



Effect of Er: YAG Laser Irradiation on Bone Metabolism-Related Factors Using Cultured Human Osteoblasts

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Abstract

Introduction: A variety of laser treatments have been applied in numerous medical fields. In dentistry, laser treatments are used for caries, root canals, and periodontal disease, as well as surgical resection. Numerous reports have recently been published on the use of lasers for bone regeneration. If laser irradiation is found to promote the activation of bone metabolism, it might also be effective for periodontal treatment, peri-implantitis, and bone regeneration. Therefore, the present *in vitro* study aimed to elucidate the mechanisms underlying the effects of erbium-doped yttrium aluminum garnet (Er: YAG) laser irradiation on the bone using osteoblast-like cells.

Methods: Osteoblast-like Saos 2 cells (5.0×10^4 cells) were seeded in 24-well plates. 24 hours after being seeded, the cells were subjected to 0.3 W, 0.6 W, and 2.0 W Er: YAG laser irradiation and then allowed to recover for 48 hours. The expression levels of bone metabolism-related factors alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteoprotegerin (OPG) were then evaluated using reverse transcription-quantitative polymerase chain reaction and western blot analyses.

Results: Saos 2 cells subjected to Er: YAG laser irradiation at 0.3 W, 0.6 W, and 2.0 W showed normal growth. When the Er: YAG laser irradiation and control groups were compared after 48 hours, increases were observed in ALP, BSP, and OPG gene and protein expression in the 2.0 W group. Similar results were obtained in the western blot analysis.

Conclusion: These findings suggest that the Er: YAG laser irradiation of osteoblast-like cells is effective for activating bone metabolism factors.

Keywords: Bone metabolism; Bone restoration; Er: YAG laser; Osteoblasts.



Introduction

A laser, which is an acronym for light amplification by stimulated emission of radiation, is artificial light made to have a single wavelength. The laser device emits this light spectrum to generate a high-density energy beam. The use of various lasers such as the erbium-doped yttrium aluminum garnet (Er: YAG) laser, the neodymium-doped yttrium aluminum garnet (Nd: YAG) laser, and the Ar gas laser depends on the medium. When classified by tissue permeability, CO₂ lasers and Er: YAG lasers are surface absorption types because energy is mainly absorbed on the tissue surface, whereas He:Ne lasers, diode lasers, Nd: YAG lasers, and Ar gas lasers absorb energy. They are classified as a tissue-transmitting type because they repeatedly scatter and reach deep into the tissue. These can be used properly according to the purpose of use and can be applied to various fields by changing the appropriate type and output of the laser. It is also used

in various applications in the medical field owing to its individual characteristics. It has applications in a wide range of fields such as distance measurement, cancer screening, pain relief, and laser hair removal. Lasers have also been used in various dental applications. Lasers have recently been applied to a variety of treatments in the field of dentistry, as evidenced by a number of clinical reports regarding caries treatment¹ and root tip resection² using the Er: YAG laser, pain relief using the CO₂ laser³ and the diode laser,⁴ and root treatment using the Nd: YAG laser.⁵ It is used to relieve pain during tooth movement, temporomandibular joint pain, and stellate ganglion blocks. However, despite these reports, the detailed mechanisms underlying these treatments remain unknown.

Dental implants are a very useful treatment for restoring occlusion in patients with missing teeth. On the other hand, implant detachment occurs for various reasons,

which is problematic.^{6,7} Depending on the severity, peri-implantitis requires patient guidance,⁸ debridement, and surgery.⁹ Treatment involving the removal of the infected site and debridement using the Er: YAG laser has been reported to be effective for peri-implantitis, and it has recently been attracting increasing attention in the field of dentistry.¹⁰⁻¹² In addition, the advantages of Er: YAG laser treatment for conventional periodontal treatment have been reported.^{13,14} These findings suggest that Er: YAG laser treatment has the potential to be effective not only for removing defective tissue but also for promoting bone and tissue healing. Irradiation with a diode laser¹⁵ or Nd: YAG laser¹⁶ has been shown to lead to the proliferation of osteoblasts. In addition, the application of Er: YAG,¹⁷ Nd: YAG,¹⁸ and diode laser irradiation¹⁹ during experimental tooth movement has been shown to accelerate tooth movement and lead to changes in bone metabolism. As described above, various lasers have been suggested to promote calcification, bone formation, and bone healing at the level of cell and animal experiments, that is, the possibility of promoting bone metabolism. Therefore, it is necessary to accumulate these basic findings and connect them in order to elucidate more detailed mechanisms. The Er: YAG laser was selected because it is frequently used in clinical dentistry for incision, caries removal, hemostasis, and periodontal treatment. Also, to investigate in more detail the effect of Er:YAG laser irradiation on the periodontal tissue, which we have previously studied.¹⁷

Given this background, the present *in vitro* study aimed to investigate the effects of Er: YAG laser irradiation on bone metabolism using human osteoblast-like cells.

Materials and Methods

Cell Culture

Experiments were conducted in Saos 2 cells (CLS; 300331/p651_Saos 2 RRID: CVCL_0548). Human-derived osteoblast-like Saos 2 cells (Riken, Japan) were first cultured in 100-mm tissue culture dishes in Dulbecco's Modified Eagle Medium (Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS; Bioserum, Melbourne, Australia) and 1% antibiotic-antimycotic mixture (Invitrogen, Carlsbad, USA), and then incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Laser Irradiation

This study utilized an Er: YAG laser (Erwin AdvErL; Morita, Japan) with a wavelength of 2940 nm and a red semiconductor laser guide light with a wavelength of 650 ± 15 nm (Table 1). No fiber tip was used on the Er: YAG laser. A handpiece was set and fixed using a stand to produce an irradiation area of 9.6 cm². The Er: YAG laser was irradiated for 30 seconds at total energy densities of 0.9375 J/cm² (0.3 W), 1.875 J/cm² (0.6 W), and 6.25 J/cm²

Table 1. The Detailed Specifications of the Er: YAG Laser Irradiation

Specifications	
Wavelength (nm)	2940
Operating mode	Pulsed
Frequency (Hz)	5–20
Pulse duration (us)	300
Laser Energy/pulse (mJ)	30–140
Average power (W)	0.15–2.8

(2.0 W) under each condition. Non-irradiated cells were used as a control.

Preliminary Experiment for Determining the Optimal Conditions

Saos 2 cells (1.0×10^5) were seeded in a 12-well plate. After reaching 60% confluency, the serum concentration in the culture medium was gradually reduced to 0%. The cells were then incubated in 0% FBS for 12 hours. and irradiated at various fluences with the Er: YAG laser. Next, at 36 hours. after Er: YAG laser irradiation, the cells were collected using phosphate-buffered saline (PBS; Mitsubishi Chemical Mediation, Japan) containing 0.2% trypsin (Nacalai Tesque, Japan) and 0.1% ethylenediaminetetraacetic acid (EDTA; Wako, Japan). Using 0.4 w/v% trypan blue solution (Wako) to count the number of viable cells revealed that the cells irradiated at an output of 2.8 W or higher were significantly reduced or killed. Therefore, for the experimental conditions, 0.3 W, 0.6 W, and 2.0 W laser irradiation groups were established, along with a control group (no irradiation) (Figure 1).

Investigation of Cell Number Change over Time by Er: YAG Laser Irradiation

Saos 2 cells were seeded in a 24-well plate at a density of 5.0×10^4 cells/well. After reaching 60% confluency, the serum concentration in the culture medium was gradually reduced to 0%, as described above. The cells were then incubated in 0% FBS for 12 hours. and irradiated at 0.9375 J/cm², 1.875 J/cm², and 6.25 J/cm². Following laser irradiation, cell culture was performed using the medium to which FBS had been added again. Next, the cells were collected at 1, 24, and 48 hours. after irradiation using PBS (Mitsubishi Chemical Mediation) containing 0.2% trypsin (Nacalai Tesque) and 0.1% EDTA (Wako). The numbers of viable cells were then counted using 0.4 w/v% trypan blue solution (Wako).

Reverse Transcription–Quantitative Polymerase Chain Reaction (RT–qPCR) Analysis

Saos 2 cells were seeded in a 6-well plate at a density of 2.0×10^5 cells/well, and after reaching 60% confluency, the serum concentration in the culture medium was gradually reduced to 0%, as described above. Next, the cells were incubated in 0% FBS for 12 hours. and

irradiated at 0.3 W (0.9375 J/cm²), 0.6 W (1.875 J/cm²), and 2.0 W (6.25 J/cm²). After laser irradiation, cell culture was performed again using the medium to which FBS had been added. The cells were collected at 48 hours after the appearance of significant changes in the preliminary study (Figure 2). RNA was then extracted using PBS containing 0.2% trypsin and 0.1% EDTA, homogenized using a syringe and a 20 G needle (both Terumo, Japan) and isolated using an RNA isolation kit (RNeasy Mini Spin Column; QIAGEN, Netherlands). Next, total RNA was quantified by reading the absorbance at 260 nm using a spectrophotometer (BioSpec-nano, Shimadzu, Japan). The cDNA was synthesized from 1 mg of total RNA using a cDNA synthesis kit (ReverTra Ace- α qPCR RT Kit; Toyobo, Japan) and a random primer (Toyobo). The PCR primer sequences for alkaline phosphatase (ALP), bone sialoprotein (BSP), osteoprotegerin (OPG), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in Table 2. For the reactions, the following conditions were used: 45 denaturation cycles at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 10 seconds. The gene expression values were then normalized to control values; the fold increase in gene expression by human-derived osteoblast-like Saos 2 cells cultured alone was monitored, and the mRNA level was normalized to that of GAPDH.

Western Blot Analysis

Saos 2 cells were seeded in a 6-well plate at a density of

2.0×10^5 cells/well, and after reaching 60% confluency, the serum concentration in the culture medium was gradually reduced to 0%, as described above. Next, the cells were incubated in 0% FBS for 12 hours and irradiated at 0.9375 J/cm², 1.875 J/cm², and 6.25 J/cm². Following laser irradiation, cell culture was performed using the medium to which FBS had been added again. The cells were collected at 48 hours after the appearance of significant changes in the preliminary study. After 48 hours, the proteins were extracted in the Triton lysis buffer (50 mM Tris-HCl [pH 7.4], 250 mM NaCl, 0.1% Triton-X buffer [Roche Diagnostics, Rotkreuz, Switzerland], 1 mM EDTA, 50 mM NaF, and protease inhibitors [Takara Bio, Japan]) and rapidly frozen in liquid nitrogen. The samples were then thawed on ice and centrifuged at 4°C at 15000 rpm for 20 minutes, and the supernatant was collected. Protein (20 μ g) was then mixed with 10% 2-mercaptoethanol and 0.01% bromophenol and denatured at 100°C for 3 minutes. Next, the Bicinchoninic acid Protein Assay Kit (Pierce, Rockford, USA) was used to determine the protein concentration, and the iBlot Gel Transfer System (Invitrogen) was used to perform the electrophoresis utilizing 10–12.5% SDS-polyacrylamide e-PAGEL® gels (ATTO, Japan). After transferring the separated proteins to polyvinylidene fluoride (PVDF) membranes immersed in Block Ace (DS Pharma Biomedical, Japan), they were incubated at room temperature for 30 min. and then at 4°C for 12 hours. in primary antibody diluted 1:1000 in CanGet Signal® Solution 1 (Toyobo). The PVDF

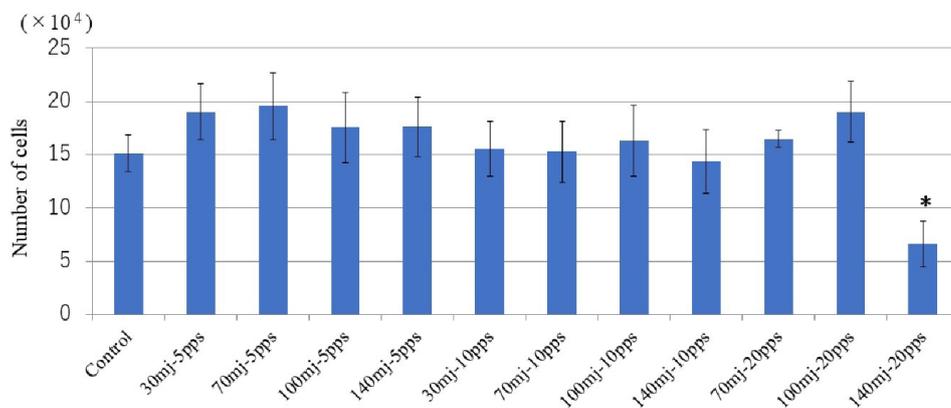


Figure 1. Viable Cell Count After Er: YAG Laser Irradiation. Human-derived osteoblast-like cells were irradiated with an Er: YAG laser, and the number of viable cells was determined after 36 hours. When the cells were irradiated with an Er: YAG laser at an output of 2.8 W or higher, a decrease in the number of viable cells was observed. Control: Control group not irradiated with the Er: YAG laser. * $P < 0.05$ vs. control, $n = 3$

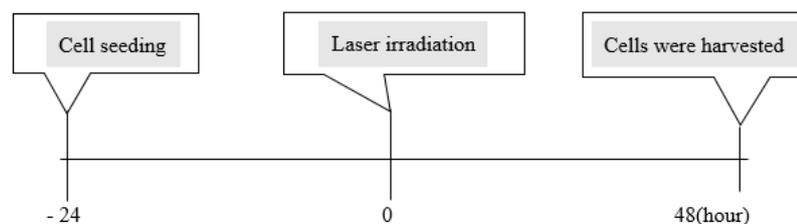


Figure 2. Time Course of Quantitative Real-Time PCR And Western Blotting

Table 2. PCR Primer Base Sequence of GAPDH, ALP, OPG, BSP

Gene	Sequence
GAPDH	Forward 5'-TGGTATCGTGAAGGACTCA-3'
	Reverse 5'-GCCATCACGCCACAGTT-3'
ALP	Forward 5'-TACAAGTGGGCGGTGA-3'
	Reverse 5'-ACAGCAGACTGCGCCTGGTAGTT-3'
OPG	Forward 5'-CTGCTGAAGTTATGGAAACAT-3'
	Reverse 5'-AGGTTAGCATGTCCAATGTG-3'
BSP	Forward 5'-GCCACAGGGTATACAGGGTTA-3'
	Reverse 5'-CTGTGCTGTTGGTACTGGTG-3'

membranes were then incubated in goat anti-mouse or anti-rabbit IRDye® (both from LI-COR Biosciences, UK) secondary antibody diluted 1:10,000 with CanGet Signal® Solution 2 (Toyobo) for 1 hr. at room temperature. The Odyssey® Imaging System (LI-COR Biosciences) was then used to detect the electrochemical fluorescence of the target antigen. The primary antibodies were ALP (Mouse Alkaline Phosphatase/ALPL Antibody; R&D Systems, MN, USA), BSP (ab33022; Abcam, Japan), OPG (H-249:sc-11383; Santa Cruz Biotechnology, Germany), and β -actin (sc-7963; Santa Cruz Biotechnology).

Statistical Analysis

The mean of all three experiments was then calculated, with the results representing the values of each experimental parameter. Data from three independent experiments are expressed as the mean and standard deviation. For group comparisons, one-way analysis of variance was used, and for differences between each experimental group and the control group, Dunnett's test was used. Values of $P < 0.05$ were considered statistically significant.

Results

Effect of Er: YAG Laser Irradiation on Cell Number

No significant differences were seen in Saos 2 cells irradiated with the Er: YAG laser in the 0.3 W, 0.6 W, or 2.0 W group, and proliferation was observed over time, as in the control group (Figure 3).

Investigation of the Effect of Er: YAG Laser Irradiation by RT-qPCR and Western Blot Analysis

Alkaline Phosphatase

The results of RT-qPCR analysis revealed that compared with the control group, ALP gene expression was significantly enhanced in the 2.0 W laser irradiation group. No significant differences compared with the control group were found in the other laser irradiation groups. Moreover, the results of the western blot analysis revealed that ALP protein expression was increased in the 2.0 W laser irradiation compared with the control group (Figure 4).

Bone Sialoprotein

Based on the results of RT-qPCR, BSP gene expression

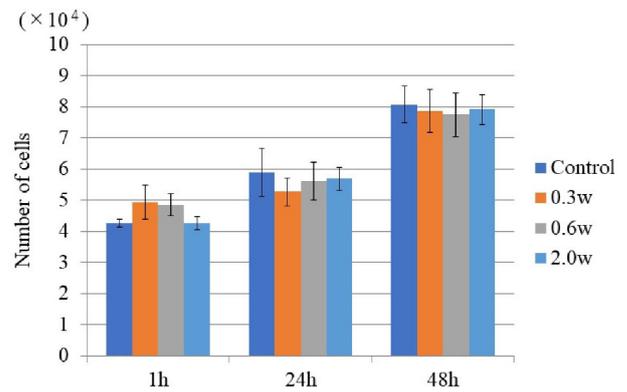


Figure 3. Time Course of the Number of Viable Cells After Er: YAG Laser Irradiation. No significant differences were found between the 0.3 W, 0.6 W, and 2.0 W groups, and proliferation was similar to that in the control group. Control: Control group not irradiated with the Er: YAG laser. 0.3 W, 0.6 W, and 2.0 W: Experimental groups irradiated with the Er: YAG laser at each output. * $P < 0.05$ vs. control, $n = 3$.

was significantly enhanced in the 0.6 W and 2.0 W laser irradiation groups compared with the control group. However, no significant difference from the control group was seen in the 0.3 W irradiation group. Moreover, the results of the western blot analysis revealed that compared with the control group, BSP protein expression was enhanced in the 0.6 W and 2.0 W laser irradiation groups (Figure 5).

Osteoprotegerin

The results of RT-qPCR analysis revealed that compared with the control group, OPG gene expression was significantly enhanced in the 2.0 W laser irradiation group. However, compared with the control group, no significant differences were found in the other laser irradiation groups. In addition, the results of the western blot analysis revealed that compared with the control group, OPG protein expression was enhanced in the 2.0 W laser irradiation group (Figure 6).

Discussion

In recent years, various studies have reported that lasers and laser irradiation affect the bones. It has been found to have positive effects on bone promotion, which requires extensive validation. This study led to a detailed in vitro elucidation of the effects of Er: YAG laser irradiation on bone metabolism during experimental tooth movement as performed in our previous study.¹⁷ In addition, the Er: YAG laser is frequently used in clinical practice for peri-implantitis, which has become a problem in recent years, and it will help to establish evidence for these treatments. In clinical practice, debridement is necessary to treat inflammatory conditions such as peri-implantitis. When performing this procedure using a laser, we must change the settings and type of laser irradiation at each step. First, to remove severely infected or necrotic tissue, we should set the laser setting at a relatively high power to cut the

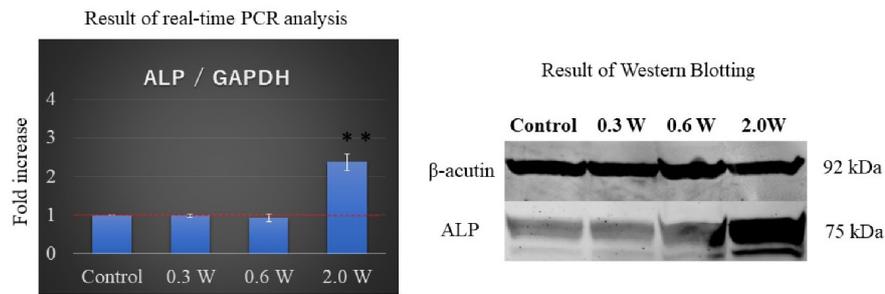


Figure 4. Results of RT-qPCR and Western Blot Analysis of ALP Expression at 24 Hours. after Er: YAG laser irradiation. According to the results of RT-qPCR, ALP gene expression was significantly enhanced in the 2.0 W laser irradiation group compared with the control group. The results of western blot analysis also showed that ALP protein expression increased in the 2.0 W laser irradiation group compared with the control group. $**P < 0.01$ vs. control, $n = 3$

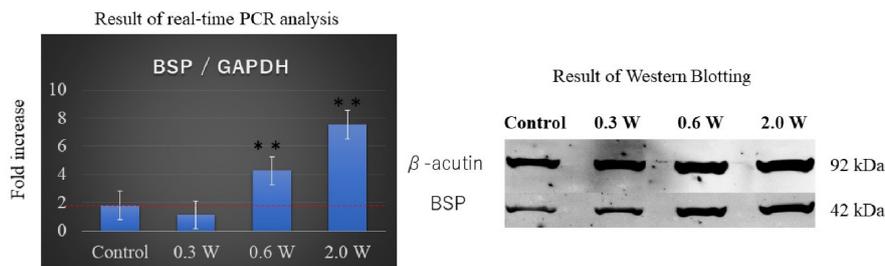


Figure 5. Results of RT-qPCR and Western Blot Analysis of BSP Expression at 24 Hours. after Er: YAG laser irradiation. According to the results of RT-qPCR, BSP gene expression was significantly enhanced in the 0.6 W and 2.0 W laser irradiation groups compared with the control group. The results of western blot analysis also showed that BSP protein expression was enhanced in the 0.6 W and 2.0 W laser irradiation groups compared with the control group. $**P < 0.01$ vs. control, $n = 3$

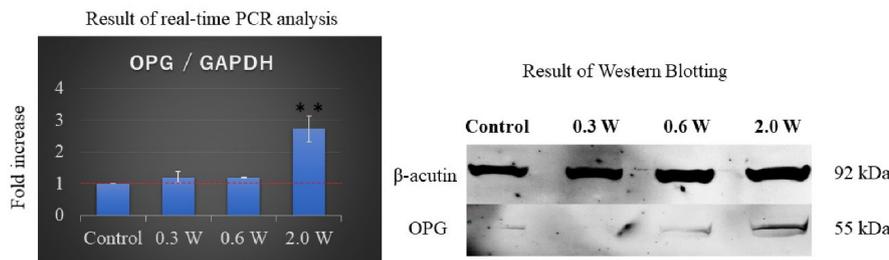


Figure 6. Results of RT-qPCR and Western Blot Analysis of OPG Expression at 24 Hours. after Er: YAG laser irradiation. According to the results of RT-qPCR, OPG gene expression was significantly enhanced in the 2.0 W laser irradiation group compared with the control group. The results of western blot analysis also showed that OPG protein expression was enhanced in the 2.0 W laser irradiation group compared with the control group. $**P < 0.01$ vs. control, $n = 3$

tissue. Second, the laser should be set at relatively low power for tissue hemostasis and wound healing. Ideally, it is desirable to have one laser irradiation setting; however, at least two steps are considered necessary. Breaking up the treatment stages into smaller steps, changing the laser irradiation settings, and creating a better treatment protocol are also considered good treatments. However, it can be clinically complicated. This study envisions and validates a relatively low-power stage aimed at tissue hemostasis and wound healing after the removal of infected or necrotic tissue.

In the present study, the number of cells was significantly reduced in the 2.8 W Er: YAG laser irradiation group compared with the control group in the preliminary experiments. This may have been because the excessive stimulation of the human osteoblast-like cells caused cell death. A similar report found that the

irradiation of MC3T3-E1 cells, which are also osteoblast-like cells, with an Er: YAG laser and with high fluence (6.7 and 8.6 J/cm²) significantly increased cytotoxicity.²⁰ Weak stimulation has been reported to promote biological and physiological responses, whereas strong stimulation has been shown to suppress them; therefore, it is considered to be harmful.^{21,22} In the present study, the same cell proliferation as that seen in the control group was observed when the output was set to 2.0 W or less. Thus, Er: YAG laser irradiation of human osteoblast-like cells at 0.3 W, 0.6 W, and 2.0 W did not affect cell proliferation compared with the control group. Er: YAG laser irradiation of rat calvaria-derived primary osteoblast-like cells has been reported not to affect cell proliferation, but to enhance calcification significantly.²³

A previous study found that ALP activity increased after osteoblasts were irradiated with an Er,Cr:YSGG

laser.²⁴ Similarly, in this experiment, ALP gene and protein expression significantly increased in the 2.0 W group. Similar to ALP, BSP is a bone-related substance. In the present study, we found that gene and protein expression significantly increased in the 0.6 W and 2.0 W groups. BSP gene expression has also been reported to be enhanced in rat osteoblast-like cells after irradiation with the same surface-absorbing CO₂ laser.²⁵ Irradiation of osteoblasts with various lasers between 600 and 1000 nm has been reported to promote osteoblast proliferation.²⁶ In the present experiment, the number of living cells did not change before and after Er: YAG laser irradiation of human osteoblasts. This is because the wavelength was different from that used in the present experiment. In addition, the laser used in this experiment is a surface absorption type laser that uses pulse oscillation, and the difference in output may have also had an effect. Irradiation of primary rat calvaria cells using a low-power Ga-Al-As laser at 1.1 J/cm² has been shown to increase OPG gene expression significantly.²⁷ Nd: YAG laser irradiation of human osteoblast-like cells was also found to increase OPG gene expression in another previous study.²⁸ In the present study, similar results were obtained using an Er: YAG laser. Although the wavelength and output of the laser are different, the pulse wave oscillation may activate the cells.

Er: YAG laser irradiation provides high reactive-level laser treatment (HLLT) by creating a range of heat in the target tissue, thereby altering or destroying its structure. Nondestructive thermal and nonthermal bioactivation occur simultaneously at the periphery of the target tissue, and this is referred to as “simultaneous low reactive-level laser treatment (LLLT).” LLLT occurs along with HLLT, and this explains some of the advantages of laser irradiation.²⁹ In other words, HLLT and LLLT occur on the tissue surface and in the surrounding tissue, respectively. Moreover, HLLT generates a photobiodestructive reaction that has been shown to induce cellular vaporization, whereas LLLT generates a photobioactive reaction that has been shown to stimulate cellular proliferation and differentiation.³⁰ Treatment of peri-implantitis with an Er: YAG laser can result in the removal of the infected layer and the occurrence of HLLT and LLLT on its surface. It has also been reported that proliferation and differentiation increased after osteoblast-like cells were cultured on titanium discs and irradiated with an Er,Cr:YSGG laser.²⁴ In addition, there is a report that Er: YAG lasers can remove plaque on titanium more efficiently than ultrasonic scalers.³¹ On the other hand, it has also been reported that culturing osteoblast-like cells on a titanium disk and irradiating them with an Er: YAG laser can adversely affect their survival rate.³² However, this is considered to depend on the irradiation conditions, especially the output.

The Er: YAG laser used in this study was a surface

absorption type. The same surface-absorbing CO₂ laser has been reported to reduce pain substances in a rat tooth movement model.³³ There are many reports that irradiating a wound with a CO₂ laser promotes healing.³⁴ It has also been reported that compared with CO₂ lasers, Er: YAG lasers are advantageous for early healing conditions in experimentally wounded models.³⁵ It would be wonderful if we could find the conditions for efficient generation of HLLT that occurs on the tissue surface and LLLT that may occur around the tissue in surface absorption lasers. However, this can be very difficult. As mentioned in the introduction, lasers have different effects and actions, depending on their types and characteristics. This is also related to the shape of the handpiece, connector, tip, output, and the heat generated during irradiation. In clinical practice, various factors, such as irradiation time, time between irradiation, distance and angle between the tip and irradiation surface, and the amount of water injected, are involved in a complex manner. Although it is difficult to completely reproduce these findings in vitro, it is necessary to conduct various studies using cells to elucidate the underlying mechanisms. Furthermore, by verifying in animal experiments, it is possible to set up a system that is quite close to the clinical setting. As mentioned above, in order to imitate the conditions of clinical laser irradiation and more complicated clinical techniques in cell experiments and animal experiments, there are a wide variety of parameters, and verification is extremely difficult. In the future, we will have to verify the extent to which errors and differences in the results due to this procedure will affect the results. In cell research, it is necessary to minimize parameters other than those to be verified.

The results of this experiment indicated that gene and protein expression increased in the bone formation and resorption systems, which may provide evidence that Er: YAG laser irradiation promotes bone healing when applied to peri-implantitis. These findings may also help explain why Er: YAG laser irradiation accelerated tooth movement in a previous study. Overall, these findings suggest that Er: YAG laser irradiation may promote bone healing in various treatment methods. In addition, there are many reports that lasers other than Er: YAG also promote osteogenesis. It is necessary to separate and verify whether the reaction is caused by the vibration of the pulse wave, by the heat generated as a side effect of the laser, or by photochemical action. We believe that further research is important in the future.

Conclusion

Er: YAG laser irradiation of cultured human osteoblasts at 2.0 W increased bone metabolism-related factors, which suggests that bone metabolism factors are activated by irradiating human osteoblast-like cells with an Er: YAG laser.

Competing Interests

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

Data Availability Statement

The data that support the findings of this study are available from the author upon reasonable request.

Ethical Approval

Not applicable.

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