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## Biofilm formation on titanium alloy and anatase-Bactercline<sup>®</sup> coated titanium healing screws: an in vivo human study

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**KEYWORDS** Abutments; Anatase; Bacterial adhesion; Dental implants; Healing screws.

### ABSTRACT

**Aim** Bacterial adherence to implants is considered to be an important event in the pathogenesis of bacterial infections. In fact, this infection process is a first stage of peri-implant mucositis and peri-implantitis, and a positive correlation has been found between oral hygiene and marginal bone loss around implants in the edentulous mandible. Surface properties of transgingival implant components are important determinants in bacterial adhesion. The purpose of this study was to characterize the biofilm formation, in vivo, on healing screws made of titanium alloy or coated with a combination of anatase and Bactercline<sup>®</sup> product.

**Materials and methods** Twenty-five patients, between 21- 37 years, in excellent systemic health, participated in this study. In each of the 25 participants, one anatase-Bactercline<sup>®</sup> coated healing screw (Test) and one titanium alloy (Ti6Al4V) healing screw (Control) were adapted to two different implants. Quantitative and qualitative biofilm formation on healing abutments was analyzed by culture method.

**Results** Bacterial adherence to the two different healing screws used in this study were compared. Statistically significant differences were found between the Control and the Test group for both aerobic and anaerobic bacterial counts ( $p < 0,05$ ). The microflora consisted both of Gram-positive and Gram-negative bacteria, and displayed a high variability. The anaerobic *S. intermedius*, potentially "pathogenic", was isolated only from the Control group. Both healing screws harbored primarily Gram-positive rods as *Actinomyces* spp, *A. naeslundii*, *A. viscosus* and the Gram-negative rods (*Fusobacterium* spp, *Prevotella* spp, *Capnocytophaga* spp) were mostly found on the Control healing screws.

**Conclusion** Anatase-Bactercline<sup>®</sup> coated healing screws reduce the number of initially adhering bacteria, formed mainly of Gram-positive microorganisms, while, on the contrary, the microflora covering the titanium alloy healing screws was, for the most part, Gram-negative.

### INTRODUCTION

Titanium dioxide exists in three different crystal lattices, anatase, rutile, and brookite (28). Normally, a stochastic distribution of two titanium-oxides (rutile and anatase) is present on the surface of the titanium, and this is responsible for the properties of the material (7). A homogeneous anatase coating can be produced around the implant surface with different systems (14, 4) and this anatase coating gives special characteristics as genetic effects on osteoblasts (15, 25). The anatase coating releases, under UV irradiation, free radicals such as  $\bullet\text{OH}$ ,  $\text{O}_2^-$ ,  $\text{HO}_2^-$ , and  $\text{H}_2\text{O}_2$  and this potent oxidizing power characteristically results in the lysis of bacteria and other organic substances (2, 24, 10, 1). Several reports have been published on the bactericidal properties of  $\text{TiO}_2$  against microorganisms such as *Escherichia coli* (9, 6), but the exact killing mechanism has not been well elucidated (9, 24). This bactericidal action could be due to the elimination of the protection of the cell wall of the bacteria, and then to an increase in the cell permeability determining a loss of intracellular contents, leading to the death of the cells (9, 24). The modification of the surfaces or the use of different materials have been shown to play a relevant role in the bacterial adhesion to implant surfaces (22, 23). Bacterial adherence to implants is considered to be an important event in the pathogenesis of bacterial infections and the infectious process can be viewed as a stepwise process in which the bacteria must first adhere to an implant surface. The failure to adhere would result in their being swept away in the fluids which constantly bathe the tissue surface. Surface properties of transgingival implant components are important determinants in bacterial adhesion. In

fact, a bacterial adhesion to implant surfaces is a first stage of peri-implant mucositis and peri-implantitis, and a positive correlation has been found between oral hygiene and marginal bone loss around implants in the edentulous mandible (8). Also other surface characteristics of the components of the transgingival element, in addition to surface roughness, seemed to be extremely important in relation to the plaque formation and may be helpful in the prevention of peri-implant soft tissue pathology (19). Different adhesion affinities of bacteria have been reported for different materials, such as for example titanium and titanium alloys (27). The purpose of this study was to characterize the biofilm formation, in vivo, on healing screws made of titanium alloy or coated with a combination of anatase and Bactercline® product.

## MATERIALS AND METHODS

### Patient selection and study design

Twenty-five patients, between the ages of 21 and 37 years, and in excellent systemic health, participated in this study. All patients gave their informed consent, and the protocol was approved by the Ethic Committee of Chieti-Pescara University, Chieti, Italy. The participants were selected on the basis of good periodontal health and no signs of mouth breathing. One week prior to the beginning of the study, supragingival plaque and calculus were professionally removed, oral hygiene procedures were established and ideal gingival health conditions were obtained in all volunteers (Loe & Silness Gingival Index = 0). None of them had used mouthrinses or had taken antibiotics during the previous six months. In each of the 25 participants, one anatase- Bactercline® coated healing screw (Test) and one titanium alloy (Ti6Al4V) healing screw (Control) were adapted to two different implants (Dental Tech, Misinto, Milano, Italy). The radiographical and surgical evaluation of the bone density at the moment of the insertion was, for all implants, D2-D3 bone quality. All these implants were left submerged, and after 6 months, a healing screw was positioned on all implants. A total of 50 healing screws (25 Test and 25 Control) were used in this study. Neither cleaning procedures nor agents for chemical plaque control were applied to the healing screws for the complete duration of the test period. After twelve days, all healing screws were removed gently, placed inside sterile plastic vials containing 1 ml of Reduced Transport Fluid (RTF), and immediately sent to the microbiological laboratory.

An additional 6 healing screws (3 Test and 3 Control) were analysed for surface characterization, and the surface roughness was evaluated under a Leo 435 VP scanning electron microscope (LEO, Cambridge, UK) and a Mitutoyo SurfTest 211 Profilometer (Mitutoyo Corporation, Tokyo, Japan): an average of 3 readings

was performed for each surface. The arithmetical mean of surface roughness of every measurement within the total distance (roughness average=Ra) was assessed. The surface roughness values were expressed as a mean  $\pm$  standard deviation. Two areas of 200 microns in diameter were evaluated for each healing screw surface.

### Preparation of anatase-Bactercline® coatings

Bactercline® is essentially an aqueous suspension of titanium dioxide nanoparticles functionalized with organic and inorganic ions with antibacterial, virucidal and fungicidal properties. (International Patent Application 1. WO 2007/122651-Functional nanomaterials with antibacterial and antiviral activity 01.11.2007 B22F 1/02 PCT/IT2006/000280 2. WO 2008/020460-Nanomaterial coatings for osseointegrated biomedical prostheses 21.02.2008 A61L 27/30 PCT/IT2006/000450).

Silver ions, stabilized in their one electron oxidized form, are covalently bound to high surface area titanium dioxide nanoparticles, acting in synergy with cationic species which improve the bactericidal, fungicidal and virucidal effect of the product. Bactercline® is a new disinfecting formulation which has passed the examination of the Italian National Institute of Health and is now classified as Presidio Medico Chirurgico N. 19258. The product was applied on the healing screws by dip coating after the deposition of an anatase layer prepared according to the procedure which follows.

TiO<sub>2</sub> colloidal solutions were prepared by hydrolysis of titanium isopropoxide, Ti (OCH<sub>2</sub>(CH<sub>3</sub>)<sub>2</sub>)<sub>4</sub>: 50 ml of titanium isopropoxide (FLUXA) was added dropwise, by means of an addition funnel, to 300 ml of deionized water acidified with 2.1 ml of 65% HNO<sub>3</sub> under vigorous stirring. During the hydrolysis a white precipitate was formed. The mixture was stirred for 8 h at 80°C. During this process the mixture was allowed to concentrate to 120 ml, corresponding to a TiO<sub>2</sub> concentration of 170 g/l. A stable colloidal solution resulted from this procedure. The size of the colloidal particles was about 8 nm and X-ray diffraction analysis showed them to consist of anatase. Fifty ml. of this solution was heated at 220°C for 12h in an autoclave. During this process the nanoparticles grew until a size of 50–100 nm.

Coating of the screws was done by dip coating into the titanium dioxide suspension of nanoparticles, followed by heating for 30 min in an oven at 450°C in the presence of oxygen. The screws were then placed in a desiccator for cooling at room temperature and finally inserted in plastic tubes.

### Microbiological procedures

Biofilm formation on healing screws was analyzed quantitatively and qualitatively by culture method. All samples were incubated in 0.25% trypsin for 45 min in a shaking water bath at 37°C. Following the trypsin step, the contents of the tube were vortexed thoroughly and

each was then subjected to a series of 10-fold dilutions in 0.1 M phosphate buffer (26). Aliquots of 100 µl from undiluted suspension and each dilution were spread in duplicate onto Columbia Blood Agar (CBA) plates (Oxoid Italia SpA, Garbagnate Milanese, Milan, Italy) and trypticase soy agar plates (Oxoid) supplemented with 5% defibrinated sheep blood (ETSA) to quantify the number of all cultivable oral bacteria, which was recorded as the colony count that formed *per ml* (CFUs/mL) on the growth plate. In particular, the CBA plates were used to cultivate anaerobic bacteria under strictly anaerobic conditions at 37°C for 7–12 days (anaerobic chamber; 80/10/10, N<sub>2</sub>/H<sub>2</sub>/CO<sub>2</sub>; Don Whitley Scientific Ltd, Shipley, UK; International PBI SpA) and the ETSA plates were incubated at 37°C for 2–4 days to cultivate aerobic bacteria. Generally, isolation of microorganisms was carried out by methods previously reported (16). In particular, for some bacterial strains, special microbiological procedures were applied. The samples were also plated onto Mitis Salivarius Agar (MSA) (Difco™, Becton, Dickinson and Company, Sparks, USA) containing 1% Chapman tellurite solution to enumerate oral *streptococci* and *enterococci*; onto MRS Agar (Difco™, BD) to assess *Lactobacillus* spp.; onto Brain Heart Infusion Agar (BHIM) (Oxoid) enriched with nalidixic acid (30mg/l) (Sigma Aldrich, Milan, Italy) e metronidazole (10mg/l) (Sigma Aldrich) to assess *Actinomyces* spp.; trypticase soy crystal violet erythromycin (4 mg/l) (CVE), to assess *Fusobacterium* spp.

The purification and characterization of clinical isolates was carried out essentially as described previously (3). Finally, a definitive identification of all representative isolates was obtained by subculturing onto Brucella blood agar (Oxoid) followed by inoculation of purified cultures onto a commercially packaged automated system (bioMérieux, Marcy-l'Etoile, France). For each microbial species, data were recorded as the count of CFUs/ml on the growth plate. The total bacterial counts, obtained by adding data of each microbiota, and the frequencies of detection for each microbiota were also reported.

### Statistical analysis

The non parametric Mann-Whitney U-test was used to evaluate the presence of statistically significant differences among the two groups. Results were presented as means ± standard errors (SE), and differences at  $p \leq 0.05$  were considered statistically significant.

## RESULTS

### Surface characterization

Grooves and ridges, typically produced during the manufacturing, were present in both types of surfaces. The surface roughness (Ra) was  $0.73 \pm 0.05 \mu$  for

the titanium healing screws and  $0.86 \pm 0.06 \mu$  for the anatase-Bacterline® coated healing screws. No statistically significant differences were present.

### Microbiological analysis

Bacterial adherence to the two different healing screws used in this study were compared. Figures 1 and 2 showed the proportional composition of the CFU of aerobic and anaerobic strains isolated from the two different implant healing screws. Statistically significant differences were found among the Control group and the Test group for both aerobic and anaerobic bacterial counts ( $p < 0.05$ ). Table 1 showed the frequencies of detection of the predominant cultivable microbiota from the two different groups. The microflora consisted both of Gram-positive and Gram-negative bacteria, and displayed a high variability. The anaerobic *S. intermedius*, potentially "pathogenic", was isolated only from the Control group (Fig. 1). Both healing screws harbored primarily Gram-positive rods (*Actinomyces* spp, *A. naeslundii*, *A. viscosus*). The Gram-negative rods (*Fusobacterium* spp, *Prevotella* spp, *Capnocytophaga* spp) were mostly found on the healing screws of the Control group (Fig. 1). The aerobic bacterial strains *S. sanguis*, *S. mitis* were predominantly isolated from the Test group. No bleeding of the peri-implant tissues was observed after the healing screws were removed from the oral cavity.

## DISCUSSION AND CONCLUSION

The quality and the quantity of plaque adhesion on the implant surfaces (transgingival abutment or healing implant components) are important in the long-term success of dental implants. A correlation between plaque accumulation and progressive bone loss around implants has been reported in experimental and clinical studies (13).

Bacterial adhesion and colonization are considered to play a key role in the pathogenesis of peri-implant disease (17). The effectiveness of systemic and local antibiotic prophylaxis of infections of implanted devices is constrained by the continual evolution of antibiotic resistant bacteria (12). In addition, adhesion-mediated infections are extremely resistant to antibiotics due to the biofilms that protect the organisms from the activity of antimicrobial agents and from host defence mechanisms. Hence, such infections frequently persist until the biomaterial is removed (3). The present study examined the influence of the surface features of healing screws on the quantitative and qualitative microbial colonization. Patients with oral two-stage implants were selected because the healing screws were replaceable without any discomfort to the subjects and because they exhibited a subgingival area with an anaerobic environment that enabled formation of the same biofilm as during development of periodontitis. The

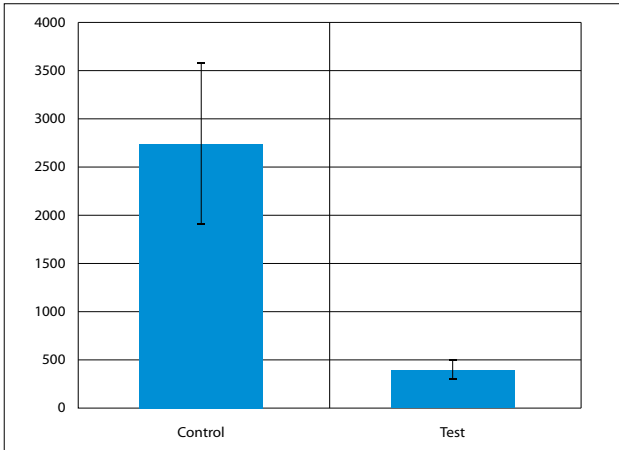


FIG. 1 Comparison of Colony-Forming Units (CFU) of aerobic strains on the Control Group and the Test Group. Statistical significance of the differences between the groups Mann-Whitney Test ( $p=0.0095$   $p<0,05$ ).

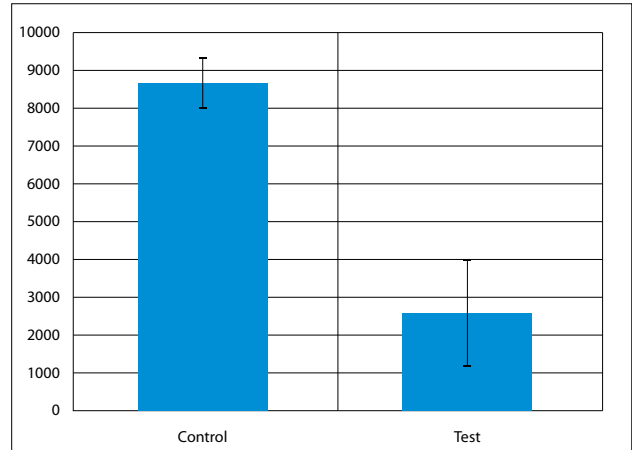


FIG. 2 Comparison of Colony-Forming Units (CFU) of anaerobic strains on the Control Group and the Test Group. Statistical significance of the differences between the groups Mann-Whitney Test ( $p=0.0485$   $p<0,05$ ).

	Control	Test
Streptococcus spp	100	50
S. sanguis	–	25
S. mitis	–	25
S. intermedius	37,5	–
Actinomyces spp	100	100
A. naeslundii	62,5	25
A. viscosus	–	25
Lactobacillus spp	–	50
Peptostreptococcus spp	–	25
Gemella morbillorum	50	–
Leptotrichia buccalis	25	25
Capnocytophaga spp	37,5	–
Eubacterium lentum	75	–
Fusobacterium spp	75	–
Prevotella spp	75	–

TABLE 1 Number of healing screws positive for the presence of each bacterial species in the different experimental groups.

present data indicated that in the anatase-Bacterline® coated healing screws an important decrease in aerobic and anaerobic bacterial counts was found with a dramatic change in plaque composition. After twelve days, the bacterial counts from the anatase-Bacterline® coated healing screws were on average 3–5 times smaller than those from the titanium alloy surfaces and these differences were statistically significant. The results of this study confirmed those previously reported

from our laboratory that lower amounts of bacteria on anatase coated titanium healing screws were found (21). Microporosities seemed to be eliminated in anatase-Bacterline® coated healing screws. In this way the number of ecological niches were reduced and this fact could explain the reduced number of microorganisms recovered from the healing abutments. It can be assumed that different dental materials exerted a strong influence on the adherent biofilm microflora. Titanium alloy healing screws always harbored a more complex microbiota, characterized by both Gram-positive and Gram-negative bacteria and for teeth, such a microbiota was considered a more mature plaque. However, diverse phenomena may have contributed to the present findings, i.e. physical parameters like chemical composition or surface roughness of the materials, preferential attraction of dead biological material by oral hard surfaces and dying of microbes after initial adhesion or a true antibacterial effect of the dental materials via leaching compounds. No overall relationship could be established between biofilm vitalities, surface coatings and biofilm heights. Quirynen et al. (2002) (17) demonstrated that without oral hygiene, there was a direct positive relationship between surface roughness and undisturbed plaque growth rate and pathogenicity. In this study, the patients maintained good oral hygiene, so one might conclude that not only the growth rate but also the reduced ability to clean a rough surface justified the use of smooth surfaces for all intraoral hard surfaces. Qualitative analysis revealed that the periodontal pathogens were not present in any sample. These results were in agreement with the finding of Heuer et al. (2007) (5) who found no *Porphyromonas gingivalis* or *Aggregatibacter actinomycetemcomitans* in healing abutments of stable osseointegrated implant surfaces after a period of 14 days, in contrast with



peri-implant lesions, in which high levels of periodontal pathogens and superinfecting bacteria were present. For colonization of healing screws surfaces with these microorganisms a long-standing biofilm and proliferation in subgingival areas seemed to be necessary. In partially edentulous patients, the composition of the subgingival microbiota was similar to teeth and implants and the transmission of bacteria from residual pockets around neighboring teeth was also possible. In the present study, none of the patients had clinical signs of periodontal disease. Therefore, cross infection between healing screws and natural teeth was unlikely. After twelve days, however, the anaerobic *S. intermedius*, potentially "pathogenic", was isolated only from Control group and Gram-negative rods (*Fusobacterium* spp, *Prevotella* spp, *Capnocytophaga* spp) were mostly found in the Control group. Many of these bacteria are known to be involved in periodontal infections.

It is therefore very important to develop screw surfaces that reduce the number of initially adhering bacteria, thereby minimizing biofilm formation and subsequent inflammation of the soft tissue. The present results demonstrated that these requirements could be satisfied by the use of anatase-Bactercline® coated healing screws. The colonization with potentially pathogens and the presence of Gram-negative bacteria could lead to a recommendation that the titanium healing screws should be removed as early as possible.

In conclusion the microflora covering the anatase-Bactercline® coated healing screws was Gram-positive, while, on the contrary, the microflora covering the titanium alloy healing screws was, for the most part, Gram-negative.

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