Journal of Photochemistry & Photobiology, B: Biology 163 (2016) 14–21

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Low intensity lasers differently induce primary human osteoblast proliferation and differentiation

Flávia A. Oliveira [a], Adriana A. Matos [a], Mariana R. Santesso [a], Cintia K. Tokuhara [a], Aline L. Leite [a], Vanderley S. Bagnato [b], Maria A.A.M. Machado [a], Camila Peres-Buzalaf [c], Rodrigo C. Oliveira [a,*]

[a] Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Bauru, SP, Brazil
[b] Departamento de Física e Ciência dos Materiais, Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, SP, Brazil
[c] Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Sagrado Coração, Bauru, SP, Brazil

ARTICLE INFO

Article history:
Received 25 April 2016
Accepted 4 August 2016
Available online 06 August 2016

Keywords:
LLLT
LLL
LED
Phototherapy
Human osteoblasts
ERK

ABSTRACT

Among various compounds used in research and clinic for degenerative bone diseases, low level laser therapy (LLLT), comprising low level lasers (LLL) and light emitting diodes (LEDs), has been investigated regarding its effects on bone metabolism. They have specific wavelengths but in general act as a cellular biomodulator, and as a therapeutic agent, rebalancing and normalizing their activity. However, they are not standardized yet, since their parameters of use are relevant for the effects and mechanisms of action. Therefore, the aim of this study was to compare the influence of two spectrums of LLL and LED phototherapy, at the same energy densities (10 and 50 J/cm^2), on human osteoblasts proliferation and differentiation. The involvement of ERK signaling on proliferation was also investigated by evaluating its activation during proliferation under different phototherapies by western blotting and CFSE-based osteoblast proliferation was measured in a presence or absence of the ERK-specific inhibitor. Osteogenic differentiation was evaluated through in vitro mineralization and gene expression of type I collagen (COL1A1) and osteonectin (SPARC) by Real Time- PCR. Increases in viable cells and proliferation were obtained after irradiation, regardless of LLLT type. However, only red at 10 J/cm^2 and infrared at both doses, but not LED, induced ERK1/2 activation. In the presence of ERK inhibitor, the LLL-induced proliferation was prevented. In addition, while COL1A1 gene expression was upregulated by red laser, SPARC does so by infrared stimulation. However, LED, at both doses, increased both COL1A1 and SPARC expression. All LLLT increased mineralization, dependent on the dose and time. Thus, LLL and LED differently modulated the metabolism of human osteoblasts, increasing proliferation by mechanism dependent or not of ERK signaling activation and osteogenic differentiation markers.

1. Introduction

Low level laser therapy (LLLT) is a treatment based on red and infrared lasers or light-emitting diodes (LEDs) that promotes changes at cellular levels through photobiomodulation. The therapeutic use of low-level laser (LLL) has become widespread in biology, dentistry and medicine. Several benefits have been accompanied by its use such as pain relief, stimulation of mitochondrial activity [1], recovery from nerve injury [2,3], angiogenesis [4], modulation of inflammation [5], vasodilation [6], and tissue healing [7]. Mechanisms involved in LLL-based tissue healing are dependent on the increased cell turnover, recruitment, proliferation and differentiation. Target cells include mesenchymal cells [8], keratinocytes, fibroblasts [9], endothelial cells [10], and osteoblast [11]. Considering the variety of laser, time of exposure, cell types and application protocols, it is still difficult to standardize positive clinical LLL based-protocols. Moreover, the fact that the mechanisms by which they play an effective role in several biological processes have not been fully understood.

In this context, several cellular and molecular mechanisms have been proposed to the LLL-induced biological responses. Data from our laboratory has determined that two different LLL, red and infrared lasers, both at a potency of 20 mW and energy density of 1.9 J/cm^2 or 3.8 J/cm^2 increases mouse preosteoblast MC3T3-E1 proliferation, alkaline phosphatase (ALP) and metalloproteinase (MMP)-2 activity at early time point but not of MMP-9 (unpublished results). Also, it is extensively described that the stimulatory effect of LLL in bone cells is dependent on intracellular signaling protein activation [12]. A recent study demonstrated that LLLT at 70 mW power and energy density of 3 J/cm^2 stimulated primary human alveolar bone-derived osteoblast differentiation when cultured on titanium discs [13].

However, there still no data about the influence of light exposition on human primary osteoblast at late periods under osteogenic stimulus. Neither the impact of LLL-induced mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinases (ERK) activation on cellular proliferation and gene transcription codifying extracellular matrix formation markers and mineralization. The aim of this study was to

* Corresponding author at: Alameda Octávio Pinheiro Brisolla, 9-75, Bauru, SP 17012-901, Brazil.
E-mail address: rodrigocardoso@usp.br (R.C. Oliveira).

http://dx.doi.org/10.1016/j.jphotobiol.2016.08.006
1011-1344/

evaluate the effects of two doses of red, infrared and LED on proliferation and differentiation of primary human osteoblast for driving bone regeneration.

2. Material and Methods

2.1. Human Primary Osteoblast Isolation and Culture

Cells were collected from bone explants during orthopedic surgeries. The procedure was approved by the local Ethics Committee, Fluminense Federal University (protocol # 232/08) and the Ethics Committee of the Bauru Dental School-University of Sao Paulo (protocol # 95/2011). Explants were treated with 0.25% collagenase for 2 h and then placed in 25 cm^2 flasks in Dulbecco's Modified Eagle Medium (DMEM) culture medium supplemented with 10% fetal bovine serum (FBS). Cells were maintained at 37 °C in an atmosphere containing 5% CO_2 and 95% air. After single cells were attached to the plastic flask, non-adherent cells were removed by changing the medium. When confluent, the cells were sub-cultured with 0.25% trypsin-EDTA. Cells of passage 9 were used for the experiments. When appropriated, cells were cultured or not in osteogenic medium containing 50 μg/mL acid ascorbic and 10 mM β-glicerophosphate in DMEM/ 10% FBS.

2.2. Phototherapy

Low intensity laser irradiations were done by using the Twin Flex Evolution diode laser (MM Optics-São Carlos, SP, Brazil). Red laser at a wavelength of 660 nm and InGaAlP active medium, infrared at a wavelength of 780 nm and GaAlAs active medium or light emitting diode (LED) at a wavelength of 637 ± 15 nm were applied to cells. They were set up as continuous mode, potency of 40 mW, power density of 1 W/cm^2, energy density of 10 and 50 J/cm^2, energy dose of 6 and 31 J, and exposure time of 10 and 50 s, respectively. All parameters are described in Table 1. Power measurements were checked by using the Laser Check PowerMeter (Coherent Inc., Santa Clara, CA, USA). The irradiation with different lasers was performed under the plate [14]. In 96 well plates, the stimulation was performed with static probe mode since the diameter of each well and the laser spot area is 6.4 mm and 4 mm^2, respectively. In 24-well plates (15.4 mm diameter), irradiation was done with a scanning mode [15]. Single or double application protocols were performed for cell viability and proliferation where the last was applied with an interval of 6 h. For differentiation and mineralization assays, the irradiation was performed every 6 days. The assays were performed at least in triplicate.

2.3. Cell Viability

The cell viability was assessed after 1, 2 and 3 days after the last irradiation by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) and Crystal violet assays. Cells were plated in 96-well plates at a density of 2×10^3 cells in DMEM/10% FBS. Upon adhesion, 5% FBS-containing medium was replaced, to highlight the effects of the phototherapies [15]. As follows, adherent cells were irradiated with single or double application of each phototherapy. Control cells were not treated but received the same experimental conditions.

For MTT assay, the cells were washed with phosphate-buffered saline (PBS) followed by the addition of solution containing 0.5 mg MTT per mL of medium. After incubation at 37 °C for 4 h protected from light, DMSO was added to the cells. The absorbance was read in the spectrophotometer (Fluostar OPTIMA, BMG Labtech, Offenburg, Germany) at 562 nm. For crystal violet assay, the cells were washed and then added 100% methanol for 10 min. After been removed, the solution containing 0.2% crystal violet in 2% ethanol was added to the cells for 3 min followed by additional wash and incubation with 0.05 mol/L in 50% ethanol solution for 10 min. The plates were read at 540 nm.

2.4. Western Blotting

Cells were plated in 24-well plates at a density of 4×10^4 cells. Adherent cells were irradiated with different phototherapies (n = 24 for each condition). After 10 min at 37 °C, the cells were lysed with buffer solution containing 50 mM Tris-HCl (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$, and 0.2% Nonidet P-40 supplemented with protease inhibitors (Roche Diagnostics) and phosphatase inhibitor (0.2 M sodium orthovanadate - Calbiochem). The lysates were pooled, sonicated and centrifuged at 1000 rpm for 10 min at 4 °C. Protein samples (40 μg) were applied to electrophoresis in Tris-HCl 10% polyacrylamide gel and subsequently transferred to PVDF membrane. It was immuno-labeled with rabbit polyclonal anti-phospho-ERK or anti-ERK primary antibodies (Cell Signaling) followed by secondary anti-rabbit IgG conjugated to HRP (Horseradish Peroxidase - Cell Signaling) and ECL reagent (enhanced chemiluminescence) detection (Amersham Biosciences). The relative densities of the bands were determined by densitometry analysis using Image J software (National Institutes of Health, NIH Image). The density values obtained were corrected by subtraction of the background values.

2.5. Proliferation Assay

Cell proliferation was performed by labeling cells with carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes) followed by flow cytometry analysis. Osteoblasts at a density of 3×10^5 (n = 3) were incubated with 10 μM CFSE for 10 min at 4 °C, according to the manufacturer's protocol. After washing with PBS, the labeled cells were plated in 24-well plates followed by double application phototherapies. When appropriate, the cells were pretreated with 20 μM ERK inhibitor (PD98059) for 30 min at 37 °C. After 3 days of the last irradiation, cells were trypsinized and acquired in FACsorting (BD Biosciences). Analyzes were performed using the CellQuest program (CellQuest software, BD).

2.6. Real Time RT-PCR

5×10^2 cells in 96-well plates in different conditions had the mRNA extracted and transcribed into cDNA using TaqMan® Gene Expression Cells-to-CT™ (Applied Biosystems) kit according to manufacturer's recommendations after 7, 14, 21 and 28 days of culture. The cDNA samples were incubated with Taqman® Gene Expression Master Mix and Taqman® Gene Expression assay (Applied Biosystems) for SPARC (secreted protein acidic and rich in cysteine-also named osteonectin) or COL1A1 (type I collagen) and were read in the Real-Time RT-PCR System ViiATM7 (Applied Biosystems). PCR amplification was performed

Table 1
Irradiation parameters.

Light source	Wavelength (nm)	Output power (mW)	Power density (W/cm^2)	Energy density (J/cm^2)	Energy dose (J)	Exposure time (s)	Application mode
Red laser (InGaAlP)	660	40	1	10	6	10	Continuous
	660	40	1	50	31	50	Continuous
Infrared (GaAlAs)	780	40	1	10	6	10	Continuous
	780	40	1	50	31	50	Continuous
LED	637 ± 15	40	1	10	6	10	Continuous
	637 ± 15	40	1	50	31	50	Continuous

using the following primer sets: SPARC, 5′-AAACCGAAGAGG-AGGTGGTG-3 (forward), 5′-GCAAAGAAGTGGCAGGAAGA-3′ (reverse); Col1A1: 5′-CAGGTCTCGGTCATGGTACCT-3′ (forward), 5′-GTCGAGGGC-CAAGACGAA-3′ (reverse); GAPDH: 5′-TGGCAAAGTGGAGATTGTTGC-3′ (forward), 5′-AAGATGGTGATGGGCTTCCCG-3′ (reverse). The GAPDH expression was used a control.

2.7. Mineralization Assay

The mineralization was assessed by Alizarin Red S Staining (Sigma) in 12-well plated cells at a density of 4×10^4 and cultivated in osteogenic medium. Red, infrared or LED irradiations were applied every 6 days. After 14, 21 and 28 days of culture, the supernatants were removed and cells were washed with PBS followed by fixation with 4% formaldehyde for 5 min at room temperature (RT). Cells were incubated with 2% alizarin solution at pH 4.2 for 10 min at RT followed by three consecutive washes with ultrapure water. Then, quantitative analysis was evaluated by the colorimetric method [13]. Dried plates were added with 10% acetic acid for 30 min under agitation at RT. The content of each well was heated at 85 °C for 10 min and then kept on ice for 5 min followed by centrifugation at 20,000*g* for 15 min. A volume of 100 μL was transferred to 96-well plates, when 10% ammonium hydroxide was added to neutralize the acid. The absorbance was measured at 405 nm.

2.8. Statistical Analysis

The results were statistically analyzed using GraphPad Prism 4 Software (La Jolla, CA, USA). Groups were subjected to parametric ANOVA One-way or Two-way, followed by Tukey's test. The significance was given when $P < 0.05$. All samples passed to normality assessed by Kolmogorov and Smirnov test. Comparisons to control cells, and to different type of LLLT (when significantly different from control cells) were adopted.

3. Results

3.1. Red, Infrared and LED Upmodulate Osteoblast Viability and Proliferation

To evaluate effectiveness of differential LLLT protocols, both single and double applications of each were used. When assessed by MTT, an increase in the cellular viability after stimulation with different phototherapies in specific time points and doses was observed. Cells singly irradiated with red laser and infrared both at 50 J/cm^2 and LED at 10 J/cm^2 had greater viability compared to control at day 1 (Fig. 1a). On day 2, the same effect was observed by LED at 10 J/cm^2, infrared at 50 J/cm^2 and red laser at both doses. However, on day 3, none LLLT increased cell viability. Cells irradiated twice presented increased of viability after red laser and LED at both doses at day 2 compared to control

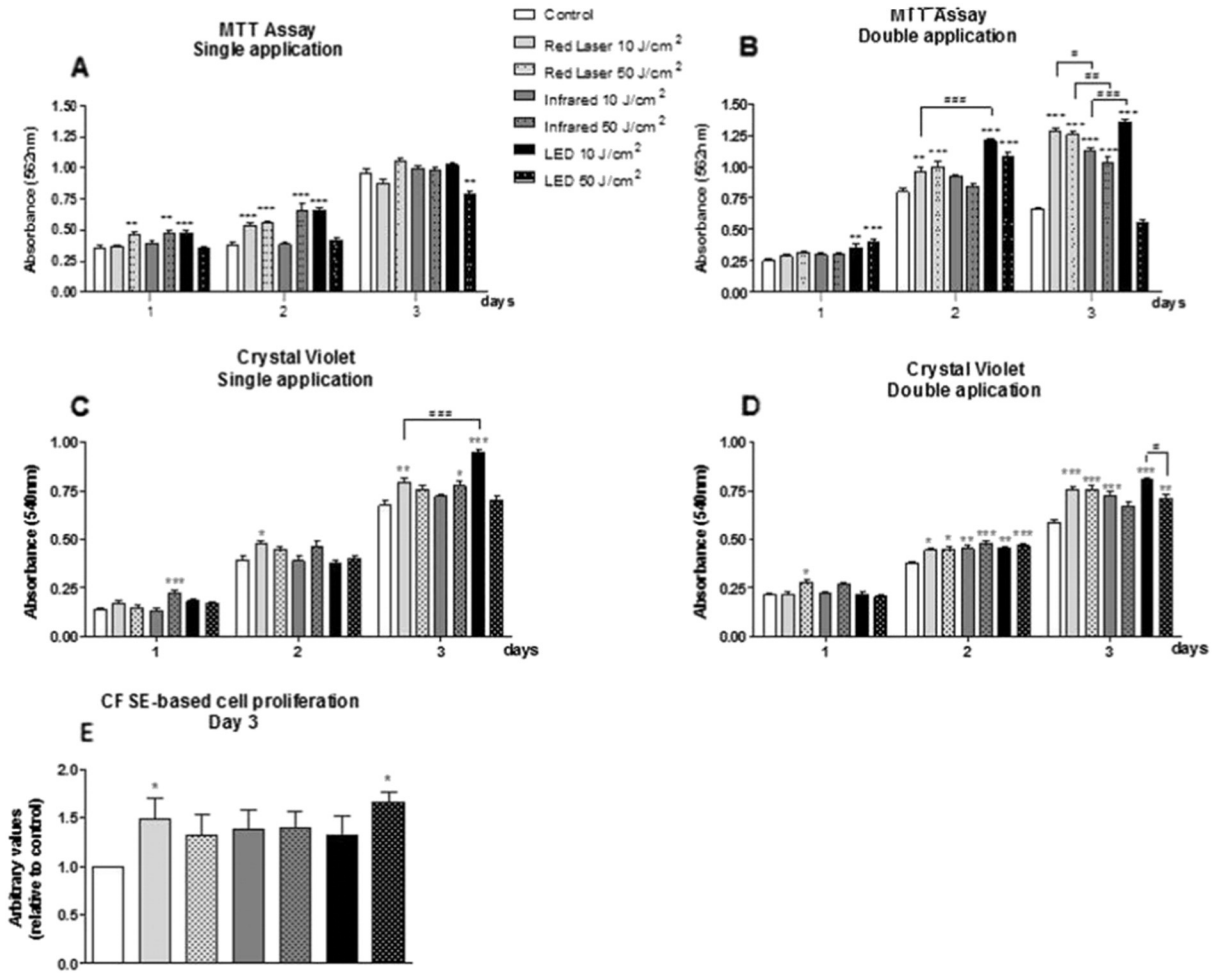

Fig. 1. LLLT effects on human osteoblast viability and proliferation evaluated by (a,b) MTT, (c,d) crystal violet and (e) CFSE-based assays. The graphs show the irradiation performed in (a,c) single or (b,d,e) double applications at the energy density of 10 or 50 J/cm^2. *P < 0.05, **P < 0.01, and ***P < 0.001 represent significant differences compared to control cells. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, and $^{\#\#\#}$P < 0.001 represent significant differences among irradiated cells. Results express the mean ± SE of (a–d) absorbance values and (e) fluorescence intensities.

non-irradiated cells, whereas the last (at 10 J/cm^2) was significantly higher than red-irradiates cells (Fig. 1b). On day 3, all LLLT increased viability compared to control, except LED at 50 J/cm^2. In addition, significant differences among LLLTs were observed, been LED at 10 J/cm^2 the most potent (Fig. 1b).

When assessed by crystal violet, we showed that single application of infrared at 50 J/cm^2 and red laser 10 J/cm^2 increased cell viability on day 1 and day 2, respectively (Fig. 1c). Also, red laser and LED both at 10 J/cm^2 and infrared at 50 J/cm^2 significantly increased viability compared to control cells (Fig. 1c). On the 2nd day, double application of all LLLTs enhanced viability compared to control cells, but no differences were found among them (Fig. 1d). And on day 3, red laser and LED at both doses and infrared at 10 J/cm^2 also increased it. Comparing doses of LED, a greater effect was observed for the lower (Fig. 1d). In general and regardless of assay type, a greater effectiveness was obtained for double applications.

Thus, we next evaluated the CFSE-labeled osteoblast proliferation in irradiated and non-irradiated cells on day 3. Compared to control, red laser at 10 J/cm^2 and LED at 50 J/cm^2 significantly increased osteoblast proliferation (Fig. 3e and supplementary Fig. 1).

3.2. Red and Infrared Lasers Enhance Proliferation Through ERK Signaling

Since LLLTs were efficient to increase viability and/or proliferation, we next evaluated the signaling mechanism by which they act. The results showed that red at 10 J/cm^2 and infrared at both doses significantly increased the phosphorylation of ERK1/2 (p-ERK) compared with control cells (Fig. 2a and b). No differences were observed among them. To verify the implication of ERK activation on LLLT-induced proliferation, we evaluated it in the presence of a specific ERK inhibitor, PD98059 compound. The results showed that inhibition of ERK prevented the positive effect of either red or infrared lasers on cell proliferation (Fig. 2c and supplementary Fig. 2).

3.3. Gene Expression of Type I Collagen and SPARC as Osteogenic Markers

To evaluate the effect of different LLLT on osteogenic differentiation, we measured expression of Col1A1 and SPARC genes. On day 7, Col1A1 mRNA increased after red laser at 10 J/cm^2 and LED at both doses compared to the control (Fig. 3a). Within 14 days, LED at 50 J/cm^2 positively modulated its expression and the lower dose enhanced on the 21st day. No differences were observed among the irradiation types and doses (Fig. 3a).

Regarding SPARC gene, infrared and LED groups, both at 50 J/cm^2, positively stimulated its expression on day 7 (Fig. 3b). LED-based irradiation also increased SPARC mRNA. On the 28th day, its expression was significantly higher under infrared and LED at 10 J/cm^2, compared to non-irradiated control cells. In addition, comparing these two types, infrared was significantly more effective than LED in increasing SPARC expression (Fig. 3b).

3.4. Effect of LLLTs on the Matrix Mineralization

Since all LLLT, at specific type, dose and time, had an impact on bone matrix formation, we analyzed if they also affect its mineralization, as follows. Calcium deposition was quantitatively measured through colorimetric analysis. On the 7th day, we showed that infrared, at 10 J/cm^2 and LED, at 50 J/cm^2, significantly augmented it when compared to control cells. On day 14, red and LED both at 50 J/cm^2 and infrared at 10 J/cm^2 presented the same effect. On day 21, red laser at both doses and infrared at 10 J/cm^2 also increased calcium deposition whereas only red at the lower dose did it on the 28th day (Fig. 4). No differences were noticed among different types of LLLTs.

4. Discussion

In the present study, we demonstrated that different LLLTs, at the same parameters, affect proliferation and differentiation of primary

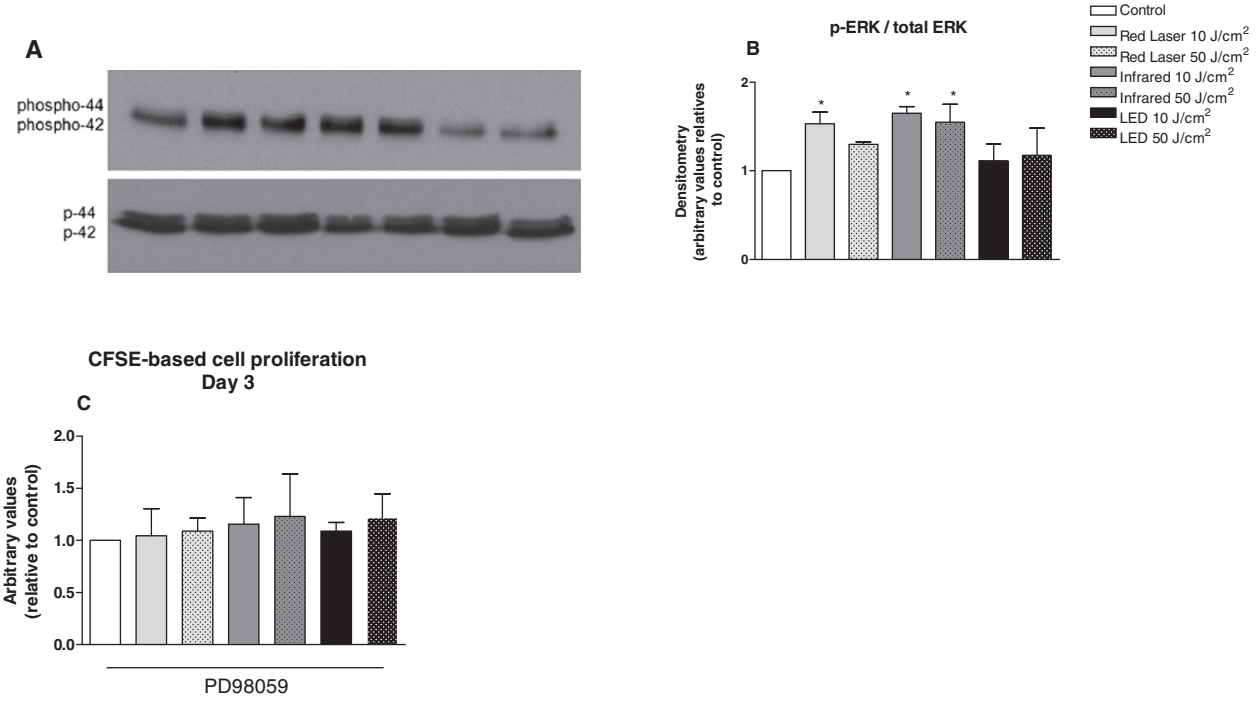

Fig. 2. ERK activation in osteoblasts treated with phototherapies. (a) Representative figure of control cells and red laser, infrared or LED irradiated at an energy density of 10 or 50 J/cm^2. (b) Densitometric analysis of western blotting. Arbitrary values are relative to obtained for control cells, adopted as 1. (c) ERK inhibition impaired increased proliferation on day 3 induced by phototherapies. *P < 0.05 represents significant differences compared to control cells. Results express the mean ± SE of arbitrary values of three experiments, taking the control cells as 1.

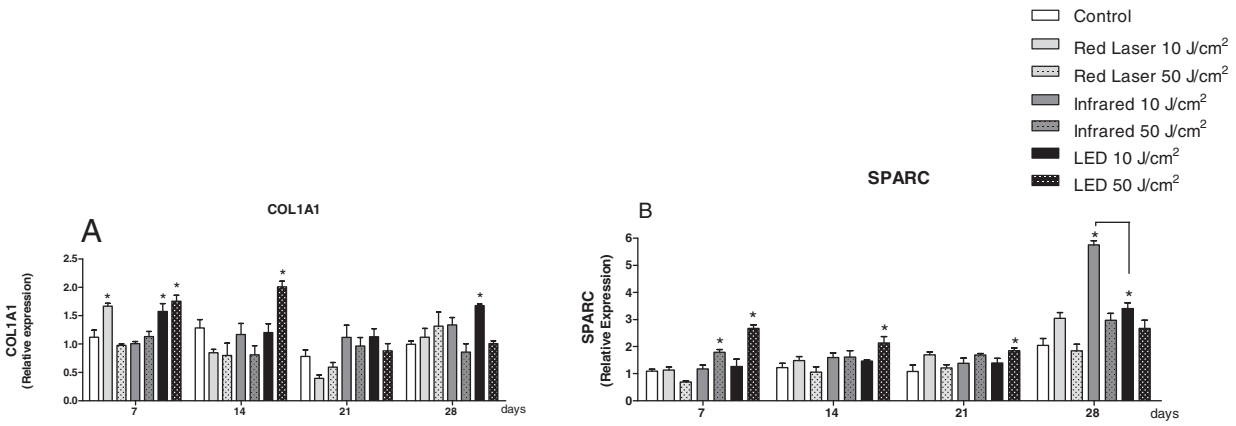

Fig. 3. LLLT effects on Col1A1 and SPARC gene expression by osteoblasts under osteogenic stimulation. Real time RT-PCR analysis for (a) Col1A1 and (b) SPARC expression were performed in cells treated or not with different phototherapies for 28 days. GAPDH gene expression was used as control. *P < 0.05 represents significant differences compared to control cells or as indicated. Results express the mean ± SE from 3 independent experiments performed in triplicate.

human osteoblasts. Although several studies have focused on this topic, the strategies for their application systems are not completely established because the parameters of use have not been standardized yet. In addition, few studies employed it on primary human osteoblasts. Accumulating evidences show that laser at low-level intensity increases proliferation of osteogenic lineage cells, including mesenchymal stem cells, preosteoblast and osteoblast [12,16,17]. Thus, LLLTs have promising effects on osteogenesis through stimulation of bone cells and bone formation [13,18–21]. But they are very restricted to their parameters, which high power densities and prolonged time of exposure may inhibit these biological responses [22]. Study showed that laser therapy with 90 and 150 J/cm^2 doses do not stimulate the proliferation and differentiation of osteoblasts [15]. Our group previously determined that red and infrared spectrums at a power potency of 20 mW and punctual application were effective in promoting murine osteoblast proliferation, but the data were restricted to the first 3 days after exposition (data not published). Here, to exacerbate the impact of LLLTs, we standardized their parameters as 40 mW of power potency and 10 and 50 J/cm^2 of density energies. And even these settings have been applied for prolonged exposure; they were able to stimulate osteogenesis in human primary osteoblasts.

This work revealed that all LLLTs, at tested settings, increased human osteoblast viability and proliferation, whereas only infrared and red spectrums do so by a mechanism dependent on ERK activation, since the enhancement of LLLT-induced cells was impaired by the pretreatment with the compound PD98059, a specific ERK inhibitor. These responses were dependent on the density energy and time of evaluation. The MAP kinases ERK1 and ERK2 are 44- and 42-kDa serine/threonine kinases respectively, are related to receptor tyrosine kinases that signals through Ras-Raf-MEK-ERK signal transduction cascade. During growth factor stimulation, ERK phosphorylation cascade is involved in processes such as cellular proliferation, differentiation and apoptosis [16]. MEK1/2 catalyzes the phosphorylation of human ERK1/2 at Tyr204/187 and then Thr202/185. The phosphorylation of both tyrosine and threonine is required for enzyme activation. Whereas the Raf kinase and MEK families have narrow substrate specificity, ERK1/2 catalyze the phosphorylation of hundreds of cytoplasmic and nuclear substrates including regulatory molecules and transcription factors [23]. It has been reported that low power laser irradiation (LPLI-He-Ne laser, 632.8 nm) induces the phosphorylation of tyrosine protein kinase receptor (TPKR) (such as c-Met, receptor of hepatocyte growth factor), previously shown to activate the MAPK/ERK pathway, and promote proliferation of the cells [24]. Once activated, TPKR activates its downstream signaling elements, like Ras/Raf/MEK/ERK, PI3K/Akt/eIF4E, PI3K/Akt/eNOS and PLC-gamma/PKC pathways. In preosteoblast, it was shown that low-level Er:YAG laser at dose of 0.7–17.2 J/cm^2 enhanced ERK phosphorylation in MC3T3-E1 cells within 5 min [12]. Also, the pretreatment with MAPK-ERK 1/2 inhibitor blocked the enhanced proliferation. An interesting fact is that LED, at 637 nm, did not increase proliferation through ERK-signaling. In fact, the effect of LLLT depends on the parameters applied (wavelength, power density, energy density, and time of exposure) [25]. Thus, it is perfect possible that red

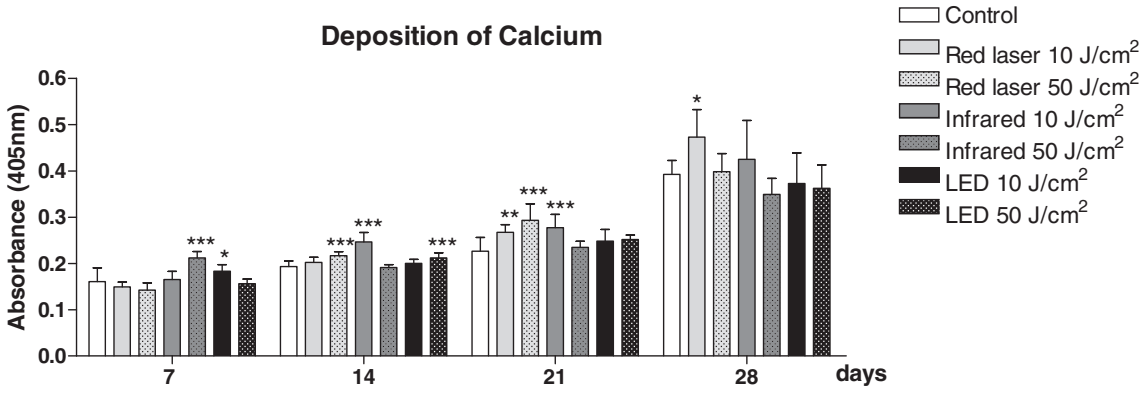

Fig. 4. LLLT effects on calcium deposition by differentiated osteoblast. Cells were irradiated as indicated and mineralization assay was performed by alizarin red staining on days 7, 14, 21 and 28. *P < 0.05, **P < 0.01, and ***P < 0.001 represent significant differences compared to control cells. Results express the mean ± SE from 3 independent experiments performed in triplicate.

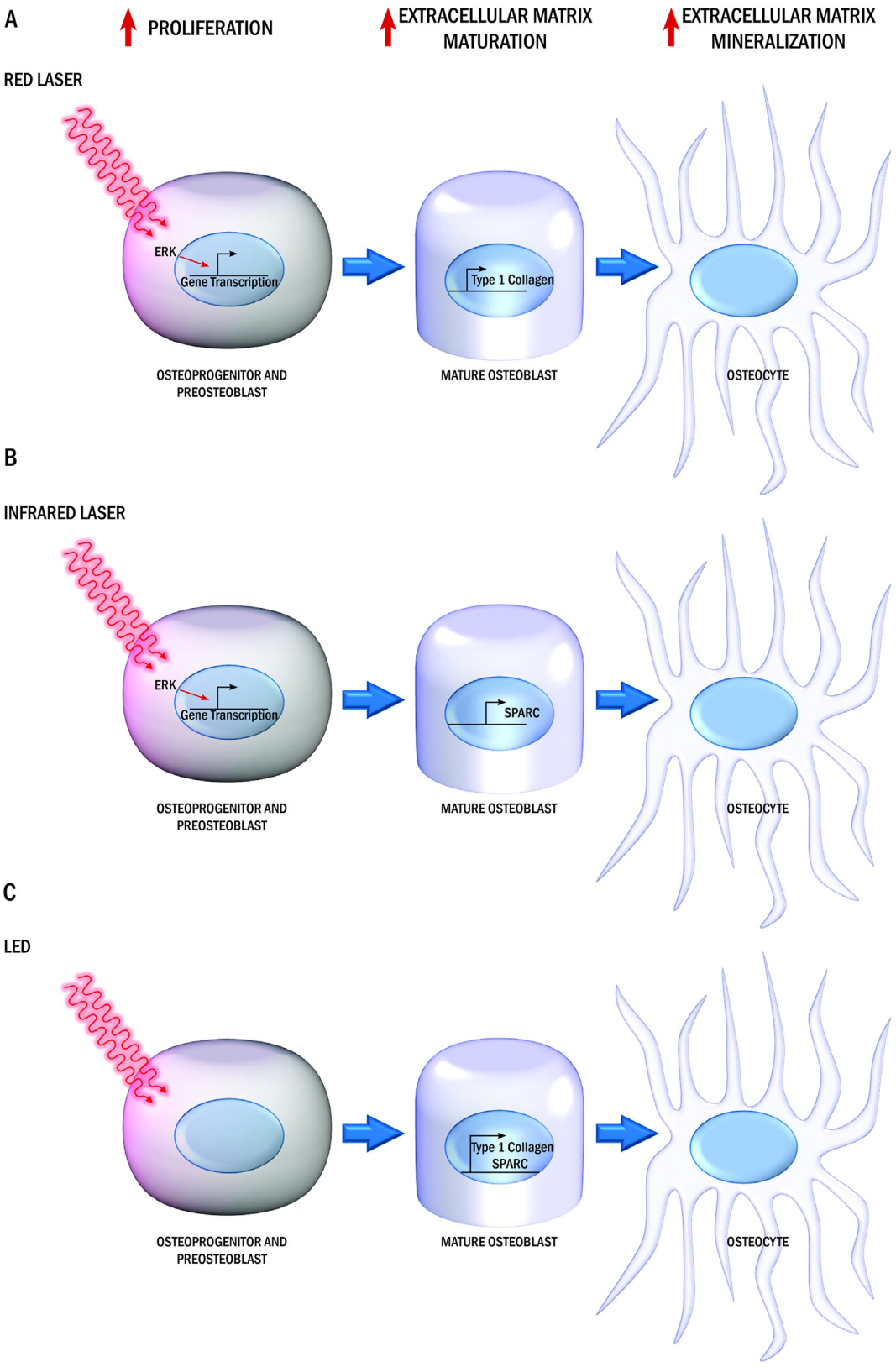

Fig. 5. Schemes of the proposed mechanisms triggered by LLLTs during osteogenic differentiation. LLLT, at different experimental designs, increases osteoblast proliferation, extracellular matrix maturation and mineralization by different mechanisms. Both red and infrared lasers, but not LED, activate ERK phosphorilation to induce preosteoblast proliferation (A, B and C). This may be involved in the cellular signaling to activate gene transcripts codifying (A) type I collagen and (B) osteonectin (SPARC) in red- and infrared-irradiated cells, respectively. (C) LED stimulation upregulates both type 1 collagen and SPARC, regardless of ERK-mediated signaling. The mechanisms by which LLLTs augment extracellular matrix mineralization are still to be determined.

laser at 660 nm but not LED at 637 nm activate differently signaling transduction in the cell, even when the difference in the wavelength is very narrow.

Our data not only corroborate these findings but also give a comparative analysis of infrared, red laser and LED on bone cells. In this context, our data showed that LED at 10 and 50 J/cm^2, revealed by MTT/crystal violet and CFSE assays, respectively, peaked in stimulating the proliferation, even in the absence of ERK activation. Although some contradictory dose-dependency data were found between these two methodologies, this can be easily explained by the fact that the first is suitable for measuring cell viability while the second is for cell proliferation. Thus, to have accurate results regarding proliferation, CFSE-label cells followed by flow cytometry were conducted, and showed that LED at 50 J/cm^2 positively affects osteoblast proliferation. This is consequence of biomodulatory effects through photochemical interactions that occur after light interaction with cell and tissues, and does not involve thermal mechanism [26]. It is possible that in an energy density of 50 J/cm^2, still considered as LLLT, a peak of temperature may occur, but this was not monitored. Some data, specially cellular viability evaluated by MTT, suggest that this may be responsible for reduced viability on cells irradiated with 50 J/cm^2. However, by CFSE method, this was not observed, which is one of the most reliable tests for measuring cellular proliferation.

Besides proliferation, LLLT potentially enhanced the osteogenic differentiation, regardless of the spectrum. This was confirmed by the up-regulated mRNA expression for osteogenic markers such as type 1 collagen (Col1A) and SPARC (osteonectin) genes in irradiated cells. While type I collagen is produced in the onset of osteogenic differentiation and constitutes the primary protein deposited into the bone matrix, osteonectin is a cysteine-rich acidic matrix-associated protein, that is tissue-specific and links the bone mineral and collagen phases, initiating active mineralization in normal skeletal tissue [27]. It belongs to a class of proteins involved in cell–extracellular matrix (ECM) interactions during tissue repair and differentiation [28]. Its role was evidenced from observation that elevated expression of SPARC occurs at early time points after injury and during remodeling of ECM [29]. The principal functions of SPARC include disrupting cell–ECM interactions, inhibiting cell-cycle progression, and regulating the expression of a number of growth factors [30], ECM proteins [31], and matrix metalloproteinases (MMPs) [32]. In this study, LED-irradiated cells at both doses were more effective in increasing the expression of these genes, but also red and infrared lasers stimulated them, dependent on the dose and time. Among the LLLTs, only infrared spectrum at lower dose exceeded the effect of LED application on SPARC expression. These data corroborates the increased mineralization of the bone matrix induced by different LLLTs. In fact, in vitro studies using LLLT on osteogenic cells suggest that it has a stimulatory effect on mineralization process [33,34]. Our data show that besides this knowing ERK-based mechanism of activation by low level lasers, it may act through transcription factor that in turn regulates type I collagen and osteonectin expression. The participation of the MAPK signaling pathway in the osteogenesis of mesenchymal stem cells (MSCs) is well related, since transcription factors such as RUNX2 is regulated by MAPK-dependent phosphorylation [35]. In this context, it was shown that the receptor tyrosine kinase, named discoidin receptor 2 (DDR2), that mediates interactions between cells and fibrillar collagens signals through both ERK1/2 and p38 MAP kinase, which stimulate osteoblast differentiation and bone formation [36]. Also, the expression levels of alkaline phosphatase and type I collagen were reduced following inhibition of ERK1/2 [37]. During differentiation, the secretion of Col1 acts though $\alpha 2\beta 1$ integrins, that in turn leads to the phosphorylation of ERK1/2 in the MAPK signaling pathway and subsequent translocalization of P-ERK1/2 to the nucleus, where it binds to Runx2 and induces gene expression of osteogenic proteins. Also, mineralization induced by beta-glycerosphosphate is induced by phosphorylation of ERK1/2 [38]. Taken together, data from the literature and ours make us to hypothesized that red laser promotes proliferation by ERK activation that in turn activates gene transcripts encoding the type 1 collagen, as in the case of red laser (Fig. 5a) and osteonectin (SPARC) in cells irradiated with infrared (Fig. 5b). On the other hand, LED irradiation increases both Col1A and SPARC expression regardless of ERK activation (Fig. 5c). A possible explanation for it is that LED, but not red and infrared laser, act dephosphorylating ERK1/2, impairing its activation. In fact, the regulatory dephosphorylation of ERK1/2 is mediated by protein-tyrosine specific phosphatases, protein-serine/threonine phosphatases, and dual specificity phosphatases and the combination of kinases and phosphatases make the overall process reversible, and thus invalidating ERK activation.

In turn, even though different mechanisms, LLLTs promotes bone matrix mineralization by dose and time-dependent manner. To better explore this hypothetical mechanism, future studies will be conduct using overexpression genes and pharmacological inhibitors. In summary, LLLTs, at these conditions, modulate the metabolism of human osteoblasts increasing their proliferation and differentiation. Although we speculated the differential role of ERK in inducing type I collagen and osteonectin expression for each spectrum, the mechanisms by which LLLTs increase in vitro mineralization remains to be determined.

Conflict of Interest Statement

The authors have declared no conflict of interest.

Acknowledgments

Prof. Dr. José Mauro Granjeiro, who kindly donated the primary human osteoblasts for this research and Profa. Dra. Ana Paula Campanelli for acquiring cells in the flow cytometry experiments. This work was supported by São Paulo State Research Support Foundation (FAPESP) (grants #2007/08367-0; #2008/11261-1; #2008/11391-2).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jphotobiol.2016.08.006.

References

[1] R. Lubart, M. Eichler, R. Lavi, H. Friedman, A. Shainberg, Low-energy laser irradiation promotes cellular redox activity, Photomed. Laser Surg. 23 (2005) 3–9.
[2] T. Akgul, M. Gulsoy, H.O. Gulcur, Effects of early and delayed laser application on nerve regeneration, Lasers Med. Sci. 29 (2014) 351–357.
[3] K.G.G. Serafim, S.P. Ramos, F.M. de Lima, M. Carandina, O. Ferrari, I.F.L. Dias, et al., Effects of 940 nm light-emitting diode (led) on sciatic nerve regeneration in rats, Lasers Med. Sci. 27 (2012) 113–119.
[4] A.S. da Rosa, A.F. dos Santos, M.M. da Silva, G.G. Facco, D.M. Perreira, A.C.A. Alves, Effects of low-level laser therapy at wavelengths of 660 and 808 nm in experimental model of osteoarthritis, Photochem. Photobiol. 88 (2012) 161–166.
[5] T.Y. Fukuda, M.M. Tanji, S.R. Silva, M.N. Sato, H. Plapler, Infrared low-level diode laser on inflammatory process modulation in mice: pro- and anti-inflammatory cytokines, Lasers Med. Sci. 28 (2013) 1305–1313.
[6] M. Carrera, M.C. Pereiraa, Bacellar de Pinho C, Medradoa ARP, Z. de Araújo Andradec, de Almeida Reis SR., Influence of 670 nm low-level laser therapy on mast cells and vascular response of cutaneous injuries, J. Photochem. Photobiol. B 98 (3) (2010) 188–192.
[7] F.G. Basso, C.F. Oliveira, C. Kurachi, J. Hebling, C.A. de Souza Costa, Biostimulatory effect of low-level laser therapy on keratinocytes in vitro, Lasers Med. Sci. 28 (2013) 367–374.
[8] F. Ginani, D.M. Soares, M.P. Barreto, C.A. Barboza, Effect of low-level laser therapy on mesenchymal stem cell proliferation: a systematic review, Lasers Med. Sci. 30 (8) (2015) 2189–2194.
[9] C.C. Martignago, R.F. Oliveira, D.A. Pires-Oliveira, P.D. Oliveira, C. Pacheco Soares, P.S. Monzani, et al., Effect of low-level laser therapy on the gene expression of collagen and vascular endothelial growth factor in a culture of fibroblast cells in mice, Lasers Med. Sci. 30 (1) (2015) 203–208.
[10] K. Góralczyk, J. Szymańska, M. Łukowicz, E. Drela, R. Kotzbach, M. Dubiel, et al., Effect of LLLT on endothelial cells culture, Lasers Med. Sci. 30 (1) (2015) 273–278.
[11] R. Amid, M. Kadkhodazadeh, M.G. Ahsaie, A. Hakakzadeh, Effect of low level laser therapy on proliferation and differentiation of the cells contributing in bone regeneration, J. Lasers Med. Sci. 5 (4) (Fall 2014) 163–170.

[12] V. Aleksic, A. Aoki, K. Iwasaki, A.A. Takasaki, C.Y. Wang, Y. Abiko, et al., Low-level Er: YAG laser irradiation enhances osteoblast proliferation through activation of MAPK/ERK, Lasers Med. Sci. 25 (4) (2010) 559–569.
[13] A.D. Petri, L.N. Teixeira, G.E. Crippa, M.M. Beloti, P.T. de Oliveira, A.L. Rosa, Effects of low-level laser therapy on human osteoblastic cells grown on titanium, Braz. Dent. J. 21 (6) (2010) 491–498.
[14] L.E. Volpato, R.C. de Oliveira, M.M. Espinosa, V.S. Bagnato, M.A. Machado, Viability of fibroblasts cultured under nutritional stress irradiated with red laser, infrared laser, and red light-emitting diode, J. Biomed. Opt. 16 (7) (2011) 075004.
[15] P.S. Pacheco, F.A. de Oliveira, R.C. Oliveira, A.C. Sant'ana, M.L. de Rezende, S.L. Greghi, C.A. Damante, Laser phototherapy at high energy densities do not stimulate pre-osteoblast growth and differentiation, Photomed. Laser Surg. 31 (5) (2013) 225–229.
[16] F.R. Grassi, F. Ciccolella, G. D'Apolito, F. Papa, A. Iuso, A.E. Salzo, et al., Effect of low-level laser irradiation on osteoblast proliferation and bone formation, J. Biol. Regul. Homeost. Agents 25 (4) (2011) 603–614.
[17] T. Kiyosaki, N. Mitsui, N. Suzuki, N. Shimizu, Low-level laser therapy stimulates mineralization via increased Runx2 expression and ERK phosphorylation in osteoblasts, Photomed. Laser Surg. 28 (Suppl. 1) (2010) S167–S172.
[18] M.M. Jawad, A. Husein, A. Azlina, M.K. Alam, R. Hassan, R. Shaari, Effect of 940 nm low-level laser therapy on osteogenesis in vitro, J. Biomed. Opt. 18 (12) (2013) 128001.
[19] S.J. Pyo, W.W. Song, I.R. Kim, B.S. Park, C.H. Kim, S.H. Shin, I.K. Chung, Y.D. Kim, Low-level laser therapy induces the expressions of BMP-2, osteocalcin, and TGF-β1 in hypoxic-cultured human osteoblasts, Lasers Med. Sci. 28 (2) (2013) 543–550.
[20] N. Bloise, G. Ceccarelli, P. Minzioni, M. Vercellino, L. Benedetti, M.G. De Angelis, M. Imbriani, L. Visai, Investigation of low-level laser therapy potentiality on proliferation and differentiation of human osteoblast-likecells in the absence/presence of osteogenic factors, J. Biomed. Opt. 18 (12) (2013) 128006.
[21] E. Stein, J. Koehn, W. Sutter, G. Wendtlandt, F. Wanschitz, D. Thurnher, M. Baghestanian, D. Turhani, Initial effects of low-level laser therapy on growth and differentiation of human osteoblast-like cells, Wien. Klin. Wochenschr. 120 (3–4) (2008) 112–117.
[22] H. Chung, T. Dai, S.K. Sharma, Y. Huang, J.D. Carroll, M.R. Hamblin, The nuts and bolts of low-level laser (light) therapy, Ann. Biomed. Eng. 40 (2012) 516–533.
[23] R. Roskoski Jr., ERK1/2 MAP kinases: structure, function, and regulation, Pharmacol. Res. 66 (2) (2012) 105–143.
[24] G. Shefer, U. Oron, A. Irintchev, A. Wernig, O. Halevy, Skeletal muscle cell activation by low-energy laser irradiation: a role for the MAPK/ERK pathway, J. Cell. Physiol. 187 (1) (2001) 73–80.
[25] J. Huang, X. Luo, J. Lu, J. Chen, C. Zuo, Y. Xiang, S. Yang, L. Tan, J. Kang, Z. Bi, IPL irradiation rejuvenates skin collagen via the bidirectional regulation of MMP-1 and TGF-β1 mediated by MAPKs in fibroblasts, Lasers Med. Sci. 26 (3) (2011) 381–387.
[26] I. Stadler, R.J. Lanzafame, P. Oskoui, R.Y. Zhang, J. Coleman, M. Whittaker, Alteration of skin temperature during low-level laser irradiation at 830 nm in a mouse model, Photomed. Laser Surg. 22 (3) (2004) 227–231.
[27] R.A. Brekken, E.H. Sage, SPARC, a matricellular protein: at the crossroads of cell-matrix, Matrix Biol. 19 (7) (2000) 569–580.
[28] A.D. Bradshaw, E.H. Sage, SPARC, a matricellular protein that functions in cellular differentiation and tissue response to injury, J. Clin. Invest. 107 (9) (2001) 1049–1054.
[29] N. Hunzelmann, M. Hafner, S. Anders, T. Krieg, R. Nischt, BM-40 (osteonectin, SPARC) is expressed both in the epidermal and in the dermal compartment of adult human skin, J. Invest. Dermatol. 110 (2) (1998) 122–126.
[30] E.W. Raines, T.F. Lane, M.L. Iruela-Arispe, R. Ross, E.H. Sage, The extracellular glycoprotein SPARC interacts with platelet-derived growth factor (PDGF)-AB and -BB and inhibits the binding of PDGF to its receptors, Proc. Natl. Acad. Sci. U. S. A. 89 (4) (1992) 1281–1285.
[31] K. Kamihagi, M. Katayama, R. Ouchi, I. Kato, Osteonectin/SPARC regulates cellular secretion rates of fibronectin and laminin extracellular matrix proteins, Biochem. Biophys. Res. Commun. 200 (1) (1994) 423–428.
[32] P.M. Tremble, T.F. Lane, E.H. Sage, Z. Werb, SPARC, a secreted protein associated with morphogenesis and tissue remodeling, induces expression of metalloproteinases in fibroblasts through a novel extracellular matrix-dependent pathway, J. Cell Biol. 121 (6) (1993) 1433–1444.
[33] E. Fukuhara, T. Goto, T. Matayoshi, S. Kobayashi, T. Takahashi, Optimal low-energy laser irradiation causes temporal G2/M arrest on rat calvarial osteoblasts, Calcif. Tissue Int. 79 (6) (2006) 443–450.
[34] H.O. Schwartz-Filho, A.C. Reimer, C. Marcantonio, E. Marcantonio Jr., R.A. Marcantonio, Effects of low-level laser therapy (685 nm) at different doses in osteogenic cell cultures, Lasers Med. Sci. 26 (4) (2011) 539–543.
[35] E. Rodríguez-Carballo, B. Gámez, F. Ventura, p38 MAPK signaling in osteoblast differentiation, Front. Cell Dev. Biol. 4 (2016) 40.
[36] C. Ge, Q. Yang, G. Zhao, H. Yu, K.L. Kirkwood, R.T. Franceschi, Interactions between extracellular signal-regulated kinase 1/2 and p38 map kinase pathways in the control of Runx2 phosphorylation and transcriptional activity, J. Bone Miner. Res. 27 (3) (2012) 538–551.
[37] Y. Li, J. Wang, G. Chen, S. Feng, P. Wang, X. Zhu, R. Zhang, Quercetin promotes the osteogenic differentiation of rat mesenchymal stem cells via mitogen-activated protein kinase signaling, Exp. Ther. Med. 9 (6) (2015) 2072–2080.
[38] J.H. Jansen, F.A. Weyts, I. Westbroek, H. Jahr, H. Chiba, H.A. Pols, J.A. Verhaar, J.P. van Leeuwen, H. Weinans, Stretch-induced phosphorylation of ERK1/2 depends on differentiation stage of osteoblasts, J. Cell. Biochem. 93 (3) (2004) 542–551.