Antibacterial effect of hydrogen peroxide - titanium dioxide suspensions in the decontamination of rough titanium surfaces

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Disclosure statement

We hereby confirm that each of the authors has read and concurs with the content in the final manuscript. The material within has not been and will not be submitted for publication elsewhere except as an abstract. All authors have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content and (3) approving the final version of the submitted manuscript.

Abstract

The chemical decontamination of infected dental implants is essential for the successful treatment of peri-implantitis. The aim of this study was to assess the antibacterial effect of a hydrogen peroxide - titanium dioxide (H_2O_2 - TiO_2) suspension against *Staphylococcus epidermidis* biofilms. Titanium (Ti) coins were inoculated with a bioluminescent *S. epidermidis* strain for 8 h and subsequently exposed to H_2O_2 with and without TiO_2 nanoparticles or chlorhexidine (CHX). Bacterial regrowth, bacterial load and viability after decontamination were analysed by continuous luminescence monitoring, live/dead staining and scanning electron microscopy. Bacterial regrowth was delayed on surfaces treated with H_2O_2 - TiO_2 compared to H_2O_2 . H_2O_2 -based treatments resulted in a lower bacterial load compared to CHX. Few viable bacteria were found on surfaces treated with H_2O_2 and H_2O_2 - TiO_2 which contrasted with a uniform layer of dead bacteria for surfaces treated with CHX. H_2O_2 - TiO_2 suspensions could therefore be considered an alternative approach in the decontamination of dental implants.

Keywords

Titanium dioxide, hydrogen peroxide, antibacterial agent, implant surface, biofilm, staphylococcus.

Introduction

Peri-implantitis is defined as an inflammatory process around an osseointegrated dental implant (Zitzmann & Berglundh 2008). It is characterized by the loss of surrounding bone and may ultimately result in total implant failure (Leonhardt et al. 2003; Quirynen et al. 2002). The formation and development of a bacterial biofilm on the implant surface has been suggested as the main causative factor of peri-implantitis (Quirynen et al. 2002; Renvert et al. 2012). Analogous to periodontitis, the infected lesion can trigger an inflammatory host response which causes the resorption of supporting alveolar bone by altering bone homeostasis (Darveau 2010; Heitz-Mayfield & Lang 2010). The microbiota around infected implants is characterised by a high prevalence of Gram-negative anaerobes similar to the pathogenesis of periodontitis (Botero et al. 2005; Heitz-Mayfield & Lang 2010; Mombelli & Lang 1998). However, several other microorganisms, such as *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Candida albicans*, can also be found at failing implants (Kronström et al. 2001; Leonhardt et al. 1999; Persson & Renvert 2014).

Most treatment strategies aim at debridement of granulation tissue and decontamination of the implant surface to prevent reinfection and promote re-osseointegration (Claffey et al. 2008; Lindhe et al. 2008; Schwarz et al. 2011). However, success rates as low as 45 % for the decontamination of dental implants have been reported for surgical treatment of peri-implantitis (Carcuac et al. 2016). Remaining bacteria and biofilm components following decontamination are considered an important factor in the re-occurrence of peri-implantitis (Kreisler et al. 2005).

The complete removal of adhering bacteria from implant surfaces, or prevention of adhesion following surgical treatment, is virtually unachievable in the heavily colonized microbial environment of the oral cavity. Antibacterial agents generally show a reduced effect on biofilms (Larsen & Fiehn 1996; Roberts & Mullany 2010; Zaura-Arite et al. 2001) and the implant shape and surface morphology further hamper successful decontamination (Carcuac et al. 2015; Carcuac et al. 2016). Mechanical debridement is one common approach to physically remove biofilm from a contaminated surface. Several techniques, such as the use of curettes, brushes and CO₂ lasers, have been suggested (Romanos et al. 2009; Schwarz et al. 2006). However, mechanical debridement alone may not be sufficient to access and decontaminate infectious sites. Therefore, adjunctive antibacterial treatment is generally recommended (Claffey et al. 2008; de Waal et al. 2013; Karring et al. 2005; Lindhe et al. 2008; Renvert et al. 2012). One

common approach is to chemically decontaminate the implant surface by the local use of antiseptics such as hydrogen peroxide (H_2O_2), chlorhexidine digluconate (CHX) or citric acid (Bürgers et al. 2012; Gustumhaugen et al. 2014; Henderson et al. 2013). The choice of the chemical agent used is often based on the experiences from therapeutical treatment of periodontitis and the most effective agent for implant decontamination has not yet been identified (Claffey et al. 2008; Romanos et al. 2015).

The adjunctive use of locally applied H_2O_2 for the chemical decontamination of infected dental implants has shown promising results, both *in vitro* (Gustumhaugen et al. 2014; Henderson et al. 2013; Ungvári et al. 2010) and *in vivo* (Gosau et al. 2010; Leonhardt et al. 2003). Aqueous H_2O_2 solutions (3 - 6 vol.%) are commonly used as an antiseptic in dental practice. H_2O_2 solutions may have several advantages over other chemical agents, such as a broad spectrum of activity against various pathogens and the absence of toxic by-products (Linley et al. 2012). The use of antiseptics in open wounds is controversial due to the cytotoxic activity of the agents which may impair wound healing (Atiyeh et al. 2009; Drosou et al. 2003; Giannelli et al. 2008). In contrast, aqueous H_2O_2 is considered safe for the use in open wounds and also has been showing to slightly enhance the proliferation of human epithelial cells (Ungvári et al. 2010).

The bactericidal effect of H_2O_2 is associated with its capacity to oxidize various cellular components through its intrinsic oxidative power and subsequent formation of free radicals (Linley et al. 2012; McDonnell & Russell 1999). It has been shown that the antibacterial effect of H_2O_2 is significantly reduced for pathogens with intrinsic defence mechanisms, in particular pathogens with high catalase activity and the ability to form a protective biofilm (Gaupp et al. 2012; Mah & O'Toole 2001; McDonnell & Russell 1999; Stewart et al. 2000). Recent studies indicate that the oxidative power of H_2O_2 can be enhanced by the addition of titanium dioxide (TiO_2) particles due to non-photocatalytic formation of oxygen centred radicals at the H_2O_2 - TiO_2 interface (Sanchez et al. 2013; Wiedmer et al. 2016). So far, only two *in vitro* studies have investigated the antibacterial effect of such H_2O_2 - TiO_2 suspensions with different outcome. Both studies assessed the antibacterial effect in a *S. epidermidis* biofilm model. Henderson et al. (2013) found an increased removal of biofilm mass for H_2O_2 - TiO_2 suspensions compared to H_2O_2 alone. The bactericidal effect was not investigated in this study. A similar study by Gustumhaugen et al. (2014) could not confirm the positive effect of TiO_2 particles in H_2O_2 in a

biofilm mass and a viability assay. Therefore, there is no conclusive evidence for the antibacterial effect of TiO₂ particles in aqueous H₂O₂ suspensions.

The main aim of the present study was to assess the antibacterial activity of H₂O₂-TiO₂ suspensions in comparison to conventional decontamination with either H₂O₂ or CHX. The use of the constitutive bioluminescent strain *Staphylococcus epidermidis* Xen43 allowed the non-destructive, real-time monitoring of bacterial regrowth on titanium implant surfaces after decontamination. Scanning electron microscopy (SEM) and confocal fluorescence microscopy after live/dead staining have been used as complementary methods to evaluate the outcome of decontamination.

Materials and Methods

Titanium coins and decontamination agents

Commercially pure (cp), machine shaped Ti coins (diameter = 6.2 mm, height = 2 mm) were surface modified to mimic a rough dental implant surface. The coins were grit-blasted with TiO₂ particles (particle size = 180 - 220 µm) and further acid etched with 0.2 vol.% hydrofluoric acid. The modified surface resembled the commercially available OsseoSpeed™ implant surface (Astra Tech AB, Mölndal, Sweden). For a detailed description of the modification procedure and a detailed characterization of the surface see (Gustumhaugen et al. 2014). A summary of all tested groups is given in Table 1. The nanoparticles (Degussa P25, Evonik Degussa GmbH, Hanau Wolfgang Germany) used for H₂O₂-TiO₂ suspensions have a primary particle size of 23 nm and consist of a mixed crystal phase of 80% anatase and 20% rutile according to the manufacturer specifications. It has also been shown that these particles occur as stable agglomerates in aqueous suspensions at physiological pH (Wiedmer et al. 2016). All chemicals were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, USA) if not stated differently.

Table 1: Control surfaces and chemical agents used to treat contaminated Ti surfaces (exposure time 1 min).

Category	Group	Description	Manufacturer
Antibacterial agents	CHX	0.2 vol.% chlorhexidine digluconate	VWR (Oslo, Norway)
	H ₂ O ₂	3 vol.% hydrogen peroxide	Sigma Aldrich (St. Louis, USA)
	H ₂ O ₂ -TiO ₂	3 vol.% hydrogen peroxide + 1 g l ⁻¹ titanium dioxide (Degussa P25)	H ₂ O ₂ : see above TiO ₂ : Evonik Degussa GmbH (Hanau Wolfgang, Germany)
Control groups	H ₂ O	sterile, ultrapure milliQ water	-
	H ₂ O-TiO ₂	sterile, ultrapure milliQ water + 1 g l ⁻¹ titanium dioxide	TiO ₂ : see above
	PBS	sterile phosphate buffered saline	VWR (Oslo, Norway)
	no bacteria	no inoculation with bacteria	-
	no treatment	no exposure to chemical agent	-

Bacterial culture and biofilm formation

In this study, the genetically modified strain *S. epidermidis* Xen 43 was used as a model for biofilm formation on Ti coins. The strain was derived from the clinical isolate *S. epidermidis* 1457, where the *luxABCDE* genes have been incorporated into the bacterial genome to create a constitutive bioluminescent strain. The engineered strain has been shown to be phenotypically equal to its parental strain (Vuong et al. 2008) and was kept as frozen stock cultures to avoid

phenotypic changes due to passages. Calibration curves were determined to correlate luminescence and colony forming units (CFU) as well as OD₆₀₀ and CFU (supplementary information, Figure SI 1).

Tryptic soy broth (TSB) was used as growth medium and incubation was performed in an aerobic atmosphere at 37°C. Bacterial stocks of *S. epidermidis* Xen 43 were stored at -80°C in 15 % glycerol. Stocks were used to prepare diluted cultures at an initial optical density at 600 nm (OD₆₀₀) of 0.05 (~ 1x10⁷ cells/ml) as determined spectrophotometrically (Biophotometer, Eppendorf, Hamburg, Germany) before the suspension was incubated for 24 h. The overnight cultures were then diluted in TSB 1:100 and cultured overnight for a second time. The second overnight cultures were diluted in TSB 1:100 for OD₆₀₀ = 0.02 - 0.03 (~ 1x10⁷ cells/ml) and used to inoculate Ti coins. Coins were placed in a 48-well micro-titre plate (Nunc, Roskilde, Denmark) and inoculated with 700 µl of bacterial suspension. The plate was then incubated for 8 h to allow colonization and biofilm formation on the Ti coins. After the incubation, all coins were transferred to a new 48-well plate and each well was filled with 500 µl sterile PBS, followed by the exposure to the antibacterial agents. A scheme of the experimental setup is given in Figure 1.

Figure 1: Scheme of the experimental setup. Ti coins were inoculated with *S. epidermidis* Xen 43 and (1) incubated for 8 h for biofilm formation. Contaminated samples were transferred to a new well and (2) exposed to chemical agents for 1 min. Decontaminated samples were rinsed with PBS and (3) reincubated in TSB for 24 h. Luminescence was recorded every 15 min during reincubation. The decontamination outcome was further assessed by SEM and fluorescence microscopy after live/dead staining.

Decontamination

All samples, except the control surfaces, were transferred to wells filled with 500 µl of chemical agents. TiO₂ containing agents were vortexed for 10 s immediately before use. The treatment was stopped after 1 min by transferring the treated coins to separate wells filled with 1 ml PBS. After transferring, all samples were carefully rinsed with sterile PBS three times to remove all chemical agents before further processing. All treatment groups were tested in three

independent experiments, each consisting of eight samples (luminescence: $n = 6$, scanning electron microscopy: $n = 1$, fluorescence microscopy: $n = 1$).

Luminescence assay

Regrowth of bacteria was measured by monitoring luminescence after decontamination. Six coins of each group were transferred to a new 96 optical well plate (Perkin Elmer, Waltham, USA) and wells were filled with 150 μ l of pre-heated TSB. Afterwards, the well plate was sealed before incubation in a multi-detection microplate reader (Synergy HT, BioTek, Winooski, USA) at 37°C. Luminescence expressed as relative light units (RLU) was recorded for 24 h in time intervals of 15 min with 10 s of moderate shaking immediately before measuring. Peaks in RLU are from now on referred to as RLU_{max} , time of reincubation until RLU_{max} is reached is referred to as $tRLU_{max}$. $tRLU_{max/2}$ is defined as the time of reincubation until 50 % of RLU_{max} is reached and was used as a reference value to assess and compare the antibacterial effect of different agents. The luminescence assay was performed in randomised triplicates for a total sample number of $n = 18$ per treatment group.

SEM and fluorescence microscopy

In addition to luminescence measurements, SEM and fluorescence microscopy after live/dead staining was performed directly after the treatment with different chemical agents. SEM imaging was performed in order to investigate the structure of the remaining biofilm after treatment with chemical agents. Samples for SEM imaging were fixated overnight in 2.5 % glutaraldehyde in 0.1 M Sørensen's phosphate buffer solution. Afterwards, samples were treated with ethanol (70 %, 80 %, 90 %, 95 % and 100 % ethanol) and further dehydrated by critical point drying before being sputtered with platinum (Pt). SEM examination was done using a field emission scanning electron microscope (S-4800, Hitachi, Tokyo, Japan), and images were taken for three randomly selected areas on the surface.

A Live/Dead BacLight bacterial viability kit (Molecular Probes Inc., Eugene, USA) was used to determine the viability of remaining bacteria directly after the exposure to different chemical agents. 6 μ l propidium iodine and 6 μ l SYTO9 were diluted in 5 ml sterile, ultrapure milliQ water. Samples were placed in a new 24-well plate and stained for 15 min in dark before they were rinsed with PBS in order to remove excess dye. Mounting oil was used to fixate a cover slip onto the Ti surfaces before imaging. A confocal fluorescent microscope (DM 6000 CFS, Leica, Wetzlar, Germany) was used to sequentially detect light emission for viable (green

fluorescent) and dead (red fluorescent) bacteria. Images were derived from a full projection of a 20 μm z-stack at a magnification of 40x taken at three randomly selected areas on the surface. Both SEM and fluorescence microscopy were performed in triplicates.

Statistical analysis

$t_{\text{RLU}_{\text{max}}/2}$ for groups which showed bacterial regrowth is presented as mean values $\pm$ standard deviation (SD). Datasets were analysed for normality (Shapiro-Wilk) and equal variance (Brown-Forsythe) before further testing. One way analysis of variance (ANOVA) was performed separately for antibacterial agents (H_2O_2 , $\text{H}_2\text{O}_2\text{-TiO}_2$ and CHX) and control agents (H_2O , $\text{H}_2\text{O-TiO}_2$ and PBS). A subsequent pairwise comparison (Holm-Sidak) between data groups was run to determine statistically significant differences ($p < 0.05$) between agents of each category. Statistical significant differences are highlighted with an asterisk.

Results

SEM

Untreated surfaces were almost completely covered with a dense biofilm of a thin biofilm of 1-3 bacterial layers, whereas the amount of bacteria was greatly reduced after the treatment with H_2O_2 or $\text{H}_2\text{O}_2\text{-TiO}_2$ (Figure 2). Both groups showed only a few remaining clusters of bacteria distributed over the coin, particular in grooves and pits of the rough surface. Further, TiO_2 agglomerates of varying size and morphology were found in all examined areas for $\text{H}_2\text{O}_2\text{-TiO}_2$. SEM images of samples exposed to CHX showed a higher number of total bacteria than surfaces treated with H_2O_2 -based agents. Bacteria were present in large colonies for CHX but the amount of bacteria was reduced compared to untreated samples. Representative SEM images of Ti surfaces before and after treatment with antibacterial agents are shown in Figure 2. SEM images for all control surfaces and control agents can be found in the supplementary information (Figure SI 2).

Figure 2: SEM (top row) and fluorescent micrographs (bottom row) of Ti surfaces before and after the treatment with different antibacterial agents. Bacteria stained green are considered viable while dead bacteria are stained red.

Fluorescence microscopy

Untreated biofilms consisted of mostly green fluorescent (viable) bacteria with only a few, isolated red fluorescent (dead) bacteria (Figure 2). Similar to SEM images, the treatment with H_2O_2 or $\text{H}_2\text{O}_2\text{-TiO}_2$ resulted in a strong reduction of the total amount of biofilm. The remaining

bacteria for these two groups were organised in clusters of mainly dead bacteria, although some viable bacteria were also present on these surfaces. TiO₂ agglomerates which remained on the surface after treatment with H₂O₂-TiO₂ were strongly green fluorescent. Live/dead staining further indicated that the high number of bacteria on surfaces treated with CHX were dead. Fluorescence microscopy images after live/dead staining before and after treatment are shown in Figure 2. Additional fluorescent images for all control surfaces and control agents can be found in the supplementary information (Figure SI 2).

Luminescence assay

A characteristic pattern for luminescence over time was found for all tested groups which showed bacteria regrowth: (1) an initial low RLU intensity was followed by (2) an exponential increase which resulted in a distinct peak (RLU_{max}) and (3) decreased until (4) a stationary level of RLU higher than (1) was reached (Figure 3). A linear correlation between CFU and RLU was valid for $t = 0 \text{ h} - t = t_{\text{RLUmax}}$ ($R^2 = 0.98$, Figure SI 3). Both, H₂O₂ and H₂O₂-TiO₂ showed bacterial regrowth within 24 h of reincubation following the characteristic curve progression described above. No regrowth was detected for CHX treated surfaces. The change in RLU intensity during reincubation after the treatment with the three antibacterial agents is plotted in Figure 3A (means, $n = 18$). The corresponding data for groups treated with control agents and for control surfaces are provided in the supplementary information (Figure SI 3).

Figure 3: *S. epidermidis* regrowth after treatment with antibacterial agents (A). Luminescence measured during reincubation of Ti surfaces following the decontamination with the tested antibacterial agents (mean, $n = 18$). Characteristic segments of the curve progression are labeled 1 - 4 and are described in the discussion. (B) Time after treatment with antibacterial agents and control groups until RLU_{max}/2 is reached (mean $\pm$ SD, $n = 18$, * if $p < 0.05$ for multiple comparison within one category).

$t_{RLU_{max}/2}$ is illustrated in Figure 3B for all treated surfaces. All antibacterial agents showed a strong delay in $t_{RLU_{max}/2}$ compared to the control agents. Importantly, the addition of TiO_2 nanoparticles in aqueous H_2O_2 solutions resulted in an increased delay that was significant ($p = 0.028$) compared to H_2O_2 alone (H_2O_2 - $TiO_2 = 13.75 \pm 1.90$ h, $H_2O_2 = 12.29 \pm 1.86$ h). No statistically significant differences in $t_{RLU_{max}/2}$ were found between surfaces which had been treated with control agents ($H_2O = 3.90 \pm 1.70$ h, H_2O - $TiO_2 = 3.79 \pm 1.59$ h and $PBS = 2.99 \pm 1.63$ h).

Discussion

The present study assessed the antibacterial effect of TiO₂ nanoparticles in H₂O₂ solutions for chemical decontamination of dental implant surfaces. The effect was tested on *S. epidermidis* which readily forms mono-species biofilms on various substrates *in vitro*. The Ti substrate was completely covered with a thin, multilayer biofilm after incubation for 8 h and subsequently exposed to different antibacterial agents for 1 min. Such a biofilm cannot entirely represent the complex structure of polymicrobial biofilms in peri-implantitis. However, *S. epidermidis* is one of the early colonizers in the formation of dental biofilm (Sbordone & Bortolaia 2003) and has been found in biofilms of failing dental implants (Leonhardt et al. 1999). Further, it is a safe and well-established model to test the antibacterial activity of chemical agents on biofilms (Bürgers et al. 2012; Gustumhaugen et al. 2014; Henderson et al. 2013).

A bioluminescence assay was used to continuously monitor the metabolic activity and potential bacterial regrowth after the reincubation of decontaminated samples. Since bioluminescence is an energy demanding process, it can be used as a valuable indicator for the metabolic activity of microorganisms (D'Souza 2001; Marincs 2000). Linear correlation between RLU and CFU during the exponential growth phase (Figure SI 2) further makes luminescence a valid measure to describe the viability of bacteria which survive chemical decontamination and regrow on the implant surface. In addition, SEM and fluorescence microscopy were used to analyse Ti surfaces immediately before and after the exposure to different chemical agents and thereby complemented the findings of the luminescence assay.

The addition of TiO₂ nanoparticles enhanced the antibacterial effect of H₂O₂ solutions and delayed the regrowth of bacteria on previously contaminated surfaces. The delay in $t_{RLU_{max}/2}$ for H₂O₂ suspensions which contained TiO₂ nanoparticles was statistically significant compared to H₂O₂ solutions of the same concentration (Figure 3B). TiO₂ nanoparticles did not show intrinsic antibacterial properties (Figure 3B), and hence, the delay in $t_{RLU_{max}/2}$ is suggested to be due to the interaction between TiO₂ and H₂O₂. The non-photocatalytic formation of oxygen centred radicals, due to the interaction of H₂O₂ and TiO₂, has previously been shown (Fenoglio et al. 2009; Sanchez et al. 2013; Wiedmer et al. 2016). These studies suggested that TiO₂ can act as a Fenton catalyst and decompose H₂O₂ into oxygen centred radicals namely superoxide (O₂^{•-}) and hydroxyl radicals (•OH). The bactericidal effect of free radicals is undisputed and several cellular components have been identified as targets for radical mediated oxidation (Gaupp et al.

2012; Halliwell & Gutteridge 2007). However, the potential formation of radicals on the TiO₂ catalyst could not abolish bacterial regrowth which was most likely due to remaining vital clusters after the decontamination (Figure 2).

One explanation for the relatively small increase in antibacterial activity may be the nature and location of the radicals formed at the H₂O₂-TiO₂ interface. Earlier results indicated that primarily superoxide radicals (O₂^{•-}) are formed at this interface and the oxidative effect is limited to the proximity of the surface (Wiedmer et al. 2016). Extracellular polymeric matrix (EPS) may protect bacteria from direct contact with TiO₂ nanoparticles and thereby hamper the antibacterial effect. Further, there is evidence that O₂^{•-} can adsorb to the metal oxide surface resulting in negative net surface charge of TiO₂ in physiological pH (Anpo et al. 1999; Wiedmer et al. 2016). This may lead to unfavourable repulsive forces between the negatively charged TiO₂ surface and the overall negatively charged bacterial wall which may further limit the bactericidal effect of formed free radicals.

The positive effect of TiO₂ nanoparticles may also be masked by the presence of a more potent catalyst than TiO₂ in the presented *in vitro* assay. Strong bubbling was observed on contaminated Ti surfaces approximately 15 s after the introduction of H₂O₂ or H₂O₂-TiO₂. This may be due to decomposition of H₂O₂ into oxygen and H₂ at the Ti substrate. Ti possesses a higher catalytic activity in the decomposition of H₂O₂ than its oxide form TiO₂ (Tengvall et al. 1989). The presence of a protective oxide layer may initially inhibit the interaction of Ti and H₂O₂ and cause the observed delay until bubbling occurred. The TiO₂ layer on top of the Ti surface is comparably thin and is likely to be broken-up by H₂O₂. The ruptured oxide layer may enable the interaction between metallic Ti and H₂O₂ which consequently leads to the observed gas evolution. This gas evolution may contribute substantially to the eradication of biofilms by physically removing bacteria and biofilm components from the surface.

The antibacterial effect of H₂O₂-TiO₂ suspensions may further be limited due to the formation of TiO₂ agglomerates (Figure 2). The occurrence of clusters in similar conditions has been described earlier for the nanoparticles used in this study (Wiedmer et al. 2016). Agglomeration reduces the number of active sites for the catalytic decomposition of H₂O₂ but more importantly may reduce the probability of contact between the bacterial wall and the oxidative TiO₂ surface. Adverse effects of TiO₂ clusters, such as the alteration of the implant surface or the cytotoxicity of released nanoparticles, may even hamper the re-osseointegration of decontaminated surfaces.

Future research is therefore necessary to clarify the role of residual TiO₂ agglomerates on the implant surface.

Compared to all other tested agents, H₂O₂ and H₂O₂-TiO₂ showed the highest capacity to eradicate biofilm and remove adherent bacteria from the surface. The ability of H₂O₂ to disrupt bacterial biofilm has been observed earlier (Gustumhaugen et al. 2014; Henderson et al. 2013; Presterl et al. 2007) and is related to oxidation of several cellular components and potentially the gas evolution described above. The removal of attached microorganisms and also endo- and exotoxins from contaminated surfaces is a key aim in surgical peri-implantitis treatment (Kreisler et al. 2005; Takasaki et al. 2007). The most common strategy to achieve this aim is to combine mechanical debridement with adjunctive use of antimicrobial agents. Currently used approaches to physically remove biofilm from contaminated surfaces are often insufficient (Schwarz et al. 2006) or fail to retain the original implant surface unaltered (Deppe et al. 2002; Kreisler et al. 2005). Therefore, the capacity of H₂O₂-based treatments to actively reduce bacteria mass may contribute to a more efficient decontamination.

In contrast to H₂O₂ and H₂O₂-TiO₂, CHX was not able to actively reduce biofilm mass (Figure 2) but was effective in preventing bacterial regrowth. This is supported by live/dead staining (Figure 2) where no vital bacteria were found on surfaces treated with this agent. CHX is frequently used in dentistry (Quirynen et al. 2000) and the strong bactericidal effect of CHX against various strains has been reported in numerous studies (Cervone et al. 1990; Ercan et al. 2006; Sassone et al. 2008; Vianna et al. 2004). The mechanism of action is linked to the rapid uptake of CHX by bacteria and the subsequent damage of the microbial membrane (McDonnell & Russell 1999). The strong bactericidal effect of CHX compared to H₂O₂ and H₂O₂-TiO₂ may be advantageous in peri-implantitis treatment, in which the inactivation of the microorganism is a key requirement (Lindhe et al. 2008). However, CHX has also been shown to be cytotoxic against different cell types which are related to early wound healing and should therefore be used cautiously in open wounds (Giannelli et al. 2008; Pucher & Daniel 1992). The inability of CHX to detach biofilm, as demonstrated by a uniform layer of dead bacteria on the CHX treated surfaces, may further be disadvantageous in peri-implantitis treatment, by forming a foundation on which other bacteria may adhere to. This is particularly relevant in view of the commonly used techniques, which often fail to remove adherent biofilm from implant surfaces (Schwarz et al. 2006).

The ultimate goal in the decontamination of dental implants is to prevent reinfection and promote re-osseointegration. Therefore, decontamination generally aims at the restoration of the original, microbe-free implant surface. The treatment with either CHX or H₂O₂-based agents could not achieve this goal in the tested *in vitro* setup. It is therefore suggested that a combined approach could be used to overcome the limitations of each agent. In a first step, H₂O₂ or H₂O₂-TiO₂ is used to detach a great amount of biofilm only leaving a few viable cells on the surface. In a second step, CHX or an equivalent bactericidal agent is used to efficiently kill remaining microbes. The consequent dead organic matter left on the implant surface may be removed by phagocytic host cells or thorough rinsing in a clinical situation. Further research is needed to assess the potentially synergistic effects of this combined strategy for implant decontamination.

Conclusion

This study investigated the antibacterial effect of H₂O₂-TiO₂ suspensions in the decontamination of Ti implant surfaces which have been inoculated with a biofilm forming *S. epidermidis* strain. The addition of TiO₂ nanoparticles enhanced the antibacterial effect of H₂O₂ and may be considered as an alternative approach for chemical decontamination in peri-implantitis treatment. Further *in vitro* studies are necessary to optimise the decontamination efficacy of H₂O₂-TiO₂ suspensions, e.g. by the use of adjunctive UV irradiation, but also to assess the potentially cytotoxic effect of TiO₂ nanoparticles which remained on the implant surface.

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Supplementary material

The supplementary information is available free of charge on the Biofouling website at DOI: [10.1080/08927014.2017.1322585](https://doi.org/10.1080/08927014.2017.1322585).

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