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# Evaluation of attachment of osteoblast-like cells based on morphology at the fixture surface of dental implants

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**Abstract**--Background and Aim: Bacteria can attach to the implant surfaces, and the rough pattern can make it difficult to clean the microorganisms and result in an inflammatory process and loss of the implant. Accordingly, the present study aimed to investigate the attachment of osteoblast-like cells based on morphology at the fixture surface of dental implants. Materials & Methods: MTT test (3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyl Tetrazoliumbromide) was used for quantitative evaluation of the effect of 980nm Diode laser irradiation and Er: YAG on the viability rate of saos-2 cells on implants. In this method, the cell viability rate is evaluated by quantifying a metabolic product and confirms the mitochondrial activity of cells, which is directly associated with cell growth and, subsequently, its viability. Results: The results of this study show that based on the MTT test, the percentage of viability of cells for implants under Diode laser irradiation was higher than the control group, but this value was not significant. This value was lower than the Er: YAG laser, which was insignificant. Conclusion: According to SEM observations, the density of cell aggregates and surface spreading of the implants were similar in the three groups.

**Keywords**-- *Dental implants, Osteoblast-like cells, Cell attachment, Laser irradiation.*

**Introduction**

Peri-implantitis is defined as an inflammatory reaction around an osseointegrated implant leading to loss of bone support (1). Peri-implantitis leads to serious diseases after implant treatment, affecting both the soft tissue and surrounding

hard tissue. The prevalence of peri-implantitis has been reported to be above 56%, leading to implant loss (1). In various studies, several risk factors such as gingival index (more than 10%) and having more than two implants were associated with peri-implant disease. Other risk factors have also been identified, such as previous periodontal disease, poor plaque control, occlusal overload, rheumatoid arthritis, and excessive alcohol consumption (2).

Although dental implants have become a predictable long-term treatment for patients, not all implant treatments are necessarily successful, and pre-implant diseases are prevalent. Early diagnosis and elimination of peri-implantitis processes will increase long-term prognosis (3). Smokers and people with a history of periodontal disease are at higher risk for peri-implantitis (4). Bacteria can attach to implant surfaces and make the basis of the treatment of peri-implantitis, and rough patterns can make the cleaning of microorganisms that is the basis of the treatment of peri-implantitis unpredictable. Therefore, the availability of a treatment that relies on the removal of bacteria is highly important.

Geminiani et al. (2012) stated that an increased temperature of more than 10 ° C could affect bone life. The results revealed a rapid increase in temperature at 810 nm, while the 980 nm diode laser increased the temperature even faster. They concluded that irradiating this laser to the implant's surface after only 10 seconds can raise the temperature by more than 10 degrees (7). In a study by Petri AD et al. (2010), osteoblastic cells derived from human alveolar bone were cultured on titanium disks for 17 days. 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 gene expression of RUN-x2, OPN and OPG was lower. This study showed that despite the transient destructive effect of the laser, the low-power laser could have advantages in line with implant osseointegration (8).

Chris Leja et al. (2013) examined the effect of Er: YAG, 2 CO and Diode lasers on increasing the temperature of intraosseous implants in vitro. They concluded that the temperature could exceed the 10-degree increase in just 18 seconds, affecting bone life (9). In a study by Ayobian et al. (2014), SLA-sterilized titanium disks were irradiated by an Er-YAG laser with a wavelength of 2.94 µm and powers of 100 mj / pulse and 60 mj / pulse. They reported after 72 hours of incubation of osteoblast-like cells and imaging by SEM and comparison with three experimental and control groups. Since 100 mj / pulse power is probably more efficient in removing microbial plaque, Er-YAG laser 100 mj / pulse irradiation is recommended for treating peri-implantitis. However, 60 mj / pulse irradiation had no adverse effect on the biocompatibility of the implant (10).

Listl et al. (2015) attempted to evaluate the cost-effectiveness of various non-surgical methods in treating peri-implantitis. In their research, they examined previous studies on pocket depth after various treatments for peri-implantitis. They stated that to obtain valid treatment recommendations for the treatment of peri-implantitis, there is a need for more comprehensive and more pathological research on various non-surgical treatments, and previous studies in this field are insufficient [11]. Natto et al. (2015) conducted a study to evaluate the effect of lasers, including Neodymium-Doped Yttrium-Aluminum-Garnet (Nd: YAG) and

Carbon dioxide (CO<sub>2</sub>) and diode and Erbium / Chromium-Doped Yttrium-Scandium-Gallium-Garnet (Er, Cr: YSGG) and Erbium-Doped Yttrium-Aluminum-Garnet (Er: YAG) in the treatment of peri-implantitis and their use in surgical and non-surgical methods.

All human studies on laser treatment of peri-implantitis between 2002 and 2014 were collected. Among them, thirteen studies were selected for this review. No human study examined the effect of Nd: YAG laser on peri-implantitis. Also, the Co<sub>2</sub> laser was reported to increase bone regeneration and safety. The 980 nm diode laser effectively affected its bactericidal effect without changing the implant surface pattern. In one case, the Er, Cr: YSGG laser failed to regenerate bone around the implant. Er: YAG laser provided a strong bactericidal against the bacteria of periodontal diseases (12).

Ayobian-Markazi N et al. (2015) compared 21 sandblasted and acid-etched (SLA) titanium disks irradiated with ER-YAG laser with 13 disks without laser irradiation. Human osteoblast-like cells were cultured on disks. Accordingly, the surface roughness in the test group was significantly reduced, and the ability to absorb moisture was increased. They concluded that the laser has no negative effect on the biocompatibility of titanium (13). Shahin Kasraei et al. (2016) evaluated the success rate of re-implantation of failed implants after their surface treatment with CO<sub>2</sub> laser. They stated that despite the increasing use of implants, many cases of failure of this treatment are still reported. In this study, 10 failed implants (for various reasons other than erosion or rupture) underwent debridement by a CO<sub>2</sub> laser and re-implanted in the maxilla of dogs. In the control group, three failed implants were used without laser irradiation and re-implanted after placement in normal saline. They concluded that the success of re-implantation of implants in dogs increased after surface debridement by CO<sub>2</sub> laser (14). Given what was stated above, this study aimed to investigate the attachment of osteoblast-like cells based on morphology at the fixture surface of dental implants (15, 16).

## 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 (10) 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 divided into case and control groups. 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 a spiral movement 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 a 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. A mounted assembly was used to irradiate the lasers uniformly on the surface of the implant.

### **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% CO<sub>2</sub> 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 is transformed into the purple metabolite of formazan, which is crystalline and insoluble.

Then, the formed crystals are dissolved in their appropriate solvent (usually DMSO), and a spectrophotometer measures the intensity of light absorption of the resulting solution at a wavelength of 500-600 nm.

The reduction in the number of viable cells in the test sample reduces 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 the 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 for 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 observation under an electron microscope. Then, the SEM device (Phenom world PRO X, The Netherlands) was connected to a vacuum pump, the samples were placed in the device, and a digital image with 2500x and 5000x 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. With a collaboration of a specialized 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

Examining the cell morphology is important since it can indicate the tendency of cells to attach to surfaces, as cells with a flat morphology have a stronger attachment to titanium surfaces compared to cells with spherical morphology due to their cytoplasmic appendages and filopodia. Morphological view of osteoblasts attaching to the surface of control and case group implants in three areas (apical, middle 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 was evaluated under an electron microscope and compared with each other.

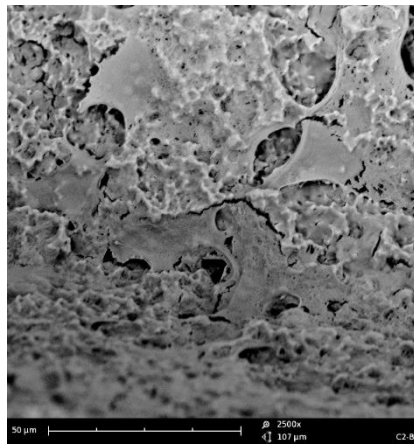


Figure 1: SEM view of osteoblasts attached to the implant surface of the control group (2500x magnification)

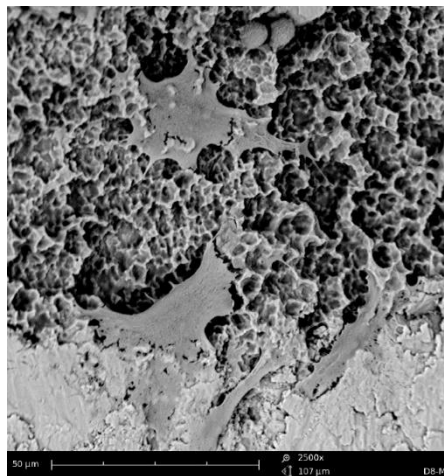


Figure 2: SEM view of osteoblasts attached to the implant surface in the Diode laser-irradiated group (2500x magnification)

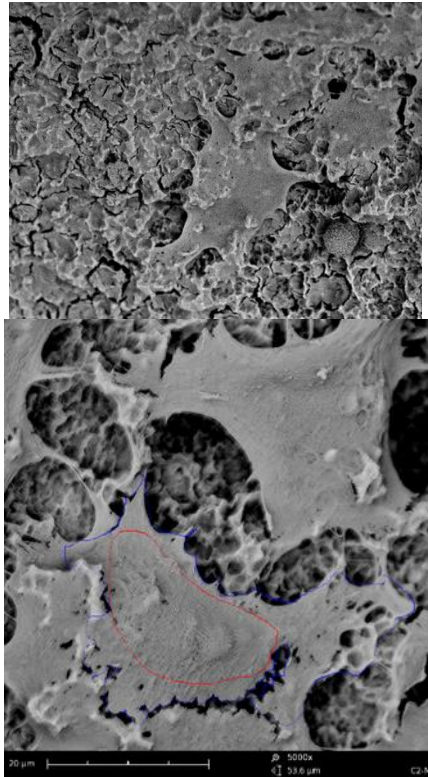


Figure 3: SEM view of osteoblasts attached to the implant surface of the Er: YAG laser-irradiated group (2500x magnification)

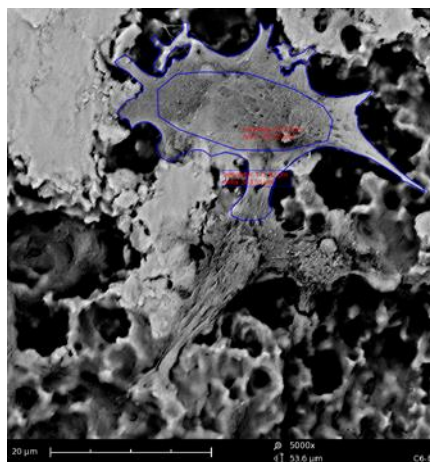


Figure 4: SEM view of osteoblasts attached to the implant surface of the control group (5000x magnification)

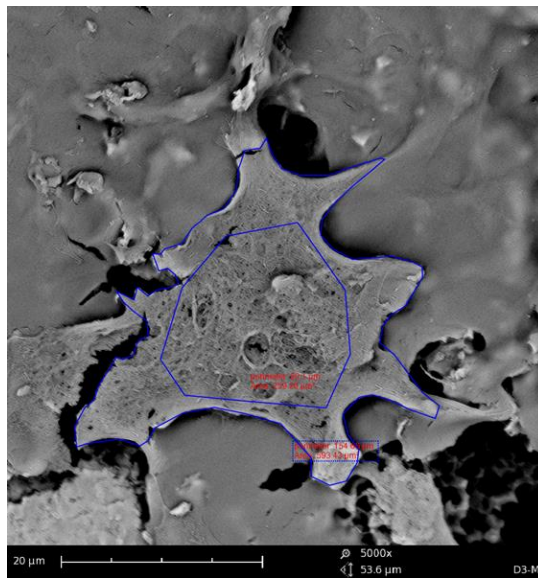
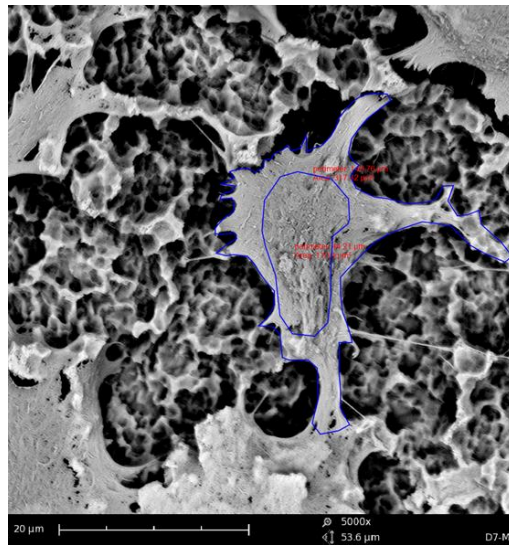


Figure 5: SEM view of osteoblasts attached to the implant surface of the Diode laser-irradiated group (5000x magnification)

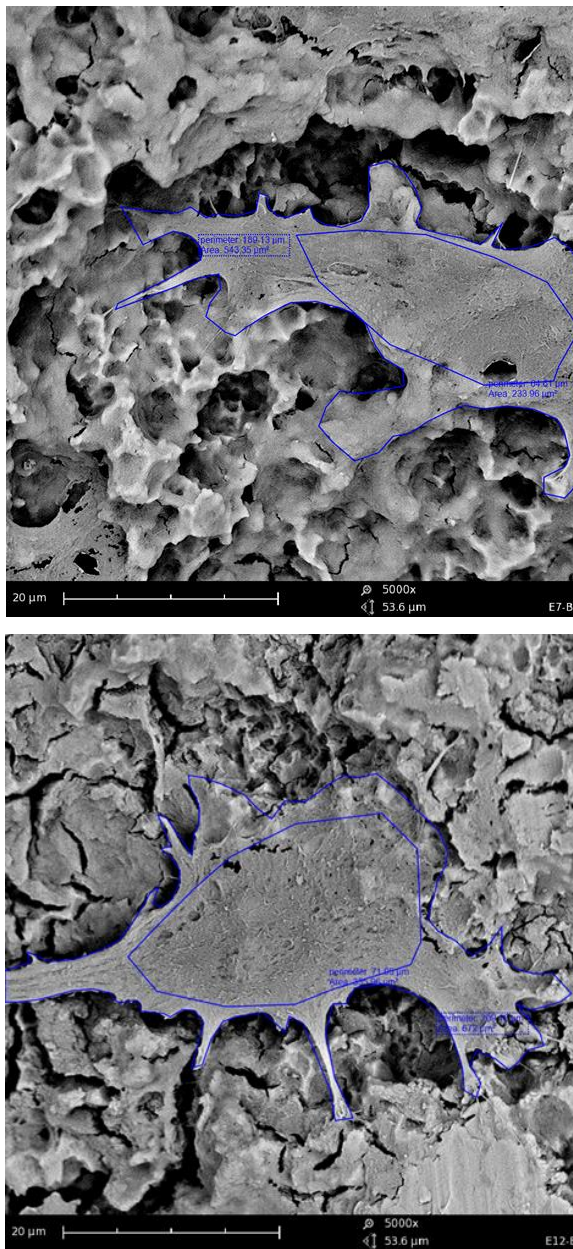


Figure 6: SEM view of osteoblasts attached to the implant surface of the ER: YAG laser-irradiated group (5000x magnification)

According to the SEM observations, the density of cell aggregates and their surface spreading were similar in the three groups, but since the data obtained from electron microscope images were qualitative and there was no possibility of quantitative comparison, using MIP® image analysis software. With a

collaboration of Asia Nahamin Pardazan's specialized image analysis clinic, the images were examined with 5000x magnification to detect cell filopodia processes. In this software, the filopodia area was measured and divided by the total area of the cell to obtain the rate of cell spreading and the attachment of cells.

Table 1: Evaluation of the mean ratio of filopodia to total cell area in images obtained from SEM in the study groups

Group	Mean (SD) ratio of filopodia to the total cell area
Control	0.23±0.67
Diode laser irradiation	0.78±0.56
Er: YAG laser irradiation	0.78±0.55

Comparison of the mean ratio of filopodia to total cell area in images obtained from SEM

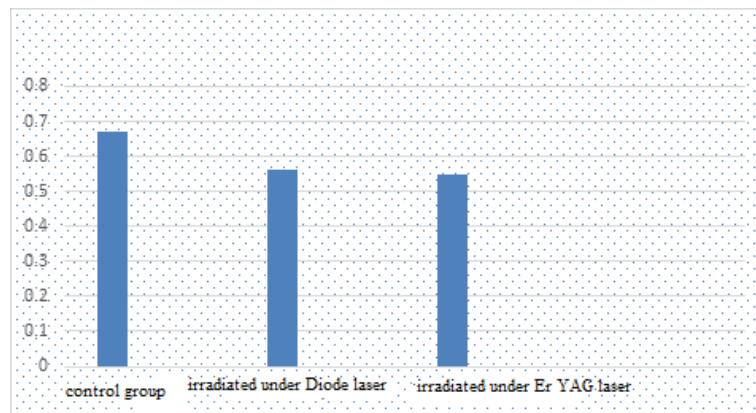


Figure 1: Comparison of the mean ratio of filopodia to total cell area in images obtained from SEM in the study groups (\*: p-value <0.05)

## Discussion

The results of this study show that based on the MTT test, the percentage of viability of cells for Diode laser irradiated implants was higher than the control group, but this value was not significant. This rate was lower than the Er: YAG laser, which is still insignificant. In morphological examinations of the cells attaching to the surface of diode laser irradiated implants, less attachment and spreading were observed than in the control group based on the results of SEM image analysis. This cell attachment rate was better than the Er: YAG laser-irradiated group. However, this difference is not statistically significant.

According to this study, the use of Diode laser does not significantly change the adaptability characteristics of the titanium SLA surface not only in terms of the

morphology of human saos-2 osteoblast-like cells cultured in the presence of titanium but also laser irradiation with Diode 980nm can increase proliferation and viability of osteoblast cells, although this increase was not significant. In the group irradiated with Er (YAG), the viability of the cells was higher than the control group. Also, this value was higher than the viability of the cells in the group irradiated with a Diode laser, but it was not statistically significant. Attachments to the surface of Er: YAG laser-irradiated implants also showed a lower rate of attachment and spreading based on the results of SEM image analysis, which was lower than the Diode laser-irradiated group, and it was statistically significant.

It may be because the Er: YAG laser ablation energy has the potential to damage the implant surface, leading to a lower tendency for osteoblast cells to attach to the irradiated surface. In the past, dentists believed that eukaryotic cells do not detect nanostructured changes in dental implant surfaces for ossification. However, recent studies have shown that ossifying cells respond to chemical and morphological changes at nano-scale on surfaces, including the arrangement of titanium dioxide nanotubes, functional peptide coatings, fluoride therapies, and calcium-phosphorus applications, and ultraviolet light performance. Some modifications at the nano-level have not yet been clinically evaluated. However, these modified dental implant surfaces at the nanoscale have shown excellent results in vitro and in vivo, and they promise potential clinical use in the future (17). The results of SEM images, EDX findings, and profilometry results in the study by Sakigan et al. (2021) showed that the Er: YAG long pulse groups and ultrasonic were the most effective for debridement of implant surfaces. Also, these two interventions have shown the closest surface topography of sandblasting, large pebble and acid-etched (SLA), as seen in virgin implants [18]. In a study conducted by Jao et al. 2022 (19), it was reported that applying a high-power laser on the surface of the implant, depending on its settings, creates topographic changes that can optimize the process of protein uptake and thus accelerate other biological processes.

## Conclusion

According to the SEM observations, the density of cell aggregates and surface spreading of the implants were similar in the three groups.

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