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A comparative study on the effect of YAG: Er and 089 nm diode laser irradiation on the attachment of osteoblast cells on the surface of dental implant fixtures

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Abstract---Background and Aim: Nowadays, lasers are the focus of attention as a new treatment in dentistry. One of the applications of lasers is their use in implant dentistry. The present study aimed to compare the effect of YAG: Er and 089 nm Diode laser irradiation on the attachment of osteoblast cells on the surface of dental implant fixtures. Materials and Methods: In the present study, 36 titanium implants were divided into three groups of 12. Twelve implants were irradiated with Er: YAG laser and 12 implants were irradiated with a diode laser, and 12 implants were not irradiated as a control group. Osteoblast cells were cultured on the samples, and the attachment and viability rate of these cells were examined by SEM electron microscopy and MTT test. Results: There was no significant difference between laser irradiated implants groups and the control group in terms of cell viability rate based on the MTT test. Regarding the attachment of osteoblast cells to the implant surface, Diode laser irradiation had no significant effect, but the Er: YAG laser had a negative effect. Conclusion: This study examined the effect of Er: YAG and Diode 980nm lasers on the implant surface and showed that laser application does not impair cell viability, although it does not have a significant positive effect on osteoblast cell viability. The ER: YAG laser also reduces attachment of the cells on the implant surface.

Keywords---*Implant, Laser, Osteoblast, Scanning electron microscope.*

Introduction

Peri-implantitis is an inflammatory reaction around the osseointegrated implant that causes loss of bone support, and if not treated properly, it will result in implant loss (1, 2). Various treatments have been presented for peri-implantitis, and detoxification is the common characteristic of all of them, which can be done in various ways. One of these methods is the use of lasers. Various studies support the success of this method in detoxifying the surface of implants, although more studies are still needed in this area. It should be noted that the surface characteristics of the implant play a major role in osseointegration and the long-term viability of the implant (3). Romanos et al. (2000) examined the effect of diode laser and Nd: YAG on titanium surfaces. Their study revealed that the diode laser, unlike the Nd: YAG laser, does not damage the titanium surface, so it can be used to treat peri-implantitis. In this study, Nd: YAG laser was used in 6, 4, and 2-watt power and diode laser were used in 15, 10, and 5-watt power. Nd: YAG laser damaged the surface even at the lowest power, but no damage was seen at different powers of the diode laser (4). Schwarz et al. (2003) examined the effect of Er: YAG laser on the surface of titanium implants. In addition to conventional treatment, they used laser and examined the effect of laser on implant surface properties and the effectiveness of this treatment in removing subgingival masses. Six implants were treated with Er:YAG laser (100 mJ / pulse and 10 Hz) and two implants were used as control. Compared to the control group, the non-laser laser method effectively removed subgingival masses without thermal damage (5).

Monzavi et al. (2003) examined the temperature changes of the implant surface during the use of Er: YAG laser. They used lasers with three different cooling systems, including air spray, air spray alone and without. Three implants were irradiated with these methods. In all three groups, the maximum temperature increase was less than 10 °C (an increase in temperature of more than 10 °C can cause irreversible damage), but temperature changes with different cooling systems showed a significant difference ($P < 0.001$). It was concluded that a laser could be a safe method for the treatment of peri-implantitis (6). Schwartz et al. (2006) examined non-surgical methods to treat advanced and moderate pre-implantation diseases. They concluded that although laser treatment of peri-implantitis lesions showed a greater reduction in gingival bleeding, these results were limited to 6 months (7).

Giannini et al. (2006) examined the effect of Nd: YAG laser on killing bacteria while maintaining the surface characteristics of the implant. They concluded that using the laser with the right parameters can kill aerobic and anaerobic bacteria while not damaging the titanium surface (8). George Romanos et al. (2006) examined the attachment of osteoblasts to titanium disks after laser irradiation. This study showed that osteoblasts might grow on the surface of titanium after CO₂ or Er, Cr: YSGG laser irradiation and laser irradiation improve osteoblast attachment and bone formation (9). Romanos et al. (2009) examined the life of implants after disinfection of the implant surface with a CO₂ laser and performed graft surgery in the damaged area. Finally, they evaluated the result as promising

and reported an improvement in long-term results of using CO2 laser in the treatment of peri-implantitis (10)

Stübinger et al. (2010) stated that although laser has become very popular in treating peri-implantitis, its exact effect on the implant surface is not yet known. They concluded that CO2 and Diode lasers do not alter the implant surface and are safe, but Er: YAG laser is only safe if used at a power less than 300 or 500 mJ / 10 Hz (11). In a study by Cei S et al. (2011), the response of primary osteoblast cells to the rate of laser-induced roughness on different titanium surfaces was examined. The results revealed the attachment of osteoblasts to all four surfaces, but in laser micro-machined surfaces with more porosity (20 µm), more osteoblast response was observed than on other surfaces and sandblasted surfaces (12). Galli et al. (2011) examined osteoblast response on three commercial titanium surfaces: machined, sandblasted, acid-etched, and plasma spraying before and after ER-YAG laser irradiation. They examined osteoblast-specific proteins, including osteocalcin and osteoprotegerin, by immunoenzymatic tests. Cell proliferation on all irradiated titanium surfaces at both 150 and 200 mJ / pulse was slower than in the control group (13). The present study aimed to compare the effects of Er: YAG laser irradiation on the titanium surface of the implant compared to the Diode laser and both of these lasers compared to the lack of laser irradiation.

Materials and Methods

The present study was an experimental and laboratory type of study. Considering the significance level of 5% and the test power of 80% and according to the results of previous studies (14) and the standard deviation $S = 0.01$ and to achieve a difference of 10% in the mean attachment in each group, 12 samples are required.

$$n = \frac{(z_{\frac{\alpha}{2}} + z_{\beta})^2 \times 2S^2}{(X_1 - X_2)^2}$$

Thirty-six titanium implant fixtures with size 10 * 4 (Dio, Busan, Republic of Korea) were prepared from Afrand Atlas Company (representative of DIO implants) with SLA surface. The samples were selected in the same size and with a cover screw and were divided into two groups of case and control. In the samples of the first group, Diode 980nm laser product of BIOLASE company (USA) with 400-micron fiber and in radiant specifications including continuous irradiation mode (according to the setting recommended by the manufacturer for using diode laser around the implant) with 2.5W power with a distance of 4 mm from the surface of the implant was used in spiral movements in the direction of the threads from the coronal to the apical area for an average of 60 seconds for each implant. To maintain a 4-mm distance, a repeatable mounted mold was used to place the laser handpiece and the implant motor driver that moves the fixture in front of the laser. Er: YAG laser irradiation by Kavo Dental GmbH, Germany KaVo KEY LASER® 3+ with a distance of 1 mm and a 90-degree angle with a constant speed of movement for one minute and a diameter of 1 mm with pulsative irradiation mode (according to the setting of the manufacturer's recommendation for the

application of laser around the implant) with a power of 1.5 W and a frequency of 30 HZ under saline cooling was used on the surface of the implants. To irradiate the lasers uniformly on the surface of the implant, a mounted assembly was used.

Cell preparation and culture

The saos-2 human bone cell line was cultured in a 75 ml cell culture flask in Dulbecco's Modified Eagles Medium (DMEM) containing 10% FBS Fetal Bovine Serum and 1% antibiotic (penicillin-streptomycin- amphotericin B). It was kept in an incubator with 5% CO₂ and 95% humidity at 37 ° C.

Culture of saos-2 cells on implants

Sterile implants were transferred to a 24-cell plate with the help of sterile forceps. The cells prepared several days ago in their best growth phase (logarithmic – health phase and complete proliferation) were removed with the help of the trypsin enzyme from their original flask. After counting with a hemocytometer slide and trypan blue dye, 40000 cells in one ml of complete cell culture medium were added to a 24-cell plate of cell culture on each implant. Then, the plate was incubated in a cell culture incubator (temperature 37 ° C and humidity 95%) for 3 days.

Evaluation of viability and attachment of cells by quantitative MTT test

To evaluate the effect of Diode 980nm and Er: YAG laser irradiation on the viability rate of saos-2 cells on implants quantitatively, the MTT test (3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyl Tetrazoliumbromide) was used. In this method, the cell viability rate is evaluated by quantifying a metabolic product and confirms the mitochondrial activity of cells, which is directly related to cell growth and cell viability. In this experiment, MTT, a yellow tetrazolium salt, is reduced by the enzyme dehydrogenase in the active mitochondria of living cells and transformed into a purple metabolite of formazan, crystalline and insoluble. Then, the formed crystals are dissolved in their appropriate solvent (usually DMSO), and the intensity of light absorption of the resulting solution is measured by a spectrophotometer at a wavelength of 500-600 nm. The reduction in the number of viable cells in the test sample leads to a reduction in its total metabolic activity. This reduction is directly related to the reduction in the formation of purple crystals and indicates the level of mitochondrial activity and thus the amount of viable or dead cells. In this study, to evaluate the viability and attachment of saos-2 cells implanted on control implants and laser-irradiated implants, 3 days after implantation, the plate was removed from the incubator, and each cell medium was completely evacuated. Then, 1 ml of medium containing 10% yellow MTT was added to each cell. The plate was returned to the incubator and incubated for 3 hours at 37 ° C. Then, each cell medium was completely evacuated and replaced with 1 ml of DMSO solvent (Dimethyl sulfoxide) to dissolve the purple crystals and obtain a purple solution. Then, 100 µl of the solution was transferred from each cell (3 repetitions) to each 96-cell plate specific for ELISA reader (Biochrom Anthos 2020, UK). The plate was placed in the ELISA reader, and the optical absorption of dyes (OD: Optical Density) was read at 570 (specific for MTT dye) and 630 nm (reference wavelength).

Preparation of samples for observation under SEM

SEM imaging was used to examine the morphology of cells attaching to the surfaces of implants. First, based on the protocol mentioned in the previous step, the implants were sterilized, and each was placed on a 24-cell culture plate. Then, 40000 cells were added to 1 cc of complete culture medium on each implant. The desired plate was incubated in a cell culture incubator for 3 days. Then, to prepare the cells attaching to the implants for electron imaging, the implants were fixed after rinsing with PBS buffer with 2.5% glutaraldehyde solution for 24 hours at refrigerator temperature. After rinsing with distilled water (3 times, each time for 10 minutes), 1% Osmium solution was added to each implant, and the samples were incubated for 2 hours in the dark at room temperature.

Then, it was rinsed with distilled water (3 times, each time for 10 minutes). Different percentages of alcohol (ethanol) were used to dry the samples. Accordingly, samples were exposed to alcohol 30% (5 minutes), 50% (5 minutes), 70% (5 minutes), 90% (5 minutes) and finally 100% (2 times, each time for 30 minutes). Finally, the samples were placed under a biological hood (Faraz Hood, Iran) for 48 hours to complete the drying process. Then, the prepared samples were sent for imaging. The implant samples were attached to the base and placed in a device with a gold cover to be prepared for observing under an electron microscope. Then, the SEM device (Phenom world PRO X, The Netherlands) was connected to a vacuum pump, and the samples were placed in the device, and a digital image with 2500 x and 5000 x magnification was taken from the three apical, medial and coronal regions.

Morphological view of osteoblasts attaching to the surface of control and case implants in three regions (apical, medial and coronal) by direct observation of the size and general view of mature cells (polyhedral shape and presence of cytoplasmic spike-like growths and the spreading of cells on the surface were evaluated under an electron microscope and compared with each other. Since the data obtained from electron microscope images were qualitative and there was no possibility of quantitative comparison, using MIP® image analysis software. In collaboration with Image Analysis Clinic, images taken at 5000x magnification were examined to detect cell filopodia processes. The ethics committee examined the project proposal and approved it with the IR code.SSU.REC.1396.126. To analyze the changes of cultured cells at the implant surface, SPSS software and ANOVA tests were used.

Results

Calculations of optical absorption (OD) numbers obtained from the purple solution of each plate cell and statistical analysis of the results were performed using ANOVA (comparison of the mean numbers of the test group with the mean numbers of the control group). According to the standard, the p-value below 0.05 was considered a criterion for a significant difference between the two groups.

Table 1
Mean difference of cell viability rate (MTT) in the studied groups

Group	n	SE $\pm$ Mean difference	P-Value (ANOVA)
Control	12	0.25 $\pm$ 1.03	P= 0.819
Diode laser irradiation	12	0.14 $\pm$ 1.04	
Er: YAG laser irradiation	12	0.15 $\pm$ 1.08	

Mean cell viability rate (MTT) in the study group

Mean cell viability rate (MTT) in the study groups

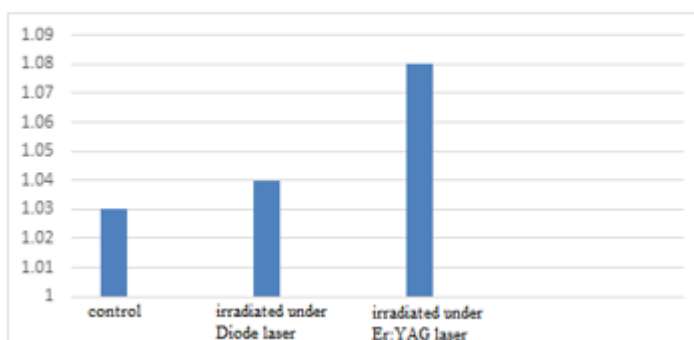


Chart 1: Mean cell viability rate (MTT) in the study groups

By calculating the percentage of cell viability (optical absorption in the test group divided by optical absorption in the control group multiplied by 100), the percentage of viability (% viability) of cells in the control group was calculated at 103%, which is different from the percentage of cell viability in test groups including Diode laser (104%) and Er: YAG laser (108%), which is not statistically significant ($p = 0.819$)

Table 2
P-value of comparison of cell attachment of irradiated laser groups with the control group and with each other

group	Compared group	P value
Control	Diode laser irradiation	p=0.055
	Er: YAG laser irradiation	p=0.026
Diode laser irradiation	Er: YAG laser irradiation	p=0.937

Diode laser-irradiated group: It was found that the topography of osteoblast cells in the Diode laser-irradiated group was not significant compared to the control group in terms of spreading and the ratio of filopodia area to total cell area (0.56 ± 0.78) compared to the control group (0.67 ± 0.23) ($p=0.055$). Er: YAG laser irradiated group: It was found that the topography of osteoblast cells in the Er: YAG laser irradiated group was significant compared to the control group in terms of spreading and the ratio of filopodia area to total cell area (0.55 ± 0.78) compared to the control group (0.67 ± 0.23) ($p=0.026$). Comparison of Er: YAG laser irradiation with diode laser: There was no significant difference between the effects of two laser irradiations on the morphology of osteoblast cells ($p=0.937$).

Discussion

The results showed that the irradiation of each of the studied lasers did not cause a significant improvement in the viability rate of osteoblast cells, but in terms of the rate of cell attachment, Er: YAG laser significantly reduces the rate of cell attachment compared to lack of laser irradiation. In a study by Ayobian et al. (2014), titanium disks sterilized by the Er-YAG laser were irradiated with a wavelength of $2.94 \mu\text{m}$ and powers of 100 mj / pulse and 60 mj / pulse. After incubation of osteoblast-like cells and imaging by SEM and comparison with the three experimental and control groups, they reported that the 100 mj / pulse power is probably more efficient in removing microbial plaque. The 100-mj / pulse Er-YAG laser irradiation is recommended for peri-implantitis treatment. However, 60 mj / pulse irradiation had no adverse effect on the biocompatibility of the implant. (14)

In the present study, investigating the cell morphology in the Er: YAG irradiated laser group showed negative results. These results are inconsistent with the study conducted by Ayobian et al. The use of image analysis software to convert qualitative data into quantitative is the advantage of the present study, and a more accurate judgment of cell attachment has been obtained compared to the Ayobian study. In the study conducted by Schwarz et al., results showed a decrease in cell viability rate at the surface of the disks of the Er: YAG laser irradiated group compared to the control group. These results are not consistent with the results of the present study in terms of cell viability, which may be due to differences in the type of viability test used to assess ATP-based luminescent cell viability and the surfaces of the studied disks were infected with microbial deposits (52). In another study by Schwarz et al. (68), the negative results of using Er: YAG laser in disinfecting the implant surface in patients with peri-implantitis were found.

In this study, the case group was treated with Er: YAG laser and the control group was treated with a plastic curette and sterile saline. The 4-year follow-up showed that the reduction in BOP and plaque index was greater among control implants than in the sham group. The present study was laboratory research, and clinical results were not examined. In the Er: YAG laser application, the cooling effect of saline on the implant surface is considered equal to the initial conditions for cleaning the implant surface, which eliminates the possibility of its effect as a confounding factor. The results of the present study can be generalized to clinical

settings due to the controllable conditions of laser application in the clinic, including the lack of increase in temperature beyond bone tolerance. In the study conducted by Galli, osteoblast response on three commercial titanium surfaces, including machined, sandblasted, acid-etched, and plasma sprayed groups, was evaluated before and after ER-YAG laser irradiation. The cell viability rate in the surfaces irradiated with laser was lower than in the control group. These results are not consistent with the results of the present study in terms of cell viability. However, it should be noted that the study of laser irradiation was performed on samples at energies less than 150 and 200 mJ, and they used osteoblast-specific proteins, including osteocalcin and osteoprotegerin, by immunoenzymatic tests to evaluate cell viability (13).

In a study by Hao (2005), a high-power diode laser (1.5KW) was used on the surfaces of SLA implants. The results of this study showed that the irradiation of this laser caused a rougher surface and increased the surface oxygen, decreased the contact angle with the surface, and increased the cell surface spreading (15). Cell proliferation and attachment increase with the wettability of the alloy. Therefore, the laser can be an effective way to improve cell attachment (15). Since similar studies have not been conducted on the effect of laser irradiation, including Diode laser with irradiation characteristics used in the clinic on increasing surface oxygen, surface energy changes, and wettability properties, their exact mechanism remains unclear, and further studies are needed. However, based on the results of the present study, the similarity of cell attachment in the control and case groups, it can be said that the 980nm Diode laser with a power of 2.5W did not change the surface energy or wettability of the implant surface.

In a review study conducted by Salah Kamel on the disinfecting effect of different types of lasers on the implant surface, it was observed that Diode lasers have a variable range of disinfection in reducing the number of bacterial colonies (CFU), which is dose-dependent and increases from 45% with a power 0.5 W to 99.9% with a power of 2.5W. Also, this study stated that when the mixture of bacterial biofilm of the peri-implantitis agent is irradiated, they show different levels of resistance (16). In our study, the effect of laser in disinfecting the implant surface was not investigated, and this requires the use of surfaces of the implant that are microbially infected or are placed under controlled bacterial culture. In the study by Petri AD et al., osteoblastic cells derived from human alveolar bone were cultured on titanium disks. It was found that the gene expression of ALP, BSP, and BMP-7 was higher in the culture medium where the laser was irradiated with low power, while it was lower in RUN-x2, OPN and OPG. This study showed that despite the transient destructive effect of the laser, the low-power laser could have advantages in line with implant osseointegration (17). In our study, no gene expression factors were used, but the efficiency of the Diode laser in enhancing osseointegration does not contradict the results of this study. However, it does not improve cell attachment.

In the study by Cei S, the response of early osteoblast cells to the level of laser-induced roughness on different surfaces of titanium was investigated. According to this study, the attachment of osteoblasts to four different titanium surfaces, including a sandblasted surface and surfaces with laser-induced micron porosity,

was compared. It was concluded that laser micro-machined surfaces with more porosity (20 μm) give a higher osteoblast response than other sandblasted surfaces (12). A significant point in this study is paying attention to micro-design created by laser irradiation. Stübinger et al. (2010) stated that although lasers are now very popular in the treatment of peri-implantitis, the exact effect on the implant surface is not yet known. In their study, they tried to compare the effect of Er: YAG, CO₂ and Diode lasers on the polished, sandblasted and acid etched surface of titanium implants. Finally, they concluded that CO₂ and Diode lasers do not alter the implant surface and are safe, but the Er: YAG laser is only safe if it has no power greater than 300 or 500 mJ / 10 Hz (11). The advantage of this study was the use of different commercial surfaces of implants. In our study, only the SLA surface was evaluated. There is no concern about the safety of using a diode laser because less power is used in the present study. The positive results of this study were not obtained for the lasers used in the present study, which its reason can be attributed to differences in the type of laser. However, the mechanism of Er, Cr: YSGG laser is comparable to Er: YAG laser in terms of effect on the surface.

A study by Jao et al. (2022) showed that high-power laser surface treatment is a promising method with a positive effect on protein uptake and osseointegration (18). Another study by Romanos examined the effect of diode lasers and Nd: YAG on titanium surfaces. The effect of both lasers on three different titanium surfaces (sandblasted, plasma-sprayed titanium and hydroxyapatite-coated titanium) was compared by scanning electron microscopy. The results of their study revealed that the diode laser, unlike the Nd: YAG laser, does not damage the titanium surface, so it can be used as a method to treat peri-implantitis and reveal the buried implant. In this study, the Nd: YAG laser at powers of 6, 4, and 2 w was used, and a diode laser was used at 15, 10, and 5 w. In Nd: YAG laser, the titanium surface was damaged even at the lowest power, but no damage was seen in the different powers of the diode laser (4). In this study, more attention was paid to the mechanical properties of the titanium surface due to laser irradiation, and cell attachment and viability, like in our study, were not presented and had less biological generalization potential. However, the use of different powers of diode lasers requires more extensive studies.

Conclusion

The present study investigated the effect of different lasers on implant surfaces and showed that the Diode laser has no effect on the viability and attachment of osteoblasts on the implant surface, and the mechanism of action of this laser in the clinic might be its antiseptic properties. Diode laser with the irradiation parameters used in this study is safe and does not cause destructive changes against biological conditions. In contrast, using Er: YAG laser can reduce cell attachment, and due to its slight positive effect on the viability of osteoblast cells, it is not recommended to improve reosseointegration.

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