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Antimicrobial effects of photodynamic therapy with antiseptics on *Staphylococcus aureus* biofilm on titanium surface

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ABSTRACT

Background: *S. aureus* biofilm plays a predominant role in the establishment and development of peri-implantitis. It is suggested to combine different modalities as peri-implantitis treatment. The aim of this study was to evaluate the disinfection efficacy of combined application of antiseptics with PDT on *Staphylococcus aureus* (*S. aureus*) biofilm formed on titanium(Ti) disks with different surface roughness.

Methods: *S. aureus* biofilm was incubated on polished and sandblasted large-grit acid-etched (SLA) Ti surfaces for 48 h. 72 contaminated Ti disks (36 polished, 36 SLA) were randomly divided into 6 different groups as follows: (a) PBS, (b) 0.2% chlorhexidine digluconate (CHX), (c) 3% hydrogen peroxide (H_2O_2), (d) PDT, (e) 0.2% CHX + PDT, and (f) 3% H_2O_2 + PDT. Colony forming unit (CFU) was measured to determine antimicrobial effects. Biofilm structure was assessed using scanning electron microscopy (SEM) and confocal laser scanning microscope (CLSM).

Results: All disinfection methods significantly reduced bacteria amounts compared to control group on both polished and SLA Ti surfaces ($P < 0.001$). PDT demonstrated stronger decontamination ability in eliminating *S. aureus* from Ti surfaces than CHX and H_2O_2 did ($P < 0.05$). The combined CHX or H_2O_2 with PDT treatment were more effective in bacterial disinfection than a single administration of these treatments ($P < 0.001$).

Conclusion: The combination of CHX or H_2O_2 administration with PDT was more effective in eradicating *S. aureus* on both polished and SLA Ti disks in comparison with either treatment alone, suggesting that combined usage of antiseptics with PDT could be a more efficient method for the treatment of peri-implantitis.

1. Introduction

During the past decades, peri-implantitis has been regarded as a main clinical challenge for dental implants [1–3]. In analogy to periodontitis affecting the periodontium of natural teeth, peri-implantitis is defined as a progressive and irreversible inflammatory process affecting implant-surrounding soft and hard tissues, leading to bone resorption and decreased osseointegration, thereby threatening the long-term survival of dental implants [1,4].The prevalence of peri-implantitis has been reported by different studies ranging from 1% to 47% [5,6]. This wide range is mainly due to varying study designs and sample sizes with different risk factors and statistic profiles [1,7,8]. According to the Consensus Report of the Sixth European Workshop in Periodontology, Lindhe & Meyle concluded an incidence of peri-implantitis between 28% and 56% [9].

The microbial colonization and biofilm formation on implant surfaces play a pivotal part in the development and progression of peri-implantitis [10]. Being a poly-microbial anaerobic infection [11], peri-implantitis has been found to harbor a spectrum of periodontopathic germs [12]. But, some other bacteria that are not part of the typical periodontal pathogenic microbiota are also involved in peri-implantitis. In particular, *Staphylococcus aureus* (*S. aureus*) appears to play a predominant role in the establishment and development of peri-implantitis. Previous studies have documented the contribution of *S.*

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aureus to the development of peri-implantitis [13,14] and the presence of *S. aureus* in the failure of implants [15]. It is revealed that staphylococcal species including *S. aureus* [15] and *S. warneri* [16] have affinity for titanium (Ti) surfaces colonization. During biofilm formation, *S. aureus* acts as an early colonizer, creating a favorable environment for the adhesion and colonization of late bacteria colonizers [17]. Therefore, it is essential to effectively eradicate *S. aureus* biofilm in peri-implantitis therapy [18,19].

Various protocols have been proposed to treat peri-implantitis. These protocols comprise non-surgical and surgical therapy. Non-surgical methods could be conducted alone or in combination with surgical methods to de-toxify the contaminated implant surface based on the severity of peri-implant disease. These conservative treatments consist of mechanical debridement, antibiotics, antiseptics and laser treatment [20,21]. It is generally accepted that the combined application of different conservative treatments could result in more efficient reductions of clinical peri-implantitis symptoms [22].

As a novel minimally invasive therapy, photodynamic therapy (PDT) has shown promising effects on the treatment of oral infectious diseases owing to its photochemical reaction [23]. The underline mechanism includes killing bacteria with reactive oxygen species (ROS) production [24], inactivating bacteria endotoxin, altering the bioactivity of lipopolysaccharide [25] and possibly promoting cell differentiation [26]. Previous studies using PDT as an adjuvant disinfection protocol in eradicating caries [27], endodontic [28,29] or periodontal [30] pathogens have achieved favorable results. PDT has also been introduced as an adjunct therapy into peri-implantitis treatment [31,32]. *In vitro* studies have demonstrated that PDT alone could dramatically but not completely eliminate *S. aureus* biofilm on Ti surface [33,34]. Combined application of PDT with other conservative therapy like debridement using Ti brush was shown to be more efficient in reducing the number of *S. aureus* in biofilm on implant surface [18]. However, some adverse consequences including implant surface alteration, antibacterial resistance and thermal damage might be induced by mechanical debridement, antibiotics, and laser administration respectively [12,35]. It is recommended to combine the decontamination effects of PDT with local application of antiseptics like hydrogen peroxide (H_2O_2) and chlorhexidine (CHX) [34]. But, no reports have been published on the concurrent administration of PDT and antiseptics on *S. aureus* biofilm on the Ti surface.

Therefore, the aim of the present *in vitro* study was to evaluate the disinfection efficacy of combined application of antiseptics with PDT on *S. aureus* biofilm attached to Ti surface. Both polished and sandblasted large-grit acid-etched (SLA) Ti surfaces were used in this study. It was expected that the combined administration of antiseptics with PDT could significantly improve the disinfection effects on the Ti surface contaminated with *S. aureus* biofilm.

2. Materials and methods

2.1. Ti specimen preparation

Polished Ti disks (Bioconcept, Jiangshu, China) (n = 36) and sandblasted large-grit acid-etched (SLA) titanium disks (Bioconcept, Jiangshu, China) (n = 36) were used in this study. Each disk had a diameter of 10.0 mm and thickness of 1.0 mm. Ti disks were sequentially sonicated with acetone, absolute ethanol, deionized water for 15 min, rinsed with distilled water and autoclaved for 15 min at 121℃.

2.2. Bacterial culture and biofilm formation

All Ti disks were dipped into artificial saliva to form acquired pellicle before inoculation. *S. aureus* strain ATCC 25923 was grown overnight in Luria-Bertani (LB) agar and then inoculated into LB broth to obtain bacteria suspension. Thereafter, suspension was diluted with PBS, washed twice, mixed by repeated vortexing and then adjusted to 1×10^8 CFU/mL using UV Spectrophotometer. Ti disks were placed in 24-well plates and incubated with 100 μL bacterial suspension supplemented with 1.5 mL culture broth to allow biofilm formation. The incubation time of *S. aureus* biofilms was set at 48 h.

2.3. Treatment protocol

After contamination with *S. aureus*, Ti disks were rinsed with PBS three times to remove loose bacteria around Ti disks before decontamination procedure. Ti disks (36 polished, 36 SLA) were randomly divided into 5 decontamination groups. Disks receiving PBS treatment served as control.

1 Negative control (n = 12) - disks were immersed in 1 mL PBS solution for 60 s.
2 0.2% CHX (n = 12) - disks were immersed in 1 mL CHX solution for 60 s and were gently rinsed with PBS solution to remove excess CHX.
3 3% H_2O_2 (n = 12) - disks were immersed in 1 mL H_2O_2 solution for 60 s and then gently rinsed with PBS solution to remove excess H_2O_2.
4 PDT treatment (n = 12) - A light emitting diode (LED) (Denfotex, England) was used as light source with output power at 595 mW and a wavelength of 635 nm [36]. Disks were placed on sterile slides and covered fully by toluidine blue O (TBO) photosensitizer (100 μg/mL) (Denfotex, England) in the dark for 60 s. Then Ti disks were irradiated by the LED with a light fiber at a distance of 5 mm at continuous mode according to the manufacturer's instruction. The irradiation time was set as 60 s and the applied output energy density was 31.5 J/cm^2.
5 CHX + PDT (n = 12) Ti disks were decontaminated using CHX for 60 s. Then, Ti disks were treated by PDT for another 60 s.
6 H_2O_2 + PDT (n = 12) Ti disks were decontaminated with H_2O_2 for 60 s, followed by PDT treatment for another 60 s.

2.4. Viability assessment

After treatment, disk was transferred into a test tube containing 1 mL PBS and vortexed for 1 min in order to detach residual biofilm from Ti disks. The samples were serially diluted in PBS solution and plated by a spiral plater. *S. aureus* biofilms were plated on LB agar plate and incubated anaerobically at 37 ℃ for 36 h. Finally, the number of colonies from the proper range will be calculated for analysis of disinfection effect.

2.5. Surface roughness and contact angle measurement

The roughness average was measured under contour measuring instrument (SE600, Kosaka, Japan) on six samples. In addition to the arithmetic mean deviation of the roughness profile (Ra), other roughness parameters maximum height of profile (Rz), root mean square deviation of roughness profile (Rq), maximum profile peak height (Rp) were also included to describe Ti surface profile in detail. Contact angle measurements were carried out by DSA25 (Kruss, Hamburg, Germany) using advance software. The sessile drop approach was used to measure contact angle and 2 μL distilled, deionized water was placed on Ti disk surface for 60 s. The median value of three measurements of each disk was recorded.

2.6. Scanning electron microscope (SEM)

Once the decontamination procedure terminated, three disks were randomly selected from each experimental group for SEM to observe the structure of the remaining biofilms attached to Ti disks. Specimens were fixed in 2.5% glutaraldehyde overnight at 4 ℃ and then dehydrated through a graded sequence of ethanol at different concentration.

Polished and SLA Ti disks were examined using SEM (Nova NanoSEM 230, FEI, Hillsboro, American) under different magnification.

2.7. Confocal laser scanning microscopy (CLSM)

To determine the viability of remaining bacteria after exposure to different treatments, a Live/Dead BacLight bacterial viability kit (Molecular Probes Inc. Eugene, OR, USA) containing two fluorescent dyes propidium iodine(PI) and SYTO9 was used. Before examination, Ti disks were stained with fluorescent dye and incubated in the dark at room temperature for 15 min. Then samples were rinsed with PBS in order to remove the extra dye and covered with mounting oil. Each disk was observed under a fluorescence microscope (Carl Zeiss Microscopy GmbH, Gottingen, Germany).

2.8. Statistical analysis

All microbiological data were recorded using $\log_{10}$. *t*-test was used to analyze surface roughness and contact angles of titanium disks used in this study. Then, the one-way ANOVA test was applied in antimicrobial activity evaluation, followed by LSD test, at a significance level of 0.05. Data were analyzed using SPSS (IBM SPSS 21.0 for Windows; IBM, Armonk, NY, USA).

3. Results

3.1. Surface properties of two Ti disk

As shown in Table 1, all surface roughness parameters including Ra, Rz, Rp and Rq values of SLA Ti disk were higher than those of polished Ti disk with significant difference ($P < 0.05$). The measurements of static contact angles of tested Ti surfaces were shown in Fig. 1. The mean values of contact angle measurement were 109.76 ± 1.46°for SLA Ti disk and 78.39 ± 0.97° for polished Ti disk. The contact angle of polished Ti disk was significantly higher than that of SLA Ti disk ($P < 0.001$).

3.2. Assessment of bactericidal efficacy on biofilms

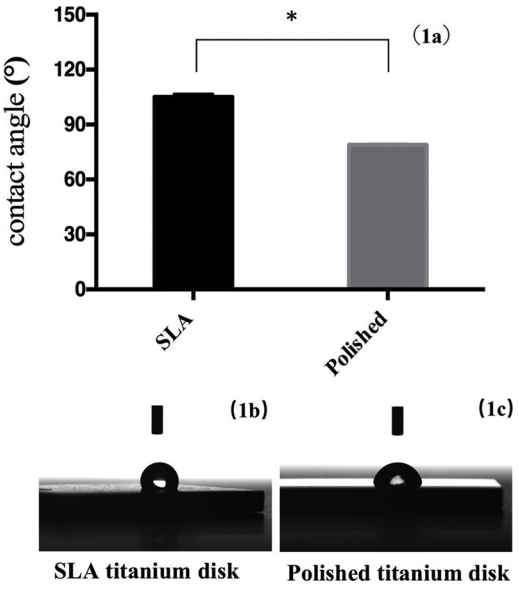
There was no significant difference in the bacteria counts of remaining *S. aureus* between the polished Ti disks and the SLA ones in either of the groups treated with PBS, CHX, H_2O_2, PDT, CHX + PDT, or H_2O_2 + PDT ($P > 0.05$) (Fig. 2a).

Fig. 2b and c presented decontamination efficacy of each treatment on *S. aureus* biofilm contaminated with Ti disks. When compared with negative control, remaining alive bacteria number in all decontamination groups were reduced significantly ($P < 0.001$). Preceding antiseptic administration (CHX or H_2O_2) with subsequent PDT was the most effective protocol, yielding approximately 3–4 $\log_{10}$ reduction of adhered bacteria number in comparison with either treatment alone ($P < 0.001$). On both polished and SLA Ti surfaces, CFU counts in the PDT group were significantly less than those in either the CHX group or H_2O_2 group ($P < 0.05$) (Fig. 2b,c).

Table 1
Surface roughness parameters of polished and SLA titanium disk. (mean standard deviation).

Surface	Ra(μm)	Rz(μm)	Rp(μm)	Rq(μm)
SLA	1.62 ± 0.13	10.95 ± 0.89	4.79 ± 0.34	2.04 ± 0.16
Polished	0.26 ± 0.11	1.86 ± 0.50	2.85 ± 0.26	0.30 ± 0.18

Ra, arithmetic mean deviation of the roughness profile; Rz, roughness parameters maximum height of profile; Rp, maximum profile peak height; Rq, root mean square deviation of roughness profile; SLA, sandblasted large-grit acid-etched.
*Statistically significant difference when compared to the polished titanium disk ($P < 0.05$; *t*-test).



*Statistically significant difference between polished titanium disk and SLA titanium disk (P<0.05; T-test).

Fig. 1. (1a) Static contact angles with sessile drop method between polished and SLA Ti disks. (1b) water drop on SLA Ti disk. (1c) water drop on polished Ti disk.

3.3. SEM observation

In PBS group, a large number of bacteria were observed on both polished and SLA Ti surfaces forming clusters (Fig. 3a and b). Dramatic decrease in bacteria amount could be detected in samples treated with PDT (Fig. 4a), H_2O_2 + PDT (Fig. 4b), and CHX + PDT (Fig. 4c). In addition, compared with the PDT group, fewer remaining bacteria were found in H_2O_2 + PDT group and CHX + PDT group (Fig. 4a,b,c).

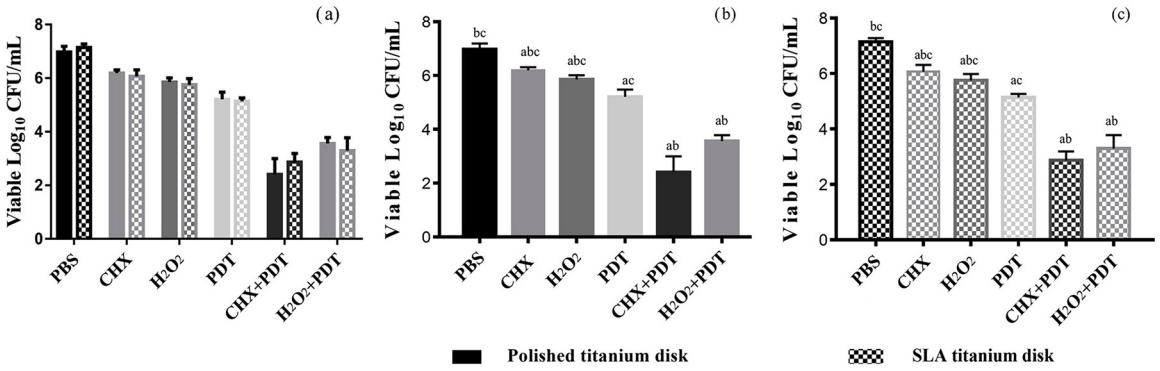
3.4. CLSM images

Viable and dead bacteria after each treatment were shown in CLSM images. As a negative control, PBS group showed largely intact *S. aureus* biofilm which was predominantly composed of living bacteria (Fig. 5a). After treatment with either 0.2% CHX, 3% H_2O_2, or PDT, damage of biofilm structure and a large number of dead cells were observed (Fig. 5b,c,d). The CHX + PDT and H_2O_2 + PDT group exhibited the most severe structural destruction and the highest proportion of dead cells. (Fig. 5e,f).

4. Discussion

To the best of our knowledge, this is the first *in vitro* study which assessed the antibacterial effects of concurrent application of antiseptics with PDT against *S. aureus* biofilm presented on the Ti surface. The hypothesis that combined administration of antiseptics with PDT could significantly improve the disinfection effects on *S. aureus* biofilm attached to Ti surface was confirmed.

In order to facilitate osseointegration and epithelial sealing, dental implants are specially designed with rough bone-contact surface and smooth abutment surface [37]. As gingival and bone tissues are both involved in peri-implantitis, it is imperative to effectively disinfect contaminated Ti surfaces with different roughness. To mimic the clinical situation, both polished and SLA Ti surfaces were used in the present study. The results of our study showed no significant difference of



[a] indicated a significant difference when compared to PBS negative control group ($P<0.001$;ANOVA/LSD).
[b] indicated a significant difference when compared to PDT group ($P<0.05$;ANOVA/LSD).
[c] indicated a significant difference when compared to combined group(CHX/ H_2O_2 +PDT) ($P<0.001$;ANOVA/LSD) .

Fig. 2. Comparison of different disinfection modalities for *Staphylococcus aureus* (*S. aureus*) formed on polished and SLA Ti disks. (2a) Bar chart illustrated the viable Log_{10} CFU amounts of *S. aureus* biofilm attached to polished and SLA Ti disk after different treatments. (2b) Log_{10} viable bacteria counts (CFU/mL) of *S. aureus* attached to polished Ti disks after different treatments. (2c) Log_{10} viable bacteria counts (CFU/mL) of *S. aureus* attached to SLA Ti disks after different treatments.

bacteria attachment between the polished and SLA Ti surfaces. Bacteria biofilm adherence is partly determined by surface morphology. Evidence has demonstrated that surface roughness positively correlates to bacteria biofilm formation [38–40]. Rimondini et al. found that a Ti surface with Ra ≤ 0.088 μm and Rz ≤ 1.027 μm dramatically inhibited biofilm formation [40]. In our study, both Ra and Rz values of either surface are above this threshold. In addition, roughness parameters including Ra, Rz, Rp, and Rq in SLA titanium disks are unanimously higher than those in polished ones. Surface wettability also has substantial impacts on oral bacterial attachment. In 1999, Drake et al. conducted an *in vitro* study evaluating the influence of wettability and roughness of Ti surfaces on primary bacterial colonization [39]. Higher degrees of bacterial colonization were detected on rough or hydrophobic (low wettability) Ti surfaces. But, most of the recent studies stated that hydrophilic (high wettability) surface facilitates bacteria attachment [38]. The effects of surface roughness and wettability might counteract each other in our study. In the present study, there was no difference of decontamination effects between smooth and rough surfaces regardless of the disinfection modalities. This is in line with previous studies [18] in which no significant differences were found between polished and SLA Ti surfaces in bacteria load reduction in *S. aureus* biofilm with 0.2% CHX or PDT. These results suggested that the topography of the Ti surface per se does not influence the outcomes of the different disinfection protocols.

Local antiseptic administration has been proposed as an effective non-surgical therapy to decontaminate the implant surface in peri-implantitis treatment. But, CHX and H_2O_2 have been found to be toxic for cells when used at high concentrations or in prolonged exposure [41]. Thus, it is suggested to combine the disinfection techniques of antiseptic administration with other conservative therapies [34].

Being minimally invasive, nontoxic and easily repeatable, PDT has been gradually applied as a promising decontamination technique in oral infectious disease treatment in recent years [42,43]. In the present study, we compared the administration of antiseptics or PDT alone in eliminating *S. aureus* biofilm from different titanium surfaces. From the results of this *in vitro* study, single application of any disinfection modality has achieved significant bacterial reduction compared to control group, but could not achieve complete eradication. In addition, it is noticeable that PDT exhibited higher antimicrobial efficiency than 0.2% CHX or 3% H_2O_2 did. Our result was in line with some previous studies [44]. In our study, *S. aureus* was used as infected bacteria. *S. aureus* is a gram-positive bacterium, which was reported to be sensitive to PDT treatment owing to its relatively porous cytoplasmic membrane [45]. By contrast, Decker EM et al. found that 0.2% CHX could more effectively kill periodontal pathogens including *P. gingivalis* than PDT [46]. As multiple factors including bacteria species, photosensitizers, PDT parameters, and biofilm attaching materials differed among these studies, the results are hardly comparable. Nevertheless, further studies are needed to optimize the antibacterial effects of PDT.

We also investigated combined usage of PDT with CHX or H_2O_2 in disinfection *S. aureus* biofilm fromTi disk surface. The results confirmed our hypothesis that pre-treatment with antiseptics could improve the efficiency of PDT treatment on *S. aureus* biofilm on the Ti surface. Until now, there are only few studies on combined treatment of PDT with antiseptics for oral infectious diseases [46–48], all of which were confined within endodontic or periodontal inflammation. An *in vitro* study conducted by Lins et al. evaluated the combined effects of methylene blue (MB)-mediated PDT with H_2O_2 and manifested significant *E. faecalis* and *P. aeruginosa* reduction [49]. The authors stated that the addition of H_2O_2 could lead to more uptake of the photosensitizer by the microorganism and enhance the efficiency of PDT. In our study, exposure to H_2O_2 prior to PDT treatment also greatly contributed to bacteria disinfection. Oxidative foaming effect of H_2O_2 could disrupt the bacteria biofilm structure thus decreasing the biofilm depth and enabling deeper penetration of photosensitizer. In addition, as antibacterial mechanism of PDT is related to reactive oxygen species (ROS) production, elevated oxygen concentration within the microenvironment in the presence of H_2O_2 could strengthen the antibacterial potential of PDT. The results of the present study also showed that pretreatment with CHX could dramatically promote the disinfection effect of PDT on Ti disks contaminated with *S. aureus.* This finding was in line with previous studies, in which combined CHX with other disinfection modalities like ultrasonics significantly decreased viable bacteria counts in various biofilms [50]. Conversely, Souza et al. investigated CHX and PDT + CHX effect on *E. faecalis* biofilm in root canals, reporting no significant difference between the two groups [51]. However, in their study, CHX and photosensitizer were mixed together, which may interfere with the effects of photosensitizer thus causing unexpected outcomes. The underline mechanism of enhanced effects of PDT with preceding CHX treatment might be explained by the cell membrane structure alteration by CHX administration. Being a cationic agent, CHX could bind to the bacterial cell wall and alter osmotic equilibrium, leading to the precipitation of cytoplasmic content and bacteria death [52]. The binding of CHX to the bacterial cell membrane could cause an increase of permeability of photosensitizer, improving antibacterial effect of PDT [53]. In addition, it is well documented that the CHX could trigger Ca^{2+} increase in previous findings [54] and it is reasonable to speculate Ca^{2+} efflux may trigger ROS production, thus

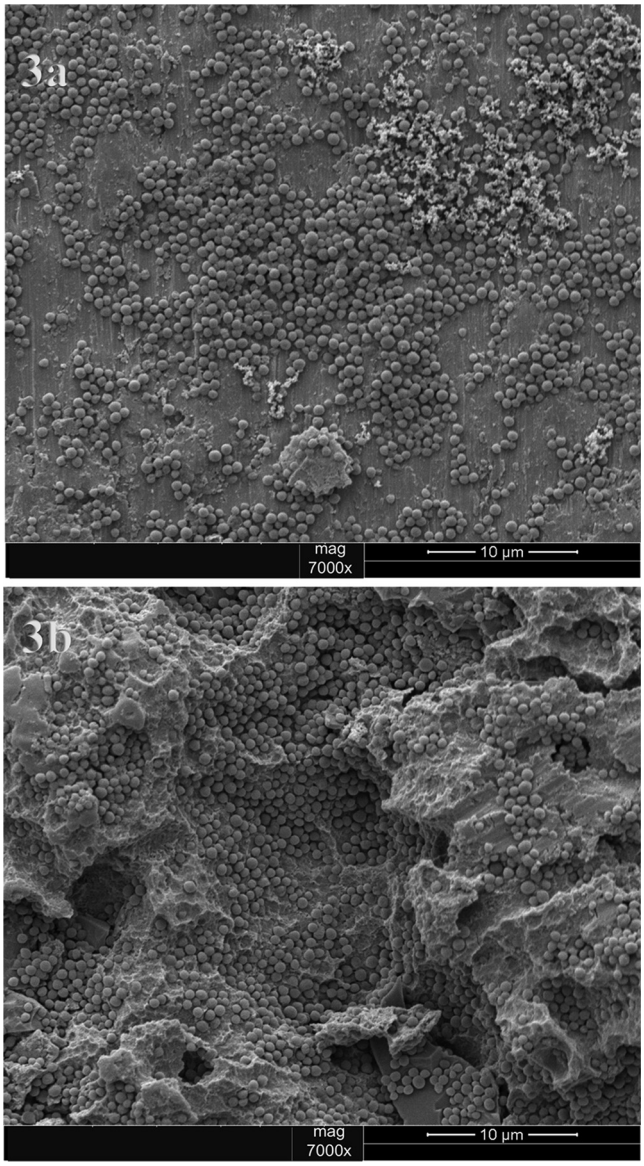


Fig. 3. Biofilm of *S.aureus* attached to polished and SLA Ti disk treated with PBS. Original magnification 7000×. (3a) Polished Ti disk treated with PBS. (3b) SLA Ti disk treated with PBS. Original magnification 7000×.

enhancing antibacterial effects of PDT treatment. Furthermore, in a recently published report, it was found that the CHX + PDT could markedly inhibit the gene expression of virulence factors related to colonization, adhesion, immunoevasion, and immunosuppression in *E. faecalis* [55]. Thus, it is plausible to propose that combined application of antiseptics with PDT may suppress gene expression associated with *S.aureus* biofilm formation. It should be noted that PDT in combination with pretreatment of CHX or H_2O_2 solution was unable to completely eradicate *S.aureus* biofilm in this *in vitro* study. This underlines that at the present time combined application of antiseptics with PDT should be regarded as an efficient adjunctive therapy to mechanical debridement in peri-implantitis treatment.

Though, there were some limitations in the present study. Firstly, it should be pointed out that mono-species bacteria biofilm was used in this *in vitro* study. As development of peri-implantitis is orchestrated by interaction of multiple pathogenic microorganisms in the complex oral environment, it would be necessary to validate the disinfection efficiency of the combined application of CHX or H_2O_2 with PDT on multi-species biofilm on Ti surfaces clinically. Secondly, our results showed that the combined application of PDT with CHX or H_2O_2 was the most effective in eradicating *S. aureus* biofilm on both polished and SLA Ti surfaces. But, isobologram analysis or combination index measurement should be conducted in further studies to determine whether the enhanced results were due to synergic or additive effects. Thirdly, we have only observed the effects of CHX or H_2O_2 with subsequent PDT on *S. aureus* biofilms. The different orders of the two disinfection protocols might give a clue of possible underlying mechanism of the decontamination effects.

5. Conclusion

The combination of CHX or H_2O_2 administration with PDT was more effective in eradicating *S. aureus* on both polished and SLA Ti disks in comparison with either treatment alone, suggesting that combined usage of antiseptics and PDT could be an efficient method for the treatment of peri-implantitis.

Declaration of interests

There is no conflict of interest to declare.

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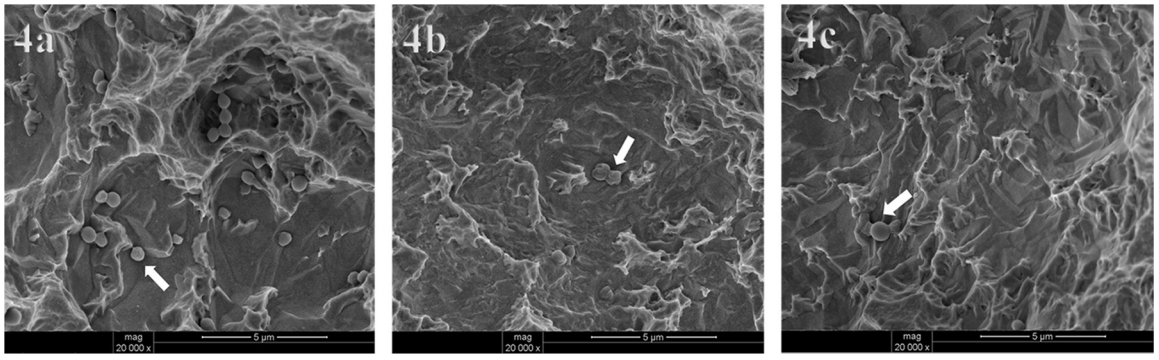

Fig. 4. *S.aureus* biofilm on SLA Ti disk after different treatments. Original magnification 20,000×. (4a) SLA Ti disks treated with PDT group. (4b) SLA Ti disks treated with preceding application of CHX and then performed PDT. (4c) SLA Ti disks treated with preceding application of H_2O_2 and then PDT. Remaining bacteria cells after treatments (white arrow). Original magnification 20,000×.

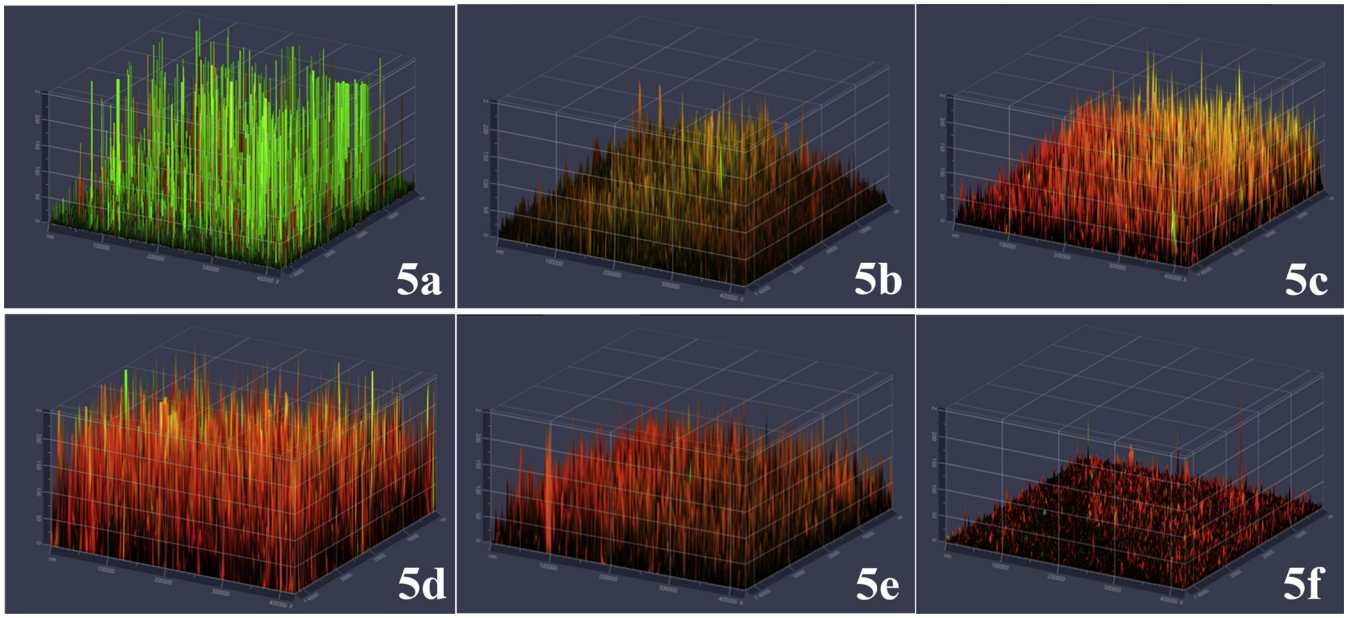

Fig. 5. 2.5-dimensional architecture of *S. aureus* biofilm under different treatments on polished Ti disks. (5a) PBS group. (5b) CHX group. (5c) H_2O_2 group. (5d) PDT group. (5e) CHX + PDT group (5f) H_2O_2 + PDT group.

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