Antibacterial Activity and Impact of Different Antiseptics on Biofilm-Contaminated Implant Surfaces

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Abstract: Several antiseptic agents have been proposed for the treatment of peri-implantitis as a complementary therapeutic strategy in addition to mechanical devices. The aim of this study was to compare six different antiseptics, as well as alternative formulations of the same chemical agent, with respect to their decontamination efficacy and impact on chemical properties of the implant surface. Titanium disks with a micro-rough surface, previously contaminated with *Porphyromonas gingivalis* and *Streptococcus mutans* biofilms, were treated for 2 min with different antiseptics (liquid sodium hypochlorite 5.25%, gel sodium hypochlorite 5.25%, liquid chlorhexidine 0.2%, gel chlorhexidine 1%, gel citric acid 40%, and gel orthophosphoric acid 37%) or sterile saline solution (control) and their antibacterial activity as well as their ability to remove biofilm were assessed by viable bacterial count and scanning electron microscopy, respectively. Spectroscopic analysis was also performed on non-contaminated disks after exposure to the antiseptics, in order to detect any change in the elemental composition of the titanium surface. All the antimicrobial formulations examined were effective against *P. gingivalis* and *S. mutans* biofilms. SEM analysis revealed however that liquid sodium hypochlorite 5.25% was more effective in dissolving biofilm residues. Spectroscopic analysis detected traces of the antiseptics, probably due to insufficient rinsing of the titanium surfaces. In conclusion, since gel formulations of these antiseptic agents possessed a similar antibacterial activity to the liquid formulations, these may be proposed as alternative treatments given their properties to avoid overflows and increase contact time without significant side effects on the bone.

Keywords: peri-implantitis; chemical treatments; antiseptics; implant surfaces

1. Introduction

Implant failure due to biological complications, such as peri-implantitis, represents a major concern in implant rehabilitation. According to a recent literature review, the prevalence of peri-implantitis is 19.83% on subject-based evaluation, making this an emerging oral health issue [1]. Despite this high frequency, standardized therapy for peri-implantitis has not been established, though several treatments have been proposed to arrest disease progression and obtain re-osseointegration [2].

According to the most widely diffused theory, the accumulation of a biofilm is the primary etiological factor of peri-implantitis [3], while different microbial species, specifically *Porphyromonas*

gingivalis and *Streptococcus mutans*, contribute to its development and progression. In fact, both microorganisms are responsible for oral diseases and, in particular, *P. gingivalis* is recognized as most closely linked to the development of peri-implantitis while *S. mutans* is known to favor the development of a biofilm coating on titanium surfaces [4]. On this matter, Mombelli and Lang [5] provided at least five lines of evidence to support the bacterial etiology of peri-implantitis, findings also confirmed by the World Workshop for the Classification of Periodontal and Peri-Implant Diseases and Conditions [6]. Due to the eminently bacterial etiology of the disease, surface decontamination is considered a cardinal principle of the therapy and a prerequisite for restoring bone defects around infected implants. Implant decontamination is based on two different but synergic strategies: mechanical debridement of the implant surface followed by chemical disinfection. This combined approach reflects the need to disrupt the adherent biofilm and to dissolve residual organic contaminants, allowing for re-osseointegration of the previously infected surfaces. However, the mechanical treatments cannot achieve complete removal of the biofilm due to implant design, surface roughness, and limited access to the infected surface [7]; therefore, a wide range of antiseptic agents have been proposed as adjuvant treatment for infected implants in order to reach inaccessible areas where mechanical treatments are ineffective.

The antimicrobial activity of antiseptics is based on different mechanisms of action and can be influenced by many factors such as formulation and bacterial load [8]. From a biological perspective, the ideal antiseptic agent should have rapid action, broad-spectrum antimicrobial activity and low toxicity on host cells, essential factors when topical antiseptics are used in close contact with the host tissue. Despite the numerous antiseptics proposed for the treatment of infected implants, to date, there is no evidence of the superiority of any specific antiseptic, the choice of which is mainly based on the individual preferences of clinicians. In the clinical practice, a certain degree of toxicity can always be expected when antiseptics are used in the surgical treatment of peri-implantitis, alongside possible alterations of the physicochemical properties of implant surfaces [9]. Indeed, these alterations have been shown to affect osteoblastic differentiation of mesenchymal stem cells, osteoblast maturation, as well as the production of factors that regulate bone formation, influencing, thereby, the process of osseointegration [9,10]. As a result, it is important to evaluate the effects of antiseptic agents on titanium surface by detecting any change in the elemental composition.

Therefore, in order to reduce the significant side effects on the bone, it might be helpful to confine the antiseptic agent to the infected surface and, in this regard, gel formulation might represent a valid approach since it avoids overflows and allows an increased contact time.

To the authors' knowledge, no study has investigated the effects of different formulations of the same chemical agent on contaminated implant surfaces. The aim of this in vitro study was to assess the antibacterial efficacy of commercially available antiseptics, including alternative formulations of the same chemical agent, on *P. gingivalis* and *S. mutans* biofilms formed on titanium disks with micro-rough surfaces. In addition, the effects of these antiseptics on the surface chemical properties were evaluated.

2. Materials and Methods

2.1. Titanium Disks

Disks of commercial pure titanium grade 4 ASTM (American Society for Testing and Materials, West Conshohocken, Pennsylvania, US) were used for the study. The disks (10 mm diameter, 1.5 mm thickness) had a moderately rough (S_a (average roughness) 1.30 µm [11]) sandblasted and acid-etched surface (Camlog Promote®, Basel, Switzerland). Each disk was sterilized by dry heat sterilization before the experimental procedure.

2.2. Antiseptic Products

Sodium hypochlorite (NaOCl), chlorhexidine (CHX), citric acid (CA), and orthophosphoric acid (OPA) were used as test antiseptics. Specifically, the following specific commercial formulations were investigated:

(1)   NaOCl 5.25%, liquid (Chloraxid, Cerkamed);

(2)   NaOCl 5.25%, gel (Chloraxid, Cerkamed);

(3)   CHX 0.2%, liquid (Curasept);

(4)   CHX 1%, gel (Curasept);

(5)   CA 40%, liquid (Cerkamed);

(6)   OPA 37%, gel (Axia etch).

2.3. Bacterial Strains and Growth Conditions

Standard reference strains of *Streptococcus mutans* ATCC 25,175 and *Porphyromonas gingivalis* ATCC 33,277 (Thermo Fisher Scientific Inc., Waltham, MA, USA) were used in this study. *S. mutans* was grown on Brain Heart Infusion agar (BHI, Oxoid, Basingstoke, UK) under aerobic conditions at 37 °C for 48 h, whereas *P. gingivalis* was grown on Tryptone Soy Agar (TSA, Oxoid, Basingstoke, UK), supplemented with 10% horse serum (Sigma-Aldrich, St. Louis, USA), hemin (5 mg/mL; AppliChem, Darmstadt, Germany), and vitamin K1 (10 mg/mL; Sigma) (complete TSA), and anaerobically incubated at 37 °C for 4–5 days, using the Anaerocult A system (Merck, Darmstadt, Germany). All strains were stored at −80 °C in BHI broth containing 20% glycerol.

2.4. Preparation of Saliva

Unstimulated saliva samples were obtained, with informed consent, from one nonsmoker healthy volunteer, who did not have active carious lesions, periodontal disease or any other oral disease.

The saliva samples were collected for one hour a day, at least 1.5 h after eating, drinking, or teeth brushing, for seven days. Subsequently, the saliva samples were pooled and clarified by centrifugation (30 min; 4 °C; 15,000× g). The supernatant obtained was pasteurized (30 min; 60 °C), re-centrifuged (30 min; 4 °C; 15,000× g), and, finally, stored at −20 °C.

The lack of any bacterial growth was evaluated by plating 100 μL of saliva on blood agar plates incubated under aerobic and anaerobic conditions at 37 °C for 48–72 h.

2.5. Biofilm Formation

Overnight cultures of *S. mutans* and *P. gingivalis* were used for the biofilm formation. Briefly, *S. mutans* cultures were grown in BHI at 37 °C, and then diluted in fresh BHI up to an optical density of 0.7 at 610 nm (~1×10^9 colony-forming unit per milliliter (CFU/mL)). *P. gingivalis* cultures were grown in BHI broth supplemented with hemin (5 mg/mL; AppliChem) and vitamin K1 (1 mg/mL; Sigma) at 37 °C. The bacterial concentration was then adjusted by measuring optical density at 620 nm to 0.08–0.1 nm (~1.2×10^8 CFU/mL).

Sterilized titanium disks were placed in 24-well cell culture plates and incubated with 400 μL of human saliva at 37 °C in an orbital shaker (75 rpm). After 4 h, the saliva was removed and the disks were washed with sterile saline solution (0.9% NaCl). For *S. mutans* biofilm formation, each saliva-coated disk was covered with 800 μL of BHI broth supplemented with 1% sucrose and incubated at 37 °C for 30 min. Next, the disks were inoculated with 100 μL of bacterial suspension and anaerobically incubated at 37 °C for 48 h. For *P. gingivalis* biofilm formation, each saliva-coated disk was pre-incubated at 37 °C for 30 min, with 1.2 mL of BHI broth supplemented as described above. The disks were then inoculated with 200 μL of bacterial suspension and incubated at 37 °C under anaerobic conditions for 48 h. The biomass of bacterial biofilm was determined by crystal violet (CV) staining, as described by Yoshida [12].

2.6. Exposure of Biofilms to Antiseptic Products

The 48 h old biofilm disks were washed three times with sterile saline solution and placed in new wells. These biofilm disks were then covered with 1 mL of the antiseptic compound or with a sterile saline solution (untreated control). After two minutes of exposure, the treated disks were

transferred to sterile tubes containing D/E neutralizing broth and glass beads, and vortexed vigorously for 2 min. Serial dilutions of the re-suspended biofilm solutions were performed in sterile saline solution. Following this, two aliquots (1 mL) from each dilution were plated in BHI agar for *S. mutans* or in complete TSA agar for *P. gingivalis* and incubated as described above. The viable bacterial count was expressed as colony-forming units (CFU)/mL.

Simultaneously, the disks were aseptically transferred in sterile Petri plates and covered with BHI agar for *S. mutans* or complete TSA for *P. gingivalis* and incubated as described above. The viable bacterial count was expressed as "CFU/disk".

The neutralizing activity of D/E was verified by the quantitative suspension test according to DIN EN 1040.

2.7. Scanning Electron Microscopy Analysis (SEM)

Biofilm-covered disks treated with antiseptic agents or sterile saline solution were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 2 h, post-fixed with 1% OsO4 in the same buffer for 1 h, dehydrated through a graded ethanol series, critical point dried with CO_2 (CPD 030 Balzers device, Bal-Tec, Balzers, Liechtenstein), and gold coated by sputtering (SCD040 Balzers device, Bal-Tec). Specimens were analyzed using a field emission gun scanning electron microscope (FEG-SEM) (Inspect FTM; FEI Company, Hillsboro, OR, USA).

2.8. Spectroscopic Analysis

Titanium surface chemical analysis was carried out using X-ray photoelectron spectroscopy (XPS) (Escalam MKII, Vacuum Generators). Three diskss for each treatment plus one untreated control were analyzed. Before treatment, each sample previously sterilized by autoclaving was given an ultrasonic bath with acetone and with 95% ethyl alcohol, each for 2 min, and air dried. Each disk was then immersed for 2 min in the antiseptic solution, subsequently washed first with 10 mL of sterile saline solution and then with 10 mL of sterile bidistilled water.

2.9. Statistical Analysis

Data were expressed as means ± standard deviation (SD) of at least three replicates from two independent experiments. Antibacterial activity was expressed by log_{10} reduction factor (RF): $RF = log_{10}n_c - log_{10}n_u$, where n_c = number of viable cells (CFU) in the re-suspended biofilm solution of untreated control, and n_u = number of viable cells (CFU) in the re-suspended biofilm solution after contact with antiseptic treatments.

Statistical analysis was performed using one-way ANOVA with a Tukey's multiple comparisons test. *p* values were considered statistically significant when $p < 0.05$. Statistical analyses and graphs were produced in GraphPad Prism software v. 7.05. The log_{10} reduction factor (RF) data were calculated in Excel (MS Excel, 2013), as described above.

3. Results

3.1. Antibacterial Activity

The antibacterial activity against *P. gingivalis* and *S. mutans* biofilms formed on human saliva-coated titanium surfaces was investigated via the total bacteria count and expressed as reduction factor (RF). As shown in Table 1, all of the antiseptic agents showed a similar antibacterial activity against *P. gingivalis* and *S. mutans* biofilm as compared to control ($p < 0.01$). Specifically, NaOCl 5.25% and OPA appeared to be more effective than the other antiseptics agents, although the differences were not statistically significant.

Table 1. Antibacterial activity of the antiseptics against *P. gingivalis* and *S. mutans* after 2 min of exposure.

Antiseptics	*P. Gingivalis* RF ± SD	*S. Mutans* RF ± SD
NaOCl 5.25% liquid	6.3 ± 0.3 [#]	7.2 ± 0.6 [*]
NaOCl 5.25% gel	6.5 ± 1.0 [#]	4.3 ± 1.7 [*]
CHX 0.2% liquid	0.9 ± 0.02 [#]	0.8 ± 0.03 [*]
CHX 1% gel	6.5 ± 1.0 [#]	0.9 ± 0.1 [*]
CA 40% liquid	5.3 ± 0.1 [#]	4.1 ± 0.5 [*]
OPA 37% gel	6.2 ± 0.2 [#]	7.1 ± 0.5 [*]

NaOCl, sodium hypochlorite; CHX, chlorhexidine; CA, citric acid; OPA, orthophosphoric acid; #, $p < 0.0001$ and *, $p < 0.01$ vs. control (NaCl 0.9%).

3.2. SEM Analysis

The photomicrographs of *P. gingivalis* and *S. mutans* biofilms following the antiseptic treatments are shown in Figure 1. The control disks revealed a different biofilm growth pattern for *S. mutans* and *P. gingivalis*: the first forming a multilayer structure, the second appearing as small clusters or isolated coccobacilli of 0.5–1 μm diameter. Microscopic analysis showed a substantial reduction of biofilm mass on all of the samples. Specifically, no bacterial aggregates were found on the contaminated titanium surfaces treated by liquid 5.25% NaOCl. Conversely, the contaminated titanium surfaces treated with the other antiseptics showed some remnants of bacterial cells.

3.3. Spectroscopic Analysis

Carbon and oxygen were the predominant elements on the surface of all samples. These elements result either from test substances or environmental contamination. Titanium was revealed in small proportions as a consequence of environmental contamination by organic compounds or alteration of the surface due to exposure to test agents. The curves of the normalized spectra of Ti2p (Figure 2) are very similar to each other, indicating that the Ti–O bond in TiO_2 (459.3 eV) is dominant in all samples. Other potential bonds formed by titanium cannot be distinguishable on the Ti2p spectra due to their small chemical shifts with respect to the Ti–O bond. Interestingly, titanium could not be detected in the sample treated with 37% OPA acid.
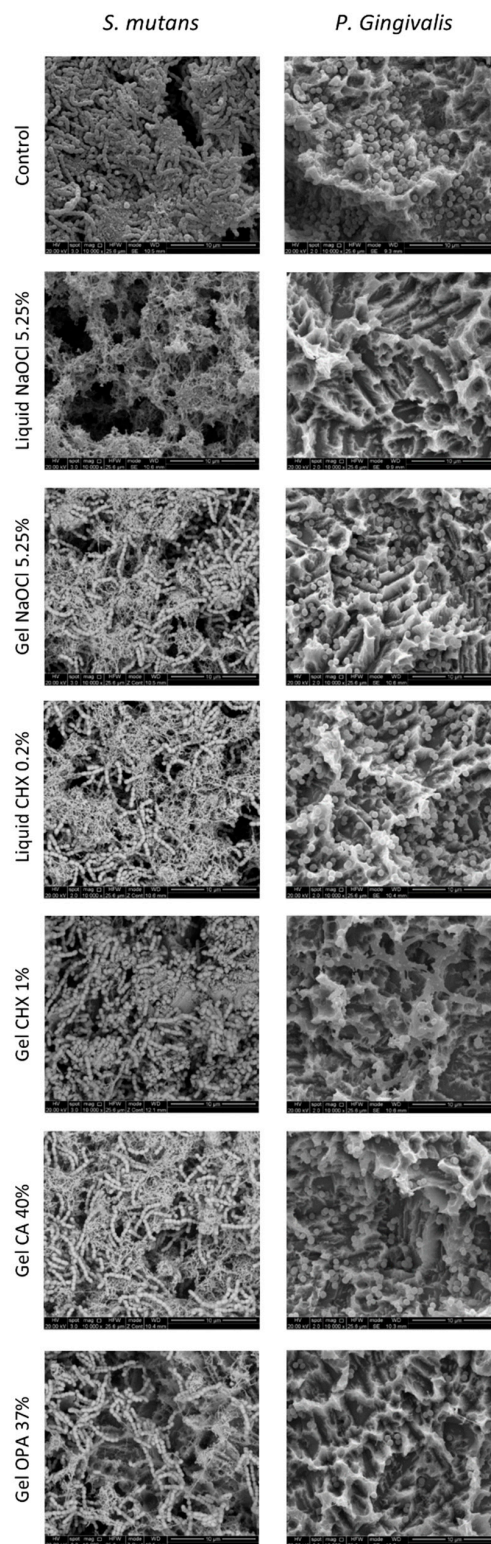
Figure 1. SEM images (magnification ×10,000) of *S. mutans* and *P. gingivalis* biofilms attached on titanium surface after exposure to antiseptics for 2 min. NaOCl, sodium hypochlorite; CHX, chlorhexidine; CA, citric acid; OPA, orthophosphoric acid; control, NaCl 0.9%.
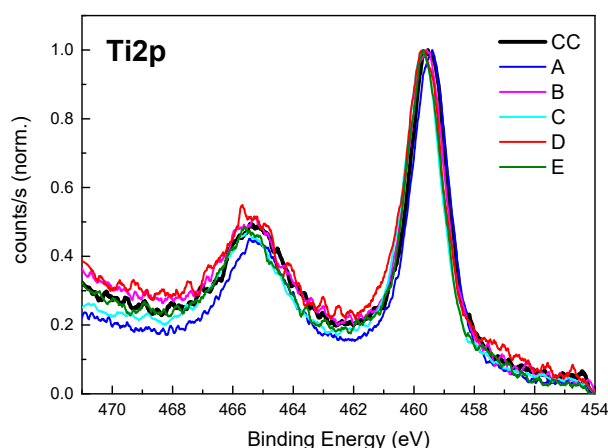
Figure 2. Normalized spectra of Ti2p of non-contaminated titanium samples after immersion in 1 mL of each antiseptic product for 2 min. (**CC**) Control; (**A**) NaOCl 5.25% liquid; (**B**) NaOCl 5.25% gel; (**C**) CHX 0.2% liquid; (**D**) CHX 1% gel; (**E**) AC 40% gel.

Spectra relative to the peak O1s (Figure 3) show very different characteristics among the samples. Each spectrum can be interpreted as a composition of peaks at the characteristic energies of the different bonds in different proportions from one sample to another. All samples show the peak corresponding to the Ti–O bond, together with the peaks of the C = O, C–O and OH bonds. Sample F, the only one that contained phosphorus, showed a predominance of the peak corresponding to the P–O bond.
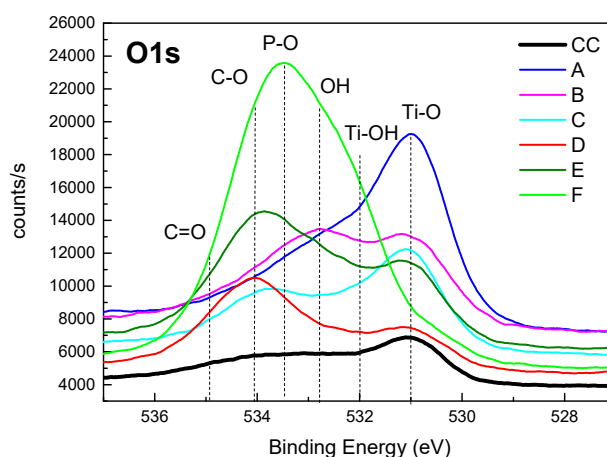

Figure 3. Normalized spectra of O1s of non-contaminated titanium samples after immersion in 1 mL of each antiseptic product for 2 min. (**CC**) Control; (**A**) NaOCl 5.25% liquid; (**B**) NaOCl 5.25% gel; (**C**) CHX 0.2% liquid; (**D**) CHX 1% gel; (**E**) AC 40% gel; (**F**) OPA 37% gel.

C1s spectra (Figure 4) also differed among the samples. These spectra are the result of the composition of numerous peaks corresponding to the energies of the different bonds indicated in the figure, and present in different proportions. In particular, O = C–O, C–C, O, C–OH, and C–NH bonds were observed. The Ti–C bond was not detected.
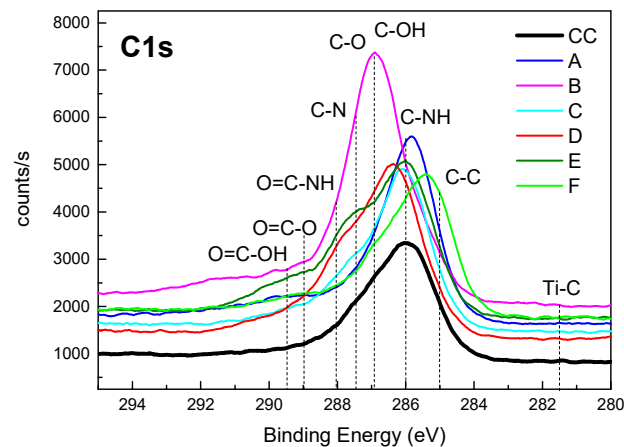
Figure 4. Normalized spectra of C1s of non-contaminated titanium samples after immersion in 1 mL of each antiseptic product for 2 min. (**CC**) Control; (**A**) NaOCl 5.25% liquid; (**B**) NaOCl 5.25% gel; (**C**) CHX 0.2% liquid; (**D**) CHX 1% gel; (**E**) AC 40% gel; (**F**) OPA 37% gel.

Sodium was detectable in all samples, in small and always different proportions. Even nitrogen was always visible, but at the limit of detectability. The chlorine signal was absent in the CC and F samples, while in the other samples it was not quantifiable. Phosphorus was present only in sample F.

4. Discussion

In this study, the decontamination efficacy of six commercially available antiseptic agents against *P. gingivalis* and *S. mutans* biofilms on micro-rough titanium disks, and their effects on surface chemical composition, were assessed.

To reach our goal, we developed an in vitro biofilm model resembling the in vivo physiological microenvironment as closely as possible. As described above, *P. gingivalis* is one of the pathogens most often associated with peri-implantitis [13], and *S. mutans* is able to form a greater quantity of biofilm, favoring the colonization of *P. gingivalis* [14]. The titanium disks were exposed to human saliva in order to reproduce the physiological protein layer known to mediate bacterial adhesion on the implant surface in vivo. Disks with moderate surface roughness (S_a:1.30 μm) were used as 95% of the surfaces in currently commercialized implant systems are composed of titanium with a moderately rough topography [15], and the greater affinity of bacteria and peri-implantitis progression for this type of surfaces is well-known [16,17].

In this study, marked antibacterial activity against *P. gingivalis* and *S. mutans* biofilms following 2 min of exposure was demonstrated for 5.25% NaOCl liquid and gel, followed by 37% OPA gel, 40% CA liquid, 1% CHX gel, and 0.2% CHX liquid formulations. However no significant difference in their antibacterial activity was observed despite 5.25% NaOCl appearing to be the most effective in removing the biofilms. Indeed, as evidenced by SEM analysis, disks treated with liquid 5.25% NaOCl showed a reduced biofilm matrix and no residual bacterial cells.

Overall, our findings are in agreement with other studies that have not found significant differences in the decontaminating efficacy between the most commonly used antiseptics [18–20].

The antimicrobial properties of NaOCl have been thoroughly investigated with respect to its endodontic application as an irrigant [21,22], while less is known about its effect on infected implants [23]. The germicidal action of NaOCl depends on its dissociation into sodium hydroxide and hypochlorous acid, which has a high oxidizing power and can easily penetrate inside bacterial cells leading to irreversible enzyme inhibition, lysis of the cytoplasmic membrane, degradation of phospholipids, and alterations of cellular metabolism [24]. However, NaOCl is highly cytotoxic for host cells, including osteoblasts and fibroblasts. In fact, following the exposure of canine bone samples to 5.25% NaOCl liquid for 30 min, Kerbl et al. [25] observed macroscopic demineralization and degradation of the organic matrix. Another study demonstrated that the toxicity of NaOCl liquid on

human bone marrow mesenchymal stem cells was proportional to its concentration and the exposure time [26]. Thus, the 5.25% NaOCl liquid formulation cannot be recommended for surgical treatment of peri-implantitis. The NaOCl gel formulation, which is equally effective against *P. gingivalis* and *S. mutans* biofilms as evidenced in our study, may however represent a potential alternative treatment. Similar results were also observed for the 37% OPA and 1% CHX gel formulations, further supporting the possible use of antiseptic gels for the treatment of infected implant surfaces, since gel formulations may limit the accidental contact of the antiseptic agent to the surrounding tissues. CHX has however also been shown to possess cytotoxic effect against host tissues [27–30], whereas OPA's cytotoxic properties have been highlighted by a single study and, hence, warrants further investigation [31].

In this regard, a more interesting finding of our study shows that 40% CA had similar antibacterial activity to the 5.25% NaOCl as well as the 37% OPA formulations. This evidence is of especial clinical importance as CA has been demonstrated to be well tolerated by host cells. One study also observed that 50% CA favored the adhesion and proliferation of pre-osteoblastic cells on bone as a result of the demineralization and consequent formation of a micro-rough substrate similar to that produced by osteoclasts [32].

In addition to the antibacterial activity toward biofilm contaminated implants, our results also evidenced that NaOCl, OPA, CA, and CHX did not produce significant alterations in surface chemical properties, avoiding potential detrimental effects on surrounding tissues [20]. Indeed, NaOCl, CA and CHX have not altered the chemical structure of the titanium oxide surface layer as evidenced by spectroscopic analysis. However, titanium could not be detected on the sample treated with OPA, suggesting the formation of a layer of corrosion products consisting of titanium oxide phosphate [33], and this deserves further investigation. Moreover, multiple elements such as carbon, chlorine, sodium, and phosphorus were detected on treated titanium disks, suggesting adsorption and/or residual traces of decontaminating substances on the surface. Titanium disk surfaces can be altered by either a direct action of the substance on the surface or, more likely, by the adsorption and permanence of the chemical agent. Our results point to a potential chemical contamination of the surface, which may be due to inadequate washing of decontaminated titanium disks. Prolonged rinsing of the treated surfaces might then be required in clinical practice. Also, we cannot exclude that the chemical surface contamination of treated titanium disks might compromise their biocompatibility with host tissues. In fact, Kotsakis et al. showed the presence of elemental contaminants of NaOCl and CHX on decontaminated surfaces that lead to a cytotoxic effect of the host tissues, potentially compromising the biocompatibility of treated surfaces [20]. In addition to chemical surface contamination by antiseptic agents, chemical alterations of the titanium oxide layer can be also caused by the peri-implantitis environment, the inflammatory process and acids originated by the bacterial metabolism [34–38]. It is not clear, however, whether local corrosion phenomena affect re-osseointegration. On this, a protocol aimed at restoring the atomic composition of the titanium oxide surface layer, based on the combined use of hydrogen peroxide and CO_2 laser, was also investigated, although with controversial results [39,40].

The main limitation of this study is that it utilizes an in vitro system that warrants caution in translating results to clinical practice as host defense mechanisms and local tissue detoxification, and the influence of blood flow should also be considered. In our study we set up a biofilm model grown on a titanium surface better to simulate the oral microenvironment, which uses human saliva-coated surfaces, the most common periodontal pathogens, anaerobic conditions during biofilm formation and routine exposure time.

In the future, ex vivo studies with multispecies biofilm will be of great help to identify the most promising antiseptic agent, in terms of antibacterial, anti-biofilm, and biocompatibility properties, for the standardization of peri-implantitis treatment.

5. Conclusions

The present in vitro study aimed to assess the decontamination efficacy and impact on surface chemical composition of six commercially available antiseptic agents on titanium disks with micro-rough surfaces contaminated with mono-species biofilms.

Overall our results showed that NaOCl, OPA, CHX, and CA possess antibacterial activity against *P. gingivalis* and *S. mutans* biofilms. Importantly, the promising antibacterial activity of gel formulations (NaOCl, OPA and CHX) suggests these as alternative approaches in clinical practice during the surgical treatment of peri-implantitis. Gel formulations, by prolonging the contact time, could be more effective toward oral pathogens and, simultaneously, could limit the accidental contact with peri-implant tissues; investigating these aspects in more detail might be of interest in future research.

More importantly, the antiseptics examined did not modify the chemical composition of the titanium disks, though the effects of OPA merits further study. Some contaminant residues were detected, however, suggesting the necessity of prolonged washing with saline solution following implant surface exposure to antiseptics. Within the limits of this study, our results indicate that these antiseptic agents may be beneficial as a complementary treatment to mechanical debridement for the elimination of biofilm on rough implant surfaces in the treatment of peri-implantitis.

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