Effect of Low Direct Current on Anaerobic Multispecies Biofilm Adhering to a Titanium Implant Surface

Philipp Sahrmann, Dr. med. dent.;* Matthias Zehnder, PD, Dr. med. dent., PhD;† Dirk Mohn, PhD;‡ André Meier;¶ Thomas Imfeld, Prof. Dr. med. dent., MBA;** Thomas Thurnheer, PhD††

ABSTRACT

Purpose: Peri-implantitis is caused by biofilm adhering to the implant. It has been shown that bactericidal electrolysis products are generated when a low direct current is applied to a titanium implant used as the anode. The hypothesis of this study was that low-current electrolysis would eradicate viable bacteria in a simulated subgingival multispecies biofilm adhering to a titanium implant surface.

Material and Methods: Biofilms consisting of eight anaerobic species were grown on pellicle-coated titanium discs with sand-blasted, acid-etched, large-grit (SLA; Straumann, Basel, Switzerland) surface. After 40.5 hours of growth, discs were treated with 10 mA for 10 minutes in an electrolytical setup with physiological saline and gelatin.

Results: Low direct current at discs used as the cathode caused a reduction of three to four orders of magnitude in viable counts, while no viable bacteria were recovered from anode discs (Mann–Whitney *U*-test, $p < .01$). Confocal laser scanning microscopy in combination with a live/dead stain showed biofilm detachment at the cathode and reduced viability at the anode.

Conclusion: Electrochemical treatment of diseased implants appears to be promising and well worth investigating further.

KEY WORDS: biofilm, disinfection, electrolysis, hypochlorite, peri-implantitis

INTRODUCTION

As has been pointed out, there is no nonsurgical gold standard treatment for peri-implantitis.[1] This is unfortunate, as the disease is widespread.[2] Peri-implantitis is caused by bacteria colonizing the implant surface.[3] Bacterial infection appears to be similar in periodontitis and peri-implantitis. Hence, most of the proposed debridement protocols for dental implants are derived from periodontal therapy.[1] However, form and surface structure of implant and tooth surface differ considerably: biofilm-bearing implant surfaces have threads, grooves, and a roughened surface. These features render mechanical cleaning impossible.[4] An innovative approach to disinfect implants by electrolysis has recently been suggested.[5] This approach takes advantage of the fact that the biofilm that causes the peri-implant disease adheres not to a biological tissue, but to an electrically conductive titanium implant. As has been shown, a low direct current applied to the implant can

*Resident dentist, Department of Preventive Dentistry, Periodontology, and Cariology, Center of Dental Medicine, University of Zurich, Zurich, Switzerland; †docent, Department of Preventive Dentistry, Periodontology, and Cariology, Center of Dental Medicine, University of Zurich, Zurich, Switzerland; ‡senior researcher, Department of Preventive Dentistry, Periodontology, and Cariology, Center of Dental Medicine, University of Zurich, Zurich, Switzerland, and Institute for Chemical and Bioengineering, Department of Chemistry and Applied Biosciences, ETH Zurich, Zurich, Switzerland; ¶technician, Institute of Oral Biology, Center of Dental Medicine, University of Zurich, Zurich, Switzerland; **professor, Department of Preventive Dentistry, Periodontology, and Cariology, Center of Dental Medicine, University of Zurich, Zurich, Switzerland; ††senior researcher, Institute of Oral Biology, Center of Dental Medicine, University of Zurich, Zurich, Switzerland

Reprint requests: Dr. Matthias Zehnder, Department of Preventive Dentistry, Periodontology and Cariology, Center for Dental Medicine, University of Zurich, Plattenstr. 11, CH-8032 Zurich, Switzerland; e-mail: matthias.zehnder@zzm.uzh.ch

DOI 10.1111/cid.12018

decontaminate its surface under laboratory conditions.[5] The former study showed that oxidizing species are generated in situ at the implant surface if it is used as the anode in a low direct current circuit. A mere alkaline environment is generated at the cathode. This might explain why disinfection was more thorough at the anode than at the cathode. In the first proof of concept study,[5] a simple single-species *Escherichia coli* biofilm was used. Peri-implantitis, however, is caused by biofilms of complex composition containing mostly anaerobic bacteria with taxa from the so-called red complex.[6]

It was the aim of the current study to test the electrolytic implant disinfection approach on a simulated subgingival multispecies biofilm, which has been shown to elicit responses associated with bone resorption in human cell cultures.[7] The hypothesis of this study was that a low direct current should suffice to eradicate viable counts on implant surfaces, and that electrolytic disinfection should be more thorough on anode implants than on cathode counterparts.

MATERIALS AND METHODS

Due to practical advantages for histological incubation and analysis,[8] discs instead of dental implants were used in this study. We used 30 titanium discs with a sand-blasted, acid-etched, large-grit titanium surface (SLA; Straumann, Basel, Switzerland) of an overall surface of 4.0 cm^2. Before treatment, the discs were numbered on their back side using a small round bur. Holes of 1.2 mm diameter were then drilled close to the disc's margin using a bur. Subsequently, discs were sterilized with ethylene oxide.

In Vitro Biofilm Generation

Biofilms were produced using a modified procedure described elsewhere.[9] In the present study, *Streptococcus oralis* SK248 (OMZ 607), *Streptococcus anginosus* ATCC 9895 (OMZ 871), *Actinomyces oris* (OMZ 745; formerly *Actinomyces naeslundii*), *Fusobacterium nucleatum* subsp. *nucleatum* OMZ 598, *Veillonella dispar* ATCC 17748^T (T = type strain), *Campylobacter rectus* OMZ 698, *Prevotella intermedia* ATCC 25611^T, and *Porphyromonas gingivalis* ATCC 33277^T were used. Biofilms were grown in 24-well polystyrene cell culture plates on the pellicle-coated titanium discs. To initiate biofilm formation, discs were covered for the first 16.5 hours with 1.6 mL of growth medium consisting of 60% saliva, 10% human serum, 30% modified fluid universal medium,[10] and 200 µL of a bacterial cell suspension prepared from equal volumes and densities ($OD_{560} = 1$) of each strain. Incubation was anaerobic at 37°C. After 16.5 hours, the inoculum suspension was removed by "dip-washing" the discs, which then were transferred into wells containing fresh growth medium and incubated for further 24 hours. During this time period, discs were again dip-washed after 20.5 and 24.5 hours. After 40.5 hours of incubation, the discs were prepared for electrolysis treatment.

Electrolysis Treatment of Biofilms

Directly before treatment, the discs were washed by gentle dipping in saline solution and placed into an electrolytic setup with sterile 0.9% NaCl solution as conductive liquid and sterile ballistic gelatin (Gelita, Eberbach, Germany) for the electrical resistance as described previously.[5] Following a randomization list, the test discs were used either as negative or positive pole ($n = 10$, each). For this purpose, the discs were suspended on custom-made, sterile, grade-2 titanium hooks to keep the clamps away from the liquid. We used a fine dental probe to insert the discs to minimize mechanical destruction of the biofilm. A device able to maintain a constant current by self-adapting voltage was used to close the electric circuit. In this study, we used a constant current of 10 mA. The voltage tuned itself in between 11 and 19 V. The treatment time for the test discs was 10 minutes each. Control discs ($n = 10$) were placed for 10 minutes into the setup without exposure to current. Both test and control discs were dipped five times in saline to remove salt and gas remnants after removal from electrolytic bath. The discs were then put into separate labeled tubes with physiological saline and subsequently processed for the microbial analysis.

Analysis of Biofilm Composition

After treatment, six discs per group were vigorously vortexed for 1 minute in 0.9% NaCl to harvest the biofilms. Four intact biofilm-discs per group proceeded to confocal analysis (see below). Serial dilutions of suspended biofilm bacteria were prepared in 0.9% NaCl and 50 µL aliquots were plated on Columbia blood agar supplemented with 5% whole human blood (to estimate total colony-forming unit [CFU], *A. naeslundii, C. rectus, S. anginosus, V. dispar*) and phosphomycin (*P. gingivalis, P. intermedia*), on mitis salivarius agar (*S. oralis*), and

on fastidious anaerobe agar with erythromycin, vancomycin, and norfloxacin (*F. nucleatum*).[10] With the exception of mitis-salivarius-agar plates (10% CO_2), plates were incubated anaerobically at 37°C for 72 hours. Species identification was achieved by observation of colony morphology. Data were scored for each species as CFU per biofilm.

All microbiological tests and analyses were performed strictly blinded to the nature of the previous treatment of the individual discs.

Confocal Laser Scanning Microscopy (CLSM) Analysis of Biofilm Structure

For CLSM, biofilms were stained using the LIVE/DEAD BacLight bacterial viability assay (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer. After 20 minutes staining, excess dye was gently aspirated from the discs without touching the biofilms. They were embedded upside down in 20 µL of Mowiol,[11] and stored at room temperature in the dark for at least 6 hours prior to microscopic examination.

Stained biofilms were examined by CLSM at randomly selected positions using a Leica TCS SP5 (Leica Microsystems, Heidelberg GmbH, Germany) with a ×20/0.8 numerical aperture (NA) and ×63/1.4 NA oil immersion objective lens in conjunction with 488 nm laser excitation and 530 nm emission filters for Syto 9 (live stain), and 561 nm laser excitation and 640 nm emission filters for propidium iodine (dead stain). Image acquisition was done in 8-line average mode and the data were processed using Imaris 7.2.2 (Bitplane AG, Zürich, Switzerland).

Statistical Analysis

Data related to CFU counts were tabulated as ranges in absolute CFU numbers. These numbers were compared between treatment groups by Mann–Whitney *U* test. The alpha-type error was set at 1%.

RESULTS

The total number of cultivable bacteria was around 10^7 on control SLA discs, which were immersed for 10 minutes in saline, but not electrochemically treated (Table 1). When a direct current of 10 mA was applied for 10 minutes, no viable bacteria could be recovered if the disc was the anode in the electrolysis setup. If the disc was the cathode, there was also a reduction of total counts by three to five orders of magnitude, but no complete eradication of viable bacteria ($p < .01$ between all three groups). There was no apparent selectivity of the treatment, as the taxa that survived at the cathode were those that grew to the highest number in the biofilm before treatment (Table 1).

CLSM images of anodic and cathodic implant surfaces showed a vast destruction of the biofilm as compared with the control surfaces (Figure 1), with some remaining islets of biofilm remnants. The detachment of biofilm was greater at the cathode, while killing was more thorough at the anode.

DISCUSSION

The current study showed that electrolysis could be an effective means to disinfect implant surfaces with complete kill of the CFUs at each of the anodic test discs, and

TABLE 1 Colony-Forming Unit Counts Recovered from SLA Implant Surfaces

Species	No Current	Anode (10 mA)	Cathode (10 mA)
All	9.5E6 – 5.5E7	ND	1.4E2 – 1.3E4
A. oris (OMZ 745)	4.0E4 – 1.6E6	ND	ND – 5.4E2
V. dispar (OMZ 493)	6.1E6 – 2.0E7	ND	6.0E1 – 9.5E3
F. nucleatum (OMZ 598)	1.1E4 – 7.2E4	ND	ND – 6.0E1
S. anginosus (OMZ 871)	1.2E6 – 1.7E7	ND	8-0E1 – 1.3E3
S. oralis (OMZ 607)	3.4E5 – 4.0E6	ND	ND – 6.4E2
P. intermedia (OMZ 278)	1.8E3 – 2.4E4	ND	ND
C. rectus (OMZ 698)	1.9E3 – 1.7E7	ND	ND
P. gingivalis (OMZ 925)	1.9E3 – 3.5E4	ND	ND

Treatment time: 10 minutes. Ranges from two triplicate experiments ($n = 6$), obtained on different experimental days.
ND = not detected; the detection limit in the current setting was 20 colony-forming units or 2.0E1.

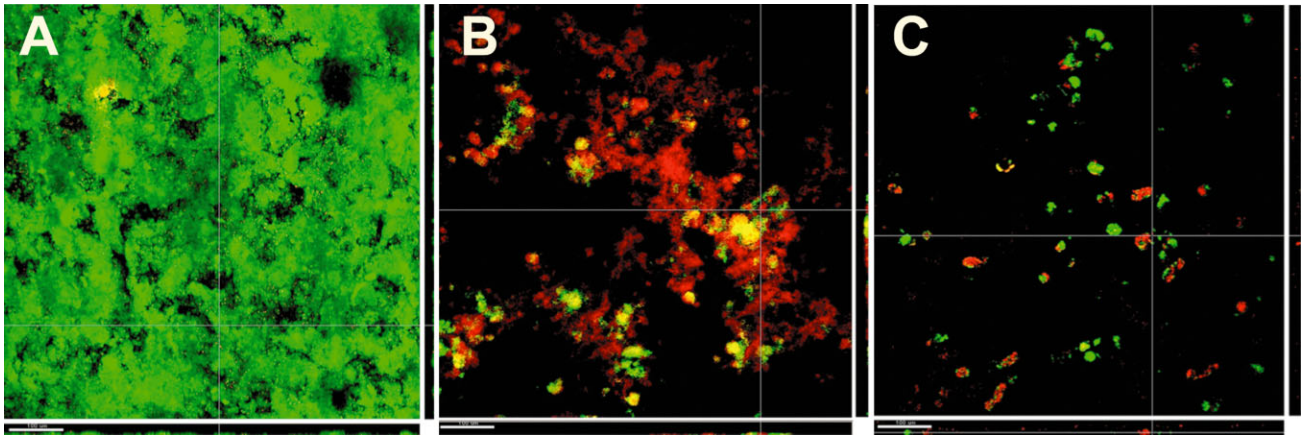

Figure 1 CLSM images of anaerobic in vitro biofilms grown on titanium discs after electrolysis treatment with 10 mA for 15 minutes and LIVE/DEAD staining. (A) control; (B) anode; (C) cathode; scale bar: 100 μm.

a reduction of total counts by three to five orders of magnitude at the cathode.

This current approach to disinfect dental implants is new, and thus needs to first be scrutinized in an in vitro environment before moving to animal and clinical experiments. Several studies with comparable biofilms proved substantial transferability from laboratory to clinics. In a similar biofilm model with supragingival taxa, that application time and concentration of disinfectants were comparable between the model and the clinic.[12] In another study, results from experimental rat caries studies were similar to those obtained by the oral in vitro biofilm.[13] Biofilms within periodontal pockets or on dental implants are formed in vivo under substantially different nutritional and environmental conditions, compared with supragingival biofilms. These anaerobic biofilms have a rather complex microbial ecology and are difficult to study as a whole due to technical limitations. To overcome this, an in vitro "anaerobic biofilm model" consisting of eight subgingival species was considered. This model uses the same batch approach as the previously mentioned supragingival biofilm model.

The current data comply well with the first publication on the issue,[5] though the multispecies biofilm used in this experiment is more complex and therefore should dispose of stronger defense mechanisms.[14,15] The finding that electrolytic treatment in a direct current setup results in a better viable count reduction at the anode as compared with the cathode is in accordance with findings of other groups.[16,17] This was explained by the generation of highly reactive chlorine species at the anode as opposed to the cathode, where hydroxyl ions and hydrogen are formed. The observation that biofilm detachment was greater at the cathode, while viability reduction was greater at the anode, is also in line with published observations on electrolytic disinfection.[17] This finding may be explained by the generation of electro-repulsive forces at the cathode. However, the lack of oxidative species at that electrode let the adhering bacteria survive.

Some differences were observed in the current study between CFU counts and the LIVE/DEAD BacLight stain. Some green coloration was still detected in biofilms at the anode after electrochemical treatment, indicating that the propidium iodide (dead stain) did not (yet) penetrate through the cell membranes of the respective bacteria. This can be explained by the different nature of the two assays. CFU counts miss out on the nongrowing or dormant microorganisms. On the other hand, the BacLight stain merely catches a moment in time, and it may well be that the green areas may have disappeared after an extended lag time. In the current study, specimens for CLSM were prepared immediately after the electrolytic treatment. In contrast, CFU counting involves multiple steps and thus assesses viability at a somewhat later stage after treatment.

It is still not known whether the treatment can be performed clinically. Some concerns relate to the current density in a clinical situation: Would the active species be generated at the site of the peri-implantitis and would the treatment be painful to the patient? The systemic danger of sending 10 mA of direct current through the human body should be minimal.[18] In the first half of the last century, direct current devices called "electrolyzers" were used clinically to disinfect the root canal system by

placing a thin positive electrode (anode) inside the canal system, which was previously filled with an electrolyte solution.[19] The cathode was a metallic hand electrode. In that treatment, electric current with 5 mA for 6 minutes was commonly used without any reported untoward effects on patients.

CONCLUSIONS

Based on the results presented here, electrochemical treatment of diseased implants appears to be promising and well worth investigating further. Future studies should address the clinical applicability and efficacy of electrolytic implant disinfection.

ACKNOWLEDGMENTS

The generous provision of SLA discs by Straumann AG, Basel, Switzerland, and the funding by the Division of Preventive Dentistry and Oral Epidemiology, University of Zurich, Center for Dental Medicine and the use of the CLSM at the Center for Microscopy and Image Analysis of the University of Zürich are kindly acknowledged.

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