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Chemical debridement of contaminated titanium surfaces: An *in vitro* study

ELISABET HENDERSON[1]*, SIGURD SCHNEIDER[1]*, FERNANDA C. PETERSEN[2], HÅVARD J. HAUGEN[3], JOHAN CASPAR WOHLFAHRT[4], KARL EKSTRAND[1] & ANDERS EKFELDT[1]

[1]*Department of Prosthetic Dentistry and Oral Function,* [2]*Department of Oral Biology,* [3]*Department of Biomaterials, and* [4]*Department of Periodontics, Faculty of Dentistry,University of Oslo, Blindern, Oslo, Norway*

Abstract

Objective. To compare the efficacy of different chemical solutions when used for chemical debridement of biofilm contaminated titanium surfaces in an in-vitro experimental study. ***Materials and methods.*** Commercially pure titanium discs with a diameter of 6.2 mm and height of 2 mm, mirror-polished with a measured surface amplitude value S_A = 0.037 μm ± 0.009 were used as test-surfaces. A biofilm was simulated with multi-layers of *Staphylococcus epidermidis* ATCC359844 covering the entire titanium surface. The chemical agents tested were: 3% H_2O_2, 0.2% Chlorhexidine, 24% EDTA-gel, 3% H_2O_2 mixed with 1.6 g/L TiO_2 and sterile saline solution. The decontamination effect was evaluated by optical density analysis using spectrophotometry and with scanning electron microscopy (SEM) images of the remaining biofilm. ***Results.*** The suspensions of 3% H_2O_2 and 1.6 g/L TiO_2 or 3% H_2O_2 alone were the most effective in removing *S. epidermidis* biofilms ($p < 0.05$), whereas 0.2% chlorhexidine or 24% EDTA gel had no significant effects. SEM images of the remaining biofilms supported the quantitative results indicating the higher efficacy of 3% H_2O_2 and 1.6 g/L TiO_2 or 3% H_2O_2 alone. It also revealed that EDTA, despite a non-significant effect on reducing the amount of established biofilms, was able to alter the biofilm architecture, as demonstrated by increased interspaced regions. ***Conclusions.*** In this *in vitro* study the decontamination potential of a suspension of 3% H_2O_2 and 1.6 g/L TiO_2 or 3% H_2O_2 alone were encouraging. Whether such procedures would have a similar effect *in vivo* remains to be determined.

Key Words: *biofilm, chemical agents, decontamination, titanium implants, titanium surface*

Introduction

Peri-implantitis is defined as an inflammatory process in the tissues surrounding an osseointegrated implant in function, resulting in loss of supporting bone [1]. In the treatment of peri-implantitis, the reduction of bacteria from the surface of dental implants is probably crucial [2–4].

Biofilm formation is a process that involves bacterial adhesion to a surface followed by cell proliferation and production of an extracellular matrix enclosing the cells [5]. The accumulation of biofilm on dental implants may lead to inflammatory responses similar to those observed in experimental gingivitis [6]. Once a biofilm has formed, it can be very difficult to clinically remove it by debridement and further decontamination of the titanium surface. Bacteria in the interior of the biofilm are usually less susceptible to antimicrobial agents and immune defence mechanisms than their planktonic counterpart [5]. The presence of micro-organisms in the peri-implant areas may lead to a persistent inflammatory stimulus and an imbalance between bacterial impact and host defence that may lead to progressive disintegration of implants [7]. The formation of biofilm on the implant surface is influenced by the surface properties of the implant, including chemical composition, surface roughness and surface free-energy [8].

Peri-implantitis may also be initiated and maintained by iatrogenic factors, e.g. cement remnants,

Correspondence: Anders Ekfeldt, Professor, odont.dr, Department of Prosthetic Dentistry and Oral Function, Institute of Clinical Dentistry, University of Oslo, PO Box 1109 Blindern, NO – 0317 Oslo, Norway. Tel: +47 22 85 20 00. Fax: +47 22 85 23 44. E-mail: anders.ekfeldt@odont.uio.no
*Both authors have contributed equally to this work

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inadequate abutments seating, over-contoured prosthetic restorations, malpositioning of the implant and other technical complications [9]. In an ideal situation the implant surface must be free from bacteria and contaminants, with conditions favouring re-osseointegration. Debridement of contaminated titanium surface proves, however, to be difficult. The cleaning procedures should remove the contaminants without changing the surface topography. The dental biofilm redevelops fast, with reports showing that bacterial numbers may reach or even exceed baseline values 2 days after professional tooth cleaning or scaling and root planning [10]. Environmental conditions are thought to influence the microbial composition of biofilms, leading to shifts that may influence the healthy balance between the host and the microbes. A goal with debridement will therefore also be to create environmental conditions that would favour a microbial composition compatible with health. Characterization of the inflammatory host response around implants and teeth in patients with peri-implantitis has shown a site-specific inflammation rather than a patient-associated specific host response [11]. Several studies illustrate that most methods used for surface debridement achieve a resolution of the inflammatory lesion in the peri-implant mucosa. However, no surface decontamination technique has been found to be superior and none of them have demonstrated re-osseointegration along the previously contaminated implant surface, despite new bone regeneration in some of the defects [12]. To eliminate infection, resolve inflammation and render the surface conductive to bone regeneration and re-osseointegration, mechanical, chemical or laser therapy and a combination of all three can be used during surgical exposure. Various protocols with: air powder abrasion, saline wash, citric acid treatment, laser therapy, peroxide treatment, ultrasonic and manual debridement and application of topical medication have been tested on ligature induced peri-implantitis in animals. The various techniques have both advantages and disadvantages, such as damage of the implant surface with mechanical or laser therapy and the inability to remove all contaminants with chemical treatment [12].

Staphylococcus epidermidis is one of the most important species associated with infections of implanted medical devices. *S. epidermidis* has also been used as a model organism in numerous studies to evaluate the effect of factors that may affect bacterial colonization of implants [13–15]. Of clinical concern is the fact that bacteria in biofilms, including *S. epidermidis*, are particularly resilient to the action of antimicrobials and immune-responses.

The aim of this *in-vitro* experimental study was to compare the efficacy of different chemical solutions when used for chemical decontamination of *S. epidermidis* biofilms formed on titanium surfaces.

Materials and methods

Titanium discs preparation and surface modification

Commercially pure (cp) titanium discs with a diameter of 6.2 mm and a height of 2 mm were ground and polished (Phoenix 4000, Buehler GmbH, Düsseldorf, Germany) in seven sequences according to Lamolle et al. [16]. After polishing, the discs were washed with NaOH at 40 vol.% and HNO_3 at 50 vol.% in an ultrasonic bath to remove contaminants, then washed with deionized water to reach a neutral pH [17] and wrapped in a sealed package and finally autoclaved, at 134°C and 2 bar.

The surface topography (S_A) of the polished discs was measured with a blue light laser interferometer (Sensofar Plμ 2300, Terrassa, Spain) with a 50× DI Nikon objective. The measured surface amplitude value was $S_A = 0.037\ \mu m \pm 0.009$ which is considered 'polished' (<0.05 μm) according to the definition of surface roughness for implants [18].

Decontamination agents

Several different chemical decontamination agents were preliminarily screened for the ability to promote debridement of *S. epidermidis* biofilms formed on titanium discs (Table I). These chemical agents were selected through a review of other studies presented in this subject [4,19–23].

Biofilm assay

Bacterial strain and culture conditions. S. epidermidis ATCC359844 type strain stored at −20°C in 15% glycerol was used in the study. Growth medium was brain heart infusion broth (BHI), with incubation at 37°C in aerobic atmosphere. Five-milliliters of BHI was initially inoculated with 10 μL of the *S. epidermidis* glycerol stock and incubated overnight.

Biofilm was allowed to form on the discs for 24 h at 37°C incubation in an aerobic atmosphere. Exposure to the chemical agents was for a period of 2 min.

Spectrophotometry measurement of biofilms

For assessment of biofilm mass, the bacterial film was stained for 10 min with a 0.1% solution of safranin. The discs were then transferred to new wells after rinsing with distilled water. Bound dye was released from stained biofilm using 30% glacial acetic acid. The optical density at 530 nm (OD 530) was measured (Synergy HT Multi-Detection Microplate Reader; Biotek, VT, Winooski, USA). The results show the relative amount of safranin

Table I. Chemical agents screened for their decontamination effect.

Chemical agent	Concentration	Manufacturer
Sodium chloride water (saline)	0.9% (NaCl)	VWR (Oslo, Norway)
Citric acid	1.8%w/v; pH2.2	Sigma-Aldrich, Chemie (Steinheim, Germany)
Hydrofluoric acid	0.2% w/v; pH 3.3	Rectapur, Prolabo (Paris, France)
Sodium lauryl sulphate (SLS)		VWR (Oslo, Norway)
SLS + Chlorhexidine digluconate	0.2%	VWR (Oslo, Norway)
Chlorhexidine digluconate	0.2%	VWR (Oslo, Norway)
Chlorhexidine digluconate +	0.2%	VWR, Oslo, Norway.
EDTA (ethylenediaminetetraacetic acid)	17%	
PrefGel ™	24% (EDTA-gel)	Straumann Institut (Basel, Switzerland)
Hydrogen peroxide (H_2O_2)	3%	Sigma-Aldrich (St Louis, MO)
H_2O_2 (3%) + 1.6 g/L TiO_2 powder		Degussa, Aeroxide, P25 (Hanau-Wolfgang, Germany)
Exposure to Ozone		HealOzone KaVo, Dental Excellence [24].

that was bound to the bacterial cells in the biofilm. A reduction in the optical density values indicates biofilm detachment from the titanium discs.

Scanning electron microscopy

The remaining biofilms were examined using a scanning electron microscope (Philips XL 30 ESEM, FEI Electron Optics, Eindhoven, The Netherlands) to investigate the effect of different chemical agents on the architecture and amount of the *S. epidermidis* biofilms. The remaining biofilm on the titanium discs was fixated in 2.5% glutaraldehyde, followed by serial dehydration steps using ethanol and coating with gold-palladium (50 nm layer) (Polaron E5100 Sputter Coater, Polaron Equipment Ltd, Watford, UK).

Preliminary screening

The polished titanium discs were divided into 13 groups, with six titanium discs in each group. Eleven decontamination agents were initially tested. The negative control disc was without inoculated bacteria and the positive control disc was inoculated with biofilm but was not decontaminated.

Statistical analysis

A power analysis was performed on the preliminary screening data in order to find the appropriate number of samples (SPSS 17.0 for Windows). One-way ANOVA was used to compare all the groups for a normalized dataset followed by pairwise multiple comparison procedures with Tukey test. If the data set failed both equality and normality test, non-parametric Kruskal-Wallis analysis of variance (ANOVA) on ranks was used instead, followed by pairwise multiple comparison procedures with Dunn's method. The significance threshold (p) was set at a 0.05 level (SigmaStat 3.5, Systat Inc, St. Louis, MO).

Results

Preliminary screen

The remaining amount of biofilms after decontamination with different chemical agents (Table I) was determined using the safranin-staining method (OD 530). Five of 11 screened chemical agents reduced *S. epidermidis* biofilms in this preliminary screen. The results revealed the presence of remaining bacteria on the titanium surface ($n = 6$) after debridement with the above chemical agents (Figure 1). Decontamination with 3 vol. % H_2O_2 resulted in reduced amounts of remaining biofilm compared to the other agents and it was significantly more effective in reducing the amount of established biofilm than Chlorhexidine 0.2 vol%.

Debridement of the biofilm with sterile saline showed little or no measurable effect. This was also observed with the following chemical agents: 1.8% citric acid; 0.2% hydroflouric acid; 1% sodiumlaurylsulphate (SLS) alone or in combination with 0.2 vol.% Chlorhexidine. A similar negligible effect was found for the 0.2 vol% Chlorhexidine alone or for a combination of 0.2 vol.% Chlorhexidine and 17% EDTA (ethylenediaminetetraacetic acid). Exposure to Ozone (HealOzone® KaVo, Dental Excellence) showed no decontamination effect on the biofilm.

Based on these preliminary results, the chemical decontamination agents listed in Table II were selected for further evaluation. The preliminary screen data was also used to find the appropriate number of samples needed for statistical power. The suitable number of test samples (titanium discs) was found to be $n = 53$.

Main study

Effect of chemical agents on biofilm mass. Decontamination with a suspension of 3% H_2O_2 and 1.6 g/L

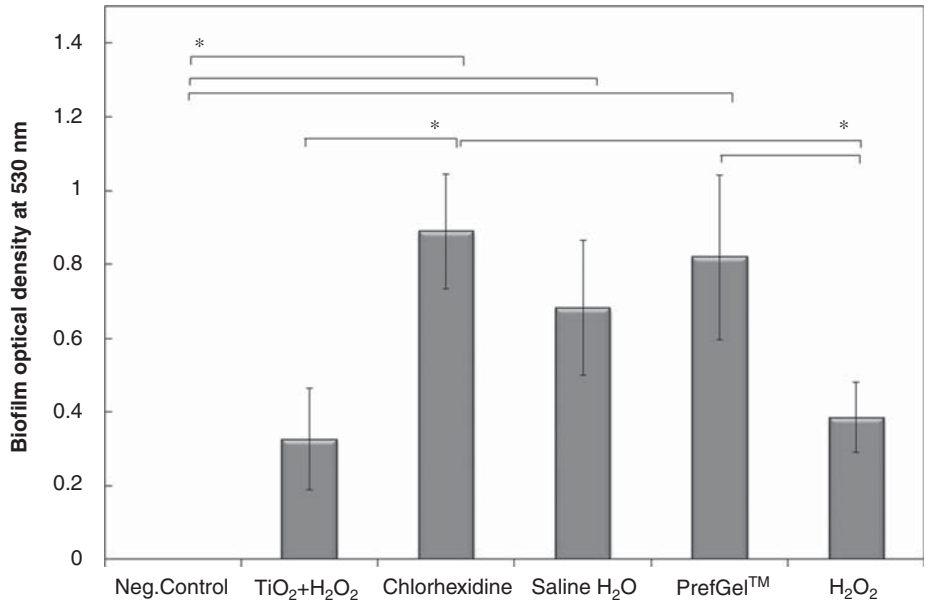

Figure 1. Preliminary screen for the presence of remaining bacteria after debridement with various chemical agents. Biofilms were stained with safranin and optical density was measured by absorbance at 530 nm (OD 530). The negative control was sample without inoculated bacteria. The significant differences ($p < 0.05$) are labeled with * ($n = 6$).

TiO_2 showed significantly less remaining bacteria compared to the other chemical agents (Figure 2). The result was comparable with the negative control discs without inoculated bacteria. Decontamination with 3 vol.% H_2O_2 also displayed significantly ($p < 0.05$) less remaining bacteria compared to Chlorhexidine 0.2 vol.%, saline H_2O or 24% EDTA-gel. Debridement with Chlorhexidine 0.2 vol.% or 24% EDTA-gel showed a minor effect on the biofilm, whereas saline H_2O showed a significantly better effect than these two chemical agents. Only two groups were found not to be significantly different: the negative control compared to a suspension of 3% H_2O_2 and 1.6 g/L TiO_2 and Chlorhexidine 0.2vol.% vs 24% EDTA-gel.

Biofilm image analysis. SEM images at 1000× (Figure 3) and 5000× (Figure 4) magnification were taken after the chemical decontamination. The lower magnification presented a better overview of the debrided titanium surface.

SEM after debridement with 3 vol.% H_2O_2 (Figures 3, 4D and E) or with a suspension of 3 vol.% H_2O_2 and 1.6 g/L TiO_2 powder displayed a reduced number of remaining bacteria and parts of the surface appeared to be cleaned. The remaining TiO_2 powder was visible on the SEM picture (Figures 3 and 4E). Debridement with saline (Figures 3 and 4A) and Chlorhexidine 0.2 vol% (Figures 2 and 3C) showed no visible effect on the biofilm and there was still a thick layer of *S. epidermidis* covering the titanium surface. However, decontamination with 24% EDTA-gel, despite a non-significant effect on reducing the amount of established biofilms, was able to alter the biofilm architecture, as demonstrated by increased interspaced regions (Figures 3 and 4B).

Discussion

The present study compared the efficacy of different chemical agents used for chemical debridement of polished titanium surfaces contaminated with a biofilm. To simplify the evaluation of useful chemical agents a single strain model of *S. epidermidis* was chosen to simulate a biofilm in combination with a polished titanium surface [15]. Of clinical concern

Table II. Decontamination agents used in the main study.

Chemical agent	Concentration	Manufacturer
Sodium chloride water (saline)	0.9% (NaCl)	VWR (Oslo, Norway)
Chlorhexidine	0.2%	VWR (Oslo, Norway)
PrefGel ™	24% (EDTA)	Straumann Institut (Basel, Switzerland)
Hydrogen peroxide (H_2O_2)	3%	Sigma-Aldrich (St Louis, MO, USA)
H_2O_2 (3%) + 1.6 g/L TiO_2 powder		Degussa, Aeroxide, P25 (Hanau-Wolfgang, Germany)

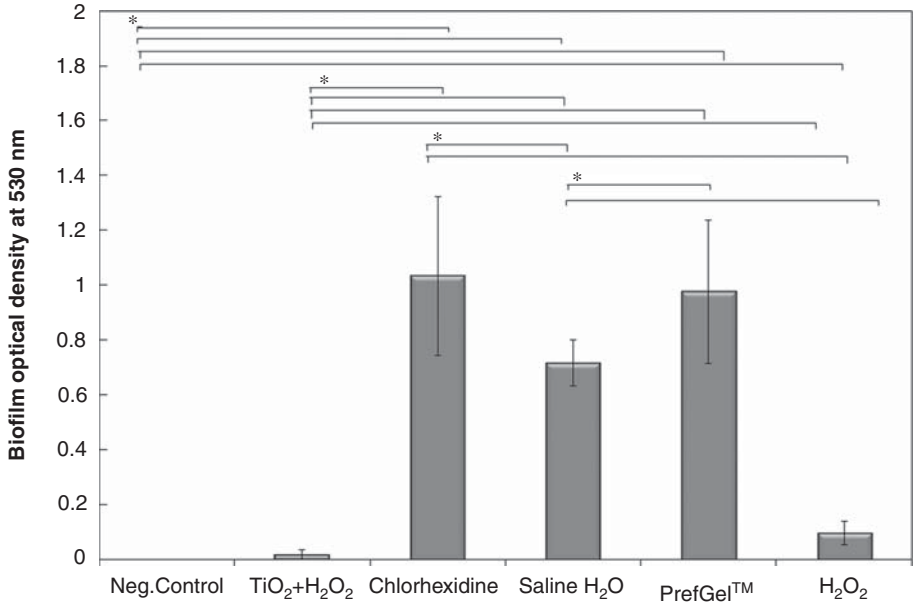

Figure 2. Remaining biofilm after exposure to different chemical agents. Biofilms were stained with safranin and optical density was measured by absorbance at 530 nm (OD 530). The negative control was the sample without inoculated bacteria. The significant groups ($p < 0.05$) are labeled with * ($n = 53$).

is the fact that bacteria in biofilms, including *S. epidermidis*, are particularly unaffected to the action of antimicrobials and immune-responses. The result in this study indicates that *S. epidermidis* binds strongly to the titanium surface and forms a stable biofilm to the surface, even to a polished surface. *S. epidermidis* is seldom presented in the microbiota found at peri-implant sites but has been reported in a high proportion of patients with peri-implant infections [2].

A suspension of 3 vol.% H_2O_2 and 1.6 g/L TiO_2 showed a significantly better decontamination effect than all other chemical agents tested (Figure 2). This was also verified with SEM (Figures 3 and 4E), displaying an almost clean titanium surface. The titanium surface needs to be washed, with sterile saline, after decontamination with this suspension in order to remove the remaining TiO_2 powder. Decontamination with 3 vol.% H_2O_2 also displayed a pronounced effect on the biofilm (Figures 2, 3

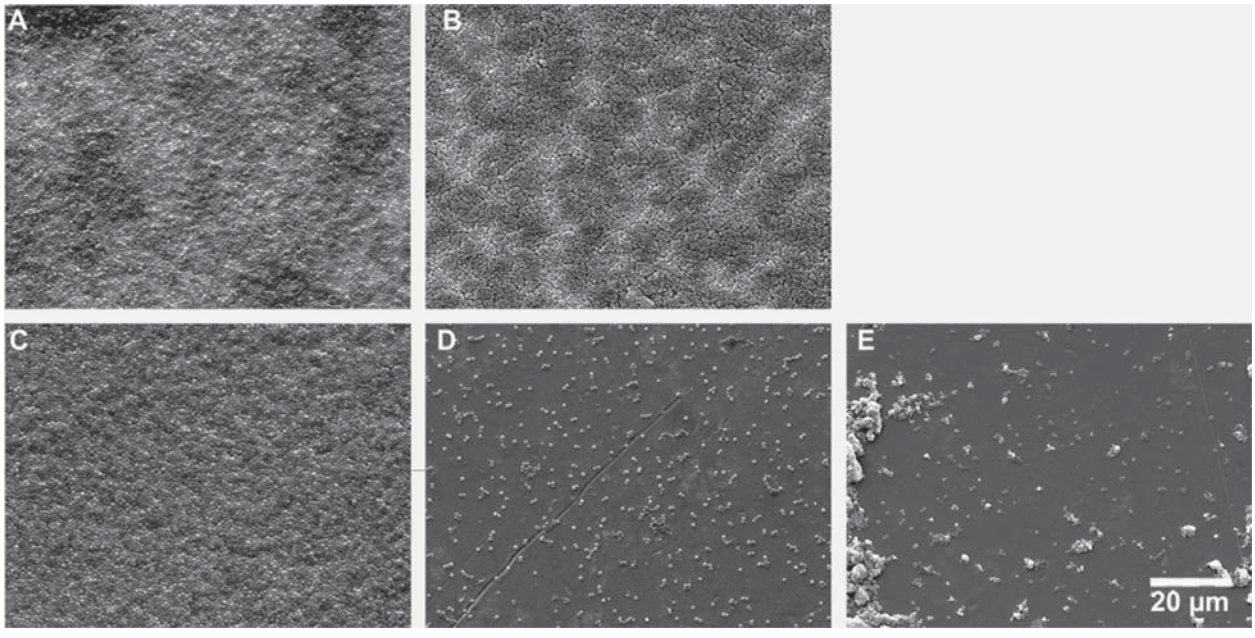

Figure 3. SEM images (1000×) of *S. epidermidis* biofilm remaining on the titanium surfaces after decontamination with (A) Saline; (B) 24% EDTA-gel; (C) Chlorhexidine 0.2 vol%; (D) 3 vol.% H_2O_2 and (E) suspension of 3 vol.% H_2O_2 and 1.6 g/L TiO_2. The remaining white granules are TiO_2.

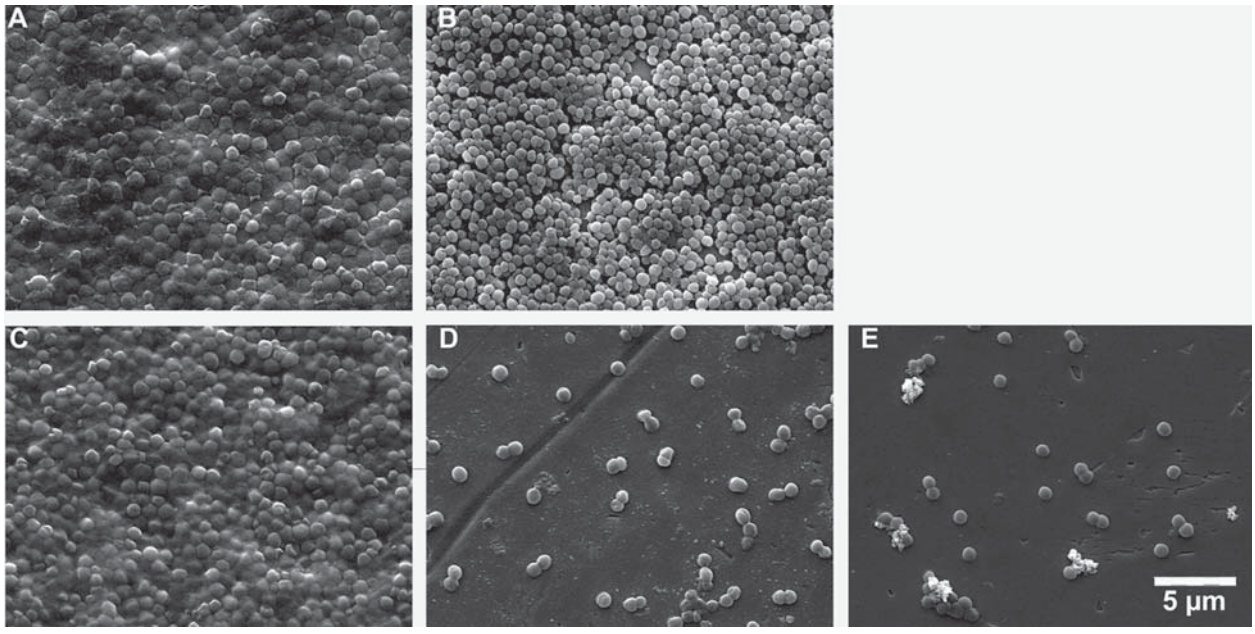

Figure 4. SEM images (5000×) of *S. epidermidis* biofilm remaining on the titanium surfaces after decontamination with (A) Saline; (B) 24% EDTA-gel; (C) Chlorhexidine vol.0.2%; (D) 3 vol.% H_2O_2, and € suspension of 3% H_2O_2 and 1.6 g/L TiO_2. The remaining white granules are TiO_2.

and 4D). It was obvious that the two solutions containing H_2O_2 decontaminated the surface more effectively than the other tested agents. Chlorhexidine 0.2 vol.% and 24% EDTA-gel did not seem to be superior to debridement with sterile saline. However, 24% EDTA-gel dissolved the biofilm on the titanium surface, but most of the biofilm seemed to remain (Figure 4B).

Hydrogen peroxide has been used as an antibacterial agent in many studies due to its oxidizing action on the bacteria and also for its mechanical effect in dislodging biofilms as a result of foaming [21]. The suspension of TiO_2 and H_2O_2 is believed to increase the antibacterial effect, as H_2O_2 has been shown to activate TiO_2 (unpublished, Haugen 2012). This may explain the additive effect with the use of 3 vol.% H_2O_2 and 1.6 g/L TiO_2 in our study.

Initially, a large number of chemical agents and solutions were tested (Table I). These agents were selected based on previous studies investigating the use of chemical agents for decontamination of titanium surfaces, both *in vitro* and *in vivo* [4,19–23]. In some other reports a CO_2 laser in combination with 3% H_2O_2 and citric acid have been used with promising results for decontamination of the microbiota attached to a titanium surface [25–28] Renvert et al. [29] found a limited and similar effect when comparing a YAG laser and an air abrasive device—PERIO-FLOW®—in treating clinical cases with peri-implantitis.

Krozer et al. [23] found exposure to gaseous ozone effective in cleaning a titanium surface. However, in the current study no such effect was noticed of gaseous ozone on the biofilm with *S. epidermidis*. One explanation could be that gaseous ozone disclosed a selective efficacy for different bacteria. In a study by Hauser-Gerspach et al. [30] *Porphyromonas gingivalis* was eliminated from titanium surfaces with gaseous ozone (HealOzone), but *Streptococcus sanguinis* was found to be more resistant. Citric acid has been used as a decontamination agent in several studies, both for peri-implant disinfection treatment and for cleaning titanium surfaces. No or a limited debridement effect was observed on the biofilm with the use of 1.8% Citric acid, but the used concentration might have been too low (authors comment). However, its bactericidal effect on adhering bacteria has also been questioned in another study using a 40% citric acid [21]. Chlorhexidine digluconate has wide-spectrum antibacterial activity against gram-positive and gram-negative bacteria and yeasts. The antibacterial mode of action is to alter the bacterial cell membrane, which results in a leakage and leads to cell death [31]. However, little or no decontamination effect was observed in this study.

Rompen et al. [32] stated that 'The ultimate goal of cleaning procedures should be to remove the contaminants and restore the elemental composition of the surface oxide without changing the surface topography'. Mouhyi et al. [25] examined failed implants with SEM viewing contamination of various sizes on the titanium surface and spectrophotometry of the same surfaces indicated high levels of carbon but almost no titanium. Decontamination of a titanium surface must also cope with the physicochemical modification of the osseointegrated interface for a possible re-ossintegration [27]. The most suitable chemical agent for implant disinfection has not

yet been found, mainly because of a lack of suitable clinical trials [21,25].

A smooth titanium surface was found to be easier to clean than a rough one in an earlier *in vitro* report [33]. In a review, Renvert et al. [34] concluded 'based on the limited data, there is no evidence that implant surface characteristics can have a significant effect on the initiation of peri-implantitis' and The seventh European workshop of periodontology made the same conclusion [9]. Narhi et al. [35] found, in an *in vitro* study, that a bioactive titanium surface did not increase the risk of microbial adhesion.

This study used a standardized technique for evaluation of the decontamination effect of different chemical agents on a titanium surface with a specified surface texture in order to reduce unknown variables of surfaces characteristics. *S. epidermidis* was chosen to simulate the biofilm as this bacteria species is fast growing, has good adhesion and is easy to reproduce for a high number of test samples. The cleaning procedures in this *in vitro* experiment are based on the chemical effect alone; no mechanical cleaning has been done. Our study only measured the amount of *S. epidermidis* remaining after decontamination and not the survival or re-growth of the bacteria.

Conclusions

In this *in vitro* study the decontamination potential of a suspension of 3% H_2O_2 and 1.6 g/L TiO_2 or 3% H_2O_2 alone was encouraging. Whether such procedures would have a similar effect *in vivo* and whether they would possibly elicit a toxic response on the host remains to be determined.

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