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Original Article

The different irradiation parameters of carbon dioxide laser effects on periodontal ligament cells

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Inflammation

Abstract *Background: /purpose*: Photobiostimulation (PBS) can affect cellular functions. The objective of the present study was to evaluate the cellular changes in periodontal ligament (PDL) cells that received different carbon dioxide (CO_2) laser irradiation parameters under negative pressure culture.

Materials and methods: The negative pressure-cultured PDL cells on normal medium and differentiation medium were subjected to continuous irradiation with a CO_2 laser at an energy density of 5 J/cm^2 or 10 J/cm^2. The irradiated PDL cells were harvested at Days 1, 5 and 7, and their viability was analyzed by the Presto Blue assay and the biologic markers alkaline phosphatase (ALP), bone sialopoietin (BSP), osteopontin (OPN), osteocalcin (OC), matrix metalloproteinase-3 (MMP-3) collagen I (Col I) and cyclooxygenase-2 (COX-2) expression by reverse transcription-polymerase chain reaction (RT–PCR).

Results: The PDL cell viability showed that the differentiation medium groups were higher than the normal culture groups. The cell morphologies were all expressed as spindle type. The inflammatory markers in the laser-irradiated groups were higher on the first day and decreased on the seventh day ($P < 0.05$). Osteogenesis markers were highly expressed at different time periods ($P < 0.05$). The Col I and OPN genes were highly expressed on the first day, and the Col I high expression lasted until the seventh day. The OC gene was highly expressed on the seventh day. The effects of PDL cultured in differential medium and normal medium were the same in the present study.

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Conclusion: A low-dose CO_2 laser continuously irradiating cultured PDL cells can induce osteogenesis and reduce cell inflammatory expression.

Introduction

PBS is defined as a low level of light irradiation to alter the chemical, physical and metabolic processes of cells or target cells. The light source may be a light-emitting diode (LED), low-level visible or near-infrared (NIR) light from a laser.[1] The photons are absorbed by mitochondria, leading to increased ATP production, modulation of reactive oxygen species, and induction of transcription factors.[2] PBS treatment of cells could result in a decreased inflammatory response, pain reduction and promote tissue regeneration by photodissociation of inhibitory nitric oxide (NO).[3–6]

Many studies have demonstrated that PBS can increase cell proliferation, adhesion, differentiation and osteogenesis product expression,[7–10] One of the PBS methods using low-level laser irradiation was found to accelerate burn wound healing by stimulating reepithelization, fibroblast proliferation, angiogenesis, granulation tissue formation, and collagen synthesis and to reduce inflammatory infiltration.[11,12] A number of investigations have focused on low-level laser therapy (LLLT) and light-emitting diodes (LEDs) to accelerate orthodontic tooth movement (OTM).[13,14] However, the conclusions of different articles are quite controversial.[15]

The PDL fiber affects post orthodontic tooth stability. The PDL fibers move in opposite configurations from compressed to stretched or from stretched to compressed conditions during the orthodontic retention period.[16] The expression of matrix metalloproteinases (MMPs) was investigated to support the biological mechanism of periodontal remodeling during OTM.[17] PBM promotes cellular viability and the expression of osteocalcin in the tension site of PDL.[18] This mechanism might increase tooth stability.

The different PBS parameters might affect the outcome of irradiation of target tissue. Adequate wave lengths and power densities (wattage and duration of laser exposure) can affect phototherapy. The laser wavelengths for dental application fall within the range of 488 to 10,600 nm.[19] The tissue interactions occur within the energy range of 1—1000 J/cm^2.[20] Thus, it is suggested that the accelerating canine retraction parameter involves PBS irradiation at 150 and 200 J/cm^2 per month and that the dentition alignment parameter is between 260 and 336 J/cm^2 per month.[21,22] To date, there is a lack of laser effect reports regarding orthodontic tooth stability. Because PDL is related to tooth stability, it is interesting and important to investigate the effects of PDL cellular changes by stimulating with different PBS parameters.

The purpose of this study was to use a negative pressure incubator to simulate the orthodontic tooth movement environment and to evaluate the effects of different PBS (low-dose laser) protocols on cultured PDL cells. The irradiated PDL cell bone formation markers and inflammation markers were analyzed.

Material and methods

Cell culture in a tension incubator

Cultured PDL cells were obtained from the Bioresource Collection and Research Center (ATCC 33277; DSM 20709, Hsinchu, Taiwan). The PDL cells were seeded at a density of 1×10^5 cells in 5 cm plates, and different media were added. The culture medium was prepared as Medium A (MA) and was composed of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 l g/mL streptomycin, and 1 mM HEPES. Medium B (MB), a differentiation medium, was composed of DMEM supplemented with 100 n mol/L dexamethasone, 50 μM l-ascorbic acid 2-phosphate and

Table 1 The compositions added in differentiation medium.

Item	Concentration	Function
dexamethasone (DEX)	100 nmol/l	DEX is one of the earliest osteogenic inducers that can control the expression of the osteogenic marker genes and has been routinely applied to direct the differentiation of multipotent mesenchymal stem cells and accelerate tissue remodeling.
β-glycerophosphate	10 mM	β-glycerophosphate an inorganic phosphate needed to produce hydroxyapatite mineral.
L-ascorbic acid 2-phosphate	50 μM	L-ascorbic acid 2-phosphate is required for efficient collagen biosynthesis
vitamin D3	50 nM	Vit D has a direct effect on mesenchymal stem cells (MSCs) in stimulating their osteogenic differentiation.

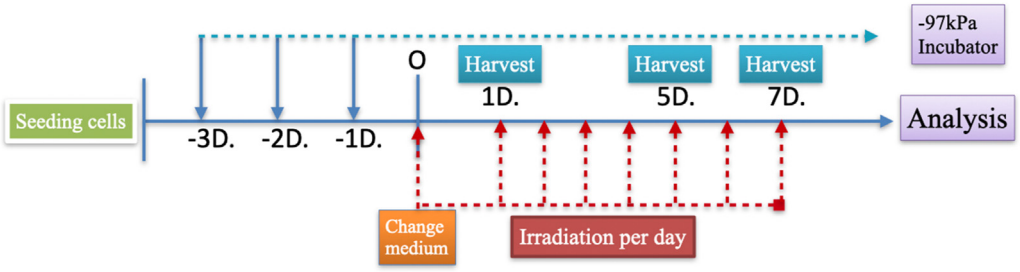

Figure 1 The protocol schedule of continuous laser irradiation per day of the cultured cells. The cultured cells were incubated in a negative −97 kPa incubator for three days, the medium was changed, and laser irradiation was continued daily. Then, irradiated cells were harvested on Day 1, Day 5 and Day 7.

Table 2 The primer used in present study.

Name	Primer sequence (5′–3′)
ALP	Forward: GCTGTAAGGACATCGCCTACCA
	Reverse:CCTGGCTTTCTCGTCACTCTCA
BSP	Forward: ATAGTAGGTGTTTAGTGGAGAG
	Reverse:ATAATCACCGAATATCTACCCTAT
OPN	Forward: ACA CTT TCA CTC CAA TCG TCC
	Reverse: TGC CCT TTC CGT TGT TCT CC
OC	Forward: TCT GAC AAA GCT TCA TGT CC
	Reverse: AAA TAG TGA TAG ATG C
Col I	Forward: TCT CCA CTC TTC TAG TTC CT
	Reverse: TTG GGT CAT TTC CAC ATG C
MMP3	Forward: CACTCACAGACCTGACTCGGTT
	Reverse: AAGCAGGATCACAGTTGGCTGG
Cox2	Forward: AACACGGACTTGCTCACTTTGTTG
	Reverse:AATGGAGGCCTTTGCCACTG

The abbreviations full name are as follows: alkaline phosphatase (ALP), bone sialopoietin (BSP), osteopontin (OPN), osteocalcin (OC), Collagen I (Col I), matrix metalloproteinase-3 (MMP-3) and cyclooxygenase-2 (COX-2).

10 mM β-glycerophosphate.[23] (Table 1) PDL cells cultured with dimethyl sulfoxide (DMSO) were used as a negative control in this study. The cultured cells of the MA group or MB group were placed in a tension incubator (TI, Model. 3618P, Lab-Line Instrument, Inc. Melrose Park, IL. USA) for 1 day under the following conditions: −97 kPa (1 Pa = 1/100,000 kg/cm^2, equal to a negative force of 97 g/mm^2), 37 °C and 5% CO_2.

The PBS parameter

After 72 h of cultivation, the new media were changed (Fig. 1). The cultured cells were irradiated using an OPE-LASER PRO laser (carbon dioxide laser, Yoshida Dental MFG Co., Tokyo. Japan) using continuous wave modulus with an output power of 1.0 W for 5 s or 10 s at an energy density of 5 J/cm^2 or 10 J/cm^2. Each plate was continuously irradiated from Day 1 to Day 7. The irradiated PDL cells were harvested at Days 1, 5 and 7, and their viability and biological markers were analyzed.

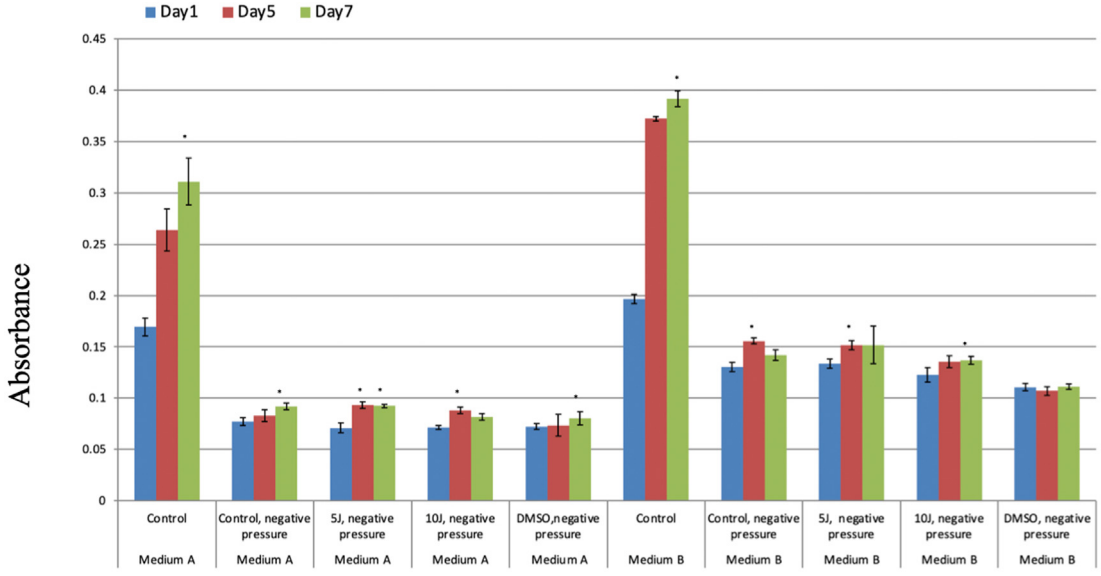

Figure 2 Periodontal ligament (PDL) cells were cultured in Dulbecco's Modified Eagle Medium (Medium A) and differentiation medium (Medium B) in a −97 kPa, 37 °C tension incubator. The PrestoBlue assay was performed to detect PDL cell viability. *The statistically significant difference of the highest value of all tested experiments ($p < 0.05$).

PDL cell viability assay

At the end of the culture period, the culture medium was discarded, and the cells were washed twice with cold phosphate buffer solution.[24] The cultured plate had added medium with a 1:9 ratio of PrestoBlue (Invitrogen Co., Grand Island, NY. USA) to fresh medium. Then, the cultured plate was placed into a normal incubator at 37 °C for 30 min. A spectrophotometer (Hitachi Co., Tokyo, Japan) at 570 nm with a reference wavelength of 600 nm was used to read the data. All of the aforementioned measurements were performed in duplicate and are expressed as the mean ± SD of three independent experiments. One-way ANOVA and Tukey's multiple comparison test were used to determine the significant differences between the investigation groups. The level of significance was set at $P < 0.05$.

Reverse transcription-polymerase chain reaction (RT—PCR)

The treated PDL cells were harvested, and inflammation and osteogenic markers were analyzed by using an RT—PCR assay. Table 2 shows the cDNA sequences of the target osteogenic markers alkaline phosphatase (ALP), bone sialopoietin (BSP), osteopontin (OPN), osteocalcin (OC), collagen I (Col I), matrix metalloproteinase-3 (MMP-3) and cyclooxygenase-2 (COX-2).

The procedure followed our laboratory's previous method.[25] Briefly, the PDL cells were harvested using 0.25% trypsin—EDTA and extracted using TRIzol reagent (Invitrogen Co.) for 10 min. The cell lysate was obtained by adding an aliquot of 200 μl of chloroform for 10 min of incubation. Then, they were centrifuged at 12000 rpm for 15 min at 4 °C. Then, 500 μl of isopropanol (Fluka Co., Buchs, Switzerland) was added to the supernatant for 10 min of incubation, followed by centrifugation at 12,000 rpm for 15 min at 4 °C. After centrifugation, the supernatant was removed, and the sample was stored at −20 °C and washed twice with 75% alcohol. The RNA in aqueous solution was stored at −20 °C and quantified by spectrophotometry at 260 nm. One microgram of total RNA in aqueous solution was reverse transcribed in a volume of 50 μl containing 400 μM of each NTP, 10 units of RNase inhibitor, 10 μl of 5XTris buffer, and 2 μl of RT—PCR mixed enzyme. Reverse transcription was set and performed at 50 °C for 30 min. After an initial denaturation step at 95 °C for 15 min, 40 PCR cycles were run,

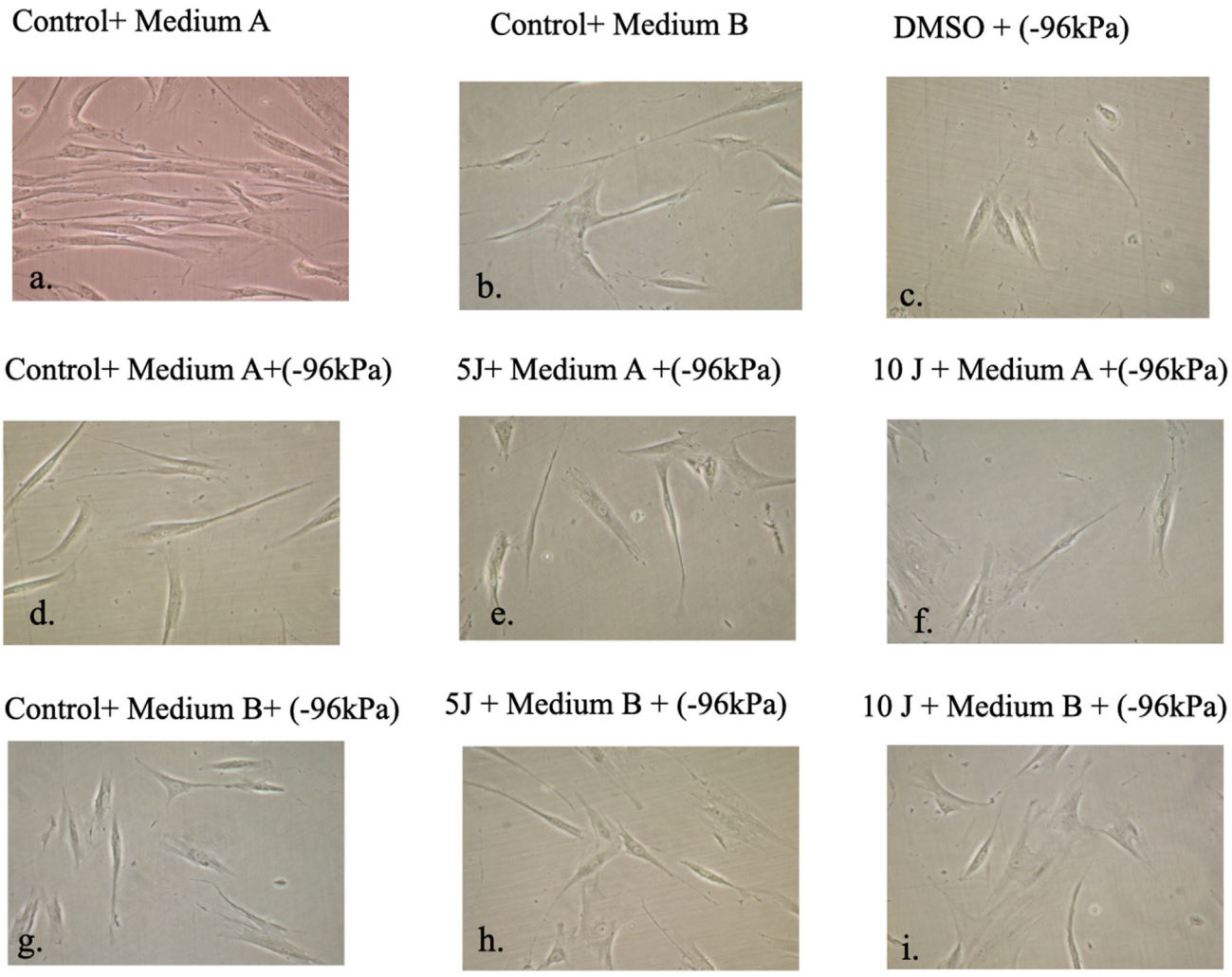

Figure 3 Microscope image (magnification 350 X) of the periodontal ligament (PDL) cell morphologies under different culture conditions at Day 1.

each consisting of denaturation at 94 °C for 1 min, annealing for 1 min, extension at 72 °C for 1 min, and then elongation at 72 °C for 10 min. The PCR products (20 μl) were separated by electrophoresis on a 2% agarose gel at 100 V cm^{-1} in 1% tris-acetate-EDTA buffer. The electrophoresis gels were stained with ethidium bromide, destained with distilled water and photographed with a charge-coupled device camera. To compare the final results, the resultant films were scanned and quantified using a densitometer and the SCION image program.

Results

Cell viability

Under medium A negative pressure conditions, the proliferation of the cultured PDL cells increased as the culture time increased (Fig. 2). However, PDL cell proliferation was decreased under MA and negative pressure conditions. At 5 J/cm^2, negative pressure and MA-cultured PDL cells showed that as the number of culture days increased, PDL cell proliferation was increased ($P < 0.05$). Under the 10 J/cm^2, negative pressure and MA conditions, Day 5 showed a higher proliferation than Days 1 and 7 ($P < 0.05$). The DMSO-treated PDL cell group in MA showed higher proliferation at Day 7 ($P < 0.05$).

The PDL cells cultured in MB showed higher proliferation than those cultured in MA. In MB, the PDL cell culture increased proliferation as the culture days increased in the control group ($P < 0.05$). In the control condition with negative pressure, the cell number increased on Day 5 but decreased on Day 7 ($P < 0.05$). Then, 5 J/cm^2 or 10 J/cm^2 of laser irradiation and negative pressure on PDL cultured in MB showed increased proliferation after Day 5 ($P < 0.05$). The DMSO-treated PDL cells in MB under negative pressure and laser irradiation showed no significant difference as the number of culture days increased ($P > 0.05$).

Cell morphology

The microscope image of the PDL cell morphologies under different culture conditions was as follows: at Day 1, PDL cell morphology was spindle-like (Fig. 3). In the control group, the PDL cells cultured in medium A or medium B were spindle fibrous in shape. The DMOS group showed only

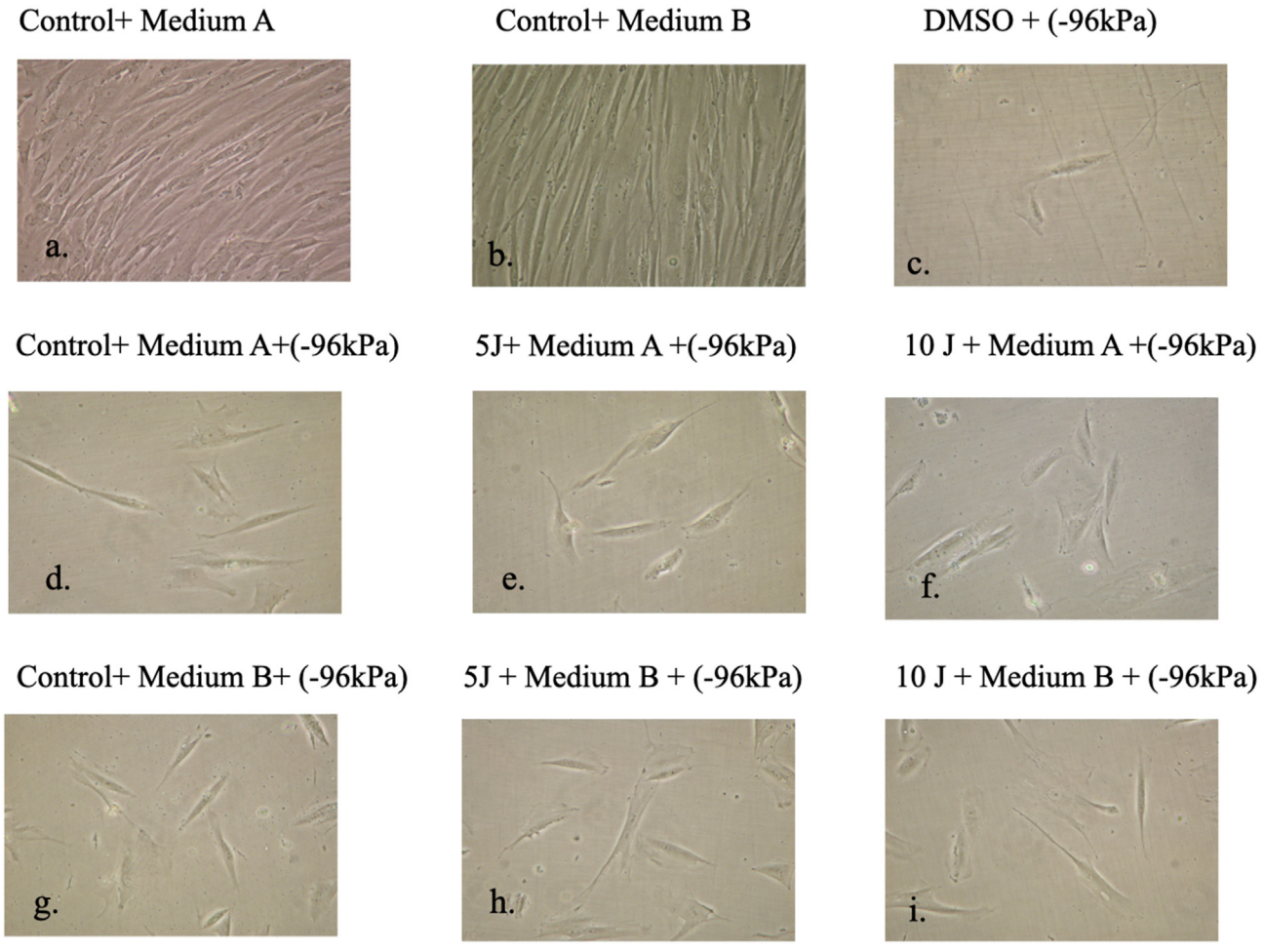

Figure 4 Microscope image (magnification 350 X) of the periodontal ligament (PDL) cell morphologies under different culture conditions at Day 5. PDL cell morphology showed spindle types. Panels a. and b. show that the cells were confluent and grew in one direction. c. The PDL cells treated with DMSO showed only a few cells.

a small number of PDL cells on the culture plate. There was no obvious change in PDL cell morphology under various culture conditions.

On Day 5, PDL cell morphology was spindle-like (Fig. 4). In the control group, the PDL cells cultured in MA (Fig. 4a) or medium B (Fig. 4b) were spindle fibrous in shape, and the cells accumulated in the same direction. The DMOS group showed only a small number of PDL cells on the culture plate.

On Day 7, PDL cell morphology was spindle-like (Fig. 5). In the control group, the PDL cells cultured in MA (Fig. 5a) or MB (Fig. 5b) were spindle fibrous but more confluent, and the cells accumulated in the same direction. In the DMOS group, the PDL cell morphology became normal. The cell numbers in Fig. 5d to i were less than that in the control.

Gene expression

The expressions of osteogenesis genes (ALP, BSP, OPN, OC and Col I) and inflammatory genes (MMP3 and COX-2) in PDL cells cultured in medium A and medium B are shown in Figs. 6—8.

On Day 1, in MA, OPN was highly expressed but without statistical significance in all groups ($P > 0.05$) (Fig. 6). Col I and COX-2 gene expression showed higher gene expression in the laser-treated group than in the unirradiated group ($P < 0.05$). In MB, the Col I gene was highly expressed but without statistical significance in all groups ($P > 0.05$). High COX-2 gene expression was found in the control with the normal pressure and without irradiation group (control -) and the control with negative pressure and without irradiation group (control +) ($P < 0.05$).

On Day 5, in MA, the Col I gene was highly expressed but without statistical significance in all groups ($P > 0.05$) (Fig. 7). OPN gene expression was significantly higher in the 10 J/cm^2-treated group ($P < 0.05$). MMP3 gene expression was significantly lower in the 5 J/cm^2-treated group ($P < 0.05$). In MB, Col I gene expression was higher in the control with the negative pressure group and 5 J/cm^2 group ($P < 0.05$). The COX-2 gene expression significantly decreased in the 5 J/cm^2 and 10 J/cm^2 laser-treated groups ($P < 0.05$).

On Day 7 in MA, OC and Col I gene expressions were high but without statistical significance in all groups ($P > 0.05$) (Fig. 8). The control group without negative pressure showed the highest MMP3 gene expression ($P < 0.05$). In MB, Col I gene expression was high but without statistical significance in all groups ($P > 0.05$). The highest OC gene

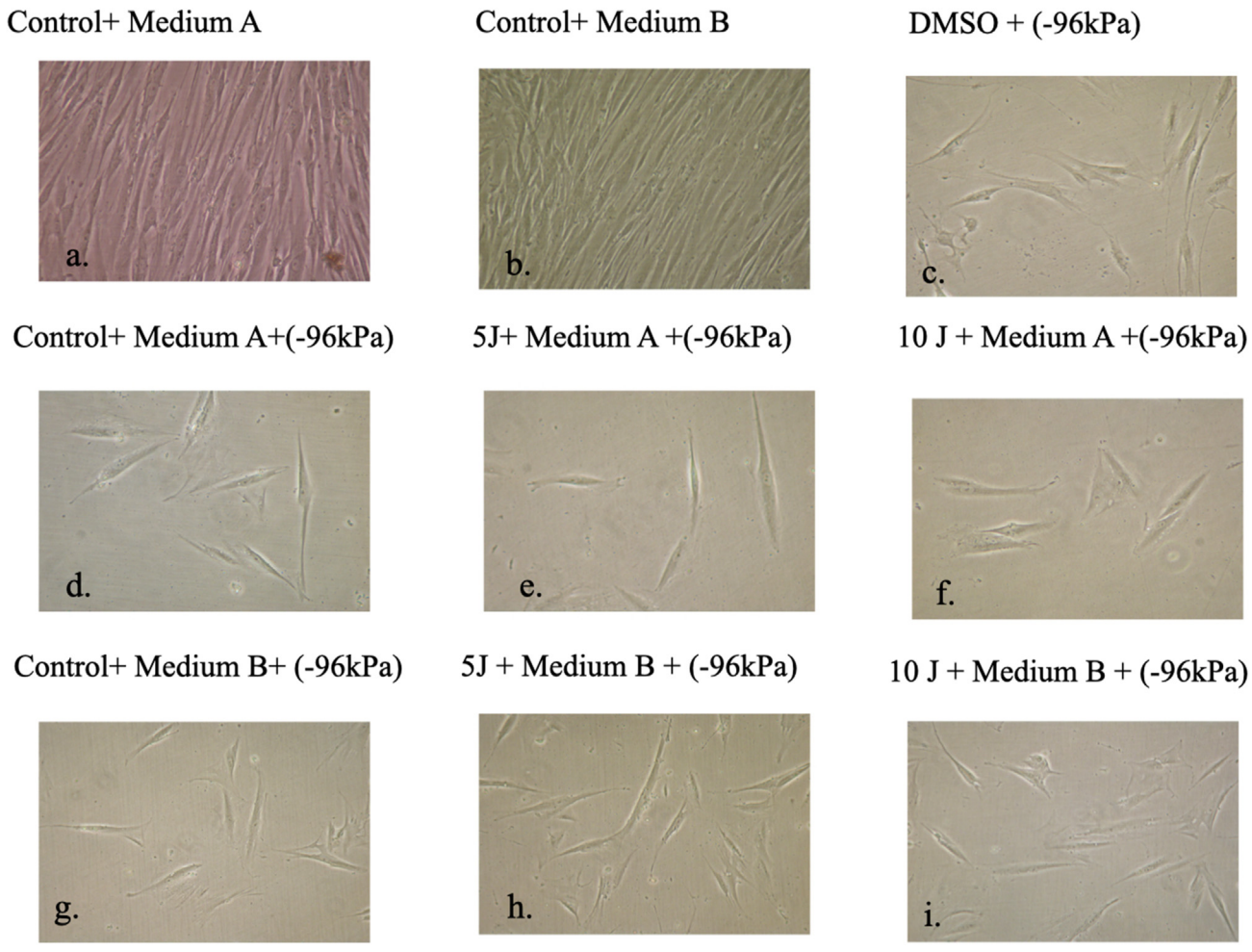

Figure 5 Microscope image (magnification 350 X) of the periodontal ligament (PDL) cell morphologies under different culture conditions at Day 7. The PDL cell morphology remained spindle-shaped.

expression was in the control group without negative pressure. The lowest OC gene expression was observed in the control group under negative pressure ($P < 0.05$). The control group without negative pressure showed the highest MMP3 gene expression ($P < 0.05$). COX-2 gene expression was lower in the 5 J/cm^2 and 10 J/cm^2 groups ($P < 0.05$).

Discussion

Previous studies have shown that low-level lasers affect fibroblast proliferation and collagen synthesis and reduce inflammation.[26—28] The present results showed that PDL cells increased proliferation as the number of culture days

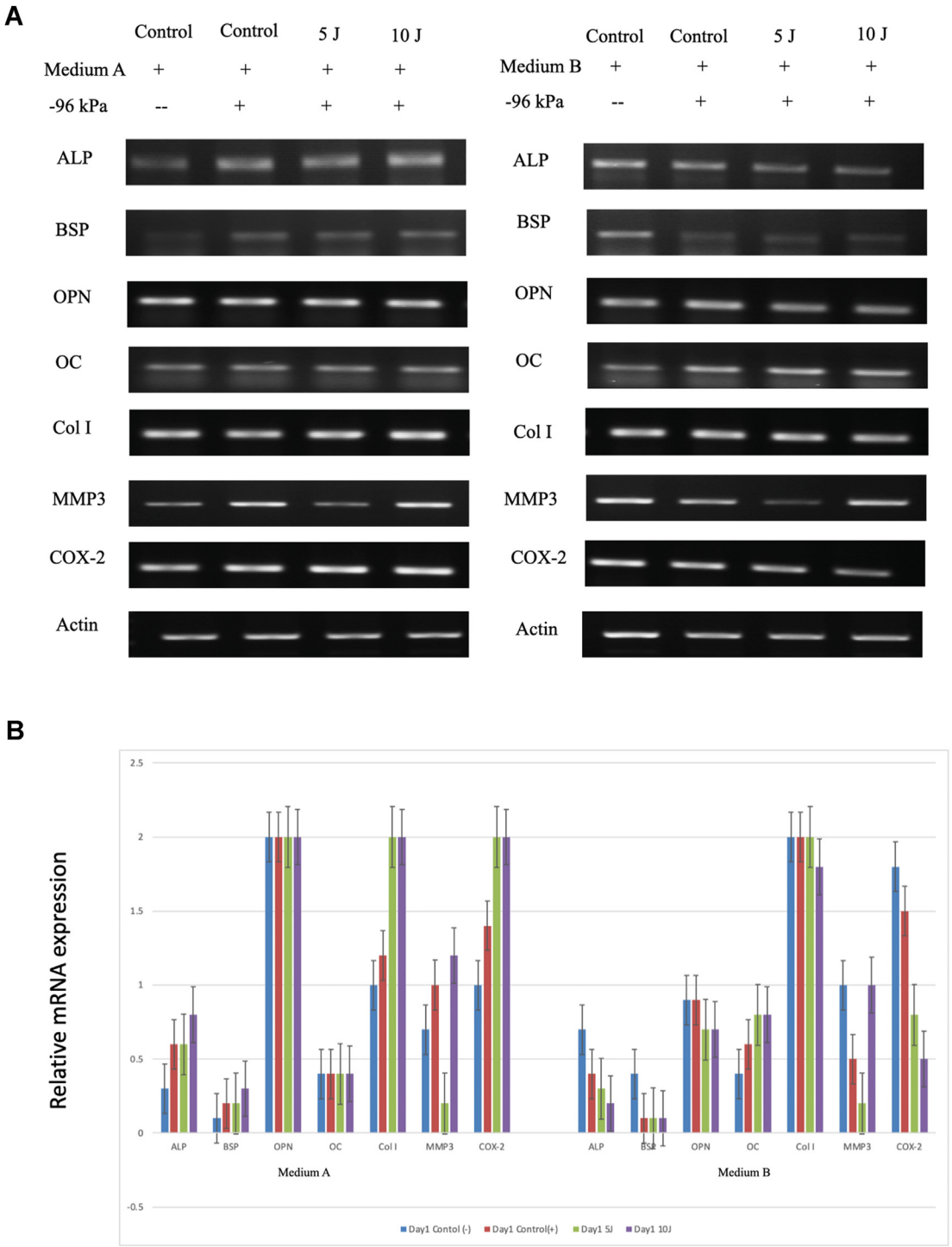

Figure 6 A. Periodontal ligament (PDL) osteogenic and inflammatory marker expression on Day 1 as assayed by RT—PCR analysis. B. Statistical analysis of the relative mRNA expression in PDL cells treated for one day with 5 J/cm^2 and 10 J/cm^2 irradiation. The OPN, Col I, and COX-2 genes were expressed at significantly higher levels in medium A. The OPN and COX-2 genes were expressed at significantly higher levels in medium B.

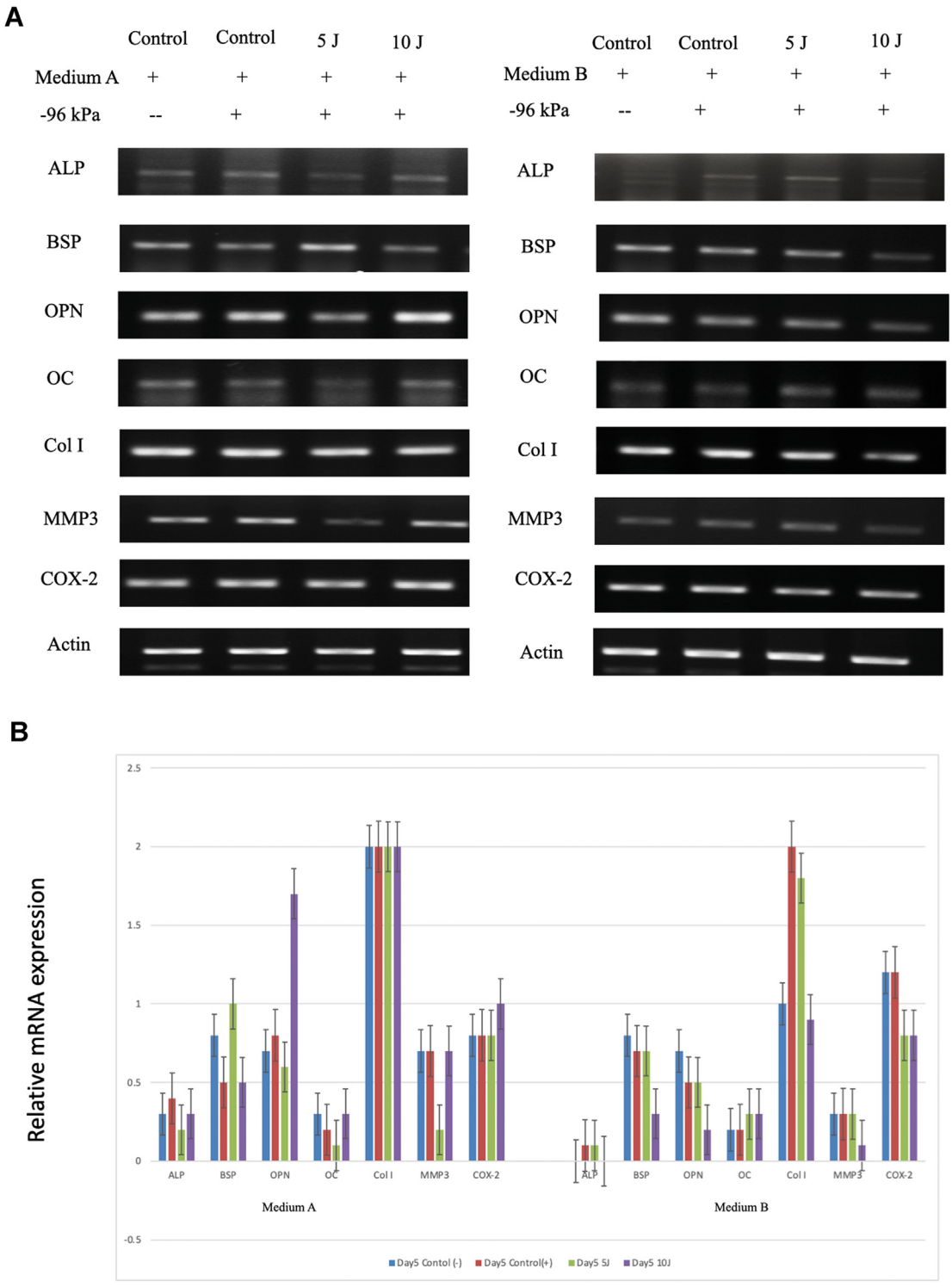

Figure 7 A. Periodontal ligament (PDL) osteogenic and inflammatory marker expression on Day 5 as assayed by RT—PCR analysis. B. Statistical analysis of the relative mRNA expression in PDL cells treated for one day with 5 J/cm^2 and 10 J/cm^2 irradiation. The Col I gene was expressed at significantly higher levels in medium A. The Col I gene was expressed at significantly higher levels in medium B.

increased, but the amount of proliferation was lower than that in the routine cultured control group (Fig. 2). The PDL cells showed a spindle-type morphology without size changes (Figs. 3—5). Although the low PBS studies showed that it can increase the cell proliferation. However, the present results showed that the low PDL cell proliferation phenomenon might be due to negative pressure being a stress or an inflammatory factor in PDL cells that can inhibit PDL cell growth.

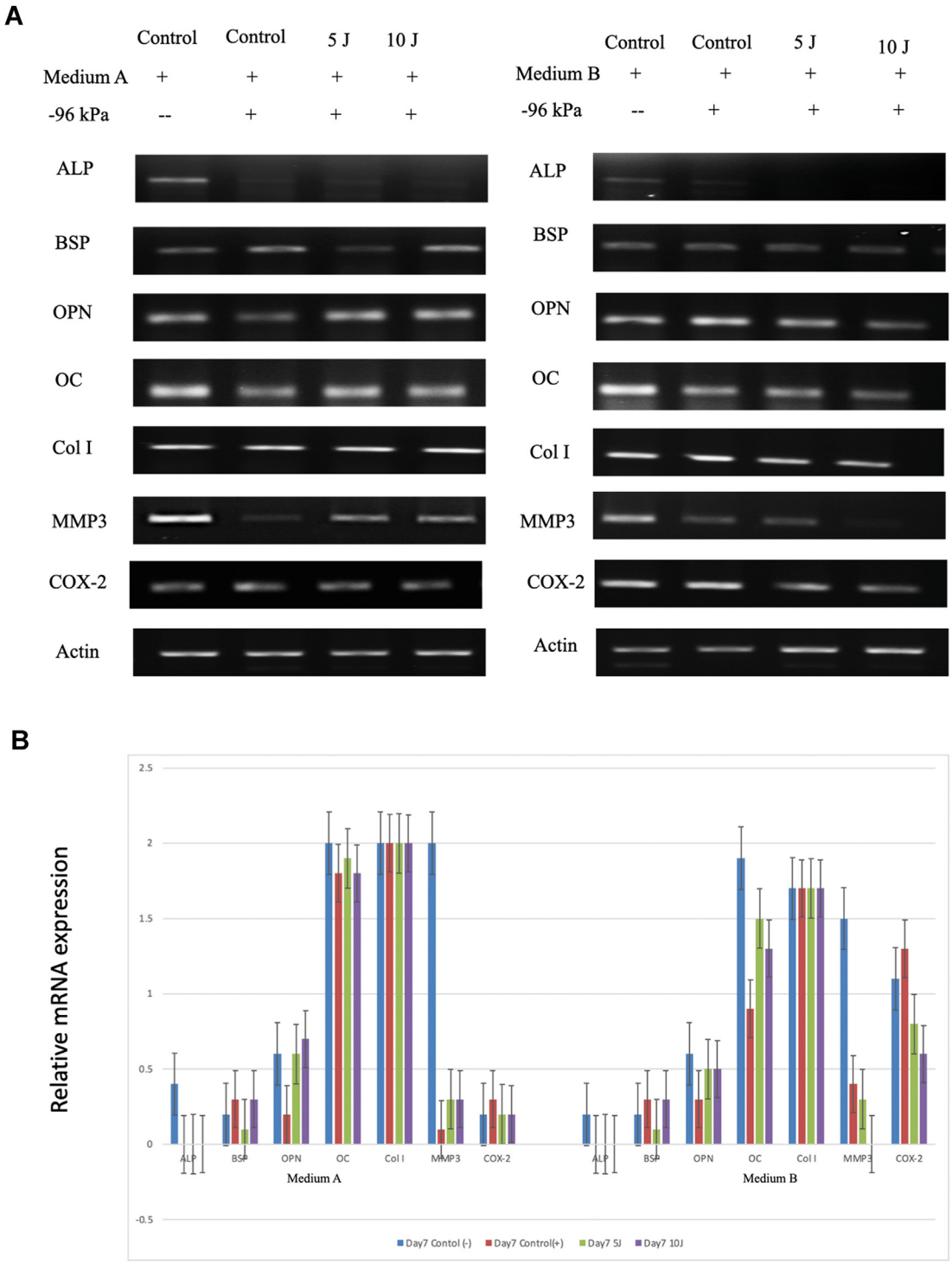

Figure 8 A. Periodontal ligament (PDL) osteogenic and inflammatory marker expression on Day 7 as assayed by RT—PCR analysis. B. Statistical analysis of the relative mRNA expression in PDL cells treated for one day with 5 J/cm^2 and 10 J/cm^2 irradiation. The OC, Col I and MMP3 genes were significantly more highly expressed in medium A. The OC, Col I and COX-2 genes were significantly more highly expressed in medium B.

Tension can affect stem cell growth and differentiation. In human keloid-derived mesenchymal stem cells (KD-MSCs), prior studies showed that tension enhances cell proliferation and collagen synthesis.[29] Our present results controversially showed that cell proliferation was higher in the control without tension pressure group than in the control with the tension pressure group. The reason might be that the present PDL cells are not of stem cell origin.

Inflammation appears as a cell is stimulated by environmental factors, such as mechanical, physical or

chemical factors. One study showed that inflammatory responses to tension may be strain-dependent when low tensile strain is anti-inflammatory and can induce magnitude-dependent anabolic signals in osteoblast-like periodontal ligament cells, culminating in the regulation of inflammatory gene transcription.[30] In this study, the negative pressure was −98 kPa, which is less than in our previous study at −100 kPa. A previous diode laser-treated PDL cell study found that the inflammation markers inducible NO synthase (iNOS), cyclooxygenase (COX-2) and interleukin (IL)-1 showed stronger expression in 5 and 10 J/cm^2 therapy at Days 1 and 5 but decreased in expression at Day 7.[31] In the present CO_2 laser-treated PDL cell study, MMP-3 and COX-2 gene expressions under PBS stimulation decreased compared with that in the control group. It showed that PBS can reduce inflammation.

The sequential action of cytokine networks and transcription factors can cause the differentiation of osteoblast lineage cells from mesenchymal precursors.[32] These osteogenic markers include ALP, Col I, BSP, OPN, and OC. The ALP gene expression in MA and MB appeared higher on the first day and decreased the ALP gene expression on the seventh day. Col I gene expression was highly expressed under all conditions from Day 1−7. Type I collagen is involved in osteogenesis. The PDL cells treated in medium B showed high Col I expression, which was high, low and high on Days 1, 5 and 7, respectively. Bone sialopoietin (BSP) is a major structural protein of the bone matrix. and links to the formation of bone-like tissue.[33] Our showed that BSP low expression was due to BSP appearing in the osteogenesis maturation stage.

The OPN gene controls osteoblast mineralization.[34] In this study, OPN gene expression decreased as the harvest days increased. The present PDL cells were not stem cells; thus, low OPN expression was expected. OC expression is similar to OPN expression, and both are expressed in the late process of bone formation.

The parameters used in this study were different from those used in a previous study.[31] The present study used a CO_2 laser, and in the past, a diode laser was used. The irradiated PDL cells were continuously irradiated for seven days, and in the past, there was only one irradiation of PDL cells in the beginning. This study did not demonstrate which laser is better. The above results showed that PDL cells can be induced to enter the osteogenesis pathway and reduce cellular inflammation. The present in vitro experimental results suggest that PBS application should be applied continuously or with interrupted irradiation of PDL cells and that the PBS dose is sufficient for clinical usage, which still requires further investigation.

Declaration of competing interest

All authors have no conflicts of interest relevant to this article.

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