UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

Í SIS DE FÁTIMA BALDERRAMA

Osteoblastic cell adhesion on implant surfaces contaminated by
Aggregatibacter actinomycetencomitans and treated by
photodynamic therapy and chemical decontamination

Adesão de células osteoblásticas em superfícies de implantes
contaminadas por *Aggregatibacter actinomycetencomitans* e
tratadas com terapia fotodinâmica e descontaminação química

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Orientador: Prof. Dr. Sebastião Luiz Aguiar Gregghi

Co-orientadora: Prof. Dr^a. Adriana Campos Passanezi Sant'Ana

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Ísis de Fátima Balderrama

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Dedicatória

DEDICATÓRIA

Dedico cada página dessa dissertação para o meu querido papito:

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(Isaías 41:10)

Abstract

ABSTRACT

Osteoblastic cell adhesion on implant surfaces contaminated by *Aggregatibacter actinomycetemcomitans* and treated by photodynamic therapy and chemical decontamination

The decontamination process of titanium implants surface is important for the successful treatment of peri-implantitis. The methods of decontamination can be classified in two major groups: chemical or physical. However, the best method of decontamination of implant surfaces is yet undertermined. The aim of this study is to analyze the effectiveness of decontamination of titanium implants surface by chemical conditioning agents and photodynamic therapy, by Scanning Electron Microscopy (SEM) and to analyze the adhesion and proliferation of osteoblastic cells on the previously decontaminated surfaces. Commercially available implants of different brands: *Biomet 3i*® (Nanotite – NT; Osseotite - OT), *Straumann*® (SLActive – SLA) and *Neodent*® (Acqua Drive CM – ACQ; Neoporos Drive CM – CM) were acquired in the market and analyzed in SEM images in 3 different areas (n= 1/group) to determine surface roughness parameters and wettability properties. After that, the surface of dental implants was inoculated with *Aggregatibacter actinomycetemcomitans* (A.a.) strains for 4 days and prepared for SEM analysis to determine the percentage area of contamination in a software for image analysis. Samples were then decontaminated by two different chemical treatments (citric acid 10% and ethylenediamine tetraacetic acid – EDTA - 24%) and photodynamic therapy (methylene blue associated with LASER), both with a 3-minutes application time. In the control group, surfaces were decontaminated with chlorhexidine 0.12% for 3 minutes. The area of decontamination was determined in ImageJ software for SEM images analysis. After decontamination, the adhesion and proliferation of human osteoblastic osteosarcoma cell lineage (Saos-2) on the surface of uncontaminated sterile implants (control; n: 1/period) and decontaminated implants (n: 3/period/group) were investigated. Saos-2 cells [5×10^4] were seeded on implant surfaces and incubated for 24h (adhesion assay) and 72h (proliferation assay), determined on SEM images. No significant differences were found among the different implants regarding roughness parameters, with exception Rv (SLA: 19.57 ± 4.01 vs. OT: 8.36 ± 7.91 ; $p=0.0031$). Chemical composition varied among implants depending on surface treatment, with all groups showing prevalence

of Titanium. Values showed greater contact angle (wettability analysis) for NT (hydrophobic) and smaller for ACQ (highly hydrophilic) ($p < 0.0001$). Nanotite/Biomet 3i® showed significantly greater percentage of area contaminated by bacteria ($68.19\% \pm 8.63\%$; $p = 0.050$; Kruskal Wallis/Dunn) than ACQ ($57.32\% \pm 5.38\%$). Osseotite/Biomet 3i® resulted in a smaller remaining contaminated area ($50.89\% \pm 9.12\%$) after decontamination treatments. Increased Saos-2 cells adhesion and proliferation were observed on SLA after 24h ($p = 0.0006$; ANOVA/Tukey) and 72h (< 0.001 ; ANOVA/Tukey). Decontaminated groups showed significantly less number of cells adhered to the surfaces at 24h and 72h than uncontaminated controls ($p < 0.005$; ANOVA post hoc Sidak). Regarding decontamination methods, no differences in the number of cells attached to implants treated by photodynamic therapy and chemical agents compared to chlorhexidine at 24h, but implants treated by photodynamic therapy and chemical agents showed greater number of cells attached after 72h. These findings suggest that surface characteristics influenced bacterial contamination and decontamination of implant surfaces; none of the decontamination methods were able to completely remove bacterial contamination, impairing cell adhesion and spreading. These findings may explain the varying clinical results of decontamination methods in re-osseointegration.

Key words: Titanium. Dental implants. Microscopy electron scanning. Decontamination. Osteoblasts

Resumo

RESUMO

Adesão de células osteoblásticas em superfícies de titânio contaminadas por *Aggregatibacter actinomycetemcomitans* e tratadas por terapia fotodinâmica e descontaminação química

O processo de descontaminação de implantes de titânio é importante para o sucesso do tratamento da peri-implantite. Os métodos de descontaminação podem ser classificados em dois maiores grupos: químico ou físico. Entretanto, o melhor método de descontaminação de superfícies de implantes está ainda indeterminado. O objetivo desse estudo é analisar a efetividade da descontaminação de implantes com superfície de titânio por agentes condicionantes químicos e terapia fotodinâmica, por Microscopia Eletrônica de Varredura (MEV) e analisar a adesão e proliferação de células osteoblásticas previamente com superfícies descontaminadas. Implantes disponíveis comercialmente de diferentes marcas: *Biomet 3i*® (Nanotite – NT; Osseotite - OT), *Straumann*® (SLActive – SLA) e *Neodent*® (Acqua Drive CM – ACQ; Neoporos Drive CM – CM) foram adquiridos no mercado e analisados em imagens de MEV em 3 diferentes áreas (n=1/grupo) para determinar o parâmetro de rugosidade da superfície e a propriedade de molhabilidade. Depois disso, a superfície dos implantes dentários foi inoculada com cepa de *Aggregatibacter actinomycetemcomitans* (A.a.) por 4 dias e preparada para análise da MEV afim de determinar a área de porcentagem da contaminação em um software de análise de imagem. Amostras foram então descontaminadas por dois diferentes tratamentos químicos (ácido cítrico 10% e ácido etilenodiamino tetra-acético-EDTA – 24%) e terapia fotodinâmica (azul de metileno associado com LASER), ambos em 3 minutos com tempo de aplicação. No grupo controle, os implantes foram descontaminados com clorexidina 0.12% por 3 minutos. A área de descontaminação foi determinada no software Image J para análise das imagens de MEV. Depois da descontaminação, a adesão e proliferação da linhagem de células de osteosarcoma osteoblástica humana (Saos-2) em superfícies não contaminadas de implantes estéreis (controle; n: 1/período) e implantes descontaminados (n: 3/período/grupo) foram investigados. Células da Saos-2 [5×10^4] foram cultivadas sobre as superfícies dos implantes e incubadas por 24 horas (ensaio de adesão) e 72 horas (ensaio de proliferação) e

determinadas em imagens de MEV. Não foram encontradas diferenças significantes entre os implantes em relação a parâmetros de rugosidade, com exceção para *Rv* (SLA: 19.57 ± 4.01 vs. OT: 8.36 ± 7.91 ; $p=0.0031$). Houve variação na composição química dos implantes de acordo com o tratamento de superfície, com todos os grupos mostrando prevalência do Titânio. Houve maior ângulo de contato (análise de molhabilidade) para NT (hidrofóbico) e menor para ACQ (altamente hidrofílico; $p<0.0001$). Nanotite/Biomet 3i® mostrou porcentagem significativamente maior de área contaminada por bactérias ($68.19\% \pm 8.63\%$; $p=0.050$; Kruskal Wallis/Dunn) que ACQ ($57.32\% \pm 5.38\%$). Osseotite/Biomet 3i® resultou em significativa menor área contaminada remanescente ($50.89\% \pm 9.12\%$) depois dos tratamentos de descontaminação. Foi observada maior adesão e proliferação de células Saos-2 em SLA após 24 ($p=0.0006$; ANOVA/Tukey) e 72 horas (<0.001 ; ANOVA/Tukey). Os grupos descontaminados mostraram número significativamente menor de células aderidas às superfícies nos dois períodos de tempo comparativamente aos controles não contaminados ($p < 0.005$; ANOVA post hoc Sidak). Em relação aos métodos de descontaminação, não foram observadas diferenças no número de células aderidas aos implantes tratados por terapia fotodinâmica e agentes químicos comparados com clorexidina em 24 horas, mas os primeiros mostraram maior número de células aderidas depois de 72 horas. Esses achados sugeriram que as características de superfície influenciaram a contaminação bacteriana e a descontaminação de superfície; nenhum método de descontaminação é capaz de remover completamente a contaminação bacteriana, dificultando a adesão e proliferação celular. Esses achados poderiam explicar a variação de resultados clínicos nos métodos de descontaminação e obtenção de re-osseointegração.

Palavras-chave: Titânio. Implantes dentários. Microscopia eletrônica de varredura. Descontaminação. Osteoblastos.

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1 Introduction

"Knowledge will bring you the opportunity
to make a difference."

Claire Fagin

1 INTRODUCTION

Osseointegration is defined as a direct contact between vital bone and implant surface at optical microscopy level (BRANEMARK et al., 1964; 1977) and describes a rigid fixation of an alloplastic material in bone, in an asymptomatic clinical situation and functional load (ALBREKTSSON, SENNERBY, 1990). Bone formation at the bone-implant interface is considered a complex physiological process that involves cell interaction, proliferation, differentiation and deposition of bone matrix (KADRA et al., 2005).

Five key successful factors for implant therapy were defined: design, material, surface properties, bone quality and surgical techniques (ALBREKTSSON, 1983). Among surface properties, one of the most important refers to surface topography, chemistry, superficial load and wettability (BUSER et al., 2004). Considering that, the topographical characteristics of implant surfaces are widely investigated (BUSER, 2001). Several changes on titanium implants surface have been proposed to achieve better clinical and histological results, but results are yet inconclusive (LANGE, 2002; WENNERBERG, ALBREKTSSON, 2009; ELIAS, MEIRELLES, 2010).

The topographic and physicochemical properties of the surface of implants are fundamental in the early stages of osseointegration (ELLINGSEN, 1998). The physicochemical properties of the oxide film and its long-term stability in biological environments are a key role for titanium implants biocompatibility (OLIVEIRA et al., 2007). Then, the surface properties of the dental implant and its physical and chemical characteristics are determined by the biocompatibility of the material, which is intimately related to cell behavior (ANSELME, 2000).

Peri-implant microbiota is influenced by the microbiota of remaining teeth and, in healthy sites, is similar to the composition of periodontally healthy sites (MOMBELLI et al., 1987). The microbial composition of peri-implant sulcus is similar to gingival sulcus of adjacent teeth, with high frequency of *P. gingivalis*, *T. forsythia*, *P. intermedia*, *P. micros* and *E. corrodens* (CANULLO et al., 2015).

As in natural teeth, the development of biofilm induces biological responses of hard and soft tissue around implants, resulting in biological complications

(CHARALAMPAKIS, BELIBASAKIS, 2015), such as pocket deepening, bleeding on probing, exudation, bone loss and clinical mobility, impairing the success of implant therapy (MOMBELLI et al., 1987; ALBREKTSSON, ISIDOR, 1994; TONETTI, 1998).

Peri-implant diseases are classified in mucositis and peri-implantitis (DERKS et al., 2015). Mucositis is defined as a reversible biofilm-related inflammation of soft peri-implant tissues without bone loss, while peri-implantitis is defined by the presence of biofilm-related irreversible inflammation characterized by deepening of peri-implant sulcus (pocket depth > 3mm), suppuration, implant mobility, bleeding on probing and progressive bone loss around implants (ALBREKTSSON, ISIDOR 1994; MOMBELLI, LANG, 1998; ZITZMANN, BERGHLUND, 2008; CHARALAMPAKIS, BELIBASAKIS, 2015).

The colonization, structure and power of bacterial biofilm on implant surfaces is influenced by implant surface roughness, chemical composition, hydrophobic properties, surface electrical charge and energy (AL-AHMAD et al., 2013; BADIHI-HAUSLICH et al., 2013). In vitro conditions differ from in vivo ones because the organisms in biofilm are characterized by cells that live in microbial communities embedded in the glycocalix (COSTERTON et al., 1999; FREIRE et al., 2011). Modifications in micro and nanotopography of dental implants were designed to increase bone-to-implant contact, but biofilm accumulation in these treated surfaces is accelerated when implant threads are exposed to oral cavity (TEUGHELS et al., 2006). Bacterial adhesion to Ti surfaces is roughness-dependent and the adhesion mechanism is influenced by ions and proteins of the initial coating derived from blood (BADIHI-HAUSLICH et al., 2013). Considering that, decontamination of texturized implant surfaces is a challenge in the treatment of peri-implant diseases related to biofilm.

Failing implants show a high prevalence of Gram-negative anaerobes, similar to periodontitis (MOMBELLI, LANG, 1998; BOTERO et al., 2005; HEITZ-MAYFIELD, LANG, 2010). Other microorganisms may be also found on implants surfaces compromised by peri-implantitis, such as *S. aureus* and *Candida albicans* (LEONHARDT et al., 1999; KRONSTRÖM et al., 2001; PERSSON, RENVERT, 2014). Recently, SIMION et al., (2016) demonstrated that a failing implant retrieved from a 40-year old woman, 4 years after its installation showed minimal plaque accumulation

and large areas free of plaque accumulation at smooth surface of titanium abutments, while the implant-abutment interface showed significant bacterial infiltration on the rough oxidized implant surface. Microorganism filling the porosity of the oxidized surface was composed of cocci, rods and filamentous bacteria. At the middle portion of the implant, large aggregates of subgingival biofilm composed mainly of rods and a filamentous bacterium was noticed.

The treatment of peri-implantitis is directed to the decontamination and/or detoxification of implant surfaces, providing adequate conditions to obtain re-osseointegration (LANG, HEITZ-MAYFIELD, 2004; CLAFFEY et al., 2008; LINDHE et al., 2008; SCHWARZ et al., 2011), which is very difficult to achieve, especially in contaminated surfaces (CARCUAC et al., 2016; WIEDMER et al., 2017). Remnants of bacterial biofilm impair re-osseointegration and results in peri-implantitis recurrence (KREISLER et al., 2005). The success of peri-implantitis treatment is therefore primarily related to implant surface (ALBOUY et al., 2011). Rough implants show higher rates of re-osseointegration when compared to machined ones (PERSSON et al., 2001). However, rougher implants tend to show biofilm accumulation which makes these areas more difficult to decontaminate (TEUGHELS et al., 2006).

Decontamination and/or detoxification of implant surfaces can be performed by mechanical and chemical methods, during non-surgical and surgical treatment. Mechanical biofilm removal can be done with manual instrumentation with metal or plastic curettes, titanium or soft brushes, ultrasound devices, air powder abrasives, laser or photodynamic therapy – PDT (MEFFERT et al., 1992; AUGTHUN et al., 1998; PEREIRA DA SILVA, 2005; SCHWARZ et al., 2006; SCHWARZ et al., 2011; SALMERON et al., 2013; MELLADO-VALERO et al., 2013; JOHN et al., 2015). Metal instruments, such as Gracey curettes and ultrasonic scalers, increase the surface roughness of implants (FOX et al., 1990; LOUROUPOULOU et al., 2012). Because of that, the use of plastic curettes and non-metal ultrasonic tips was proposed to reduce damage to implant surfaces during instrumentation, as well as to avoid contamination of implant surfaces with other metals (DO, KLOKKEVOLD 2015¹). However, nonmetal

¹ Do JH, Klokkevold PR. Supportive implant treatment. In: Newman MG, Takei H, Klokkevold PR, Carranza FA. Carranza's Clinical Periodontology. 12ed. Elsevier Saunders. Ch. 83, p. 805-812.

curettes do not allow effective plaque and calculus removal (RAMAGLIA et al., 2006) and some instruments cannot be sharpened.

Louropoulou et al., (2012) performed a systematic review about the effect of different instrumentals on implant surface characteristics (smooth and rough). The majority of selected studies have performed through SEM assessment; and burs were the choice instrumental to regularization on rough surface implants; plastic instrumentals do not damage the surface characteristics, therefore metal instrumentals caused greater damage on smooth surface.

Mechanical instrumentation of tinted implants mounted in resin casts simulating a peri-implantitis defect with Gracey curettes, ultrasound and air powder abrasive showed remnants of ink at all groups, greater for curettes than for ultrasound and air powder abrasive, which showed the best cleaned surfaces after treatment (SAHRMANN et al., 2015). The removal of biofilm from experimentally induced peri-implantitis around implants installed in dogs' mandible by the use of rotating titanium brushes associated with sodium hypochlorite and chlorhexidine (TBH) or chlorhexidine only (TB) and the use of an ultrasonic device associated with chlorhexidine (US) resulted in statistically improvements of all clinical parameters, without significant differences between groups in the formation of woven bone. These findings suggested that mechanical removal of biofilm from contaminated surfaces was effective to promote re-osseointegration, independent from the use of sodium hypochlorite (CARRAL et al., 2016).

Chemical decontamination of implant surfaces was proposed to promote a cleaner surface, allowing cell adhesion and re-osseointegration. Different substances were proposed to promote surface decontamination, including the use of acid (citric acid, phosphoric acid), chelating (EDTA) or antibiotic solutions (tetracycline) (MEFFERT et al., 1992; SCULEAN et al., 2004; ROCCUZZO et al., 2011; SUBRAMANI, WISMEIJER, 2012; PARMA-BENFENATI et al., 2015; HENTENAAR et al., 2017).

Citric acid has been used as chemical agent for implant decontamination, showing the greatest potential for biofilm removal of contaminated implants in vitro (NTROUKA et al., 2011) and favoring cell adhesion (ALHAG et al., 2008; UNGVARI et al., 2010). Lubin et al., (2014) inoculated *P. gingivalis* in 88 titanium implant disks,

which were decontaminated with EDTA, citric acid and tetracycline. After that, implant disks were placed in osteoblastic cell cultures. Citric acid and tetracycline were more effective for disinfection of implants discs.

Dostie et al., (2017) have investigated the effects of disinfecting implant surfaces inoculated with multispecies oral biofilms, cultured anaerobically for 21 days. Disks were rinsed with 0.9% NaCl, exposed for 2 minutes with tetracycline paste, 1% chlorhexidine gel (CHX), 35% phosphoric acid gel (Etch) or a chemical formula containing 0.3% cetrimide, 0.1% CHX and 0.5% EDTA, followed by a new rinsing with 0.9% NaCl. The majority of biofilm was removed with NaCl first rinsing; however, persistence of bacteria was observed in all specimens. None of the solutions was significantly more effective for implant surface contamination than double-rinsing with NaCl. CHX and EDTA resulted in significant reduction in the number of viable bacteria, although small, suggesting antibacterial effects.

By the other side, three implants with severe bone loss in a male patient were treated by mechanical debridement, chemical decontamination and implantoplasty. Implants were removed after 4 months and the growth and biofilm formation were measured by spectrophotometry and scanning electron microscopy after 48hs of incubation. Results showed an average of *S. mutans* planktonic growth over the implants of 0.21 nm (mechanical debridement), 0.16nm (mechanical decontamination), and 0.15nm (implantoplasty) (GEREMIAS et al., 2017).

Peri-implantitis lesions were treated with resective surgical treatment aimed at peri-implant granulation tissue removal, bone recontouring and pocket elimination in 28 patients (53 implants). Decontamination was performed with mechanical biofilm removal and 35% phosphoric acid conditioning or sterile saline. A significant reduction in total bacterial counts on implant surface was found for both groups, with greater reduction for the phosphoric acid. At 3 months after surgery, 75% of the implants in the control group and 63.3% of the implants in the test group showed disease resolution, with no significant differences in clinical and microbiological outcomes between groups (HENTENAAR et al., 2017).

Experimentally-induced peri-implantitis around implants positioned in the mandibles of beagle dogs were treated by Er: YAG laser, PDT, titanium burs alone and titanium burs associated with citric acid. Greater improvement of vertical bone height

was observed with the use of titanium burs associated with citric acid, which also showed better bone-to-implant contact than PDT and bur-alone group (HTET et al., 2016).

Diode laser with different wavelengths (810 or 980nm), with or without photosensitization, were effective in the decontamination of machined sterile implants placed into sterile porcine bone blocks with standardized coronal angular bony defects and inoculated with *S. sanguis* and without dangerous increase of temperature (VALENTE et al., 2016). Besides the bactericidal effect, the application of diode laser irradiation both continuous and pulse modes resulted in inhibition of LPS-induced macrophage activation and consequent blunting of the inflammatory response (GIANNELLI et al., 2016). Strever et al., (2017) inoculated sandblasted, acid-etched, large grit (SLA), calcium-phosphate coated (CaP), anodized and machined titanium disks with a single strain of *Porphyromonas gingivalis*. Disks were treated by Er, Cr: YSGG laser. The amount of bacterial contamination/removal was investigated by confocal microscopy and SEM. The results showed bacterial contamination at all specimens, which required more laser power for removal at SLA and CaP. Bacterial decontamination was effective for all specimens, without physical damage to implants surface. CO₂ laser associated or not with hydrogen peroxide was shown to be effective in the decontamination of implant surfaces (MOUHYI, 2000).

To investigate if the decontamination of titanium implants with chemical agents would alter titanium physicochemistry and consequently compromise cellular response, KOTSAKIS et al., (2016) promoted the contamination of grit-blasted acid-etched titanium disks with biofilms grown from in vivo peri-implant plaque samples. Disks were decontaminated by burnishing with 0.12% chlorhexidine, 20% citric acid, 24% EDTA/1.5% NaCl or sterile saline. Chemical agents resulted in reduction in bacterial counts, confirming the antimicrobial properties of chemical agents. X-ray photoelectron spectroscopy detected elemental contaminants associated with chemical agents that significantly altered wettability compared with sterile implants, used as controls. Increased cell counts were found in the saline-treated group compared with chlorhexidine. No association between antimicrobial effect and cell counts was found.

Considering the varying results observed in current literature, the protocol to decontaminate titanium surfaces are yet to be determined (GOSAU et al., 2010). A recently published systematic review (SUAREZ-LOPEZ DEL AMO et al., 2016) on the non-surgical treatment of peri-implant mucositis and peri-implantitis reported different methods of decontamination, including self-performed cleaning techniques, laser, photodynamic therapy, supra and submucosal debridement and use of air-abrasive devices. Mechanical treatment of mucositis was effective, but modest and non-predictable outcomes were expected for peri-implantitis lesions. Variable results were found due to different peri-implant diseases definitions, treatment approaches, different implant designs and surfaces and defect characteristics. Similar findings were described in a systematic review including 25 animal studies, which failed to identify a predictable method of accomplishing complete resolution of the peri-implant defect and re-osseointegration of previously contaminated surfaces (RENVERT et al., 2009). Valderrama and Wilson Jr., (2013) concluded that the complete elimination of the biofilms is difficult to achieve. All therapies were found to induce changes in the chemical and physical properties of the implants surface, with partial re-osseointegration after detoxification being reported in animals. Considering that, further investigations are necessary to evaluate the validity and reliability of the techniques at longer periods of follow-up.

The aim of this study is to investigate if commercially available implants with different treatment surfaces are more prone to bacterial accumulation; to evaluate the effectivity of implant surfaces decontamination by physical and chemical methods; to investigate if decontamination methods modify implant surfaces; and to investigate osteoblastic cell adhesion after decontamination. Implant surface properties are influenced not only by microtopography or nanotopography, but also by macrotopography. Most in vitro studies investigating the effects of decontamination methods of implant surfaces are performed in titanium disks (GOSAU et al., 2010; UNGUVARI et al., 2010; BÜRGERS et al., 2012; SALMERON et al., 2013; AL-AHMAD et al., 2013; BADIHI-HAUSLICH et al., 2013; LUBIN et al., 2014; SÁNCHEZ et al., 2014; JOHN et al., 2015; WHEELIS et al., 2016; KOTSAKIS et al., 2016; GIANNELLI et al., 2017; STREVER et al., 2017), that does not reproduce the macro and microtopography characteristics of implants. For these reasons, in this study, we have

used implants of different treatment surfaces acquired in the market to investigate both bacterial contamination and decontamination methods, influencing cell adhesion.

2 Article I

"You see things; and you say 'Why'?
But I dream things that never were;
and I say 'Why not?'"

George Bernard Shaw

2 ARTICLE I

The article I presented in this Dissertation was written according to the instructions and guidelines for article submission (to be submitted).

Title: Bacterial adhesion to implant surfaces negatively affects osteoblastic cells adhesion and spreading after mechanical and chemical decontamination procedures

Running title: *Biofilm impairs cell adhesion on implant surfaces*

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ABSTRACT

Purpose: To investigate the influence of implant surface treatment in biofilm formation and in osteoblastic cells adhesion and spreading after decontamination procedures. **Materials and Methods:** Commercially implants of different brands and surface characteristics were acquired in the market: *Biomet 3i*® Nanotite (NT) and Osseotite (OT), *Straumann*® SLActive (SLA) and *Neodent*® Acqua Drive (ACQ) and Neoporos Drive CM (CM). Physical and chemical properties of the implants were investigated by scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDS) and wettability (WETT). Implants were inoculated with *Aggregatibacter actinomycetemcomitans* strains for 4 days and prepared for SEM analysis to determine the percentage of contaminated area. After that, samples were decontaminated by chemical methods. The percentage of remaining contaminated area was determined in SEM images by ImageJ software. The number of human osteoblastic osteosarcoma cells (Saos-2) adhered on the surface of uncontaminated (control; n: 1/period) and decontaminated implants (n: 3/period/group) were investigated after 24h and 72h in SEM images. **Results:** No significant differences were found in roughness parameters among implants, except for *Rv*. ACQ was found to be highly hydrophilic and NT was the most hydrophobic implant. ACQ showed significantly smaller contaminated area than NT, while OT showed a greater decontaminated area (50.89 ± 9.12) after chemical and physical treatments. Increased variation of Saos-2 cells adhesion and proliferation were observed on CM implant surface ($p < 0.05$; paired t test). All decontaminated groups showed significantly less number of cells adhered to the surfaces at 24h and 72h than the respective uncontaminated control ($p < 0.0001$; ANOVA post hoc Sidak). **Conclusions:** These findings suggest that decontamination methods were not able to completely remove bacterial biofilm probably influenced by surface characteristics, impairing cell adhesion and spreading. **Key words:** dental implants; decontamination; osteoblasts; implant surface; roughness

3 Article II

"We talk on principal, but act on motivation."
Walter Savage Landor

3 ARTICLE II

The article II presented in this Dissertation was written according to the instructions and guidelines for article submission (to be submitted).

Title: Effectiveness of implant surface decontamination and osteoblast growth on moderate rough implants

Running title: *Moderate rough implants impairs methods of decontamination*

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ABSTRACT

Aim: To analyze the effectiveness of decontamination of moderate rough implants and to determine osteoblast cell adhesion and proliferation on the decontaminated surfaces. **Material and methods:** Five types of implants were acquired in the market: Biomet 3i® Nanotite and Osseotite; Straumann® SLActive; Neodent® Acqua Drive CM and Neoporos Drive CM. Implant surfaces were analyzed in scanning electron microscopy (SEM) images to determine surface roughness parameters (n= 1/group). Implants (n= 5/implant type) were then inoculated with *Aggregatibacter actinomycetencomitans*. The contaminated area was determined in SEM images. Decontamination was performed by photodynamic therapy (PDT), citric acid and EDTA (PDT+AC/EDTA; n= 15) or 0.12% chlorhexidine (control; n= 15). Uncontaminated, sterile implants were used as negative controls. The remaining contaminated area was determined in SEM images before incubation with osteoblastic cells (SAOS-2) to determine adhesion (24h; n= 15) and proliferation (72 h; n= 30). **Results:** No significant differences were found among the different implants regarding mean surface roughness (Ra). Both decontamination methods resulted in the presence of a remaining contaminated area, with no differences between groups. Uncontaminated implants showed higher number of cells at 24 (p< 0.0001) and 72h (p< 0.0001). No differences in the number of cells attached to PDT+AC/EDTA and CHX were found at 24h (p= 0.37). At 72h, a significantly higher number of cells were attached to PDT+AC/EDTA vs. CHX (p= 0.006). **Conclusions:** These findings suggest that the treatment by PDT+AC/EDTA is significantly more efficient for decontamination of implant surfaces than rinsing of implant surfaces with 0.12% CHX.

Key words: Saos-2 cells; peri-implantitis; titanium surface; treatment of surface; SEM.

4 Discussion

"Science is always wrong.
It never solves a problem without creating ten more."

George Bernard Shaw

4 DISCUSSION

The objectives of this research were to investigate if different surface treatment of titanium implants influence bacterial adhesion; if decontamination procedures are effective in the removal of bacterial contamination; and if osteoblastic cells adhesion and proliferation would be impaired after surface decontamination. Our findings have shown that all implants investigated were minimally to moderately rough, without significant differences in *Ra* value, according to the methodology used in this study.

Roughness parameters were determined at SEM images by the plugin SurfCharJ (ImageJ, NIH, Bethesda, USA). This software was initially developed to the analysis of supercalendered papers in which surface representations are horizontally aligned by subtracting a regression plane from surface (CHINGA et al., 2007), and it was found to be more suitable than other mathematical software for quantitative analysis of surface roughness of titanium alloy structures, providing information on global and local roughness analysis, gradient analysis, domain segmentation, surface leveling and directional analysis (SAFDAR, 2010). The disadvantage of this analysis is that it does not measure roughness directly from implant surface, and that the results obtained cannot be compared to other reports in literature, considering that reference values are yet unknown. Considering that, the assumption that we have investigated only minimally to moderate rough implants is based on literature reports (ROSA et al., 2013; SARTORETTO et al., 2015) that have investigated the implants used in our study. Besides that, no difference was found among groups in *Ra* values, which indicates that roughness characteristics were homogeneous within the samples investigated.

The colonization, structure and power of bacterial biofilm on implant surfaces is dependent on roughness characteristics, chemical composition, wettability, surface electrical charge and energy (AL-AHMAD et al., 2013; BADHIHI HAUSLICH et al., 2013). Rougher implants are more prone to biofilm accumulation than smoother ones (PERSSON et al., 2001; AL-AHMAD et al., 2013), especially when implant threads are exposed to the oral cavity (TEUGHELS et al., 2006; BADHIHI HAUSLICH et al., 2013). Considering that, surface roughness and structure are considered as local factors for the development of peri-implantitis (RENVERT, QUIRYNEN, 2015).

Bacterial adhesion to Ti surfaces is roughness-dependent, although not influencing plaque composition. Greater roughness provides larger surfaces and additional niches to bacterial adhesion, reduces shearing forces and desorption of bacteria during early phases of adhesion (AL-AHMAD et al., 2013).

De novo formation of bacteria on titanium implants surface is influenced by free energy, roughness and chemical characteristics of dental implants. In a systematic review of the literature, TEUGHELS et al., (2006) concluded that both an increase in surface roughness above the Ra threshold of 0.2 mm and/or the surface-free energy (which is directly related to wettability) facilitates biofilm formation on restorative materials. Biofilm formation is also influenced by the chemical composition of the biomaterial, and comparisons in biofilm formation on different transmucosal implant surfaces are scarce. According to their findings, complete colonization occurs faster in rough than in smooth surfaces (within 3 days). Besides that, high wettability grooves and pits 'protect' bacteria against shear forces. Rough surfaces are more difficult to clean, leading to a rapid biofilm re-growth, which is the main cause of development or progression of peri-implantitis (KREISLER et al., 2005).

We have evaluated roughness parameters, chemical surface characteristics, bacterial adhesion and decontamination properties on the implant surface itself, which is relevant for clinical use (CONSERVA et al., 2013). Most studies investigate bacterial adhesion and decontamination procedures in titanium disks, which does not reproduce the macro- and micro- or nano-structure of dental implants (GOSAU et al., 2010; UNGVARI et al., 2010; BÜRGERS et al., 2012; SALMERON et al., 2013; AL-AHMAD et al., 2013; HAUSLICH et al., 2013; LUBIN et al., 2014; SÁNCHEZ et al., 2014; JOHN et al., 2015; WHEELIS et al., 2016; KOTSAKIS et al., 2016; GIANNELLI et al., 2017; STREVER et al., 2017).

Chemical composition of surface layer varied between implants, probably influenced by the surface preparation methods and impurities (SMITH et al., 1992). Two implants showed only Ti (NT and CM); OT showed the presence of Ti, P, Ca, O, Al and V; SLA showed the presence of T, Na, Cl, Ca, Br and Zr; ACQ showed the presence of Ti, Na, Cl and Al. Surface chemical composition results in different reactions from the surrounding media (ALBREKTSSON, WENNERBERG, 2004), influencing cell and bacterial behavior (ANSELME et al., 2010).

Although the surface topographic characteristics are chemically similar to the bulk, the surface itself may induce differences in surface chemistry or energy, such as an increase in hydrophobicity (ANSELME et al., 2010). For small surface energies, increases in surface roughness impairs cell adhesion; in moderate or intermediate surface energy, increases in surface roughness has a minor effect on cell adhesion; in large surface energy, optimal roughness maximizes cell adhesion (DECUZZI, FERRARI, 2010). Considering that, surface topography and physical-chemical properties of biomaterials influence both cell and bacterial adhesion (ANSELME et al., 2010).

Hydrophilic surfaces enhance the early stages of cell adhesion, proliferation, differentiation and bone mineralization (ERIKSSON et al., 2004; BORNSTEIN et al., 2008), promoting earlier osseointegration (LANG et al., 2011). Surface energy of the implant is related to wettability and can be indirectly measured by the liquid-solid contact angle – CA (sessile-drop contact angle) (GITTENS et al., 2014), as performed in this study.

Three implants were hydrophilic (ACQ, CM and SLA) and the remaining two were hydrophobic (NT and OT), considering as hydrophilic those implants in which the contact angle observed by the sessile-drop contact angle analysis is $< 90^\circ$. ACQ can be considered as superhydrophilic, since CA is close to 0° .

The lesser percentage of contaminated area (CtA) was found at ACQ, which was the most hydrophilic implant and the greatest contamination area was observed at NT, which was the most hydrophobic implant of our sample. Since all implants presented similar roughness characteristics, differences in the contamination between implants could be related to hydrophilic or hydrophobic properties (GITTENS et al., 2014). Considering that, it can be assumed that hydrophilic surfaces tend to accumulate lesser amount of dental plaque than hydrophobic surfaces.

In the present study, the highest contamination by *A. actinomycetencomitans* was observed in NT and OT. These results are opposite to other report in literature (RODRIGUEZ Y BAENA et al., 2012), who showed a significantly less contaminated area in OT and even lesser on NT than on machined implants.

By the other side, the remaining contaminated area (rCtA) was greater at CM and SLA (which were hydrophilic), while NT, OT and ACQ showed the lesser amount of rCtA, suggesting that wettability would influence not only biofilm formation, as well as its removal. Similar results were described by LUBIN et al., (2014), who showed that OT disks were easier to decontaminate than NT. Taking that into account, it can be suggested that different solutions may have different outcomes depending on surface characteristics.

Surface topography and physical-chemical properties of biomaterials may be modified by mechanical and/or chemical treatments performed to decontaminate implant surfaces (RENVERT et al., 2011). Since that, it is important to determine the effects of decontamination procedures on the adhesion and proliferation of osteoblastic cells. Despite of decontamination procedures, all implants showed a remaining contaminated area that may have affected cell adhesion and proliferation, since uncontaminated sterile implants showed significantly higher number of cells at 24h and 72h.

These findings are in agreement with other studies describing partial decontamination of implant surfaces after the application of different agents (SCHWARZ et al., 2006; BÜRGERS et al., 2012; LUBIN et al., 2014; DOSTIE et al., 2017). Bürgers et al., (2012) showed that decontamination of titanium disks previously contaminated with monocultures of *S. epidermidis*, *S. sanguinis* and *C. albicans* were partially effective, except for sodium hypochlorite, which is toxic to patients and cannot be used in clinical practice. Re-growth of *P. gingivalis* on Osseotite and Nanotite disks after disinfection with laser, EDTA, citric acid and tetracycline showed with differences between OT and NT (LUBIN et al., 2014). These findings may indicate that implant surfaces should be decontaminated by more adequate chemical agents, since the treatment of peri-implantitis seems to be directly influenced by surface characteristics (ALBOUY et al., 2011).

The presence of threads may have difficult cleaning effects and reduce the ability of removing bacterial contamination (AL-HASHEDI et al., 2017). To the best of our knowledge, no study investigated the adhesion and proliferation of osteoblastic cells directly on implant surface after contamination with bacteria. One study has used implants provided by the manufacturer and contaminated with saliva to investigate the

effects of aPDT and laser on decontamination of surfaces (MAROTTI et al., 2013), with better effects obtained by PDT compared to the use of laser without dye or the topical application of chlorhexidine 0.12%. Osteoblasts were cultivated on OT and NT disks intentionally inoculated with bacteria after the treatment with EDTA, tetracycline, citric acid or Nd: YAG. The results showed that citric acid and tetracycline were the most effective solutions for decontamination of implant surfaces (LUBIN et al., 2014).

Since osteoblasts were able to attach and proliferate on decontaminated surfaces with remnants of bacterial contamination, it may be considered a bactericidal effect of PDT+AC/EDTA, which showed a significantly higher proliferation rate than CHX. Studies investigating chemotherapeutic agents to disinfect single- or multi-species contaminated disks showed a decreased proportion in viable bacteria after treatment, indicating its antimicrobial or bactericidal effect (GOSAU et al., 2010; KOTSAKIS et al., 2016; WIEDMER et al., 2017; DOSTIE et al., 2017).

The growth of osteoblasts on implant surfaces is influenced by disinfection methods altering implant surface characteristics and by the toxicity of the substances (OLIVEIRA et al., 2014; LUBIN et al., 2014; UNGVÁRI et al., 2010; KOTSAKIS et al., 2016). Kotsakis et al., (2016) have demonstrated that chemotherapeutics residuals were detected by X-ray photoelectron spectroscopy and significantly altered wettability compared to controls, resulting in cytotoxic effects that affected osteoblastic response.

Although in 24h no differences in the number of cells attached were found between PDT+AC/EDTA and CHX, in the first group a significant increase in the number of attached cells was seen at 72h, indicating that CHX may compromise the biocompatibility of titanium surfaces, impairing cell adhesion and proliferation (KOTSAKIS et al., 2016). Indeed, the choice of the chemotherapeutic agent to be used to disinfect implant surfaces should be directed towards the preservation of titanium properties as well. CHX presents substantivity and is slowly released from implant surfaces, similar to natural tooth (KOSLOVSKY et al., 2007; RYU et al., 2015), impairing cell proliferation (KOSLOVSKY et al., 2007). Our findings are similar to other in literature (KOSLOVSKY et al., 2007; RYU et al., 2015; KOTSAKIS et al., 2016), although a consensus on the best protocol to decontaminate implant surfaces is yet to be established (GOSAU et al., 2010).

Antimicrobial photodynamic therapy (aPDT) protocol is based on photochemical properties of exogenous non-toxic compounds (phenothiazinic dyes) which are absorbed by bacteria and subsequently activated by laser (WAINWRIGHT, 2005), being a potential therapeutic approach to decontamination of implant surfaces during the treatment of peri-implant diseases (DORTBUDAK, 2001; CHAN et al., 2003). Giannelli et al., (2016, 2017) investigated the *in vitro* efficacy of PDT with methylene blue photoactivated with 635 nm diode laser and 405 nm violet-blue LED phototreatment in the reduction of *S. aureus* single-species bacterial biofilm and LPS from *E. coli* adherent to implant surfaces, before seeding with macrophages to quantify LPS-driven inflammatory cell activation. Both treatments resulted in significant reduction of viable bacteria and inhibited LPS bioactivity. The viability, attachment, proliferation and differentiation of Saos-2 osteoblasts on new and treated disks were also investigated by Giannelli et al., (2017) after the treatment with ultrasonic scalers or Er: YAG laser. Both resulted in reduction of contaminated area, but ultrasound produced marked surface changes and incomplete removal of titanium dioxide layer and remnants of plaque accumulation. The treatment with Er: YAG did not produce significant alterations in implant surface and was compatible with osteoblastic cell growth, adhesion and viability.

The effects of aPDT for decontamination of implant disks was previously investigated by our research group (SALMERON et al., 2013), in animal models. Smooth or rough implant disks contaminated with *in situ* dental plaque were treated by aPDT, toluidine blue (TBO) or low-intensity laser (LIL) and posteriorly inserted subcutaneously in rats. The inflammatory tissue reaction was significantly greater at rougher than at smoother implants. aPDT resulted in significantly smaller inflammatory reaction after 7 days, suggesting its role on decontamination of implant surfaces.

A single-specie *A. actinomycetencomitans* biofilm was developed based on the findings of Freire et al., (2011). After contaminating titanium cover screws with *A. actinomycetencomitans* strain, samples were inoculated in animals and triggered an inflammatory lesion characterized by bleeding, ulceration, soft tissue necrosis, hyperplasia and implant loss, indicating its potential to develop a pathogenic biofilm on titanium surfaces. *A. actinomycetencomitans* is able to contaminate titanium-abutment interface (CANULLO et al., 2015, 2016). A recent study showed that *A. actinomycetencomitans* are consistently associated with both periodontitis and peri-

implantitis, while *P. gingivalis* and *F. nucleatum* are consistently associated with periodontitis only (ZHUANG et al., 2016; WINKELHOFF, WOLF, 2000; PERSSON, RENVERT, 2014). Besides that, some studies perform in situ contamination of titanium disks (SALMERON et al., 2013; JOHN et al., 2015), or collect subgingival plaque that is grown anaerobically to later contaminate titanium surfaces (KOTSAKIS et al., 2016; DOSTIE et al., 2017). We have used 10mm-long implants, which makes in situ contamination not possible, as well as anaerobic growth of subgingival plaque collected in situ. Other studies have also used single-species biofilms to investigate the efficacy of disinfection methods, but none with *A. actinomycetencomitans* (BÜRGERS et al., 2012; LUBIN et al., 2014; WIEDMER et al., 2017; GIANNELLI et al., 2017; STREVER et al., 2017).

The effects of different surface and decontamination treatments were investigated by the incubation of implants with Saos-2 cells, from an osteoblastic cell lineage. An *in vitro* study has investigated the adhesion of Saos-2 cells on micro-rough titanium disks (surface sandblasted with aluminum oxide beads) mechanically treated by plastic curettes, air-abrasive device, titanium brush and implantoplasty. These procedures resulted in surface alterations that affected cell viability, phenotype and morphology (TOMA et al., 2016).

Screw-shaped titanium dental implants with different surface treatments affected the adhesion and proliferation of Saos-2 cells *in vitro*. Grit-blasted and acid-etched surface of Straumann implant showed the highest Ra value and the lowest rate of proliferation and ALP activity. Sandblasted surfaces of Ankylos and turned surfaces of MKIII (NobelBiocare) induced differentiation of Saos-2 cells (CONSERVA et al., 2013). Oxidized titanium disks (TiUnite, Nobel Biocare) were treated by diode laser GaAlaAs and chlorhexidine digluconate and incubated with Saos-2 cells. Laser treatment did not affect cell proliferation, but CHX induced a reduction in cell viability, alteration in morphology and reduced ability of cell adhesion to treated surfaces (CHELLINI et al., 2017), as observed in our study.

A limitation of this study is that treatments were performed as a group, and it is not possible to determine to which of the substances the decontamination effects are related with. Considering that, further studies are necessary to better investigate the

effects of different chemical agents and protocols in the decontamination of titanium implants surface.

5 Conclusions

**"Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less."**

Marie Curie

5 CONCLUSIONS

Based on the results obtained and within the limitations of this research, it was concluded that:

- All implants investigated are minimally to moderately rough, without significant differences in *Ra* values;
- There are differences in the chemical composition of surface layer of the implants, depending on surface treatment;
- One implant (Acqua, Neodent®) is highly hydrophilic; two implants (SLA, Straumann® and Neoporos Drive CM, Neodent®) are hydrophilic; and two implants (Nanotite and Osseotite, 3i®) are hydrophobic;
- Adhesion of *Aggregatibacter Actinomycetemcomitans* is significantly reduced in highly hydrophilic implants (commercial brand: ACQUA, Neodent®) *in vitro*, independent from macrogeometry or roughness characteristics;
- Mechanical and chemical decontamination methods are only partially effective for surface decontamination, considering that all samples showed residual contaminated area after decontamination procedures;
- Residual bacterial contamination impairs adhesion and spreading of osteoblastic cells;
- Antimicrobial photodynamic therapy and chemical conditioning (citric acid 10% and EDTA 24%) are significantly more efficient for implant surfaces decontamination than chlorhexidine 0.12%.

Further studies are necessary to determine the best method for decontamination of implants with different surface treatment, chemistry, surface energy and wettability.

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APPENDIX

Appendix I: **DECLARATION OF THE USE OF THE ARTICLE IN DISSERTATION****DECLARATION OF THE USE OF THE ARTICLE IN DISSERTATION**

We hereby declare that we aware that the articles "Bacterial adhesion to implant surfaces negatively affects osteoblastic cells adhesion and spreading after mechanical and chemical decontamination procedures" and "Effectiveness of implant surface decontamination and osteoblast growth on moderate rough implants" will be included in the Dissertation of the student Ísis de Fátima Balderrama were not used and may not be used in other works of Graduate Programs at the Bauru Dental School, University of São Paulo.

Bauru, January 18th of 2018.

Ísis de Fátima Balderrama

Signature

Sebastião Luiz Aguiar Greghi

Signature

Adriana Campos Passanezi Sant'Ana

Signature