and chemical aspects of a new porous hydroxyapatite. *Anal Bioanal Chem* 2005;381(3):634-8. DOI: 10.1007/s00216-004-2957-7.
33. Aleksic V, Aoki A, Iwasaki K, Takasaki AA, Wang CY, Abiko Y, Ishikawa I, Izumi Y. Low-level Er:YAG laser irradiation enhances osteoblast proliferation through activation of MAPK/ERK. *Lasers Med Sci* 2010;25(4):559-69. DOI: 10.1007/s10103-010-0761-5.
34. Stein A, Benayahu D, Maltz L, Oron U. Low-level laser irradiation promotes proliferation and differentiation of human osteoblasts *in vitro*. *Photomed Laser Surg* 2005;23(2):161-6. DOI: 10.1089/pho.2005.23.161.
35. Saracino S, Mozzati M, Martinasso G, Pol R, Canuto RA, Muzio G. Superpulsed laser irradiation increases osteoblast activity via modulation of bone morphogenetic factors. *Lasers Surg Med* 2009;41(4):298-304. DOI: 10.1002/lsm.20762.
36. Huang TH, Lu YC, Kao CT. Low-level diode laser therapy reduces lipopolysaccharide (LPS)-induced bone cell inflammation. *Lasers Med Sci* 2012;27(3):621-7. DOI: 10.1007/s10103-011-1006-y.
37. Fujihara NA, Hiraki KRN, Marques MM. Irradiation at 780 nm increases proliferation rate of osteoblasts independently of dexamethasone presence. *Lasers Surg Med* 2006;38(4):332-6. DOI: 10.1002/lsm.20298.
38. Pires Oliveira DA, de Oliveira RF, Zangaro RA, Soares CP. Evaluation of low-level laser therapy of osteoblastic cells. *Photomed Laser Surg* 2008;26(4):401-4. DOI: 10.1089/pho.2007.2101.
39. Coombe AR, Ho CT, Darendeliler MA, Hunter N, Philips JR, Chapple CC, Yum LW. The effects of low-level laser irradiation on osteoblastic cells. *Clin Orthod Res* 2001;4(1):3-14.
40. Almeida-Lopes L, Rigau J, Zangaro RA, Guidugli-Neto J, Jaeger MM. Comparison of the low-level laser therapy effects on cultured human gingival fibroblasts proliferation using different irradiance and same fluence. *Lasers Surg Med* 2001;29(2):179-84. DOI: 10.1002/lsm.1107.
41. Hans HFI, Breugel V, Bar D. Power density and exposure time of He-Ne laser irradiation are more important that total energy dose in photo-biomodulation of human fibroblasts in vitro. *Lasers Surg Med* 1992;12(5):528-37. DOI: 10.1002/lsm.1900120512.