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Investigation of the antibacterial effect of laser irradiation and chemical agent on human oral biofilms contaminated titanium discs

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ABSTRACT

Introduction: A main challenge in treatment of peri-implant disease is the effective decontamination of the implant surface. This challenge has always been a problem, associated with the treatment of these diseases with regard to the difficulty in removing and eliminating bacterial biofilm from the surface of dental implants, especially rough surfaces. The aim of this in-vivo study was to evaluate the effect of five different antimicrobial methods in reducing bacteria adhering to titanium surfaces.

Materials and methods: In the present in-vivo study, the contaminated discs, except for the negative control group, randomly underwent one of five treatments: Erbium: Yattrium Aluminum Garnet (Er-YAG) laser, plastic curette, 0.12% chlorhexidine, aPDT, and 810 nm diode laser. A pectrophotometer was used to measure Optical Density (OD) in case of aerobic microorganisms. Colony-Forming Units (CFUs) were used for anaerobic bacteria. Then, all the analyses were carried out at a significance level of $\alpha = 0.05$ through SPSS software.

Findings: One-way analysis of variance (ANOVA) of aerobic bacteria showed a significant difference among 6 groups in terms of OD variations during a 0–24 h time interval ($P < 0.001$). The results of Kruskal-Wallis test were used to investigate the effect of study methods on anaerobic bacteria after 48 h, and the results showed a significant difference among 6 groups in terms of CFUs ($P < 0.001$).

Conclusion: The results of the present study showed that all five mechanicals (plastic curette), chemical (CHX), laser (810 nm diode and Er: YAG), and aPDT methods could reduce oral biofilms from roughed surfaces of titanium discs. Er: YAG laser and plastic curette had the highest and the lowest effects respectively.

1. Introduction

Implant treatment is widely used in the world and gradually becomes the "gold standard" of prosthetic treatments [1,2]. However, the occurrence of peri-implant diseases is also increasing [3]. Among the etiologic factors for the peri-implant diseases, the bacterial adhesion to the implant surface is a major reason for implant-related problems that can lead to implant failure [4]. Therefore, in the treatment of periodontal diseases, the elimination of this bacterial biofilm and the peri-implant calculus is critical to the prevention and treatment of peri-implant inflammations [5]. The main challenge in treating peri-implant diseases is the effective decontamination of the implant surface [6]. Many methods have already been proposed to decontaminate the implant surface, and thus, to treat the peri-implant diseases. They include the use of an air-powered abrasive system, citric acid, mechanical cleaning of metal and plastic curettes and ultrasonic devices [7–9], along with topical and systemic antibiotics [10]. However, none of these methods were able to completely eliminate the bacteria from the implant surface [11]. Non-metallic tools were unable to remove the calcium deposits and bacterial plaques from the implant surface [12]. Air-powered- abrasive systems effectively decontaminates the implant surface, although it results in certain microscopic surface changes and there are some limitations [13]. Chlorhexidine (CHX) has been widely used as an antiseptic material for the treatment of periodontal and peri-implant diseases. None of the previous studies have shown any evidence indicating that CHX has an advantage over other

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decontaminants. However, its microbiological benefits have been reported in all studies [14]. In recent decades, high-power dental lasers with thermal effects and antibacterial photodynamic therapy (aPDT) have been used for decontamination of dental implant surfaces to treat peri-implant diseases. These lasers, which include Nd:YAG, Diode, Co2, and Erbium family [15], have been reported to be effective in cleaning implant surfaces [16]. However, in-vitro studies have shown that, among various lasers, only CO2, Diode, and Erbium family lasers may be suitable for radiation to the implant surfaces because their wavelengths are absorbed slightly by titanium, and thus, the implant's body temperature will not increase significantly [17,18]. An important point regarding the use of lasers in the treatment of peri-implant diseases is their ability to remove bacterial biofilms, calculus and debris from the dental implant surface in addition to their bactericidal property. It thus seems that only Erbium family lasers such as Er: YAG, ErCr: YSGG have such capability [18–20]. CO2 and diode lasers should be used with mechanical cleansing tools [21–23]. Er.YAG laser is used as an effective and safe device for the treatment of peri-implantitis with regard to its high bactericidal potentiality and minimal changes made in the morphological characteristics of the fixture [18]. Recently, diode lasers with varying wavelengths have been increasingly used to treat periodontal and peri-implant diseases. Clinical research provides evidence that the diode laser with photo thermal effects can be used as an effective tool for the treatment of periodontitis and peri-implantitis [24]. Another method of implant surface decontamination is using antibacterial photodynamic therapy (aPDT) with conventional mechanical therapy. aPDT is a non-invasive method based on the use of a photosensitizer (PS), usually a dye, which is activated by light with a specific wavelength and can destroy bacteria by creating free oxygen radicals [25,26]. Several types of dyes have been used as photosensitizer, such as toluidine blue, radachlorin indocyanin green, methylen blue, porphyrin and its derivatives [27]. These dyes can be activated with an optical source within the visible red spectrum (630–670 nm) or infrared range (810 nm) [28,29]. Many studies have shown the bactericidal effects of aPDT using photochemical effects as an adjunctive therapy, along with conventional treatments without any damage to the titanium implant surface [30–32]. Considering that none of the implant roughed surface decontamination methods have been definitely confirmed so far, the aim of this in-vivo study was to evaluate the effect of five different antimicrobial methods in reducing the bacteria sticking to SLA titanium surfaces.

2. Material and methods

In this in-vivo study, 6 patients with mild to moderate periodontitis (3 men and 3 women) with an age range of 34–65 years and a mean age of 49.5 years, were voluntarily enrolled in the study. The consent form was signed by the patients before the study. The inclusion criteria included mild to moderate periodontitis, based on clinical examinations and radiographic evaluation, being non- smokers, no-use of antibiotics or antibacterial mouthwashes in the last 12 months, and having good general health. In this study, 72 (n = 72) titanium discs (Snucone Co., Daegu, Korea) with a roughed surface of SLA type and dimensions of 5.3*1.5 were used. Discs were later divided into two control groups and four experimental groups (n = 12 per group) (Table 1).

Table 1
Groups studied.

Groups (n = 12per group)	Decontamination Method
Group 1 (Neg Con)	Not decontaminated
Group 2 (Pos Con)	Er:YAG Laser
Group 3	Plastic Curette
Group 4	CHX 0.12% + Plastic Curette
Group 5	aPDT + Plastic Curette
Group 6	Laser 810 + Plastic Curette

3. Contamination stage

In order to contaminate the surfaces of the discs with the oral biofilm, patients' maxilla was first molded and an intra-oral maxillary splint was then prepared [33,34]. Then, six discs and a total of 12 discs were fixed on each side of each splint using glue wax at the buccal surface of the lateral teeth to the second molar. Before being inserted in the patients' mouths, discs were washed with acetone fluid, and then, normal saline and sterilized in an autoclave at a temperature of 121 °C for 15 min [35]. Patients were asked to hold their splint in their mouths for 24 h [33,36,37], and remove it from their mouth only during the time to eat and drink and put in a phosphate buffered saline (PBS) solution, which had already been handed over to them in a sterile container at the same time. They were also requested not to use toothbrush, toothpaste or mouthwash, and wash their mouth only with tap water after each meal.

4. Decontamination stage

After being removed from the patient's mouth, the splint was first washed using sterile normal saline and rinsed gently so as to wash bacteria or free debris. Then, the following methods were randomly and in a blinded manner carried out on each contaminated disc, except for the negative control group.

1-Negative Control Group

No action was taken to decontaminate the surface of contaminated discs in this group.

2-Er-YAG Laser Group

An Er: YAG laser device (Fotona, Fidelis plus, Ljubljana, Slovenia) with a wavelength of 2940 nm was used. Laser parameters were set at 100 mJ/pulse (15.7Jcm2), 10 Hz [37,38]. The laser beam was delivered onto the implant surfaces by an optic fiber tip with a diameter of 900 μm under water irrigation (5 ml / min) at a distance of 0.5–1 mm from the disc surface with a 900 radiation angle and up-and-down and side-to-side motions. It was in such a way that all of the disk surface areas were radiated in an overlapping manner. Radiation time was considered to be 1 min [16,17,24].

3-Plastic curette group

A plastic curette (Implacare TM, Hu-Friedy, Chicago, IL) was used to perform scaling on the disk surface and remove oral biofilm for one minute. The curette- disc surface contact angle was adjusted to 70°. At the end of the mechanical debridement, each disc was washed with a sterile physiological serum of 5 ml [33]. In addition, the plastic curette was also used in other studied groups, except for the Er: YAG group for mechanical debridement, prior to each method in the same way. It should be noted that all steps were calibrated and performed by a trained expert.

4-Chlorhexidine (CHX) Group

First, a mechanical debridement was performed by curettes, and the surface of each was cleaned by a sterile cotton pellet, impregnated with 0.12% chlorhexidine (Lacer SA Barcelona, Spain) pericoxidine as burnishing for one minute. It was ultimately washed by sterile normal saline (5 ml) (14)

5- aPDT Group

After performing mechanical debridement with plastic curette, each disc was immersed in a container containing the photosensitizer Lasers HF Paro-PDT (Hager & Werken, Gmbh&Co.KG DuiSburg, Germany), along with tolonium chloride, for 3 min, according to the manufacturer's instruction. Then, it was exposed to a 660-nm laser diode (, ASTAR Co, Bielsko-Biala, Poland) polaris for 1 min and was set perpendicular to the disk surface with a power of 40 mw [31]. The probe tip was kept at one cm from the surface of each disk in such way that the spot size of 6 mm in diameter and the energy density of 5 J/cm2 were created on the discsurface.

6-810-nm diode laser group

Radiation was carried out by 810-nm GaAlAs laser having a power

of 1 W (Fox A.R.C. Laser, Gmbh Germany) in the form of continuous wave (CW) method of radiation, and the time was completely similar to group 2 (Er:YAG).

5. Laboratory stage

Sampling was performed on the disc surface immediately after the decontamination process. An anaerobic culture was performed by first taking the microbial specimen from the surface of each disc by a sterile swab, which was then transferred to an anaerobic culture medium. For this purpose, plates containing 5% of (Colombia-Agar & Hemin & Vitk & Blood) were used. When the sampling process was completed, all the plates were placed in an anaerobic culture medium jar. The jar was opened and the colony-forming units / CFUs were counted for each of the studied groups 48 h later. However, in the case of aerobic culture media, the sterilized swab was used to carry out the sampling on the surface of each disc and the specimens were transferred to test tubes containing Tripotocase Soy Broth (TSB) solution. Then, the test tubes were sent to the microbiological laboratory to determine the OD. The degree of OD of the bacterial suspension was measured at 0 and 24-h time interval with a wavelength of 620 nm in TSB using a spectrophotometer (PG Instrument Ltd England). Aerobic bacteria-containing tubes were kept in the incubator at a temperature of 37 °C at the above time intervals [39–41]. It should be noted that in order to blind the study process in the laboratory stage, each studied group was encoded and identified by the letters A to F in such a way that the observers were blind concerning the groupings and interventions.

6. Data analysis stage

All analyses were carried out at the significance level of $\alpha = 0.05$ through SPSS ver. 22 (IBM, Armon, NY, USA). In order to compare the OD variation, Kolmogorov-Smirnov test was used to check normality of data distribution, and Levene's test ($P > 0.05$) was also applied to investigate the data homogeneity ($P < 0.05$) through one-way ANOVA and post-Hoc Scheffe method. To compare the number of colonies in the groups and considering the lack of normal data distribution in the Kolmogorov-Smirnov test ($P < 0.05$), Kruskal-Wallis Test and Mann-Whitney Test were used with regard to the Dunn test and BonFerroni correction for the significance level.

7. Results

Spectrophotometric changes at 0 and 24-h time interval were used to investigate the effect of the studied methods on aerobic microorganisms. One-way analysis of variance (ANOVA) showed that significant difference between the 6 groups in terms of changes occurred during 0 and 24-h interval ($P < 0.001$). Scheffe's test was performed to compare methods in a pair wise manner (Table 2).The results showed that there is a significant difference between manual curette with CHX, aPDT and Er: YAG, as well as between the 810-nm laser with aPDT and Er: YAG. The important point is that the results of aPDT and Er:YAG methods are very similar to each other and do not differ significantly (Fig. 1). Colonial-Forming Units (CFUs) were used to investigate on the effects of study methods on anaerobic microorganisms 48 h later. Kruskal Wallis test showed a significant difference between 6 groups in terms of level of CFUs ($P < 0.001$) (Fig. 2). Also, Mann-Whitney test was performed to compare the groups. The results of the pairwise comparisons of groups show that significant difference between manual curette with all other methods and it has a very weak effect on the decontamination of anaerobic bacteria. There is a significant difference between the 810 nm laser with the other four groups; in the sense that it has a better result than manual curette; but it has lower effect than CHX, aPDT, and Er:YAG. However, there was no significant difference between the three methods of CHX, aPDT and Er: YAG and had very similar their effect on anaerobic bacteria (Table 2).

Table 2
Comparison of the effect of different decontamination methods on aerobic and anaerobic bacteria.

Studied groups	OD variations during 24 hours (Aerobic bacteria)	CFUswithin48 hours (AnaerobicBacteria)
Curette – Co-N	< 0.001	1.00
Laser810 – Co-N	< 0.001	< 0.060
CHX– Co- N	< 0.001	< 0.001
aPDT– Co- N	< 0.001	< 0.001
Er-YAG– Co- N	< 0.001	< 0.001
Laser 810 – Curette	0.762	0.060
CHX– Curette	0.01	< 0.001
aPDT– Curette	< 0.001	< 0.001
Er-YAG– Curette	< 0.001	< 0.001
CHX– Laser 810	0.001	< 0.001
aPDT– Laser 810	0.007	< 0.001
Er-YAG– Laser 810	0.000	< 0.001
aPDT– CHX	0.404	0.494
Er-YAG– CHX	0.164	0.04
Er-YAG– aPDT	1	0.01

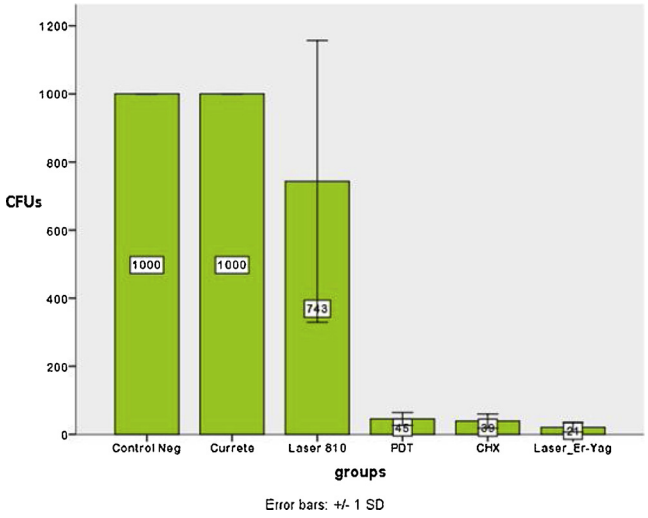

Fig. 1. Comparison of the effects of decontamination methods on the number of aerobic bacteria. OD = Optical Density.

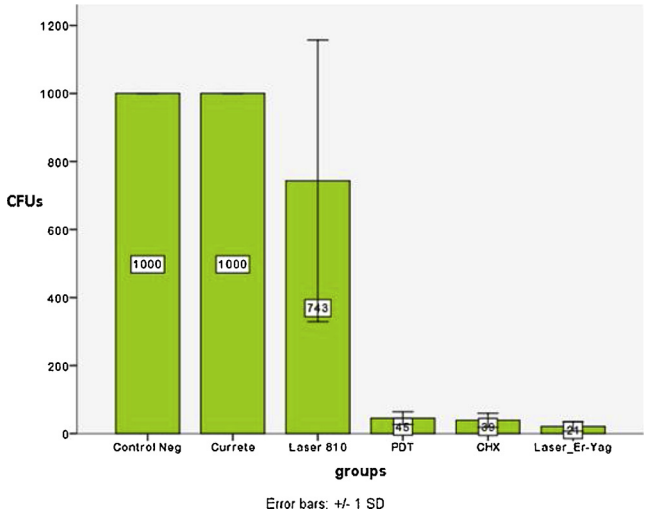

Fig. 2. Comparison of the effects of decontamination methods on the number of anaerobic bacteria. CFU = Colony-Forming Unit.

8. Discussion

The aim of this study was to assess the potential of several mechanical, chemical, laser and aPDT methods for decontamination of roughed titanium surfaces contaminated by oral bacterial biofilms. For this purpose, different biofilm models have been tested and of bacteria and the surface of the dental implants [38,39], it provides the opportunity to evaluate implant surfaces in real clinical conditions [33]. The results of the present study showed significant difference between treatment groups with negative control in terms of OD variations in the aerobic bacteria group during 0 to 24-h interval ($P < 0.001$). Also, the results of investigating the effect of the studied therapeutic methods on anaerobic microorganisms after 48 h and comparing CFUs between 5 treatment groups with negative control group showed that there was no significant difference between the plastic curette and 810-nm laser groups with negative control group ($P > 0.05$); but there is a significant difference between the three groups of Er:YAG, PDT and CHX lasers with negative control group ($P < 0.001$) (Table 2). The comparison of all treatment methods in both groups of aerobic and anaerobic bacteria showed that Er: YAG laser and plastic curette had the highest and lowest effect on the cleaning of titanium surfaces, respectivly. However, none of the cleaning methods could completely eliminate surfaces of specimens from both aerobic and anaerobic bacteria (Figs. 1 and 2). The results of the present study showed that plastic curette had the least effect on the removal of both types of aerobic and anaerobic bacteria from the surfaces of titanium disks. This result is consistent with previous studies that reported this method had poor efficiency in removing dental biofilms from smooth and roughed surfaces [42,43], which is may be due to the flexibility of the curette tip as compared to metal curettes. It has been shown that metal curettes have a higher potential for removing dental biofilm from titanium surfaces, but these curettes have their own defects such as causing more surface roughness and damage to the morphology of the implant surface [38]. The inefficiency of the plastic curette is also attributed to their repeated use and the blunt side which, in turn, reduces its efficiency. In addition, instrumentation with plastic curette on roughed surface dental implants causing dplastic particles to be remained on surfaces that are not easily cleaned and may interfere with reosseo integration [37]. In contrast, the data of the present study show that Er:YAG laser has the greatest effect on the removal of oral biofilm from the surface of titanium specimens (Figs. 1 and 2). Er:YAGLaser, as one of lasers used in the dentistry, has been considered as a promising therapeutic approach to disinfecting peri-dental implant surfaces [44]. Kreisler et al. showed in their study that Er:YAG laser has a high potential for reducing the number of bacteria from implant surfaces, which is consistent with the results of the present study [18]. It has been reported that the Er: YAG Laser has the ability to remove biofilms from smooth and roughed surfaces of titanium implants in such way that they significantly improve the clinical parameters and the new bone formation on the implant surface [19,20]. Despite these capabilities, some reports indicate that Er: YAGs Laser is not able to completely remove dental biofilms from roughed titanium surfaces [[45,46].Chen et al. reported in an in vitro study that Er: YAG laser radiation with parameters similar to these used in the present study (100 mJ. pluse, 10 Hz) led to no obvious damage on titanium surfaces, but they could not fully decontaminate the implant surfaces and attributed it, based on the SEM images, to the presence of a number of bacteria in the valleys and undercuts in the roughed surfaces of the implants [45]. However, it has been reported that removing more than 96% of biofilms from the implant surface seems to be sufficient to achieve the peri-implant clinical health [46]. But, Giannelli et al., in a recent study, using Er: YAG laser radiation on the surface of titanium discs with 150 mJ. 12 Hz parameters, compared with 80 mJ. 12 Hz parameters, managed to almost completely carryout debridement on the treated surfaces in such way that the remaining plaque areas in 150 mJ. 12 Hz and 80 mJ. 12 Hz groups were reduced from 76.5% to 0.03% and 32.2%, respectively. It is noteworthy that the SEM images obtained in that study showed that there are no indications of melting and other heat-induced deformations in the 150 mJ. 12 Hz group [47]. Although some studies have suggested that Er: YAG is the most promising method for cleaning dental implant surfaces, some limitations, such as the cost of this system and the need for climatic equipment, prevent its extensive use.Therefore, other easy to use methods are considered. According to pairwise comparison of the therapeutic methods in the present study, the aPDT was and there is no even significant difference between the aPDT and Er: YAG methods in terms of their OD level in the aerobic bacteria group ($P = 1$). This result suggests that the aPDT method used in conjunction with a mechanical method (plastic curette) is capable of removing bacteria from oral biofilm-contaminated titanium surfaces, which is consistent with some previous studies [30,31]. In the present study, we used a PS containing a tolonium chloride compound that is activated with a 660-nm diode laser. But some studies, which have questioned the effect of aPDT or failed to achieve result from it, attributed it to probabilities such as the mistake of choosing the wavelength used or other parameters related to laser light, or the way dye is used. For example, Bombeccari et al. used photosensitizer (PS) of Toluidine blue and 810-nm laser radiation. They reported that they have not achieved any results from the application of the aPDT method [48]; while the peak absorption of the toluidine blue substance occurs at 635 nm wavelength [49]. Valente et al., also used Indocyanine Green, and two 810 mm and 980 mm wavelengths. They also reported that they have not achieved any results from the application of the aPDT method [50]. Occasionally, false positive results may also be obtained, such as Htet et al.'s study, in which false positive results were obtained in case of Toluidine blue and 830-nm laser radiation [51]. In conclusion, photodynamic therapy, when used as an adjunct, along with conventional treatments, can increase the effect of standard antibacterial therapy [52]. Figs. 1 and 2 show that the CHX method, although significantly different from the Er: YAG laser, and had poorer efficiency, but its effect was significantly higher than that of the plastic curette and 810-nm laser, and was not significantly different from aPDT ($P < 0.05$). This result is consistent with the study carried out by Marotti et al., who also compared the 0.12 CHX with the aPDT method and reported that both methods were effective in reducing bacterial levels from roughed dental implant surfaces; however, there is no significant difference between the two methods. In that study, 660-nm laser radiation was applied to Methylen Blue for 3 and 5 min, but there was no significant difference between the two radiation times [30]. However, Saffarpour et al. compared 2% CHX with aPDT and Er: YAG laser, and reported that CHX had more pronounced antibacterial effect than the other two methods [53], which could be due to high concentrations of CHX. CHX has cytotoxic effects, and some studies have reported its cytotoxic effects on host cells, including macrophages, endothelial cells, fibroblasts, and osteoblasts, among which osteoblasts are the most sensitive cells to CHX [54,55].Therefore, it is logical to use CHX with the lowest effective concentration on microorganisms. The most commonly CHX concentrations used in various studies included as 0.12% or 0.2% [20]. Treje et al. reported no difference between the two treatment groups after comparing 0.12% or 0.2% CHX with mechanical therapy and evaluating clinical and histological parameters [56]. It seems that further studies should be conducted to determine the appropriate antibacterial concentration of CHX with the least toxic effects. However, regarding the photothermal role of 810-nm diode laser in decontaminating titanium specimens in the present study, the comparison of OD and CFUs values shows that the 810-nm laser had weaker performance than all other methods, except for the plastic curette ($P < 0.05$). In a study on titanium discs infected with *S. sanguinis*, Kreisler et al. reported that 809-.

However, it was less effective than 0.2% CHX for one minute [24]. The results of our previous clinical trial and the microbiological study that were performed to treat primary peri-implantitis lesions, and compare 810-nm laser alone and aPDT (810-nm laser radiation and EmonDo photosensitizer methods) indicate improvement in clinical

parameters in both groups during a three-month period but there was no significant difference between them. However, the results of the real-time PCR-based microbiology assay showed that the aPDT method reduced the number of A-actionomycetemcomitans, *P. gingivalis* and *Tannerella frosythia*, while the 810-nm laser radiation only reduced the *P. gingivalis* bacterium [57]. Some other studies also have reported the positive effect of 810-nm laser, as an adjunctive technique following conventional treatments, in improving periodontal indices, and introduced 810-nm laser as an ideal and valuable tool for treating peri-implant diseases [58–60]. However, by using inappropriate parameters there is a potential risk of damage to the surface structure of the implant when using this laser in nonsurgical treatments as compared to aPDT. So, there seems to be few clinical studies on the use of diode lasers, specifically 810-nm diodes in the treatment of peri-implant diseases and the decontamination of implant surfaces. Therefore, it is suggested that, further studies should be conducted over a longer time period (at least one year), in the future, to find out the capabilities of diode lasers, Limitations of this study were low number of patients who were volunteer for sampling and keeping implant disks in their mouth more than 24 h, financial problems and less compliance of some patients who were excluded from study.

9. Conclusion

The results of the present study showed that all types of mechanical (plastic curette), chemical (CHX, lasers (810 nm diode and Er: YAG) and aPDT (photosensitizer + 66onm diode laser) methods can reduce the oral biofilm from titanium discs with roughed surfaces (SLA).Plastic curette and Er: YAG laser had the least and the greatest effects in this regard, respectively. However, with regard to the results obtained, the aPDT method did not differ much from the Er: YAG laser in decontaminating the titanium surfaces. Thus, it is recommended that this method to be considered and applied given its safe nature.

More clinical researches are needed to evaluate the efficacy of these methods in patients with chronic peri-implantitis.

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