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ORIGINAL ARTICLE

Photobiomodulation with single and combination laser wavelengths on bone marrow mesenchymal stem cells: proliferation and differentiation to bone or cartilage

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Abstract

Tissue engineering aims to take advantage of the ability of undifferentiated stem cells to differentiate into multiple cell types to repair damaged tissue. Photobiomodulation uses either lasers or light-emitting diodes to promote stem cell proliferation and differentiation. The present study aimed to investigate single and dual combinations of laser wavelengths on mesenchymal stem cells (MSCs). MSCs were derived from rabbit iliac bone marrow. One control and eight laser irradiated groups were designated as Infrared (IR, 810 nm), Red (R, 660 nm), Green (G, 532 nm), Blue (B, 485 nm), IR–R, IR–B, R–G, and B–G. Irradiation was repeated daily for 21 days and cell proliferation, osseous, or cartilaginous differentiation was then measured. RT-PCR biomarkers were SOX9, aggrecan, COL 2, and COL 10 expression for cartilage and ALP, COL 1, and osteocalcin expression for bone. Cellular proliferation was increased in all irradiated groups except G. All cartilage markers were significantly increased by IR and IR–B except COL 10 which was suppressed by IR–B combination. ALP expression was highest in R and IR groups during osseous differentiation. ALP was decreased by combinations of IR with B and with R, and also by G alone. R and B–G groups showed stimulated COL 1 expression; however, COL 1 was suppressed in IR–B, IR–R, and G groups. IR significantly increased osteocalcin expression, but in B, B–G, and G groups it was reduced. Cartilage differentiation was stimulated by IR and IR–B laser irradiation. The effects of single or combined laser irradiation were not clear-cut on osseous differentiation. Stimulatory effects on osteogenesis were seen for R and IR lasers, while G laser had inhibitory effects.

Keywords Photobiomodulation · Low level light therapy · Mesenchymal stem cells, bone · Cartilage · Comparison of wavelengths

Introduction

Tissue engineering involves the addition, stimulation, differentiation, and guiding of cells with the goal of reconstructing impaired or damaged tissues. Three critical factors play a significant role in a successful tissue regeneration: cells, scaffolds, and signaling mediators such as growth factors. Stem cells are widely used in tissue

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engineering [1, 2]. These cells have an infinite capacity for self-renewal, and can differentiate to various different types of target tissue depending on the cues they are exposed to [3]. Mesenchymal stem cells (MSCs) are multipotent undifferentiated cells which can be harvested from many different tissues. Bone marrow is one of the most important sources for the harvest of MSCs [4]. Bone marrow MSCs (BMSCs) are a heterogenous population with a high capacity for replication. They are pluripotent stem cells which can differentiate into osteoblasts, chondroblasts, adipocytes, etc. [3, 4]. BMSCs are being investigated to treat pediatric high risk leukemia, osteogenesis imperfecta, mucopolysaccharidosis, graft-versus-host disease, myocardial infarction, immune deficiencies, and metabolic disorders [3, 5, 6]. As mentioned above, MSCs have a critical role to play in tissue engineering procedures. The proliferation and differentiation of MSCs can, to some extent, be considered to be separate programmable processes, and controlling these processes in a predictable manner is crucial to satisfactory regeneration of the desired tissue type to be replaced or repaired.

Photobiomodulation (PBM) describes the use of lasers or light-emitting diodes (LEDs) to stimulate, repair, and regenerate cells or tissues [7, 8]. The PBM effects depend on the absorption of photons by chromophores within cells or tissues. This can be influenced by parameters like wavelength, energy density, mode of irradiation, etc. The wavelength of lasers used in PBM can range from 400 to 1100 nm, which covers the whole spectrum from visible blue to invisible infrared. "PBM therapy" is the new and more accurate term for what was formerly known as "low level light therapy" (LLLT). This term was added to the Medical Subject Headings (MeSH) database in 2016. PBM can be defined as "a nonthermal process involving endogenous chromophores eliciting photophysical (i.e., linear and nonlinear) and photochemical events at various biological scales" [9]. PBM can promote or suppress specific cellular process such as proliferation or differentiation depending on the dose (biphasic dose response). PBM can act as a physical stimulus, which can promote or inhibit signaling mechanisms concerned with growth factor activity and cellular metabolic activities. Different laser wavelengths with different energy densities or targeting different chromophores, can initiate various cellular responses. Therefore, a combination of lasers may produce novel effects on cell proliferation and differentiation compared to using single wavelengths alone. Our goal was to compare single lasers with four different wavelengths, with combinations using two different wavelengths, looking at the proliferation of MSCs and their differentiation into bone or cartilage. We chose to use rabbit MSCs because a future study would investigate PBM in an animal model constructed in rabbits.

Materials and methods

Isolation and culture of rabbit mesenchymal stem cells

Animal experiments were carried out under a protocol [10] approved by the IACUC of Royan Institute for Stem Cell Biology and Technology. MSCs were isolated from rabbit bone marrow following a detailed protocol described previously [11]. Briefly, 8 to 12 month-old rabbits were anesthetized by intramuscular injection of 50 mg/kg ketamine hydrochloride (100 mg/mL, Alfasan, Woerden-Holland) and 10 mg/kg xylazine hydrochloride (20 mg/mL, Alfasan, Woerden-Holland). After shaving and disinfecting the region, almost 3 mL of bone marrow was aspirated from the humerus of the animals using a Jamshidi aspiration needle and a 10 mL syringe containing 3000 U of heparin. All procedures were carefully performed under sterile conditions to avoid bacterial infection of the samples. The marrow was slowly flushed out of the bones and suspended in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) supplemented with 15% fetal calf serum (FCS; Gibco), 100 U/ml penicillin (Sigma), and 100 mg/ml streptomycin. The mononuclear cell fraction was plated in a 75-cm^2 culture flask and incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 3 weeks. Cells were subsequently expanded through several passages and cells at passage 3 were used for the experiments. For the experiments, the cells were seeded at low density.

Cell proliferation (MTT) assay

To measure and compare the proliferation rates of cells, 5×10^4 cells from each group (passage 2–3) were cultivated in 10-cm^2 plastic dishes for 10 days to measure the fold-increase in cell number. The MTT assay was used to measure the proliferation capacities of the cells in each groups, the medium was exchanged with 300 mL of fresh DMEM and 20 μL of MTT solution (5 mg/mL). Cells were then incubated at 37 °C for 2–3 h then the supernatant was discarded and 200 μL of dimethyl sulfoxide (DMSO, Sigma, Germany) was subsequently added. Absorbance was recorded at 540–630 nm. The rates of cell growth were calculated by constructing growth curves.

Assessment of rMSCs differentiation

MSCs were evaluated for their capacity to differentiate to cells from the skeletal lineage (i.e., osteoblasts, chondrocytes, and adipocytes). Based on the literature, complete osteogenesis includes two phases, an early and a late phase. The early phase comprises ECM secretion, and the later phase comprises mineralization. These are complete at approximately 3 weeks. Therefore, we collected our cells after 3 weeks of differentiation to ensure that osteogenesis was complete. Rabbit mesenchymal stem cells (rMSCs) were trypsinized and seeded in

six-well culture plates. Osteogenic differentiation was induced by incubating the cells in osteogenic culture medium (DMEM supplemented with 10% FBS, 10 mM β-glycerophosphate, 0.2 mM ascorbic acid, and 1 nM dexamethasone) for 3 weeks. Osteogenesis was examined using 1% Alizarin red staining (Sigma). For adipogenic differentiation, the culture media was exchanged to adipogenic inductive medium containing DMEM supplemented with 10% FBS, 0.5 mM indomethacin, l mM ascorbic acid, and 1 μM dexamethasone, for 21 days. Lipid droplets in the cells were visualized by oil red O staining solution (Sigma). A micro mass culture system was used to induce chondrogenic differentiation of MSCs as previously described [12]. Briefly, about 2.5×10^5 passage-3 MSCs were pelleted under 1200 g for 5 min and cultured in chondrogenic medium (Lunza, Switzerland) for 21 days at 37 °C, 5% CO_2; with medium changed twice weekly. Chondrogenic differentiation was assessed by toluidine blue staining of pellets.

Photobiomodulation therapy

PBM for bone and cartilage differentiation was initiated at t = 0 (time of differentiation) and then continued every other day for 3 weeks. Different wavelengths of visible and infrared diode laser light in continuous emission mode all at the same fluence of 4 J/cm2 per session were used.

Lasers

Lasers were designated as Infrared (IR) 810 nm (THOR Photomedicine Ltd. UK); Red (R) 660 nm (THOR Photomedicine Ltd.); Green (G) 532 nm (LASER SYSTEM Ltd., Iran); and Blue (B) 485 nm (LASER SYSTEM Ltd.); and combination groups included IR-R, IR-B, R-G, and B-G. The illumination time for each group considering the power and irradiation area to produce the same 4 J/cm^2 were as follows: IR 3 s, R 24 s, G 15 s, and B 15. Lasers were irradiated sequentially and the order of irradiation was as specified in the group name; for example, in IR–R group, cells were irradiated first by IR laser and then by R laser. Combination groups received 4 J/cm^2 of each wavelength for a total of 8 J/cm^2. The relevant parameters are given in Table 1.

qRT-PCR measurement

The expression level of osteogenic, chondrogenic, and adipogenic related genes was evaluated using the qRT-PCR technique. Total RNA was extracted from cells using TRI Reagent® (Sigma-Aldrich, T9424). cDNA was produced by the RevertAid First Strand cDNA Synthesis Kit (Fermantas, K1632) according to the manufacturer's instructions.

Duplicate qRT-PCR reactions were performed with the SYBR Green Master Mix (Applied Biosystems Life Technologies, Inc., REF 4367659) with a real-time PCR system (Applied Biosystems ABI Step one plus) and analyzed with Step one software (Applied Biosystems; Step one software version 2.1). The samples were taken from three independent biological replicates. The expression level of target genes was normalized to GAPDH as a reference gene. Analysis was performed by the comparative ΔΔCT method. Primer sequences are listed in Table 2.

Statistical analysis

Statistical analyses were carried out on datasets consisting of at least three independent experiments, using an unpaired Student's t test comparing two groups and one-way ANOVA for comparing all groups together; with GraphPad Prism software (GraphPad, San Diego, CA, USA). All data are expressed as the mean ± SD.

Results

Characteristics, morphology, and proliferation of rMSCs

Plastic-adherent cells with a typical fibroblastoid-like shape were isolated and expanded from bone marrow of rabbits (Fig. 1). The initial colonies from rMSCs appeared within 2 to 5 days after plating.

Table 1 Laser parameters

	IR laser, IR	Red laser, R	Green laser, G	Blue laser, B
Wavelength	810 nm	660 nm	532 nm	475 nm
Power	200 mW	30 mW	30 mW	30 mW
Power density	1333 mW/cm^2	167 mW/cm^2	266 mW/cm^2	266 mW/cm^2
Illumination time	3 s	24 s	15 s	15 s
Fluence	4 J/cm^2	4 J/cm^2	4 J/cm^2	4 J/cm^2
Spot size	0.150 cm^2	0.180 cm^2	0.113 cm^2	0.113 cm^2

Table 2 Description of rabbit primer sequences used in qRT-PCR

Gene	Gene symbol	Sequence (5–3)	Annealing time (°C)	Amplicon size (bp)
Osteocalcin	OCN	F: 5' ACA AGA GAT TCA GCG ACT 3' R: 5′ GGT TCT TGG CTT CCT GTT TC 3'	57	126
Collagen 1	COL 1	F: 5′ GGT GCT GCT GGT AAA GAA GG 3' R: 5' GTC TAC CCA AAG CAC CAG 3′	60	245
Alkaline phosphatase	ALP	F: 5' ACTTTGTCTGGAAC CGCACT 3′ R: 5' GTGGTCAATCCTGC CTCCT 3'	58	215
Sex-determining region Y box 9	SOX9	F: 5' AAGATGACCGACGA GCAG 3' R: 5' GGCTTGTTCTTGCT GGAG 3'	60	232
Collagen 2	COL 2	F: 5' GTGGAAGAGCGGTG ACTA 3' R: 5' TAGGTGATGTTCTG GGAGC 3'	60	250
Aggrecan	Aggrecan	F: 5' GGAGGTCGTGGTGA AAGGTG 3' R: 5' CAGAGGAGATGGAG GGTGAG 3'	61	255
Collagen 10	COL 10	F: AGTTCTTCATTCCC TATGCCA R: CAATGTCTCCTTTC GGTCCA		
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	F: 5' CACCCACTCCTCTA CCTTCG 3' R: 5' GGTCTGGGATGGAA ACTGTG	57	141

Differentiation potential of rMSCs into skeletal lineage

To confirm the phenotype of isolated rMSCs and their differentiation into the two branches of the skeletal lineage, we assessed both histopathological staining and qRT-PCR gene expression. Differentiation of MSCs to the osteoblastic lineage was measured by alizarin red staining and qRT-PCR. The alizarin red results confirmed the presence of calcium minerals in the extracellular matrix of rMSCs. Mineral deposition started at day 5 and increased progressively up to day 21 (Fig. 2A, a). Real time PCR analysis of osteogenic-related genes indicated that there were significant differences in the expression levels of the COL 2, OCN, and OPN genes in rMSCs before and after differentiation (Fig. 2B, a). Oil red O staining and qRT-PCR established adipogenic differentiation of rMSCs. Oil droplets were observed

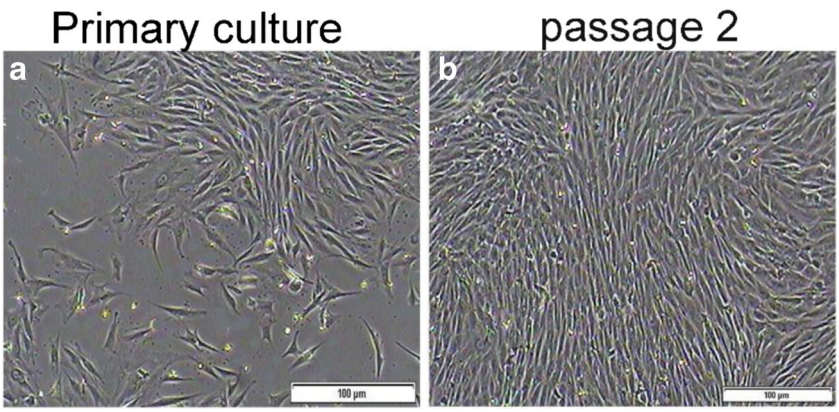

Fig. 1 Characterization of rMSCs. The figure shows the morphology of primary (**a**) and passage 2 culture (**b**) of bone marrow-derived MSCs

in the cytoplasm of differentiated cells after 3 weeks of culture (Fig. 2 A, c). Analysis of adipogenic-related genes such as Lpl, Ppar-G, and adiponectin showed a highly significant expression level of these genes in differentiated MSCs compared to the control group (Fig. 2B, c).

The ability of rMSCs to undergo chondrogenic differentiation was measured by toluidine blue staining and also by qRT-PCR of the COL 2, aggrecan, and Sox9 genes. After 21 days, toluidine blue-stained areas indicated the presence of sulfated proteoglycans in rMSCs (Fig. 2A, b). Analysis of genes involved in chondrogenesis showed that rMSCs expressed comparable level of COL 2and Sox9 (Fig. 2B, b).

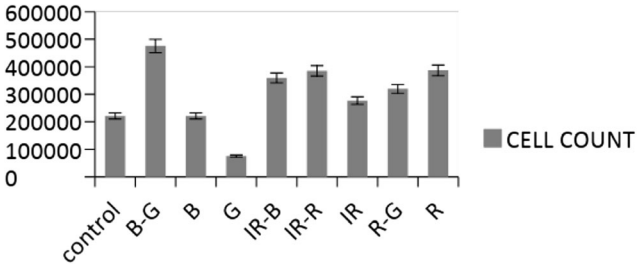

Fig. 3 Evaluation of rabbit BMSCs proliferation after laser irradiation during 10 days. Initial cell count was 50×10^3. B–G lasers combination increased cellular proliferation significantly compared to control group. ($p < 0.05$)

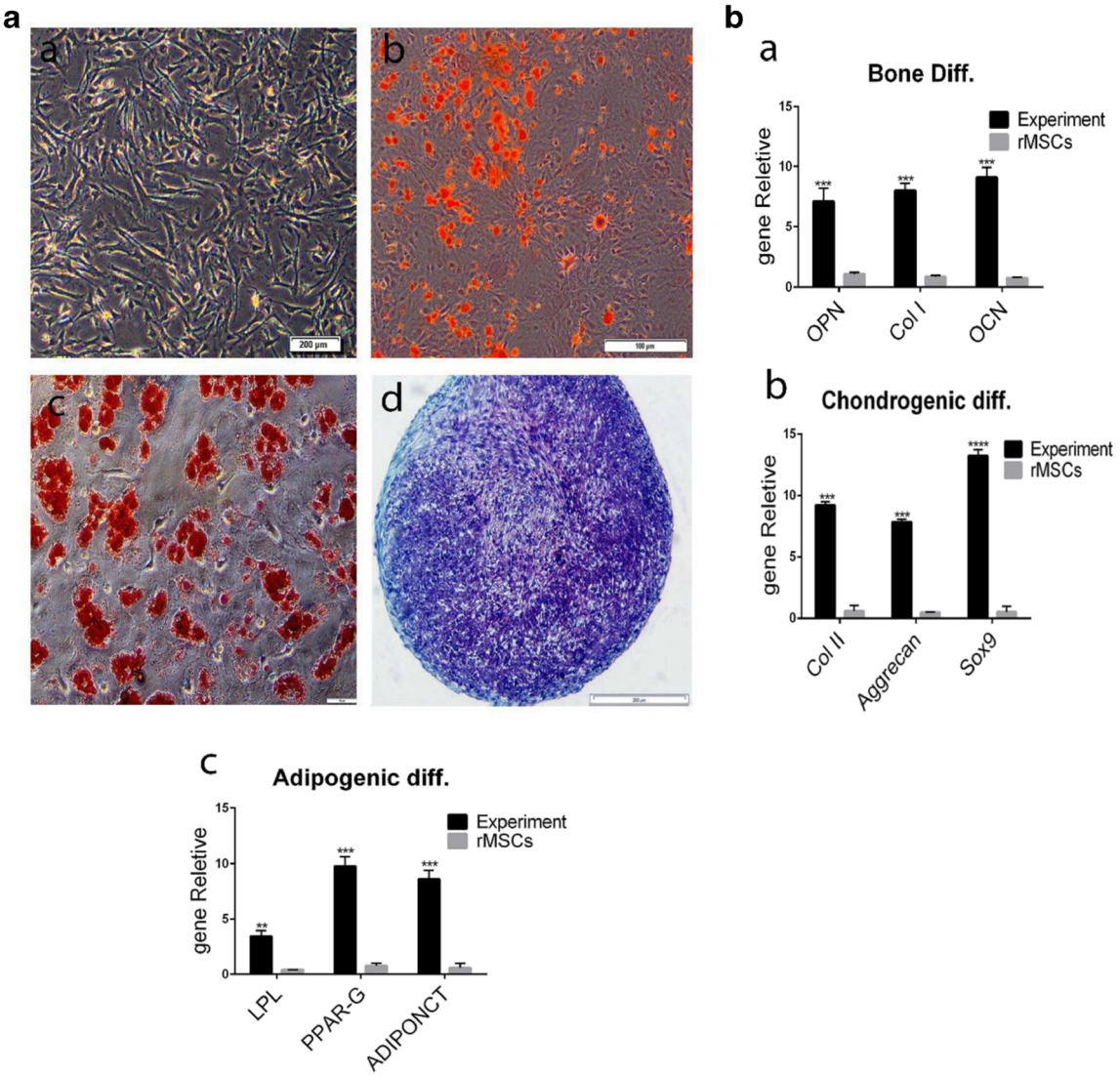

Fig. 2 Potential differentiation of rMSCs into cells of skeletal lineages. (**a**) The image shows differentiation potential of rMSCs to osteoblasts, chondrocytes and adipocytes. Undifferentiated rMSCs as a control group and differentiated cells as experimental groups. Control group (**a**); cells differentiated into osteoblasts stained with Alizarin red-S (**b**); cells differentiated into adipocytes stained with Oli red (**c**); and cells differentiated into chondroblasts stained with Toluidine blue (**d**). (**b**) qRT-PCR data of rMSCs differentiated to cells of skeletal lineage. qRT-PCR result for osteogenic differentiation (i.e., COL 1, OCN, and OPN) (**a**), qRT-PCR result for chondrogenic differentiation (i.e., COL 2, Sox9, and aggrecan) (**b**), and qRT-PCR result for adipogenic differentiation (i.e., LPL, adiponectin, and Ppar-G) (**c**) were obtained after 21 days. Data are presented as means ± SD ($n = 3$) ($p < 0.05$)

Cellular proliferation

Cellular proliferation was evaluated after a period of 10 days (Fig. 3). The B–G laser combination led to the highest increase in cellular numbers, while G laser alone decreased MSC proliferation, and B laser alone had no effect. There was no significant difference between control group and other groups except B-G group. R and IR lasers both increased cellular proliferation with R better than IR. Combination of IR laser with either B or R lasers increased cell proliferation. R–G combination increased cellular proliferation compared with control, but to a lesser extent than R laser alone. A suppressive effect of G laser may have led to this result.

Osteogenic differentiation

Osteogenic differentiation requires maturation of the matrix and subsequent mineralization to initiate bone production. Alkaline phosphatase expression (ALP) increases immediately after cellular proliferation. In the mineralization stage, osteocalcin expression increases. Alkaline phosphatase (ALP) was increased by all laser groups except G, IR–B, and IR–R (Fig. 4). IR–B and IR–R groups decreased ALP to the lowest level and G laser was next. IR–B and G groups showed an insignificant decrease in APL compared with control group, while IR–R combination did not show any significant difference. It appeared that IR combined with other wavelengths (R or B) decreased ALP expression despite an increase with IR alone. All other groups increased APL insignificantly. R laser increased APL (5 times higher than control), while IR laser was close (4.5 times). The expression levels of all the genes were remarkably similar whether measured at 12 days or at 24 days.

Figure 5 shows levels of collagen type 1 (COL 1) gene expression which is also expressed during osteogenic differentiation. Combinations of IR laser (IR–B, IR–R) and also G laser alone insignificantly suppressed COL 1. There was no significant difference between IR–R and control groups. IR and B alone increased osteogenic differentiation, while R and B–G increased COL 1 dramatically (21 and 16.5 times higher than control).

At the final stage of bone differentiation, osteocalcin is expressed. IR laser increased OCN expression at 24 days but not at 12 days (Fig. 6). Besides that, the only significant changes were an increase with R–G and a decrease with G alone.

There were some similarities between the three markers. IR alone and R alone tended to show increases, while G alone, IR–R and IR–B showed decreases. Overall, IR laser had a better effect from beginning to end, while R laser had a better effect at beginning.

Chondrogenic differentiation

Overall, the IR laser had highest stimulatory effect on chondrogenesis-related gene expression (Fig. 7), and on COL 10 in particular. All laser groups had stimulatory effects on COL 10, except IR–R. IR, G, and B alone, and IR–B increased the other markers (SOX9, aggrecan, and COL 2).

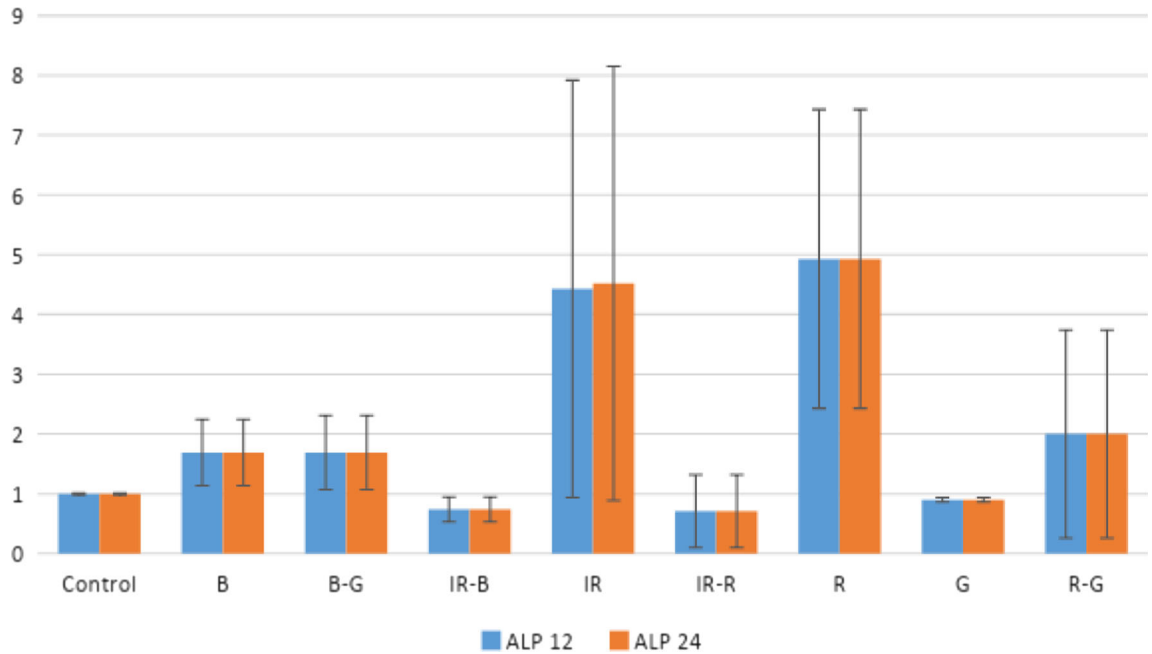

Fig. 4 Alkaline phosphatase gene expression after different laser irradiations during osseous differentiation (day 12 and 24). there was not any significant difference between groups. (p value > 0.05). Experiments were repeated 3 times

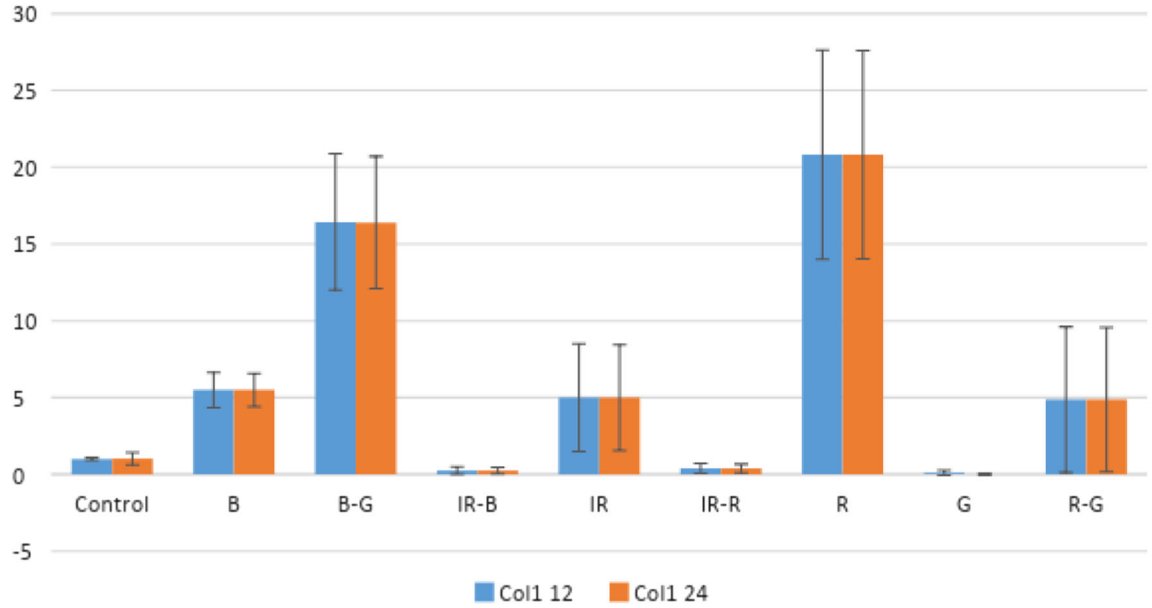

Fig. 5 Collagen type 1 gene expression after different laser irradiations during osseous differentiation (day 12 and 24). B–G and R laser groups had significant differences compared to control groups (p value < 0.05). Experiments were repeated 3 times

Discussion

Tissue engineering is concerned with the repair or regeneration of damaged or diseased biological tissues. Three critical factors play a role in this regard: cells, scaffolds, and signaling molecules. Since satisfactory tissue repair requires the participation of many different cell types, an efficient way to accomplish this goal is to employ stem cells that can differentiate into many different daughter cells. MSCs are a common type of stem cell used in tissue engineering. These cells usually have low yield and do not have a high proliferative rate when cultured in vitro. This slow proliferation rate can hinder clinical applications, when procedures must wait for enough cells to be produced. Moreover, the differentiation of MSCs into target cell types in governed by a range of different cues or stimuli, such as various chemicals, growth factors, mild forms of cell stress, or physical interventions such as lasers or PBM.

Many parameters can influence the response of cells to lasers or PBM. Besides the energy density, power density, and mode of irradiation (continuous or pulsed), the most important single

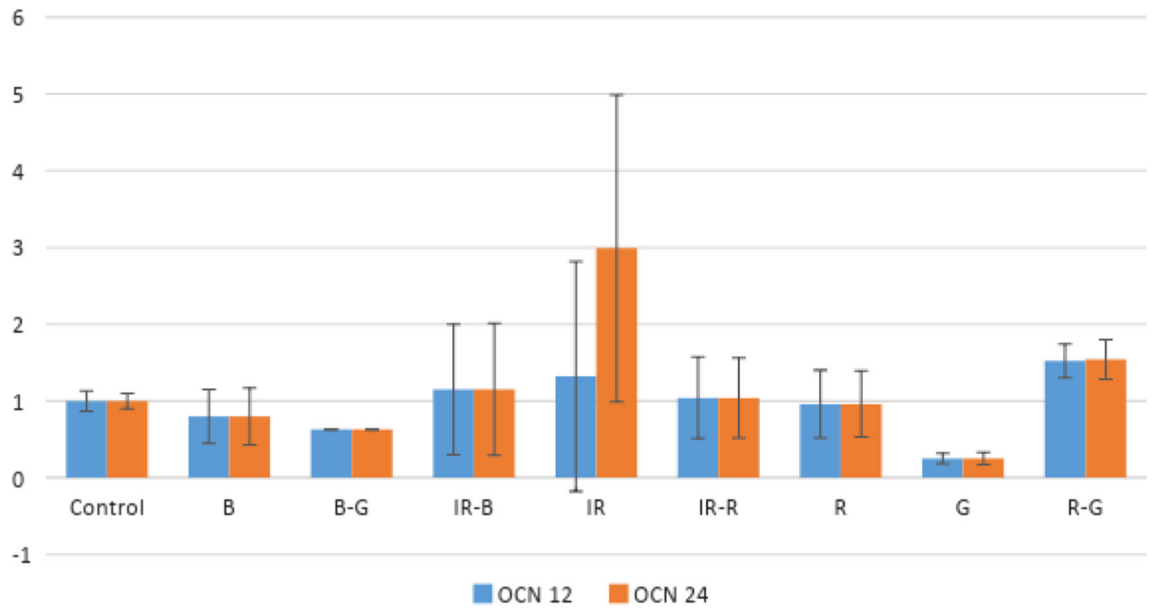

Fig. 6 Osteocalcin gene expression after different laser irradiations during osseous differentiation (day 12 and 24). There were no significant difference between control group and other groups. (p value > 0.05). Experiments were repeated 3 times

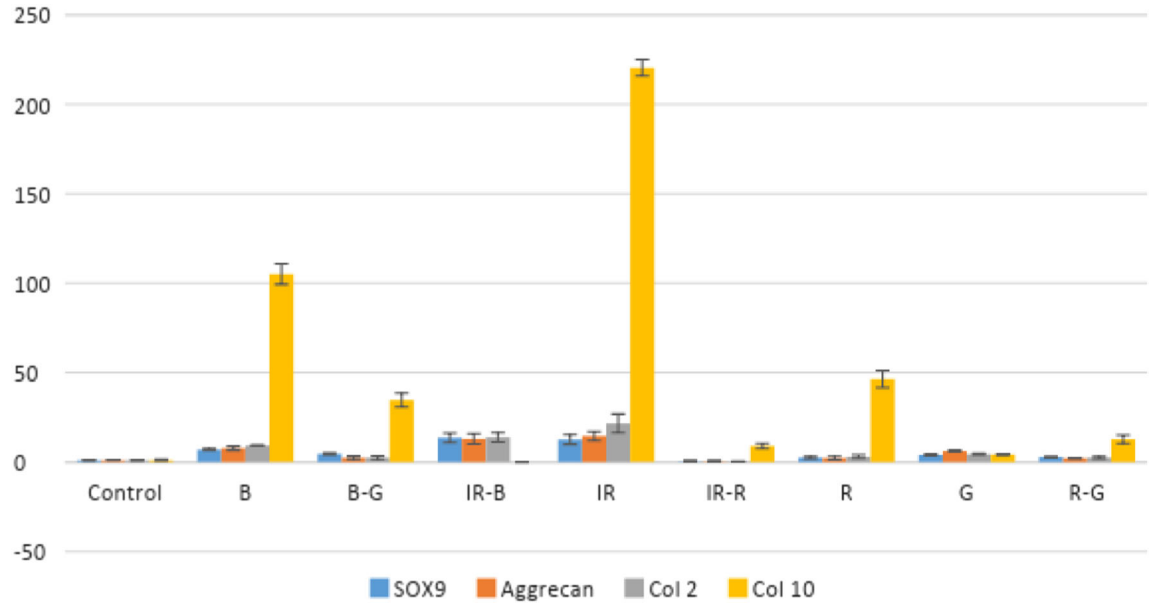

Fig. 7 Sox9, Aggrecan, COL 2, and COL 10 gene expression after different laser irradiations during osseous differentiation. IR, IR–B, and B groups had significant differences in Sox9, Aggrecan, and COL 2 compared to control group. G group had significant difference in Aggrecan with control group. B, B–G, IR, and R groups had significant difference in COL 10 (p value < 0.05). Experiments were repeated 3 times

parameter is the wavelength. The focus of this study was to compare the effects of four different laser wavelengths on MSC proliferation and differentiation. Importantly, we wished to test the combination of two different wavelengths, which has seldom been done before. It was decided to use the same fluence (4 J/cm^2) of each wavelength for a total of 8 J/cm^2 in the combination groups. This may have been an important factor considering the biphasic dose response that operates in PBM [13, 14]. It is possible that even if a beneficial effect of combining two different wavelengths did in fact exist, it may not have been apparent because the total dose of 8 J/cm^2 exceeded the peak of the combined dose response curve. In retrospect, it may have been preferable to use 2 J/cm^2 of each wavelength and to have kept the total dose the same (4 J/cm^2) in both the single and dual wavelength groups.

Overall, we found that the most effective laser wavelength for affecting both proliferation and differentiation of MSCs was IR closely followed by R. G was probably the worst single wavelength. Combinations of two wavelengths were generally less effective than either IR or R alone, and interestingly, the IR–R combination appeared to be a lot worse. In some cases, the B–G combination was better than either wavelength alone.

Green laser has been successfully used in treatment of vascular lesions like hemangioma [15, 16] and has also been used for tooth bleaching, oral soft tissue surgery, or killing bacteria in root canals or periodontal lesions [17–19].

Merigo et al. [20] evaluated G laser (potassium-titanyl-phosphate or KTP, 532 nm) at 4 J/cm^2, three times a week) for osteogenic differentiation of mouse bone marrow stromal cells (BMSCs). They claimed that G laser had a positive effect in this regard which was in conflict with our results. They found that G laser had no effect on the cellular proliferation, which also was in contrast with our study that found suppression of proliferation. We found that G laser could suppress cellular proliferation and osteogenic differentiation; however, it could promote chondrogenic differentiation.

In 2016, Soltani et al. [21] showed that R and G lasers can increase proliferation of human umbilical cord matrix-derived mesenchymal stem cells (hUCM). G laser was more effective than R laser. This was also opposite to our results with G laser; however, the cell types were different.

In agreement with our results, Wang and colleagues [22] showed that B (415 nm) and G (540 nm) lasers inhibited proliferation of human adipose-derived stem cells (hASCs), while R (660 nm) and IR (810 nm) lasers stimulated proliferation. They also found that B and G lasers were better than R and IR lasers for stimulating differentiation of hASCs into osteogenic lineage cells [23]. This is in partial agreement and partial disagreement with the present results.

Our results suggest that if it is desired to achieve differentiation into cartilage without any osteogenic differentiation, G laser would be the preferred wavelength.

In a literature review by Amid et al. in 2014 [24] and a systematic review by Ginani et al. [25], the stimulatory effect of R and IR lasers was confirmed on both proliferation and osteogenic differentiation of stem cells. This was in agreement with our results. By contrast, Renno et al. [26] showed a single exposure of 830 nm IR laser at 10 J/cm2 fluence and inhibited osteoblast proliferation compared to control group. Bouvet-Gerbettaz et al. [27] showed that exposure of IR laser (808 nm) at 4 J/cm^2 three times a week did not alter proliferation and differentiation of murine bone marrow stem cells (Table 3).

Table 3 Literature review of PBM and LLLT on bone marrow stem cells

No.	Author and year	Type of laser	Type of irradiation	Type of cells	Criteria	Main results
1	Bloise et al.2013 [28]	659 nm	Single transverse-mode Power output 10 mW Fluence 1, 3 J/cm2	Human osteoblast-like cell line (Saos-2 cell line)	Proliferation differentiation	LLLT enhanced Saos2 cells proliferation and maturation.
2	Renno et al. 2007 [29]	670-nm, 780-nm, and 830-nm	Single exposure Power output 10 mW Fluence 0.5, 1, 5, and 10 J/cm2	Neonatal, murine, calvarial, osteoblastic (MC3T3), and human osteosarcoma (MG63) cell lines	Cell proliferation Alkaline phosphatase activity	Cell lines responded differently to specific wavelength and dose. Osteoblastic proliferation and ALP activity were significantly increased by 830 nm wavelength at 10 J/cm2
3	Stein et al. 2005 [30]	He-Ne laser (632 nm)	Power output 10 mW Fluence 0.43 J/cm2	Human osteoblast cell line	Cell proliferation Differentiation	LLLT increased cellular proliferation and differentiation of osteoblasts. ALP activity was twice higher in irradiated group.
4	Wu et al. 2012 [31]	635 nm	Single irradiation Power 60 mW Fluence 0.5 J/cm2	Murine bone marrow	Proliferation Gene expression	Bone marrow proliferation was increased significantly by laser at 2, 4, and 6 days later of irradiation
5	Li et al. 2006 [32]	630	Single irradiation or daily irradiation for 5 days Power 2.8, 4.25, and 8.86 mW Energy density 1.5 and 2.5 J/cm2	Murine bone marrow	Proliferation	Single dose irradiation did not show significant increase in cell proliferation, but daily doses did at 5 days.
6	Hou et al. 2008 [33]	635 nm	Single irradiation Power 60 mW Fluence 0.5, 1, 2, and 5 J/cm2	Murine bone marrow	Proliferation	Cellular proliferation was increased significantly by laser irradiation and 0.5 J/cm2 was optimal in this regard.
7	Horvat-Karajz et al. 2009 [34]	660 nm	Power 60 mW Fluence 1.9 and 3.8 J/cm2	Murine bone marrow	Proliferation	Lower doses had biostimulatory effect in adverse cell proliferation was inhibited after 48 h at higher doses (11.7 J/cm2)
8	Giannelli et al. 2013 [35]	635 nm	Single irradiation Power 89 mW Fluence 0.3 J/cm2	Murine bone marrow	Proliferation	Diode laser increased cell proliferation significantly at 72 h after irradiation
9	Wang et al. 2012 [36]	635 nm	Single irradiation Power 60 mW Fluence 0.5 J/cm2	Murine bone marrow	Proliferation	Laser irradiation promotes proliferation process 2 and 4 days after of exposure compare to control group.
10	Migliario et al.2014 [37]	980 nm	Continuous mode Power outputs 1–50 J Fluence 1.57, 7.87, 15.74, 39.37, and 78.75 J/cm2	Murine preosteoblasts MC3T3 cells	Cell proliferation	LLLT increased proliferation significantly by 5–15 J energy output. While higher energies (25–50 J) had inhibitory effect on the osteoblast proliferation.
11	Jawad et al.2013 [38]	940 nm	Continuous mode Power outputs 100, 200, 300 mW	Human fetal osteoblast cell line	Proliferation differentiation (ALP and osteocalcin activity)	100 and 200 mW powers promoted cell differentiation significantly however 300 mW stimulated osteoblast proliferation.
12	Renno et al. 2010 [26]	830 nm	Single exposure Power output 30 mW	Osteoblastic (MC3T3) cell line	Proliferation	Laser irradiation reduced osteoblast proliferation compared to control group.

Table 3 (continued)

No.	Author and year	Type of laser	Type of irradiation	Type of cells	Criteria	Main results
			Fluence 10 J/cm2			
13	Bouvet-Gerbettaz et al. 2009 [27]	808 nm	Continuous mode Fluence 4 J/cm2 Three times a week	Murine bone marrow cell	Bone cell proliferation, osteoblastic and osteoclastic differentiation	Infrared laser did not alter proliferation and differentiation compared to control group
14	Hamajima et al. 2003 [39]	830 nm	Continuous mode Power output 500 mW Fluence 7.64 J/cm2	Mouse calvaria derived osteoblastic cell line, MC3T3-E1	Bone formation	Diode laser can promote bone formation by increasing osteoglycin expression
15	Tuby et al. [40]	804 nm	Single irradiation Power 400 mW Fluence 1 and 3 J/cm2	Murine bone marrow	Cell proliferation	LLLT promoted MSCs proliferation at all intervals for both doses studied (1 and 3 J/cm2)
16	Soleimani et al. [41]	810 nm	1, 3, and 5 days after incubation Power 50 mW Fluence 2 and 4 J/cm2	Human bone marrow	Proliferation Differentiation	Cell proliferation was enhanced by doses of 2, 3, and 4 J/cm2 but 6 J/cm2 gave no difference. ALP activity was increased significantly by laser irradiation.
17	Fekrazad et al. 2015 [11]	810 nm	Power: 200 mW Fluence 4 J/cm2 Power density 0.2 W/cm^2 20 s per day for 3 week	Rabbit bone marrow mesenchymal stem cells	Healing of artificial calvarial defects	LLLT significantly increased new bone formation relative to control group but had no synergistic effect in conjunction with MSCs in bone formation.
18	Fekrazad et al. 2016 [7]	810 nm	Power: 30 mW Fluence 8.5 J/cm2 20 s per day for 3 week	Rabbit bone marrow mesenchymal stem cells	Healing of artificial osteochondral defects	There was better healing by LLLT compared with BMSCs alone, with higher bone formation rather than cartilage formation
19	Aleksic et al. 2010 [42]	Er:YAG laser (2940 n-m)	Pulsed radiation Energy/pulse output 30–350 mJ Fluence 0.7–17.2 J/cm2	Mouse-derived osteoblastic cell line MC3T3-E1	Cell proliferation Cell death Mitogen-activated protein kinase (MAPK) pathways	Er:YAG laser may be able to promote bone healing following periodontal and peri implant therapy.

In a comparative study, Wang and colleagues [43] found that hASCs had better osteogenic differentiation by B (420 nm) and G (540 nm) lasers than R (660 nm) or IR (810 nm).

We were not able to find any studies on PubMed database about combination of different laser wavelengths on MSCs differentiation either to chondrocytes or osteoblasts in vitro. Moreover there were no pre-clinical or clinical studies either. There have been a few studies on combination laser wavelengths used for treatment of skin disorders like wound healing in bacterially contaminated cutaneous wounds [44], or in psoriasis [45]. These studies generally used a combination of R and IR lasers. They concluded that combination laser therapy could be an improvement in anti-inflammatory effects and wound healing compared to single wavelengths [46]. Our study is probably the first to evaluate the effect of a combination of different laser wavelengths on MSC proliferation and differentiation. Combination of IR–R suppressed collagenous differentiation, while IR alone can stimulate it. R–G combination stimulated cartilage formation although G laser alone had a better effect than combined therapy. R and IR lasers alone each stimulated osteogenic differentiation, however IR–R combination suppressed it.

Conclusion

Our results show that R and IR lasers stimulated the proliferation of rabbit BMSCs and modulated differentiation into bone and cartilage. The G laser inhibited cell proliferation and osseous differentiation while it stimulated cartilagenous differentiation. Combination lasers had different effects that could not be predicted from the effects of each wavelength alone. It is possible that the combined laser fluences exceeded the peak of the biphasic dose response.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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