Effect of erbium-doped yttrium-aluminum-garnet laser on surface characteristics and biocompatibility of resorbable blast media titanium

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Er:YAG laser irradiation can modify the surface characteristics of titanium surface. This study aimed to evaluate the influence of Er:YAG laser irradiation on mechanical, chemical, and biological characteristics of resorbable blast media (RBM) titanium surfaces. RBM disks were divided into 4 groups. Control groups were nonirradiated RBM disks. Laser irradiated groups were divided into the RBM-50, RBM-100, and RBM-250 group which irradiated Er:YAG laser at energy level 50, 100, and 250 mJ/pulse, respectively. In scanning electron microscopy (SEM) images, whereas the RBM-50 group showed slightly melted surface area, RBM-100 and RBM-250 groups showed broken areas caused by Er:YAG laser. Laser irradiated groups had significantly higher roughness than the control group. The roughness of the RBM-50 group was comparable with the RBM-100 group and significantly lower than the RBM-250 group ($p<0.05$). In X-ray diffractometer analysis of the laser irradiated group, Ti (002) (rutile) proportion to Ti (101) (anatase) was decreased according to energy level. In X-ray photoelectron spectroscopy analysis, RBM-50 and RBM-100 groups show the crystal change from acidic Ti-OH to basic Ti-OH. MC3T3-E1 cells of all groups adhered and proliferated well on the surfaces and their morphology was not different between groups in SEM images. The alkaline phosphatase activity levels of RBM-50 and RBM-100 groups showed higher than those of the control group ($p<0.05$). Therefore, within the limitations of this study, Er:YAG laser irradiation at low energy levels can modify mechanical and chemical characteristics of the RBM surface, leading to improve the biological responses of osteoblastic cells on the surface.

Key Words: Biocompatibility; Er:YAG laser; RBM titanium surface; Surface modification

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Introduction

Titanium implant has been common choice for fully or partially edentulous patients. Since titanium implant has been used, constant efforts for improve osseointegration,

functional attachment of bone to dental implants, cause improvements in surface properties, design, and chemical composition.

One of effort to improve osseointegration is alteration of surface roughness. Many studies have found that rough

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implant surface has a better and an accelerated osseointegration than smooth implant surface [1]. Applying either additive or subtractive techniques have been utilized to modify topography of implant surface. Surfaces modified with additive techniques show decline in their survival rate [2,3], thus surfaces modified using subtractive techniques, like resorbable blast media (RBM) surface and sand blasted and etched (SLA), have been more widely adopted [4].

RBM surface has been used widely in clinical practice. RBM is formed through propelling resorbable coarse bio-ceramics, calcium phosphate, particles on titanium for roughness followed by passivation process in order to eliminate contaminating debris after blasting. This procedure does not involve acid-etching, and therefore does not affect the fatigue strength of the implant surface [5,6]. RBM implants show more alkaline phosphatase positive osteoblasts and be in direct contact with bone than machined implant [7].

Erbium-doped yttrium-aluminum-garnet (Er:YAG) laser can treat titanium surfaces effectively without damaging not only titanium surface but also adjacent tissues [8-10]. Furthermore, Er:YAG laser can enhance biocompatibility of titanium surfaces [11], improve osseointegration [9]. For that reason, Er:YAG laser irradiation on implant surface anticipated improve surface characteristics and biological response. Another study also showed Er:YAG laser irradiation improve surface characteristics and biocompatibility of MC3T3-E1 cell on smooth titanium surface [12].

Several studies have shown that irradiation of Er:YAG laser on titanium surface modifies the surface characteristics of titanium by increasing wettability, decreasing surface roughness of titanium surface and affecting the proliferation rate of cells and it can be speculated that this improves the osseointegration of implants. However, most studies on laser irradiation on titanium surface were related with smooth surface or other lasers and studies related with irradiation of Er:YAG laser on rough surface titanium was limited. So far, however, study about mechanical, chemical and biological effect of Er:YAG laser on implant with RBM surface is insufficient. Thus the purpose of this study was to assess the influence of Er:YAG laser irradiation on mechanical, chemical and biological characteristics of RBM titanium in vitro.

Materials and Methods

Laser device

An Er:YAG laser (Anybeam; BnB system, Seoul, Korea) with cylindrical-shaped optical fiber at a wavelength of 2940 nm was used. This laser equipment has 15-400 mJ/pulse output energy, 5-30 pulse rate. For this study, laser irradiated with pulse frequency kept constant to 25 Hz.

RBM disks preparation

RBM disks with 15 mm diameter and 2 mm thickness of pure titanium (cp-Ti, grade II, ASTM) was manufactured from Titanium Center in Gwangju, Korea. RBM surface was treated with biodegradable hydroxyapatite powder (MCD powder; Hi-Med, Old Bethpage, NY, USA) by mixing an average particle size of 100-150 μm and 90 μm with a portion of 50/50 wt% under 4 atm and followed by 10 minutes treatment with 20% HNO_3 solution. Ultrasonic cleaning was done under 70% methanol for 10 minutes and was washed by distilled water for 20 minutes in an ultrasonic bath (Bandelin; Sonorex, Berlin, Germany). The cleaning cycle of disks were performed 3 times. And then, the disks were rinsed with distilled water and dried in a laminar air flow [13].

Laser irradiation

RBM disks divided into 4 groups: 1) control group: no laser irradiation, 2) RBM-50 group: 50 mJ/pulse (energy used for peri-implantitis treatment) laser irradiation, 3) RBM-100 group: 100 mJ/pulse (energy used for soft tissue treatment) laser irradiation, 4) RBM-250 group: 250 mJ/pulse (energy used for hard tissue treatment) laser irradiation. The energy used for the treatment was determined according to a study by Matsuyama et al. [14].

The laser was irradiated under water manual irrigation for cooling. The laser irradiated perpendicular from the disk surface through optic fiber at 5 mm distance from disk surface. The laser irradiation was performed in zigzags for 120 seconds.

Observation of surface morphology

An ion sputter coater (E-1030; Hitachi, Tokyo, Japan) sputtered gold-palladium on all polished disks. And then, the disks were observed by scanning electron microscopy (SEM, S-4700; Hitachi). SEM images of all group were taken twice at $\times 100$, $\times 1,000$ magnifications.

Evaluation of roughness

Electronic portable surface roughness tester (Diavite DH-7; Asmeto AG, Basel, Switzerland) were used to measure surface roughness. Surface roughness (R_a) was calculated by the mean value of five perpendicular measurements from different area at surface of each disk.

Evaluation of chemical composition and crystal form

X-ray diffractometer (XRD: D/MAX Ultima III; Rigaku, Tokyo, Japan) was performed to evaluate change of chemical composition and crystal form after Er:YAG laser irradiation. A $\text{CuK}\alpha$ incident radiation of current of 40 mA and a tube voltage of 40 kV was used. The scanning speed was $2^\circ/\text{min}$ and scanning angle ranged between 20° and 90° .

Evaluation of oxide and hydroxide group

X-ray photoelectron spectroscopy (XPS: Multilab 2000 system; SSK Corp., Waltham, MA, USA) was performed to observe surface characteristics and chemical states of each group. The hydroxide group and the oxide group of each surface could be detected by result of XPS.

Cell culture

All disks for biologic study were placed in the bottom of 12-well culture dishes under aseptic conditions. The disks washed with 70% ethanol 3 times, followed exposed to UV light for 1 hour and dried in a laminar air flow.

MC3T3-E1 cells (ATCC, Rockville, MD, USA), derived from mouse calvarium tissue, were cultured in alpha minimum essential medium (α -MEM media; invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% heat-inactivat-

ed fetal bovine serum (FBS; Invitrogen Corp.), 100 $\mu\text{g/mL}$ penicillin, and 100 $\mu\text{g/mL}$ streptomycin at 37°C in humidified atmosphere of 5% CO_2 -95% air.

Observation of osteoblastic cells using SEM

To evaluate the morphology of attachment and early growth of MC3T3-E1 cell, disks seeded with MC3T3-E1 cell were observed using SEM (S-4700; Hitachi) image.

MC3T3-E1 cells were seeded at a density of 1×10^4 cells/mL with α -MEM media containing 10% FBS. The cells were incubated for 2 days. And then, the cells were rinsed with phosphate buffer saline (PBS), followed fixed with 2.5% glutaraldehyde in 100 mM cacodylate buffer (Sigma, St. Louis, MO, USA) and dehydrated in increasing concentrations of ethanol (30%, 60%, 95%, and 100%). Disks were immersed in hexamethyldisilazane (Sigma) for 15 minutes and mounted on aluminum stubs immediately followed by coating with Au/Pd alloy.

SEM image was taken twice in each group at $\times 100$, $\times 250$ magnifications.

Alkaline phosphatase (ALP) activity test

ALP is an early marker of the osteoblastic cell differentiation [15]. ALP activity test was performed to evaluate the osteoblastic cell differentiation on RBM surface.

MC3T3-E1 cells were seeded on RBM disks placed in a 12-well plate at a density of 1×10^4 cell/mL in alpha-MEM solution containing 10% FBS, and incubated for 24 hours. And then, culture medium was change into 10% FBS, ascorbate 40 $\mu\text{g/mL}$ and 20 $\mu\text{g/mL}$ β -glycerol phosphate to induce differentiation into osteoblasts. At day 7, ALP activity was determinated. Briefly, the cells were lysed in Triton 0.1% (Triton X-100; Invitrogen Corp.) in PBS (Invitrogen Corp.), then frozen at -70°C and thawed. Subsequently, 100 μL of the cell lysates was mixed with 200 μL of 10 mM p-nitrophenol phosphate (Sigma) and 100 μL of 1.5 M 2-amino-2-methyl-1-propanol buffer (Sigma), and then incubated for 60 minutes in an oven at 60°C . The ALP activity was measured by absorbance at 405 nm with a spectrophotometer (SmartSpec; Bio-Rad, Hercules, CA, USA). All ALP test was done triplicate.

Statistical analysis

This study used the statistical program called SPSS software package (ver. 12.0; SPSS Inc., Chicago, IL, USA) to analyze roughness test and ALP activity with one-way ANOVA. Potential group comparison was performed by using Mann-Whitney U-tests. The results were considered statistically significant when p -values were less than 0.05.

Results

Observation of surface morphology using SEM

The surface morphology of all groups was shown in Fig. 1.

In low magnification ($\times 100$), surface didn't show a big difference between groups. However, in high magnification ($\times 1,000$), whereas RBM-50 group show slight melted area, RBM-100 and RBM-250 groups show broken area made by Er:YAG laser unlike RBM-50 group. And, RBM-250 group show more clear broken structure than RBM-100 group.

Roughness test

The result of roughness test in all groups was shown in Table 1.

Roughness (R_a) of control group, RBM-50 group, RBM-

100 group, and RBM-250 group was $1.08 \pm 0.11 \mu\text{m}$, $1.15 \pm 0.10 \mu\text{m}$, $1.20 \pm 0.08 \mu\text{m}$, and $1.31 \pm 0.10 \mu\text{m}$, respectively. Laser irradiated groups had statistically significant higher R_a than control group ($p < 0.05$). R_a of RBM-50 group and RBM-100 group was not different significantly. RBM-250 group had statistically significant higher R_a than RBM-50 and RBM-100 groups ($p < 0.05$).

XRD analysis

The XRD patterns of all groups were shown in Fig. 2. XRD pattern can show crystal structure change of specimen. No apparent difference in surface components be-

Table 1. Roughness values of control and Er:YAG laser irradiated groups

Group	Value	
Control	$1.08 \pm 0.11 \mu\text{m}$	
RBM-50	$1.15 \pm 0.10 \mu\text{m}$	
RBM-100	$1.20 \pm 0.08 \mu\text{m}$	
RBM-250	$1.31 \pm 0.10 \mu\text{m}$	

Values are presented as mean $\pm$ standard deviation. Laser irradiated groups had significant higher R_a than control group.

Er:YAG, erbium-doped yttrium-aluminum-garnet; RBM, resorbable blast media.

*Indicate different statistically ($p < 0.05$). p -value was < 0.05 between control, and RBM-250 groups.

Fig. 1. SEM photomicrographs of control and Er:YAG laser irradiated groups. RBM-50 group show slight melted area, RBM-100 and RBM-250 groups show broken area made by Er:YAG laser (A) control group, (B) RBM-50 group, (C) RBM-100 group, and (D) RBM-250 group (A-D: $\times 100$, A'-D': $\times 1,000$ magnification; white arrow: melted area caused by Er:YAG laser irradiation, black arrow: broken area caused by Er:YAG laser irradiation). SEM, scanning electron microscopy; Er:YAG, erbium-doped yttrium-aluminum-garnet; RBM, resorbable blast media.

Fig. 2. XRD patterns of control and Er:YAG laser irradiated groups. No apparent difference in surface components between groups was observed. Energy level increases, proportion of T(002) to T(101) phase was decreased. XRD, X-ray diffractometer; Er:YAG, erbium-doped yttrium-aluminum-garnet; RBM, resorbable blast media.

tween groups was observed. Whereas T(002) indicate TiO_2 in the rutile phase, T(101) indicate TiO_2 in the anatase phase. Thus, in this study, as the energy level increases, proportion of rutile phase to anatase phase was decreased.

The rutile fraction in the RBM surface was calculated by the following equation.

$$X = (1 + 0.8I_A/I_R)^{-1}$$

In equation, X means rutile fraction. I_A and I_R means peak intensity of anatase phase, T(101), and rutile phase, T(002), respectively [16].

XPS analysis

XPS analysis can reveal the surface oxygen and hydroxide crystal change. Fig. 3A–D show the O1s XPS spectra for the surface of groups. The skewed peaks reflects the alteration of oxygen group according to different irradiation energy levels. The O1s XPS spectra were classified into three Gaussian component peaks. The peaks were TiO_2 , acidic TiOH and basic Ti-OH . Of these, acidic TiOH and basic Ti-OH were two hydroxide groups of titanium dioxide surfaces.

Fig. 4 shows the percentage of three oxygen groups according to Er:YAG laser irradiation. The proportion of

TiO_2 , Ti-OH , TiOH of RBM-50 and RBM-100 group were increased compared to control group. On the other hand, RBM-250 group show lower the proportion of TiO_2 , Ti-OH , TiOH .

SEM analysis of osteoblastic cells

The SEM images of attachment of the MC3T3-E1 cell, immortalized cell-line such as pre-osteoblast, to surface of all group after 48 hours culturing are shown in Fig. 5. SEM image of all groups did not exhibit any differences on morphology and attachment pattern of the growing of cells between groups. All groups exhibited a layer of cells, dendritic shape, with cytoplasmic extensions connected with one another.

ALP activity

The results of ALP test were shown in Fig. 6. RBM-50 group and RBM-100 group showed statistically significant higher ALP activities of than control group ($p < 0.05$).

Discussion

From implant inserted on edentulous alveolar ridge, the most important topic of implant dentistry was osseointegration. To achieve improved osseointegration, various efforts such as surface alteration conducted.

Rough surfaces for improved osseointegration show a higher degree of early plaque formation and bacterial adhesion, leading to peri-implantitis and finally implant failure [17]. Also, rough surface is difficult to cleaning affected implant effectively [18]. Thus, because rough surface is common used nowadays, control of peri-implantitis is increasingly common and difficult.

Taniguchi et al. [19] reported that Er:YAG laser irradiation with water coolant decrease the oxygen and carbon content. The result indicate that the laser remove hydrocarbons which inhibit osseointegration from the titanium surface [19]. Moreover, many studies showed that Er:YAG laser irradiation on titanium surfaces improve cell response and enhance osseointegration [20–23].

In the preset study, RBM titanium surfaces were tested.

Fig. 3. The electron binding energy of control and Er:YAG laser irradiated groups (A) control group, (B) RBM-50 group, (C) RBM-100 group, and (D) RBM-250 group. The peaks were 529.9 eV; oxygen in surface oxide lattices (TiO_2), 531.1 eV; oxygen in the hydroxyl residue of acidic titanium hydroxyl groups (acidic TiOH , bridging OH group) and 532.4 eV; oxygen in the hydroxyl residue of basic titanium hydroxyl groups (basic Ti-OH , terminal OH group). Er:YAG, erbium-doped yttrium-aluminum-garnet; RBM, resorbable blast media.

RBM fabricated with coarsely ground calcium phosphate, which form rough surface without contaminating debris after blasting. Wennerberg et al. [24] concluded that adequate rough RBM implant show higher removal torque and more bone-to-metal contact. And the RBM surfaces could be considered more osteoconductive than the machined surfaces [5,6,25]. However, reports concerned about influence of Er:YAG laser on RBM titanium are little. Therefore the purpose of this in vitro study was to evaluate alteration of surface characteristics and cellular response of RBM implant after Er:YAG laser irradiation.

Because Er:YAG laser is absorbed into water but not absorbed in titanium due to its wavelength, this laser can be used for both hard and soft tissue treatment effectively [26,27] and application of the laser at low density have not

shown to alter titanium surface [8,11]. Kreisler et al. [11] also reported that Er:YAG laser remove cytotoxic bacterial components effectively from titanium without visible alterations microscopically of the implant surface. Schwarz et al. [28] reported that Er:YAG laser irradiation didn't induce visible changes on diverse titanium surfaces. On the other hand, Taniguchi et al. [19] reported Er:YAG laser (40 mJ/pulse, 30 Hz) induce slight melting of microstructural edge of RBM implant. In multiple studies, wide range of Er:YAG laser was irradiated and various results have been reported. In our present study, we reviewed several studies and determined the energy levels such as 50 mJ/pulse energy level for peri-implantitis treatment, 100 mJ/pulse energy level for soft tissue treatment and 250 mJ/pulse energy level for hard tissue treatment. SEM analysis of this study revealed

that whereas RBM-50 group showed slight melting area, RBM-100 and RBM-250 groups showed broken area made by Er:YAG laser. Thus, energy density lower than 50 mJ/pulse at 25 Hz is considered to be generally recommended for RBM surface.

Moderately rough surfaces (S_a 1.0–2.0 μm) has shown improved bone biological response and mechanical properties than not only very rough surface ($S_a > 2.0 \mu\text{m}$) but also smooth surface ($S_a < 0.5 \mu\text{m}$) [29,30]. Surface roughness

of all group (R_a) were within R_a 1.0–2.0 μm . R_a represents a profile of 2-dimensional line while S_a is a parameter of 3-dimension. Although R_a and S_a parameters cannot be directly correlated, studies show R_a gives a good estimate of S_a [31]. Thus, RBM surface irradiated by Er:YAG laser within 250 mJ/pulse have adequate roughness for osseointegration.

Previous study, used titanium disk and profilometer, showed that surface roughness values of the non-irradiated

Fig. 4. Percentage of three oxygen species of control and Er:YAG laser irradiated groups. The proportion of TiO₂, Ti-OH, TiOH of RBM-50 and RBM-100 group were increased compared to control group. On the other hand, RBM-250 group show lower the proportion of TiO₂, Ti-OH, TiOH. Er:YAG, erbium-doped yttrium-aluminum-garnet; RBM, resorbable blast media.

Fig. 6. ALP activity of control and Er:YAG laser irradiated groups. RBM-50 group and RBM-100 group were statistical significant higher ALP activities of than control group. ALP, alkaline phosphatase; Er:YAG, erbium-doped yttrium-aluminum-garnet; RBM, resorbable blast media. *Statistical significant to control group ($p < 0.05$).

Fig. 5. SEM image of MC3T3-E1 cell attachment (48 hours observation). All group exhibited a layer of cells, dendritic shape, with cytoplasmic extensions connected with one another. (A) Control group, (B) RBM-50 group, (C) RBM-100 group, and (D) RBM-250 group (A–D: $\times 100$, A'–D': $\times 250$ magnification; black arrow: broken area caused by Er:YAG laser irradiation). SEM, scanning electron microscopy; Er:YAG, erbium-doped yttrium-aluminum-garnet; RBM, resorbable blast media.

control group and Er:YAG laser (100 mJ/pulse, 10 Hz) irradiated groups were 1.661 and 1.564 μm , respectively and the difference was statistically significant [7]. Another study, used implant and white light interferometer, indicate that Er:YAG laser irradiation (60-, 100-, 140-, or 180 mJ/pulse, 10 Hz) nonsignificantly decreased the surface roughness of anodic oxidized surface and SLA titanium surfaces [32]. Other study reported that Er:YAG laser irradiation at 300 mJ/10 Hz caused decreased S_a and S_k values on the SLA surfaces, and irradiation with 500 mJ/10 Hz reduced the S_a and S_k values even more, but the difference was not significant. Surface roughness of SLA titanium surface decreased with increased laser settings [33]. Whereas, this study showed surface roughness of laser irradiated groups were significantly higher than control group and surface roughness of RBM-250 group was significantly higher than RBM-50 and RBM-100 groups. This result could be attributed to fusion or broken of RBM titanium surface by heat from laser irradiation. Different energy density, pulse, titanium surface, methods used for measuring surface roughness, measurement scale (S_a , S_t , R_a , etc.), and limited number of measurement could result in conflicting data.

Titanium dioxide, the only naturally occurring oxide of titanium at atmospheric pressure, have three polymorphs: rutile, anatase and brookite. Among them, atomic arrangements of both stable rutile and metastable anatase may provide appropriate for epitaxial growth of apatite crystals. However, these two crystal structure have different electronic binding energy, thus different reactivity. The crystallographic matching between the anatase and apatite crystal planes are speculated more favorable than the corresponding rutile and apatite planes. Thus changes from rutile phase to anatase phase accelerate the apatite formation consequently [34]. Wu and Nancollas [35] also reported apatite nucleation on metal oxides in terms of surface tension and suggested that anatase structure have higher apatite-forming ability. This high reactivity accelerate adhesion and proliferation of osteoblastic cells on the titanium surface [36]. In this study, as energy of Er:YAG laser irradiation increased, rutile fraction decreased. Thus, Er:YAG laser irradiation enhance bioactivity by rutile-anatase transformation. In order to further accurate analysis of surface structure (the particle size, crystallinity and morphology of samples),

Transmission Electron Microscope analysis is required.

To observe the change of chemical composition, XPS analysis was conducted. The Ti-OH groups react with calcium ions to form calcium titanate. The calcium titanate adsorb not only phosphate but also calcium ions, followed by formation of apatite nucleation layer. Calcium and phosphate ions in the fluid is consumed continuously for growing the apatite nuclei. This apatite layer is favorable to the attachment as well as proliferation of the human osteoblast cells [37]. RBM-50 group and RBM-100 group show higher basic Ti-OH proportion, whereas RBM-250 group show lower Ti-OH proportion than control group. Thus, Er:YAG laser irradiation below 100 mJ expected to higher cell response.

MC3T3-E1 cell, an osteoblast precursor cell line, was used for the testing of surface biocompatibility. From SEM images taken after aforementioned cell seeded, dendritic cells with numerous filopodia on surface of all group were observed. Because filopodia is a feature of cellular adhesion and osteoblast maturation leading to promotes bone formation adjacent to treated surfaces [28,38], assumption that cells are good at spreading and attachment to surface of all group could be possible. In accordance with our findings, previous study [39] evaluate the responses of SaOs-2 cells, like human osteoblast, to SLA surface irradiated by Er:YAG laser and reported that the cells exhibited flattened, polygonal shapes with numerous filopodia and growth patterns of proliferation rates show no differences between control group and test group. This conflicting result may because different titanium surfaces were studied.

Biocompatibility of Er:YAG laser irradiation on RBM surface analyzed by ALP test. ALP activity of RBM-50 group and RBM-100 group after 7 day were higher than that of control group statistical significantly ($p < 0.05$). This finding is consistent with present result of XPS test. The reason why ALP activity of RBM-50 group and RBM-100 group was improved is assumed that increase of Ti-OH group in SBF play important role in formation of apatite, which promote differentiation of osteoblast as previously mentioned. That is, Er:YAG laser irradiation change structure, characteristic and chemical composition of RBM surface and thus would be positive on osteogenic response of MC3T3-E1 cell.

Er:YAG laser irradiation of 250 mJ/pulse caused severe structural change of RBM surface thus rather induce negative result on mechanical, chemical and biological characteristics on the surface. There was wide gap between laser irradiation energy level of groups. Thus, further study with segmentalized energy level within 0 and 100 mJ is needed.

Within the limits of this study, it may be concluded that Er:YAG laser irradiation can improve the biologic responses of osteoblastic cells on RBM surface by modification of mechanical and chemical characteristics of the surface. Irradiation with Er:Yag laser below 100 mJ/pulse level increases ALP activity and induce crystal change from acidic form (TiOH) to basic form (Ti-OH) with no significant difference in roughness. Er:YAG laser with proper energy level must be irradiated carefully on RBM surface.

Conflict of Interest

The authors declare that they have no competing interests.

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