Lasers Med Sci (2012) 27:777–783
DOI 10.1007/s10103-011-0968-0

ORIGINAL ARTICLE

Effect of low-level laser therapy after rapid maxillary expansion on proliferation and differentiation of osteoblastic cells

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Received: 6 September 2010 / Accepted: 8 July 2011 / Published online: 13 August 2011

Abstract The aim of this study was to investigate the osteoblastic activity of cells derived from the midpalatal suture upon treatment with low-level laser therapy (LLLT) after rapid maxillary expansion (RME). A total of 30 rats were divided into two groups: experimental I (15 rats with RME without LLLT) and experimental II (15 rats with RME + LLLT). The rats were euthanized at 24 h, 48 h, and 7 days after RME, when the osteoblastic cells derived from the rats' midpalatal suture were explanted. These cells were cultured for periods up to 17 days, and then in vitro osteogenesis parameters and gene expression markers were evaluated. The cellular doubling time in the proliferative stage (3–7 days) was decreased in cultured cells harvested from the midpalatal suture at 24 and 48 h after RME + LLLT, as indicated by the increased growth of the cells in a culture. Alkaline phosphatase activity at days 7 and 14 of the culture was increased by LLLT in cells explanted from the midpalatal suture at 24 and 48 h and 7 days after RME. The mineralization at day 17 was increased by LLLT after RME in all periods. Results from the real-time PCR demonstrated that cells harvested from the LLLT after RME group showed higher levels of ALP, Runx2, osteocalcin, type I collagen, and bone sialoprotein mRNA than control cells. More pronounced effects on ALP activity, mineralization, and gene expression of bone markers were observed at 48 h after RME and LLLT. These results indicate that the LLLT applied after RME is able to increase the proliferation and the expression of an osteoblastic phenotype in cells derived from the midpalatal suture.

Keywords Culture cells · LLLT · Osteoblasts · Rapid maxillary expansion · Real-time PCR

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Introduction

Biostimulatory effects of low-level laser therapy (LLLT) on bone cells have been reported in in vivo [1–4] and in vitro studies [3, 5–7]. There is evidence that LLLT induces cell proliferation in rat calvaria [7] and accelerates fracture consolidation [4, 8, 9]. In dental practice, LLLT applied to the alveolar bone after tooth extraction is able to stimulate angiogenesis and increase collagen fiber deposition and bone cell proliferation at the site, accelerating new bone formation [10]. In orthodontic treatments, authors of previous studies have also observed that LLLT accelerates bone regeneration in the midpalatal suture after rapid maxillary expansion (RME) in rats [11]. Several studies have pointed out that LLLT can accelerate bone formation by increasing osteoblastic activity, vascularization, and organization of collagen fibers [7, 9, 12, 13].

Considering that osteoblasts are responsible for bone formation, it is possible that LLLT exerts a stimulatory effect on osteoblastic activity when it is applied to bone tissue; and effects of LLLT applied directly to osteoblast cultures have corroborated this hypothesis. LLLT stimulates cellular proliferation, bone nodule formation, alkaline

phosphatase activity, and osteocalcin gene expression of osteoblastic cells at the early proliferative stage in vitro [7]. Additionally, LLLT has been demonstrated to increase DNA and RNA synthesis, bone-like nodule formation, and osteocalcin and osteopontin gene expression [8, 13, 14]. Furthermore, the level of ALP activity has been shown to significantly increase after LLLT [15].

Considering the stimulatory effects of LLLT on bone regeneration after RME and its direct effect on osteoblastic cells demonstrated by in vitro studies, the aim of the present study was to evaluate the osteoblastic activity of cells harvested from the midpalatal suture that were treated with LLLT after RME.

Materials and methods

This study was approved by the Committee of Ethics in Animal Research, School of Dentistry, Ribeirão Preto/ University of São Paulo, Brazil. A total of 30 Wistar Albinus male rats were used for the study. They were housed in an air-conditioned room with an automatically controlled temperature (20–23°C), a 12/12-h light/dark cycle and 50% relative humidity, and they received a standard pelleted laboratory diet and water ad libitum. The rats were divided by blind randomization methods into two groups: experimental I (15 adult rats with midpalatal suture expansion without low-level laser irradiation) and experimental II (15 adult rats with midpalatal suture expansion associated with low-level laser irradiation).

Anesthetic procedures and maxillary expansion procedure

For all procedures, the animals were intramuscularly anesthetized with a combination of ketamine (Agener®, 40 mg/kg) and xylazine (Syntec®, 20 mg/kg) at a 1:2 ratio (1 ml/kg body weight). The experimental animals were submitted to rapid maxillary expansion (RME) alone or in association with low-level laser irradiation (RME + LLLT). The immediate expansion of the midpalatal suture was performed by inserting a 1.5-mm-thick circular metal ring fabricated from a 0.5-mm-diameter stainless-steel orthodontic wire (Dental Morelli Ltda, Sorocaba, SP, Brazil) between the maxillary incisors according to the methods described by Sawada and Shimizu [16]. This appliance was kept in position with a light-cured adhesive (3M Unitek, Monrovia, CA).

Laser devices and laser irradiation procedures

A gallium-aluminum-arsenide (AsGaAl) diode laser device (Photon Laser, DMC Equipaments, São Carlos, SP, Brazil; λ 830ŋm, 30 mW, θ 1 mm, CW and 0.00785 cm^2 area) was used as the lower-level laser source in this study. The irradiation was administered under anesthesia by placing the end of the optical fiber tip in contact with and aligned perpendicular to the palatal mucosa at the midline and median points between the anterior edges of the incisors and the incisive papilla. Irradiation was performed immediately after expansion, corresponding to a total energy dosage of 160 J/cm^2, and a single application lasting 0.42 s. The treatment regime for the sham groups (experimental I) was the same as for the experimental II group, except that the laser device was not switched on. Five rats from each group were euthanized at 24 and 48 h and at 7 days with an overdose of the ketamine and xylazine anesthetic. After the treatments, the palatal bone fragments were processed for cell culture experiments.

Cell culture experiments

Rat palatal bone fragments (explants) were harvested from the midline suture (between the incisors, below the incisive papilla, region that received the irradiation), and osteoblastic cells were obtained by enzymatic digestion using collagenase type II (Gibco, Life Technologies, Grand Island, NY) as previously described [17]. These cells were cultured in α-minimum essential medium (Gibco), supplemented with a 10% fetal bovine serum (Gibco), 50 μg/ml gentamicin (Gibco), 0.3 μg/ml fungizone (Gibco), 10^{-7} M dexamethasone (Sigma, St. Louis, MO), 5 μg/ml ascorbic acid (Gibco), and 7 mM β-glycerophosphate (Sigma). Subconfluent cells in the primary culture were harvested after treatment with 1 mM ethylenediamine tetraacetic acid (EDTA) (Gibco) and 0.25% Trypsin (Gibco) and subcultured in 24-well culture plates (Falcon, Franklin Lakes, NJ) at a cell density of 2×104 cells/well. During the culture period, cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air; the medium was changed every 3– 4 days.

Culture growth

Cells were subcultured for 3 and 7 days and enzymatically released (1 mM EDTA—Gibco, 1.3 mg/ml collagenase–Gibco, and 0.25% Trypsin–Gibco). Viable and non-viable cells were detected by Trypan blue (Sigma) and counted using a hemocytometer (Housser Scientific Company, Horsham, PA). The number of viable cells was used to calculate the doubling time in hours between 3 and 7 days [18]; the doubling time was expressed as a percentage of the control.

ALP activity

ALP activity was assayed by the release of thymolphthalein from thymolphthalein monophosphate using a commercial

kit (Labtest Diagnostica SA, MG, Brazil) at day 7 and 14 of the subculture. Briefly, the culture medium was removed, the wells were washed three times with phosphate-buffered saline (Gibco) at 37°C and filled with 2 ml of deionized water, and then the cultures were submitted to five cycles of thermal-shock (alternating temperatures between 15 min at 37°C and 20 min at −20°C). Next, 50 μl of thymolphthalein monophosphate was mixed with 0.5 ml of a diethanolamine buffer, 0.3 mmol/ml, pH 10.1, and left for 2 min at 37°C. After this period, the mixture was added to 50 μl of the sample from each well. This mixture stood for 10 min at 37 ºC, and then 2 ml of a solution of 0.09 mmol/ml Na_2CO_3 and 0.25 mmol/ml NaOH were added to develop color. After 30 min, absorbance was measured at 590 nm, and ALP activity was calculated from a thymolphthalein standard curve to give a range from 0.012 to 0.40 μmol thymolphthalein/h/ml. ALP activity was normalized by the total protein content measured at 7 and 14 days, and these results were expressed as a percentage of the control.

Matrix mineralization

Matrix mineralization was detected at day 17 of the subculture by Alizarin Red S (Sigma), which stains areas rich in calcium. Attached cells were fixed in 10% formalin for 2 h at RT. After fixation, the specimens were dehydrated through a graded series of alcohol and stained with 2% Alizarin Red S (Sigma) with a pH 4.2 for 10 min. The calcium content was evaluated using a colorimetric method as previously described [19]. Briefly, 280 μl of 10% acetic acid was added to each well stained with Alizarin Red S, and the plate was incubated at RT for 30 min with shaking. This solution was transferred to a microcentrifuge tube and vortexed for 1 min. The slurry was overlaid with 100 μl mineral oil (Sigma), heated to exactly 85°C for 10 min, and transferred to ice for 5 min. The slurry was then centrifuged at 20,000×*g* for 15 min, and 100 μl of supernatant was transferred to a new microcentrifuge tube. Then, 40 μl 10% ammonium hydroxide was added to neutralize the acid. Each sample containing 140 μl was read at 405 nm in a 96-well format using opaque-walled transparent-bottomed plates (Fisher Scientific) on the μQuant plate reader (Biotek). Data were analyzed as absorbance and expressed as a percentage of the control.

RNA extraction and quantitative real-time PCR

The primer sequences, the predicted amplicon sizes, and the annealing and melting temperatures were designed using the Primer-Express software (Applied Biosystems, Foster City, CA) and are depicted in Table 1. Total RNA from osteoblasts was extracted using the Promega RNA extraction kit (Promega, Madison, WI), according to the manufacturer's instructions. The concentration of RNA was determined by optical density at a wavelength of 260 nm, using the GeneQuant (Amersham Biosciences, Piscataway, NJ). Complementary DNA (cDNA) was synthesized using 1 μg of RNA in a reverse transcription reaction (High Capacity, Applied Biosystems). Real-time PCR quantitative mRNA analyses were performed in an ABI Prism 7500 (Applied Biosystems, Warrington, UK). SybrGreen PCR MasterMix (Applied Biosystems), specific primers and 2.5 ng of cDNA were used in each reaction. The standard PCR conditions were 95°C (10 min) and 40 cycles of 94°C (1 min), 56°C (1 min), and 72°C (2 min), followed by the standard denaturation curve. For mRNA analysis, the relative level of gene expression was calculated in reference to both GADPH expression in the sample and its respective control using the cycle threshold (Ct) method [17].

Statistical analysis

For each experiment, all parameters and treatments were assayed in quintuplicate, with the exception of the real-time results, which were assayed in triplicate. All results were

Table 1 Primers sequences and reaction properties

Target*	Sense and anti-sense sequences	T.A. (°C)	T.M. (°C)	Bp
GADPH	AAATGCTTCTAGGCGGACTG GGTTTTGTCAAAGAAAGGGTG	58	78	60
ALP	CCAACTCATTTGTGCCAGAG CAGGGCATTTTTCAAGGTCTC	57	74	75
RUNX2	CACAAACAACCACAGAACCAC TTGCTGTCCTCCTGGAGAAA	56	77	137
OC	GCAGACACCATGAGGACCCT CCGGAGTCTATTCACCACCTTACTG	61	83	153
BSP	CTACTTTTATCCTCCTCTGAAACGGTT GCTAGCGGTTACCCCTGAGA	59	81	202
COL	CCAACGAGATCGAGCTCAGG GACTGTCTTGCCCCAAGTTCC	61	83	113

**GADPH* glyceraldehyde 3-phosphate dehydrogenase; *ALP* alkaline phosphatase; *RUNX2* runt-related transcription factor 2; *OC* osteocalcin; *BSP* bone sialoprotein; *COL* type I collagen.

TA annealing temperature; *TM* melting temperature; *bp* product size

expressed as the mean±standard deviation. The Kruskal–Wallis test, followed by the Fisher test, was performed to assess the significance of the parameters expressed as a percentage of the control as well as the significance of the real-time PCR results. Differences with values of $p<0.05$ were considered statistically significant.

Results

The doubling time, analyzed between 3 and 7 days, was reduced by LLLT in cultured cells harvested 24 and 48 h after RME, which indicates an increase in the cell proliferation rate (Fig. 1). However, LLLT increased the doubling time in cultured cells that were harvested 7 days after RME, indicating an inhibitory effect on the proliferation rate in this experimental group (Fig. 1).

In all groups evaluated, LLLT increased the ALP activity in cells cultures at days 7 and 14. Among experimental groups at day 7 of the subculture, the cells harvested 24 h after RME presented the most pronounced ALP activity. In contrast, the most pronounced effect at day 14 of the subculture was observed in the group harvested 48 h after RME (Fig. 2).

The mineralization analyses indicated that at day 17 of the subculture, cells from the rats submitted to LLLT exhibited a considerable increase in mineralization in all experimental groups, as demonstrated by a colorimetric assay (Fig. 3). The effect of LLLT on mineralization was marked on cultured cells explanted at 48 h after RME.

An osteoblastic phenotype in these cells was confirmed at the transcriptional level by analyzing the mRNA expression of the genes encoding ALP, Runx2, OC, COL, and BSP in cultured cells either treated or not treated with LLLT. These results indicate that LLLT induced a significant increase in the mRNA expression of ALP, Runx2, OC, COL, and BSP when compared to cultured cells from the

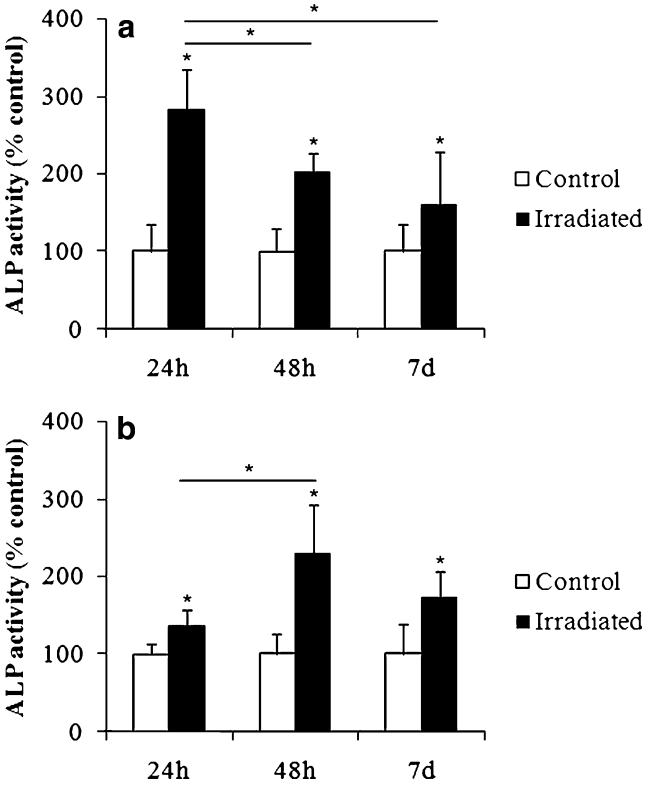

Fig. 2 Effect of LLLT after RME on ALP activity in cells derived from the palatal suture. Effect of LLLT after RME on ALP activity in osteoblastic cells derived from the palatal suture at 24 and 48 h and at 7 days after RME and LLLT. The cells were cultured for 7 days (**a**) and 14 days (**b**). Data are expressed as a percentage of the control. *$p<0.05$ when comparing the treatment to its respective control at the same point

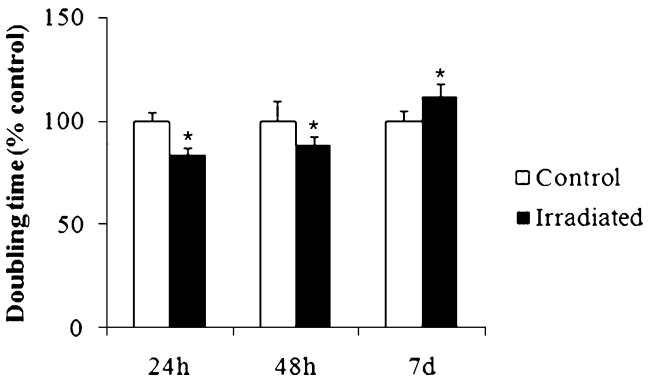

Fig. 1 Effect of LLLT after RME on cultures derived from the palatal suture. Effect of LLLT after RME on doubling time in osteoblastic culture derived from the palatal suture at 24 h, 48 h, and 7 days after RME and LLLT. The cells were cultured for 3–7 days (proliferative stage). Data are expressed as a percentage of the control. *$p<0.05$ when comparing the treatment to its respective control at the same point

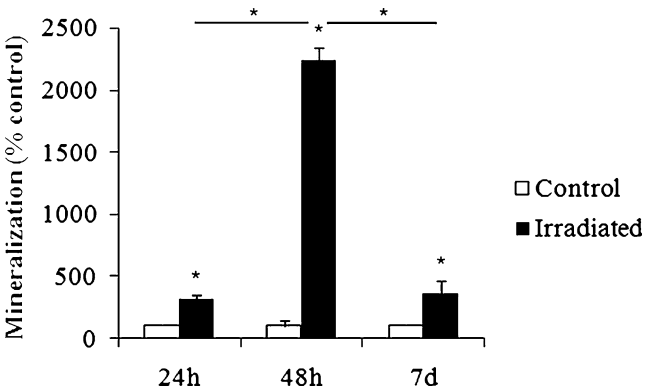

Fig. 3 Effect of LLLT after RME on bone-like formation in cultured cells derived from the palatal suture. Effect of LLLT after RME on mineralization in osteoblastic cultures cells derived from the palatine suture at 24 and 48 h and at 7 days after RME and LLLT. The cells were cultured for 17 days. Data are expressed as a percentage of the control. *$p<0.05$ when comparing the treatment to its respective control at the same point

sham groups. These results were consistent among the cells harvested 24 and 48 h and at 7 days after RME (Fig. 4).

Discussion

The present study indicates that local LLLT applied after RME has a stimulatory effect on proliferation and differentiation in rat osteoblastic cells derived from the palatal suture. These results were demonstrated by several in vitro assays. Cells isolated from the palatal suture after exposure to LLLT showed an increase in the proliferation rate, ALP activity, mineralization, and gene expression of osteoblastic markers, such as ALP, Runx2, OC, COL, and BSP. A single LLLT stimulus after RME induced changes in the osteoblastic activity that persisted for an extensive period of time, as demonstrated by the cells harvested 24 and 48 h and at 7 days after RME.

Cells derived from the palatal suture were able to proliferate and express ALP activity and mineralized the matrix independently of treatment, suggesting that the explanted cells exhibited an osteoblastic phenotype. The LLLT applied in vivo on the palatine suture after RME induced an increase in vitro in the proliferation of osteoblastic cells. This result suggests that the stimulatory

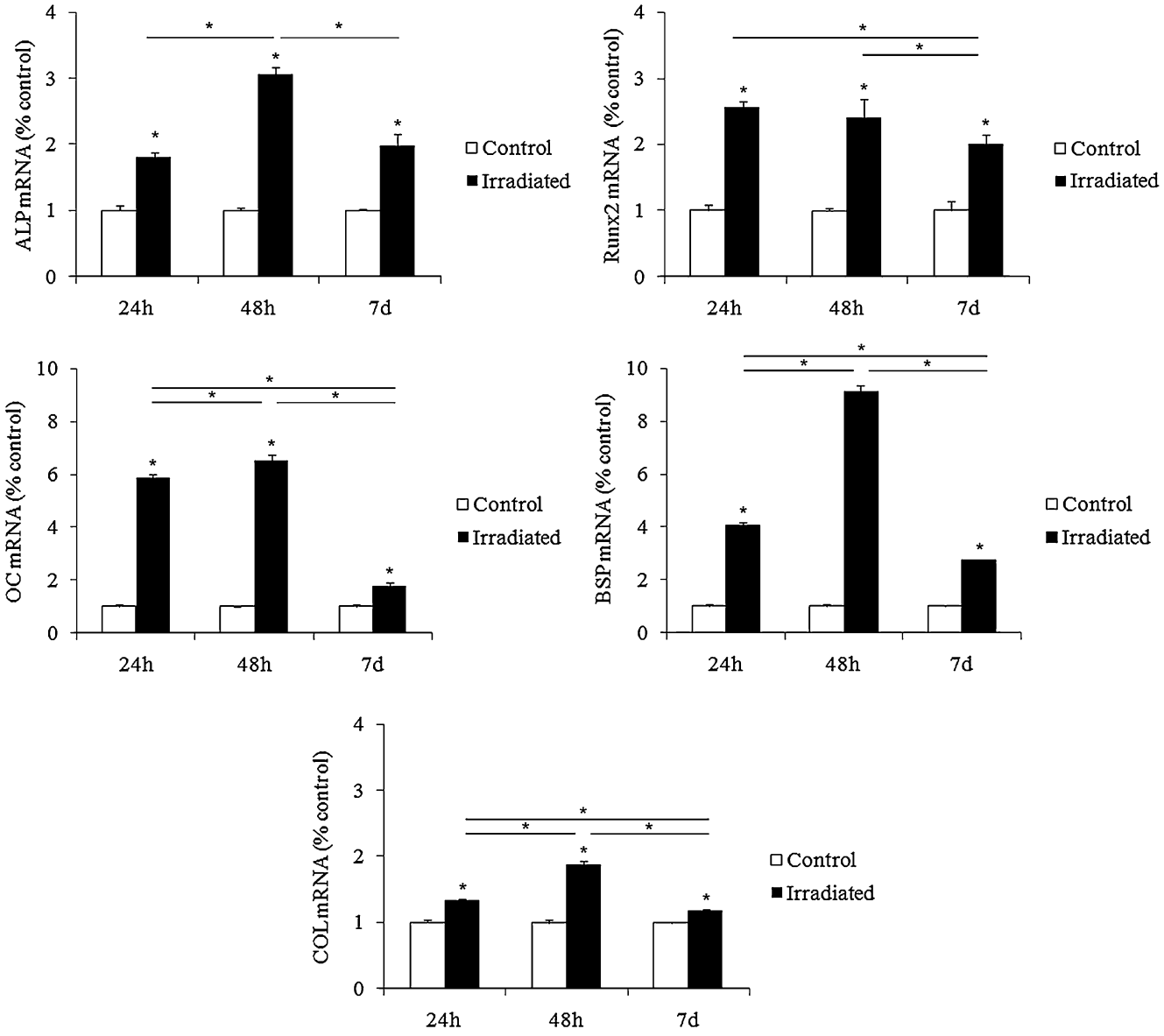

Fig. 4 Effect of LLLT after RME on mRNA of bone markers in cultured cells derived from the palatal suture. Effect of LLLT after RME on ALP, Runx2, OC, BSP, and COL mRNA in osteoblastic cultures cells derived from the palatal suture at 24 and 48 h and at 7 days after RME and LLLT. Data are expressed as a percentage of the control. *$p<0.05$ when comparing the treatment to its respective control at the same point

effect of LLLT on bone formation in the palatal suture after RME [11] could be due, at least in part, to an increase in the cellular proliferation rate. This hypothesis is corroborated by an increase in the proliferation observed after the direct application of LLLT on osteoblastic cells derived from rat calvaria [7], mouse calvaria (MC3T3) [7, 13, 19] and human cell lines [15, 20]. Conversely, no significant effect of LLLT has been reported in cells from rats [21]. The controversy regarding the effects of LLLT on proliferation could be related to variations between different studies in the total dose of irradiation or the irradiation time [5, 22–25].

The increase in the ALP activity, gene expression of osteoblastic markers, and mineralization indicated an increase in the expression of an osteoblastic phenotype in the palatal suture cells submitted to LLLT after RME. These findings indicate that the increase in the bone formation induced by LLLT after RME observed by other authors [11] could also be induced by enhancing osteoblastic differentiation. This notion is supported by higher levels of ALP protein activity and mRNAs encoding ALP and RUNX2 than control cells. Additionally, our study observed an increase in the mRNA expression of genes encoding matrix proteins, such as COL, OC, and BSP. The increases in the expression of these genes could mediate the increase in the matrix mineralization formation as evidenced in the cultured palatal suture cells submitted to LLLT after RME.

This study pioneered the use of in vitro assays to demonstrate an increase in the osteoblastic activity caused by in vivo applications of LLLT on bone. Others studies have shown similar enhancements in the ALP activity and matrix formation after LLLT applied directly to osteoblastic cells in culture [5, 7, 26, 27]. The similarities of these results indicate that LLLT is able to induce an increase in the osteoblastic activity whether applied in vivo on bone or in vitro on isolated cells.

In general, the stimulatory effects of LLLT on the proliferation rate and differentiation and mineralization were more pronounced in osteoblastic cells explanted 48 h after the RME and laser stimulus. Considering the different periods that cells were explanted in this study, it is possible suppose that the peak of the effects of LLLT on cell activity can occur in vivo around 48 h after the stimulus and that these effects persist in vitro for all 17 days of the culture period.

Conclusions

In conclusion, our findings indicated that the in vivo stimulatory effects on bone formation observed in the midpalatal suture after RME following LLLT can be mediated, at least in part, by an increase in the proliferation and expression of an osteoblastic phenotype in the cells derived from the palatal suture and submitted to the laser stimulus.

Acknowledgments We are deeply grateful to Roger Rodrigo Fernandes for excellent laboratory assistance at the Cell Culture Laboratory, School of Dentistry of Ribeirao Preto, University of São Paulo. We gratefully acknowledge the Brazilian agencies CAPES/FAPESP for financial support.

Author disclosure statement No competing financial interests exist.

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