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Bacterial interactions with biomaterials metallic surfaces

Interações bacterianas com biomateriais metálicos

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ABSTRACT

The loss of a metallic implant can be caused by the presence of sessile bacteria, biofilm formation and infection. The aim of this study was to evaluate bacterial adhesion on pure titanium used in dentistry. The experiment was conducted in a liquid culture medium, Mueller Hinton, and suspension of *Streptococcus mutans* contained in Falcon tubes to assess qualitative and quantitative bacterial adhesion to metallic biomaterial smooth and rough surface, which was obtained after treatment with Nd: YAG laser. The adhesion test used bacteriological culture procedures. *S. mutans* were grown for various periods of time in the presence of metal discs of pure titanium. The qualitative test was analyzed by scanning electron microscope (SEM) and quantitatively by calculating the count of viable cells after culture plates containing solid medium. The SEM results showed the presence of isolated and grouped cells consistent with the morphological structure of cocci adhered on both surfaces and the counting of viable cells was similar for smooth and rough surface, the 0.05 level averages showed no significant differences. Comparing the smooth and rough surfaces (modified by laser beam application of high intensity Nd: YAG) laser was no significant reduction in the number of bacteria adhered to the roughened surface, suggesting that physical modification creates a favorable surface for the adherence of *S. mutans*, which may be prerequisite for the development of biofilms on dental surgical implants and the pathogenesis of infection.

Keywords: *Streptococcus mutans*; Bacterial adhesion; Commercially pure titanium; Laser beam Nd:YAG; Biofilm.

RESUMO

A perda de um implante metálico pode ser causada pela presença de bactérias sésseis, formação do biofilme e infecção. O objetivo deste estudo foi o de avaliar a aderência bacteriana sobre titânio puro usado na Odontologia. O experimento foi realizado em meio de cultura líquido, Mueller Hinton e suspensão de *Streptococcus mutans* contido em tubos Falcon, para avaliar qualitativa e quantitativamente a aderência bacteriana ao biomaterial metálico de superfície lisa e rugosa, a qual foi obtida após tratamento com laser Nd:YAG. A prova de aderência usou procedimentos de cultura bacteriológica. *S. mutans* foram crescidos durante vários períodos de tempo em presença de discos metálicos de titânio puro. O teste qualitativo foi analisado por microscópio eletrônico de varredura (SEM) e o quantitativo por cálculo da contagem das células viáveis após cultura em placas contendo meio sólido. Os resultados de SEM mostraram células bacterianas (*S. mutans*) sésseis isoladas e agrupadas, compatíveis com a estrutura morfológica de cocos aderidos nas superfícies lisa e rugosa. A contagem das células viáveis foi semelhante para superfície lisa e rugosa, a nível 0,05 as médias não mostraram diferenças significantes. Comparando-se as superfícies lisa e rugosa (modificada por aplicação de feixe de laser de alta intensidade Nd:YAG) não foi observada redução significante no número de bactérias aderidas à superfície rugosa, o que sugere que a modificação física cria uma superfície favorável à aderência de S. mutans, a qual pode ser pré-requisito para o desenvolvimento de biofilmes em implantes dentários cirúrgicos e à patogênese da infecção.

Palavras-chave: *Streptococcus mutans*; Aderência bacteriana; Titânio comercialmente puro; Laser Nd:YAG; biofilme.

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INTRODUCTION

Nowadays metallic biomaterials are used to restore human body functions, such as dental restorations and implants[1]. The success of a dental implant depends on the bone integration with the surrounding tissue, and then the surfaces are prepared to encourage the integration and not the prevention of bacterial colonization[2,3]. Bacteria embedded in biofilm change its biological behavior and become less susceptible to agents, such as antibiotics, antiseptics and immune system, so it can be a clinical problem, due to systemic infections[4,5]. An accumulation of bacteria on the teeth, dentures or endosseous implants allow the formation of thick biofilms[6,7]. These solid surfaces can allow the oral streptococci colonization, such as *Streptococcus mutans* (*S. mutans*) and form biofilm, which can be associated with caries and implants loss[8]. The commercially pure titanium (cpTi) a metallic biomaterial is applied as oral implant due to its biocompatibility and osseointegration[9]. The biological performance of cpTi, in relation tissue integration, can be improved with a laser beam (Nd:YAG-*Neoymium – Yttrium Aluminum Garnet*)[10]. Sometimes, an implant failure is due to biofilm formation and not due to mechanical adjustments. The aim of this study was to evaluate bacterial adhesion on commercially pure titanium used in dentistry for provide knowledge to the implant field.

MATERIAL AND METHODS

Preparation of Metallic Coupons

The test discs used (12 mm in diameter, 0.2 mm in thickness) were commercially pure titanium, as seen in Table 1 and 2, according ASTM F67-88. The cpTi discs were provided by Group of Materials, Institute of Chemistry, Campus of Araraquara, UNESP. One surface was smoothed in a polishing machine (MAXIPLAN®) and water abrasive sandpaper (3M®) particle size 180, rinsed with distilled water and dried at room temperature. The machined titanium was used as control. And, another one, considered rough surface, received a laser treatment that was carried out by means a pulsed Nd:YAG laser (DigiLaser, DML-100)[11], as seen in optical images in Figures 1 and 2. Titanium discs were cleaned in ultrasonic bath with mild detergent solutions distilled-water, acetone, ethanol and ultra pure water and dried at room temperature. After laser application, the cpTi coupons were autoclaved at 121°C and then immersed in the bacterial suspension. The coupons surfaces were observed by scanning electron microscopy (JEOL-JSM, T330A-Japan), and the wettability of the surfaces was determined by means of goniometer OCA-20 Contact Angle System (DataPhysics Instruments) at room temperature. A profilometer (Mitutoyo SJ-301) was used to measure the surface roughness and results were expressed in μm as average roughness R_a (arithmetic mean of sampling area roughness)[11].

Table 1: Maximum composition of commercially pure titanium. ASTM F67-88.

Elements (% max)	G1	G2	G3	G4
N	0.03	0.03	0.05	0.05
C	0.10	0.10	0.10	0.10
H	0.015	0.015	0.015	0.015
Fe	0.20	0.30	0.30	0.50
O	0.18	0.25	0.35	0.40
Ti	balance	balance	balance	balance

Legend: G=grade

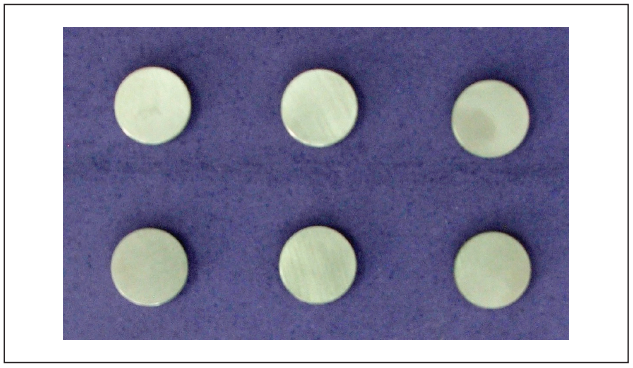

Figure 1: Image of cpTi polished discs (12 × 12 mm) before any treatment.

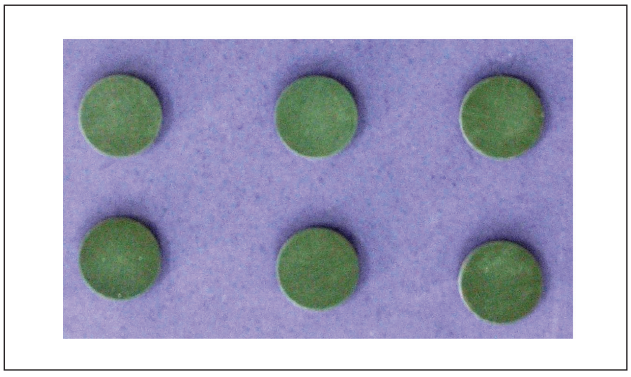

Figure 2: Image of cpTi discs after laser Nd:YAG treatment.

Bacterial Strain and Adherence Assay.

Streptococcus mutans (*S. mutans*) reference 25175 from Instituto Adolfo Lutz (São Paulo) was selected for the in vitro assays. The bacteria obtained from stock was transferred into

Table 2: Maximum permissible concentration of elements for commercially pure titanium according to ASTM F67-88 (% m / m).

Elements	C	Fe	O	H	N	Ti
CpTi	0.10	0.30	0.25	0.015	0.03	balance

tubes containing 7 mL of Thioglycollate medium U.S.P. (Oxoid, England) and incubated at 35-37ºC for 24 hours. One aliquot was used to inoculate 5 mL of BHI (Brain Heart Infusion, Oxoid, England) until to obtain a suspension of approximately 10^8 cells/mL of *Streptococcus mutans*, an optical density of 1.0 at 540nm. Thereafter, 200 µL of this cell suspension was transferred to conical tubes of polystyrene (Falcon), sterilized, containing 15 mL of Mueller-Hinton broth and the coupons. They were incubated at 37ºC in bacteriological incubator with constant shaking (100 rpm) for 1, 6, 24, 48 and 72 hours. Then, the coupons were washed with 5 mL of sterile 0.9% NaCl solution, and introduced, separately, in a sterile glass tube with 5 mL of sterile 0.9% NaCl solution and subjected to ultrasonic bath cleaner (Unique, Indaiatuba, Brazil) at frequency of 40kHz for 8 minutes in order to leaves bacteria in suspension. The coupons were removed from the 0.9% NaCl solution and examined by means of scanning electron microscopy (SEM), and the bacterial suspension was serially diluted in 0.9% NaCl solution and seeded according to bacteriological techniques.

Electron Microscopic Procedures

The samples discs of the cpTi were fixed with 2.5% glutaradehyde in 0.1M phosphate buffer (pH7.1) for 15 min, dehydrated with series of aqueous ethanol solutions (15, 30, 50, 95 and 100%) for 15 min each, and dried at 37ºC, coated with gold (1KV, 15mAp, S150 B Edwards) during 2 minutes and examined by using a JEOL-JSM (T330A) SEM.

Viability Assay

An aliquot (0.1 mL) of each dilution of the bacterial suspension was seeded on TSA (*Tryptic Soy Agar*). Plates were incubated at 37ºC in bacteriological incubator during 24 h. The bacterial growth on each plate was counted, calculated and the total colony forming units expressed in CFU / mL.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA). Student t-test was conducted to compare the samples. Statistical analysis used OriginPro software (version 5.0) at a confidence interval of 95%.

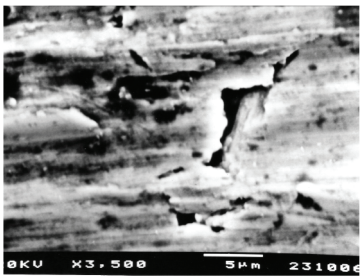

Figure 3: Topographical characteristic of cpTi surface before treatment with laser beam Nd:YAG and contact with *S. mutans* (x3,500) observed by SEM (JEOL-JSM T330A-Japan)

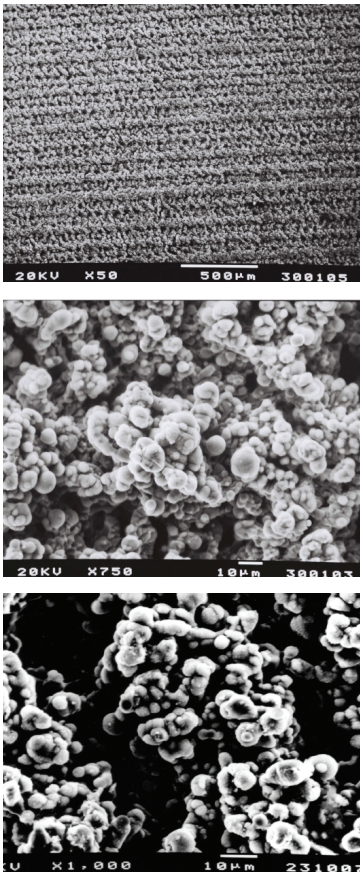

Figure 4: Topographical characteristic of cpTi surface after treatment with laser beam Nd:YAG before contact with *S. mutans*: A-x50; B-x750 and C-x1000, as observed by SEM (JEOL-JSM T330A-Japan).

RESULTS AND DISCUSSION

The topographical characteristics of smooth and rough cpTi surfaces examined by scanning electron microscope (SEM) before contact with *S. mutans*, is seen in Figures 3 and 4. After bacterial contact with cpTi discs during several periods of time (1, 6, 24, 48 and 72 hours) one can observe that *S. mutans* are adherent on both surfaces in all periods of time. This fact is shown as viable cells after have been retrieved from both surfaces (Table 3). Bacterial adhesion to surfaces and the formation of clusters is related with the ability of survival, persistence and virulence of the microorganisms within the biofilm[12]. Some strains of bacteria, such as *S. mutans* secrete extracellular polysaccharides (EPS) matrix in dental biofilms. The exopolysaccharides produced are insoluble and soluble glucans that allow the accumulation of *S. mutans* on hard surfaces[12]. The qualitative aspect of bacterial adhesion can be seen in Figure 5 (A, B), a representative example of micrographs demonstrating grouped coccus adherent onto smooth and rough surfaces of the cpTi following 48 hours bacterial exposure. One can note that the grooves formed on

cpTi discs, after the laser application, the adherent bacteria is protected from shear forces and the biofilm formed is thicker than that formed on smooth surface (Figure 5B). In this case, shear forces were occasioned by sonication bath, used to retrieve cells for viability analysis. As mentioned in the literature, it is well known that bacteria retained on grooves in rough surface can be protected of shear forces[13]. Quantitative analysis, in the present study, shown that there was no statistical difference between the bacterial adhesion over these surfaces. Surface roughness evaluation shown that Ra for smooth surface was 0.33 ± 0.06μm, and for rough surface was 1.38±0.23μm. Quirynen et al. reported that in a study *in vivo* the surface roughness below 0.2 μm Ra did not interfere with bacterial adhesion[14,15,16].

The wettability of smooth and rough surfaces evaluated by contact angle measurement showed that both are hydrophilic. The contact angle for smooth surface was 75.26º ± 0.70, and for rough surface was <7º. Furthermore, the smooth surface of cpTi is considered of medium wettability (θ = 75°), and the roughened surface of high wettability (θ <7°), because the water drop was spread over all canaliculi created by application of Nd:YAG laser. In the present research *S. mutans* has the ability to adhere to hydrophilic surfaces. Furthermore, the surface roughness increases the surface area available for bacterial adhesion. Wettability data shows that there is a statistical difference (P<0.01) between the smooth surface (>65°) and the roughened surface (<65°). Vogler et al (1998)[17] reported that on surfaces that water contact angle is greater than 65º is called hydrophobic and less than 65° is considered hydrophilic.

Table 3: Viable cells of *S. mutans* (Cfu/mL) retrieved from cpTi smooth and rough surfaces

Period of time (h)	Viable cells: cfu/mL from smooth surfaces	Viable cells: cfu/mL from rough surfaces
1	7.00×10^3	1.40×10^4
6	4.90×10^5	5.30×10^5
24	1.02×10^6	2.80×10^6
48	2.80×10^6	1.28×10^6
72	4.00×10^6	6.80×10^5

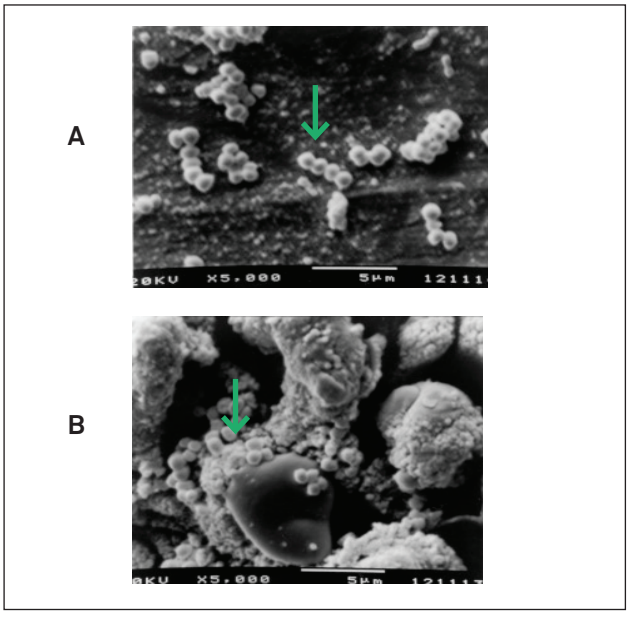

Figure 5: Scanning electron micrograph of cpTi surfaces with adhered *S. mutans* cells after 48 h exposure to Mueller Hinton Broth, as shown by arrow. A) cpTi smooth surface showing the attachment, accumulation and formation of *S. mutans* biofilm. B) cpTi rough surface showing *S. mutans* adhesion and biofilm formation. Original magnification:x5000, as observed by SEM (JEOL-JSM T330A-Japan).

The parameters that may explain the bacterial adhesion to solid surface include the physical and chemical interactions, such as hydrophobicity[18] and thermodynamics[19]. The surface characteristics of the solid substratum and the adhering bacteria can affect the adherence process[20]. Aykent et al.[21] observed a positive correlation between surface roughness and *S. mutans* adhesion. Several studies shown the oral bacterial adherence on rough surfaces of dental implant biomaterials[22,23]. The concern with the implant surface that comes in contact with the host tissue is with the osseointegration, and nevertheless there are no studies on bacterial adhesion and his effects on titanium surface modified by Nd:YAG laser process. In the present research *S. mutans* adhesion to the smooth and rough surfaces had a similar pattern. Viable cell counts of *S. mutans* retrieved from smooth surface showed that the average standard deviation was equal to $1.66 \pm 1.67 \times 10^6$ and from cpTi rough surface was equal to $1.06 \pm 1.07 \times 10^6$. One Way - ANOVA showed that the 0.05 level averages are not significantly different. The viable cells in biofilms of *S. mutans* are a significant factor in the pathogenesis of dental plaque[24]. Data from the present study showed that *S. mutans* adhesion and viable counts in the biofilm are related, according with Steinberg and Eyal[24]. Several studies have shown that *S. mutans* has been used in tests on bacterial adhesion because it is the main organism in the process of dental caries and, in dental biofilm formation, in which several bacterial species in the human oral cavity are involved[25,26,27]. The present investigation evaluate the adhesion of *S. mutans* on smooth and rough surfaces, after immersion in a liquid culture inoculated with *S. mutans* and a pH 7.0, under this condition both surfaces permitted the bacterial adhesion in a medium proper for bacterial growth. The bacterial cells do not need proteins from physiological fluids to bind to biomaterial surfaces. The explanation of the bacterial attachment in culture broth may be due to physicochemical interactions of the surface and extracellular polysaccharide production from bacteria[28]. The present research demonstrated *S. mutans* adhesion on both surfaces of cpTi.

CONCLUSIONS

The laser Nd:YAG was applied to modify the surface of the commercially pure titanium for improving the osseointegration in dental implants to preserve bone healing, by the other hand

the prevention of bacterial adhesion is also very important. The presence of bacteria on the implant surface may lead to infection, which can be a risk factor for the implant failure. This means that rough surfaces of metallic biomaterials can facilitate bacterial colonization and biofilm formation in the oral environment and become a niche of persistent infection.

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