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ORIGINAL ARTICLE

Scanning electron microscopy and microbiological approaches for the evaluation of salivary microorganisms behaviour on anatase titanium surfaces: In vitro study

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Summary Implantology research framed the implant surface as a key element for a good and sustainable osseointegration of an implant fixture. The aim of this study was to analyze the antibacterial properties of anatase-coated titanium healing screws through microbiological and scanning electron microscopy. The comparison of the bacterial colonies growth between the anatase-coated titanium healing screws and non-coated titanium healing screws showed comparable antibacterial properties, without significant statistical differences. The scanning electron microscopy observations confirmed the microbiological study. These data, also considering previous reports on the positive effects on osteoblasts genetic expressions, might suggest a use of the anatase-coated titanium healing screws to preserve the tissues surrounding implants from microbial attacks.

Introduction

The research in implant dentistry mainly focused on implant titanium surfaces, identified as the key elements to reach the osseointegration [1—3]. The evolution of the implant surface treatments has led to the development of two types of surfaces: (i) one with a convex profile and (ii) one with a concave profile [4,5]. The convex profile shows a rough surface with prominent protrusions made of titanium particles and realized with additional techniques such as the titanium plasma sprayed (TPS) surfaces. The latter presents cavities and holes, resulting from a removal or subtraction tech-

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nique, such as sandblasting and/or acid-etching (SLA). The search of the ideal implant surface leads to the development and the testing of many different techniques for surface treatment (mechanical, chemical surfaces alteration, coating techniques, anodization technique) [6]. Mechanical treatment defines two types of surfaces: machined and sandblasted . The machined surfaces appear geometrically simple. The sandblasted surfaces are regular and geometrically more complex than the machined ones. Chemical treatments modify the initial surface by immersion of the titanium alloy samples in a polar solution, which etching action creates cavities with diameter ranging from 0.5 to 2 μm.

In an alkaline solution, the high pH leads to the formation and deposition of titanium salts on the surface [7—9]. Concerning the coating techniques, two different methods are available: the titanium plasma sprayed, consisting in the addiction of titanium particles and the hydroxyapatites coating, consisting in the deposition of calcium-phosphates [10].

Titanium oxidation (TiOx) is considered as a complex form of various oxides. One of the methods used to obtain the titanium oxidation is anodization.

Anodization consists in the electrochemical oxidation of the superficial layers of the titanium, resulting in the formation of titanium dioxide (TiO_2), useful for its bactericidal and biological properties. The research regarding osseointegration aims to increase the implant survival rate, and to reach at the collar level a long-lasting sealing. In this vein, one of the mineral elements added to the healing fixture, representing the connection between the oral habitat and the inserted fixtures, is the anatase, a form of titanium dioxide (TiO_2) [11]. The aim of this study was to compare microbiologically and morphologically the biofilm aggregation on anatase titanium surfaces and on non-treated titanium surfaces.

Material and methods

Eight healing screws, kindly provided by MaCo Dental care were used. Four screws samples presented a surface in TA6V (titanium alloy grade 5-Ti AL6V4 ELI) and four had a surface in anodized titanium.

The anodization of the titanium surface included the following steps:

- ultrasonic bath with a basic surface-active agent at 50 °C for 10 minutes;
- rinsing in distilled water for 10 minutes at 50 °C;
- pickling with a HNO_3 at 10% and HF at 2.5% solution for 2 minutes;
- ultrasonic bath in distilled water for 10 minutes at room temperature;
- drying by means of ethanol and nitrogen gas;
- anodic passivation by the slow immersion of the titanium in a solution of 15% cloridric acid for 10 seconds with setting the potential at 3.9 V and placing the material on the anode;
- distilled water rinse at room temperature;
- drying by means of ethanol and nitrogen gas;
- staining of the surface by means of anodization process.

The microorganisms used for the experiment derived from a saliva sample. The use of natural saliva was preferred to the artificial one to evaluate the eventual adhesion of a mixed microbial population and not of a particular selected bacterial strain. In addition, the electrochemical behaviour of natural saliva is not completely imitable by the available artificial saliva solutions [12]. Therefore, in order to study the behaviour of bacterial adhesion as much as possible similar to the in vivo situation, we used a natural saliva sample.

The source of saliva was a healthy, 27 years, female volunteer. The general and oral health status of the volunteer was assessed by a dentist.

The criteria inclusions for the recruitment of the volunteer were:

- good oral hygiene level;
- Periodontal Screening and Recording (PSR) Index 0;
- good general health with reference to American Society of Anesthesiologists Physical Classification System.

The biofilm and microbiological formation on the screws was evaluated using culture-dependent techniques. Then the surfaces were observed at the Scanning Electron Microscopy (SEM) to evaluate eventual bacterial residuals on the tested surfaces.

Microbiological protocol

Ten millilitres of saliva were sampled early in the morning before brushing teeth, closed in a sterile vial and transported in dry ice. The saliva sample was enriched with broth medium (Liofilchem® s.r.l., Italy). The microbial culture was performed according the techniques described by Lodish et al. [13], free from antibiotics to allow the development of aerobic bacterial strains on the titanium surfaces. Each group of screws were immerged in 3 millilitres of enriched saliva and incubated at 37 °C for 7 days in aerobic conditions.

After the 7 days of incubation, the screws were immerged in a phosphate-buffered saline (PBS) solution and then sonicated for 10 minutes at 60 Hz and 100 Watts. Subsequently, the PBS solution was plated on blood agar and incubated at 37 °C for 7 days in aerobic conditions. The colonies were enumerated and analyzed by MALDI-TOF, as described before [14].

Three millilitres of enriched saliva were cultured on blood-agar medium and incubated in aerobic condition for 7 days and used as control to investigate the aerobic microbiological population. The colonies were analyzed by MALDI-TOF.

The colonies enumeration of the two types of surfaces was statistically compared by means of parametric test (*t*-student), considering significant a *P*-value < 0.05. The statistical analyzes were performed using the software SAS University Studio.

SEM analysis

Protocol of the samples' preparation was followed as described before [15—17]. Briefly, after exposure to the microbiological culture, 2 anodized healing screws and 2

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