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Antimicrobial Agents Used in the Treatment of Peri-Implantitis Alter the Physicochemistry and Cytocompatibility of Titanium Surfaces

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Background: Chemotherapeutic agents (ChAs) are considered an integral part of current treatment protocols for the decontamination of titanium (Ti) implants with peri-implantitis, based on their antimicrobial effect. Despite the proven antimicrobial effect of ChAs on titanium-bound biofilms, previous studies have elucidated an unexpected disassociation between bacterial reduction and biologically acceptable treatment outcomes. In this study we hypothesized that ChAs residues alter Ti physicochemistry, and thus compromise cellular response to decontaminated surfaces.

Methods: Grit-blasted acid-etched Ti discs were contaminated with multi-species microcosms grown from in vivo peri-implant plaque samples. To simulate implant decontamination, we burnished the contaminated discs with either 0.12%-chlorhexidine, 20%-citric acid, 24%-EDTA/1.5%-NaOCl, or sterile saline, and assessed surface physicochemical properties. Sterile untreated surfaces were the controls. Biologic effects of decontamination were assessed via cell proliferation and differentiation assays.

Results: Bacterial counts after decontamination confirmed that the ChAs were antimicrobial. XPS invariably detected elemental contaminants associated with each ChA molecule or salt that significantly altered wettability compared to controls. Notably, all surfaces with ChA-residues showed some cytotoxic effect compared to controls ($p<.05$). Increased cell counts were consistently found in the saline-treated group compared to chlorhexidine ($p=.03$). Interestingly, no association was found between antimicrobial effect and cell counts ($p>.05$).

Conclusion: ChA-specific residues that were left on the Ti surfaces altered titanium physical properties and adversely affected osteoblastic response irrespective of their observed antimicrobial effect. Chlorhexidine may compromise the biocompatibility of titanium surfaces, and its use is not recommended to detoxify implants. Sterile saline, citric acid and NaOCl-EDTA may be proposed for use in the treatment of peri-implantitis. Contrary to previous studies that recommended the selection of ChAs for the decontamination of Ti implants according to their antimicrobial effects, we demonstrated that the restoration of the biocompatibility of contaminated titanium surfaces is also contingent upon the preservation of titanium material properties.

MESH TERMS:

biofilms; titanium; Peri-Implantitis; decontamination; chlorhexidine; osteoblasts.

Even though dental implants are a successful treatment modality, a number of implants are lost due to peri-implantitis even after years of successful osseointegration.[1] The number of implants that are affected depends on the definition of peri-implantitis in each study with prevalence estimates of peri-implantitis ranging between 7.8 to 43.3% on an implant level.[2, 3] Bacterial contamination of the implant surface is considered as a prerequisite for peri-implant inflammation and current evidence shows that the host

response to microbial biofilm formation on implant surfaces includes a series of inflammatory reactions that play a major role in the etiology of peri-implantitis.[4] Consequently, the removal of bacterial biofilms has been considered as a fundamental step in the therapy of peri-implantitis and the majority of current research efforts are focused on identifying potent antimicrobial agents for implant surface decontamination.[2]

In an attempt to determine an efficacious treatment protocol for the removal of bacterial biofilms and thus resolution of peri-implantitis a variety of mechanical means and chemotherapeutic agents, as well as combinations of the above, have been proposed in the literature.[5-9] Mechanical treatment with curettes has shown limited potential in removing bacterial biofilms around implants due to the screw-shaped design and surface roughness of dental implants.[10-12] The use of gauzes or cotton pellets soaked in chemotherapeutic agents (ChAs) for burnishing the implant surface has shown favorable outcomes for reduction of bacterial levels that seem to be dependent on the modification of the titanium surface and the selection of the ChA.[12-14] Nonetheless, currently available information challenges whether efficacious removal of bacterial biofilms alone is sufficient for the treatment of peri-implantitis.[6, 15, 16]

In parallel with the investigation of the antimicrobial efficacy of implant decontaminating agents, studies have investigated the effect of these agents on the physicochemical characteristics of the decontaminated surfaces.[17-19] There is preliminary information showing that decontamination approaches may alter the material properties of titanium surfaces.[15, 20, 21] It is well established that bacterial contamination of an implant's titanium surface, even in thin layers, will diminish its surface energy and negatively affect cell attachment on its surface.[22-24] Results from previous studies have shown that certain decontamination approaches that may be efficacious in reducing bacterial load may not necessarily be able to restore the elemental composition of an implant, or may even further compromise titanium material properties.[17,18] In an in vitro study assessing the chemical effect of citric acid, hydrogen peroxide and other ChAs during burnishing of titanium surfaces the authors found that these decontamination protocols led to signs of oxide layer damage in a pH-related manner.[18] In the same study, traces of titanium were found on the cotton swabs following decontamination that were indicative of titanium dissolution.[18]

Collectively, previous studies have indicated that oral biofilm formation on titanium surfaces interferes with cell adhesion and proliferation, but its removal or reduction alone may not be sufficient to render these surfaces biologically acceptable. There is existing, but limited, information to support that titanium surface alterations induced by decontamination interventions lead to compromised biologic response during the healing phase.[18, 21, 25]. In the present study we hypothesized that this reported lack of association between antimicrobial and biologic effects occurs due to physichochemical alterations of the titanium surface caused by ChAs. To investigate this hypothesis we assessed the physicochemical properties of clinically-relevant titanium surfaces decontaminated with various ChAs and their effects on cell response.

MATERIALS & METHODS

For the development of a clinically-relevant *in vitro* peri-implantitis model we contaminated 10 mm-diameter grit-blasted acid-etched titanium disc surfaces with a multi-species biofilm and assessed their physicochemical characteristics and biocompatibility following their decontamination using clinically available ChAs. All experiments were performed in duplicate.

Microcosm Biofilm

To better simulate clinical conditions, a microcosm biofilm model was utilized for the contamination of titanium discs. For collection of the initial plaque inoculum, one volunteer with active peri-implantitis presenting with bleeding on probing and suppuration (maximum probing depth: 7 mm; radiographic bone loss on vertical bitewing radiograph: 5.5 mm) was sampled. The volunteer was a medically healthy, non-smoking 59 year-old male that had not taken antibiotics during the last 6 months. Following approval from the University of Minnesota Institutional Review Board, a submucosal peri-implant plaque sample was collected during a scheduled surgical peri-implantitis treatment appointment. Briefly, following initial flap reflection the implant area was isolated with cotton pellets to avoid blood contamination and plaque samples were collected with sterile plastic curettes from the buccal implant threads. The plaque sample was vortexed for 30 seconds and suspended in 10ml of growth medium, while a portion was retained for DNA extraction for microbial identification (Sample T1). The growth medium was basal mucin medium (BMM), and bacteria were cultured overnight in anaerobic conditions (5% H_2, 5% CO2, 90% N_2) to support the growth of anaerobes that are associated with peri-implant plaque samples.[26, 27] A sample of the overnight culture was obtained for DNA extraction for microbial identification from the microcosm model (Sample T2). The remainder was pelleted and re-suspended in BMM with 20% glycerol and stored at -80°C.[27] Previous work from our group has shown that microcosms grown from frozen stocks adequately reproduce the microbiota of the original microcosm.[27]

Microbial Analysis

DNA was extracted from Samples T1 and T2, and analyzed to ascertain the presence of putative peri-implant pathogens, and assess the retention of oral bacterial taxa from the original plaques sample in our BMM-based microcosm model. The microbial analysis was performed according to the Human Oral Microbe Identification using Next Generation Sequencing (HOMINGS) protocol.[28] HOMINGS parses Illumina sequence data from the V3-V4 region of total sample 16sDNA against sequences that have been validated for species-level identification of approximately 600 oral taxa.[28] DNA extracts were stored at -80°C and shipped on dry ice to the HOMINGS analysis core at the Forsyth Institute.

Imaging methods were also utilized to assess biofilm formation on the titanium discs. For assessment of bacterial viability and biofilm growth, selected contaminated disc samples were stained with calcein-AM and propidium iodide staining[§] for observation under confocal microscopy, or were fixed in 2% Glutaraldehyde, 0.1M Sodium Cacodylate and 0.15% Alcian Blue and prepared for scanning electron microscopy

(SEM). The samples were dehydrated in ascending grades of ethanol. The dehydrated samples then received gold-sputter coating for SEM observation and were evaluated at various magnifications with a field-emission scanning electron microscope.

Titanium Disc Contamination

Titanium (Ti) discs 10mm in diameter with grit-blasted and fluoride-etched modified surfaces corresponding to a commercially available implant surface modification ‖ were utilized. Ti discs with rough surfaces were selected; 1) to better simulate commercially available implant surfaces and; 2) because rough surfaces have increased plaque retention and are less resistant to plaque removal as compared to machined surfaces.[29]

Prior to contamination, disks were ultrasonicated in cyclohexane for 10 min, and rinsed with distilled water and acetone, followed by drying with N_2 gas. For titanium disc contamination, a portion of the frozen stock was re-suspended in BMM and cultured anaerobically overnight at 37°C. The approximate number of bacteria in the liquid culture was estimated by measuring the optical density at 600 nm (OD_{600}). Following pilot experiments using varying bacterial concentration and incubation time parameters, optimal biofilm growth was observed with a 1:25 dilution ratio of aliquots obtained at OD_{600}= 0.2, after inoculating the contaminated Ti discs in the anaerobic chamber for 48h at 37°C. For additional description of the pilot experiments see supplementary Figure 1 in the online *Journal of Periodontology*. Those growth conditions were then utilized for all experimental groups.

Decontamination Methods

Following contamination of the discs with the peri-implant microcosm biofilm we utilized clinically available and commonly used ChAs to decontaminate the titanium surfaces. Following a standard clinical approach, we burnished contaminated disc for 20 seconds each with sterile cotton pellets moistened in either:

- 0.12%-chlorhexidine solution (CHX group)
- 20%-citric acid gel followed by removal with sterile saline (CA group)
- 24%-EDTA/1.5%-sodium hypochlorite (NaOCl-EDTA group), or
- 0.9% NaCl - sterile saline (SS group)

Sterile titanium discs with the same grit-blasted and fluoride-etched modification were utilized as controls. Discs were autoclaved prior to cell cultures.

To assess the antimicrobial effect of each ChA we counted colony-forming units (CFUs) from n=3 decontaminated titanium discs from each group in duplicate and compared to the CFUs counted from contaminated, untreated samples. For the CFUs, we placed the discs in 1ml of sterile PBS and dispersed by sonification. We then prepared tenfold serial dilutions in sterile PBS and plated them on non-selective agar medium containing sheep blood. Blood Agar plates were prepared combining 3% (w/v) Todd-Hewitt Broth (30g), 1.5%(w/v) agar (15g), dH2O to 1L, autoclaving and then adding 5% sterile defibrinated sheep blood (50ml) Heim/Menodine stock (5µg/ml. 0.5µg/ml) 10ml to pour the plates. The plates were incubated anaerobically for 72h at 37°C, and CFUs were calculated and expressed on a logarithmic scale (logCFUs) for analysis.

Surface Characterization

X-ray photoelectron spectroscopy (XPS). To assess ChA-induced chemical alterations on the treated titanium surfaces following decontamination, we ultrasonicated n=3 samples per group for 5 min (plus sterile disc controls), rinsed in DI-water and acetone and dried in N_2. XPS was performed (SSX-100, Al Kα x-ray, 1mm spot size, 35° take-off angle) to characterize the atomic composition of the surface. Survey scans (0-1100 binding energy, 4scans/sample) were done at 1eV step-size. The peak fittings and quantification of surface chemical composition were conducted using specialized software provided with the XPS system, and data were presented in atomic percentage charts.

Wettability assay. Wettability assays were performed to assess the physical-chemical properties of the ChA-treated samples. Water contact angle measurements by the sessile drop method were performed on n=3 decontaminated titanium discs per group using a contact angle analyzer with appropriate software¶. Deionized water was used as the wetting liquid with a drop volume of 2µl. Control sterile untreated surfaces were also tested in all experiments.

Cell Culture

To assess biocompatibility following decontamination procedures, we performed cell proliferation and differentiation assays utilizing murine osteoblasts of the MC3T3-E1 line, as previously described.[30] Briefly, MC3T3-E1 osteoblasts from the 5th or 6th passage were cultured in α-MEM supplemented with fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) at 37°C in a humidified atmosphere of 5% CO2. For all assays, cells were trypsinized and seeded on either sterile control or decontaminated and autoclaved titanium discs placed in 24 well-plates. Cells in 2D culture (tissue culture plates-TCP) served as controls.

Cell proliferation. Cells were seeded on the titanium surfaces at a density of 5,000 cells/well and cultured for 3 and 5 days. Cells were fixed on the surfaces with 4% paraformaldehyde in PBS for 20 min in dry ice. After fixing, cells were permeabilized and incubated in DAPI nucleus stain (4',6-diamidino-2-phenylindole 1:1,500), and cell proliferation was assessed by counting the number of cell nuclei per image frame at each period of culture under fluorescent microscopy# using image analysis software**.[30] Four fields per sample and three samples per group were analyzed at each time point.

Cell differentiation. The MC3T3-E1 cells were cultured on the decontaminated titanium samples at a density of 20,000 cells/well. After reaching 80% confluency, cells were cultured in osteogenic media (α-MEM supplemented with 10% FBS, 1% P/S, 50 µg/ml l-ascorbic acid, and 10 mM β-glycerophospate). After 7 and 14 days of osteogenic differentiation, samples were collected for assessing alkaline phosphatase (ALP) activity as a marker of early differentiation and osteocalcin (OCN) expression after 7, 14 and 21 as a marker of late differentiation utilizing ELISA murine assays. Briefly, for the ALP activity cultures were treated with ALP lysis buffer containing 1% Triton X-100, 0.1 mM $MgCl_2$ and 150 mM Tris–base at pH 10.5 for 10 min at room temperature. Then samples were centrifuged at 3000 rpm for 10 min and supernatants were collected and

added into a flat-bottom 96 well plate with AMP reaction buffer containing 2M-$MgCl_2$, p-nitrophenyl phosphate and diluted AMP in mili Q water (1:4 concentration). Samples were incubated at 37 ˚C for 2 hours for colorimetric determination of the product (p-nitrophenol).[30] After the incubation period, 1M NaOH solution was added and the absorbance was measured at λ= 410 nm. The absorbance was normalized with total protein concentration using BSA as standard.

Osteocalcin expression in the culture medium was determined using a Mouse Osteocalcin Elisa Kit according to manufacturer's instructions. Briefly, 25 μL of medium and 100μL OCN antiserum were added in a microtiter plate and incubated in 37˚C for 1 day. Then, wells were washed with 1X PBS three times followed by the addition of streptavidin–horseradish peroxidase and incubation at 37˚C for another 30 min. Hydrogen peroxide solution and 3,3′,5,5′-tetramethylbenzidin were added in each well for 15 min. Stop solution was added in each well to quench the reaction and the absorbance of each well was read at λ=450nm and normalized using total protein content.[30] Three samples per group were evaluated at each time point.

Statistical Analysis

Summary statistics were presented using means (SDs), or percentages as appropriate. Data relative to antimicrobial effect; water contact angles; cell proliferation; and cell differentiation (dependent variables) were analyzed statistically using linear generalized estimated equation (GEE) models to account for experiments performed in duplicate. To assess antimicrobial efficacy as a potential predictor for cell proliferation, we constructed another GEE model with cell proliferation as the dependent variable and the logCFU/ml count for each ChA as a covariate. Tukey's HSD post-hoc tests were performed to assess pairwise differences among groups adjusting for multiple comparisons.

RESULTS

Validation of the Contamination Model

Our microcosm model led to the *in vitro* formation of mature layered multi-species biofilms on clinically-relevant grit-blasted and fluoride-etched modified Ti discs (Figure 1). Confocal microscopy revealed that 48h mature biofilms covered the Ti surface uniformly, and produced multi-layered bacterial biofilms almost exclusively composed of viable bacteria. Figure 2 shows a confocal microscopy image obtained at X60 magnification of multispecies biofilm formed after 48h of anaerobic culturing. 16s rDNA analysis of the microcosm inoculums found 44 distinct oral taxa frequently encountered in peri-implantitis plaque samples, including genera such as Actinomyces, Campylobacter, Eubacterium, Fusobacterium, Parvimonas and Streptococcus.[31] There was high similarity in species composition between the original inoculum and the expanded culture (Jaccard similarity coefficient = 0.918) showing that BMM was an adequate medium for the retention of several oral taxa that are associated with human peri-implantitis lesions. (see supplementary Fig. 2 in online *Journal of Periodontology*)

Assessment of Decontamination Effects

Reduction in microbial load. CFU counts after decontamination confirmed that the ChAs were antimicrobial. The antimicrobial effect of the tested ChAs was greater for the CA ($p<.001$) and NaOCl-EDTA ($p=.001$) groups, followed by the CHX group ($p=0.132$), as compared with the contaminated controls. SS only had minimal antimicrobial efficacy ($p=0.996$). (Figure 3).

Surface chemical modification following decontamination. XPS analysis found that sterile control titanium surfaces showed characteristic C1s (285 eV), Ti 2p (460 eV) and O1s (530 eV) peaks, while analysis of the decontaminated discs detected characteristic elemental contaminants associated with each ChA molecule or salt (Figure 4a). In detail, discs in the SS group (molecular composition of the ChA: NaCl) showed traces of Na and Cl, in the NaOCl-EDTA group (molecular composition of the ChA: NaOCl – $C_{10}H_{16}N_2O_8$) traces of Na, N and increased percentage of atomic C and the highest percentage of O traces among all test groups, in the CA group (molecular composition of the ChA: $C_6H_8O_7$) increased percentage of atomic C and minerals associated with the use of saline for removal of the gel and for the CHX group (molecular composition of the ChA: $C_{22}H_{30}Cl_2N_{10}$) high traces of C and N. Figure 4b displays the semi-quantitative elemental composition of the XPS survey spectra. Presence of N signal in CA spectra can be associated to minimal remnants of bacteria after decontamination. XPS has a detection depth of 30 Å; thus, the Ti signal diminished as contaminants were accumulated on the decontaminated surfaces.

Water contact angle measurements suggested that the cleaning effects on the original sterile control surfaces; i.e., mostly removal of carbonaceous molecules, in combination with the contamination of the decontaminated surfaces from using the different ChAs significantly altered the physical-chemical nature of the decontaminated surfaces. The average water contact angle for the control surfaces was 107.35° (9.97), while all groups treated with ChAs showed significantly reduced contact angles; i.e., increased wettability compared to controls. Notable differences in water contact angles on surfaces treated with different ChAs were also assessed. Surfaces in the NaOCl-EDTA group had the highest wettability with water contact angle as low as 16.50° (6.22) ($p<.001$). (see supplementary Fig. 3 in online *Journal of Periodontology*)

Cell Proliferation and Differentiation

Assessment of proliferation of MC3T3-E1 murine osteoblasts on the decontaminated surfaces showed that all surfaces with ChA residues showed some cytotoxic effect as the number of grown cells was significantly reduced compared to sterile controls at both 3- and 5-days ($p<.05$). The number of cells proliferating on the decontaminated titanium surfaces at 3- and at 5-days varied per group from SS having the largest number of cells, to CHX having the least number of cells (Figure 5a) At the 3-day timepoint the only significant difference in pairwise comparisons among the test groups was identified between the SS [1462.06 cells/frame (238.57)] and CHX [362.50 cells/frame (250.57)] groups ($p=0.033$). This difference persisted at the 5-day timepoint as well ($p<.001$). At 5-days, significantly increased cell count was also found in the CA-treated ($p=.003$) and

NaOCl-EDTA-treated (p<.001) groups as compared to CHX. (Figure 5a,b) ALP and OCN differentiation assays showed that surviving cells on the decontaminated surfaces were able to differentiate comparably to controls. Differences among the groups were not significant either for ALP (all p-val>.062) or OCN (all p-val>.218) at the various time points (Figure 5c).

When the antimicrobial effect of the ChAs was assessed as a predictor for cell proliferation on the decontaminated titanium surfaces, no association was found between logCFU/ml and cell counts either at 3-days (p=0.338) or at 5-days (p=0.420) (Figure 6)

DISCUSSION

The goal of this study was to assess whether the ChA residues on Ti surfaces after decontamination alter Ti physicochemistry and affect the biological response to the implant surface. In order to investigate our hypotheses we developed and validated a microcosm biofilm model. Our contamination protocol led to the reliable formation of layered, multi-species, microcosm biofilm models from *in vivo* samples composed of numerous oral taxa that have been previously associated with human peri-implantitis.[31] Previous studies have shown that multispecies biofilms are more resistant to the antimicrobial effect of ChAs.[32, 33] Thus, our microcosm biofilm contamination protocol enabled us to assess the antimicrobial effect of the investigated ChAs using relevant surrogates for implant surface decontamination in established peri-implantits. Our results showed that the use of selected ChAs, such as a combination of NaOCl-EDTA had a significant antimicrobial effect against multi-species biofilms. CHX on the contrary only had a mild, non-significant antimicrobial effect. This is consistent with findings from other research groups that have shown a weak antimicrobial effect of CHX against bacteria that are not planktonic, but part of organized biofilms on titanium surfaces.[32, 33, 34] Nonetheless, the antimicrobial effect of the ChA did not have any impact on the biologic response of the treated surfaces.

This disassociation between the bactericidal effect of the ChAs used in the treatment of peri-implantitis and the biological response of the treated surfaces is a paradox that has also been underlined in previous investigations.[15, 25] Schwarz et al.[15, 25] previously observed in a series of reports that plaque removal efficacy of various mechanical methods used for the treatment of peri-implantitis failed to predict the biologic response of decontaminated titanium surfaces and did not restore their biocompatibility. These findings along with our experimental findings led us to investigate additional variables that could better predict the biological response of treated Ti surfaces than antimicrobial efficacy. The investigation of the elemental composition of the treated surfaces revealed that the use of ChAs to burnish the Ti surface invariably left contaminants. These contaminants were ChA-specific and had a significant effect on the physical properties of the tested surfaces. Notably, all tested surfaces exhibited diminished Ti signals and increased signals of absorbed carbonated molecules as compared to control surfaces. These results are consistent with a previous report that found that the presence of contaminants from abrasives, or CA on contaminated titanium implants showed lower levels of titanium compared to controls.[20] When viewed through the prism of cytocompatibility, these carbonaceous surface contaminants may be responsible for the observed reduced cell viability on the treated surfaces by reducing Ti surface energy.[35]

Overall, decontaminated surfaces showed higher wettability than control surfaces in our experiments. Although hydrophilic surfaces can promote an environment conducive to osseointegration by improving osteoblast maturation[36,37], the specific effect of wettability on osteoblast proliferation and differentiation is controversial. Changes in surface wettability are obtained by changing micro/nanotopography[36] of the substrate and/or addition/removal of specific chemical components[37,38], as it is the case in our work. Thus, changes in cell response cannot be exclusively attributed to the assessed changes in surface wettability.

The adverse biologic response noted in this study was most detrimental when CHX was tested as the ChA of choice. CHX's cytotoxicity against osteoblasts has been also elucidated by other groups.[39,40] Previous studies found that the purportedly favorable CHX adsorption on dental tissues and gradual release, i.e. "substantitivity", also applies around implants.[41,42] Consistent with our XPS findings of residual CHX-related elements on the titanium surface, Kozlovsky et al. have shown that a considerable percentage of the available CHX that contacts a titanium surface gets adsorbed and is gradually released over at least 24 hours.[42] The persistence of CHX on the titanium surface in association with its known cytotoxicity may explain our results that shown that growth of cells was hindered on the CHX-treated samples.[39,41,42]

Cell proliferation on CA and NaOCl-EDTA surfaces was higher than on CHX surfaces and the differences were statistically significant after 5-days of cell culture. These findings are consistent with the results of Guimaraes et al. who showed that the inhibitory effect of CA on osteoblastic cell proliferation is transient and cell response is restored after approximately 5 days.[43] The combination of NaOCl-EDTA that was utilized in our study was based on previous investigations where it was used for regenerative endodontic procedures and was found to exhibit selective cytotoxicity in these concentrations.[44] Based on our results this combination is more promising for its use in peri-implantitis than CHX. Early and late stages of cell differentiation among the different treated surfaces showed no significant differences with respect to controls. Overall, these results indicate that the cytotoxic effects of the decontaminated surfaces, such as CHX, were only evident during the cell proliferation stage, but cells that were able to proliferate on the treated surfaces demonstrated osteoblastic phenotypic behavior comparable to that of controls.

Interestingly, there was a trend for consistently favorable cellular response with SS compared to the remaining test groups. Given that the antimicrobial effect of SS was minimal and can be most likely attributed to the effect of burnishing on the contaminated surface, these results highlight the increased significance of cytocompatibility versus antimicrobial effect in the selection of ChA in the treatment of peri-implantitis. Although the presented experimental protocol closely captured the peri-implantitis microbiome and applied decontamination methods on clinically-relevant surfaces utilizing robust methodology, our results should be viewed considering the limitations of translating *in vitro* findings. For example, in clinical practice rinsing of surfaces following use of antimicrobial agents could alter the observed biologic response and warrants further investigation. Nonetheless, rinsing following use of antimicrobial agents on titanium surface cannot eliminate their adsorption on titanium[42], thus the effect of rinsing is not

expected to negate the ChA-related observed effect on titanium physicochemistry and cytocompatibility.

CONCLUSION

Utilizing a multi-species microcosm biolfilm model grown from peri-implant plaque samples, we showed that ChAs frequently used in the treatment of peri-implantitis leave surface contaminants, elicit physichochemical alterations on Ti surfaces and adversely affect osteoblastic response. The use of CHX is not recommended because it produced cytotoxic effects on the decontaminated surfaces and may compromise the biocompatibility of titanium surfaces. SS, CA and NaOCl-EDTA are effective in restoring biocompatibility, and can be proposed for use in the treatment of peri-implantitis.

The ideal ChA for clinical use should exhibit selective cytotoxicity and maintain a balance between bacterial decontamination, cell proliferation and differentiation on the implant surface.

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Figure 1.

Overview of the experimental protocol; Ti disc stereoscopic image shows erythrosine staining of the titanium-bound biofilm.

Figure 2

Left: *Laser confocal microscopy image obtained at X60 magnification of multispecies biofilm formed after 48h of anaerobic culturing. The majority of the bacterial observed are live (green color) with a few dead (red color) bacteria visible . In this sample, the thickness of the biofilm was measured at 25μm. (Stain: Calcein-AM –green, and propidium iodide --red).*

Right: *Field-emission SEM images of a contaminated Ti disc showing various bacterial morphotypes included in the poly-species microcosm biofilm (X5,000 magnification, left; X10,000 magnification, right). These findings are consistent with the findings of the 16S rDNA microarray that identified 44 distinct bacterial taxa in our biofilm samples.*

Figure 3

Barchart showing residual microbial count after decontamination of each group as compared to contaminated controls. Dark blue bars show groups that had a significant difference in logCFU/ml counts as compared to controls.

Figure 4

XPS surface chemical composition analysis. ***4a****. Representative survey spectra from each group showing the elemental composition of the titanium samples following decontamination.* ***4b.*** *Atomic percentage charts derived from the XPS survey spectra revealing the ChA-specific contamination of the treated titanium surfaces. Error bars depict standard errors per group.*

Figure 5

a. Fluorescent microscope images (X10) showing osteoblasts grown for 3-days on the decontaminated samples. Cells nuclei were stained with DAPI (blue). Note the reduced cell count in all test groups as compared to control after 3-days of proliferation.

b. Quantitative results of the 3- and 5-day (blue and red bars, respectively) cell proliferation assays. Cell proliferation depicted as mean number of cells/frame for each group; error bars depict standard errors per group. All test groups exhibited reduced cell count as compared to control after 3 and 5-days of proliferation.

c. Quantitative results of the cell differentiation assays. Both ALP (early mineralization) and OC (late mineralization) assays confirmed the ability of attached cells to differentiate comparably to controls.

Figure 6

Cell counts at 3- and 5-days versus antimicrobial effect.

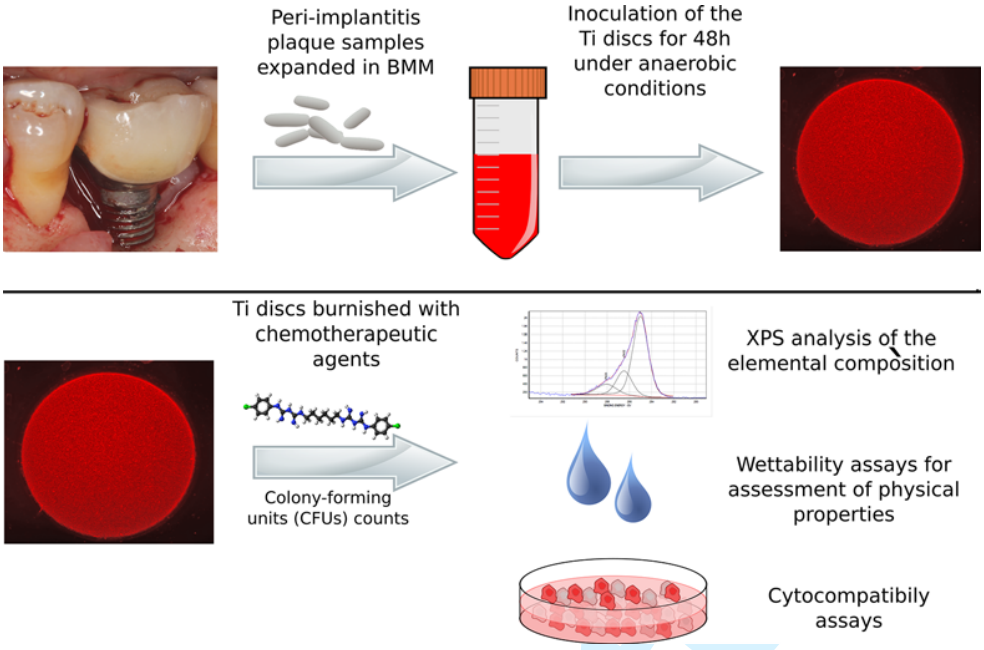
The barplots are showing the mean number of cells/frame (SE) for each group, while the incorporated heatmap depicts the logCFU/ml as a measure of the antimicrobial effect of each ChA. Note that the variation in color among the groups that are depicted in ascending order of cell count does not follow any specific pattern showing no association between cell counts and antimicrobial effect (all p-val>0.338). The higher the color intensity indicates a higher logCFU/ml counted after decontamination, which indicates a lower decontamination effect.

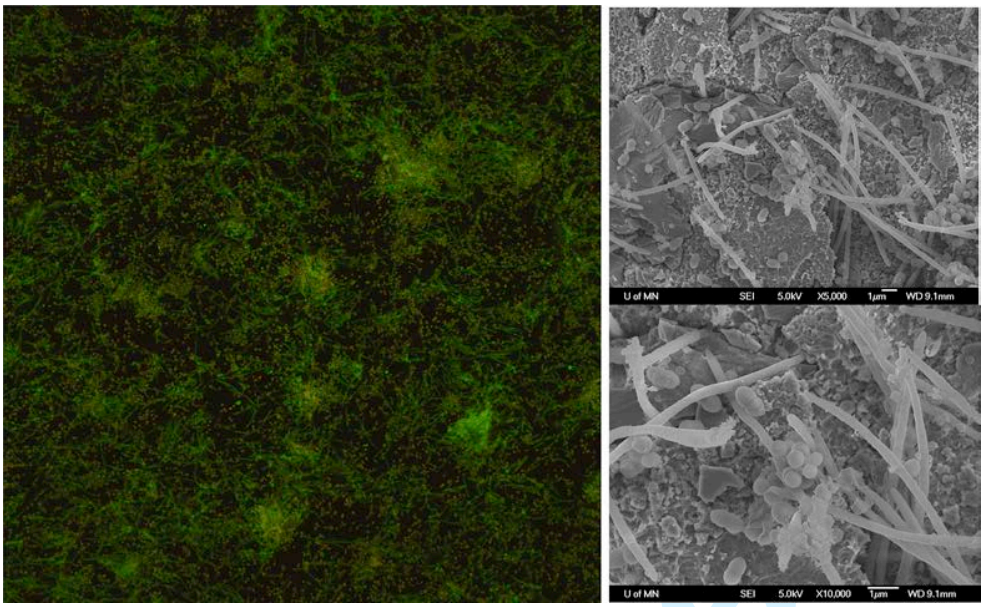
§ LIVE/DEAD® BacLight™ Bacterial Viability Kit, Life technologies, Grand Island, NY

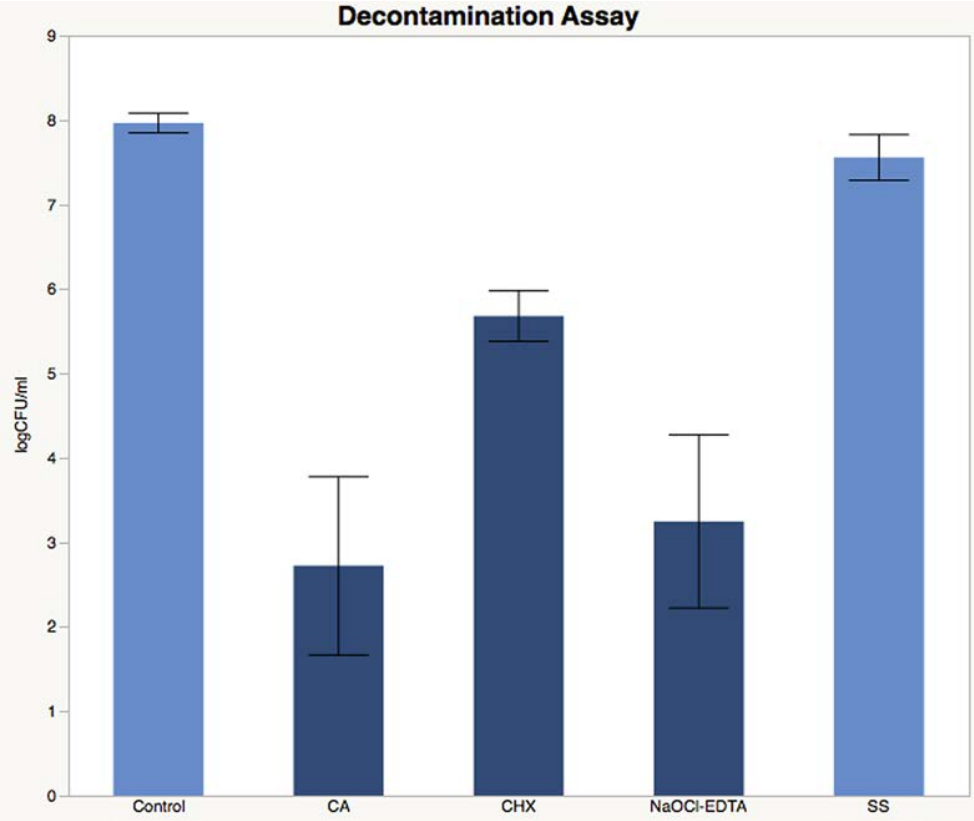
‖OsseoSpeed™, Dentsply International, York, PA

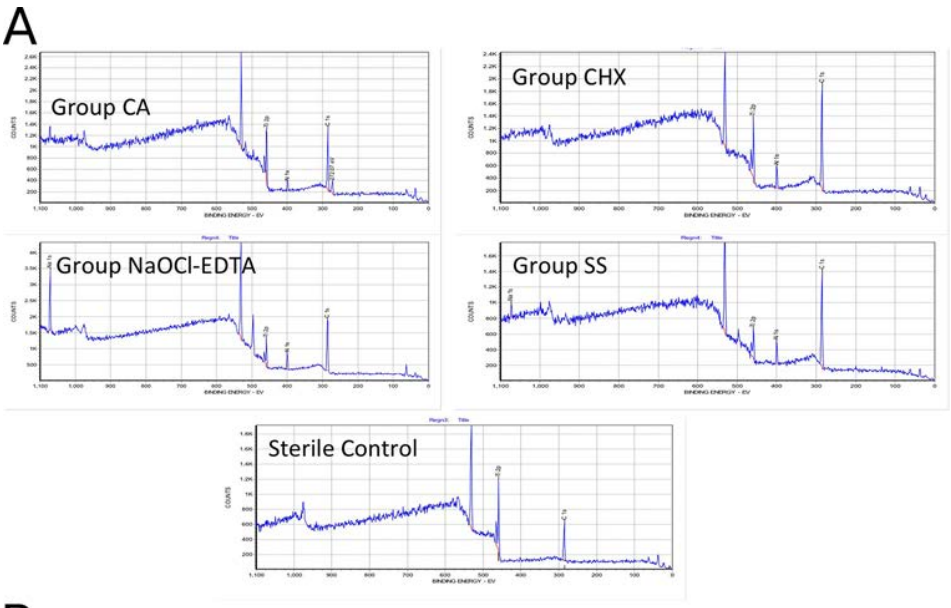
¶DM- CE1 & FAMAS, Kyowa Interface Science, Japan

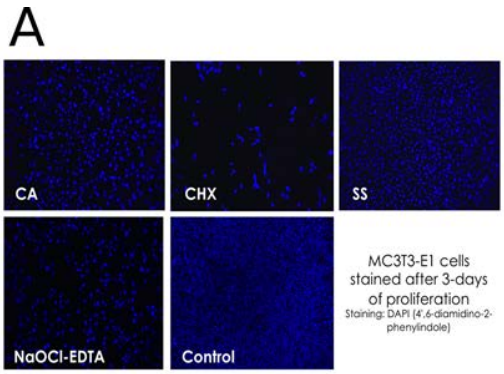
#Eclipse E800, Nikon, Japan, **Image J, NIH, Bethesda, MD

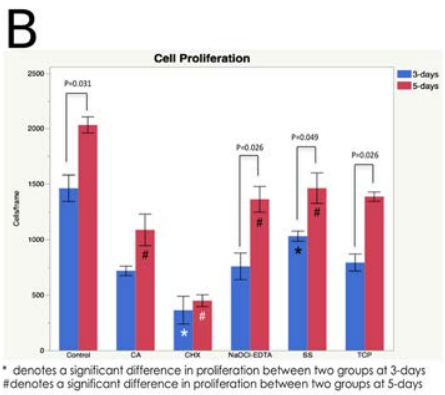

Peri-implantitis plaque samples expanded in BMM
Inoculation of the Ti discs for 48h under anaerobic conditions
Ti discs burnished with chemotherapeutic agents
Colony-forming units (CFUs) counts
XPS analysis of the elemental composition
Wettability assays for assessment of physical properties
Cytocompatibily assays

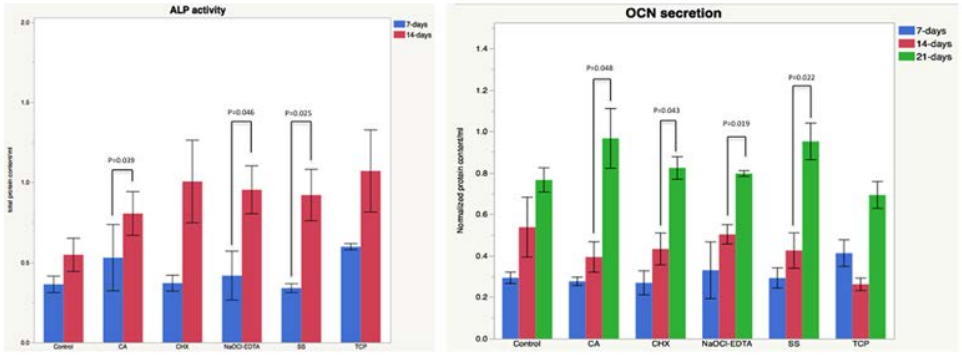

U of MN SEI 5.0kV X5,000 1μm WD 9.1mm
U of MN SEI 5.0kV X10,000 1μm WD 9.1mm

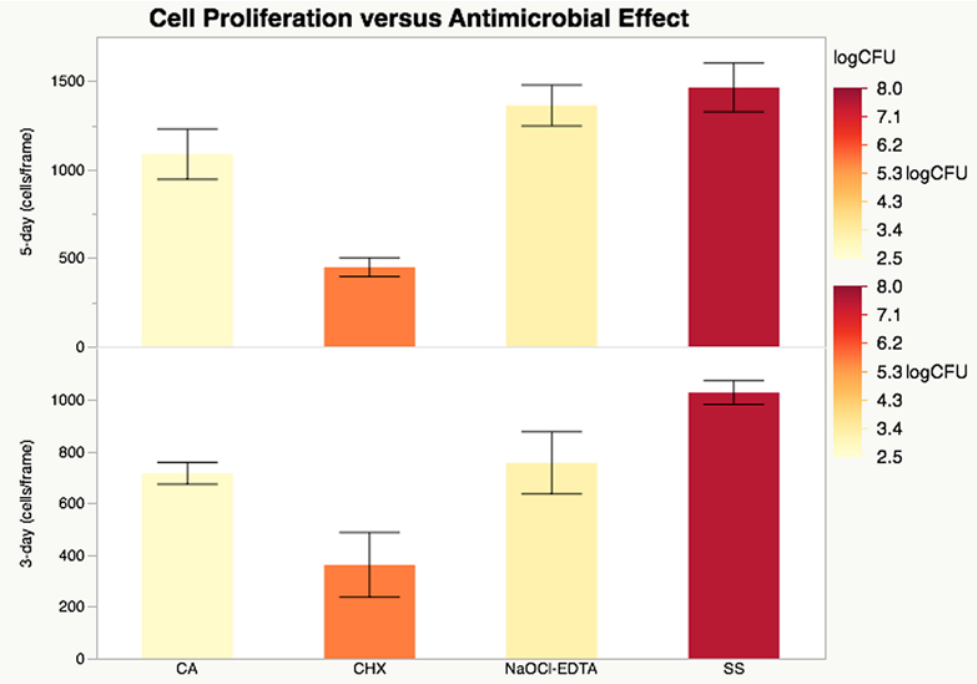

Decontamination Assay
logCFU/ml
9
8
7
6
5
4
3
2
1
0
Control
CA
CHX
NaOCl-EDTA
SS


A
Group CA
Group CHX
Group NaOCl-EDTA
Group SS
Sterile Control
B
Group CA
Group CHX
Sterile Control
Group NaOCl-EDTA
Group SS
Molecular Composition of the ChAs
• CA: C6H8O7
• CHX: C22H30Cl2N10
• SS: NaCl
• NaOCl-EDTA: NaOCl – C10H16N2O8
Atomic Percentage (%)


A
CA
CHX
SS
NaOCl-EDTA
Control
MC3T3-E1 cells stained after 3-days of proliferation
Staining: DAPI (4',6-diamidino-2-phenylindole)


B
Cell Proliferation
3-days
5-days
P=0.031
P=0.026
P=0.049
P=0.026
Control
CA
CHX
NaOCl-EDTA
SS
TCP
* denotes a significant difference in proliferation between two groups at 3-days
#denotes a significant difference in proliferation between two groups at 5-days


C
ALP activity
7-days
14-days
OCN secretion
7-days
14-days
21-days
Control
CA
CHX
NaOCl-EDTA
SS
TCP


Cell Proliferation versus Antimicrobial Effect
5-day (cells/frame)
1500
1000
500
0
3-day (cells/frame)
1000
800
600
400
200
0
CA
CHX
NaOCl-EDTA
SS
logCFU
8.0
7.1
6.2
5.3 logCFU
4.3
3.4
2.5
8.0
7.1
6.2
5.3 logCFU
4.3
3.4
2.5