Journal of Lasers in Medical Sciences

J Lasers Med Sci 2024;15:e45

Original Article

http://journals.sbmu.ac.ir/jlms

doi 10.34172/jlms.2024.45

Photobiomodulation Modulates Proliferation and Gene Expression Related to Calcium Signaling in Human Osteoblast Cells

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Received: April 16, 2024
Accepted: July 29, 2024
ePublished: September 14, 2024

Abstract

Introduction: Photobiomodulation with low-level laser treatment can enhance bone formation by stimulating the cell division of osteoblasts and increasing the amount of protein deposition, thus encouraging the formation of new bone. The aim of this study was to evaluate the effects of photobiomodulation with a low-level laser on proliferation and gene expression related to calcium signaling in human osteoblasts.

Methods: Osteoblastic cell lines of the hFOB1.19 lineage, human osteoblasts, were grown and assigned into two groups, control (C; n = 78 cultured wells) and photobiomodulation (L; n = 78 cultured wells) with n = 6 per day of the experimental period. Cells were cultured (immature at 34 ºC), and after maturation at 37 ºC, group L cells were exposed to laser irradiation with a low-level laser device (gallium and aluminum arsenide), at a wavelength of 808 nm, a power output of 200 mW, and a power density of 200 mW/cm^2. The energy delivered to the cells was 37 J/cm^2, with a beam area of 0.02 mm^2 and an exposure time of 5 seconds. This treatment was applied daily for a period of 13 days. Following this, the number of cells was counted, and RNA was isolated, measured, and then converted into cDNA for further quantification using a comparative Ct method with real-time polymerase chain reaction. The results were then subjected to statistical analysis through a Mann-Whitney test, with a significance level of $P < 0.05$.

Results: The cell count in the L group (37.25x10 ± 4 ± 22.02) was statistically higher compared to the control group (22.75x10 ± 4 ± 7.660) with a P value of 0.0259. The values of $2^{-}\Delta\Delta$Ct for S100A6, plasma membrane calcium ATPase (PMCA), and calmodulin genes indicated hyper-expression on the thirteenth day, while the osteocalcin gene showed hypo-expression.

Conclusion: The study suggests that the photobiomodulation mechanism with a low-level laser may regulate gene expression in human osteoblasts in a dose-dependent and cumulative manner.

Keywords: Photobiomodulation; Osteoblasts; Gene expression; Quantitative real-time PCR

Introduction

Bone tissue homeostasis is maintained by progenitor and resorptive mechanisms that are directly or indirectly mediated by hormones, intracellular signals, and/or ions that modulate the synthesis, activation, and expression of genes. The regulation of genes and proteins necessary for bone mineralization relies on calcium signaling, transport, and secretion by osteoblasts and osteoprogenitor cells.[1,2]

These transport mechanisms can occur through cells (transcellular) and around cells (paracellular) and are mediated by membrane structures and binding transport proteins.[3,4] Genes encoding calcium transport include S100A6 (S100 calcium binding protein A6),[5] CAM2 (calcium/calmodulin dependent protein kinase II beta),[6] PMCA1b (ATPase plasma membrane Ca2 + transporting 1),[7] and osteocalcin (BGLAP, bone gamma carboxyglutamate protein).[8]

Osteoblasts require ATP and must express several genes to calcium transport, including S100A6, PMCA, and osteocalcin.[5-8] These mechanisms rely on calcium signaling, a fundamental ion in cell physiology that is involved in various processes in mammalian cells, such as bone formation.[2]

These calcium transporters can be regulated by chemical agents, caloric restriction, aging, presence of microRNAs, and physical agents such as electromagnetic fields, microcurrents, and low-level laser photobiomodulation (PBM).[9-13]

PBM with low energy doses is able to bio-modulate cellular activity, such as inducing a greater release of

Please cite this article as follows: Bomfim FRCd, Sella VRG, Thomasini RL, Plapler H. Photobiomodulation modulates proliferation and gene expression related to calcium signaling in human osteoblast cells. *J Lasers Med Sci.* 2024;15:e45. doi:10.34172/jlms.2024.45.

calcium into the cytoplasm and increasing intracellular pH, both of which are responsible for triggering mitogenic signals and increasing cell proliferation. The infrared laser acts directly on the mitochondria, which are its main chromophores, resulting in the transduction of cellular signals such as DNA and RNA synthesis and cell adhesion.[14,15]

Multiple research studies indicate that PBM exposure enhances protein levels in osteoblasts, thereby impacting bone cell function[13] and bone repair.[16] Also, in a study by our group, anti-inflammatory activities and anticipation of the bone healing process were observed based on increased protein expression.[17]

PBM also affects the influx and increase of Ca^{2+} concentrations,[18] modulates Ca^{2+}-dependent endoplasmic reticulum pathways,[19] stimulates mitochondrial Ca^{2+} pathways by releasing reactive oxygen species (ROS), and modifies the plasma membrane calcium ATPase (PMCA), which acts as a pump related to the major Ca^{2+} release mechanisms.[20]

This study is the first to evaluate the effects of PBM using LLLT at these specific parameters and application days on cell proliferation and the expression of genes associated with calcium signaling in adult human osteoblast cells. The study aimed to evaluate the effects of low-level infrared laser PBM on cell proliferation and gene expression related to calcium signaling in adult human osteoblast cells.

Materials and Methods

Cell Culture

Cell line hFOB 1.19 (ATCC® CRL-11372™) of human osteoblast was acquired from the American Type Culture Collection (ATCC), provided by the renal biology laboratory of São Paulo University (USP-SP) and cultured according to Harris et al.[21] Cells were grown in a six-well plate with a growth area of 9.07 cm^2, and 10^4 cells were seeded per well with 2 mL of Ham's F12 Dulbecco's modified medium and Eagle's medium in a 1:1 mixture, without phenol red, plus 2.5 mM L-glutamine, fetal bovine serum (10% - Gibco), geneticin - G418 (50 mg/mL - Sigma Chemical Co., St. Louis, MO, USA) and fungizone (250 µg/mL - Sigma Chemical Co, St. Louis, MO, USA) at 34 °C and 5% CO_2, with the culture medium changed every 3 days. When 70% confluence was reached in each well of each plate, the oven temperature was adjusted to 39 °C for differentiation of the cell line according to ATCC instructions. After 4 days at 39 °C, the osteoblasts acquired an adult phenotype and the laser group (L) was irradiated. During the experiment, the control group (C) followed an identical procedure with the device in the off position. The groups were as follows: C (n = 78; n = 6 per day) without low-level laser application and L (n = 78; n = 6 per day) with low-level laser application.

Photobiomodulation - Low-Level Laser Irradiation

Group L was treated daily with PBM with a low-level laser applied directly on the top of the cell surface using a laser device of gallium aluminum arsenide (GaAlAs, Magnus Plus, DMC *Equipment*, São Carlos, Brazil), operating in the infrared continuous mode at a wavelength of 808 nm, delivering a nominal power of 200 mW, energy density of 0.2 W/cm^2, fluence of 37 J/cm^2, beam area of 0.02 mm^2, with a duration of 5 seconds, each session delivering 1 J of energy. This treatment was carried out for a total of 13 days (Figure 1)[22]. Group C followed a similar protocol, with the laser device in the off position throughout the duration of the treatment.

Sample Collection

The samples were collected daily for 13 days from each of the six well plates following the groups division (n = 6 samples for each day) for cell counting and RNA extraction. The cells were treated with 0.25% trypsin-EDTA without phenol for 10 minutes at 39ºC. Aliquots of 1 mL were inserted into microtubes, and RNAlater® (Sigma-Aldrich) was added to stabilize the RNA and frozen at -80 ºC. An aliquot of 100 µL of the sample was used for cell counting.

Cell Counting (Proliferation)

Counts were performed daily for 13 days, and 10 µL of the cell aliquot was used for cell proliferation by the number of viable cells. These were performed in a Neubauer chamber in the four distal quadrants with trypan blue dye (Sigma Aldrich, Merck KGaA, Darmstadt, Germany) in a 1:1 ratio, and the following formula was used for counting according to Peres and Curi[23]: Q1 + Q2 + Q3 + Q4 X10000 = nº cells/mL, where Q refers to each quadrant.

RNA Extraction

The cells were isolated for RNA extraction with a

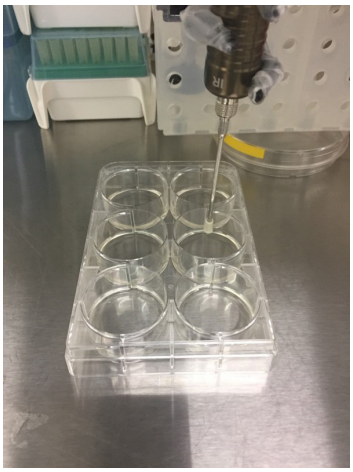

Figure 1. Photobiomodulation Therapy on the Irradiated Group on the Top of the Cell Plate With Six Wells. PBM was performed for thirteen consecutive days

high purity RNA isolation kit, provided by Roche Applied Science (Mannheim, Germany). In summary, following the thawing process, the cells were subjected to centrifugation at 12 000 rpm for 10 minutes at 4 ºC to separate the RNA from the solution and precipitate it. The cells were subjected to successive centrifugations in a column containing a filter with wash buffer solution. To elute the RNA, we added 50 µL of the elution buffer to the upper reservoir of the column, centrifuged at 10 000 rpm for 1 minute, and we stored the samples at -80 °C.

Synthesis of cDNA

Complementary DNA (cDNA) was synthesized from 5 ng of RNA from each sample plus reagents from the reverse transcription kit (Platus Transcriber RNaseH-cDNa First Strand Kit, Sinapse Inc, Hollywood, Florida, USA) at final concentrations: Reaction buffer (1X), Oligo dT primer (1X), dNTP mix (1 mM), H Minus reverse transcriptase (10 U/µL), RNAse inhibitors (1 U/µL), and Mili-Q nuclease-free water q.s.p. 20 µL. The samples were homogenized, centrifuged at 12 000 rpm for 3 minutes, incubated at 42 ºC for 60 minutes, and then enzymatically inactivated at 70 ºC for 5 minutes. The cDNA was quantified from 1 µL of the sample using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and the 260 nm/280 nm ratio was automatically calculated by integrated software.

Quantitative Real Time Polymerase Chain Reaction

The kinetics of gene expression was assessed by real-time polymerase chain reaction (qPCR) using Syber Green technology with primer sequences (Invitrogen Technologies®) for β-actin, S100A6, PMCA1b, osteocalcin, and calmodulin genes (Table 1) constructed according to Lefever et al.[24] The reaction was performed with 1 µL cDNA and 9 µL master mix (6 µL Syber Green PCR Master Mix® 1X, 0.5 µL forward sequence and 0.5 µL reverse sequence of each primer pair, and 2 µL sterile Mili-Q water). All qPCR reactions used initial denaturation temperatures of 95 ºC for 3 minutes, denaturation at 95 ºC for 15 seconds, annealing (variable temperature) for 45 seconds, and extension at 72 ºC for 1 minute for a total of 40 cycles (Table 1). All reactions were performed in triplicate. qPCR was performed on the StepOnePlus™ system (Applied Biosystems®, Life Technologies Corporation) with integrated AB Prism StepOne software, and gene expression was evaluated by using the comparative Ct (cycle threshold) technique (2^{-} ΔΔCt) between groups C and L. Beta actin was used as a housekeeping gene.

Statistical analysis

The GraphPad Prism 5.0 for Windows was used for statistical analysis of the results obtained from the cell counts (Two-way ANOVA test) and the gene expression of 2^{-}ΔΔCt between the S100A6, PMCA1b, osteocalcin, calmodulin and β-actin genes using the Mann-Whitney non-parametric test for independent samples, with a significance level of 5% ($P < 0.05$) for both.

Results

Cell Morphology and Proliferation (Viability)

Osteoblasts cells showed a typical mature phenotype and cell counting showed a lower number of cells in the control group (Figure 2A) compared to the PBM group (Figure 2B). Cell proliferation values (median ± standard deviation) showed in PBM group higher number of cells $37.25x10^4 \pm 22.02$ compared to the control group $22.75x10^4 \pm 7.660$ cells, with statistical differences present during the periods analyzed ($P = 0.0259$) (Figure 2C).

Gene Expression by Real Time-PCR

The evaluation of gene expression kinetics by qPCR (2^{-} ΔΔCt technique) was performed during the experimental period between the cells of the control group and the PBM group, and the results were described in terms of median, minimum and maximum values. The S100A6 gene showed differences on days 2, 4 and 13, with higher expression in the control group compared to the laser group (Figure 3A). The expression of the PMCA1b gene showed significant differences on days 2 and 4 in the control cells, while on day 13 the irradiated cells showed

Table 1. Primers Sequences, Concentrations and Annealing Temperatures

Primer	Sequence	nM Concentration	Annealing Temperature (ºC)
β-actin	Forward: 5′ - GTCCTCTCCCAAGTCCACAC -3′	50	55
	Reverse: 5′ - GGGAGACCAAAAGCCTTCAT -3′	50	
S100A6	Forward: 5′ - GTGGAAGATGGCCACGAG -3′	50	50
	Reverse: 5′ - CTGCGACACAGCCCATC -3′	50	
ATP2B1 (PMCA1b)	Forward: 5′ - GGTTTTTGGCTTTTTAGGAGG -3′	50	50
	Reverse: 5′ - CCAAATTGAAAACATCTCCCA -3′	50	
BGLAP (Osteocalcin)	Forward: 5′ - CGCCTGGGTCTCTTCACTAC - 3′	300	50
	Reverse: 5′ - CTCACACTCCTCGCCCTATT -3′	50	
Calmodulin	Forward: 5′ - GGCATTCCGAGTCTTTGACAA - 3′	300	57
	Reverse: 5′ - CCGTCTCCATCAATATCTGCT -3′	900	

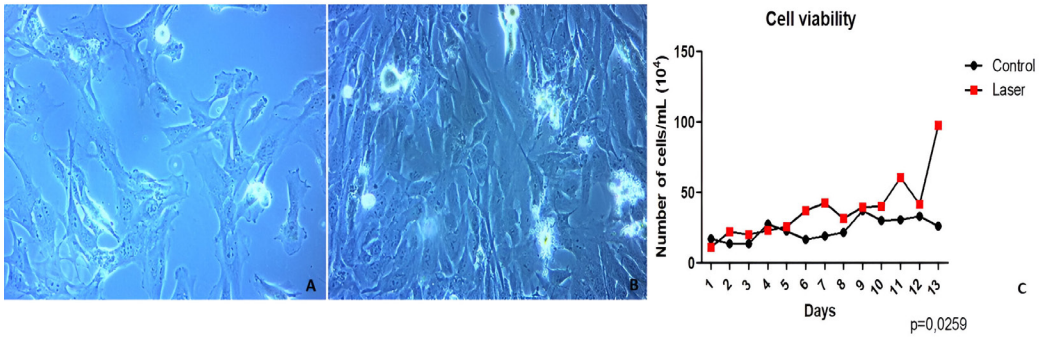

Figure 2. Mature Osteoblast Cell Morphology Without PBM (A) and After PBM Therapy (B) (figures magnification: 20x, bar scale 200 µm). The number of cells after tripan blue assay (C)

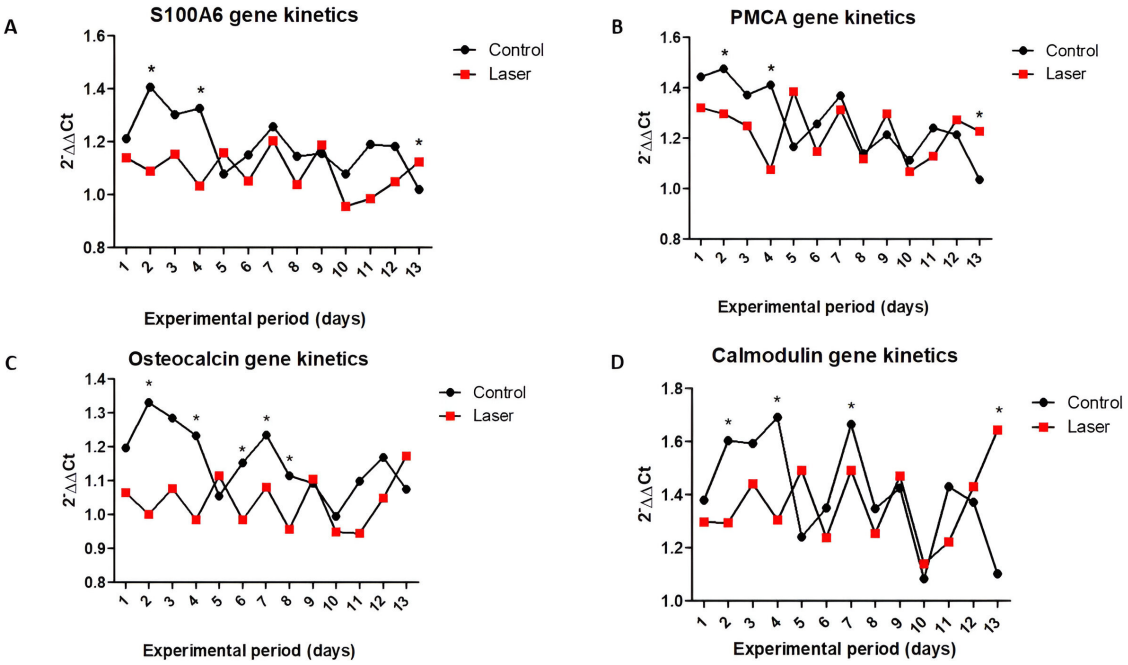

Figure 3. Kinetics of Gene Expression After 13 Days of PBM Treatment Versus No Treatment of the Analyzed Genes, S100A6 (A), PMCA (B), Osteocalcin (C) and Calmodulin (D). Mann-Whitney non-parametric test for independent samples *$P<0.05$

higher values compared to the control (Figure 3B). The osteocalcin gene did not show higher expression in irradiated cells, but control cells showed significant differences on days 2, 4, 6, 7 and 8 (Figure 3C). Analysis of the calmodulin gene followed the other genes on days 2, 4 and 7, with the control cells showing greater expression, and only on day 13 were differences observed in the irradiated cells (Figure 3D).

Discussion

The osteoblast cells used in our study, hFOB1. 19 (Human Fetal Osteoblastic), are immortalized cells with SV40 viral antigen, which, due to their histogenic origin, showed a typical phenotype of fetal osteoblasts, like fibroblasts, cultured at 34ºC, whereas the cells exposed to temperatures of 39 ºC showed a typical adult/mature osteoblast phenotype, with the presence of a large cytoplasm. At temperatures below 39 °C, the process of cell division is prolonged, leading to an enhancement in differentiation and the generation of a more advanced osteoblast phenotype. In our research, cell cultures are cultivated from various morphological stages of the cells, with the capacity for differentiation into mature osteoblasts being directly associated with the expression of the characteristic osteoblast phenotype.[25]

Studies using cell culture with hFOB 1.19 cells evaluating gene expression are varied,[26-28] but this is the first study involving a low-level laser and gene expression of the targets. In our study, when the S100A6 gene irradiated with the low-level laser was analyzed, a significant difference in gene hyperexpression was observed only on the thirteenth day. In the other periods, the expression was similar between the groups or, as observed on days 2 and 4, there was hypoexpression compared to the non-irradiated cells in the control group.

The gene plays a crucial part in the growth of fibroblasts

and osteoblasts. However, when the gene is deactivated, the growth of these cells slows down. Conversely, hyperexpression of the S100A6 gene leads to more osteoblast growth,[29-32] although its role, gene expression and mechanisms of response to PBM application remain unknown. The search for an understanding of the mechanisms of expression and interaction of this gene is due to the fact that its expression is linked to cell proliferation, its presence at the beginning of osteogenesis, and the regulation of intracellular calcium.[33] Therefore, it was expected that PBM with a low-level laser would promote the hyperexpression of this gene in osteoblasts at the beginning of the application, but this did not occur until the thirteenth day.[34]

As occurred with the S100A6 gene, it was observed for the PMCA1b and CaMKII genes that the irradiated group showed hyper-expression on day thirteen compared to the control. This suggests that PBM played a role in the signaling, active transport and release of calcium from the endoplasmic reticulum, which regulates the activity of concentration gradients and the proliferation of osteoblasts.[6,35-37]

These results are related to a study that showed the accumulative effects of low doses on cell proliferation, as the doses previously applied continue to produce effects.[37] Therefore, in our study, when the thirteenth day of the experiment was evaluated, the cells received 13 J of energy, which could have resulted in a rise in the release of calcium, the activation of calcium pumps, and the production of calcium binders.

In a study in which the number of bone cells was monitored daily for 10 days after irradiation with an 830 nm laser, 90 mW power, and energy doses of 0.5, 1, 2, and 4 J, it was found that the number of cells showed a tendency to increase from day 6 when 1 J of energy was used, but in general, there was no increase in the number of cells.[38] In the results of our study with 808 nm PBM, 200 mW power and 1 J energy, there was a tendency for an increase between days 6 and 7, although the values were statistically significant on the thirteenth day. This study showed discrepant values of the dose compared to those used by our research group, in which the parameters of PBM showed positive results before the thirteenth day.[17]

Irradiation with a wavelength of 808 nm was chosen because of its ability to penetrate deeper than wavelengths in the infrared range, such as 980 nm.[39] The selection of power was made with the objective of achieving the highest energy level within the shortest timeframe, that is, five seconds, within the cells. This decision was predicated on findings from prior research.[17,40,41] An irradiation point was designated at the top of the plate, directly on the culture medium and the cells, to avoid losses due to absorption and scattering by the plate, although the plate was colorless.

Studies to understand cellular mechanisms using in vitro methods have in common the use of cells that have not undergone cellular injury,[26,27,42,43] as in our study, where the cells did not undergo stress induction.

In the assessment of both *in vivo* and *in vitro* effects, it was also noted that there was an enhancement in the proliferation and growth of osteoblasts, as well as an increase in growth factors and cofactors associated with the bone mineralization process, a fact related to the light modulatory properties of PBM, as in a study by our group using the same wavelength, power and fluence parameters as PBM,[17] or using 780 nm PBM, 20 mW power, 0.16 J total energy, and a time of 8 seconds.[42]

However, these data contradict the effects that laser irradiation promotes; as such, it proves to be notably efficacious in its application to healing sites, including bone fractures, whereas its efficacy diminishes when utilized on normal tissue. This is attributable to PBM functioning as a regulatory signal, enhancing the proliferation of cells that are found in lower concentrations of oxygen, pH, or in the absence of nutrients.[43]

With regard to the osteocalcin gene, it was expected that it would be hyper-expressed over the same time period as the S100A6, CAMKII and PMCA1b genes, since osteocalcin, together with calcium signaled by the genes, is involved in the bone mineralization process[44,45] and is expressed in the late stages of osteoblastic differentiation.[46-49]

Our results are consistent in comparison with studies that used PBM at 4 J/cm^2 with $\lambda = 660$ nm and 4 J/cm^2 with $\lambda = 808$ nm, since there was no increase in osteocalcin expression.[50,51] This may be related to the fact that osteocalcin is not dependent on it for the mineralization process to occur, and it may also act as a negative regulator of bone formation.[52,53]

Data from our study showed hypo-expression or similar expression to the control group in the irradiated cells throughout the experimental period; these results are not consistent with those described in the literature, which reports that mature osteoblasts are responsible for gene and protein expression.[54-56]

The hypoexpression of the S100A6, PMCA and calmodulin genes on days 2 and 4 is noteworthy as they all share common hypoexpression during this period. These findings may be related to adaptation and the cell cycle. A limited number of research efforts have aimed to explore how PBM with LLLT impacts the cell division process, such as the mechanisms of influence of PBM on the different time periods of cells. Among the possible cell cycle mechanisms, PBM may regulate proliferation through the hyperexpression of mitosis-activated protein kinase 11 (MAPK11),[57] breakpoint cluster regions[58] or serum response factors, which contribute to gene-stimulated transcriptional induction during the transition of the cell cycle from G0 to G1.[59-61]

Another hypothesis is that on day 2 of culture, the cells

go from G0-G1 to S phase when they absorb the light, which shows the hypoexpression of the gene, since in this phase the DNA would be synthesized, as well as on day 4 when the cells are again in the S phase of synthesis. After this period, irradiated cells do not affect gene expression.[62] This hypothesis is related to the study of Ramos Silva et al.[63] Examining fibroblasts and immortal cancer cells showed that on the fourth day of PBM using a power level of 40 mW, duration of 90 seconds, and 3.6 J of energy, there was a rise in the cell count during the S and G2 phases, alongside a reduction in the number of cells transitioning from the G0-G1 phase. According to Ling et al,[64] after the cells were exposed to ultraviolet light and treated with PBM, there was an increase in the S phase of the cells but a decrease in the G0-G1 phase, indicating a decrease in the induced arrest of the cell cycle.

Our results differ from what is described in the literature regarding osteocalcin, where several studies show hyperexpression and protein deposition and its association with improved bone healing and shorter repair time.[17,37,38] We observed the hypoexpression of osteocalcin, which caught our attention, especially when evaluating the other genes, because there was an increase in gene expression at different times, especially on day 13, when there was the greatest proliferation of osteoblasts.

The genes studied are part of a network associated with different calcium-dependent pathways. Using the GeneMANIA platform,[65] we observed physical interaction and co-expression between S100A6, PMCA1b, calmodulin and osteocalcin, co-localization between PMCA1b and calmodulin, protein domains between S100A6, PMCA1b and calmodulin, and expression pathways between S100A6, PMCA1b and osteocalcin genes.

We observed that the irradiated cells did not show senescence changes due to the time the cells were cultured (thirteen days, mature phase), especially because a specific pathway such as opsin3 (Opn3) and/or changes in calcium concentration. In our study, no inflammatory changes were observed when morphological aspects were assessed after PBM with low-level laser irradiation. Also these cells do not suffer stress during histogenesis, once they are not mesenchymal stem/stromal cells (MSCs). These cells are immortalized primary osteoblasts with T Antigens of Simian Virus 40 (SV40 large T antigen).[66-68]

As regards the limitations of our study, we had studied just some genes related to calcium signaling, and *in vitro* studies do not show all physiological pathways related to new bone formation and bone healing.

Conclusion

Considering that PBM with a low-level laser showed a tendency to increase S100A6, PMCA1b, and calmodulin genes in human osteoblasts from day 10, it could be suggested that the mechanism of action of PBM on calcium signaling is dose-dependent and cumulative. PBM did not modulate osteocalcin, and osteocalcin is not exclusively responsible for bone mineralization. PBM behaves differently *in vivo* and *in vitro*, especially when the irradiated cells suffer injury or stress.

Acknowledgments

The authors thank the Renal Physiology Laboratory of USP-SP for cell donation.

Authors' Contribution

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Funding acquisition: Fernando Russo Costa do Bomfim and Hélio Plapler.
Investigation: Fernando Russo Costa do Bomfim and Valéria Regina Gonzalez Sella.
Methodology: Fernando Russo Costa do Bomfim.
Project administration: Fernando Russo Costa do Bomfim and Hélio Plapler.
Software: Fernando Russo Costa do Bomfim and Ronaldo Luis Thomasini.
Supervision: Hélio Plapler.
Validation: Hélio Plapler.
Writing–original draft: Fernando Russo Costa do Bomfim.
Writing–review editing: Fernando Russo Costa do Bomfim.

Competing Interests

All authors declare that they have no conflicts of interest.

Data Availability Statement

The information that backs up the findings of this research can be obtained from the corresponding author when there is a justifiable inquiry.

Ethical Approval

All experimental procedures were performed in accordance with the ethical guidelines established by the National Commission for Animal Welfare (COBEA), Brazil. The study was submitted and approved by the Institutional Animal Care and Ethics Committee of UNIFESP/EPM, protocol number 4174131114/2014.

Funding

This study was financially supported by FAPESP (grant 2015/02818-6) and Fundação Hermínio Ometto. This study was also financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

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