Aydın Dental Journal

Journal homepage: http://dergipark.ulakbim.gov.tr/adj

ER,CR:YSGG LASER AS A SURFACE DETOXIFICATION METHOD IN ENHANCEMENT OF OSSEOINTEGRATION

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ABSTRACT

Purpose: The aim of the current study was to establish protocols for Erbium Chromium-doped Yttrium-Scandium-Gallium-Garnet (Er,Cr:YSGG) laser application for detoxification of implant surface, preservation of surface biocompatibility and enhancement of osseointegration. In this purpose, four different variables including power (W), frequency (Hz), distance (mm) and duration (sn) were investigated at 3 different levels.

Material and Methods: Grade 5 titanium discs infected by S.aureus were detoxified with Er, Cr: YSGG laser according to various protocols. After laser application, surface morphology and surface roughness of titanium discs as well as cellular morphology and proliferation of osteoblasts-like cells at the end of 24 and 48 hours (SaOs-2 cell culture) were examined.

Results: The most remarkable changes on the surface of titanium discs were observed in group Test 8 (3 W-25 Hz-2 mm-45 sn) which was exposed to the highest power density (W/cm2).. In this protocol, melting and flattening on the surface was observed most prominently and surface roughness (Ra) was lowest. Proliferation indicators in groups Test 1 and Test 7 were found to be statistically significantly increased compared to the control group at the end of 48 hours. Furthermore, Ra values of these 2 groups (Test 1 and Test 7) were similar to that of control group.

Discussion: To conclude, our results have shown that power intensity, which is linked with distance, was the leading parameter for alteration of surface morphology. We suggest that cellular proliferation during reosseointegration is facilitated by conditions that maintain surface roughness in its initial form and amplify surface biocompatibility.

Keywords: *Periimplantitis, Detoxification, Er,Cr:YSGG, Osteoblast, Osseointegration, Titanium Disc.*

ÖZET

Amaç: Bu çalışmanın amacı; enfekte implant yüzeyini en uygun şekilde detoksifiye edecek ve aynı zamanda yüzey biyouyumluluğunu koruyarak, iyileşme sürecinde osteoblastların yeniden osseointegrasyonunu kolaylaştıracak Er,Cr:YSGG lazer uygulama protokollerini ortaya koymaktır. Bu amaçla lazer ile ilgili dört farklı değişken (güç-W, frekans-Hz, mesafe-mm ve süre-sn.) üç farklı düzeyde incelendi.

Materyal ve Metot: S.aureus ile enfecte edilen Grade 5 titanium diskler Erbium Chromium-doped Yttrium-Scandium-Gallium-Garnet (Er,Cr:YSGG) lazer ile farklı protokollerde detoksifiye edildi. Lazer uygulamasından sonra, titanyum disklerin yüzey morfolojileri, yüzey pürüzlülükleri, 24 saat ve 48 saat sonundaki osteoblast hücre proliferasyonları (SaOs-2 hücre kültürü) ve osteoblast hücre morfolojileri incelendi.

Bulgular: Çalışma sonucunda; titanyum disk yüzeyinde en fazla morfolojik değişikliğe neden olan protokolün güç yoğunluğunun (W/cm2) en fazla olduğu test 8 grubu (3 W- 25 Hz-2 mm-45 sn) olduğu görüldü. Bu protokolde yüzeydeki ergime ve düzleşmenin en fazla, yüzey pürüzlülük değerinin (Ra) ise en düşük olduğu belirlendi. Hücresel proliferasyon değerleri incelendiğinde, 48 saat sonundaki proliferasyon değerlerine göre test 1 ve test 7 gruplarındaki proliferasyon değerlerinin kontrol grubuna göre istatistiksel olarak anlamlı derecede arttığı gösterildi. Ayrıca, bu iki test grubunun (test 1 ve test 7) Ra değerleri incelendiğinde, kontrol grubuna oldukça benzer Ra değerlerine sahip oldukları belirlendi.

Sonuç: Sonuçta, yüzey morfolojisinin değişiminde en etkili parametrenin güç yoğunluğu olduğu bununda doğrudan uygulama mesafesi ile ilgili olduğu görüldü. Ayrıca yüzey pürüzlülüğünü neredeyse değiştirmeden, ilk haline yakın olacak şekilde koruyarak, yüzey biyouyumluluğunu arttıran uygulama koşullarının reosseointegrayon sürecinde hücresel proliferasyona olumlu katkı sağladığı görüldü.

Anahtar kelimeler: *Periimplantitis, Detoksification, Er,Cr:YSGG, Osteoblast, Osseointegrasyon, Titanium Disk*

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INTRODUCTION

Peri-implant diseases are infectious diseases that affect the peri-implant tissues. A lot of bacterial species such as Staphyloccoccus spp., gram-negative anaerobic periopathogens and opportunistic microorganisms mentioned to play a role in peri-implantitis[1]. In fact, S. aereus can be detected on titanium based dental implants, within an hour following surgical insertion[2]. Cleansing of remnants on the surface and detoxification of implant surface are crucial in the treatment of periimplantitis. Preservation of titanium surface and maintenance of biocompatibility are important points during detoxification of implant surface. Various techniques including mechanical cleansing with plastic and titanium surface curettes, ultrasonic devices, citric acid application and laser treatment are currently used for surface detoxification[3]. Contemporarily, a popular treatment in periimplantitis is laser application [4, 5].

In dentistry practice, laser application has been used as an effective therapeutic modality for a long time. This method possesses both advantages and disadvantages and the most notable advantages offered by laser in periodontal surgery is coagulation, vaporization, sterilization and its selective effect on tissues. On the other hand, long duration of procedure and increased cost are disadvantages[6].

The current practice of treatment in periimplantitis includes many types of laser such as CO2, Er:YAG, Nd:YAG and Er,Cr:YSGG. Maintenance of biocompatibility of the implant surface during detoxification by laser is critical for the reosseointegration process in the healing period. However, laser treatment protocols that provide a safe and effective detoxification without damage on the implant surface have not been established yet [7-9].

The objective of the present study was to determine the principles for safe and effective management of perimplantitis using application of Er,Cr:YSGG laser on titanium surface using a model that simulates of infected peri-implant surface by using S. aereus - one of the early detected bacteria at dental implant surface-.

MATERIALS AND METHODS

In this study, a total of 40 grade 5 titanium discs (Implance, AGS Medikal, Turkey) having a diameter of 10 mm and a thickness of 1 mm with Resorbable Blast Media (RBM) surface were utilized. These discs were packed separately before sterilization in the autoclave. Ten of these discs were used for examination with scanning electron microscopy (SEM) for evaluation of surface morphology and surface roughness after laser application and the remaining 30 discs were used for cell culture study.

1. Experimental infection of discs with S. Aureus

Staphylococcus aureus ATCC 25923-standart strain-, has been growth on blood agar 37°C, then one colony inoculated into brain-heart infusion liquid medium, and incubated for 18 hours at 37°C. Bacterial working suspension in Phosphate Buffered Saline (PBS) was prepared at concentration of McFarland 0.5 turbidity (1.5x108bacteria/ml) using the broth culture, next the suspension was washed twice with PBS. Ten microliters liquid from S.aureus suspansion (1.5x108 bacteria/ml) that reconstituted using PBS was spreaded on titanium disc surfaces and waited for 30

minutes at Class II working cabin for getting dry.

2. Laser procedure

In the current study, we used Er,Cr:YSGG laser (Biolase, California). Laser energy was applied using G4 type tips applied at an angle of 90° to the titanium disc under cooling with 15% water and 30% air and H mode. After placement of titanium discs on sterile glass, laser was applied after adjustment of appropriate angle, position, distance and dose (Fig. 1).

Figure 1. The laser treatment of Ti dics

In this experimental design, we would have to repeat the tests in 81 (34) different settings in order to compare 4 factors (power, frequency, distance and duration) with variables at 3 different levels (1-2-3W, 20-25-30 Hz, 2-4-6mm and 15-30-45 sn). To reduce this number, we alternatively used Taguschi method, which is a combination of mathematical and statistical techniques 10. Accordingly, 10 groups including 9 experimental groups and 1 control group were constituted using ANOVA program. Protocols used in test groups and laser energy values used for these protocols are presented in Table 1 and Table 2, respectively.

Table 1. Laser Protocols

Groups	Protocols
Test group 1	1 W-20 Hz-2 mm-15 sn
Test group 2	1 W-25 Hz-4 mm-30 sn
Test group 3	1 W-30 Hz-6 mm-45 sn
Test group 4	2 W-20 Hz-4 mm-45 sn
Test group 5	2 W-25 Hz-6 mm-15 sn
Test group 6	2 W-30 Hz-2 mm-30 sn
Test group 7	3 W-20 Hz-6 mm-30 sn
Test group 8	3 W-25 Hz-2 mm-45 sn
Test group 9	3 W-30 Hz-4 mm-15 sn
Control	No laser application

Table 2. Laser Energy Values

	Spot Size (2r)	Spot Area (mm2)	Power Density (W/ mm2)	Energy Density (W*sn/ mm2)	Pulse energy (W/Hz)	Pulse Duration (µs)
Test 1	0.80	0.50	1.99	29.85	50	140
Test 2	0.90	0.63	1.57	47.18	40	140
Test 3	1.05	0.86	1.15	51.99	33	140
Test 4	1.30	1.32	1.50	67.84	100	140
Test 5	1.40	1.53	1.29	19.49	80	140
Test 6	0.90	0.63	3.14	94.36	67	140
Test 7	2.10	3.46	0.86	25.99	150	140
Test 8	1.00	0.78	3.82	171.97	120	140
Test 9	1.40	1.53	1.94	29.24	100	140

3. Examination of surface roughness

Ten out of 40 titanium discs were used for assessment of surface roughness under examination with SEM. Mean surface roughness (Ra) was measured with a profilometer (MarSurf PS1, Germany) at the center of the disc, laser treated area. For each group, Ra values were calculated as the average of 3 consecutive measurements.

4. Scanning electron microscopic examination

Any morphological changes such as melting, scratch and crack occurring after laser application were examined and documented

with SEM (EVO LS10; Zeiss, Cambridge, UK) under X2000 magnification.

5. Osteoblast cell culture

Human osteoblast-like cells SaOs-2 (ATCC85-HTB) purchased from an American tissue bank were incubated in 10% fetal bovine serum (FBS, Lonza, USA), and Minimum Essential Medium Alpha (α-MEM, Lonza, USA) containing 2 mm L-glutamine, penicillin/streptomycine and fungisone using T75 culture flasks in 5% CO2 incubator at 37 °C. When cellular proliferation covered 80%-90% of culture flask surface, culture medium was removed and cells were irrigated with phosphate buffered saline (PBS) solution devoid of calcium and magnesium. After preparation of a suspension using trypsin/EDTA (Lonza, USA), cell count was performed. Each disc was placed on the wells of a 24 well plate culture and put in incubator following 6.4X104 of cell suspension. A glass slide of 1 cm diameter was used as a positive control. Since surface area of a disc is 0.785 cm2, cell count per disc is 3.2X104.

After 24 and 48 hours, discs were removed using a sterile pincette and put into wells of 24 well plate culture containing 0.5 ml of 2% PBS added α-MEM culture medium. Into each well, 0.05 ml of 3-(4,5-dimethyl-thiazol-2)-2,5-diphenyl-SH-tetrazolium bromide (MTT) solution was added and mixed slowly. The culture plate was kept at 5% CO2 incubator at 37°C for 3 hours. At the end of this period, MTT solution was completely eliminated with a micropipet, 0.5 ml dimethyl sulfoxide was put and incubated for 30 minutes. To measure the absorbance of blue-violet color, samples of 0.1 ml was collected and placed in wells of 96-well culture area and read by microplate reader (Tecan Sunrise) at 570/630 nm.

6. Examination of cellular morphology of osteoblasts

After 48 hours, culture medium in wells was removed, and discs were gently washed with PBS and subsequently fixed with 3% formaldehyde in PBS (pH 7.4) for 30 min at room temperature. Discs were dried by dry air and sputtered with gold prior to SEM examination and microscopic evaluation was performed under 2000 magnification.

Statistical analysis

Data was analyzed using Statistical Package for the Social Science program version 13.0 (SPSS; Chicago, IL) for Windows. A p value ≤ 0.05 was considered as statistically significant.

Comparison of test groups and the control group was implemented with Mann-Whitney U test. Surface roughness (Ra) and cell proliferation values for every test group were compared with control group in terms of mean absolute percentage error.

RESULTS

Images were taken for assessment of changes on the surface of titanium discs after laser application with Er,Cr:YSGG. Magnification of 2000 was used for viewing under SEM. Assessment of these images revealed that the most remarkable surface changes were evident in the group Test 8. Flattening of the surface was seen in conjunction with melting, scratch and pitting. Melting and pitting on the surface of discs were less noteworthy in the other test groups.

In the group Test 1, melting and scarce number of pits were detected compared to the control group. No cracks were seen on the surface. Test 2 exhibited melting and roughness on the

surface. In Test 3, only melting was evident on the surface. Test 4 demonstrated melting and pitting in addition to a few cracks.

Test 5 displayed mild degrees of melting on the surface compared to the control group. In Test 6, melting and pits were observed on the surface but there were no cracks. Test 7 demonstrated a few pits on the surface. Test 8 has shown remarkable melting and deep scratches on the surface. Test 9 exhibited only a few findings consistent with melting and other findings were similar to the control group. Consequently, experimental groups else than group Test 8 yielded similar findings with the control group in terms of macro changes in surface morphology (Fig. 2, 3) (Table 3).

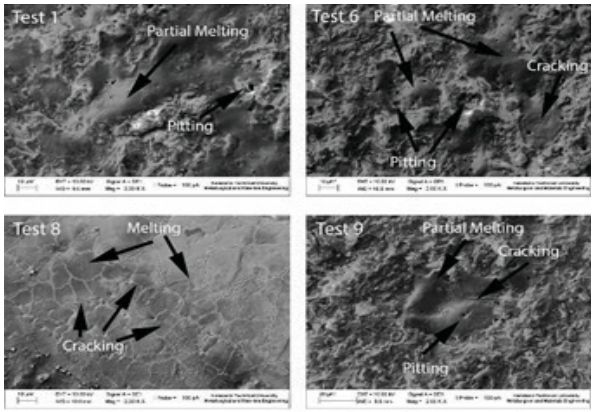

Figure 2. SEM images of some of the test groups

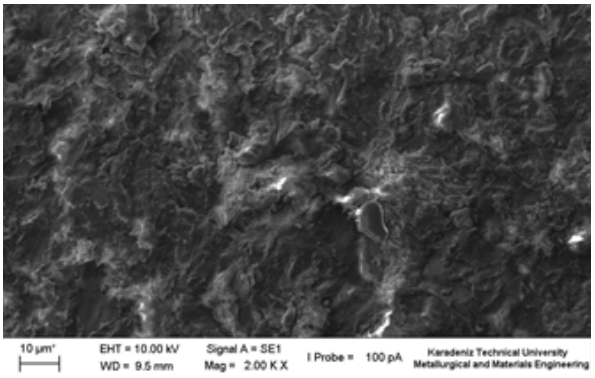

Figure 3. SEM image of control group

Table 3. Morphological Changes on Ti dics surfaces of Test Groups

Test Groups	Morphological Changes
Test 1	Surface melting and a few pits
Test 2	Surface melting and roughness
Test 3	Melting
Test 4	Melting, pitting, and a few cracks
Test 5	Mild melting
Test 6	Melting, pitting , and a few cracks
Test 7	A few pits
Test 8	Marked melting and deep scratches
Test 9	Partial melting, cracking and pitting

Table 4 presents surface roughness (Ra) after laser application. In groups Test 1 and Test 7, Ra values were similar to that of control group which has not been exposed to laser. No statistically significant difference was noted between control group and test groups with respect to Ra values.

Table 4. Mean Ra Values

	Ra (nm)
Test 1	1.320±0.48
Test 2	1.129±0.52
Test 3	1.279±0.47
Test 4	1.252±0.96
Test 5	1.230±0.33
Test 6	1.189±0.84
Test 7	1.302±0.27
Test 8	1.120±0.37
Test 9	1.237±0.22
Control	1.360±0.96

Evaluation of cellular morphology after an incubation of 48 hours, SaOs-2 cells were attached on the surface in a similar fashion with the control group. In this aspect, no

differences were detected between test groups and the control group.
Moreover, cells having spindle-like structures were encountered in test and control groups (Fig. 4, 5).

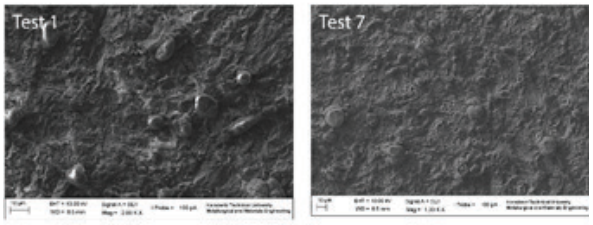

Figure 4. Cellular morphology of some of the test groups

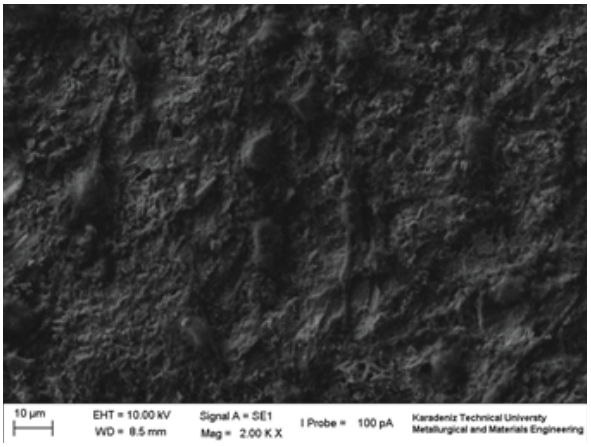

Figure 5. Cellular morphology of control group

Proliferation values at the end of 24 hours is demonstrated in Table 5. There was no statistically significant difference between test groups and control in terms of proliferation indicators ($p \geq 0.05$).

Proliferation values at the end of 48 hours is presented in Table 6. In this aspect, Test 1 and Test 7 displayed remarkably higher proliferation values compared to the control group. There was no statistically significant difference between the control group and other test groups. Comparison of proliferation in two different periods is established in Table 5 and 6.

Table 5. Proliferation Values at the end of 24 hours

Groups	OD values	Sig.(2-tailed)
Test 1	0.322±0.003	p≥0.05
Test 2	0.369±0.002	p≥0.05
Test 3	0.224±0.003	p≥0.05
Test 4	0.268±0.002	p≥0.05
Test 5	0.348±0.003	p≥0.05
Test 6	0.376±0.006	p≥0.05
Test 7	0.359±0.001	p≥0.05
Test 8	0.368±0.001	p≥0.05
Test 9	0.373±0.003	p≥0.05
Control	0.345±0.002	

*p<0.05 is statistically difference

Table 6. Proliferation Values at the end of 48 hours

Groups	OD values	Sig.(2-tailed)
Test 1	0.536±0.013	p=0.029*
Test 2	0.538±0.034	p≥0.05
Test 3	0.519±0.011	p≥0.05
Test 4	0.467±0.008	p≥0.05
Test 5	0.466±0.006	p≥0.05
Test 6	0.506±0.015	p≥0.05
Test 7	0.564±0.011	p=0.021*
Test 8	0.514±0.016	p≥0.05
Test 9	0.508±0.017	p≥0.05
Control	0.480±0.014	

*p<0.05 is statistically difference

DISCUSSION

Effective cleansing of the implant surface, avoidance of production of heat on the surface and maintenance of surface biocompatibility are key points for a successful osseointegration process in the healing period during periimplantitis treatment[8, 11-13].

Few reports have been published on the use of Er,Cr:YSGG in the treatment of periodontitis[14-20]. No consensus exists on the standardization of the measures such as power, frequency and distance of application and only 2 studies have focused on all of these variables[19, 20].

Miller et al. suggested that decontamination of titanium surface with Er,Cr:YSGG laser was a reliable and effective method 12. Interestingly, they reported no change was observed in the surface morphology despite the high power (6 W) and long duration (3 minutes) of application. This difference may be attributed to the variability in type of tip used and duration of the procedure. In addition, no data is available on the distance of laser application in that trial. On the same topic, Ercan et al. proposed that the most important determinant affecting the changes on surface morphology was intensity of power (W/mm2) and power is directly influenced by the distance[19,20]. They concluded that optimization of laser protocols was crucial for establishment of safe and effective treatment regimen besides preservation of the surface titanium discs.

Kreisler et al. have investigated the impacts of Er:YAG laser on implant surfaces[21]. Consequently, not only decontamination was satisfactory for 3 types of titanium discs at 2 energy levels, but also no excessive heat formation was observed nor there were any remarkable changes on surface morphology. In harmony with their study design, we contaminated sterile implant surfaces with bacteria (S.aureus) to simulate the clinical situation. However, we did not perform any biofilm only put bacteria on the surface and wait approximately for an hour. We only want to benefit the presence of bacteria on surface because we know that any cells found on the surface will absorb the some of the laser energy. We did not perform any microbiological analysis and this is one of the limitations of this study. The microbiological analysis of the discs after laser treatment will be another important study.

One of the most remarkable results of our study is that; the groups test 1 and test 7 had similar Ra values with control group and the proliferations were statistically significantly increased in comparison to the control group at the end of 48 hours. Surface roughness is important osseointegration and many studies have been conducted to investigate the effects of the impacts of surface roughness on proliferation and morphology of osteoblasts [22-25]. Wenneberg et al. have classified Ra values and they determined Ra<0.5 μm as smooth, Ra between 0.5-1 μm as minimally rough, Ra between 1-2 μm as moderately rough and Ra>2 μm as rough[22]. They claimed that the most appropriate Ra value for reosseointegration was detected in moderately rough (1-2 μm) surfaces. In agreement with this data, Shalabi et al. reported that contact of bone implant was improved on moderately rough surfaces[23]. The Ra value during detoxification procedure, may has a critical role in cellular proliferation. Alternatively, this increase in proliferation was attributed to the settlement of osteoblasts into the pits formed after laser application on the surface and surface energy may be

changed. Another reason may be dampening due to flattening of sharp and pointed edges allowing easier attachment of osteoblasts on larger surfaces. However, more studies with long time follow-up periods are needed to determine the factors that affect the osteoblast behavior.

Park et al. employed 3 different types of laser (Er,Cr:YSGG, Er:YAG, CO2) at various power levels (1, 2, 3, 4 and 5 W) to titanium discs and assessed the changes on surface 17. Their results demonstrated that Er,Cr:YSGG should be used maximally at a level of 3 W. Therefore, we have determined the maximal level of power in our study as 3 W.

In 2007, Huang et al. employed Er,Cr:YSGG laser at different energy levels (125 j/mm2 and 10 j/mm2) on titanium discs polished for improvement of initial biocompatibility[14]. The distance between the laser tip and titanium surface was set as 2 mm. Melting and surface roughness were found to be increased with higher levels of power applied on discs. In both experimental groups, CPI (cell proliferation index) was statistically significantly increased compared to the control group ($p<0.001$), while there was no difference between two experimental groups. Evaluation of cellular morphology at the end of first day demonstrated that spindle like cellular morphology of cell bodies in the group receiving 190 j/m2 was more evident compared to control group[14]. These findings may imply that Er,Cr:YSGG laser amplifies surface biocompatibility. In our study, since no difference was determined between experimental groups and control group at the end of first day with respect to cellular proliferation, laser application seems not to have any adverse effects on cellular proliferation.

In another study published by Ayobian et al. in 2015, impacts of Er:YAG laser on roughness, dampening and biocompatibility of titanium discs with SLA surface[26]. Evaluation of cellular proliferation in SaOs-2 culture at the end of 5 days revealed that cellular proliferation was more prominent in the group receiving laser treatment. Results of this study are in harmony with our data since we noted that Ra values were lower and cellular proliferation was significantly increased in groups Test 1 and Test 7 at the end of 48 hours.

Limitation of the present study is lack of evaluation of cellular activity. Long term trials focusing on proliferation, adhesion and cellular activity are warranted for understanding cellular biocompatibility and osteoblast cell behaviors in periimplantitis after detoxification.

Acknowledgments:
The authors report no conflict of interest.
The authors thanks to Dr. Kurtuluş Buruk for his supports during microbiological part.

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