Osteoblast differentiation of bone marrow stromal cells by femtosecond laser bone ablation

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Abstract: This study examines the osteogenic effect of femtosecond laser bone ablation on bone mesenchymal stromal cells (BMSCs). Three-week old Sprague-Dawley (SD) rats were selected for experiments. Right tibias were ablated by a 10-W femtosecond laser (treated group), whereas left tibias were not subjected to laser ablation (control group). After ablation, BMSCs of both tibias were cultured and purified separately. Cell proliferation was then analyzed, as well as the expressions of RNA and several proteins (alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2) and osteocalcin (OCN)). The results suggest that femtosecond laser ablation promotes the differentiation of BMSCs and up-regulates the expression of ALP, RUNX2, and OCN, without affecting BMSC proliferation.

1. Introduction

The use of stem cells to restore the structure and function of damaged tissues or organs has undergone rapid development in recent years [1,2]. Many studies have shown that one of the most important applications of bone mesenchymal stromal cells (BMSCs) is for bone regeneration [3,4]. As such, stem cell research is predominantly concerned with identifying surgical procedures and supporting therapies that can improve and enhance the effectiveness of various tissues repair and regeneration techniques.

The use of low-energy light intensity within the visible red and near infrared portion of the electromagnetic spectrum has been shown to stimulate irradiated cellular activity [5]. This phenomenon has been referred to as photobiomodulation (PBM) [5,6]. Many studies have reported that the effectiveness of PBM on BMSCs is related to the modulation of gene expression, proliferation, osteogenic differentiation [7], and bone formation [8]. Although a lack of ablation ability limits the applications of low-level lasers, rapid developments in laser technology have produced the femtosecond laser, which avoids thermal damage and cracking during bone ablation.

Experiments by Cangueiro et al. [9] showed that bones ablated by femtosecond laser did not exhibit melting, carbonization, or microcracking and that their composition was preserved except in the immediate vicinity of the ablated zone where collagen degradation and mineral re-crystallization was observed. Moreover, Girard et al. [10] observed a significant reduction in femtosecond laser enzymatic denaturation in experiments that examined the extracellular and intracellular enzyme activity of bone tissue after femtosecond laser ablation. Furthermore, Lo et al. [11] used a femtosecond laser to ablate a mouse skull and found that wounds ablated by femtosecond laser exhibited better healing at 2, 4, and 6 weeks and less damage to surrounding tissue than those ablated by traditional methods. Despite this reduction in damage to adjacent

tissues, a slight delay has been observed in wound healing in mouse skulls resected with femtosecond lasers compared to conventional tools [12]. Martin et al. [13] also reported delayed healing of bone damage caused by femtosecond laser ablation but observed no statistical difference in the healing time between femtosecond laser ablation and traditional methods. Although these previous studies reported the bone healing effects of femtosecond laser ablation, the relationship between the effects of femtosecond laser ablation and bone marrow mesenchymal stem cells in cytology has rarely been studied. Therefore, this study explores the osteogenic effect of femtosecond laser bone ablation on BMSCs.

2. Methods

2.1. Isolated culture and purification of mesenchymal stem cells

Femtosecond laser ablation was conducted with a computer-controlled 1030-nm femtosecond laser system (Tangerine, Amplitude system, France) and a self-developed automatic tooth preparation robot [14] (Fig. 1). Three three-week-old Sprague-Dawley (SD) rats were anesthetized by chloral hydrate (1 mg/ml) and incisions were made along the leg axis to expose the tibia. The right tibias of rats ablated by femtosecond laser (Fig. 2(a)) at a power of 10 W for 10 s (pulse frequency: 200 kHz, pulse energy: 50 μJ, focus diameter: 80 um, fluence: 1 J/cm^2, scan speed: 2000 mm/s, line distance: 0.019 mm, and pulse distance: 0.01 mm) were regarded as the treated group, whereas left tibias that did not accept laser ablation were considered the control group. The path of laser ablation is shown in Fig. 2(b). After ablation, the rats were sacrificed and the bilateral tibias were extracted. A sterilized scalpel was used to remove soft tissues and connective tissues on an ultra-clean workbench. Epiphyses in both ends of the tibias were cut to expose the bone marrow cavity. An alpha-modified minimum essential medium (α-MEM) was extracted using a 1-mL syringe to wash the bone marrow cavity repeatedly until the bone marrow contents pooled and plated on 35-mm culture dishes. Cells were grown in a α-MEM culture medium containing 10% heat-inactivated-fetal calf serum (HIFCS), penicillin (100 U/ml), and streptomycin (50 μg/ml) (all from Invitrogen, Gibco BRL, America) for 10 days at 37 °C in a humidified atmosphere of 5% CO_2 in order to generate monolayers of nonhematopoietic adherent cells (referred to as "BMSCs") [15]. The culture medium was replaced every three days. This investigation was approved by the Biomedical Ethics Committee of Peking University (No. LA2017281).
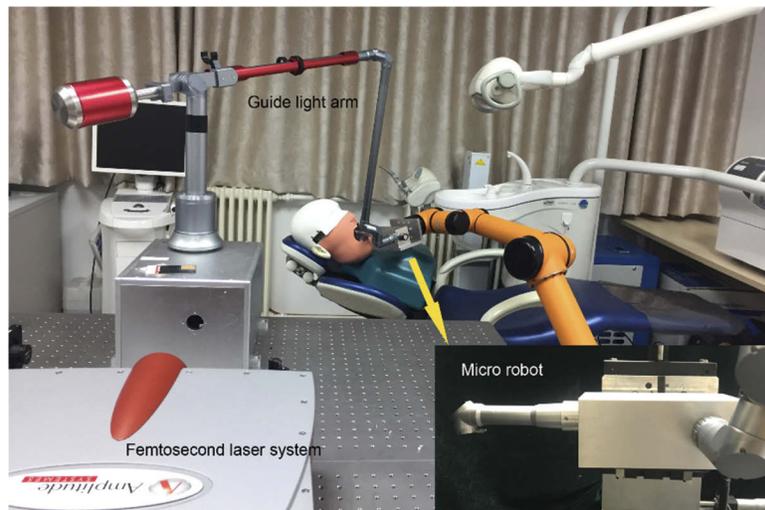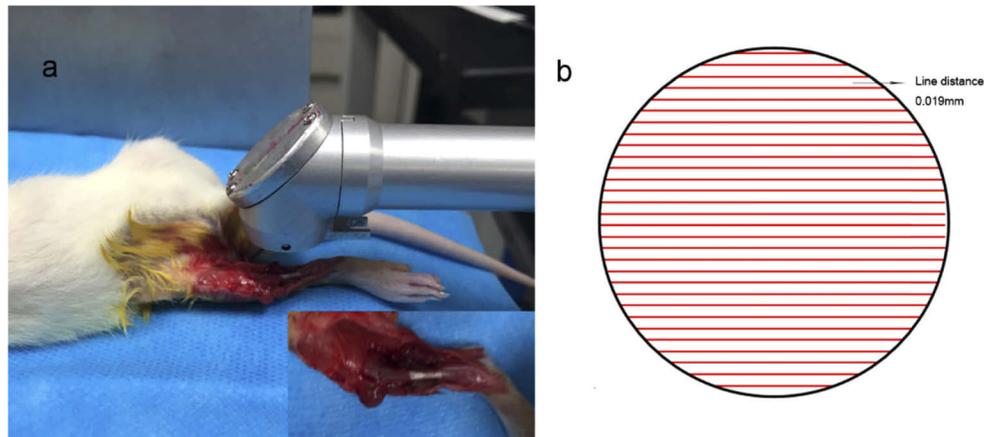

Fig. 1. Photograph of the automatic tooth preparation robot.

Fig. 2. (a) The right tibia of a rat ablated by femtosecond laser and (b) the path of laser ablation.

2.2. BMSC proliferation assay

The cells were seeded in 96 well plates at a cell density of 2×10^4 cells/well. At certain time points (1, 3, 5, and 7 days), the attached cells were incubated with Cell Counting Kit-8 (CCK-8, Dojindo, Japan) reagent. The relative cell number was determined by measuring the absorbance at 450 nm and calculating the optical density (OD) values to construct growth curves.

2.3. Alkaline phosphatase assay

At the end of day 7, the cells incubated in the osteogenic medium were fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature. Alkaline phosphatase (ALP) staining was then performed with a commercial kit (Beyotime Biotechnology) according to the manufacturer's instructions.

2.4. Alizarin red staining

Bone mineralization was visualized with alizarin red staining at day 14. Attached cells were washed with phosphate buffered saline (PBS) and fixed in 4% formalin for 10 min at room temperature. The formalin was then removed and the cells were washed twice with distilled water. 2% alizarin Red S (Sigma) solution was used to stain the cells for 5 min. Excess solution was removed and the cells were washed with distilled water three times. Calcification deposits were photographed by optical microscopy (SZ2-LGB, OLYMPUS CORPORATION, Japan).

2.5. Rt-PCR analysis

To better characterize the cellular influence, relative gene expression levels between the control groups and the groups treated by laser ablation were evaluated by reverse transcription-polymerase chain reaction (Rt-PCR). Total RNA was isolated using a High Pure RNA Isolation Kit (Roche) and cDNA synthesis was performed with a Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to the manufacturer's instructions after osteogenic induction for 1, 3, 7, and 14 days. The cell phenotype markers employed in this study were alkaline phosphatase (ALP), the runt-related transcription factor 2 (RUNX2), and osteocalcin (OCN). All samples were normalized to total RNA content and to the performance of the housekeeping gene, GAPDH. Fold differences in gene expression were calculated using the delta-delta Ct method ($2\Delta\Delta Ct$) [16]. The LightCycler 480 Multiwell plate 96 program was set at 95 °C for 10 min followed by

45 cycles of denaturation at 95 °C for 10 s, and annealing at 60 °C for 30 s followed by 30 s of cooling at 40 °C. The primer pairs were as follows:

RUNX2-F AAC AGC AC AGC AGC AGC AG
RUNX2-R GCA CGG AGC ACA GGA AGT TGG
ALP-F CAC GGC GTC CAT GAG CAG AAC
ALP-R CAG GCA CAG TGG TCA AGG TTG G
OCN-F AGA CTC CGG CGC TAC CTC AAC
OCN-R GGC GTC CTG GAA GCC AAT GTG

2.6. Western blotting

BMSCs were collected after osteogenic induction for 1, 3, 7, and 14 days, the cells were lysed, and the total cell protein was extracted and quantified. Polyacrylamide gel electrophoresis was performed and, after membrane transfer, the proteins were sealed using 5% nonfat milk for 1 h. The primary antibodies of target proteins were incubated and sealed overnight at 4 °C. The next day, after membrane washing with a mixture of tris-buffered saline (TBS) and Tween 20 (TBST), the secondary antibodies were incubated at room temperature for 1 h, and the luminous liquid was prepared using the isometric mixture of Solution A and B. Chemiluminescence apparatus was used to detect the expression of proteins. The bands' density was densitometrically quantified by Quantity One.

2.7. Statistical analysis

All data were analyzed using Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY USA). The independent-samples t-test was used for intergroup comparisons, where $p < 0.05$ was considered statistically significant. The results are representative of those obtained by independent experiments repeated at least three times (samples per group n = 3).

3. Results

3.1. Cell proliferation

The cells were monitored daily using an inverted microscope, which revealed that femtosecond laser ablation did not cause any change in cell morphology. The growth curves of the control
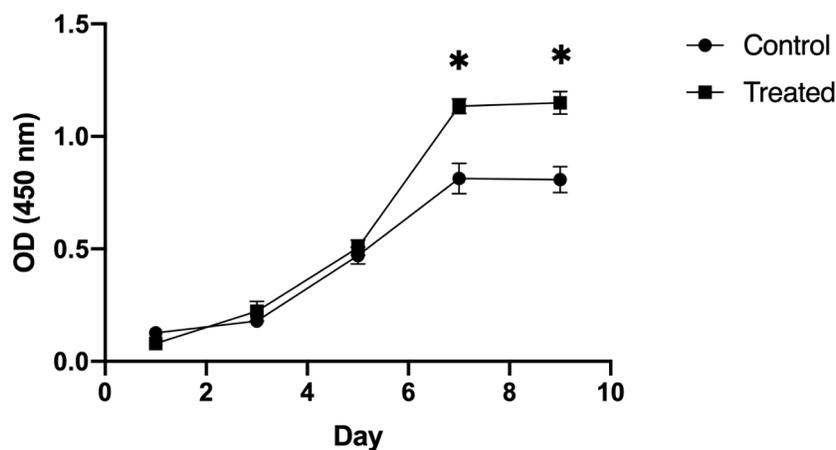

Fig. 3. Growth curves of BMSCs at day 1, 3, 5, 7, and 9. Significant differences were observed between groups on day 7 and 9 ($p < 0.05$).

and treated groups were all logarithmic (Fig. 3), with both reaching a plateau on day 7, and cell proliferation was slower in the control group than in the treated group on day 7 and 9 ($p < 0.05$).

3.2. Osteogenic differentiation of BMSCs

Normal cultured cells were evenly distributed and exhibited a long shuttle-type morphology. Seven days after the osteogenic induction of BMSCs, the ALP stain revealed the formation of a large number of ALP precipitates between cells. Fourteen days after cell osteogenic induction, the alizarin red S stain revealed a large number of mineralized nodules between cells, the number of which was smaller in the control group than in the treated group (Fig. 4).
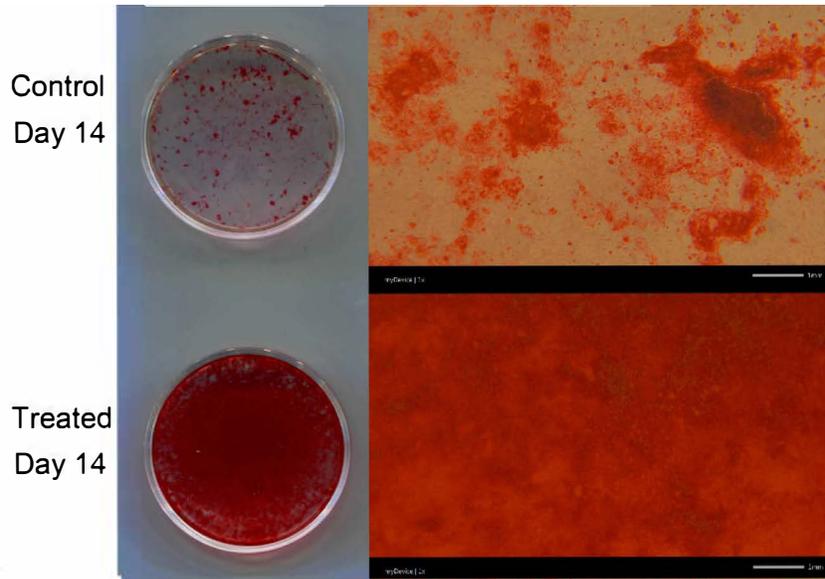

Fig. 4. Alizarin red S stain results on day 14 for the control group (upper panel) and treated group (lower panel). Scale bar = 1 mm.

3.3. RT-PCR

The relative gene expressions normalized to GAPDH on day 1, 3, 7, and 14 after laser application are shown in Fig. 5. A statistically significant up-regulation of ALP was observed in the treated group on day 7 and 14 ($p < 0.05$). No evident fold-increase was detected for the RUNX2 gene between laser-treated and control groups, whereas up-regulation of RUNX2 was detected earlier in the treated group than in the control group on day 7 ($p < 0.05$). Statistically significant up-regulation of OCN was observed on day 1, 3, 7, and 14 ($p < 0.05$).

3.4. Western blotting

The results of gel electrophoresis are shown in Fig. 6. After 7 days of culture, ALP activity in the treated group was higher than that in the control group. On day 14, ALP activity exhibited a greater increase in the treated group ($p < 0.05$). In addition, the relative expressions of Runx2 and OCN were higher in the treated group than in the control group after seven days of culture, and OCN was higher in the treated group after 14 days ($p < 0.05$). Thus, the expression levels of osteogenesis-related genes ALP, RUNX2, and OCN were significantly higher in the group that received femtosecond laser ablation than in the control group.
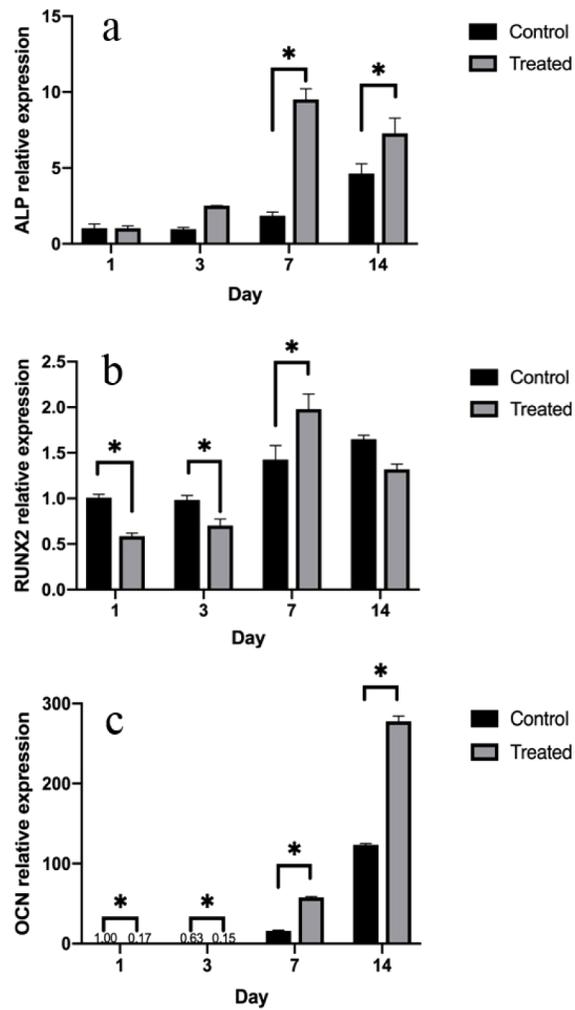
Fig. 5. Time-dependent expression of (a) ALP, (b) RUNX2, and (c) OCN in the BMSCs of control and treated groups according to the reverse transcription-polymerase chain reaction. Error bars represent ± SE (*$p < 0.05$).
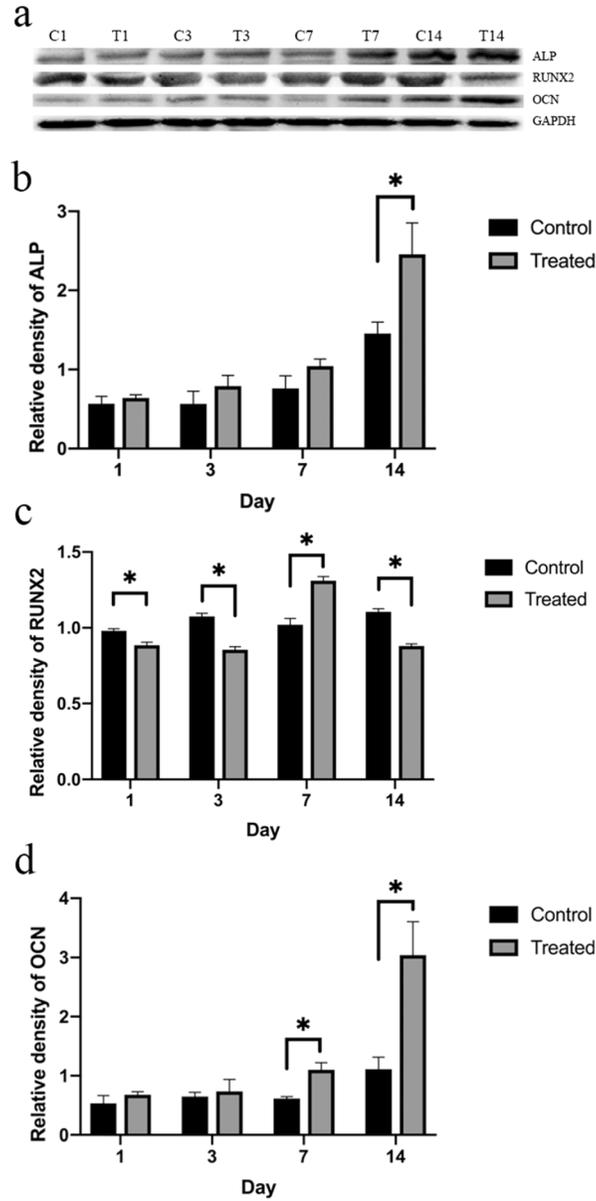
Fig. 6. Time-dependent expression of (a) ALP, (b) RUNX2, and (c) OCN in the BMSCs of control and treated groups according to Western blotting analysis. Error bars represent ± SE (*$p < 0.05$).

4.　Discussion

BMSCs, which were first discovered in bone marrow and subsequently found to be widely distributed throughout the body, have been widely studied due to their advantages of convenient material extraction, rapid expansion, autologous transplantation, and osteogenic differentiation potential [17]. Femtosecond laser ablation has been investigated in various fields of medicine for many years; however, the underlying mechanisms remain uncertain. It is difficult to compare the efficacy of femtosecond laser ablation because of the wide variation in the methods and parameters employed in previous studies. In this in vitro study, BMSCs in bones were ablated with 1030-nm femtosecond lasers at a power of 10 W, which is much greater than that employed in other studies with low-level lasers [18,19]. Therefore, this study employed high-power femtosecond laser ablation to investigate its effect on BMSCs in greater detail.

BMSCs were isolated and purified by the adherence method. In our study, the induction of BMSCs to osteoblast precursors was demonstrated by an increase of RUNX2. The multi-directional differentiation ability of mesenchymal stem cells is also one of the gold standards for identifying BMSCs [20]. Seven days after the osteogenic induction and culture of BMSCs, the ALP stain results showed that ALP activity was enhanced and the cells were stained blue, suggesting that osteogenic differentiation of BMSCs into osteoblasts had been induced. Fourteen days after osteogenic induction, alizarin red stain results revealed a large number of mineralized nodules deposited in the intercellular substance and stained red, indicating that osteogenic differentiation of BMSCs into osteocytes had been induced. The CCK8 results demonstrated that the cell viability and proliferation in treated groups were better than those in control groups after femtosecond laser ablation. The results of this study are better than and differ from those of several reports that showed no significant biostimulatory effect on cell proliferation [18], in which the Nd:YAG laser used to stimulate cells was different from femtosecond laser, these two kinds of laser might have different biological effects on cells, and the mechanism was the next objective we would study. When both cell viability and alizarin red staining results are examined, it appears that the significant increase of calcium deposition in the treated group was not attributable to a general increase in cell number, but rather to an increase in the percentage of cells exhibiting mineralization.

The above results reveal that BMSCs were differentiated into osteoblasts and osteocytes, and that femtosecond laser ablation can significantly accelerate the mineralization of osteoblasts. Considering that the formation of mineralization nodes most accurately reflects the mineralization capacity of cells, it is suggested that a 10-W femtosecond laser is appropriate for osteogenic differentiation of BMSCs.

To further elucidate the role of the femtosecond laser, the expressions of genes ALP, OCN, and RUNX2, which promote osteogenesis, were detected. ALP is an early indicator and functional marker of osteoblast differentiation, which plays an important role in bone mineralization and bone formation [21]. The activity of ALP increases as osteoblasts begin to differentiate. The results of this study showed that the relative expression of ALP increased at four time points in the treated group, indicating that femtosecond laser ablation promoted the early expression of ALP.

RUNX2 is an important transcription factor that reacts to the differentiation of chondrocytes and osteoblasts. In the early stage of osteoblast differentiation, RUNX2 predominantly regulates the expression of bone matrix proteins; conversely, it is not a key transcription factor in the mature stage of bone formation [22]. The results of Rt-PCR and Western blot showed that the relative expression of RUNX2 was higher on day 7 in the treated group, indicating that femtosecond laser ablation accelerated the expression of early RUNX2. Komori et al. [23] explored the role of RUNX2 in bone formation. After reviewing related literature, they proposed that RUNX2 plays a role in the differentiation of immature osteoblasts, but osteoblasts must be down-regulated to

further differentiate and mature; therefore, RUNX2 expression decreases in the late stage of bone formation. This was also verified by the results from day 14 in the treated group.

OCN is a non-collagen protein in bone mineralized tissues. OCN mRNA transcription and protein synthesis is one of the main indicators of osteoblast differentiation and maturation into the mineralization phase, which are considered late markers of osteoblast differentiation and maturation. The results showed that the relative expression of OCN in the treated group was lower at day 1 and 3 but increased significantly at day 7 and day as bone mineralization and osteoblast differentiation improved compared to the control group; these results are similar to those of previous research [23].

The RT-PCR and Western blotting data revealed that OCN and ALP were the dominant proteins expressed at day 14; therefore, mineralization nodules were substantially more abundant in treated groups than control groups and early up-regulation of RUNX2 may also have occurred. Combining the results of this study and other investigations, it is concluded that femtosecond laser ablation at the studied energy dose did not affect osteoblast viability but promoted the differentiation of BMSCs into osteoblasts; thus, it contributed to bone formation.

Plasma-mediated laser ablation is used as a non-mechanical alternative to bone resection [24], which has the advantage of reducing the amount of energy applied to the tissues. As the pulse duration decreases from nanoseconds to femtoseconds, the fluence threshold is reduced by three orders of magnitude, therefore minimizing thermo-mechanical damage [25]. The results of this experiment indicate that cell damage by femtosecond laser ablation using compatible parameters would be within an acceptable range.

The results of this experiment provide some evidence for the effects of femtosecond laser ablation on the osteogenic differentiation of BMSCs as well as the corresponding mechanism. This points to numerous potential applications of femtosecond laser ablation; however, adequate and reliable evidence is necessary for future surgical use. Although femtosecond laser ablation appears to contribute to bone healing, researchers do not yet fully understand the mechanism. Moreover, the parameters of femtosecond laser ablation may contribute to different biological effects. As the healing and repair of bone is a complex process, other techniques should be considered in combination with femtosecond laser ablation.

5. Conclusion

In conclusion, this study proves that 1030-nm femtosecond laser ablation of bones at a power of 10 W promotes BMSCs differentiation toward osteogenesis through the accelerated expression of ALP, RUNX2, and OCN.

Disclosures

The authors declare no conflicts of interest.

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