

Antiseptics and microcosm biofilm formation on titanium surfaces

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Abstract: Oral rehabilitation with osseointegrated implants is a way to restore esthetics and masticatory function in edentulous patients, but bacterial colonization around the implants may lead to mucositis or peri-implantitis and consequent implant loss. Peri-implantitis is the main complication of oral rehabilitation with dental implants and, therefore, it is necessary to take into account the potential effects of antiseptics such as chlorhexidine (CHX), chloramine T (CHT), triclosan (TRI), and essential oils (EO) on bacterial adhesion and on biofilm formation. To assess the action of these substances, we used the microcosm technique, in which the oral environment and periodontal conditions are simulated *in vitro* on titanium discs with different surface treatments (smooth surface - SS, acid-etched smooth surface - AESS, sand-blasted surface - SBS, and sand-blasted and acid-etched surface - SBAES). Roughness measurements yielded the following results: SS: 0.47 μm , AESS: 0.43 μm , SB: 0.79 μm , and SBAES: 0.72 μm . There was statistical difference only between SBS and AESS. There was no statistical difference among antiseptic treatments. However, EO and CHT showed lower bacterial counts compared with the saline solution treatment (control group). Thus, the current gold standard (CHX) did not outperform CHT and EO, which were efficient in reducing the biofilm biomass compared with saline solution.

Keywords: Peri-Implantitis; Biofilms; Titanium.

Introduction

Dental implants offer an alternative so that patients' rehabilitation and esthetic needs can be met, but failures have been reported and attributed to peri-implantitis.^{1,2} Described as an inflammatory disease that affects tissues in the vicinity of the osseointegrated implant, peri-implantitis leads to bone loss³ and subsequent implant loss; however, bacterial adhesion and biofilm accumulation are the major causes of this disease.^{4,5}

The basic treatment for periodontitis and peri-implantitis consists of debridement of the affected surface (dental implant).^{6,7} Implant surfaces are highly microstructured and macrostructured so that they increase osseointegration, but rough surfaces facilitate microbial adhesion and formation of complex biofilms, hindering the debridement of implant surfaces.^{4,6,7} Additional therapy with antibiotics and antiseptics has been proposed for the removal of pathogenic biofilms and improvement of nonsurgical treatment outcomes.^{6,7,8,9}



The healing potential of peri-implant defects after suppression of peri-implant microbial biofilm has been reported; nevertheless, treatment recommendations and the most appropriate chemical agent for decontamination of implants are yet unsatisfying.^{10,6,7,8}

Gosau et al.¹¹ investigated the effects of sodium hypochlorite, hydrogen peroxide, chlorhexidine digluconate, citric acid, essential oils, and triclosan on *in vivo* biofilms in healthy individuals, concluding that antiseptics had bactericidal effects on microbial adhesion, but interindividual variations hampered the interpretation of results. Moreover, the microbiota on teeth or implants under healthy conditions has qualitative and quantitative differences from those observed on sites affected by peri-implantitis or periodontitis.¹²

Filoche et al.¹³ used the microcosm technique to assess biomass and bacterial viability after oral antiseptic treatments, concluding that biomass was reduced by all tested solutions, but viability remained unchanged. Other authors have used this technique with patients' saliva since it simulates the oral cavity and is therefore appropriate for investigations in the field of cariology and for tests with antiseptic substances.^{14,15,16}

In the present study, we assessed the effect of four antiseptics against microcosm biofilm on titanium surfaces with different treatments.

Methodology

The study protocol (no. 135.946) was approved by the Research Ethics Committee of *Universidade de Passo Fundo* - UPF.

Titanium specimens. Sterile titanium specimens were donated by Titanium Fix (Titanium Fix, São José dos Campos, Brazil) in discs measuring 5.5 mm in diameter and 2 mm in thickness. The following types of surface were analyzed: smooth surface (SS), acid-etched smooth surface (AESS), sand-blasted surface (SBS), and sand-blasted and acid-etched surface (SBAES).

Surface roughness measurement. Surface roughness was measured by a SurrCode SE1200 profilometer (Kosakalab, Tokyo, Japan), calibrated with V 200, H 25 mm/ λ c and λ c 0.25 mm, and average

roughness (Ra) was considered to be that provided by the equipment.

Microcosm biofilm formation. For *in vitro* biofilm formation by the microcosm technique, we used the model described by van de Sande et al.¹⁷ Saliva was collected from a non-smoking volunteer with periodontal disease who had not taken antibiotics in the previous month, who had abstained from oral hygiene in the past 24 h, and who had fasted for 2 h prior to collection, being then processed. This patient signed a written informed consent form authorizing his participation in the study. 90 mL of saliva stimulated with paraffin (Parafilm®, Bemis Company, Oshkosh, USA) was collected into a sterile graded collector and transported to the laboratory under refrigeration, stored in a sterile vessel and homogenized in a vortex mixer.¹⁸ After collection, the patient was referred to free-of-charge periodontal treatment at the Dental School of *Universidade Federal de Pelotas* - UFPel.

Artificial saliva (DMM) preparation. The defined medium mucin (DMM) was obtained as proposed by Wong and Sissons,¹⁶ consisting of porcine gastric mucin (2.5 g/l), urea (1.0 mmol/l), salts in mmol/l (CaCl₂: 1.0; MgCl₂: 0.2; KH₂PO₄: 3.5; K₂HPO₄: 1.5; NaCl: 10.0; KCl: 15.0; NH₄Cl: 2.0), 21 free amino acids, 17 vitamins, and growth factors. The medium contains amino acids for the protein/peptide equivalent (in mmol/l), whose concentrations are based on that of human saliva: alanine (1.95), arginine (1.30), asparagine (1.73), aspartic acid (1.52), cysteine (0.05), glutamic acid (5.41), glutamine (3.03), glycine (1.95), histidine (1.08), isoleucine (2.38), leucine (3.68), serine (3.46), threonine (1.08), tryptophan (0.43), tyrosine (2.17), valine (2.38), and casein (5.0 g/l).

Biofilm growth. The patient's saliva was inoculated onto microplated titanium specimens using 400 μ L per well and incubated in a microbiological incubator at 37°C for 1 hour. Thereafter, the saliva was aspirated from the well bottoms and 1.8 mL of artificial saliva was added to each microplate. The plates were incubated at 37°C for 24 h in anaerobic jars (Anaerobac® - Probac do Brasil Produtos Bacteriológicos Ltda., São Paulo, Brazil) and biofilms were formed independently on titanium specimens. After that, the specimens were transferred with sterile tweezers to a new plate

containing DMM and kept under anaerobic conditions (Anaerobac® jar) and allowed to rest for 24 hour.

Treatment with antiseptic substances. After 48 h of incubation, the specimens were transferred with sterile tweezers to a new plate containing 2 mL of each antiseptic and maintained in contact for 60 seconds. The tested antiseptics were: chlorhexidine 0.12% (Periogard - Colgate-Palmolive Company, São Paulo, Brazil), chloramine T (Trihydral - Perland Pharmacos Ltda., Londrina, Brazil), triclosan (Plax - Colgate-Palmolive Company, São Paulo, Brazil), and essential oils containing eucalyptol, thymol, methyl salicylate, and menthol (Listerine - Johnson & Johnson do Brasil Ind. e Com. de Produtos para Saúde Ltda., São Paulo, Brazil). Saline solution 0.9% (Laboratório Arboreto, Juiz de Fora, Brazil) was used for the control group.

Quantification of viable cells. After treatment with antiseptic substances, the specimens were removed from the wells with sterile tweezers, and non-adherent cells were washed off with 2 mL of sterile saline solution in microplates. The specimens, following the order of treatment of surface and of antiseptic, were placed in microtubes containing 1 mL of saline solution, stored on ice, homogenized in a vortex mixer (Phoenix Modelo AP 56 - Phoenix Indústria e Comércio de Equipamentos Científicos Ltda, Araraquara, Brazil) and sonicated (Sonicador Vibra Cell - Sonics and Materials, Danbury, USA) at 30 W using three pulses of 10 s at a 5-second interval in order to obtain a homogenous biofilm suspension.¹⁹

The suspensions were diluted in saline solution up to 10^{-7} and inoculated in duplicate in blood agar for the count of total microorganisms under anaerobic conditions (Anaerobac®) at 37°C for 96 hours. Colony-forming units were counted and the results were expressed in CFU/mm².¹⁹

Statistical analysis. The CFU data were analyzed using two-way ANOVA at a 5% significance level and the data between the groups were compared by Tukey's test. The software used was SigmaPlot Version 11.0, from Systat Software Inc., San Jose, USA.

Results

The following surface roughness values were obtained: 0.47 µm for SS, 0.43 µm for AESS, 0.79 µm for SBS, and 0.72 µm for SBAES.

There was a significant difference concerning antiseptic agents ($p = 0.003$) and titanium surfaces ($p = 0.015$), but the interaction between these factors was not statistically significant ($p = 0.718$). After 48 h of biofilm growth, a thick biomass was observed on the titanium surfaces. Bacterial counts were higher on SBS, on SBAES, and on SS than on AESS. There was statistical difference only between SBS and AESS (Table).

There was no statistical difference among antiseptics in the groups treated with triclosan (TRI), chlorhexidine (CHX), essential oils (EO), and chloramine T (CHT). However, there were statistical differences between EO and CHT when compared with saline solution (Figure).

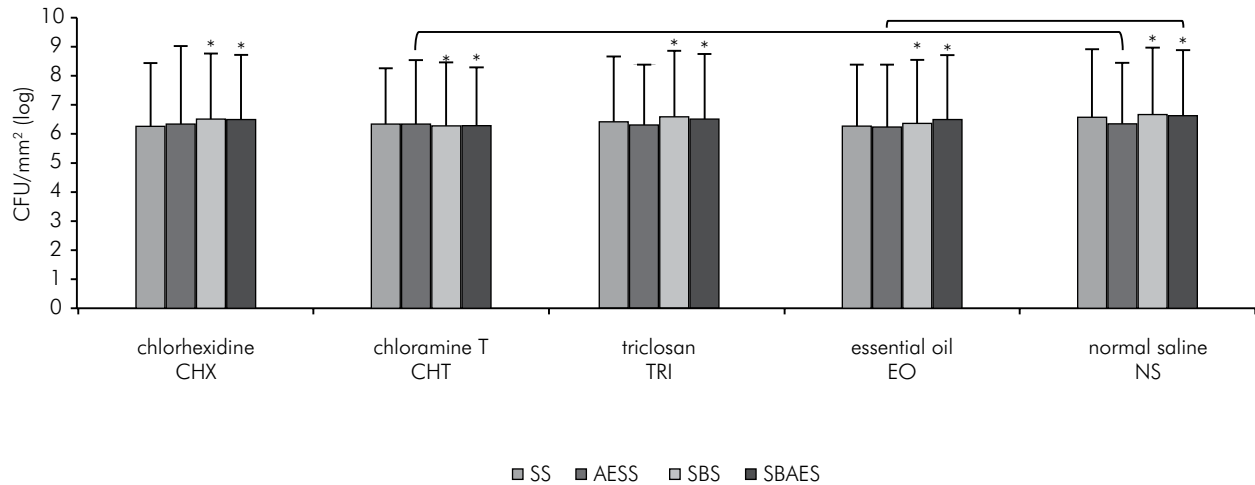
Discussion

Berglundh et al.²⁰ investigated SBAES in spontaneous progression of peri-implantitis in dogs and concluded that progression of peri-implantitis is more pronounced on rough surfaces than on smooth ones. In this context, Pongnarisorn et al.²¹ assessed the effects of different surface treatments on the transmucosal area of implants (machined, acid-etched, and anodized) and suggested that the development of implant-associated inflammation is not dependent upon the type of surface or roughness, but rather upon the presence of bacterial plaque. The authors also mention that the type of surface does not interfere with the quality of the inflammatory infiltrate, with predominance of T cells in all cases, and does not interfere with the microbiota around the implant, although the presence of notches in the subgingival area predisposes to plaque accumulation, thereby increasing the inflammatory infiltrate.

Table. Average bacterial counts (SE) on titanium surfaces submitted to different treatments.

Surface	CFU/ mm ² (log)
Sand-blasted (SBS)	8.16 (0.09)a
Sand-blasted and acid-etched (SBAES)	8.05 (0.87)a
Smooth (SS)	8.01 (0.86)ab
Acid-etched smooth (AESS)	7.97 (0.86)b

Values in the column bearing the same letter are statistically similar ($p > 0.05$).



Note: AESS: acid-etched smooth surface; SBAES: sand-blasted and acid-etched surface; SBS: sand-blasted surface; SS: smooth surface. Considering the treatment performed, the asterisks show statistical difference between titanium surfaces. Considering titanium surfaces, the brackets represent statistical difference between antiseptic treatments ($p < 0.05$).

Figure. Average bacterial counts (SE) considering antiseptic treatments and titanium surfaces.

Chlorhexidine was not so good as the other substances used. For over three decades, CHX had been the gold standard, compared with other chemical agents, because it prevented the formation of dental biofilm;²² however, the present study demonstrates that it is less efficient in reducing microbial colonization. One of the advantages of its use is its broad spectrum and its prolonged and continuous substantivity, even in the presence of blood and other bodily fluids.²³ It produces adverse effects, but no systemic toxicity has been reported so far,²⁴ as it is poorly absorbed by the gastrointestinal tract. Nevertheless, its prolonged use may cause temporary clinical side effects, such as extrinsic staining of teeth, of restorations, and of the tongue, desquamation of the oral mucosa and, occasionally, allergic reactions.

Hanke,²⁵ Sweet et al.,²⁶ and Rams et al.⁷ comment that CHT significantly reduces bacterial colonization, especially in peri-implantitis and in post-extraction bacteremia. In the present study, CHT was found to have the same microbial growth reduction as that provided by CHX and was effective when compared with the control group (Figure). The group treated with EO had fewer bacterial counts than those treated with saline solution, which is in line with the results obtained by Bugno et al.,²⁷ who found that the antimicrobial and antifungal activity of essential oils was better than that of CHX. However, Monfrin and Ribeiro²⁸ and Moreira et al.²⁹

observed that Listerine was less efficient in reducing the microbiota in the saliva, which is inconsistent, who mention that essential oils in long-term clinical trials were efficient and safe.

Even though there was no statistically significant difference between the assessed antiseptics, when an implantologist recommends a mouthwash solution, he/she may choose one that is good at reducing bacterial count and has no adverse effects in the long run. There appears to be consensus agreement that the use of prophylactic antiseptics should be a complement rather than a substitute for conventional mechanical methods, thus adding to and trying to eliminate the deficiencies of mechanical oral hygiene habits.⁶

Conclusion

Different antiseptics reduce the amount of bacteria in titanium implants, but CHX, the current gold standard, was not as good against microcosm biofilm formation as CHT and EO, which were efficient in reducing the biofilm biomass compared with saline solution.

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