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High-power light emitting diode irradiation promotes hard tissue differentiation of human dental pulp stem cells

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Antibacterial photodynamic therapy using light emitting diodes (LEDs) has been shown effective against periodontitis and peri-implantitis. In recent years, there has been research on the application of photodynamic therapy using LEDs in regenerative medicine. It uses "light" as a method of host activation, which is one of the three elements of regeneration. We reported that irradiation of human periodontal ligament stem cells with high-power red LEDs under appropriate irradiation conditions promotes hard tissue differentiation. It has also been confirmed that LEDs can be applied to bone marrow mesenchymal cells and osteoblasts to promote hard tissue differentiation. We investigated cell proliferation and hard tissue differentiation of human dental pulp stem cells, as one of the cell sources of undifferentiated mesenchymal stem cells, in order to clarify the effect of high-power red LEDs on hard tissue regeneration. (J Osaka Dent Univ 2022; 56: 203-208)

Key words: Light emitting diode; Dental pulp; Differentiation

INTRODUCTION

In recent years, light has been used in various roles[1-8] in medical treatment of the human body, and phototherapy, which is frequently used to complement this treatment, has been attracting attention. Niels Finsen[1] first reported the application of phototherapy over a century ago after it was first used for the treatment of dermatological disorders. Phototherapy induces several different effects, including cell proliferation,[2] wound healing,[3] pain relief,[4] and anti-inflammation treatment.[5] Previous studies in the field of dental research have also shown the potential beneficial effects of phototherapy for the treatment of oral mucositis,[6] candidosis,[7] and dentin hypersensitivity.[8]

Antibacterial photodynamic therapy using light emitting diodes has been shown effective against periodontitis and peri-implantitis, and their clinical application has been used in combination with a photosensitizer such as methylene blue.[9] In recent years, there has been research on the application of photodynamic therapy using LEDs in regenerative medicine. This method uses "light" as one method of host activation, which is one of the three elements of regeneration. Phototherapy or low-level light therapy (LLLT) uses low-power lasers or LEDs of less than 500 mW in the red to near-infrared (NIR) spectrum (600-1100 nm) to promote tissue repair, reduce inflammation, and relieve pain in a range of health fields.[10] We reported that irradiation of human periodontal ligament stem cells with high-power red LEDs under appropriate irradiation conditions promotes hard tissue differentiation.[11] This is an application of LEDs that does not require photosensitizers. We have also reported that LEDs have been applied to bone marrow mesenchymal cells and osteoblasts to promote hard tissue differentiation.[12]

Dental pulp plays a key role in body defense functions, such as sensing stimuli to the tooth, and has the ability to form secondary dentin that inhibits

bacterial invasion.[13] Human dental pulp stem cells (HDPSCs) are often used in tissue engineering due to their multipotency.[14] As a cell source of undifferentiated mesenchymal stem cells, they are a candidate for cell therapy to regenerate hard tissue during periodontal regenerative therapy and implant surgical operations. We investigated the effect of high-power, red LEDs on cell proliferation, and the osteogenic differentiation and mineralization of HDPSCs.

MATERIALS AND METHODS

Culture of human dental pulp stem cells

HDPSCs (Lonza Walkersville, Walkersville, MD, USA) were cultured in mesenchymal stem cell growth medium with a 10% supplementary mix in 75 cm^2 culture dishes at 37°C with 5% CO_2. The culture medium was changed every 3 days. All experiments were performed using cells at passages 3-5. Osteogenic differentiation assays were performed using osteogenic medium (OM) containing 50 μM L-ascorbic acid 2-phosphate (Nacalai Tesque, Kyoto, Japan), 10 mM β-glycerophosphate (Wako Pure Chemical Industries, Tokyo, Japan), and 10 nM dexamethasone (Wako Pure Chemical Industries).

Irradiation procedure

This study employed an LZ 1-00 R 205 Deep Red LED prototype emitter (LedEngin, Santa Clara, CA, USA) that emits red light specifically at wavelengths of 600 to 700 nm with a peak at 650 nm. The intensity of radiant energy was confirmed by a Nova II power meter (Ophir, North Andover, MA, USA). The distance from the LED to the cell layer was 40 mm, while the spot size was 4 cm^2. The intensity at well level was 200 mW/cm^2. Radiant exposure can be calculated by multiplying the intensity by the exposure time. The total radiant exposures were at 6 J/cm^2 for 30 s with continuous output. Over these irradiation periods, there were no detectable temperature changes compared to the controls.

Alkaline phosphatase activity assay and staining

HDPSCs were seeded into 24-well plates at a density of 4×10^4 cells/well and cultured to confluence in a normal culture medium. The culture medium was then replaced with osteogenic medium. After 12 hours, the cells were subjected to irradiation. After 1 or 2 weeks, the cells were washed and fixed and stained with alkaline phosphatase (ALP) with an Alkaline Phosphatase Staining Kit (Cosmo Bio, Tokyo, Japan). For measurement of ALP activity, cells were washed with phosphate-buffered saline (PBS) and lysed in 0.2 % Triton X-100, and the ALP activity was quantified by one-step p-nitrophenyl phosphate (pNPP; Thermo Fisher Scientific, Rockford, IL, USA). ALP activity was normalized to the amount of DNA. DNA content was measured by a DNA assay kit (Thermo Fisher Scientific), and data were analyzed using SoftMax Pro software (Molecular Devices, LLC., Sunnyvale, CA, USA).

Mineralization assay and Alizarin red staining

Calcium depositions in the extracellular matrix were observed by Alizarin Red S staining. Following the same seeding, growth, and irradiation procedures described for ALP, HDPSCs were cultured in osteogenic differentiation medium for 1 and 2 weeks. The cells were then washed with PBS and fixed in 70% ethanol for 10 minutes at −20°C. The HDPSCs were stained with a solution of 1% Alizarin Red S (Wako Pure Chemical Industries) for 3 minutes at room temperature and then washed with PBS. Extracellular calcium depositions were measured after dissolving precipitates with 10% formic acid. The amount of calcium was measured using a calcium detection kit (Wako Pure Chemical Industries) following the manufacturer's instructions. Absorbance was measured at 610 nm using a multimicrotiter reader.

Osteocalcin enzyme-linked immunosorbent assay (ELISA)

The sandwich enzyme immune assay used in this study was specific for human osteocalcin and measured its levels directly in cell osteogenic cul-

ture supernatants after 1 and 2 weeks of culture following the same seeding, growth, and irradiation procedures described for ALP. Osteocalcin (OCN) levels in the culture supernatant were quantified with an OCN detection kit (GLA-type Osteocalcin EIA Kit; Takara Bio, Shiga, Japan), following the manufacturer's instructions.

Quantitative real-time polymerase chain reaction

Gene expression changes of *Runx2* mRNA was determined by quantitative real-time polymerase chain reaction (PCR) in osteogenic cultures grown for 1 and 2 weeks. Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands), and 10 μL RNA from each sample was reverse transcribed into cDNA using a PrimeScript RT reagent kit (Takara Bio). All real-time PCR assays were performed using the TaqMan Real-Time PCR assay system (Thermo Fisher Scientific) following the manufacturer's instructions. Gene expression levels were calculated using the ΔΔCt method[15] and normalized to GAPDH expression.

Immunofluorescence staining

HDPSCs were seeded in 24-well plates at 5×10^4 cells/mL in 1 mL normal culture medium. After culturing of 1 day for cell adhesion, the cells were serum starved in osteogenic medium for 12 hours and then subjected to irradiation at the optimal LED energy density of 6 J/cm^2. After irradiation, the cells were fixed with 4% paraformaldehyde, washed with PBS, and permeabilized with 0.5% Triton X-100 diluted in PBS. Blocking was performed with a 3% bovine serum albumin-phosphate buffer solution (Sigma-Aldrich) diluted in PBS and then incubated overnight at 4°C with diluted primary rabbit antibodies against dentin sialophosphoprotein (DSPP). Immunofluorescence staining was performed using Alexa Fluor 488® (Thermo Fisher Scientific). Nuclei were then stained using DAPI (Dojindo Laboratory, Kumamoto, Japan) and images were captured using a confocal laser scanning microscope (Zeiss International, Optical and Optoelectronic Technology, Oberkochen, Germany).

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics Ver. 17 (IBM, Chicago, IL, USA). One-way analysis of variance followed by Tukey's post-hoc test was used to determine significance at the 5% level.

RESULTS

Production of alkaline phosphatase activity and staining

Intracellular ALP production in the 6 J/cm^2 LED irradiation group was significantly enhanced compared with non-irradiation group (Fig. 1 A), and ALP stain-

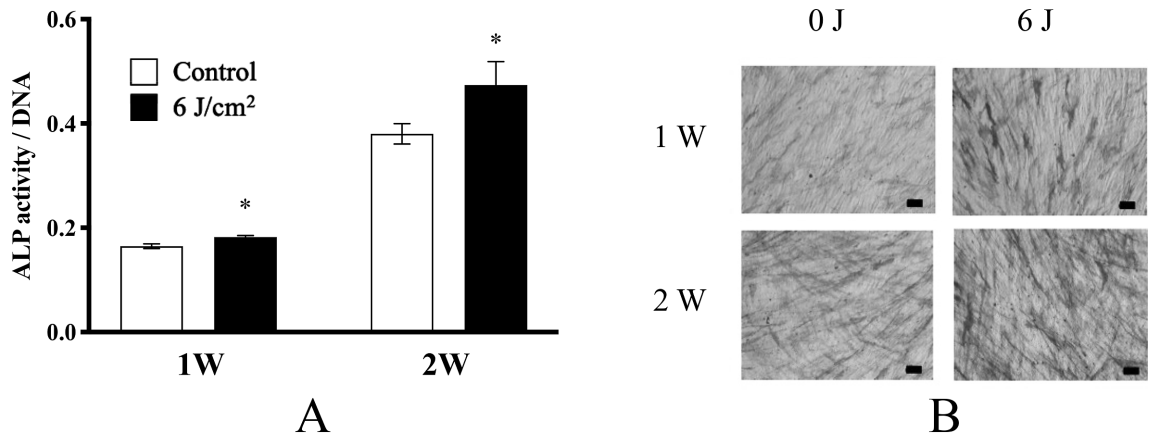

Fig. 1 (A) high-intensity red LED irradiation promotion of ALP activity by ALP activity measured at 1 and 2 weeks. The amount of ALP was normalized to the amount of DNA to normalize ALP activity. (B) ALP staining of HDPSCs after LED irradiation and culture for 1 and 2 weeks (*$p < 0.05$ vs control, Bar: 100 μm).

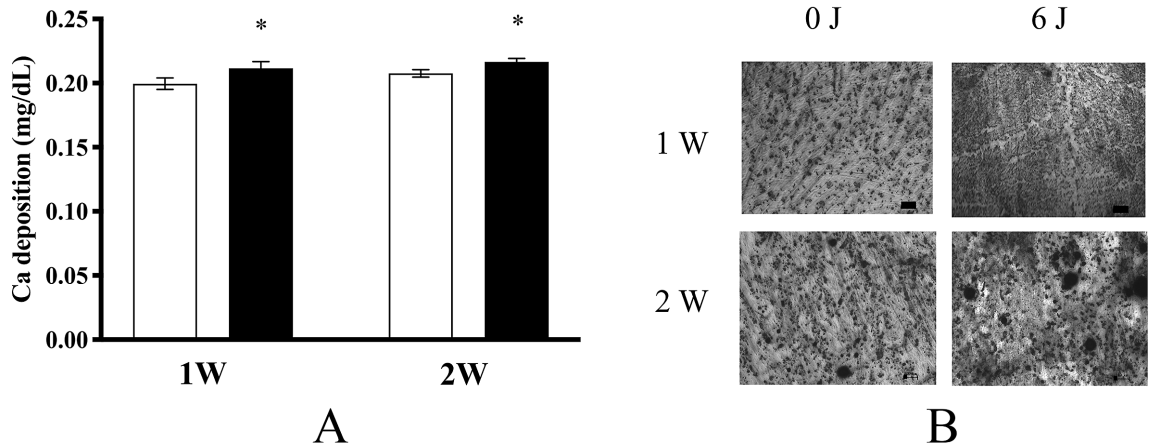

Fig. 2 (A) Enhancement of mineralization around HDPSCs by high-intensity red LED irradiation. Extracellular calcium deposition was measured at 1 and 2 weeks. (B) Calcified nodules formed by Alizarin red staining at 1 and 2 weeks (*p＜0.05 vs control, Bar: 100 μm).

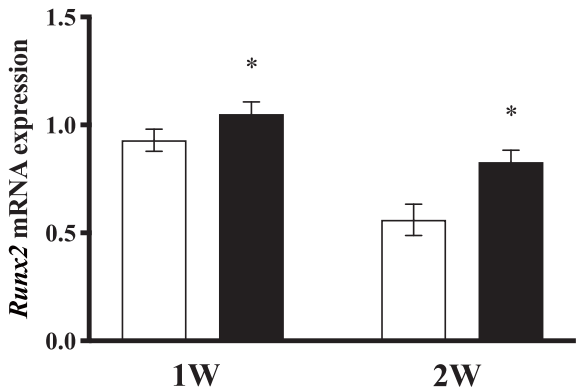

Fig. 3 Elevation of *Runx2* mRNA expression in HDPSCs by high-intensity red LED irradiation at 1 and 2 weeks (*p＜0.05 vs control).

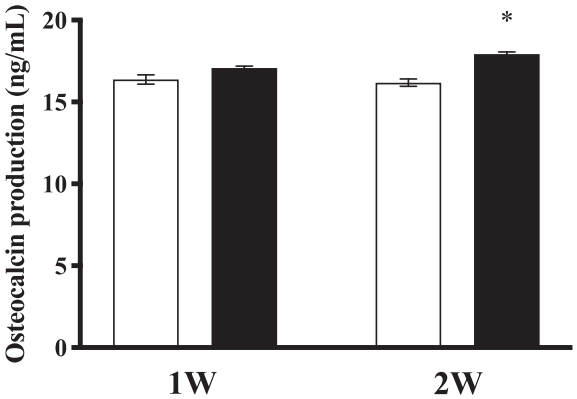

Fig. 4 Promotion of the production of osteocalcin (OCN) from HDPSCs by high-intensity red LED irradiation at 1 and 2 weeks (*p＜0.05 vs control).

ing showed that the LED irradiation group was stained darker (Fig. 1 B).

Calcium deposition and Alizarin red staining

Extracellular calcium deposition from the 6 J/cm^2 LED irradiation group was significantly enhanced compared with the non-irradiation group (Fig. 2 A), and the Alizarin red staining showed that the LED irradiation group was stained darker (Fig. 2 B).

Osteocalcin production

Osteocalcin secretion in the 6 J/cm^2 LED irradiation group was significantly increased at 1 and 2 weeks compared with the non-irradiation group (Fig. 3).

***Runx2* mRNA expression**

Runx2 mRNA in the 6 J/cm^2 LED irradiation group was significantly enhanced at 1 and 2 weeks compared with the control group (Fig. 4).

Dentin sialophosphoprotein production

LED irradiation significantly promoted production of dentin sialophosphoprotein at 1 and 2 weeks. The 2-week group stained strong positively compared with 1-week group (Fig. 5).

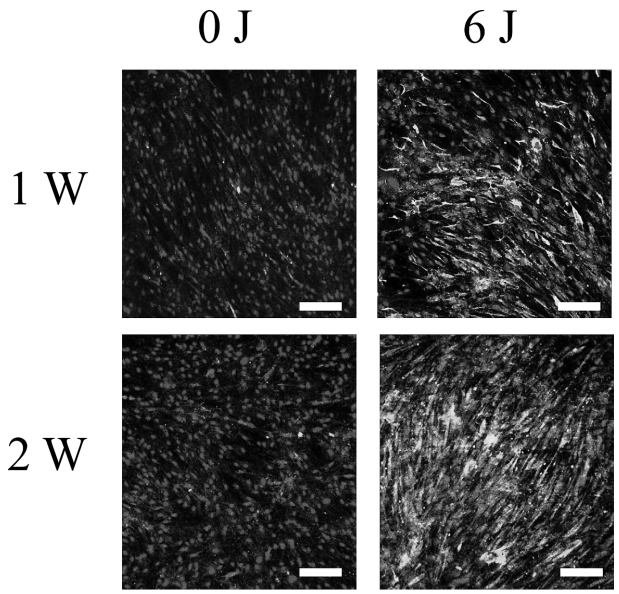

Fig. 5 Expression of dentin sialophosphoprotein (DSPP) by immuno fluorosecene. High-intensity red LED irradiation promoted the expression of stained DSPP at 1 and 2 weeks. (Bar: 100 μm).

DISCUSSION

The results of this study indicated that high-intensity, red LED irradiation promotes the osteogenic differentiation and mineralization of HDPSCs. Pulp tissue has the property of differentiating into hard tissue. The regenerative ability makes secondary dentin and creates a cell source for the treatment of hard tissue regeneration in the oral cavity. Considering that regeneration of hard tissue is mainly enhanced by cell osteogenic differentiation,[16] the present study investigated the effects of high-intensity LEDs on osteogenic differentiation and mineralization of HDPSCs. ALP, as a marker of osteogenic phenotype, indicates the activity of the hard tissue formation process.[17] The noncollagen OCN in the bone matrix is a late marker for osteoblast differentiation.[18] We measured ALP activity, the degree of ALP staining, and OCN secretion in this study and confirmed that the high-intensity LEDs affected the osteogenic differentiation of HDPSCs throughout the early to late stages. Alizarin red staining is used to determine the presence of calcified nodules in the bone matrix;[19] the amount of calcium was measured using a calcium quantification kit. The results of this study suggest that high-intensity LED irradiation promotes the mineralization of HDPSCs.

Runx2 is one of the essential transcription factors during the early stages of osteogenic differentiation.[20] Runx2 further promotes the differentiation of the cells into immature osteoblasts after differentiating into preosteoblasts.[21] The expression of Runx2 induces mesenchymal stem cells to commit to the osteoblastic lineage, differentiating into mature osteoblasts, and terminally differentiating into osteocytes.[21] The COL1A1 gene encodes the pro-alpha 1 chains of type I collagen, which is found in most connective tissues, including cartilage, and plays an important role in bone formation.[22] Our results showed that high-intensity LED irradiation enhanced the expression of transcription factors necessary for osteogenic differentiation and bone formation.

Dentin sialophosphoprotein is a precursor protein for other proteins found in the teeth. It is produced by odontoblasts in dental pulp, and in smaller quantities by osteoblasts and osteocytes. It is required for the normal mineralization of teeth.[24] The results of this study suggest that high-intensity LEDs promote the expression of dentin sialophosphoprotein. Turrioni *et al.*[25] reported that infrared LED irradiation was capable of biostimulating cultured stem cells from human exfoliated deciduous teeth by increasing the expression and synthesis of proteins associated with the mineralization of tissue such as as ALP, collagen, and dentin sialophosphoprotein. Although the properties of the cells may be slightly different, it is consistent with our results that hard tissue differentiation is promoted by irradiating stem cells from an extracted tooth with an LED.

In summary, we found that high-intensity, red LED irradiation promotes the osteogenic differentiation of DPSCs. We believe that high-intensity, red LED irradiation is useful for the formation of tertiary dentin, which will further promote the capacity for hard tissue to differentiate and calcify.

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declare that they have no conflicts of interest related to this study.

REFERENCES

1. Roelandts R. A new light on Niels Finsen, a century after his Nobel Prize. *Photodermatol Photoimmunol Photomed* 2005; **21**: 115-117.
2. Kreisler M, Christoffers AB, Willershausen B, d'Hoedt B. Effect of low-level GaAlAs laser irradiation on the proliferation rate of human periodontal ligament fibroblasts: an *in vitro* study. *J Clin Periodontol* 2003; **30**: 353-358.
3. Hopkins JT, McLoda TA, Seegmiller JG, David Baxter G. Low-level laser therapy facilitates superficial wound healing in humans: a triple-blind, sham-controlled study. *J Athl Train* 2004; **39**: 223-229.
4. Chow RT, Heller GZ, Barnsley L. The effect of 300 mW, 830 nm laser on chronic neck pain: a double-blind, randomized, placebo-controlled study. *Pain* 2006; **124**: 201-210.
5. Huang TH, Lu YC, Kao CT. Low-level diode laser therapy reduces lipopolysaccharide (LPS)-induced bone cell inflammation. *Lasers Med Sci* 2012; **27**: 621-627.
6. Gautam AP, Fernandes DJ, Vidyasagar MS, Maiya AG, Vadhiraja BM. Low level laser therapy for concurrent chemoradiotherapy induced oral mucositis in head and neck cancer patients—a triple blinded randomized controlled trial. *Radiother Oncol* 2012; **104**: 349-354.
7. Mima EG, Pavarina AC, Dovigo LN, Costa CA, Karachi C, Bagnato VS. Susceptibility of Candida albicans to photodynamic therapy in a murine model of oral candidosis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2010; **109**: 392-401.
8. Flecha OD, Azevedo CG, Matos FR, Vieira-Barbose NM, Ramos-Jorge ML, Goncalves PF, Koga Slive EM. Cyanoacrylate versus laser in the treatment of dentin hypersensitivity: a controlled, randomized, double-masked and non-inferiority clinical trial. *J Periodontol* 2013; **84**: 287-294.
9. Umeda M, Tsuno A, Okagami Y, Tsuchiya F, Izumi Y, Ishikawa I. Bactericidal effects of a high-power, red light-emitting diode on two periodontopathic bacteria in antimicrobial photodynamic therapy *in vitro. J Investig Clin Dent* 2011; **2**: 268-274.
10. 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**: 237-249.
11. Yamauchi N, Taguchi Y, Kato H, Umeda M. High-power, red light-emitting diode irradiation enhances proliferation, osteogenic differentiation, and mineralization of human periodontal ligament stem cells via ERK signaling pathway. *J Periodontol* 2018; **89**: 351-360.
12. Ruan Y, Kato H, Taguchi Y, Yamauchi N, Umeda M. Irradiation by high-intensity red light-emitting diode enhances human bone marrow mesenchymal stem cells osteogenic differentiation and mineralization through Wnt/β-catenin signaling pathway. *Lasers Med Sci* 2021; **36**: 55-65.
13. Yu C, Abbott PV. An overview of the dental pulp: its functions and responses to injury. *Aust Dent J* 2007; **52**: S 4-16.
14. Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S. Stem cell properties of human dental pulp stem cells. *J Dent Res* 2002; **81**: 531-535.
15. Finke B, Luethen F, Schroeder K, Nebe JB, Rychly J, Liefeith K, Bader R, Walschus U, Lucke S, Schlosser M, Neumann HG, Ohl A, Weltmann KD. The effect of positively charged plasma polymerization on initial osteoblastic focal adhesion on titanium surfaces. *Biomaterials* 2007; **28**: 4521-4534.
16. Yamamoto Y, Ohsaki Y, Goto T, Nakasima A, Iijima T. Effects of static magnetic fields on bone formation in rat osteoblast cultures. *J Dent Res* 2003; **82**: 962-966.
17. Aubin JE, Liu F, Malaval L, Gupta AK. Osteoblast and chondroblast differentiation. *Bone* 1995; **17**: 77-83.
18. Ikeda T, Nomura S, Yamaguchi A, Suda T, Yoshiki S. *In situ* hybridization of bone matrix proteins in undecalcified adult rat bone sections. *J Histochem Cytochem* 1992; **40**: 1079-1088.
19. Katayama N, Kato H, Taguchi Y, Takara A, Makoto U. The effects of synthetic oligopeptide derived from enamel matrix derivative on cell proliferation and osteoblastic differentiation of human mesenchymal stem cells. *Int J Mol Sci* 2014; **15**: 14026-14043.
20. Rutkovskiy A, Stensløkken KO, Vaage IJ. Osteoblast differentiation at a glance. *Med Sci Monit Basic Res* 2016; **22**: 95-106.
21. McCarthy TL, Ji C, Chen Y, Kim KK, Imagawa M, Ito Y. Runt domain factor (Runx)-dependent effects on CCAAT/enhancer-binding protein δ expression and activity in osteoblasts. *J Biol Chem* 2000; **275**: 21746-21753.
22. Karsenty G, Park R-W Regulation of type I collagen genes expression. *Int Rev Immunol* 1995; **12**: 177-185.
23. Yamamoto R, Oida S, Yamakoshi Y. Dentin sialophosphoprotein-derived proteins in the dental pulp. *J Dent Res* 2015; **94**: 1120-1127.
24. Turrioni AP, Basso FG, Montoro LA, Almeida Lde F, Costa CA, Hebling J. Phototherapy up-regulates dentin matrix proteins expression and synthesis by stem cells from human-exfoliated deciduous teeth. *J Dent* 2014; **42**: 1292-1299.