Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2022**; 27(2): 062
http://doi.org/10.31083/j.fbl2702062

Original Research

Effect of Low-Level Er: YAG (2940 nm) laser irradiation on the photobiomodulation of mitogen-activated protein kinase cellular signaling pathway of rodent cementoblasts

Jiawen Yong[1,2,*,†], Pingping Li[3,4,†], Isaac Kably Mizrahi[1], Rene Franzen[1,5], Sabine Groeger[2], Sabine Ruf[2], Norbert Gutknecht[1,5], Márcia Martins Marques[1,6]

[1]AALZ–Aachen Dental Laser Center, RWTH Aachen University, 52074 Aachen, Germany
[2]Department of Orthodontics, Faculty of Medicine, Justus Liebig University Giessen, D-35392 Giessen, Germany
[3]Department of VIP center, School and Hospital of Stomatology, Cheeloo College of medicine, Shandong University, 250012 Jinan, Shandong, China
[4]Shandong Key Laboratory of Oral Tissue Regeneration & Shandong Engineering Laboratory for Dental Materials and Oral Tissue Regeneration, 250012 Jinan, Shandong, China
[5]Department of Conservative Dentistry, Periodontology and Preventive Dentistry, RWTH Aachen University, 52074 Aachen, Germany
[6]School of Dentistry, Ibirapuera University, 04661-100 Sao Paulo, Brazil
*Correspondence: Jiawen.Yong@dentist.med.uni-giessen.de; jiawen.yong@rwth-aachen.de (Jiawen Yong)
[†]These authors contributed equally.

Academic Editor: Graham Pawelec
Submitted: 10 December 2021 Revised: 5 January 2022 Accepted: 17 January 2022 Published: 14 February 2022

Abstract

Backgrounds: Dental avulsion due to trauma, especially in young patients, is a worldwide problem, requiring tooth replacement. Delayed replantation could cause tooth loss when the cementum is severely damaged. A small number of studies has reported that photobiomodulation (PBM) therapy using Er: YAG laser irradiation activates cellular signaling responses in different cell types, resulting in a variety of favorable biological effects. The aim of this *in vitro* study was to evaluate the potential biostimulatory effect of low-level Er: YAG laser irradiation on the biological responses of cultured mouse cementoblasts (OCCM-30), including the mitogen-activated protein kinases (MAPKs). **Methods**: OCCM-30 cells were exposed to 2940 nm Er: YAG laser irradiation for 15 s at 0.34 W (pulse duration of 100 or 1000 μs, 17 mJ/pulse) at energy densities of 1 or 2 J/cm^2. Irradiated and non-irradiated OCCM-30 cells were tested for migration (Scratch assay), proliferation (MTS assay) and functional differentiation (Alizarin Red S assay). *Lumican* (*Lum*) and *Fibromodulin* (*Fmod*) gene expression, and activation of MAPKs, were assessed by RT-PCR and Western blotting, respectively. **Results**: Low-level Er: YAG laser irradiation at 2 J/cm^2 and pulse duration of 1000 μs resulted in the highest migration rate and proliferation. Moreover, the pulse duration irradiation of 100 μs increased *Lum* expression. *Fmod* expression was increased after 1000 μs pulse duration laser stimulation. Low-level Er: YAG laser irradiation increased the mineralization of OCCM-30 cells after 7 days and activated ERK1/2, P38 and JNK signaling. **Conclusions**: Low-level Er: YAG laser irradiation induces OCCM-30 cell migration, proliferation and differentiation, and activates the MAPK signaling pathway.

Keywords: Er: YAG laser; Cementoblasts; Migration; Proliferation; Cementogenesis; MAPK

1. Introduction

Dental avulsion due to trauma, especially in young patients, is a worldwide problem, which demands tooth replacement by either tooth replantation or placement of dental implants or prosthodontics. It is known that delayed replantation after avulsion may result in tooth loss because of root resorption [1]. So-called replacement resorption or inflammatory resorption is a probable adverse outcome after replantation of a tooth [2]. It is most likely that root resorption happens in cases where periodontal ligament (PDL) and cementum have been severely damaged [2]. To prevent or delay root resorption, the International Association of Dental Traumatology (IADT) has proposed different treatment methods for the root surface of avulsed teeth before replantation [3]. These different root surface treatment modalities include among others fluorides, steroids, sodium alendronate, enamel matrix derivatives, fibroblast growth factor (FGF) [4,5].

Viable cementoblasts and/or intact molecules associated with these cells are likely to be actively involved in recruiting pre-cementoblasts that will form new cementum which is critical for structurally and functionally re-establishing a sound PDL attachment [6]. Thus, to achieve a successful tooth replantation cellular viability and function of periodontal tissues, such as cementoblasts and PDL, must be preserved [7]. In this sense, photobiomodulation (PBM) therapy applied with low-level lasers could be an adjunctive therapy because PBM improves cellular proliferation, migration, differentiation [8,9], growth factor secretion [10], among others cellular biological activities [11]. Thus, PBM applied to be root surface could possibly improve the prognosis of tooth replantation. In fact, in re-

cent year, many laser-based root surface treatments, mostly using high-power lasers like erbium-dopped yttrium aluminum garnet (Er: YAG) and neodymium-dopped YAG (Nd: YAG) lasers, have attracted attention [12].

Some studies have reported that Er: YAG laser irradiation, applied with low-level energies, activates cellular signaling responses in different cell types resulting in a variety of favorable biological effects [13–17]. Schwarz *et al.* (2004) [13] showed that Er: YAG laser irradiation at an energy density of 5.1–12.7 J/cm^2 significantly decreases mitochondrial activity in Saos-2 osteoblasts. Another study reported that Er: YAG laser irradiation does not influence cell proliferation but significantly promotes mineralization of primary osteoblast-like cells [17]. Irradiation at an energy density of 3.3 J/cm^2 significantly promotes mineralization of primary osteoblast-like cells after osteogenesis induction of 7 days, possibly via enhanced *OCN* expression [16].

Low-level Er: YAG laser irradiation enhances osteoblast proliferation through activation of ERK1/2 [18]. Low-level Er: YAG laser irradiation also improves proliferation and calcification of osteoblast-like cells [17] as well as proliferation of cultured human gingival fibroblasts [14]. Finally, it has been demonstrated that low-level Er: YAG laser irradiation increases prostaglandin E production via the induction of cyclooxygenase-2 mRNA in human gingival fibroblasts [15]. Recently, Lin *et al.* (2021) [19] also reported that Er: YAG laser irradiation at 4.2 J/cm^2 promotes human periodontal ligament fibroblasts (PDLFs) cell migration and proliferation through Galectin-7 induction. However, the effects of the low-level Er: YAG laser irradiation on cementoblasts, which are the cells of importance for the tooth replantation success, remain unclear. Thus, it is necessary to understand the molecular biological effects of low-level Er: YAG laser irradiation on this cell type. Therefore, the aim of this *in vitro* study was to evaluate the potential photobiomodulatory effect of low-level Er: YAG laser irradiation on the biological response of cultured mouse cementoblasts, including mitogen-activated protein kinases (MAPKs) signaling molecules.

2. Material and methods

2.1. Cell culture

OCCM-30 cell line was provided by Prof. M. Somerman (NIH, NIDCR, Bethesda, Maryland, USA) and cultured according to a previous description [20]. Briefly, cells were maintained in cell culture growing medium composed by α-MEM (11095-080, Gibco, Invitrogen, Paisley, UK) growing medium containing 10% Fetal Bovine Serum (FBS) (10270-106, Gibco) and 1% Penicillin/Streptomycin (15140-122, Gibco) and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were seeded into 6-wells plates (657160, Greiner Bio-One, Frickenhausen, Germany) at a density of 3×10^5 cells/well.

Table 1. Power parameter of Er: YAG laser.

Parameter (units)	Values
Wavelength (nm)	2940
Operating Mode	Pulsed
Frequency (Hz)	20
Pulse Duration (μs)	1000 100
Duty Cycle	2% 0.2%
Spot Area of Tip (cm^2)	1.69
Cell Area (cm^2/well)	9.6
Energy Density (J/cm^2)	2
Number of irradiation sessions	1
Intervals between sessions	-
Irradiation Time (s)	15
Power Average (W)	0.34
Power Peak (W)	17 170
Energy Level (mJ/pulse)	17

For inducing cementogenesis and performing mineralization assay, mineralizing medium composed by 10 mM β-Glycerophosphate (#35675, Calbiochem, Darmstadt, Germany) and 50 μg/mL Ascorbic Acid (6288.1, Roth, Karlsruhe, Germany) added to cell culture growing medium was used for 7 days and 14 days after reaching confluence. The mineralizing medium was changed twice a week.

2.2 Laser application

The Er: YAG laser apparatus (Cat No.: MCL31 Dermablate; Asclepion Laser Technologies, Jena, Germany) from the department of Periodontology of the University of Aachen, emitting at a wavelength of 2940 nm was employed in this study. In order to equalize the profile of the laser beam, laser irradiation was performed perpendicularly to the bottom of the culture dish at a distance of 25–30 cm, by means of a handpiece attached to the end of the articulated arm, as described previously (Fig. 1) [18]. The cultured cells were irradiated just once without interval. The serum-free medium was removed immediately before irradiation and all irradiations were performed in the absence of culture medium. The output parameter settings were determined based on previous findings in the literature [18] and a pilot study [14]. The irradiations were done using the pulsed model and 20 Hz on the panel, with an irradiation time of 15 s (Table 1). The actual energy level reaching at the cell surface was 17 mJ/pulse, resulting in a total energy density of 2 J/cm^2. Immediately after irradiation, the appropriate serum-free medium was re-added to the dish.

2.3 Cell migration assay

In order to analyze the cell migration ability, a previously established wound healing assay was applied. Briefly, OCCM-30 cells were plated at a density of 8×10^3 cells/well in 6-wells plates (657160, Greiner Bio-One), incubated in cell culture growing medium until 100% con-

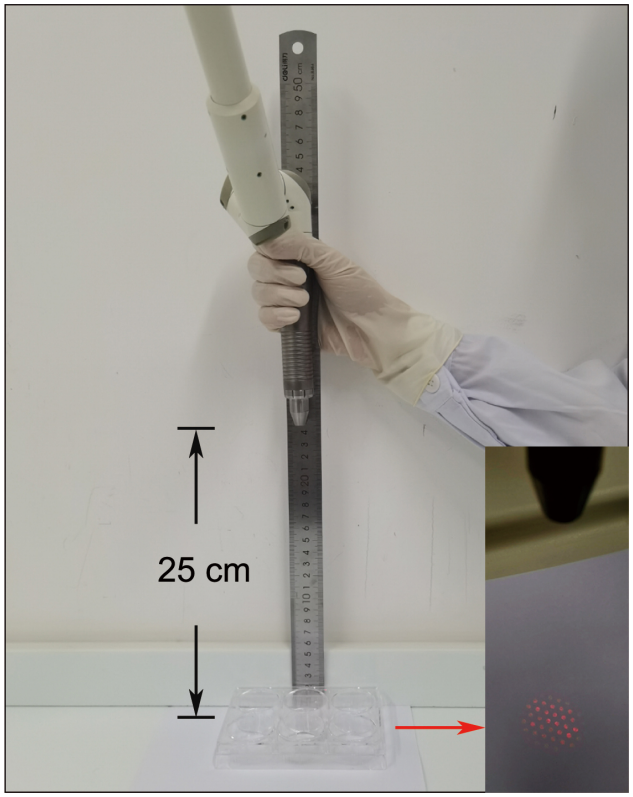

Fig. 1. Schematic illustration of the low-level Er: YAG laser irradiation procedure. The Er: YAG laser device was equipped and performed perpendicularly to the bottom of the culture dish at a distance of 25 cm (black arrow). Energy densities were generated in a round homogeneous spot with a diameter of 35 mm (red arrow).

fluence. Cells were then preincubated for 12 h with starvation medium and wounded by scratch using a 100 μL tip forming a single standardized scratch (cell-free area) in the center of the cell layer. Then, all non-adherent OCCM-30 cells were washed with PBS. The wounded cell monolayers were incubated for 12 h without, respectively after stimulated by Er: YAG (Table 1). Wounded-area images were taken immediately after wounding and 12 h after scratching. The wounded cell monolayers were photographed at 100 $\times$ magnification (Leica, Wetzlar, Germany) and the percentages of wound closure area between cell layer borders was analyzed and calculated over time using the Image J software (National Institutes of Health, NIH, USA).

2.4 Cell proliferation assay

The viability and proliferation of OCCM-30 cementoblasts were examined by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reduction assay (CellTiter 96$^{@}$ Aqueous One Solution Cell Proliferation Assay, Promega, Walldorf, Germany) according to manufacturer's instructions. Briefly, OCCM-30 cells at a density of 5 $\times$ 10^3 cells/well at passages 3–5 were seeded in 96-wells plates (655180, Greiner Bio-One) containing 5% FBS for 4 h to allow adherence. Thereafter, cells were washed twice with PBS and then irradiated or not. Then, the cultures were maintained in starvation medium (α-MEM containing 0.5% FBS and 1% Penicillin/Streptomycin) over a period of 24 h. Then, at each 3 h until 12 h, 20 μL of the MTS solution was added into each well and cells were incubated during 2 h at 37°C in a 5% CO_2 atmosphere. Plates were read by 490 nm using a 96-well micro-plate reader (BioTek, Winooski, VT, USA) to measure the amount of formazan by cellular reduction of MTS.

2.5 Quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR)

Cells were kept overnight in starvation medium. Different pulse duration parameters of Er: YAG laser were used to stimulate cells. Total RNA was isolated using the NucleoSpin$^{@}$ RNA Kit (740955.50, MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's recommended protocol. RNA concentrations were measured using a spectrophotometer (Nanodrop2000, ThermoFisher Scientific, Germany). cDNA was synthesized using the InnuSCRIPT Reverse Transcriptase kit (845-RT-6000100, Analytik Jena, Jena, Germany) and performed on CFX96^{TM} System Cycler (Bio-Rad, Feldkirchen, Germany).

The SsoAdvanced^{TM} Universal SYBR$^{@}$ Green Supermix (1723271, Bio-Rad) was used in each reaction setup. The primers employed were Mouse Lumican (qMmuCED0048548) and Fibromodulin (qMmuCED0049464) from Bio-Rad. GAPDH (qMmuCED0027497, Bio-Rad) was used as housekeeping Gene. Results were analyzed using the Bio-Rad CFX96^{TM} Manager 3.1 software (Bio-Rad, Germany). The temperature profile of the RT-PCR reaction was 95 °C for 15 min, 40 cycles of denaturation at 94 °C for 15 s, and annealing and extension for 60 °C for 30 s.

2.6 Differentiation assay (Alizarin Red S assay)

OCCM-30 cells were seeded to 6-well plates at a density of 3 $\times$ 10^5 cells /well using α-MEM containing 10% FBS and 1% penicillin/streptomycin. Upon confluence, the culture medium was replaced by mineralizing medium. The cells were irradiated three times a week and left untreated in the control group, respectively. The amount of mineralization in these cell monolayers was analyzed by the Alizarin red assay, as described previously [21–23]. Briefly, cultured cells were fixed with ice-cold 70% Ethanol for 10 min and stained with 1% Alizarin Red S solution (A5533, Sigma-Aldrich, Taufirchen, Germany) during 5 min at room temperature. Mineralized nodule formation was assessed in an inverted phase contrast microscopy (Leica, Wetzlar, Germany) using the LASV4.8 software (Leica, Wetzlar, Germany). To quantify the amount of calcium accumulation in the mineralized extracellular matrix,

Alizarin Red S-stained cultures were dissolved using 100 mM cetylpyridinium chloride (6004-24-6, Sigma-Aldrich) for 1 h to release calcium-bound dye into the solution. The absorbance of the released dye was measured at 490 nm using a spectrophotometer (xMarkTM, Microplate Absorbance Spectrophotometer, 1681150, BioRad).

2.7 Western blot analysis

Cells were irradiated or not after serum-free starvation overnight. 200 μL/well protein lysis RIPA buffer (89901, ThermoFisher Scientific, Waltham, USA) supplied with Protease Inhibitor (78442, ThermoFisher Scientific) was used for protein extraction. Protein concentrations in the cellular lysate were measured using Pierce^{TM} BCA Protein Assay Kit (23225, ThermoFisher Scientific) [24]. Further, 20 μg protein samples per lane were separated using 10% SDS-PAGE gel by electrophoresis and transferred to a Nitrocellulose membrane. The membranes were blocked with Blocking Buffer (37587, ThermoFisher) for 1 h at room temperature and incubated in primary antibodies for ERK1/2 (ab196883, Abcam, China); phospho-ERK1/2 (ab214362, Abcam, China); p54/p56 JNK (#9252, CST, China), phospho-JNK (ab4821, Abcam); P38 MAPK (ab4822, Abcam); phospho-P38 MAPK Alpha (#4511, CST), and β-actin (ab8227, Abcam) at a concentration of 1:1000. The secondary antibodies employed were Polyclonal Goat Anti-Rabbit (ab6721, Abcam) at a concentration of 1:2000. The band signal was then detected with X-ray Amersham Hyperfilm (28906836, GE Healthcare, Hamburg, Germany) utilizing Amersham ECL Western Blotting Detection Reagents (9838243, GE Healthcare) and visualized using OPTIMAX X-Ray Film Processor (11701-9806-3716, PROTEC GmbH).

The intensity of phosphorylated MAP kinase (p-MAPK) was measured using the NIH Image J program and normalized to the control group, which was considered as 1.0.

2.8 Statistical analysis

Statistical analysis was performed using the GraphPad Prism 8.0 software (GraphPad software, California, USA). All values are expressed as the mean $\pm$ standard deviation (SD) and were analyzed using Student *t*-test for unpaired samples to determine the statistically significant differences between groups. Differences were considered statistically significant at a *p*-value of <0.05. The experiments were repeated successfully at least 3 times.

3. Results

3.1 Er: YAG laser irradiation at low-level energy densities promotes the migration of cementoblasts

Firstly, we investigated the effect of Er: YAG laser irradiation with a constant pulse frequency of 20 Hz and irradiation time of 15 s at different energy densities (1 or 2 J/cm^2) and different pulse durations (100 μs and 1000 μs) on the migration of OCCM-30 cells at one time period (12 h). The quantitative analysis showed that the highest migration rate was observed in cells irradiated with 2 J/cm^2 and pulse duration of 1000 μs. When the pulse duration was 100 μs the cells irradiated with 2 J/cm^2 presented a significantly higher migration rate than the group treated with 1 J/cm^2 irradiation and the control group (Fig. 2A–B).

Secondly, we followed the migration rate of OCCM-30 cells under the influence of different pulse durations (100 μs and 1000 μs) at a fixed energy density (2 J/cm^2) each 3 h until 12 h. The results showed that after 3 h incubation the 100 μs pulse duration irradiation led to a more pronounced migration compared to 1000 μs pulse duration irradiation group (Fig. 1C–D). After 6 h the migration was the same in both pulsed groups. After 9 h incubation, the 1000 μs pulse duration irradiation increased the migration rate significantly (Fig. 2C–D).

3.2 Low-level Er: YAG laser irradiation at pulse duration of 1000 μs increases the proliferation of OCCM-30 cells

The effect of different pulse duration (100 μs and 1000 μs) at a fixed energy density (2 J/cm^2) on the proliferation of cementoblasts was assessed by the MTS assay. Proliferation of cells irradiated with 100 μs were similar to that of control cells. Significant higher proliferation rates of cementoblasts were observed in cells irradiated with 1000 μs pulse duration in comparison to the non-irradiated control group (Fig. 3A).

The total RNA was collected at 1 and 2 h after laser irradiation. qRT-PCR showed significant increase of mRNA expression of *Lumican* (*Lum*) in cells irradiated with 100 μs at both times, whereas irradiations of 1000 μs led to expressions similar to that of control cells (Fig. 3B). *Fibromodulin* (*Fmod*) expression at 2 h observation time was significantly smaller in cells irradiated with 100 μs, and significantly higher when the irradiations were of 1000 μs pulse duration than those of control cells (Fig. 3C).

3.3 Low-level Er: YAG laser irradiation promotes functional differentiation (mineralization) of OCCM-30 cells

Next, we performed a mineralization assay to evaluate the effect of different pulse duration (100 μs and 1000 μs) on cementoblasts under a constant parameter with energy density of 2 J/cm^2. The Alizarin Red S staining showed that the amount of mineralization was significantly higher (**$p < 0.01$) in OCCM-30 cells irradiated at both pulse duration of Er: YAG irradiation after day 7 compared to non-irradiated control cells (Fig. 4A–C). At 14 days all groups presented similar amount of mineralized extracellular matrix (ECM; Fig. 4A,B and D).

3.4 Low-level Er: YAG laser irradiation activates the MAP kinase signaling pathway in cementoblasts

To determine whether or not the MAPK pathway would be involved in the low-level Er: YAG irradiation

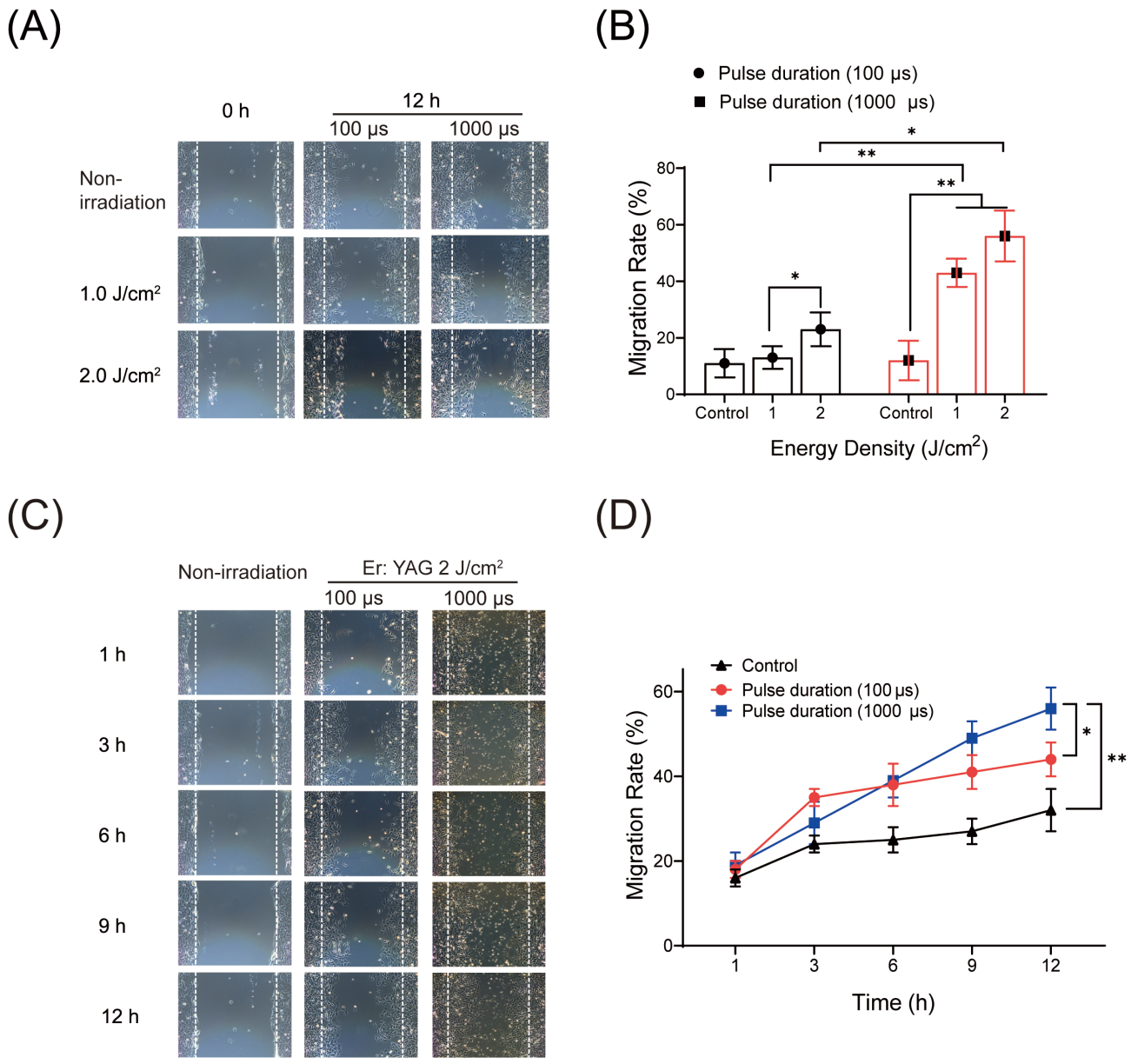

Fig. 2. Effects of low-level Er: YAG laser irradiation at 1 and 2 J/cm^2 on the migration of cementoblasts over time. (A) Illustrative phase photomicrographs showing the migration of cementoblasts (OCCM-30 cells) after low-level Er: YAG laser irradiation at the different energy densities (1 J/cm^2 and 2 J/cm^2) at the cell monolayer wounded area at 0 and 12 h after standard scratching using a 100 μL pipet tip. The dotted lines indicate the wound edge at the beginning and at the end of the experiment. (Magnification of 100 ×). (B) Graphic representation of the percentages of migration rates measured over a period of 12 h in cultures irradiated with 1 J/cm^2 and 2 J/cm^2 using pulses of 100 μs (black columns) and 1000 μs (red columns). Data are presented as mean percentages of migration rates. (C) Illustrative phase photomicrographs of OCCM-30 cells showing the migration effect of Er: YAG irradiation at energy density of 2 J/cm^2 on OCCM-30 cells wounded monolayers between 1 to 12 h after standard scratching using a 100 μL pipet tip. The dotted lines indicate the wound edge at the beginning and at the end of the experiment. (Magnification of 100 ×). (D) Graphic representation of OCCM-30 cells migration rates measured over a period of 12 h. Data are presented as mean percentages of wound closure. Bars are standard deviation (SD): *$p < 0.05$; **$p < 0.01$. These results are from the 3 different experiments.

effects on the OCCM-30 cells, we checked the expression of phosphorylated MAP kinase protein. Based on the Western Blot results, irradiation at both pulse duration was found to enhance ERK1/2, JNK and P38 phosphorylation as compared to the control as soon as 5 min after irradiation (Fig. 5). These increased phosphorylation of P38 was observed from 10 to 30 min, with a peak at 10 min, when 100 μs pulse duration was applied (Fig. 5A and B) and very

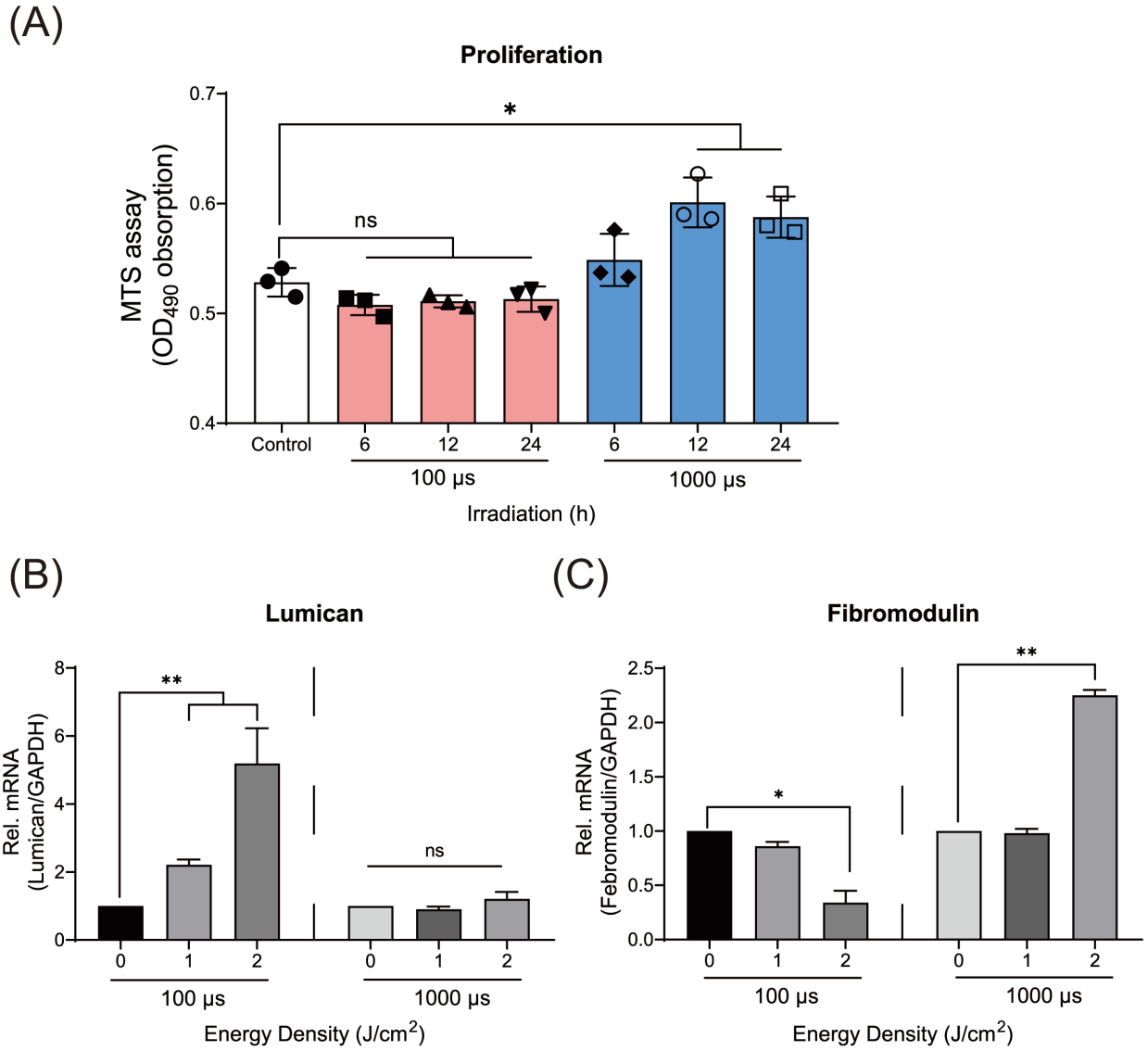

Fig. 3. Effects of low-level Er: YAG laser irradiation on proliferation of cementoblasts. (A) Graphic representation of the MTS assay. The highest response of OCCM-30 cells to the Er: YAG irradiation at 2 J/cm^2 energy density was observed in the group irradiated with 1000 μs at 12 and 24 h. (B) Graphic representation of the qRT-PCR analysis of mRNA expression of Lum in OCCM-30 cells irradiated by low-level Er: YAG laser at an energy density of 2 J/cm^2. The highest Lum expression was observed in OCCM-30 cells irradiated with 100 μs pulse duration. (C) Expression of Fmod was significantly smaller than all other groups when the OCCM-30 cells were irradiated with100 μs pulse at 2 h, and significantly higher than control when the 1000 μs. Values are expressed as mean (Bars are SD of the relative quantity): $p > 0.05$; *$p < 0.05$; **$p < 0.01$ when compared with control. These results are from 3 different experiments.

shortly (just at 5 min) after 1000 μs pulse duration irradiation (Fig. 5C and D). The enhancement of ERK1/2 and JNK phosphorylation was observed from 5 min until 10 and 15 min, respectively, with peaks at 5 min, when 100 μs pulse duration was applied (Fig. 5B). With a 1000 μs pulse duration irradiation the enhancement of ERK1/2 and JNK phosphorylation was longer from 5 to 60 or 45 min, respectively, with peaks at 30 min (Fig. 5D).

4. Discussion

In the present study, we demonstrated that low-level Er: YAG laser at specific irradiation parameters improved the migration, proliferation and functional differentiation of OCCM-30 cells along with the regulation of mRNA expression of *Lum* and *Fmod* genes. Moreover, low-level Er: YAG laser irradiation activated the MAP kinase signaling pathway of the cultured mouse cementoblasts.

In our study three important parameters were considered, the energy output, pulse repetition rate and irradiation

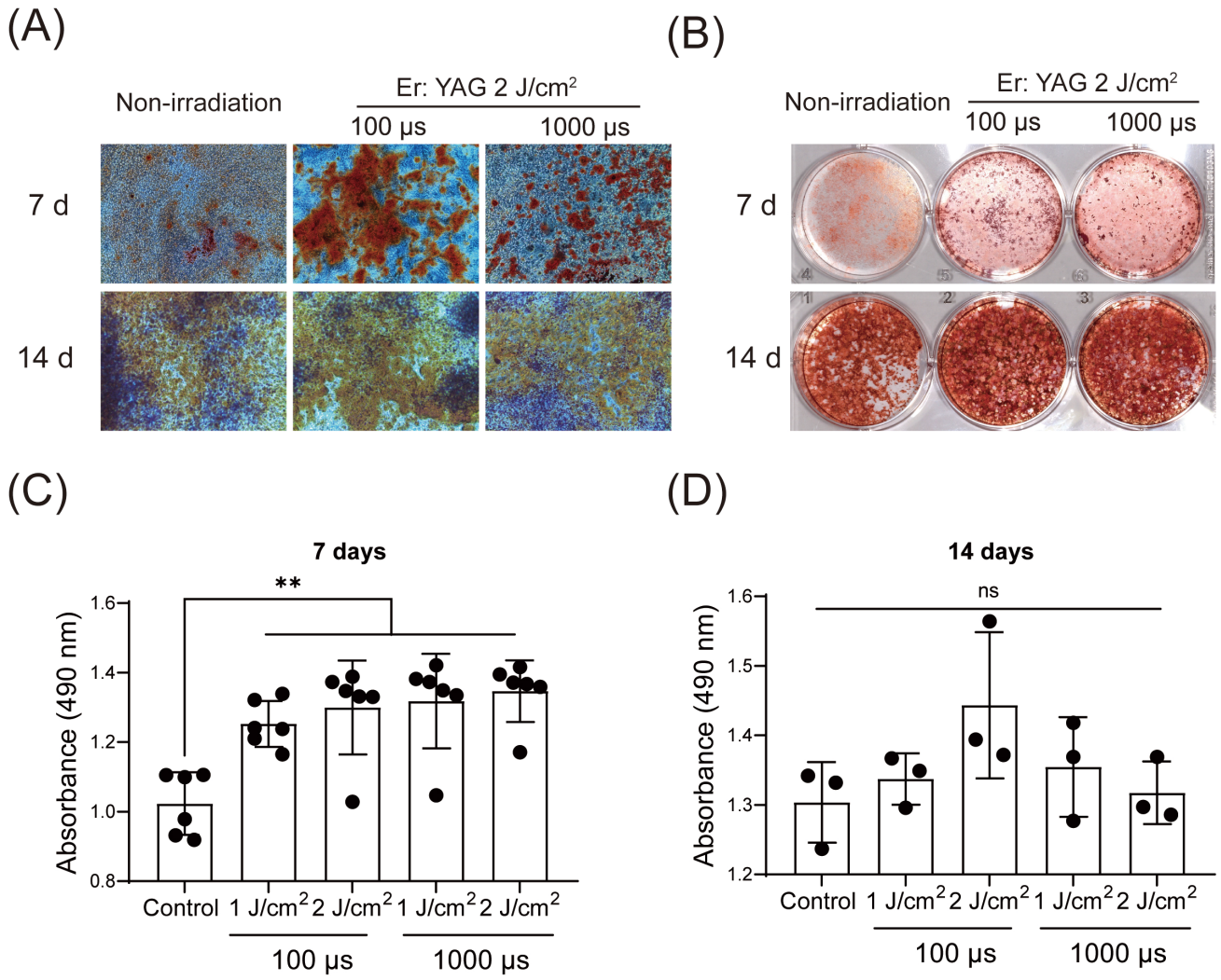

Fig. 4. Effects of low-level Er: YAG laser irradiation on mineralization of cementoblasts. (A) Illustrative phase photomicrographs of OCCM-30 cells submitted to different pulse duration parameters of Er: YAG irradiation at 2 J/cm^2 every other day for 15 s after 7 and 14 days. Extracellular mineralization nodules of the matrix were determined by Alizarin Red S staining. (Magnification of 10 ×). (B) Representative photographs of cell culture plates with OCCM-30 cells submitted to the Alizarin Red S. (C) Graphic representation of the quantification of Alizarin Red S staining extracted with cetylpyridinium chloride at 7 days of mineralization induction. Mineralization was significantly increased in both pulse duration of 100 μs and 1000 μs treatment compared to control. (D) Graphic representation of the quantification of Alizarin Red S staining extracted with cetylpyridinium chloride at 14 days of mineralization induction showing similar results in all groups. Values are expressed as means (Bars are SD) of the relative quantity: ns (not significant), $p > 0.05$; *$p <$ 0.05; **$p < 0.01$ when compared with control. The result was the representative of 3 different experiments.

time, which were predetermined according to results of previous studies [18]. An energy output of 170 mJ/pulse was fixed for the Er: YAG laser, and 25–30 cm of irradiation distance was determined based on previous studies [14,15]. An irradiation time of 15 s and a pulse repetition rate of 20 Hz was selected as the most safe and effective parameter, which is used for hard tissue [25]. After determining the basic setting for laser device, the effects of different pulse duration of low-level Er: YAG laser were evaluated by changing the pulse duration parameter while keeping the other parameters fixed.

Firstly, we demonstrated that OCCM-30 cells irradiated with low-level Er: YAG laser exhibited an increased proliferation and migration response to 1.0 or 2.0 J/cm^2 energy density. Especially, treatment with the Er: YAG laser at 2.0 J/cm^2 energy density promoted cementoblasts proliferation significantly. These data indicate that Er: YAG laser irradiation at 2.0 J/cm^2 had a higher efficiency for cementoblasts activation in these experiments. From these results, we adopted an irradiation energy density of 2.0 J/cm^2 for subsequent experiments and for the western blot analyses to investigate the activation of signaling molecules.

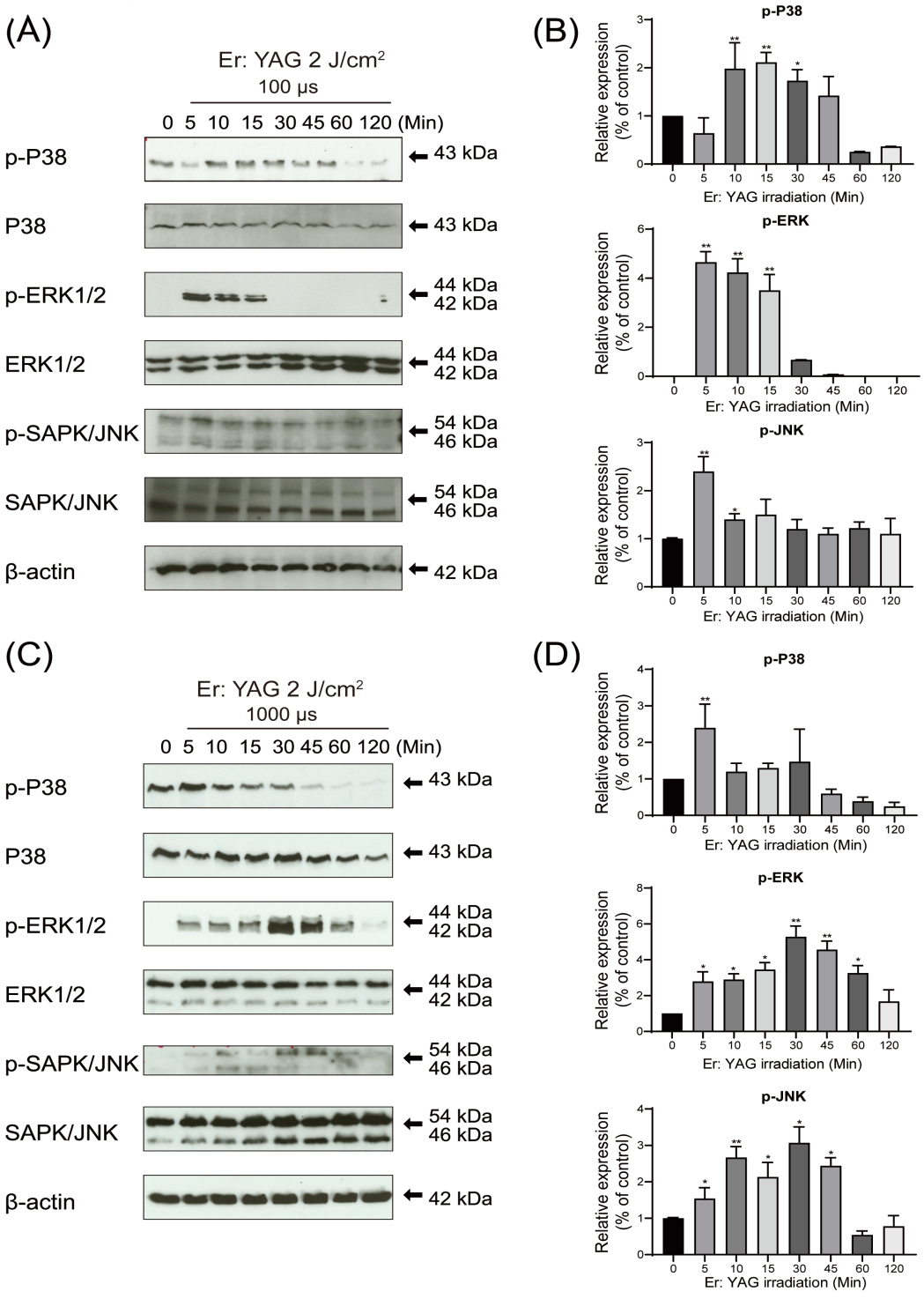

Fig. 5. Effects of low-level Er: YAG laser irradiation on the MAP kinase activation in cementoblasts. (A, C) Photographs of the gels: OCCM-30 cells were stimulated with 100 μs (A) and 1000 μs (C) pulse duration of low-level Er: YAG laser irradiation and collected after indicated time points. Cell lysates were subjected to Western Blot and incubated with JNK (46 and 54 kDa), ERK1/2 (42 and 44 kDa), P38 (42 kDa) and their phosphorylated form primary antibody. β-actin (42 kDa) served as a loading control. (B, D) Graphic representation of the relative expression of P38 (42 kDa), ERK1/2 (42 and 44 kDa) and JNK (46 and 54 kDa) normalized to the control group of OCCM-30 cells were stimulated with by 100 μs (B) and 1,000 μs (D) pulse duration of low-level Er: YAG laser irradiation. Values are expressed as means (Bars are SD) of the relative expression: $p > 0.05$; *$p < 0.05$; **$p < 0.01$ when compared with control. The result was the representative of 3 different experiments.

The effects of PBM on different cells and tissues have been studied by several researchers *in vitro* and *in vivo* showing the ability of this therapy to increase migration, proliferation and also cell differentiation depending on the applied irradiation parameters [26–32]. Also, studies were conducted to investigate the effects of PBM therapy on cell survival at different time intervals between alveolar tooth avulsion and replantation [33]. For instance, Kim *et al*. (2017) [34] reported that PBM with lasers with four wavelengths (415, 525, 660, and 830 nm) at different low energy levels variably promoted the proliferation and migration of human outer root sheath cells. Alsulaimani *et al*. [33] (2015) using an *in vivo* rat model revealed that two weeks treatment of daily application of PBM therapy (Ga-Al-As, at a wavelength of 830 nm) significantly increased dental root cementum thickness in rats as determined histologically. Pourzarandian *et al*. (2005) [14] reported that low-level Er: YAG laser irradiation at an energy density of 3.37 J/cm^2 enhances human gingival fibroblasts proliferation.

Within our knowledge up to now, only one study has described the effects of laser irradiation on cementoblasts [35]. It reported that diode laser irradiation (940 nm, 0.3 W in continuous wave, 18 J/cm^2) significantly delayed the decrease trend in cell proliferation of cementoblasts up to 96 h compared to the untreated control group. Additionally, the expression of *Integrin Binding Sialoprotein* (*BSP*) and *Osteocalcin* (*OCN*), which are transcripts required for cementum formation and the expression of *Bone Morphogenetic Proteins* (*BMP*) -2, -3 were increased [35]. These results suggested that biostimulation of the periodontal attachment apparatus by low-level can be used for regenerative periodontal therapies for activating cementoblasts [21–23,36].

Cementogenesis, including the deposition of extracellular matrix (ECM) and its subsequent mineralization, is an important process during cementum repair, homeostasis maintenance and periodontium regeneration [37]. The cementum matrix is composed of a variety of macromolecules presented in the ECM including proteoglycan such as *Lumican* (*Lum*) and *Fibromodulin* (*Fmod*), both of which were first found in bovine cementum [38] and are likely to play a regulatory role with respect to cementum homeostasis [38]. *Fmod*, a small leucine-rich proteoglycan, is involved in migration of periodontal cells and in the regulation of collagen organization of the pericellular and extracellular matrix *in vivo* [39]. Thus, it has been proven to be one of the significant components of the ECM synthesized during cementogenesis [39]. *Lum*, another small leucine-rich proteoglycan, has been shown to promote wound healing *in vitro* [40]. From our present data, the up-regulated expression of *Lum* along with the down-regulated expression of *Fmod* observed when low-level Er: YAG when applied at 100 μs pulse duration had no effect on cell proliferation. On the other hand, the up-regulated expression of *Fmod* when 1000 μs pulse duration was applied seemed to be related with the significant increase in cell proliferation. With shorter pulse duration (100 μs), the proliferation was not changed significantly. Considering that 100 μs pulse duration induced weaker activation of ERK1/2 and shorter peak of JNK signaling, the proliferation possibly resulted from the regulation of MAPK pathways. This has been observed in another cell type by Azizulkarim *et al*. (2018) [41], which showed that short pulse duration inhibits the HeLa cells proliferation rate.

The expression of *Fmod* is reported to promote not only proliferation, but also migration and cementogenesis [39]. Then, 1000 μs of pulse duration could upregulate the OCCM-30 cells mineralized extracellular matrix, which showed clear dependence on longer pulse duration.

Previous *in vitro* study has shown that 100 μs pulse duration of low-level Nd: YAG laser irradiation is short enough to enhance osteoblast cell migration and contribute to the regeneration of bone tissues [42]. But we found that rapidly migration rate occurred with longer pulse duration (1000 μs). This may due to the stronger and longer activation of ERK1/2 and JNK signalings in response to 1000 μs pulse duration.

In the current experiment using low-level Er: YAG laser irradiation, irradiated cultures, independently of pulse duration, showed higher cementogenesis induction at 7 days when compared with the control group. These results appear to be in agreement with previous *in vivo* studies showing higher or favorable bone tissue regeneration following periodontal Er: YAG therapy [25]. In addition, previous *in vitro* studies using low-level diode laser irradiation have shown laser-induced promotion of bone formation through accelerated cellular proliferation and differentiation, higher ALP activity and subsequent *OCN* expression in rat calvarial cells [43]. Stein *et al*. (2005) [44] have observed that low-level He-Ne laser irradiation induced a higher proliferation and differentiation of human osteoblasts followed by enhanced osteogenic marker expression.

Further, low-level Er: YAG laser irradiation is able to activate various cellular signaling responses in different cell types, mostly osteoblasts and fibroblasts, resulting in positive biological effects [13–17]. Previous studies also reported that triggered MAPKs have strong relation with cell proliferation [45–47]. Upon extracellular stimulation, by for example PBM, MAPKs serine/threonine kinases phosphorylate their corresponding specific substrates at serine and/or threonine residues [48]. Thereafter, the phosphorylation events can differently modulate substrates and through this the entire signaling cascade activity. Thus, the MAPK signaling pathways are likely to be involved in migration, proliferation and apoptosis.

The present results revealed, that the MAPK pathway on the protein level was activated in OCCM-30 cells in response to low-level Er: YAG irradiation. Phosphorylated ERK1/2 and JNK were strongly increased with long

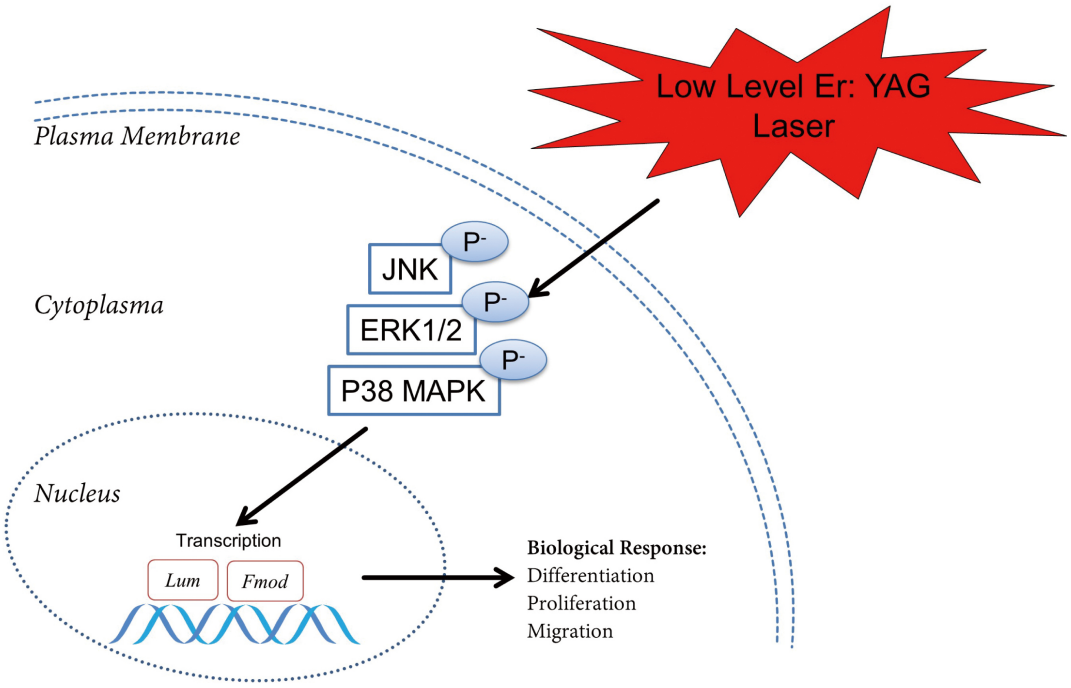

Fig. 6. Graphic representation of the possible mode of action of the irradiation on the MAP kinase activation in cementoblasts. The proposed model describing that low-level Er: YAG laser irradiation mediates the proliferation, migration and differentiation of cementoblasts and actives the MAPK signaling pathway.

duration of expression after stimulation with higher pulse duration of low-level Er: YAG laser irradiation (Fig. 6). These results suggest that Er: YAG laser-induced cementoblasts migration, proliferation and mineralization may require ERK1/2, P38 or JNK activation, which needs to be further investigated and verified by relevant inhibitors. Moreover, the peaks of expression of the phosphorylated proteins were observed as soon as 5 min after the PBM stimuli for ERK1/2 and JNK and 10 min for P38. These findings indicate the possibility of faster cementum repair following low-level Er: YAG laser therapy, providing novel mechanistic insights into the positive effects of Er: YAG laser on cementoblasts biological response.

To our knowledge, until now there is no research aiming to investigate the different pulse duration on cementoblasts. Thus, this would be the first study to report that different effects caused by two pulse durations, which may due to the different activation levels of MAPK signaling pathways, resulting in different biological responses in cementoblasts. Here we have shown that the effects of 100 and 1000 μsec are different. Proliferation and migration rates presented direct relation to the size of the pulse (e.g., large pulse induced higher rates). On the other hand, differentiation showed not to be dependent on the pulse duration, as both pulse duration were able to induce formation of similar amount of mineralized ECM and upregulated all MAPKs signaling molecules studied, although with different patterns. Thus, it is difficult to draw an explanation for such findings once under the premise of same average power and duty cycle, the pulse duration is negatively correlated to the peak power. Short pulse duration may protect illuminated samples from overheating, by allowing them to cool down between bursts of light. Long pulse duration may deliver damaging total emission, even if the peak power is moderate. However, we did not observe significant differences on temperature between two pulse durations (data not shown). This leads open the question as to which kind of pulse durations of Er: YAG laser treatment might be benefit to the hemeostasis of cementoblasts or without adversely affecting cementoblasts.

Our western blot results are in line with previous research showing that PBM with several lasers activates signaling molecules [18,49,50]. For instance, Miyata *et al.* (2010) [18] reported that ERK1/2 plays a role in the increased proliferation of human dental pulp cell following low-level diode laser irradiation. Shefer *et al.* (2006) [49] reported that biostimulatory effect of PBM using a He-Ne laser (wavelength of 632.8 nm) induced skeletal muscle cell activation by ERK1/2. Miyata *et al.* (2006) [50] demonstrated that low-level diode laser (Ga-Al-As, wavelength of 810 nm) laser irradiation is able to active ERK1/2 to play an increased proliferation effect on human dental pulp cells.

The present study suggested a strong relationship between cementoblast proliferation, migration and mineralization in response to low-level Er: YAG laser irradiation with activation of ERK1/2, P38 and JNK. However, one of the limitations of the present study is the lack of a MAPK inhibition assay that could better clarify the involvement of

MAPK pathways in the laser-induced biological response. Thus, further detailed investigation is required to better understand the potential involvement of these pathways in the positive effects of low-level Er: YAG laser irradiation on cementoblasts biology.

5. Conclusions

In conclusion, the results of the present study show that low-level Er: YAG laser irradiation influences OCCM-30 cells migration, proliferation and differentiation and activates the ERK1/2, P38 and JNK signaling depending on the pulse duration. Thus, it could be considered as an adjunctive therapy to be applied in some clinical situations involving dental roots, such as tooth replantation.

Author contributions

JY, PL—Conceptualization; Data curation; Formal analysis; Software; Writing - original draft. IKM—Data curation; Formal analysis; Investigation; Methodology. RF, SG, SR, NG, MMM—Funding acquisition; Supervision; Validation; Writing - review & editing.

Ethics approval and consent to participate

Not applicable.

Acknowledgment

We sincerely thank J. Deschner and M. Nokhbehsaim (Department of Periodontology, University of Bonn, Germany) and M. Somerman, (Laboratory of Oral Connective Tissue Biology, NIH, Bethesda, MD, USA) to provide OCCM-30 cells. We also thank Stefan Gruemer (Department of Periodontology of the University of Aachen, Germany) and P. P. Li (Department of VIP center, University of Shandong, China) for their kind devices provision.

Funding

We receive financial support from China Scholarship Council (CSC). MMM is supported by CNPq (#306423/2018-9).

Conflict of interest

The authors declare no conflict of interest.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

References

[1] Souza BDM, Dutra KL, Kuntze MM, Bortoluzzi EA, Flores-Mir C, Reyes-Carmona J, *et al*. Incidence of Root Resorption after the Replantation of Avulsed Teeth: a Meta-analysis. Journal of Endodontics. 2018; 44: 1216–1227.

[2] Müller DD, Bissinger R, Reymus M, Bücher K, Hickel R, Kühnisch J. Survival and complication analyses of avulsed and replanted permanent teeth. Scientific Reports. 2020; 10: 2841.

[3] Fouad AF, Abbott PV, Tsilingaridis G, Cohenca N, Lauridsen E, Bourguignon C, *et al*. International Association of Dental Traumatology guidelines for the management of traumatic dental injuries: 2. Avulsion of permanent teeth. Dental Traumatology. 2020; 36: 331–342.

[4] Tuna EB, Yaman D, Yamamato S. What is the Best Root Surface Treatment for Avulsed Teeth? The Open Dentistry Journal. 2014; 8: 175–179.

[5] Mori GG, Janjacomo DMDM, Nunes DC, Castilho LR. Effect of zoledronic acid used in the root surface treatment of late replanted teeth: a study in rats. Brazilian Dental Journal. 2010; 21: 452–457.

[6] Boyd DH, Kinirons MJ, Gregg TA. A prospective study of factors affecting survival of replanted permanent incisors in children. International Journal of Paediatric Dentistry. 2000; 10: 200–205.

[7] Fujita Y, Shiono Y, Maki K. Knowledge of emergency management of avulsed tooth among Japanese dental students. BMC Oral Health. 2014; 14: 34.

[8] Amaroli A, Colombo E, Zekiy A, Aicardi S, Benedicenti S, De Angelis N. Interaction between Laser Light and Osteoblasts: Photobiomodulation as a Trend in the Management of Socket Bone Preservation-A Review. Biology. 2020; 9: 409.

[9] Cardoso MV, do Vale Placa R, Sant'Ana ACP, Greghi SLA, Zangrando MSR, de Rezende MLR, *et al*. Laser and LED photobiomodulation effects in osteogenic or regular medium on rat calvaria osteoblasts obtained by newly forming bone technique. Lasers in Medical Science. 2021; 36: 541–553.

[10] Diniz IMA, Carreira ACO, Sipert CR, Uehara CM, Moreira MSN, Freire L, *et al*. Photobiomodulation of mesenchymal stem cells encapsulated in an injectable rhBMP4-loaded hydrogel directs hard tissue bioengineering. Journal of Cellular Physiology. 2018; 233: 4907–4918.

[11] Bölükbaşı Ateş G, Ak A, Garipcan B, Gülsoy M. Photobiomodulation effects on osteogenic differentiation of adipose-derived stem cells. Cytotechnology. 2020; 72: 247–258.

[12] de Carvalho FB, Andrade AS, Barbosa AF, Aguiar MC, Cangussu MC, Pinheiro AL, *et al*. Evaluation of laser phototherapy (lambda 780 nm) after dental replantation in rats. Dent Traumatol. 2016; 32: 488-494.

[13] Schwarz F, Rothamel D, Herten M, Bieling K, Scherbaum W, Becker J. Effects of an Er:YAG laser on mitochondrial activity of human osteosarcoma-derived osteoblasts in vitro. Lasers in Medical Science. 2004; 19: 37–40.

[14] Pourzarandian A, Watanabe H, Ruwanpura SMPM, Aoki A, Ishikawa I. Effect of low-level Er:YAG laser irradiation on cultured human gingival fibroblasts. Journal of Periodontology. 2005; 76: 187–193.

[15] Pourzarandian A, Watanabe H, Ruwanpura SMPM, Aoki A, Noguchi K, Ishikawa I. Er:YAG laser irradiation increases prostaglandin E production via the induction of cyclooxygenase-2 mRNA in human gingival fibroblasts. Journal of Periodontal Research. 2005; 40: 182–186.

[16] Kawaki H, Kubota S, Suzuki A, Suzuki M, Kohsaka K, Hoshi K, *et al*. Differential roles of CCN family proteins during osteoblast differentiation: Involvement of Smad and MAPK signaling pathways. Bone. 2011; 49: 975–989.

[17] Niimi H, Ohsugi Y, Katagiri S, Watanabe K, Hatasa M, Shimohira T, et al. Effects of Low-Level Er:YAG Laser Irradiation on Proliferation and Calcification of Primary Osteoblast-Like Cells Isolated From Rat Calvaria. Front Cell Dev Biol. 2020; 8: 459.

[18] Aleksic V, Aoki A, Iwasaki K, Takasaki AA, Wang C, Abiko Y, *et al*. Low-level Er:YAG laser irradiation enhances osteoblast proliferation through activation of MAPK/ERK. Lasers in Medical Science. 2010; 25: 559–569.

[19] Lin T, Yu C, Liu C, Hsieh P, Liao Y, Yu C, *et al*. Er:YAG laser promotes proliferation and wound healing capacity of human periodontal ligament fibroblasts through Galectin-7 induction. Journal of the Formosan Medical Association. 2021; 120: 388–394.
[20] D'Errico JA, Berry JE, Ouyang H, Strayhorn CL, Windle JJ, Somerman MJ. Employing a Transgenic Animal Model to Obtain Cementoblasts in Vitro. Journal of Periodontology. 2000; 71: 63–72.
[21] Yong J, von Bremen J, Ruiz-Heiland G, Ruf S. Adiponectin Interacts In-Vitro With Cementoblasts Influencing Cell Migration, Proliferation and Cementogenesis Partly Through the MAPK Signaling Pathway. Front Pharmacol. 2020; 11: 585346.
[22] Yong J, von Bremen J, Ruiz-Heiland G, Ruf S. Adiponectin as Well as Compressive Forces Regulate in vitro beta-Catenin Expression on Cementoblasts via Mitogen-Activated Protein Kinase Signaling Activation. Front Cell Dev Biol. 2021; 9: 645005.
[23] Yong J, Bremen J, Groeger S, Ruiz-Heiland G, Ruf S. Hypoxia-inducible factor 1-alpha acts as a bridge factor for crosstalk between ERK12 and caspases in hypoxia-induced apoptosis of cementoblasts. Journal of Cellular and Molecular Medicine. 2021; 25: 9710–9723.
[24] Cortés-Ríos J, Zárate AM, Figueroa JD, Medina J, Fuentes-Lemus E, Rodríguez-Fernández M, *et al*. Protein quantification by bicinchoninic acid (BCA) assay follows complex kinetics and can be performed at short incubation times. Analytical Biochemistry. 2020; 608: 113904.
[25] Mizutani K, Aoki A, Takasaki AA, Kinoshita A, Hayashi C, Oda S, *et al*. Periodontal tissue healing following flap surgery using an Er:YAG laser in dogs. Lasers in Surgery and Medicine. 2006; 38: 314–324.
[26] Garrido PR, Pedroni ACF, Cury DP, Moreira MS, Rosin F, Sarra G, *et al*. Effects of photobiomodulation therapy on the extracellular matrix of human dental pulp cell sheets. Journal of Photochemistry and Photobiology. B, Biology. 2019; 194: 149–157.
[27] Marques MM, Diniz IMA, de Cara SPHM, Pedroni ACF, Abe GL, D'Almeida-Couto RS, *et al*. Photobiomodulation of Dental Derived Mesenchymal Stem Cells: a Systematic Review. Photomedicine and Laser Surgery. 2016; 34: 500–508.
[28] Alves FAM, Marques MM, Cavalcanti SCSXB, Pedroni ACF, Ferraz EP, Miniello TG, *et al*. Photobiomodulation as adjunctive therapy for guided bone regeneration. a microCT study in osteoporotic rat model. Journal of Photochemistry and Photobiology B: Biology. 2020; 213: 112053.
[29] Arany PR, Nayak RS, Hallikerimath S, Limaye AM, Kale AD, Kondaiah P. Activation of latent TGF-beta1 by low-power laser in vitro correlates with increased TGF-beta1 levels in laser-enhanced oral wound healing. Wound Repair Regen. 2007; 15: 866-874.
[30] Choi K, Kang B, Kim H, Lee S, Bae S, Kweon O, *et al*. Low-level laser therapy promotes the osteogenic potential of adipose-derived mesenchymal stem cells seeded on an acellular dermal matrix. Journal of Biomedical Materials Research. Part B, Applied Biomaterials. 2013; 101: 919–928.
[31] Moreira MS, Diniz IM, Rodrigues MFSD, de Carvalho RA, de Almeida Carrer FC, Neves II, *et al*. *In vivo* experimental model of orthotopic dental pulp regeneration under the influence of photobiomodulation therapy. Journal of Photochemistry and Photobiology. B, Biology. 2017; 166: 180–186.
[32] Moura-Netto C, Ferreira LS, Maranduba CM, Mello-Moura ACV, Marques MM. Low-intensity laser phototherapy enhances the proliferation of dental pulp stem cells under nutritional deficiency. Brazilian Oral Research. 2016; 30: S1806-83242016000100265.
[33] Alsulaimani M, Doschak M, Dederich D, Flores-Mir C. Effect of low-level laser therapy on dental root cementum remodeling in rats. Orthodontics & Craniofacial Research. 2015; 18: 109–116.
[34] Kim JE, Woo YJ, Sohn KM, Jeong KH, Kang H. Wnt/beta-catenin and ERK pathway activation: A possible mechanism of photobiomodulation therapy with light-emitting diodes that regulate the proliferation of human outer root sheath cells. Lasers in Surgery and Medicine. 2017; 49: 940–947.
[35] Bozkurt SB, Hakki EE, Kayis SA, Dundar N, Hakki SS. Biostimulation with diode laser positively regulates cementoblast functions, in vitro. Lasers in Medical Science. 2017; 32: 911–919.
[36] Yong J, Elisabeth Groeger S, Ruf S, Ruiz-Heiland G. Influence of leptin and compression in GAS-6 mediated homeostasis of periodontal ligament cell. Oral Diseases. 2021. (in press)
[37] Cheng H, Caterson B, Neame PJ, Lester GE, Yamauchi M. Differential distribution of lumican and fibromodulin in tooth cementum. Connective Tissue Research. 1996; 34: 87–96.
[38] Cheng H, Caterson B, Yamauchi M. Identification and immunolocalization of chondroitin sulfate proteoglycans in tooth cementum. Connective Tissue Research. 1999; 40: 37–47.
[39] Matias MA, Li H, Young WG, Bartold PM. Immunohistochemical localization of fibromodulin in the periodontium during cementogenesis and root formation in the rat molar. Journal of Periodontal Research. 2003; 38: 502–507.
[40] Karamanou K, Perrot G, Maquart F, Brézillon S. Lumican as a multivalent effector in wound healing. Advanced Drug Delivery Reviews. 2018; 129: 344–351.
[41] Azizulkarim AH, Jamil MMA, Adon MN, Ahmad MS, Sharif JM, Ngadi MA, *et al*. Investigation on Suitability of Pulse Duration Parameter for HeLa Cells Line Proliferation Properties towards Anti-Cancer Application. Journal of Physics: Conference Series. 2018; 1019: 012082.
[42] Tsuka Y, Kunimatsu R, Gunji H, Nakajima K, Kimura A, Hiraki T, *et al*. Effects of Nd:YAG low-level laser irradiation on cultured human osteoblasts migration and ATP production: in vitro study. Lasers in Medical Science. 2019; 34: 55–60.
[43] Ozawa Y, Shimizu N, Kariya G, Abiko Y. Low-energy laser irradiation stimulates bone nodule formation at early stages of cell culture in rat calvarial cells. Bone. 1998; 22: 347–354.
[44] Stein A, Benayahu D, Maltz L, Oron U. Low-level laser irradiation promotes proliferation and differentiation of human osteoblasts in vitro. Photomedicine and Laser Surgery. 2005; 23: 161–166.
[45] Werlen G, Hausmann B, Naeher D, Palmer E. Signaling life and death in the thymus: timing is everything. Science. 2003; 299: 1859–1863.
[46] Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science. 2002; 298: 1911–1912.
[47] Wada T, Penninger JM. Mitogen-activated protein kinases in apoptosis regulation. Oncogene. 2004; 23: 2838–2849.
[48] Campbell JS, Seger R, Graves JD, Graves LM, Jensen AM, Krebs EG. The MAP kinase cascade. Recent Progress in Hormone Research. 1995; 50: 131–159.
[49] Shefer G, Oron U, Irintchev A, Wernig A, Halevy O. Skeletal muscle cell activation by low-energy laser irradiation: a role for the MAPK/ERK pathway. Journal of Cellular Physiology. 2001; 187: 73–80.
[50] Miyata H, Genma T, Ohshima M, Yamaguchi Y, Hayashi M, Takeichi O, *et al.* Mitogen-activated protein kinase/extracellular signal-regulated protein kinase activation of cultured human dental pulp cells by low-power gallium-aluminium-arsenic laser irradiation. International Endodontic Journal. 2006; 39: 238–244.