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Efficacy of Er:YAG Laser in Decontamination of Dental Implants: an in-vitro Study

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ABSTRACT

Aim: The primary aim of this prospective randomized controlled clinical trial was to compare the efficacy of biofilm removal from dental implant-like flat titanium surfaces by Erbium-doped yttrium-aluminum-garnet (Er:YAG) laser, titanium brush, and carbon fiber curette. The secondary aim was to evaluate the amount of residual live biofilm in the same treatment groups. **Materials & Methods:** Eight study subjects who fit the inclusion criteria were recruited in the study. A custom mouth appliance that holds 8 sand-blasted and acid etched titanium discs was fabricated for each subject. Subjects were asked to wear this appliance for 72 hours to allow for biofilm development on the discs. After retrieving the appliance, the discs were removed and randomized to one of four treatment groups: 1) Er:YAG laser, 2) titanium brush, 3) carbon fiber curette and 4) no treatment (control). The discs were stained with LIVE/DEAD *Bac*Light dye and the residual biofilm was visualized under fluorescence microscopy. Quantification of residual biofilm was performed using imageJ analysis software and expressed as percentage surface area. **Results:** A total of 59 titanium discs were retrieved. The percentage of titanium disc area covered by biofilm was 74.03 ± 21.61%, 32.78 ± 24%, 11.80 ± 10.29% and 20.14 ± 19.15% in the control, Er:YAG, titanium brush and carbon fiber curette group, respectively (Mean ± SD). The biofilm-covered area significantly decreased in each of the 3 treatment groups when compared to the untreated control ($p<0.008$). Comparisons between the Er:YAG laser, titanium brush and carbon fiber curette treatment groups were not statistically significant. The percentage of titanium disc area covered by live biofilm for the control group (Mean ± SD: 33.56 ± 19.21), the Er:YAG group (Mean ± SD: 24.28 ± 16.31), the titanium brush group (Mean ± SD: 10.91 ± 9.86), the carbon fiber curette group (Mean ± SD: 19.68 ± 18.68). For residual live biofilm

area percentage, no statistically significant difference was found between the groups.

Conclusions: Er:YAG laser is an effective method in reducing the bacterial biofilm on titanium discs. However, on a threadless titanium surface Er:YAG laser did not exhibit significantly greater efficacy in biofilm removal compared to commonly used titanium brushes or carbon fiber curettes.

DEDICATION

I dedicate my thesis to my country, the Kingdom of Saudi Arabia

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SEM: Scanning electron microscopy.

Er:YAG: Erbium-doped yttrium-aluminum-garnet.

CO_2: Carbon dioxide.

GaAlAs: Gallium-aluminum-arsenide.

Nd:YAG: Neodymium-doped yttrium-aluminum-garnet.

Er,Cr:YSGG: Erbium- and chromium-doped yttrium-scandium-gallium-garnet.

ICF: Informed consent form.

PI: Propidium iodide.

PBS: phosphate-buffered saline.

SD: Standard deviation.

IQR: Interquartile range.

Ti: Titanium.

Efficacy of Er:YAG Laser in Decontamination of Dental Implants: an in-vitro Study

The advent of oral implants has revolutionized dental practice and industry as a whole. Implants provide a solution for tooth loss that is predictable with long-term success.[2] A success rate of 90% has been reported for both fully or partially edentulous restorations.[3-6] As for such success, dental implants have become the mainstay of prosthetic treatment planning and oral rehabilitation. Different implant systems have been developed with various sizes, shapes, surfaces, and prosthetic components.[7]

The biological principle behind success of dental implants is the concept of osseointegration. Osseointegration is defined as a close structural adaptation between the implant and surround bone at light microscopic level.[8] This structural union enables the implant to withstand functional load. Different surface treatments have been introduced to create implant roughened surface and promote osseointegration. Acid etching is a process where a strong acid is employed to create a rough surface and increase surface area. Another method is blasting where different materials are projected to the implant through a nozzle with high velocity to create a roughened surface. These materials include titanium oxide, aluminum oxide, or hydroxyapatite. Plasma spraying employs titanium particles sprayed on the implant surface under elevated temperatures. These methods can be combined together to provide a unique surface for each implant company. New methods are being introduced for every new generation of dental implant.[7]

As well as teeth, dental implants are not immune to inflammatory lesions. The first European Workshop on Periodontology identified two disease entities surrounding implants. They have been recognized as peri-implantitis and peri-implant mucositis.[9] Peri-implant mucositis is a reversible disease confined with the soft tissues around the implant, while peri-implantitis is designated when the disease progressed with loss of supporting bone around the

implant.[9] Heitz-Mayfield[10] identified several diagnostic criteria for peri-implant diseases such as probing depth, bleeding on probing, suppuration, attachment level changes, mobility, and radiographic crestal bone changes. They also presented diabetes, smoking, and history of periodontitis as risk factors in developing peri-implantitis. The Consensus Report of the Sixth European Workshop in Periodontology[11] in 2008 appraised the prevalence of peri-implant mucositis to be 50% of implants and 80% of subjects. Peri-implantitis was approximated to be 28% and in 56% of subjects. Mombelli et al.[12] in 2012 estimated the prevalence of peri-implantitis to affect 10% of implants and 20% of the patients in a time frame of 5-10 years after implantation. In their meta-analysis, Derks et al.[13] in 2015 estimated the prevalence of peri-implantitis to be 22%, and 43% for per-implant mucositis. These high prevalence rates led to development of different methods for implant surface decontamination.[14]

Bacterial colonization on the implant surface is the main causative factor in development of peri-implant diseases.[15-19] The rough surface of dental implant is known to promote osseointegration. However, it can also serve as a niche for bacterial colonization. Formation of adherent bacterial biofilm on titanium surface is critical for disease development.[20] The literature provides evidence of peri-implantitis' microbial etiology that is very similar to advanced periodontitis, with high levels of spirochetes and non-motile anaerobic Gram-negative bacterium (*P. gingivalis, T. forsythia, T. denticola, P. intermedia,* and *A. Actinomycetemcomitans*).[21, 22] In a systematic review, Lafaurie et al.[23] reported similarities in microbial composition but with differences in frequencies. *P. gingivalis* was similar in both diseases, whereas *P. intermedia*, *C. rectus*, *T. forsythia* were more frequent in periodontitis. *P. aeruginosa*, *S. aureus*, *C. albicans* and enteric rods were more frequent in peri-implantitis flora. Al-Ahmad et al.[24] took samples of diseased biofilm and showed higher

relative abundance of *Bacteroidetes*, and *Fusobacterium* species. Also, they detected putative periodontal red complex *P. gingivalis* and *T. forsythia* in significantly higher levels. Another study by Dingsdag et al.[25] showed increased abundances of gram negative *Prevotella*, *Fusobacterium*, *Treponema*, and *Veillonellaceae* in diseased implants. On the other hand, a decrease in gram positive *Actinomyces*, *Corynebacterium* was detected. The presence of multiple components on a single dental implant, i.e. fixture, abutment, and crown, accumulate more plaque than natural dentition.[26, 27] A study by Pontoriero et al.[28] demonstrated a cause and effect relationship between disease development and plaque accumulation.

If the disease activity is confined to the soft tissue, complete reversal to health can be expected with proper plaque control and management of contributing factors.[29, 30] When disease progresses to involve osseous structures, clinicians are faced with the options of debridement, regenerative, or resective surgical procedures. If the disease activity is advanced, removing the implant can also be considered.[31, 32] Surgical treatment will depend on bone morphology of the defect, containment of bone graft material, esthetic concerns, and the ability to maintain adequate plaque control.[33] Regeneration around implants follows the principles of guided tissue regeneration. Therefore, the procedure is more predictable if the defect is intrabony or circumferential.[33] If regeneration is not feasible, a resective approach can be utilized. This includes gingivectomy, osteoectomy, osteoplasty, and implantoplasty.[34] However, this approach should be carefully evaluated since it may lead to unaesthetic outcome or decrease in mechanical strength in case of implantoplasty.[35] No matter the clinical procedure chosen, surface decontamination forms the foundation of any peri-implant disease treatment. [20] Nevertheless, complete debridement is not a simple task.

Hand instruments used to remove plaque and calculus still serve as the primary periodontal therapy. Based on the extensive evidence supporting hand instrumentation in treatment of periodontitis, hand instruments were suggested for peri-implant diseases. Scalers of different materials were made specifically for implant surfaces. These scalers were made with similar or lower external hardness, in order not to produce scratches on the implant surface and not to make the implant surface more plaque retentive. Furthermore, a damaged surface impairs adhesion of fibroblasts and lowers biocompatibility of the implant.[36, 37] Fox et al.[37] studied the effect of stainless steel, titanium, and plastic curettes on implant surface roughness by scanning electron microscopy (SEM). Surprisingly, the roughest surface was produced by the titanium curettes followed by stainless steel then plastic. The plastic surface alteration was determined to be insignificant. Homiak et al.[38] in their SEM study on implant abutments confirmed that stainless steel instruments produced a rougher surface, whereas plastic scalers rounded off sharp edges and smoothened the surface but changes were not extremely dramatic. Carbon fiber curettes are another alternative to plastic curettes, which do not significantly damage the implant surface.[39] Strooker et al.[40] reported decrease in gingival index scores and number of bacterial colonies in patients treated by mechanical instrumentation using carbon fiber curettes.

Karring et al.[41] showed that mechanical non-surgical therapy was not capable of treating peri-implantitis. Persson et al.[42] compared using curettes to ultrasonic devices, and reported failure of both measures to resolve the disease. Renvert et al.[20] reported that mechanical non-surgical therapy is effective in peri-implant mucositis cases only, and results could be enhanced by the adjunctive use of antimicrobial rinses. No treatment protocols have received full consensus as a standard of care, as most treatments were unable to achieve

complete resolution of inflammation or surface decontamination.[20, 43, 44] Therefore, different modalities of treatment have been developed to overcome inadequacies such as sustained antibiotics release, antiseptics, air abrasion, and laser therapy.[20]

Antibiotic therapy has been suggested for peri-implantitis therapy. However, a single antibiotic therapy may not yield satisfactory results.[45, 46] Rams et al.[45] evaluated the antibiotic resistance of the peri-implantitis microbiota. Pathogens were resistant to amoxicillin, clindamycin, doxycycline, or metronidazole, but not to a combination of amoxicillin and metronidazole. Van Winkelhoff et al.[47] necessitate susceptibility testing to determine antibiotic efficacy. The issue of antibiotic resistance has emphasized restrictions over the wide use of antibiotics for treatment of peri-implantitis.[46, 47] The use of antibiotics should be limited to conditions that have benefits that outweigh the risks involved. Hence, systemic antibiotics should be limited to the acute-phase of peri-implantitis infection rather than as a treatment of choice.[46, 47]

Commonly, lasers are named after their active element that undergoes quantum transitions that result in energy emission. Some of the examples include erbium-doped yttrium–aluminum–garnet (Er:YAG), carbon dioxide (CO_2), gallium–aluminum– arsenide (GaAlAs), diode, neodymium-doped yttrium–aluminum–garnet (Nd:YAG), and erbium- and chromium-doped yttrium–scandium–gallium–garnet (Er,Cr:YSGG).[48, 49] The first laser was developed in 1960 by Maiman[50], where crystal medium of ruby stimulated by energy emitted a coherent radiant light. Laser is primarily an energy coherent unidirectional beam of one color and one wavelength. The photons can be collimated and focused to an intense beam that shows little divergence. The beam will interact with the target with absorption, reflection, or scattering.[50] When the target is a biological tissue, mostly absorption occurs

with little scattering in cases of deep tissue penetration. This absorption interaction will produce heat that may result in coagulation, incision, excision, vaporization, or simply warming the tissues.[50] This degree of absorption depends on various parameters such as power, wavelength, pulse duration, duration of exposure, energy density, and optical properties of the target tissue.[50]

The primary variable that determines the degree of absorption is the wavelength and its interaction with the target tissue.[50] Each wavelength is absorbed in a greater or lesser extent in water, pigmentation, and hydroxyapatite. For example, bone tissue comprises around 70% of inorganic minerals, whereas in gingiva contains 70% of water.[50] These structural properties will render some types of lasers useful and some not. CO_2 laser is recommended for soft tissue procedures because of its high absorption coefficient in water. On the other hand, Er:YAG and Er,CR:YSGG are highly absorbed in both water and hydroxyapatite; therefore, they are suitable for both soft and hard tissue procedures.[50]

Goldman et al.[51] in 1964 reported the first use of lasers in dentistry. In a lab setting, ruby laser was emitted on carious lesions of natural teeth. The laser managed to completely ablate the carious tissue and was advocated as an alternative for areas with difficult accessibility. Stern and Sognnaes[52] suggested that lased enamel surfaces are more resistant to oral environmental influences compared to unlased enamel. In terms of soft tissue, lasers have been used to make incisions, tissue ablation and degranulation, and coagulation and improve wound healing.[50] Fontana et al.[53] measured a 10-11° increase in bone surface temperature with diode laser.

Laser therapy has been advocated for implant surface decontamination.[48, 49] Handful

of laser types have been evaluated for use around implants. CO_2, diode, and Er:YAG lasers have been implicated for irradiation of implant surfaces because they are poorly absorbed by the implant and do not raise surface temperature.[54] Romanos et al.[55] observed complete regeneration after decontamination of the failing implant with CO_2 laser. Birang et al.[56] reported improved clinical parameters with low-level diode laser and significant decreases in *P. gingivalis*.

Er:YAG laser has a high absorption rate in both hydroxyapatite and water. Due to this property Er:YAG laser was advocated for calculus, smear layer removal, cementum and cementum-bound endotoxins, and bone removal.[50, 57-63] Additionally, minimal heat damage is observed on the target tissue.[50, 63, 64] Schwarz et al.[59] demonstrated similar efficacy of subgingival calculus removal obtained by scaling and root planing and Er:YAG laser. Sasaki et al.[65 66] in a rat experimental model demonstrated similar osteotomy capabilities of Er:YAG compared with rotary burs with no evidence of melting or carbonization. Monzavi et al.[67] reported a maximum temperature rise of lower than 10° with Er:YAG laser and concluded that no thermal damage is to be expected with such lasers. In addition, Feist et al.[68] showed enhanced gingival fibroblasts growth on diseased root surfaces irradiated by Er:YAG laser with 60mJ/pulse compared to hand instrumentation with gracey curette.

The Er:YAG laser seems to be ideal for decontamination of implants as its primarily absorbed by water. This characteristic results in bacteria vaporization and minimal implant surface damage.[69, 70] Several studies were carried out on the effectiveness of Er:YAG laser in surface decontamination. Kreisler et al.[71] incubated *S. sanguinis* over titanium discs and irradiated them with power 60 and 120 mJ/pulse at 10 Hz. Their results reported reduction ranging from 98.3% to 99.94%. Matsuyama et al.[72] evaluated Er:YAG in six contaminated

healing abutments. They showed that Er:YAG at 30 mJ/pulse and 30 Hz was effective in plaque removal on implant abutments. Quaranta et al.[73] explored different types of implant surfaces contaminated with *P. gingivalis*. They reported values of 90.9% for titanium plasma spray implants, and 98.3% for sandblasted and etched implants. Sennhenn-Kirchner et al.[74] evaluated efficacy against *Candida albicans* incubation. They found that Er:YAG laser destroyed fungal cells to a huge degree on direct contact. Efficacy of decontamination was ranging from 59% with 80 mJ/pulse[75] to 99.94% with 120 mJ/pulse.[71] Schwarz et al.[76] in a canine study showed Er:YAG to promote re-osseointegration more than ultrasonics or plastic curettes. Nevins et al.[77] in a canine study provided histological evidence of reduced gingival inflammation with new bone formation and enhanced bone-to-implant contact in surgical treatment with Er:YAG laser. Yamamoto and Tanabe[78] reported that Er:YAG laser removed an even layer of titanium oxide from TiUnite-surfaces with limited heating of irradiated implant.

Studies evaluated efficacy of decontamination using scanning light microscopy, SEM, counting the colony forming units, bacterial smears, and photometric XTT–formazan evaluations.[48] Molecular Probes' LIVE/DEAD® *Bac*Light™ Bacterial Viability Kits provide a two-color fluorescence assay of bacterial viability. It allows investigators to measurably differentiate live and dead bacteria, even within a mixed population.[79]

Since no consensus treatment for peri-implantitis has yet emerged, there is an urgent need for quantitative evaluation of the available treatment options. This study was designed to compare the biofilm removal efficiency of Er:YAG laser, titanium brushes, and carbon fiber curettes. Our goal was to determine whether Er:YAG laser significantly improves

titanium surface decontamination. Our findings may contribute to the development of novel and efficacious treatment modalities for peri-implantitis.

Aim and Hypothesis

The primary aim of this prospective randomized controlled clinical trial was to compare the efficacy of biofilm removal from dental implant-like titanium surfaces by Er:YAG laser, titanium brush and carbon fiber curette. The hypothesis was that Er:YAG laser removes biofilm more efficaciously than titanium brush or carbon fiber curette.

The secondary aim was to evaluate the surface area percentage of live bacteria in biofilm in treatment groups using the LIVE/DEAD *Bac*Light dye stain. The secondary hypothesis was that Er:YAG laser kills biofilm bacteria in the residual biofilm more effectively than titanium brush or carbon fiber curette.

Research Design

This study was designed to investigate the efficacy of biofilm decontamination by Er:YAG laser compared to titanium brushes and carbon fiber curettes. The first phase was clinical wherein experimental subjects were recruited. Full periodontal examination was performed and alginate impressions were taken. A custom appliance that carries multiple titanium discs was fabricated. Subjects were instructed to wear this appliance for 72 hours to allow development of a natural bacterial biofilm on the disc surface. The second part of the study was done after collecting the discs from the subjects and exposing them *ex vivo* to one of four treatment groups: 1) Er:YAG laser, 2) titanium brush, 3) carbon fiber curette and 4) no treatment (control). The discs were stained with LIVE/DEAD *Bac*Light dye and the residual biofilm was visualized under fluorescence microscopy. Quantification of residual biofilm was performed using imageJ analysis software. Statistical methods were used to compare the study groups, and to determine if statically significant difference exists between the groups. The primary outcome of the study was the percent area of the titanium disc covered by the biofilm. The secondary outcome was the percent area of live bacteria in the biofilm.

Materials and Methods

This randomized clinical trial study was conducted in the department of Periodontology, Tufts University School of Dental Medicine (TUSDM). Tufts University Health Sciences Campus Institutional Review Board (IRB) approved the study design (IRB: 12380). Subjects were recruited from patients, staff, and students presented to TUSDM from January 2017 to March 2018.

Sample size calculations and randomization

A power calculation was performed with the statistical software nQuery Advisor (Version 7.0). Using the results of Schwarz et al.[80] for anticipated means and standard deviations, and assuming that the mean for the combination group was equal to the mean of the Er:YAG laser group, a sample of 8 subjects, allowing for 25% dropout with a final projected sample size of 6 (8 discs per subject, 48 total discs), was sufficient for a Type I error rate of 5% and a power over 99%.

Discs retrieved from each occlusal appliance were randomized over the 4 treatment groups using the "sample" function of the statistical software package R (Version 3.1.2), so that each subject contributed two discs to each treatment group, i.e., each subject provided two discs for the Er:YAG group, two for the titanium brush group, two for the carbon fiber curettes group, and two for the control group.

Inclusion criteria

- Adults (18 years or older).
- Presence of enough teeth to support the occlusal appliance.

- English proficiency.
- Subjects with a periodontal diagnosis of health, gingivitis, or slight chronic periodontitis. Slight chronic periodontitis is demarcated by 1-2mm loss of attachment. Subjects were not stratified based on oral health.

Exclusion criteria

- Subjects who had lost multiple teeth in the maxilla that prevents providing a stable occlusal appliance.
- Subjects exhibiting a periodontal diagnosis of moderate or severe chronic periodontitis.
- Subjects who had used antibiotics in the last six months.
- Subjects who are unable to wear an occlusal appliance due to sleep apnea or chronic obstructive pulmonary disease.
- Subjects with debilitating or uncontrolled medical conditions, including but not limited to cancers, hematologic disorders, immunosuppression, or severe cardiovascular disease or uncontrolled diabetes.
- Subjects who perform any form of smoking.
- Subjects who self-reported as pregnant.
- Subjects who are aware of allergic reactions to acrylic or titanium
- Subjects who were unable or unwilling to sign the informed consent form (ICF).
- Subjects may not participate in this study if they were concurrently participate in another research study.

Study procedures

Visit 1: Screening and consent

Table 1 illustrates the study timeline. Subjects were asked to read the informed consent form (ICF). Subjects were given full time to answer any questions. If a subject decided to participate, he or she was asked to sign the ICF. A copy of the ICF was given to the subject. Subjects were informed that they could withdraw from the study anytime they wished. They were also informed that investigators might terminate subjects if they no longer fulfilled inclusion criteria, if an exclusion criterion was met, or if they did not show up for a scheduled visit.

Medical history and demographic information was collected. To determine eligibility periodontal examination was performed according to standard of care, including measuring pocket depth, recession, attachment level, and bleeding on probing. If full periodontal examination was performed at TUSDM no more than 6 months ago, then existing records were used to determine eligibility. As periodontal measurements were not a primary outcome, existing periodontal measurements collected by other TUSDM dentists were used.

Inclusion and exclusion criteria were considered and eligibility for the study was determined at this visit. If the subject qualified for the study, alginate impression (Jeltrate Plus Dustless, Dentsply Sirona. York, PA. USA) for the maxillary teeth was taken using stock plastic trays (Plasdent Corporation, Pomona, CA. USA) according to subject's arch size in conformance with standard of care practice.

After Visit 1 (in the laboratory): impression pouring, cast, and appliance fabrication

After the visit, alginate impressions were disinfected and poured in the dental laboratory using dental stone. Subjects were not required to stay for the length of time of laboratory procedures. Dental stone was allowed to set for 45 minutes before cast retrieval. After retrieval, casts were trimmed according to laboratory standards. Titanium discs (5mm diameter, 1 mm thick, Straumann, Andover, MA. USA) were adapted on the buccal surface of the right and left premolar and molar areas with a total of eight discs per subject. The discs were secured with cyanoacrylate glue at the gingival margin.

A computerized positive pressure thermal-molding machine (Biostar, Great Lakes Orthodontics, Tonawanda, NY. USA) was used to fabricate a 1-mm thick mouth appliance on top of the cast, covering the attached titanium discs. The appliance was removed from the cast and trimmed to proper size. The discs were dislodged from the appliance and new titanium discs of the same size and count were secured in the mouth appliance disc compartments with surgical adhesive (PeriAcryl, Salvin Dental Specialties, Charlotte, NC. USA). The final shape of the appliance resembled an occlusal nightguard. The mouth appliance was disinfected with 0.12% chlorhexidine. Figure 1 shows a subject wearing the appliance.

Visit 2: Appliance delivery

At visit 2 the medical history was reviewed and eligibility criteria were re-evaluated. The appliance was inserted into the subject's mouth and adjusted for complete fitting and comfort. Intra-oral photographs were taken in frontal view of the subject's teeth and gums while wearing the appliance.

Subjects were asked to use the appliance for 72 hours continuously except during eating. Subjects were instructed to brush their teeth twice daily without the use of toothpaste and to floss once daily. Mouth rinses were prohibited. These instructions were given verbally with demonstration facing a mirror. A written instructions form was also given.

Subjects were informed of the possibility of dislodgement of the titanium discs during insertion/removal of the appliance. If the disc was dislodged, subjects had to be cautious not to swallow the disc. If that were to happen, the subjects were informed to go to the nearest emergency room. If the discs fell outside the subject's mouth, the subject was informed to not put it back and keep it aside.

Visit 3: Appliance retrieval after 72 hours

Medical history was reviewed. Eligibility criteria were evaluated. The appliance was retrieved from the subjects and stored until analysis. Biofilm removal and analysis was done ex vivo right after subject's dismissal from the chair.

After Visit 3 (in the laboratory): group assignment, experimental treatments, and measurements

After the visit, titanium discs were separated from the appliance. Subjects were not required to stay for the length of time of laboratory procedures. Care was taken not to disturb the surface biofilm. The intraoral locations of the discs were recorded. Discs retrieved from each occlusal appliance were randomized over the 4 treatment groups according to the methods described in the randomization section. Each group of discs (2 per group) were fixed over a microscope glass slides. The slides were washed once with saline using a syringe to remove food debris. The slides were stored in saline at room temperature to prevent

desiccation of the biofilm. Each treatment was done for a period of one minute. Er:YAG laser with a wavelength of 2940 nm (AdvErL EVO, Morita, Japan) was used in a non-contact mode using an energy setting of 80mJ/pulse at 20 pps using R600T tip with water spray according to manufacturer's recommendation. Titanium brush (Salvin RotoBrush. Salvin, Charlotte, NC. USA) that fits to a latch type handpiece with water spray was used for the second group. Carbon fiber curette (ImplaKlean Implant Deplaqer, PacDent, Brea, CA. USA) was used for the third group. The control group was left without treatment.

After treatment, the discs were stained using the LIVE/DEAD *Bac*Light dye Kit (Thermo Fisher Scientific, Waltham, MA. USA). An additional sterile disc was stained to serve as a negative control. Equal volumes (1.5 μl) of component A (SYTO 9 dye) and component B (propidium iodide [PI]) were combined in a microfuge tube containing 1 ml of phosphate-buffered saline (PBS). The dye mixture was added to the discs and incubated at room temperature in total darkness for 15 minutes. Two washes were done after with PBS using a 1 ml pipette to remove excess staining. *Bac*Light mounting media was used and glass coverslip was placed. The discs were observed using a fluorescence microscope equipped with FITC/TRITC filter sets (Zeiss Axiovert 200, Carl Zeiss AG, Oberkochen, Germany). Images with 4X magnification were taken at the center of the discs with FITC and with TRITC filters without changing the location of the disc. SYTO 9 images (green) expose the SYTO 9 dye that has the ability to enter all cells, live or dead using the FITC filter. The PI images expose the propidium iodide that only enters cells with compromised membranes (damaged or dead cells) using the TRITC filter.

Image analysis

Using digital image analysis software (ImageJ, NIH), the process started by separating the color channels for each image (red, green, blue). Respective color for SYTO 9 (green) and PI (red) were chosen for analysis. Afterwards, the image was converted to a binary black and white image. It was noted while observing the negative controls (sterilized discs) under fluorescent microscope that the disc material withholds some of the dye material. Therefore, an effort was made to eliminate this background noise digitally by applying a color threshold. For the SYTO 9 images, threshold was determined by eliminating 99.8% of the color in negative controls (n=6). The mean threshold of the six negative controls was equivalent to 32 units on a 256 scale. This threshold was applied for all SYTO 9 images of the study. Subsequently, percent coverage of the biofilm-covered area (signal intensity >32 on a 256 scale) was calculated. For the PI images, the threshold was developed by adjusting the Pi percent coverage to be less than the SYTO 9 counterpart. After that, the maximum threshold found was applied to the whole PI images. This threshold was equivalent to 69 on a 256 scale. The percent coverage of SYTO 9 image was subtracted by the PI image to give the percent coverage of live bacteria in biofilm, which constitutes the secondary outcome of the study.

Statistical analysis

Descriptive statistics (means, standard deviations, medians, inter-quartile ranges, minima, and maxima) were computed by group. The Shapiro-Wilk test was conducted to evaluate the normality of data. Due to non-normally distributed data, Friedman's test was used to compare the groups; the Wilcoxon signed-rank test alongside the Bonferroni correction was performed for post-hoc tests. $P<0.05$ was considered statistically significant, with the exception of tests in which the Bonferroni correction was used. The analysis was conducted using SPSS Version 24.

Results

Study subjects

A total of 8 subjects with 64 titanium discs were included in the study. There were 16 discs for each group. There were 3 males and 5 females with an age range of 29 to 34. The subject age mean ± SD was 31.63 ± 1.58 years. In terms of race, there were 4 (50%) Asians, 2 (25%) Hispanics, 1 (12.5%) Indian, and 1 (12.5%) White. Table 2 illustrates demographics of subjects. No adverse reactions were observed in study except disc dislodgement from the appliance (n=4). One disc was dislodged with subject #1, and 3 discs with subject #3. All dislodged discs were brought back by the subjects. Dislodged discs were excluded from data analysis.

SYTO 9 biofilm imaging

A total of 59 images were obtained for evaluation. In addition to the images of the 4 discs dislodged from subjects' appliances, 1 image was not included due to improper magnification. Thus, 5 in total were missing.

The percentage of titanium disc area covered by biofilm as determined by the SYTO 9 signal for the control group ranged from 46.68% to 98.79% (Mean ± SD: 74.03 ± 21.61), whereas the biofilm area in the Er:YAG group ranged from 12.62% to 73.72% (Mean ± SD: 32.78 ± 24.00%). The biofilm area in the titanium brush group ranged from 0.46% to 35.11% (Mean ± SD: 11.80 ± 10.29%). Lastly, the biofilm area in the carbon fiber curette group ranged from 1.75% to 52.73% (Mean ± SD: 20.14 ± 19.15%). Table 3 shows descriptive statistics for SYTO 9 images. Figure 2 displays examples of SYTO 9 images.

The Shapiro-Wilk test was used to evaluate the normality of the data. Results were statistically significant in 2 of the groups ($p<0.05$), indicating a non-normal distribution. Subsequently, Friedman's test was used to compare the 4 groups and showed a statistically significant difference between the groups ($p<0.001$). The Wilcoxon signed-rank test with Bonferroni correction was used in post-hoc testing. Statistically significant differences were observed between the control group and each of the 3 treatment groups ($p=0.008$). Comparisons between the treatment groups were not statistically significant (Er:YAG-brush $p=0.078$, Er:YAG-curette $p=0.383$, brush-curette $p=0.313$). Figure 3 is a side-by-side boxplot depicting analyses of SYTO 9 staining images.

Propidium iodide (PI) biofilm imaging

Similarly to SYTO 9 images, 59 PI images were available for analysis. As mentioned in Materials and Methods, the live biofilm percent coverage was obtained by subtracting the values of Pi images from SYTO 9 images. Figure 4 displays PI images of the same discs shown in Figure 2.

The percentage of titanium disc area covered by live biofilm for the control group ranged from 0.73% to 53.72% (Mean ± SD: 33.56 ± 19.21). The live biofilm area for the Er:YAG group ranged from 10.36% to 59.66% (Mean ± SD: 24.28 ± 16.31). The live biofilm area for the titanium brush group ranged from 0.40% to 33.02% (Mean ± SD: 10.91 ± 9.86). Lastly, the live biofilm area for the carbon fiber curette group ranged from 1.65% to 50.42% (Mean ± SD: 19.68 ± 18.68). Table 4 shows descriptive statistics for PI images.

As with the data from the SYTO 9 images, the Shapiro-Wilk test was statistically significant in 2 of the groups ($p<0.05$), indicating non-normality. Subsequently, Friedman's test was used to compare the 4 groups in live biofilm. No statistically significant difference was found ($p=0.08$). Figure 5 is a side-by-side boxplot depicting analyses of PI staining images.

Discussion

The objective of this study was to compare the percentage surface area of residual biofilm after subjecting the titanium discs to Er:YAG laser, titanium brush, or carbon fiber curette. Er:YAG, titanium brush, and carbon fiber curette were all effective in removing biofilm compared to control. However, no statistical significance was found between the three treatment groups. Nonetheless, differences were observed between the groups that may imply clinical significance. The rank order of efficacy in biofilm removal was in favor of titanium brush, followed by carbon fiber curette, and lastly Er:YAG laser. The small sample size of this study may be limited in showing statistically significant differences between the groups.

A major issue faced in this study was using flat titanium discs to simulate peri-implantitis with threaded implants. The outcome of this study showed that Er:YAG may not be better on flat surfaces. However, literature suggests it may provide better results on threaded implants since it has better access to the depth of threads, which may be unreachable by other treatment methods. Hakki et al.[81] examined failed threaded implants treated with several methods, including Er:YAG, using SEM. They concluded that Er:YAG was superior to different curettes, titanium brush, ultrasonic scalers, and air abrasives. They also hypothesized that Er:YAG will have more accessibility in situations of intrabony defects. This also indicate better accessibility of Er:YAG in clinical situations. Takasaki et al.[82] in an experimentally induced peri-implantitis animal study reported effective implant surface debridement by Er:YAG laser with greater bone-to-implant contact histologically compared to plastic curette treated group. It was noted that Er:YAG was used in a contact mode in contrast to our study. Human studies were also reported in the literature to evaluate

clinical parameters. Schwarz et al.[83] tested on 20 patients the efficacy of Er:YAG compared to plastic curettes and chlorhexidine digluconate 0.2% in terms of BOP and CAL. They concluded that the Er:YAG group resulted in statistically significant reduction in BOP after 6 months. However, no significant differences were observed between the 2 groups in terms of CAL. All of the formerly mentioned studies were done on threaded implants supporting the ability of Er:YAG to decontaminate threaded implants. Our study supports this assumption as well; but however, did not support superiority of Er:YAG over titanium brush or carbon fiber curette.

Several other studies have also compared the efficacy of Er:YAG on flat titanium discs similar to our study. Eick et al.[84] in an in vitro study cultivated biofilm on roughened SLA titanium specimens. Er:YAG resulted in statistically significantly superior biofilm removal compared to titanium curettes with or without photodynamic therapy. In contrast to our study, the treatments were done in an artificially created periodontal pocket, which may have provided superior access to Er:YAG. Tosun et al.[75] carried out a similar study on Er:YAG and SLA titanium discs contaminated with *S. aureus*. They reported the ability of Er:YAG laser to eliminate 99% to 100% of bacteria. This higher reduction rate compared to our study may possibly be attributed to the low single species biofilm incubation time of 10 minutes in Tosun et al. compared to our 3 days intraoral multispecies biofilm development. Schwarz et al.[80] in another study of Er:YAG laser on similar titanium discs placed in subjects' mouths for 24 hours reported Er:YAG is most suitable for the removal of biofilms grown on SLA titanium implants compared to ultrasonic device or plastic curettes with chlorhexidine. Differences between this study and ours include subjects wearing the stent for 24 hours compared to 72 hours in our study. Also, the positions of titanium discs

were placed on the palate instead of the buccal position in ours. Additionally, Er:YAG was used in contact mode rather than non contact mode in our study. Lastly, they used light microscope instead of fluorescent microscope used in our study.

With all the abovementioned positive studies supporting Er:YAG, other studies reported inferiority or no differences compared to other methods of decontamination. Saffarpour et al.[85] in a lab study on threaded implants cultured strains of *A. actinomycetemcomitans* and treated them with 4 treatment groups including Er:YAG, photodynamic therapy, diode laser, and chlorhexidine 2%. They reported a statistically significant reduction compared to the control group. However, they reported Er:YAG as lowest in reduction of colony forming units. Schmage et al.[86] in a study on flat SLA titanium discs reported ultrasonic devices and air abrasives provided better cleaning scores on SEM than Er:YAG laser. Schwarz et al.[89] in a human clinical study evaluated surgically the clinical parameters of decontamination of Er:YAG to plastic curettes and cotton pellets with saline. In 4 years outcome, they reported superiority of plastic curette protocol over Er:YAG in terms of BOP and CAL.

Several studies have reported the bactericidal effect of Er:YAG laser. Tosun et al.[75] reported the capacity of Er:YAG to almost eliminate 100% of *S. aureus* contaminated SLA titanium discs. Kreisler et al.[71] evaluated the antibacterial effect of the Er:YAG laser on *S. sanguinis* contaminated on titanium discs with several surface morphologies. The antibacterial efficacies ranged from 98.39% to 99.6% with the 60-mJ laser. They determined antibacterial effect of Er:YAG can be achieved even at low energy densities. In our study, propidium iodide images were taken to visualize cells with compromised membranes (damaged or dead cells). No statistically significant differences were observed between the

groups. Efforts were made to maintain the discs in a moist environment until the staining stage. However, dehydration of samples may have contributed to the insignificant results. Moreover, it was noted higher intensities of the PI dye in some of the discs. This may be explained either by prolonged incubation periods or excessive concentration of the PI dye, or insufficient washing afterwards. Also, contamination with salivary components such as red or white blood cells may have added to this intensity. Furthermore, it was reported by manufacturer that some live cells might take up PI by engulfment. Efforts were made to adjust this intensity using the digital software program to reach the aforementioned results.

One of the limitations of the study is the inability of fluorescence microscopy to detect surface alterations of titanium discs after treatments. Several studies have reported damage or remnants to titanium surfaces. Hakki et al.[81] reported that titanium brushes left scratches on the implant surface, while, carbon fiber curettes left carbon remnants. Another concern would be the application time period of different treatments. In our study we standardized 60 seconds for each treatment. Whereas in other studies such as Hakki et al.[81] they used 120 seconds for Er:YAG laser. This may be a source of another bias since no protocol was found of recommended times periods of use. Moreover, the use of Er:YAG laser equipment is technique-sensitive and influenced by the operator's level of experience. Another drawback of the study was that the titanium discs' material display some degree of non-specific stains when observed under fluorescence microscopy. This problem has been dealt with by digitally developing a color threshold based on visual comparison as described in the Materials and Methods. This digital alteration may have affected the final outcomes of the study.

Efforts were made in this study to mimic clinical conditions as much as possible by developing biofilm inside subjects' mouth. Efforts were also made to ensure randomization and standardization of the in vitro portion of study. Thus, results may be indicative of results expected from each treatment. More controlled studies are needed before definite conclusions and clinical protocols can be established.

Several drawbacks were faced when evaluating Er:YAG in the scientific literature such as high heterogeneity in studies and most were in vitro studies. Also, inconsistency was observed in titanium surfaces used in studies and inconsistency of setup settings of Er:YAG. In some studies[70, 71, 87], Er:YAG shows very promising results. However, many studies failed to reach conclusive results and recommendations. Therefore, Er:YAG laser therapy is still considered an experimental therapeutic when it comes to peri-implantitis treatment. More controlled randomized clinical trials are recommended to reach a decision on whether Er:YAG is effective and should be the gold standard of care in peri-implantitis cases.

Conclusion

Within the limitations of the study, Er:YAG laser was found to be an effective method in reducing the bacterial biofilm on titanium discs. However, on a threadless titanium surface Er:YAG laser did not exhibit significantly greater efficacy in biofilm removal compared to commonly used titanium brushes or carbon fiber curettes.

APPENDICES

Appendix A: Tables

Appendix B: Figures

Appendix A: Tables

Table 1 Steps flow chart

Appointment / Procedures	Visit 1 Screening/ Baseline	Visit 2 Baseline (Treatment Day 0)	Visit 3 Retrieval (Treatment Day 3)
Consent Form	X		
Demographics	X		
Medical/Dental History	X	X	X
Inclusion/Exclusion Criteria	X	X	X
Oral Examination	X		
Digital Photography		X	
Alginate Impressions	X		
Compensation			X
Appliance Delivery		X	
Appliance Instructions		X	
Appliance Retrieval			X

Table 2 Study demographics

Demographic		Number	Percentage
Males		3	37.5
Females		5	62.5
Age		Mean ± SD: 31.63 ± 1.58	
Race	Asian	4	50
	Hispanic	2	25
	Indian	1	12.5
	White	1	12.5

Table 3 Biofilm covered area (%) with SYTO 9 staining

	Mean (SD)	Median (IQR)	Minimum	Maximum	P-value*
Control	74.03 (21.61)	71.79 (44.65)	46.68	98.79	<0.001
Laser	32.78 (24.00)	23.55 (43.69)	12.62	73.72	
Brush	11.80 (10.29)	10.13 (6.97)	0.46	35.11	
Curette	20.14 (19.15)	11.87 (33.75)	1.75	52.73	

* Value given in table represents the p-value of Friedman's test. In post-hoc tests (Wilcoxon signed-rank test with Bonferroni correction) comparisons between the control group and all other groups were statistically significant (p=0.008). Comparisons between the treatment groups were not statistically significant.

Table 4 Live biofilm covered area (%) with PI staining

	Mean (SD)	Median (IQR)	Minimum	Maximum	P-value*
Control	33.56 (19.21)	38.14 (34.70)	0.73	53.72	0.08
Laser	24.28 (16.31)	18.43 (19.22)	10.36	59.66	
Brush	10.91 (9.86)	9.29 (8.12)	0.40	33.02	
Curette	19.68 (18.68)	11.76 (48.77)	1.65	50.42	

* Value given in table represents the p-value of Friedman's test. No statistically significant difference was noted between the groups.

Appendix B: Figures

Figure 1 Subject wearing the study appliance with titanium disc attached

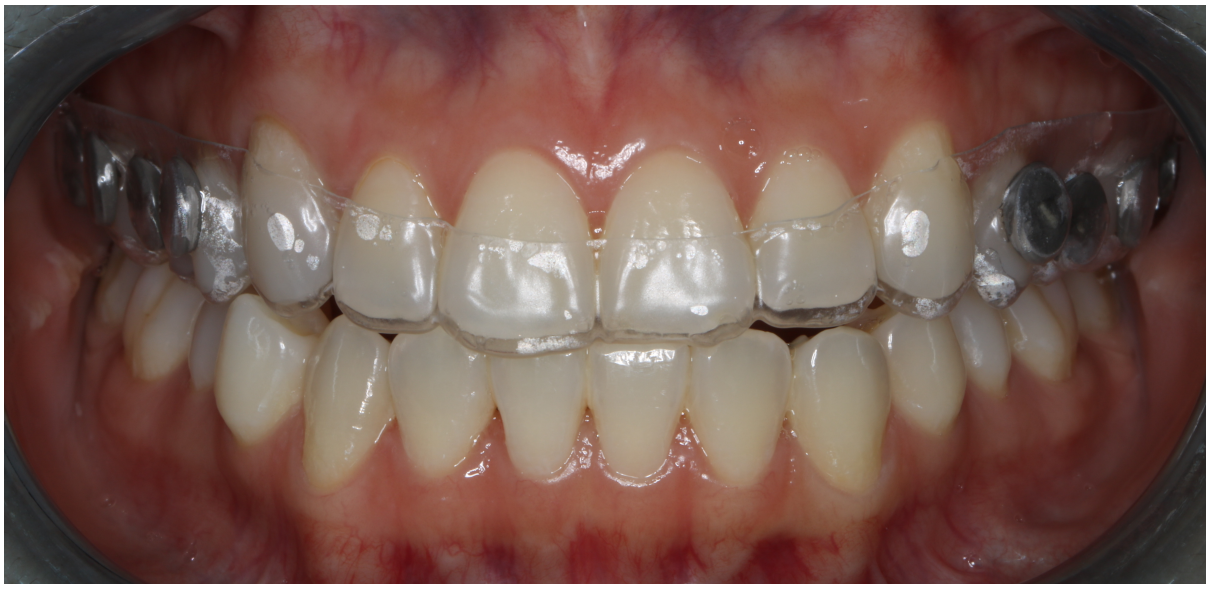


Figure 2 Examples of SYTO 9 images. A) control; B) Er:YAG; C) titanium brush; D) carbon fiber curette; E) negative control.

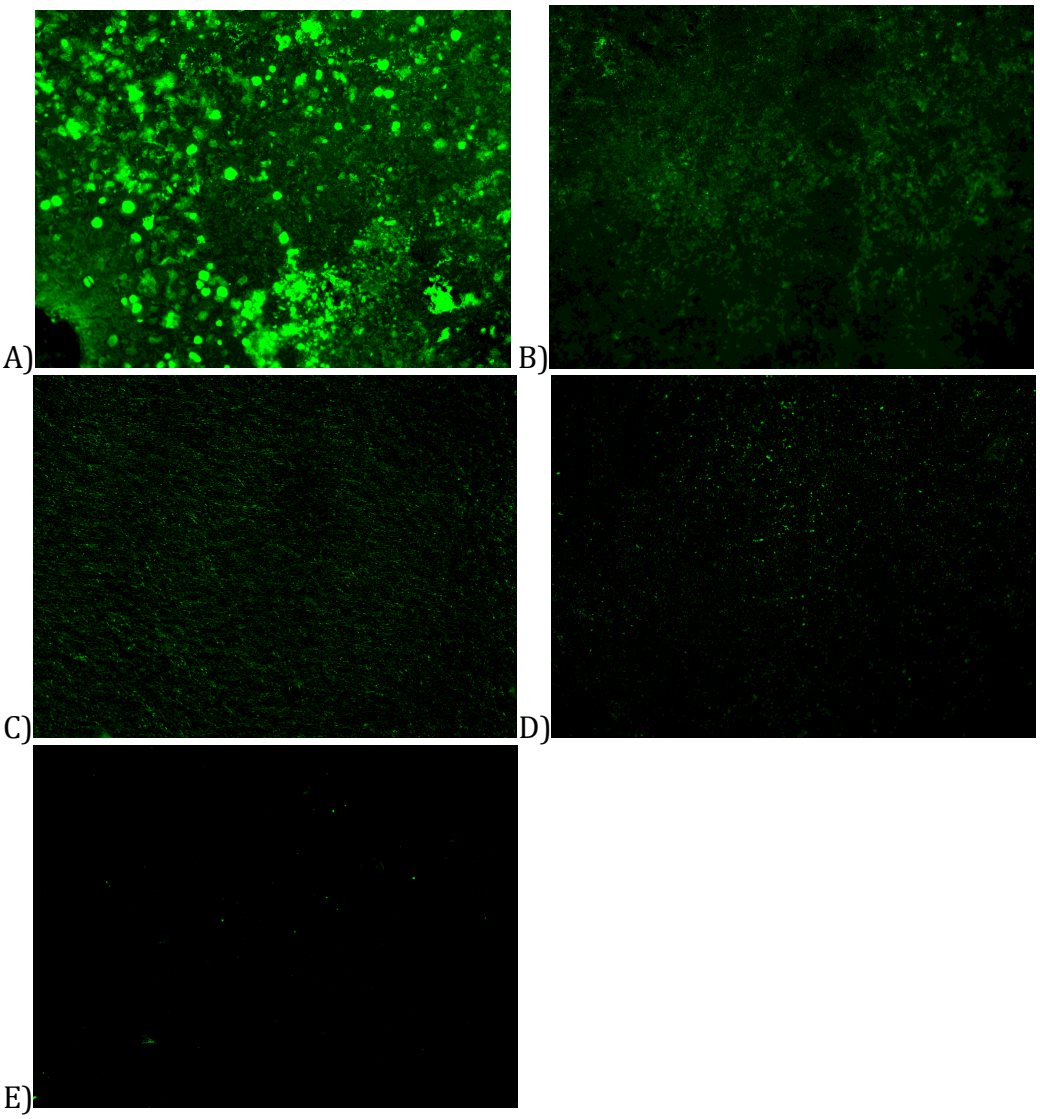


Figure 3 Side-by-side boxplots depicting analyses of SYTO 9 staining images

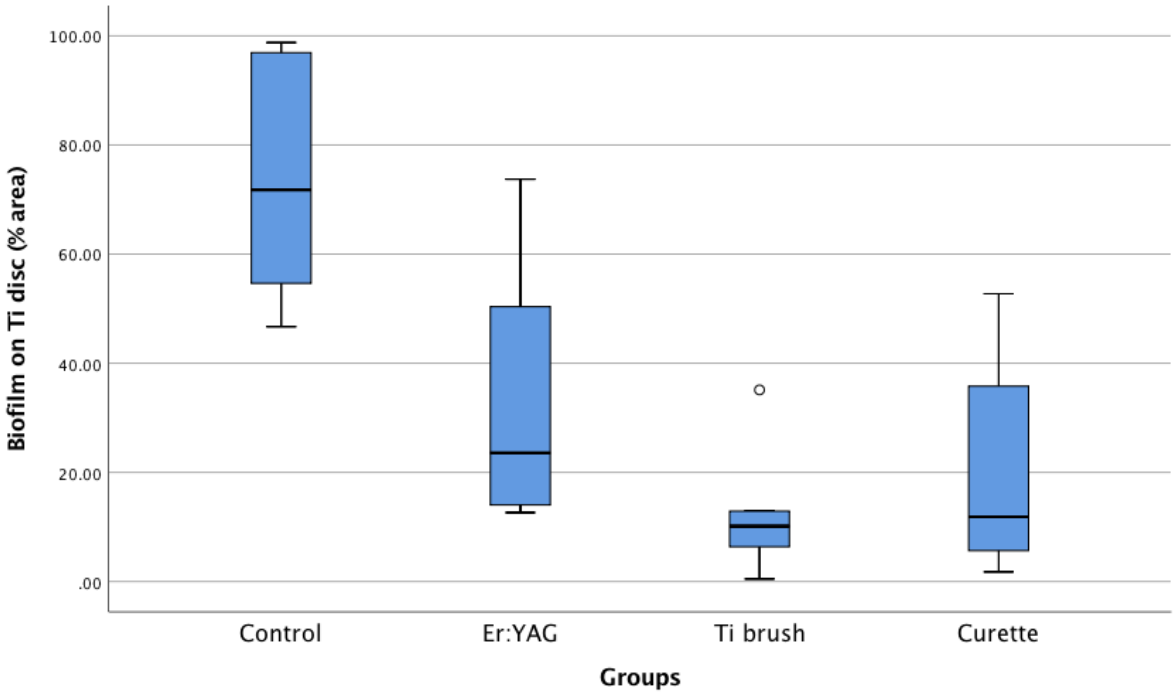


Figure 4 Examples of Pi images from their SYTO 9 counter part shown in figure 2. A) control; B) Er:YAG; C) titanium brush; D) carbon fiber curette.

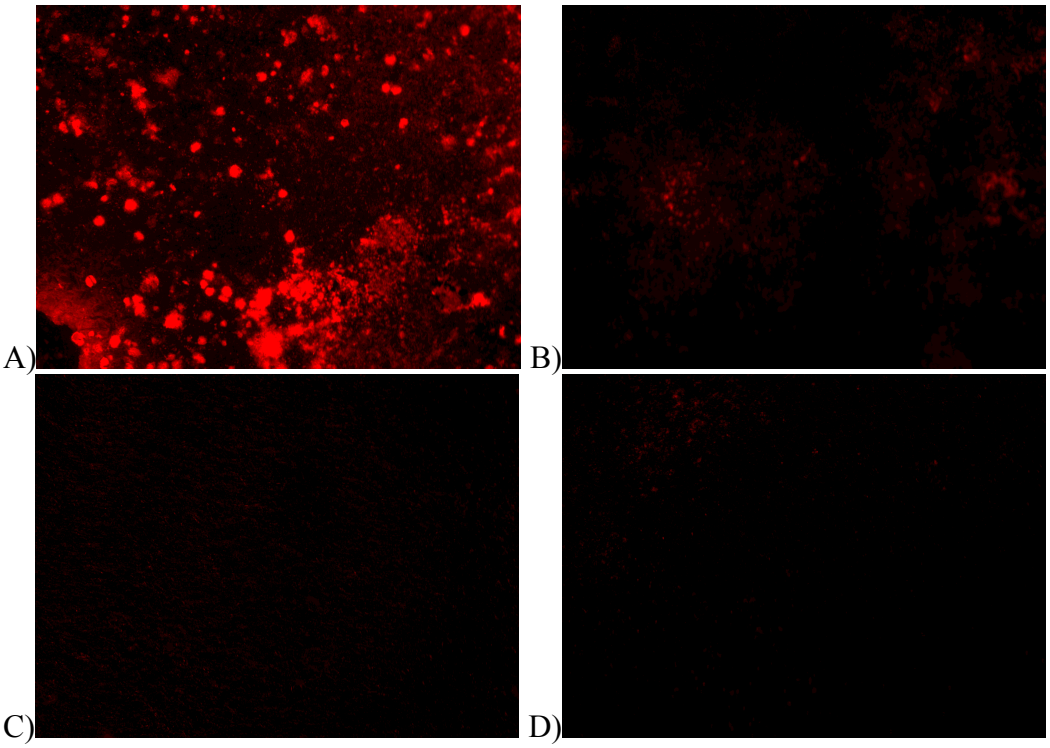
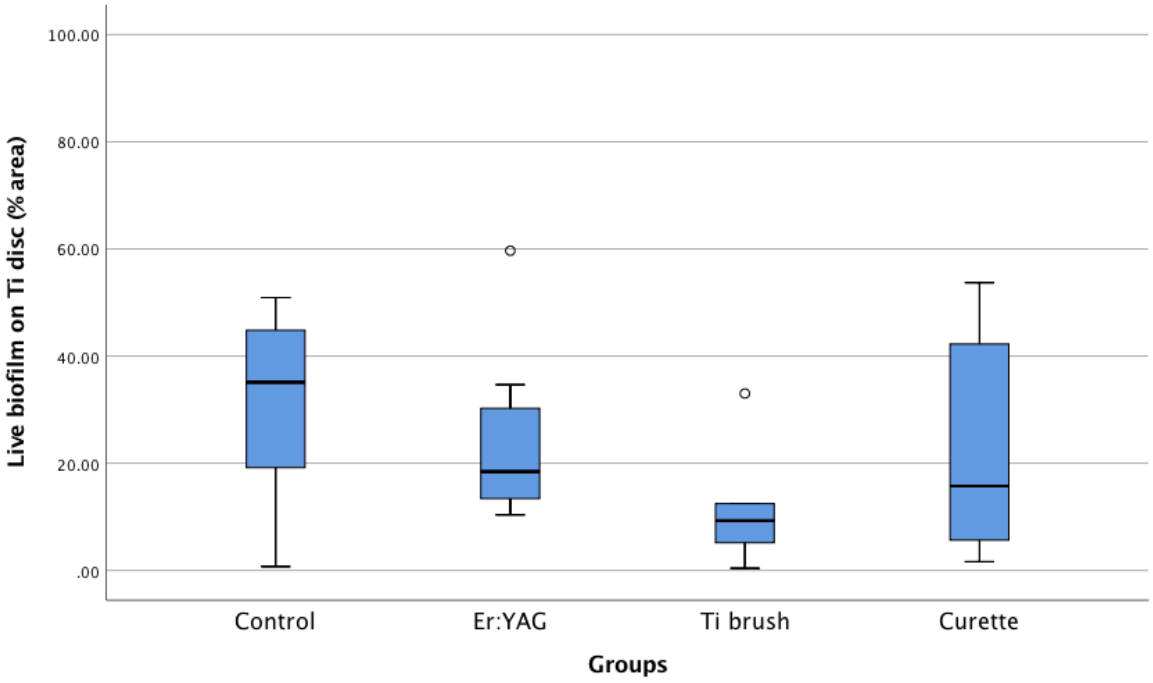


Figure 5 Side-by-side boxplots depicting analyses of PI staining images.



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