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Initial oral biofilm formation on titanium implants with different surface treatments: An *in vivo* study

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ABSTRACT

Objective: The aim of this study was to examine *in vivo* the initial bacterial adhesion on titanium implants with different surface treatments.
Design: Ten subjects wore oral splints containing machined pure titanium disks (Ti-M), acid-etched titanium (Ti-AE) and anodized and laser irradiated disks (Ti-AL) for 24 h. After this period, disks were removed from the splints and adherent bacteria were quantified by an enzymatic assay to assess total viable bacteria and by Real Time PCR to evaluate total bacteria and *Streptococcus oralis* levels. Additionally, the initial adherent microorganisms were visualized by scanning electron microscopy (SEM). Titanium surface morphology was verified using SEM, and roughness was evaluated by profilometer analysis.
Results: Regarding titanium surface roughness, Ti-AL (1.423 ± 0.397) showed significantly higher Ra values than did Ti-M (0.771 ± 0.182) and Ti-AE (0.735 ± 0.196) ($p < 0.05$, ANOVA – Tahame). Ti-AE and Ti-AL presented roughened micro-structure surfaces characterized by open pores, whereas Ti-M showed long grooves alternating with planed areas. Comparing the Ti-M, Ti-AE and Ti-AL groups for viable bacteria (MTT assay), total bacteria and *S. oralis* quantification (qPCR), no significant differences were observed among these three groups ($p > 0.05$, ANOVA – Tahame). SEM images showed similar bacterial adhesion on the three titanium surfaces, predominantly characterized by cocci and several bacilli, indicating an initial colonization of the oral biofilm.
Conclusion: In conclusion, roughness and microtopography did not stimulate initial biofilm formation on titanium surfaces with different surface treatments.

1. Introduction

Dental implants are routinely used to replace lost teeth and restore aesthetic function, phonetics and mastication (Astrand, Ahlqvist, Gunne, & Nilson, 2008; Lekholm et al., 1999; Lekholm, Grondahl, & Jemt, 2006). The success of implants depends on the integration of the implant to the bone and mucosal connective tissue as well as the absence of inflammation and infection in the surrounding tissues (Burgers et al., 2010).

Titanium is a biocompatible material and has been widely used in dental implants. Various treatments on the surfaces of titanium implants have been used to improve the rate of osseointegration (Albrektsson and Wennerberg, 2004; Meirelles, Arvidsson, Albrektsson, & Wennerberg, 2007; Schwartz-Filho, Morandini, Ramos-Junior, Jimbo, & Santos, 2012) and to stimulate proper interactions between the implant and the oral mucosa (Wennerberg et al., 2011). However, these modifications generally promote alterations in roughness, surface free energy, wettability, and

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chemical composition, which may lead to increased bacterial adhesion and biofilm formation (Al-Ahmad et al., 2013; Burgers et al., 2010; Rasperini, Maglione, Cocconcelli, & Simion, 1998; Teughels, Van Assche, Sliepen, & Quirynen, 2006). Among the surface properties that can interfere with bacterial adhesion, surface roughness has been shown to be the most relevant (Teughels et al., 2006). However, it is still debatable whether and to what extent roughness can affect biofilm formation (Teughels et al., 2006; Schmidlin et al., 2013).

Although implant surfaces are sterile, once they are introduced to the oral cavity, bacteria will adhere to the surrounding salivary pellicle (Elter et al., 2008). This initial bacterial adhesion can develop to a mature biofilm as favored by a proper environment, which can lead to shifts in the composition and virulence of microorganisms. The biofilm composition and virulence together with an immune-inflammatory response can cause *peri*-implantitis and *peri*-implant mucositis (Mombelli and Decaillet, 2011).

Despite trying to mimic the conditions of the oral cavity, *in vitro* experiments do not adequately represent the characteristics of this cavity considering the diversity of microorganisms, the presence of saliva and shearing forces, the host immune response and the characteristics of individual patients. It is known that initial bacterial adhesion is essential to determine the organization, diversity and strength of the biofilm (Busscher, Bos, & van der Mei, 1995; Kolenbrander, Andersen, Kazmerzak, & Wu, 1999; Marsh and Devine, 2011). Therefore, *in vivo* studies of adhesion and biofilm formation are needed to understand the interaction between bacteria and implant surfaces.

The development of implant surfaces that promote improved osseointegration without strengthening bacterial adhesion is important to the clinical success of implants. Among other surface treatments are the acid-etching and anodized and laser irradiated treatments, which have been used to modify the surface of implants in order to improve integration of implant to the bone. Therefore, the aim of the present study was to evaluate *in vivo* the initial biofilm formation on acid-etched and anodized and laser irradiated surfaces compared with machine-treated surfaces. Titanium surface morphology was determined using scanning electron microscopy (SEM) and roughness was evaluated by profilometer analysis. Additionally, the initial adherent microorganisms were visualized by SEM and quantified by an enzymatic assay (MTT assay) and Real Time PCR.

2. Material and methods

2.1. Titanium specimens and surface characterization

Three different types of titanium specimens in disks 2 mm in thickness and 10 mm in diameter were provided by Conexão Implant Systems (São Paulo, Brazil). Machined pure titanium disks (Ti-M) were used as controls, while acid-etched titanium (Ti-AE) and anodized and laser irradiated disks (Ti-AL) were used in the experimental groups. Acid-etched titanium is used for the Master Porous® implant system, and anodized and laser irradiated titanium is used for the Vulcano Actives® dental implant system.

The surface roughness of all specimens used in the *in vivo* experiments (n = 20) was determined with a Mitutoyo Surftest-211 Surface Roughness Tester Profilometer (Kawasaki, Kanagawa, Japan). Measurements were performed using a cut-off value of 0.5 mm (λc) and a speed of 0.1 mm/s. Three measurements were performed in the longitudinal direction and three in the transversal direction, and the scanning area was the limit of the disk diameter (Duarte, Reis, de Freitas, & Ota-Tsuzuki, 2009). The Roughness Average (Ra) parameter measures the average surface roughness analyzed by considering the peaks and valleys in the midline. The average roughness depth (Rz) parameter is defined as the difference between the five highest peaks and the five lowest peaks.

Scanning electron microscopy (SEM) was performed to visualize the titanium surfaces (Quanta 650 FEG ™, FEI Company, Japan). Three specimens for each group were fixed on metal stubs and imaged with a magnification of ×2.500, ×5.000 and ×10.000.

2.2. In vivo *bacterial adhesion assay*

After ethical approval by the Ethics Committee of the Tiradentes University (protocol # 250511 – Aracaju, Sergipe, Brazil), informed written consent was provided by all subjects. Ten healthy subjects were selected to participate in the study. Subjects had overall satisfactory health (absence of endocrine disorders; hormonal, hematologic, immune, or nutritional changes; or any diseases or drugs that alter salivary flow), salivary flow of 1,5 mL/min and excellent oral conditions (no carious lesions and periodontally healthy). Those individuals who had less than 4 mm probing depth and who did not present clinical attachment loss and gingival inflammation were considered to be periodontally healthy (Lopez, Smith, & Gutierrez, 2002). Individuals who had used antibiotics or antibacterial mouth rinses in the last six months prior to the study were not included.

For the *in vivo* bacterial adhesion assay, subjects wore an acrylic splint in the upper jaw for 24 h. A disk of each of the three titanium specimens (Ti-M, Ti-AE and Ti-AL) was fixed on each buccal side of the splint (right and left), in the region of the premolars and molars, to avoid biofilm disruption by tongue and cheeks (Fig. 1). Specimens were fixed with light-cured resin (Filtek P60, 3 M Espe, Saint Paul, MN, USA). Prior to use, the splints were disinfected by ultrasonication and immersion in a 1% sodium hypochlorite solution for ten mins. After that, the splints were washed three times in sterile distilled water for one min to remove any residual hypochlorite.

The splints were worn for 24 h and subjects were instructed to only remove the splints during meals and tooth brushing. During splint use, subjects were instructed to maintain their eating habits and oral hygiene routines (Grossner-Schreiber et al., 2001). After this period, the disks were carefully removed from the splints and gently washed 2 times in NaCl 0.9% (w/v) to remove non-adhered cells. Some of the disks (n = 3) were used for microscopic qualitative analysis by Scanning Electron Microscopy (SEM). The other disks were placed in polystyrene tubes containing saline solution and then vortexed for 1 min to detach the bacteria. This suspension was then used for bacterial quantification by the MTT assay and Real Time PCR (n = 17).

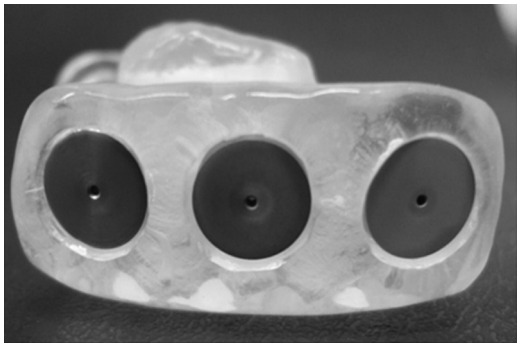

Fig. 1. Buccal view of the acrylic splint with the three titanium specimens set in niches.