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FULL PAPER

The effect of the electromagnetic field on metabolic-active bacterial biofilm experimentally-induced on titanium dental implants

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SUMMARY

Microbial biofilm is of paramount importance in the development of mucositis or peri-implantitis in patients with dental implants. This study was designed to investigate whether an electromagnetic field at high frequency waves directly applied on 33 titanium implants could remove experimentally-induced *Enterococcus faecalis* bacterial biofilm. A specially designed device (X-IMPLANT) was used to generate the electromagnetic field, with output power of 8 W, supply frequency (action/pause) 3/2s, and an output frequency of 625±5% kHz in plastic devices containing the biofilm-covered implants immersed in sterile saline. The bacterial biofilm on both treated and untreated control implants was quantitatively measured by phenol red-based Bio-Timer-Assay reagent. The kinetic analysis of the curves showed that the electrical treatment generated by the X-IMPLANT device completely removed the bacterial biofilm after 30 minutes of treatment ($p<0.01$). Elimination of the biofilm was also confirmed by chromatic observation in the macro-method. Our data seem to indicate that the procedure could be considered for clinical application in peri-implantitis to counteract bacterial biofilm on dental implants.

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INTRODUCTION

Microbial biofilm on dental implants can lead to long-term inflammation of the peri-implant tissues and induce the onset of mucositis or peri-implantitis (Sanz *et al*., 2012). Various strategies have been explored to counteract bacterial biofilms layered on dental implants, such as mechanical debridement, pharmaceutical therapy, subgingival irrigation with chlorhexidine or antibiotics, various chemical agents or laser beams, and surgical procedures (Sahrmann *et al*., 2020). In the absence of therapy, the progressive destruction of the tissues that support an implant can cause implant failure and, consequently, removal of the medical device could be the only therapeutic approach (Lindhe *et al*., 2008). This study was designed to check whether the use of high frequency electromagnetic waves generated directly on the implant by a specially designed apparatus (X-IMPLANT) could remove bacterial biofilm. In the experimental procedures, a strong bacterial biofilm produced by *Enterococcus faecalis* ATCC29212 was layered on the implants, which were successively treated in a treatment chamber by an electric current produced by the X-IMPLANT machine. The bacterial biofilm on both the treated and control untreated implants was then measured by means of Bio-Timer-Assay, a method that is able to evaluate the bacterial biomass embedded in the biofilm by means of the metabolic requirements of the germs (Pantanella *et al*., 2008; Romeo *et al*., 2015; Rosa *et al*., 2017; Olivi *et al*., 2021). Evidence confirms that the electric treatment performed by the X-IMPLANT system completely removed the bacterial biofilm.

Key words:
Dental implants, biofilm, electromagnetic field, mucositis, peri-implantitis, X-IMPLANT.

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MATERIALS AND METHODS

A total of 33 titanium implants (etched titan surface, diameter 4.2 mm, 10 mm length, MIS Dental Implants, Israel) were used. All the implants were sterilized by autoclave (121°C/15 min) before the experimental procedures. *Enterococcus faecalis* (ATCC

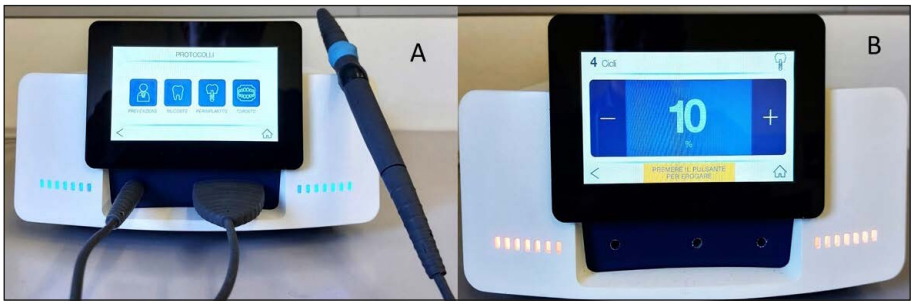

Figure 1 - *The X-IMPLANT machine (a) and a protocol available, in terms of timing and intensity of the currents by the device (b).*

29212) strain was used to generate a stable bacterial biofilm on the implants. The strain was plated on 5% sheep blood Columbia agar plate (ThermoFisher Scientific, US) to check strain purity before the experiments. Ten colonies were cultured in 5 mL of Brain Hearth Infusion (BHI) broth (Oxoid ThermoFisher Scientific, US) for 2 hours at 37°C to obtain a culture in the logarithmic phase of growth. The bacterial culture was washed in saline, adjusted to 0.5 OD of McFarland scale, diluted 1:1000 in BHI broth and thereafter used as the inoculum to generate growing biofilm on the implants. To allow formation of bacterial biofilm, the implants were incubated in 0.9 ml of bacterial *E. faecalis* inoculum in 1.5 ml sterile vials (Eppendorf Safe-Lock Tubes, Eppendorf, Italy) in upright position for 48 hours at 37°C, for 24 hours in static phase, and then for 24 hours under dynamic phase with gentle shaking. After incubation, the implants were washed three times in sterile 0.9 % NaCl solution to remove the planktonic form of non-adherent bacteria. The implants were divided into three different groups, eleven implants per group. The first group was treated with 900 µL of the *E. faecalis* suspension described above without electrical treatment; the second group was treated with 900 µL of *E. faecalis* suspension and subsequently treated with the electrical procedure; the third group with 900 µl of sterile BHI. The contaminated implants, immersed in 100 µl of sterile 0.9% NaCl solution, underwent electrical treatment using the "Peri-implantitis protocol" of the X-IMPLANT instrument (XIMPLANT S.r.l., Florence, Italy, *Figures 1a* and *1b*). The treatment consisted of four electrical current cycles, each at 90° from the previous one, applied with output power of 8 W, supply frequency (action/pause) 3/2s, and output frequency of 625±5% kHz. A homemade polystyrene-based treatment chamber was designed to ensure electrical flow, and a voltmeter was installed in the circuit. Even in the absence of correct flow of the electrical current in the treatment chamber, the samples were processed, and the event was recorded (*Figure 2*). To quantitatively evaluate the biofilm produced on dental implants, BioTimer Assay (BTA) was employed (Pantanella *et al*., 2008; Olivi *et al*., 2021; Rosa *et al*., 2017; Romeo *et al*., 2015). BTA measures bacterial metabolism by employing phenol red-BTA reagent (PR-BTA), whose colour shifts red-to-yellow due to bacterial metabolism (Pantanella *et al*., 2011). The time (in hours) required for the reagent shift was inversely related to the *E. faecalis* concentration in the bacterial biomass independent of planktonic or biofilm lifestyle (Pantanella *et al*., 2013; Romeo *et al*., 2014). To correlate the time for the reagent shift and the bacterial concentration, serial dilutions (from 10^8 to 10^2 CFUs/mL) of *E. faecalis* planktonic suspensions in PR-BTA were performed in a 96-well polystyrene micro-plate (Corning Incorporated, NY, USA). The time required for the colour switch was measured by kinetic detection of the colour change at wavelengths of 450 nm and 630 nm in a microplate reader (EL808, Biotex Instruments, Winooski, VT, USA). Absorbance values were recorded every 15 minutes for a 24-hour period. To draw the correlation line linking the time for colour shifts and bacterial concentrations, the Δ-ODs (450-630 nm) were considered and plotted versus the $\log_{10}$ of CFUs of the initial bacterial concentration at Time 0. The equation and the linear correlation coefficient describing the correlation line were calculated on the whole data set and were: $y=-0.1816x+5.4529$, $R^2=0.948$. As the equation correlates the time required to shift BTA reagent colour and planktonic bacteria, the number of biofilm *E. faecalis* was reported as planktonic-equivalent CFUs (PE-CFUs). To evaluate the biofilm developed on the implants, each implant was placed in an Eppendorf vial containing 1mL of PR-BTA reagent and vigorously vortexed for 60 seconds. Three aliquots of BTA reagent for each implant were then placed in a sterile 96-well polystyrene micro-plate (Corning Incorporated, NY, USA) and incubated in the microplate plate

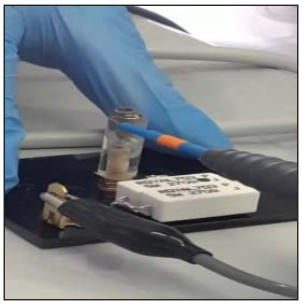

Figure 2 - *Application of the electrical procedure on the surface of an E. faecalis contaminated implant in homemade polystyrene-based chambers.*

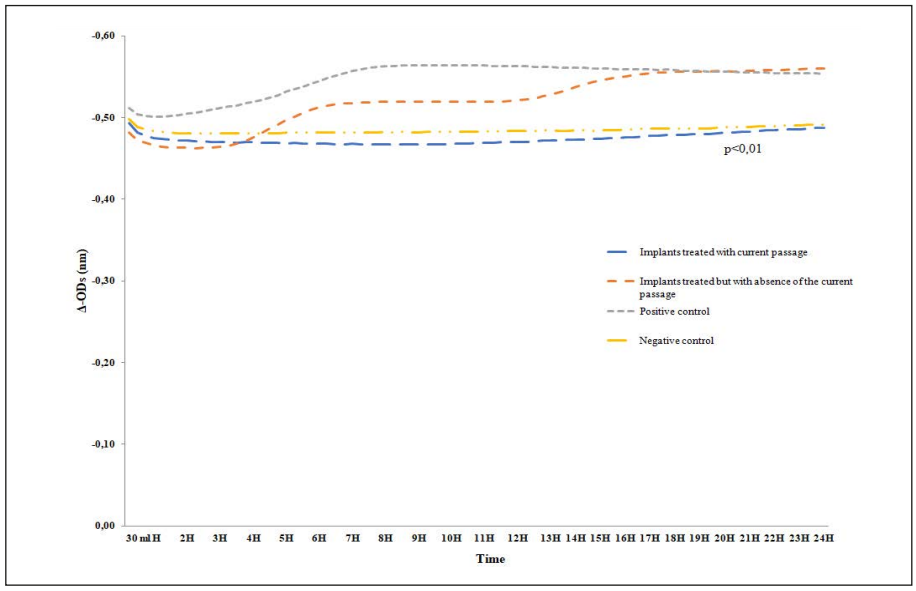

Figure 3 - *The kinetics of biofilm production. The curves of the implants in which we recorded electrical flow showed kinetics identical to the negative controls, highlighting the elimination of the biofilm during the 24 hours of observation ($p<0.01$) as early as 30 minutes after treatment. The implants in which there was no current flow showed the same kinetics as the untreated devices.*

reader. In addition, 100 µl of a solution of *E. faecalis* bacterial suspension ($3x10^5$ cfu/ml in BTA reagent) and 100 µl of sterile BTA were added to each plate for positive planktonic growth and negative reagent control, respectively. Bacterial load was calculated by evaluating the time required to obtain a Δ-OD (450-630 nm) of -0.05 from that of Time 0 and the above-reported equation. Finally, the implants were added with fresh BTA reagent and incubated for three days to confirm the colour shift and to evaluate the experimental procedures. To evaluate if the difference in bacterial load average was statistically significant, the results are expressed as mean values obtained from at least three independent experiments. Student T test was employed on bacterial load average at each time point to determine significance. P values ≤0.5 were considered significant.

RESULTS

The kinetics of biofilm production as evaluated by the BTA technique showed the production of biofilm on all 11 non-electrically-treated implants, soon after 4 hours of incubation. The production curve increased rapidly, reaching maximum values after three hours from the inoculum, with sustained bacterial metabolism during the following 14 hours (*Figure 3*). The average of the final bacterial load for the untreated positive implants, belonging to the first study group, was 4.632±0.425 PE-CFU in a range very similar to that of the planktonic positive growth control (5.075±0.05 PE-CFU), witnessing very efficient deposition of biofilm on the implants. The curves of the devices that underwent electrical treatment (second group) showed different kinetics depending on the correct functioning of the homemade treatment chambers: the voltmeter displayed electric flow in

Figure 4 - *Chromatic observation in the macro-method procedure after 72 h. First vial from the left: sample of BTA reagent from untreated implant; central vial: BTA reagent from the implant after electrical treatment; last vial on the right: negative control.*

eight out of 11 chambers (72.7%), while in two (18%) there was absence of current flow and in one (0.9%) a partial current flow. The kinetics of the curves of the 8 implants in which we recorded electric flow showed kinetics identical to the negative controls, highlighting the elimination of the biofilm during the 24 hours of observation ($p<0.01$) (*Figure 3*). Intriguingly, we recorded complete elimination of the biofilm as early as 30 minutes after treatment, with sustained clearance until the end of the reading. Furthermore, elimination of the biofilm was also confirmed by chromatic observation in the macro-method, in which no colour change was detected even in the 72 subsequent hours (*Figure 4*), witnessing complete biomass eradication. The implants in which there was no current flow showed the same kinetics as the untreated devices, while the only sample in which there was partial current flow a reduced biofilm production reduction was observed, with bacterial load of 2.98 PE-CFU.

DISCUSSION

A number of studies have tried to compare the microbial profiles of peri-implantitis with those of periodontitis and healthy implants, but a specific profile

exclusively or predominantly of peri-implantitis microbiota has not yet been recognised, and more than 400 different microbial species have been identified in the analysis of endodontic samples (Sahrmann *et al*., 2020). In our study, we tested the biofilm produced on implants by *Enterococcus faecalis*. Our choice was based on the observation that such bacterial species was very frequently isolated and identified in both endodontic infections (Rôças *et al*., 2004) and in peri-implantitis treatment failures (Lindhe *et al*., 2008). *E. faecalis,* in fact, has a marked ability to adhere and invade the dentinal tubes, to produce biofilm, to survive with minimal nutrition condition in the presence of wide variations of pH and temperature, and to resist the medicaments used in endodontic procedures (Jhajharia *et al*., 2015). Indeed, as observed in our experiments, *E. faecalis* was able to produce a stable biofilm on the implants soon after four hours. Numerous assays have been used to test biofilm on abiotic surfaces, employing reagents such as crystal violet, tetrazolium salt, resazurin, or dimethyl-methylene blue. Unfortunately, these methods have not always produced comparable results. Furthermore, the determination of PE-CFUs and the growth of biofilm over time is not always guaranteed. In our study, we elicited *E. faecalis* mature biofilm directly on titanium implants by culturing the bacteria on the devices for 48 hours at 37°C, and measured the PE-CFU on the implants by the BioTimer assay. With this method, we were able to measure the experimentally induced biofilm over time, exploiting the possibility of kinetic reading performed every 15 minutes for 24 hours on both electrically treated and untreated implants, as well as on the control devices.

Since the Food and Drug Administration (FDA) approved the pulsed electromagnetic field in 1979 to stimulate bone growth after implants, the positive effects of electric fields in various medical applications have been reported in the literature. In fact, electricity was shown to accelerate bone formation after implant placement (Song *et al*., 2009; Bins-Ely *et al*., 2017, Bins-Ely *et al*., 2020), to enhance the effect of some antibiotics on biofilms (Tagbo *et al*., 2012), and to display anti-inflammatory effect and good antibacterial potential both in infected tissues (Barki *et al*., 2019; van der Borden et al., 2004) and in peri-implantitis (Schneider *et al*., 2018). In our study, the X-IMPLANT instrument was used to generate the electric field, dispensing four cycles of electrical current directly on the implants. Our data showed that such treatment led to the complete and fast removal of the experimentally induced biofilm on the implants, as witnessed in experiments in which we observed the absence of PE-CFUs soon after 30 minutes. Moreover, the elimination of biofilm was stable, as observed in the kinetic measurement lasting 24 hours, and this effect was confirmed during the subsequent 72 hours by the chromatic observation in the macro-method. However, when there was a failure in the correct application of the electric field in three implants due to a malfunctioning of the home-made treatment chamber, we observed that production of the bacterial biofilm was similar if not identical to that of the untreated implants and of the planktonic positive growth control. Therefore, it seems that correct application of the electric field is mandatory for complete elimination of the bacterial biofilm on the implants. This approach might influence the application of the method in the clinical management of peri-implantitis, especially on titanium oxide implants, a relatively active metal, with the standard electrochemical potential of -1.628 V (Pozhitkov *et al*., 2015). Furthermore, the electrical conductivity of the mouth has to be considered (Freĭdin *et al*., 1990), together with the known capacity of the bacteria to transfer electrons (Rosenbaum *et al*., 2011; Kalathil *et al*., 2013). It therefore seems that any lack of electrical impulse conduction should be avoided if the procedure is to be used to treat peri-implantitis.

In conclusion, our experiments confirmed that the four electrical current cycles produced by the X-IMPLANT machine completely removed the *E. faecalis* (ATCC 29212) bacterial biofilm from the implants. The absence of PE-CFU and the persistence of this result over time might allow speculation that the instrument has good potential to control the bacterial biofilm of both dormant and active growing persistent bacterial cells on infected implants. However, the functionality of the process seemed to be limited when the electric field was not efficiently applied. Even though the data in our study were obtained *in vitro*, the X-IMPLANT procedure might be considered for clinical application in peri-implantitis to counteract bacterial biofilm on the surfaces of dental implants.

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