Universidade Estadual de Campinas
Faculdade de Odontologia de Piracicaba
Área de Prótese Dental

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Características de superfícies, adsorção de proteínas e aderência bacteriana em diferentes materiais odontológicos

Tese de Doutorado apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas para obtenção do Título de Doutor em Clínica Odontológica – Área de Prótese Dental

Piracicaba
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Orientadora: Profª. Drª. Altair A. Del Bel Cury
Co-orientador: Prof. Dr. Pedro Luiz Rosalen
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Este exemplar foi devidamente corrigido, de acordo com a resolução CCPG 036/83.
CPG, 06 / 06 / 2006

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Assinatura do Orientador

Piracicaba

2006

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DA FACULDADE DE ODONTOLOGIA DE PIRACICABA
Bibliotecário: Marilene Girello – CRB-8ª. / 6159

L628c

Lima, Emilena Maria Castor Xisto.
Características de superfícies, adsorção de proteínas e aderência bacteriana em diferentes materiais odontológicos. / Emilena Maria Castor Xisto Lima. – Piracicaba, SP : [s.n.], 2006.

Orientadores: Altair Antoninha Del Bel Cury, Pedro Luiz Rosalen, Hyun Koo.
Tese (Doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Implantes dentários. 2. Zircônio. I. Del Bel Cury, Altair Antoninha. II. Rosalen, Pedro Luiz. III. Koo, Hyun. IV. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. V. Título.

(mg/fop)

Título em inglês: Surface characteristics, proteins adsorption and bacterial adherence on different dental materials
Palavras-chave em inglês (Keywords): 1. Dental implants. 2. Zirconium
Área de concentração: Prótese Dental
Titulação: Doutor em Clínica Odontológica
Banca examinadora: Cínthia Pereira Machado Tabchoury, Dalva Cruz Laganá, Eduardo Passos Rocha, Pedro Luiz Rosalen, Vinicius Carvalho Porto
Data da defesa: 19/05/2006

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A Comissão Julgadora dos trabalhos de Defesa de Tese de DOUTORADO, em sessão pública realizada em 19 de Maio de 2006, considerou a candidata EMILENA MARIA CASTOR XISTO LIMA aprovada.

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PROFa. DRa. DALVA CRUZ LAGANÁ

PROFa. DRa. CINTHIA PEREIRA MACHADO TABCHOURY

Dedico este trabalho:

A Deus, pela minha existência e por guiar sempre meus passos nessa caminhada.

A minha Rosa, pelo seu amor de mãe imensurável, sempre me apoiando e dando força em todos os momentos.

Ao meu pai, pelo incentivo dedicado a minha formação.

Ao meu irmão Carlinhos, por fazer parte da minha historia.

Saudades dos que se foram .... tenho certeza que estão sempre olhando por mim.

Agradeço aos meus avós, ao meu padrinho Castor e ao meu querido irmão Rossini, um exemplo de ser humano, durante sua trajetória, com você eu aprendi a valorizar ainda mais as coisas simples e preciosas da vida. Obrigada!

Oh! Senhor
Eu ainda me lembro
Daquele loirinho
branquinho, gordinho,
Na praia a brincar,
Toquinha multicolorida
Na cabecinha pelada,
Sol a respaldar.

Anjinho de Deus
Inocência sem par
Quanta ternura
Me punha a pensar
Quanto futuro Terá!

No entanto, tempo atroz!
Roubou em momento vil e veloz
O presente vive,
Como se passado fosse,
Sempre a relembrar;

Em raros momentos
Aquele instante vem,
Sem felicidade...
Só o quadro tenho na mente
Tentando criar
A alegria daquela criança
Na beira do mar (Castor Xisto).

Agradecimentos especiais

À minha orientadora, *Profª. Drª. Altair A. Del Bel Cury,* pelo seu exemplo e dedicação ao trabalho, pelas oportunidades concedidas e por auxiliar no meu desenvolvimento profissional e pessoal.

Aos meus co-orientadores, *Prof. Dr. Hyun Koo e Prof. Dr. Pedro Luiz Rosalen,* pela dedicação e atenção dispensadas durante a realização deste trabalho.

Agradecimentos

A Universidade Estadual de Campinas na pessoa de seu Magnífico Reitor, *Prof. Dr. José Tadeu Jorge.*

A Faculdade de Odontologia de Piracicaba – UNICAMP, representada por seu Diretor *Prof. Dr. Thales da Rocha Mattos Filho.*

Ao CNPq, Conselho Nacional de Desenvolvimento Científico e Tecnológico, pela concessão da bolsa durante o doutorado.

A CAPES, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, pela concessão da bolsa PDEE para realização do estagio no exterior.

Ao Coordenador de Pós-Graduação da Faculdade de Odontologia de Piracicaba, *Prof. Dr. Pedro Luiz Rosalen.*

Ao Coordenador do Programa de Pós-graduação em Clínica Odontológica, *Prof. Dr. Roger William Moreira.*

Aos professores *Dr. Jaime A. Cury* e *Dr^a. Cínthia P. M. Tabchoury* por permitirem o uso do laboratório de Bioquímica Oral e pelos conhecimentos transmitidos.

A *Prof^a. Dr^a. Renata Cunha Matheus Rodrigues Garcia,* pela atenção e ensinamentos concedidos durante o curso

À *Dona Joselena Casati Lodi*, técnica do laboratório de Prótese Parcial Removível, por todo carinho e atenção dispensados aos alunos.

Aos meus amigos especiais em Piracicaba *Adriana, Andréa, Ângela, Blanca Liliana, Belkys, Bruno, Daniela, Edwin, Fernanda, Juliana, Joane, Karina, Leonardo, Manoel, Margarete, Noéli, Patrícia, Priscila, Ricardo, Rosena, Samira, Sílvia, Tatiana, Viviane* e *Wander*, por me proporcionarem momentos felizes e pela amizade que levarei sempre em meu coração.

A todos os colegas do curso de Pós-Graduação em Clínica Odontológica e do Curso de Pós-graduação em Odontologia, pela agradável convivência.

Aos meus amigos Bonnie, Daliana, Daniela, Érica, Julia, Juliana, Michel, Regiane, Rodrigo, Simone, Tarso, Tarsinho e Tom, pelo apoio e companhia durante o período de estágio em Rochester, tornando a minha estadia bem agradável.

Ao Prof. Dr. William Bowen, Kathy Scott-Anne, Anne-Vacca Smith, Stacy e Jennifer pela agradável convivência e conhecimentos compartilhados.

Ao corpo docente do Programa de Pós-Graduação em Clínica Odontológica da Faculdade de Odontologia de Piracicaba – UNICAMP pelos conhecimentos compartilhados durante o curso, fundamentais à minha formação.

Às funcionárias da Secretaria de Pós-Graduação *Érica A. Pinho Sinhoreti e Raquel Q. M. César Sacchi,* da Secretaria de Pós-Graduação em Clínica Odontológica, *Mônica Aparecida Barnabé* e do Departamento de Prótese e Periodontia, *Shirley Rosana Sbravatti Moreto* e *Juliana de Souza*.

A técnica do Laboratório de Farmacologia, *Eliane Franco,* pela dedicação e auxílio durante o desenvolvimento desse trabalho.

Ao técnico do Laboratório de Bioquímica Oral, *José Alfredo da Silva*, pela presteza.

À funcionária do Comitê de Ética em Pesquisa desta instituição, *Leni Cecília Rosilho*.

À bibliotecária *Marilene Girello*, pelo auxílio durante a formatação desta tese.

SUMÁRIO

Resumo: O uso de implantes osseointegrados para substituição de dentes perdidos é uma alternativa importante na odontologia atual. Uma das condições relacionada ao sucesso nos procedimentos de implante é fornecida pelas propriedades de superfície dos implantes e componentes protéticos que influenciam não só na biocompatibilidade, mas também na adesão bacteriana e colonização. A qualidade de superfície pode ser determinada pela combinação das propriedades física, química, mecânica e de estrutura de superfície. Dentre elas, a energia livre de superfície e rugosidade superficial tem efeito significante no processo de adesão. Considerando que cada biomaterial pode apresentar propriedades físicas/químicas e energias de superfícies diferentes, a adsorção dos componentes salivares/soro sanguíneo presentes na película adquirida é provavelmente específica aquela superfície. Esses componentes mediam a aderência bacteriana inicial nas superfícies dos dentes e materiais restauradores, sendo a aderência reconhecida como primeiro passo para formação do biofilme. O acúmulo de biofilme pode levar ao desenvolvimento de lesões inflamatórias na mucosa adjacente e reabsorção óssea, aumentando o risco de falhas dos implantes. Assim, os propósitos destes trabalhos foram: I) avaliar as características de superfície de abutments (Ti-6Al-4V e Tilite) e II) caracterizar o perfil de adsorção dos componentes salivares/ soro sanguíneo e a aderência bacteriana de *Streptococcus mutans* e *Actinomyces naeslundii* sobre titânio (Ti) e cerâmica zirconia (Zi) usados na confecção de abutments de implantes. Como resultados das pesquisas observamos que as ligas de Ti-6Al-4V e Tilite apresentaram diferenças nas características de superfícies, exceto para dureza de superfície e que o titânio e a cerâmica zircônia apresentaram propriedades biológicas similares em termos de adsorção de proteínas e aderência bacteriana. Entretanto, esses materiais apresentaram-se diferentes quanto à adesão bacteriana quando comparados com a hidroxiapatita, principalmente para *A. naeslundii*.

Abstract: The use of osseointegrated implants for replacing missing teeth is an important alternative in current dentistry. One of the most important conditions, which relates to the future success of implant procedures, is provided by the surface properties of the implant and its prosthetic abutments. Since these components penetrate through the gingival mucosa and are also exposed to the oral cavity, they play an important role not only for the biocompatibility but also for the bacterial adhesion and stagnation. Surface quality of a biomaterial can be measured by a combination of physical, chemical and mechanical properties and its surface structure. Surface free energy and surface roughness have a significant impact in the adhesion process. Since each biomaterial has different physical/chemical properties and surfaces energies, adsorption of salivary/serum components to each surface is likely to be specific to that surface and they mediate the initial bacterial adherence to the tooth surface and restorative materials. The bacterial adherence is recognized as the first step of the biofilm formation, which accumulation may lead to inflammatory lesions in the adjacent mucosa and bone resorption, increasing the risk of implant failure. Thus, the purposes of these studies were: I) evaluate surface characteristics of implant abutments (Ti-6Al-4V and Tilite) and II) characterize the profile of salivary/serum components adsorption, and *Streptococcus mutans* and *Actinomyces naeslundii* adherence on titanium (Ti) and ceramic zirconia (Zi) used for manufacturing implant abutments. The results revealed that the Ti-6Al-4V and Tilite alloys showed differences on the surface characteristics, except to surface hardness and titanium and zirconia ceramic displayed similar biological properties in terms of protein adsorption and bacterial adherence. However, the abutment materials showed subtle, but significant differences on the bacterial binding pattern when compared to HA (surrogate tooth enamel), especially for *A. naeslundii.*

1. INTRODUÇÃO GERAL

O uso de implantes osseointegrados para substituição de dentes perdidos é uma alternativa importante na odontologia atual. O sucesso clínico e biológico demonstrado em estudos prospectivos tem levado a uma grande aceitação e utilização de implantes dentais na reabilitação protética de pacientes edêntulos totais e parciais, podendo devolver-lhes satisfatoriamente função e estética (Branemark *et al.*, 1985; Albrektsson *et al.*, 1988; Sanz *et al.*, 1990).

Uma das condições mais importantes relacionadas ao sucesso nos procedimentos de implante é fornecida pelas propriedades de superfície dos implantes e componentes protéticos (Kasemo, 1983). A qualidade de superfície pode ser determinada pela combinação das propriedades física, química, mecânica e de estrutura de superfície (Wenneberg *et al.*, 1993). As características de superfície dos componentes protéticos influenciam não só na biocompatibilidade, mas também na adesão bacteriana e colonização (Sawase *et al.*, 2000).

Os implantes orais e seus componentes protéticos apresentam comunicação com a cavidade oral através da mucosa ou tecidos gengivais e, portanto, fornecem uma superfície dura que pode interagir com bactérias nativas do hospedeiro. A formação da película adquirida e da placa bacteriana é um processo sítio-específico que depende tanto das propriedades de superfícies como da cobertura dos materiais (Steinberg, *et. al.*, 1995). Parâmetros como energia e rugosidade de superfície tem efeito significante no processo de adesão. A rugosidade interfere na formação e maturação do biofilme, potencializando a área disponível para a adesão bacteriana em até três vezes. (Quirynen & Bollen, 1995). Outra característica importante é a dureza de superfície, que é um indicador de resistência ao riscamento relacionados aos procedimentos de higiene oral profissional e habitual (Quirynen *et al.*, 1994). Além disso, as proteínas salivares e do soro sanguíneo na película adquirida formada sobre as superfícies dentais e materiais restauradores são componentes críticos que mediam a aderência bacteriana inicial sobre essas superfícies duras.

O desenvolvimento da placa bacteriana nas superfícies dentais e dos materiais restauradores inicia com a formação da película adquirida, que consiste primariamente de glicoproteínas, mucina e enzimas presentes na saliva. A adesão da

bactéria oral na superfície do dente envolve um processo altamente seletivo de interações específicas entre os receptores na película salivar aderida ao dente e a superfície bacteriana (Marsh, 2003).

A aderência bacteriana é reconhecida como o primeiro passo para formação do biofilme, cujo acúmulo pode levar ao desenvolvimento de lesões inflamatórias na mucosa adjacente e reabsorção óssea, aumentando o risco de falhas dos implantes. Desta forma, é importante o desenvolvimento de abutments de implantes cuja superfície restrinja a aderência bacteriana inicial, minimizando a formação do biofilme e subsequente inflamação nos tecidos moles, mas que ao mesmo tempo permita a adesão epitelial (Sawase *et al.*, 2000; Groβner-Scheireber *et al.*, 2001).

Os diferentes materiais utilizados para a confecção de implantes e componentes protéticos promovem aderência seletiva durante formação da placa bacteriana inicial (Rasperini *et al.*, 1998). Considerando que cada biomaterial pode apresentar propriedades físicas/químicas e energias de superfícies diferentes, a adsorção dos componentes salivares/soro sanguíneo é provavelmente específica aquela superfície. O titânio apresenta propriedades de superfície únicas comparadas com outros metais e materiais restauradores usados na cavidade oral, podendo ser colonizado por microrganismos que diferem tanto qualitativamente como quantitativamente. Recentemente, o Tilite (Ni-Cr-Ti) foi introduzido e proposto como uma liga alternativa aos materiais atualmente utilizados na confecção de abutments. Também muita atenção tem sido dada a outros materiais como a cerâmica à base de zircônia comumente utilizada em ortopedia que combina biocompatibilidade, estética satisfatória e resistência a fraturas. Entretanto, não há informações disponíveis sobre como as bactérias colonizam as superfícies destes materiais, apesar de tais interações poderem influenciar no sucesso de seu uso (Piconi *et al.*, 1999; Rimondini *et al.*, 2002).

Tendo em vista as propriedades de superfície dos materiais e a natureza seletiva da adsorção de proteínas salivares, poderá haver modificações na capacidade de aderência de colonizadores primários da placa dental bem como na colonização das bactérias subseqüentes sobre diferentes tipos de abutments ou materiais restauradores. Assim, os propósitos destes trabalhos foram: I) avaliar as características de superfície de

abutments (Ti-6Al-4V e Tilite) e II) caracterizar o perfil de adsorção dos componentes salivares/ soro sanguíneo e a aderência bacteriana de *Streptococcus mutans* e *Actinomyces naeslundii* sobre titânio e cerâmica usados na confecção de abutments de implantes.

Este trabalho foi realizado no formato alternativo, conforme deliberação numero 001/98 da Comissão Central de Pós-Graduação (CCPG) da Universidade Estadual de Campinas (UNICAMP). O artigo apresentado no capítulo 1 foi submetido ao periódico Brazilian Oral Research (Anexo 2) e o artigo no capítulo 2 será submetido ao periódico Clinical Oral Implants Research .

2. CAPÍTULO 1

Prosthodontics

Title: Evaluation of surface characteristics of Ti-6Al-4V and Tilite alloys

Título: Avaliação das características de superfícies das ligas de Ti-6Al-4V e Tilite

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ABSTRACT: The aim of this study was to evaluate surface free energy (SFE), surface roughness (SR) and surface hardness (SH) of two materials commercially available to fabricate dental implant abutments. In addition, the specimens were investigated by scanning electron microscopy (SEM) to determine the surface morphology. Twenty five discs (5 x 2mm) of Ti-6Al-4V and Tilite (Ni-Cr-Ti) alloys were used in this study. The surface free energy was determined by the contact angle formed between a drop of distilled deionized water and the surface of each material. The surface roughness was measured using a mechanical profilometer and the surface hardness was evaluated by means of Vickers hardness micro indentation test. Data from SFE, SR and SH were analyzed using one-way ANOVA followed by Student's t test ($p<0.05$). Statistical differences ($p<0.05$) for SFE and SR were found between Ti-6Al-4V (36.2 $erg.cm^{-2}$; 0.2 μm) and Tilite (30.9 $erg.cm^{-2}$; 0.16 μm). The surface hardness values of Ti-6Al-4V (325.0 Kg/mm^2) and Tilite (324.3 Kg/mm^2) were not statistically significant ($p>0.05$). Evaluations by SEM revealed different surface morphology. In conclusion, Ti-6Al-4V and Tilite alloys showed differences on the surface characteristics, except to surface hardness, suggesting that both alloys are suitable for manufacturing implant abutments. However, further studies are necessary to elucidate the biological responses to implant abutments of these materials.

DESCRIPTORS: surfaces properties; abutments; implants; titanium; tilite alloy.

RESUMO: o objetivo deste estudo foi avaliar a energia livre de superfície (ELS) rugosidade superficial (RS) e dureza de superfície (DS) de dois materiais disponíveis comercialmente para fabricação de abutments de implante. Além disso, os espécimes foram investigados por microscopia eletrônica de varredura (MEV) para determinar a morfologia de superfície. Vinte e cinco discos de Ti-6Al-4V e Tilite (Ni-Cr-Ti) (5 x 2mm) foram usados nesse estudo. A energia de superfície foi determinada pela mensuração do ângulo de contato formado entre a gota de água destilada e deionizada e o espécime de cada material. A rugosidade superficial foi medida como uso do rugosímetro e a dureza de superfície foi avaliada por meio de teste de endentação de microdureza Vickers. Os dados foram analisados usando ANOVA seguido pelo test T de Student ($p<0,05$). Foram encontradas diferenças estatisticamente significantes ($p<0,05$) para os valores de ELS e RS entre Ti-6Al-4V (36,2 $erg.cm^{-2}$; 0,2 μm) e Tilite (30,9 $erg.cm^{-2}$; 0,16 μm). Os valores de dureza do Ti-6Al-4V (325,0 Kg/mm^2) e Tilite (324,3 Kg/mm^2) não apresentaram diferenças estatisticamente significantes ($P>0,05$). A MEV revelou morfologia de superfícies diferentes. Concluiu-se que as ligas de Ti-6Al-4V e Tilite mostraram diferenças nas características de superfícies, exceto para dureza de superfície, sugerindo que para os aspectos estudados ambas são adequadas para a confecção de abutments. Entretanto, estudos são necessários para evidenciar as respostas biológicas aos abutments de implantes confeccionados com essas ligas.

DESCRITORES: Propriedades de superfícies; abutments; implantes; titânio; liga tilite.

INTRODUCTION

One of the most important conditions, which relates to the future success of implant procedures, is provided by the surface properties of the implant and its prosthetic abutments[8]. Since these components penetrate through the gingival mucosa and are also exposed to the oral cavity, they play an important role not only for the biocompatibility but also for the bacterial adhesion and stagnation[18].

Surface quality of a biomaterial can be measured by a combination of physical, chemical and mechanical properties and its surface structure[22]. The adhesion of microorganisms to solid intra oral substrata involves interactions between their surface components, being influenced by their hydrophobicity and surface free energy (SFE) values[13]. SFE have a significant impact in the adhesion process since high SFE substrata attracts more microorganisms than low energetic materials and the bacterial adhesion seemed weak on surfaces with a low SFE[13].

As for surface roughness (SR), some studies have reported a strong positive relationship between SR and the rate of supragingival bacteria[14,15,17]. Moreover, the SR interferes on biofilm formation and maturation, and increases the area available for adhesion by a factor 2 to 3[12,15,17], which may lead to severe problems with mucositis and peri-implantitis[22]. Another important surface characteristic of implant components is surface hardness (SH), which may indicate the resistance against roughening during professional or habitual oral hygiene procedures[16].

A large number of implant systems are available, and most of them are manufactured of titanium, due to its low toxicity and high biocompatibility[8]. In addition to titanium, Tilite alloy (Ni-Cr-Ti) has been recently introduced as an alternative to current available materials. Although there are none available data comparing surface characteristics of Tilite to Ti-6Al-4V, it has been stated that Tilite provides satisfactory marginal adaptation when used as superstructure for one-piece implant-supported prosthesis[6].

Since there is a positive correlation between the surface characteristics of prosthetic implant components and plaque colonization, the aim of this study was to compare the

surface characteristics and microstructures of two commercially available materials, Ti-6Al-4V and Tilite alloy (Ni-Cr-Ti), used to fabricate implant abutments.

MATERIALS AND METHODS

Twenty five discs measuring 5 mm in diameter and 2 mm in thickness of Ti-6Al-4V (Sandinox, São Paulo, Brazil) and Tilite (composition: 76% Ni, 13.5% Cr, 6% Mo, 4%Ti) (Talladium Inc., Valencia, CA, USA) were used in this study. First, the SFE (erg cm^{-2}) was calculated with the deposition of 15 μL of distilled water on each specimen. It was made in triplicate. The image of each sessile drop was captured using a digital camera (Mavika CD 350, Sony, Tokyo, Japan) immediately after its deposition and the mean value of contact angles were measured using Autocad 2005 (Auto Desk, Sankt Augustin, USA)[11]. Hence, surface free energy was calculated using cos Θ of contact angles, according to Minagi *et al.*[11] (1985).

Following this, the SR was determined using a mechanical profilometer with a radius tip of 2 μm, under a measuring force of 0.7mN and sensitive of 0.01 μm (Surfcorder SE 1700 – Kosaka, Tokyo, Japan) calibrated at sample length of 0.25 mm, spread of 2.0 mm of measure and speed of $0.5mms^{-1}$. Six readings were performed on each specimen and an average (R_a) was determined. These profilometric traces were taken from the edge of specimen, in the middle and at the bottom part.

The SH was evaluated by means of Vickers hardness micro indentation test (Shimadzu model HMV 2000, Kyoto, Japan). Specimens were ground with 320, 400 and 600, 1000 and 1200-grit silicon carbide papers (Carbimet, Buehler, Lake Bluff, IL) in a polishing machine Arotec APL-4 (Arotec, São Paulo, Brazil) under refrigeration followed by polishing cloths and 3 μm diamond suspension (Metadi diamond suspension, Buehler, Lake Bluff, IL). The Vickers hardness of each sample was calculated by means of three indentations with a distance of 150 μm between each indentation, with a load of 50 g for 10 s.

In addition, the surface morphology of each material was examined by scanning electron microscopy (SEM) with LEO 435 VP (Carl Zeiss SMT, Oberkochen, Germany). The beam acceleration voltage was 15kV and at 500× magnification.

Statistical Analysis

All the data were analyzed using SPSS version 13.0 (SPSS Inc. Chicago, Illinois) software, being first checked for normality, using Kolmogorov-Smirnov test and for variance homogeneity, using Levene test. After that, the data were analyzed using one-way ANOVA followed by Student's t test set at $P= .05$.

RESULTS

The SFE, SR and SH mean values for each material are presented in table 1. The SFE and SR of Ti-6Al-4V were significantly higher ($p<0.05$) than to Tilite. There were no statistical differences for surface hardness ($p>0.05$) between the materials.

Table 1 - Mean values and standard deviations (n=25) of Surface Free Energy (erg.cm^{-2}), Surface Roughness (μm) and Microhardness (Vickers Kg/mm^2) of Ti-6Al-4V and Tilite alloys.

Materials	Surface Free Energy	Surface Roughness	Microhardness
Ti-6Al-4V	36.2 (3.9) [a]	0.20 (0.05) [a]	325.0 (10.1) [a]
Tilite	30.9 (3.5) [b]	0.16 (0.03) [b]	324.3 (14.9) [a]

Means values followed by distinct letters differ statistically ($p<0.05$).

The SEM micrographs (Figures 1 A-B and 2 A-B) revealed that the surface morphology of the materials are clearly distinct. The Ti-6Al-4V showed well-defined unidirectional microstructure with irregular scratches, small pits and turning marks (Figures 1A-B) whereas the Tilite (Figures 2 A-B) showed a well-defined surface texture with an equally smooth surface and only minor irregularities and light scratches.

DISCUSSION

The surface free energy of a solid surface gives a direct measure of intermolecular interactions at interfaces and has a strong influence on wetting, adsorption and adhesion behavior[24]. SFE and wettability of various materials can be determined by measuring the contact angle formed by a range of liquids on a given surface, using several diverse approaches[5,19]. In the present study, Ti-6Al-4V had higher SFE compared to Tilite, meaning that the former has a greater potential for initial bacteria colonization[13]. However, only distilled deionized water was used as the liquid with known surface tension (72.8 erg.cm^2) in contact angle measurements, which could be a limitation for further discussions, since it does not allow measurement of polar and non-polar (or dispersion) components of surface energy[5].

Surface roughness has a major impact on the microbial colonization[16]. Preferential retention occurs on rough surfaces since bacteria on such surfaces are more protected against shear forces and oral hygiene measures, thus giving the entrapped microbial cells time to become irreversibly attached to a surface[12]. Moreover, Breck *et al.*[2] (1983) stated that the proliferation of the initially adhering microorganisms accounts for the major part of the microbial mass increase during early biofilm formation which may explain the role of surface roughness in initial biofilm formation[1]. SR values found in this study were 0.16 μm for the Tilite alloy and 0.2 μm for Ti-6Al-4V (table 1). These results are in accordance with the range found on commercially available implant components[18,22,23]. Some *in vivo* studies suggested a threshold surface roughness for bacterial retention (0.2 μm) below which no further reduction in bacterial accumulation could be expected[1]. Thus, every dental material needs its own treatment modality in order to obtain and maintain a surface as smooth as possible.

SEM revealed different surface morphologies to the materials (Figures 1 A-B and 2 A-B). Based on the surface areas investigated, the density of the local defects (merely deviation from a uniform surface structure) appeared to be higher on Ti-6Al-4V surface compared to the Tilite surface, but no quantitative analysis was performed.

According to information given by manufacturers, the Ti-6Al-4V is subjected to a chemical etching process whereas the Tilite doesn't receive any chemical treatment. Carlsson *et al.*[3] (1988) and Carr *et al.*[4] (1997) stated that the use of the acid etch, such as the hydrochloride, sulfuric and nitric acid, produces micro cavities on the surface that varied according to the type, concentration and temperature of the acid, which may produce an unsuitable rough surface and affect the resistance of the material[21].

Surface hardness may explain the risk for surface roughening of the abutments of several implants systems during professional cleaning or even during habitual oral hygiene procedures[1,7]. For SH, there were no statistically differences between Ti-6Al-4V and Tilite alloys (table 1). The SH values found in this study are in accordance with Quirynen *et al.*[16] (1994) that observed Vickers hardness values for abutments varying from 154 for Branemark to 340 for Steri-OSS.

Considering professional cleansing, special care should be taken when ultrasonic scaling, metal instruments[20] and air powder abrasive systems are used, because these instruments lead to an increasing surface roughness. According to McCollum *et al.*[9] (1992) only methods that do not damage the abutment surface or enhance biofilm accumulation should be used for maintenance and prophylaxis. Meschenmoser *et al.*[10] (1996) stated that the only instrument recommended for professional hygiene is the plastic curet. Although there are no studies about the influence of professional hygiene methods on surface roughness and structural modifications of implant abutments made of Tilite, due to similarities on the surface hardness values, it probably presents the same behavior as Ti-6Al-4V.

CONCLUSION

Within the limits of this study, it can be concluded that Ti-6Al-4V and Tilite alloys showed differences on the surface characteristics, except to surface hardness. However, further studies are necessary to elucidate the biological responses to implant abutments of these materials.

ACKNOWLEDGEMENTS

The authors thank to Neodent (Neodent, Curitiba, PR, Brazil) for providing the Ti-6Al-4V and Tilite discs used in this study.

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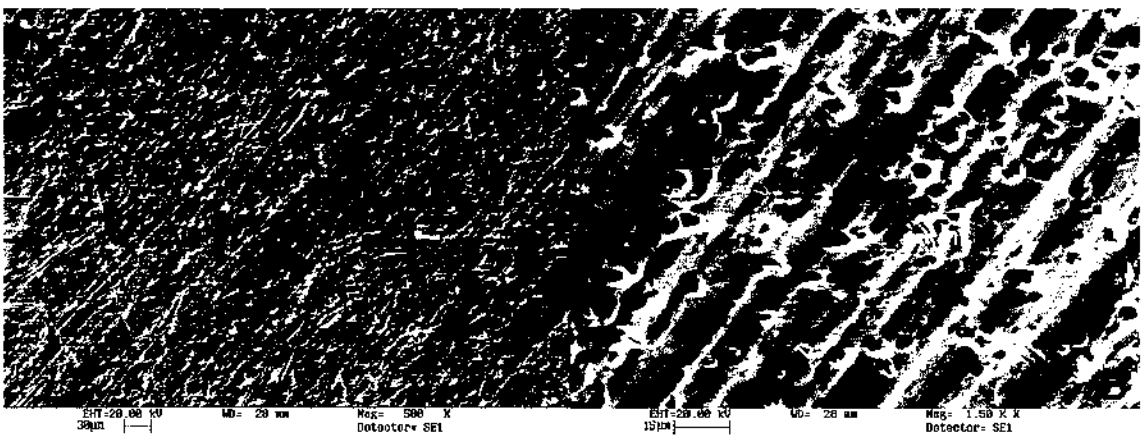
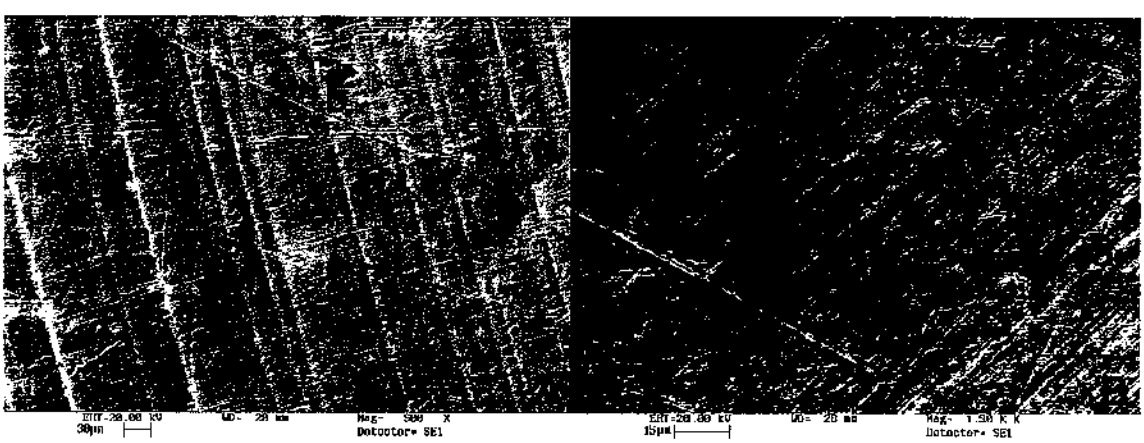


Figures 1. **A-B** – SEM of Ti-6Al-4V alloy surface at 500X and 1500X magnification; well-defined unidirectional microstructure with irregular scratches and defects result of machining (**a**) and small pits (**a**).



Figures 2. **A-B** – SEM of Tilite alloy surface at 500X and 1500X magnification; well-defined surface texture with an equally smooth surface and only minor irregularities result of machining (**a**) and light scratches (**b**).

3. CAPÍTULO 2

Adsorption of salivary and serum proteins, and bacterial adherence on titanium and zirconia ceramic surfaces

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Running title: Adsorption of proteins and bacterial adherence on dental materials

Key-words: Bacterial adherence; titanium; zirconia; salivary pellicle; serum proteins.

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Lima EMCX, Koo H, Vacca-Smith AM, Del Bel Cury AA, Rosalen PL, Bowen WH. Adsorption of salivary and serum proteins, and bacterial adherence on titanium and zirconia ceramic surfaces *(Clin Oral Impl Res)*

Abstract: The aim of this study was to determine the pattern of salivary and serum proteins present in pellicles formed on titanium (Ti) and zirconia ceramic (Zi) surfaces, and their ability to adhere bacterial cells. In addition, these parameters were compared to those of hydroxyapatite (HA). The pellicles were formed *in vitro* by incubating the materials with whole saliva, serum or saliva + serum. Protein composition in each of the pellicles was explored by SDS-PAGE and immunodetection. The adherence of radiolabeled *Streptococcus mutans* and *Actinomyces naeslundii* to uncoated surfaces and experimental pellicles was determined by means of scintillation counting. Statistical analyses were done using ANOVA and Tukey-Kramer HSD employing a significance level fixed at $P<0.05$. The electrophoretic analysis of the pellicles formed on HA, Ti and Zi revealed subtle differences in the pattern of the protein bands. Pellicle components identified included amylase, IgA, GTF B, IgG, albumin, fibronectin and fibrinogen. The number of *S. mutans* cells adhered to uncoated Ti and Zi was significantly higher than those adhered to HA ($P<0.05$). In contrast, lower number of *A. naeslundii* cells adhered to uncoated Ti and Zi than to HA ($P<0.05$). However, the differences of the levels of bacterial adherence to HA and Ti/Zi surfaces were greatly reduced in the presence of experimental pellicles, especially for *S. mutans*. The data showed that Ti and Zi display similar biological properties in terms of pellicle composition and bacterial adherence; the dental materials showed subtle, but significant differences on the levels of bacterial binding when compared to HA.

Introduction

Oral implants provide exposed hard surfaces that bind salivary and serum proteins resulting in formation of an acquired pellicle (Wolinsky et al. 1989; GroBner-Schreiber et al. 2001). Salivary and serum proteins in acquired pellicle may mediate the initial bacterial adherence to the tooth surface and restorative materials (Gibbons 1980; Marsh 2003; Carlen et al. 2003; Sardin et al. 2004). The adherence of microorganisms to these hard surfaces is recognized as the first step of dental biofilm formation (Gibbons 1980; Schilling & Bowen 1992; Wolinsky et al. 1989; Scheie 1994; Marsh 1994). The biofilm accumulation could lead to inflammation of the adjacent mucosa and, subsequently bone resorption, increasing the risk of implant failure (Ericsson et al. 1992; Lindhe et al. 1992; Abrahamsson et al. 1998).

Titanium is one of the most commonly used materials in dental implants because of its biocompatibility (Steinberg et al. 1995). Recently, zirconia ceramic has been introduced by many manufacturers to provide clinicians with abutments of enhanced esthetics with similar biocompatibility to those made with titanium (Rimondini et al. 2002). However, each dental implant material has distinct chemical and surface energy properties, which may provide distinct patterns of protein adsorption and bacterial binding (Rolla et al. 1983; Rasperini et al. 1998; Sardin et al. 2004). Although these interactions may interfere with the success or clinical failure of tissue integration (Piconi et al. 1999; Rimondini et al. 2002), sparse attention has been given to studies examining the adsorption of salivary/serum proteins and bacterial adherence on these hard surfaces. Development of dental implant materials that do not favor the initial bacterial binding would attenuate biofilm formation and subsequent inflammation of the soft tissues, and simultaneously allow epithelial and connective tissue attachment to the implant surface (Sawase et al. 2000; Groβner-Scheireber et al. 2001).

Therefore, the aim of this study was to determine the pattern of salivary and serum proteins present in pellicles formed on titanium (Ti) and zirconia ceramic (Zi) surfaces, and their ability to adhere bacterial cells. In addition, the protein adsorption and bacterial binding profiles were compared to those of hydroxyapatite (a tooth enamel surrogate).

Materials and methods

Saliva and serum

Saliva and serum samples were collected from one donor according to protocols approved by Research and Ethics Committee of University of Rochester Medical Center, NY and Faculty of Dentistry of Piracicaba, State University of Campinas. Stimulated whole saliva (SWS) was collected on ice after chewing paraffin film, and it was clarified by centrifugation at 3,800 g, 4°C, 10 min (Koo et al. 2000). Serum, without any additives, was obtained immediately after blood collection according to standard protocols used by the Clinical Laboratories of the University of Rochester Medical Center. The clarified SWS and serum were immediately used for pellicle formation and bacterial adherence assays.

Test Materials

Hydroxyapatite (Clarkson Chromatography Products. Inc., USA), titanium (Conexão Sistemas de Protéses Ltda., SP, Brazil) and zirconia ceramic (Nobel Biocare, Goteborg, Sweden) discs measuring 10mm in diameter and 2 mm in thickness (approximately 220 mm^2 of total surface area) were used in this study. The titanium (Ti) and zirconia ceramic (Zi) are two commercially available materials used for manufacturing implant abutments. Hydroxyapatite (HA) discs were used to mimic tooth (enamel) surface.

Bacterial Strains

Streptococcus mutans UA159 and *Actinomyces naeslundii* ATCC 12104, which are know to bind to acquired pellicle (Gibbons 1980; Schilling & Bowen 1992; Steinberg et al. 1993; Marsh 2003), were used for the adherence assays. The cultures were stored at -80°C in ultrafiltered (Prep/Scale; Millipore, MA) tryptone-yeast extract broth (2.5% tryptone and 1.5% yeast extract, pH 7.0) (Schilling & Bowen 1988) containing 20% glycerol.

Bacterial adherence assays were conducted using *S. mutans* UA159 and *A. naeslundii* ATCC 12104 grown in ultrafiltered (Prep/Scale; Millipore, MA) tryptone-yeast extract broth (2.5% tryptone and 1.5% yeast extract, pH 7.0) containing 185 kBq/ml ^{3}H-thymidine (Perkin Elmer Life and Analytical Sciences, Boston, MA, U.S.A) for 24 hours at 37°C, 5% CO_2 (Schilling & Bowen 1992; Koo et al. 2006). After incubation, the

radiolabeled bacteria were harvested by centrifugation (9,500 g, 10 min), washed and sonicated to obtain single-cell suspension (Schilling & Bowen 1992).

Experimental pellicle formation

The experimental pellicles were formed by incubating Ti, Zi or HA discs in one of the following solutions: 1) SWS diluted 1:1 (v/v) with adsorption buffer (50 mM KCl, 1.0 mM KPO_4, 1.0 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH 6.5); 2) SWS diluted 1:1 (v/v) with serum; 3) Serum diluted 1:1 (v/v) with adsorption buffer. Following incubation at 37°C with rocking for 1 h, the discs were removed and dip-washed twice in ultra pure milliQ-H_2O to remove loosely bound components. Subsequently, the experimental pellicle was removed from the disc surface by treatment with sodium dodecyl sulfate (SDS) sample buffer [Laemmli Sample Buffer, pH 6.8, 0.0625 M Tris (Base), 2% Sodium Dodecyl Sulfate (SDS), 10% Glycerin, 0,0500 M DL-Dithioithreitol (DL-DTT); Laemmli 1970] as detailed by Vacca-Smith & Bowen (2000); this procedure removes all the proteins from the disc surface as determined experimentally (Vacca-Smith & Bowen 2000).

SDS-PAGE and Western Blots analyses

The extracted pellicle samples were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (Western-Blot) analyses. All the samples were subjected to SDS-PAGE (Laemmli 1970) using either 8% or 10% resolving gel cast on vertical slab gel units (Hoefer Mighty Small 245 Dual gel Caster, Amersham Pharmacia Biotech), followed by sequential staining with silver nitrate as detailed elsewhere (Morrissey 1981; Vacca-Smith & Bowen 2000). Pre-stained molecular-weight standards (Bio-Rad Laboratories, Hercules, CA, USA) were used to estimate the approximate sizes of proteins.

Western blots were performed to identify the salivary and serum components in each of the samples (Towbin et al. 1979; Vacca-Smith & Bowen 2000). The extracted experimental samples were loaded on to a PAGE gel (8%, 10% and 12%) and separated by electrophoresis (Laemmli 1970). The gels were transferred to PVDF membranes (PALL Life Sciences Biotrace ™ PVDF, East Hills, NY, USA) and were probed with appropriate

antiserum/antibody according to the method of Towbin et al. (1979). The blots were developed with BCIP/NBT phosphatase substrate system kit according to the manufacturer's instructions (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA).

Monoclonal antibodies (cystatin S, cystatin SN and GTF B) were kindly provided by the Center for Oral Biology, University of Rochester Medical Center, NY, USA. Commercially obtained antibodies included antibody to IgA, albumin, fibronectin, fibrinogen, IgG, amylase (Sigma, Saint Louis, Missouri, USA). Secondary antiserum used included goat antirabbit, rabbit antigoat and rabbit antimouse IgG-alkaline phosphatase conjugate (Sigma, Saint Louis, Missouri, USA).

Bacterial adherence to experimental pellicles

Uncoated or experimental pellicle-coated HA, Ti or Zi discs were incubated with 2.5 ml of [^{3}H]thymidine-labeled *S.mutans* or *A. naeslundii* cells suspension (containing 1.0 $\times$ 10^9 cells/ml) at 37°C with rocking for 1h. Subsequently, the discs were washed twice in adsorption buffer to remove loosely bound cells, and the number of adherent bacteria was determined by liquid scintillation counting (Schilling & Bowen 1992). Bacterial adherence was calculated as the number of adherent cells divided per area (mm^2) of the disc surface. All the assays were performed using duplicate samples of each material in at least three different experiments.

Statistical analysis

The statistical analysis was done using SAS software (SAS Institute Inc., version 3.1, Cary, NC, USA) employing a significance level fixed at $P<0.05$. An ANOVA was used to test the null hypothesis of no difference among the materials. Tukey-Kramer HSD test was then used for post-ANOVA comparisons.

Results

SDS-PAGE and Western Blot

In general, the number of detectable protein bands visualized by 8% and 10% SDS-PAGE in serum-pellicles (9-14 bands) was greater than in saliva-pellicles (6-9); the electrophoretograms from saliva + serum pellicles displayed 11-12 protein bands (data not shown). However, the electrophoretic analysis of the saliva- and saliva + serum-pellicles from HA, Zi and Ti surfaces revealed only subtle differences in the number and pattern of the protein bands among the materials (data not shown); serum-pellicles from HA surface displayed 2 and 5 additional protein bands (in 10% SDS-PAGE) than those from Ti and Zi surfaces, respectively.

The pellicle components formed on each of the dental materials (HA, Ti and Zi) were identified by immunodetection, and the data are shown in Table 1. The proteins IgA, GTF B, IgG, fibronectin and fibrinogen were detected in all of the experimental pellicles formed on each of the materials. Albumin was not detected in salivary pellicle whereas amylase was absent in serum pellicle. In addition, cystatin S and cystatin SN were not detected in any of the experimental pellicles (Table 1).

Bacterial Adherence on Dental Materials

The bacterial adherence of *S. mutans* and *A. naeslundii* cells on non-coated and pellicle-coated materials are shown in Tables 2 and 3.

The number of *S. mutans* cells adhered to non-coated Ti and Zi was significantly higher than to non-coated HA ($P<0.05$) (Table 2). However, the number of cells adhered to saliva and saliva + serum derived pellicles was similar among the different materials. In contrast, more bacterial cells adhered to serum-coated Ti and Zi than to serum-coated HA.

The adherence of *S. mutans* cells to each of the materials was affected by the presence of experimental pellicles. The number of cells adhered to pellicle-coated surfaces was significantly lower than to those uncoated ($P<0.05$); except to serum-coated Ti surface.

In contrast to *S. mutans*, fewer *A. naeslundii* cells adhered to Ti and Zi than to HA irrespective of whether the surface of each materials were non-coated or coated with the experimental pellicles, with exception to Zi coated with saliva and saliva + serum pellicles (Table 3).

No significant differences were detected between the numbers of cells (either *S. mutans* or *A. naeslundii*) adhered to Ti and to Zi ($P>0.05$).

Discussion

Initial adherence of oral bacteria on the hard surfaces *in vivo* is preceded by the formation of an acquired pellicle by selective adsorption of components from saliva and gingival exudates (Hay 1967; Vacca-Smith & Bowen 2000; Marsh 2003; Yao et al. 2003; Sardin et al. 2004). Implants and dental restorative materials with distinct surface characteristics than those from the tooth enamel may affect pellicle formation and the subsequent bacterial adherence and colonization (Rasperini et al. 1998; Carlen et al. 2001). In this study, both the bacterial adherence and the electrophoretic pattern of proteins in experimental pellicles formed on different materials were determined.

The SDS-PAGE and immunobloting data revealed few qualitative differences of the composition of proteins of the pellicles formed on HA, Ti and Zi surfaces. The proteins identified in our experimental pellicles on hydroxyapatite discs were similar to those found by Rolla et al. (1983) and Vacca-Smith & Bowen (2000), which includes GTF B and amylase. Previous studies have shown that α-amylase, albumin and IgG are the main proteins adsorbed on titanium surfaces (Ellingsen 1991; Steinberg et al. 1995; Kohavi et al. 1995; Kohavi et al. 1997) whereas IgA, amylase, albumin and fibrinogen were identified on zirconia ceramics discs incubated in saliva and plasma (Milleding et al. 2001), which confirm our findings. In this study, we have shown that all the proteins identified in the experimental pellicles formed on HA were also found on Ti and Zi. The initial adsorption of salivary/serum proteins on hard surfaces in the oral cavity involves electrostatic interactions between acidic and basic components and the surface of tooth and other dental materials. Zirconia ceramic is an amphoteric metal oxide which exhibits both anion- and cation exchange properties depending on the pH and composition of the buffer (Rigney et

al. 1990). Although titanium is a metal, it is coated by a layer of surface oxide, mainly as titanium dioxide, which has physical/chemical characteristics more closely related to ceramic than to metals (Ellingsen 1991; Steinberg et al. 1995). Hydroxyapatite is also amphoteric due to both phosphate groups and calcium atoms being exposed on its surface and therefore both acidic and basic components bind to this mineral (Gibbons et al., 1980), but it has a net negative charge because there are more phosphate groups than calcium atoms exposed. These observations may explain, in part, the similar protein binding properties between Ti and Zi, and the subtle differences when compared to HA (especially in serum pellicles). However, it is noteworthy that the pellicle composition on these materials may be variable depending on individual (subject to subject) variations; the saliva and serum samples used in this study were collected from one healthy donor.

For the adherence assays, *S. mutans* and *A. naeslundii* were selected because these organisms can bind to the hard surfaces in the oral cavity (e.g. tooth surface) through various specific interactions, such as between bacterial adhesins and receptors in the acquired pellicle, and glucan mediated processes (Gibbons, 1980; Schilling & Bowen 1992; Steinberg et al. 1993; Vacca-Smith & Bowen 1998; Marsh 2003); *S. mutans* is also involved with biofilm formation and accumulation on the tooth surface (Schilling and Bowen, 1992).

The adherence of bacterial cells on the surface of the materials was variable depending on: 1) type of dental materials, 2) bacterial strains, and 3) the presence or absence of the experimental pellicles. The bacterial adherence on hard surfaces in the oral cavity is mediated by non-specific (e.g. electrostatic attractions and hydrophobic interactions) and highly specific (e.g. adhesin-receptor interaction) processes (Quirynen & Bollen 1995; Marsh 2004; Sardin et al. 2004). It is noteworthy that more cells of *S. mutans* and less of *A. naeslundii* adhered to uncoated Ti and Zi when compared to HA. In contrast, Ti and Zi showed similar number of adherent bacterial cells likely due to comparable physical/chemical attributes between zirconia ceramic and titanium dioxide surfaces (Ellingsen 1991). However, several other physical factors, such as surface roughness, distance of the bacteria to the surface, the ionic strength of the surrounding liquid medium, and the surface-free energy of the bacterium can influence the initial bacterial adhesion

(Busscher & Weerkamp 1987, Quirynen & Bollen 1995). Further studies on the bacterial binding affinity to each of the materials and their surface chemistries shall elucidate the exact mechanisms involved in the interaction of *S. mutans* and *A. naeslundii* to Ti and Zi.

Interestingly, Ti, Zi and HA surfaces coated with experimental pellicles adhered similar numbers of *S. mutans* cells, with exception of serum-only pellicle. The salivary and serum proteins in pellicles are known to act as receptors for bacterial adhesion through specific interactions with bacterial adhesins (Wolisnsky et al. 1989, Murray et al. 1992, Scannapieco et al. 1993, Marsh 2003). However, the electrophoretic patterns of proteins in saliva and saliva+serum pellicles formed on each of the materials was similar, which could explain, in part, the lack of detectable differences in the number of *S. mutans* cells on these surfaces. In addition, saliva coating may reduce the surface-free energy of the underlying materials, thereby, decreasing the binding affinity of *S. mutans* to these surfaces (Weerkamp et al. 1985; Quirynen & Bollen 1995; Ahn et al. 2002); the presence of salivary pellicle clearly decreased the number of *S. mutans* cells attached to apatitic surface confirming previous observations by Clark et al. 1978. In this study, the serum pellicle also decreased the number of *S. mutans* cells adhered on HA surfaces. It is known that serum proteins, such as albumin have the ability to mediate adherence of oral bacteria to the tooth surface in close proximity to the gingival crevice (Carlen et al. 2003); the adhesion of oral streptococci to albumin-coated HA surfaces decreases significantly (Gibbons & Etherden 1985). On the other hand, serum derived pellicle did not affect significantly the bacterial adherence on Ti. Whether this phenomenon is a result of the presence of specific serum proteins and their epitopes (which were not detected in this study) that bind *S. mutans* awaits further evaluation.

In contrast to *S. mutans*, the number of *A. naeslundii* cells adhered equally well irrespective of whether the surface of each of the materials (HA, Zi or Ti) was uncoated or coated with experimental pellicles. In general, HA adhered more cells than Ti/Zi; the subtle differences of the profile of bacterial adherence between HA and Ti/Zi were maintained in the presence of the various experimental pellicles, especially Ti. Previous studies have shown higher number of *A. viscosus* cells bound to the saliva-coated enamel than to Ti surface (Wolinsky et al. 1989; Steinberg et al. 1998) which are in agreement with our

findings. It is apparent that the range of binding sites present for *A. naeslundii* is slightly greater on experimental pellicles formed on HA surface than on Ti, and to a lesser extent Zi. Although major qualitative differences in the protein composition of the experimental pellicles were not detected in this study, quantitative differences of some specific proteins in the pellicles could explain the distinct pattern of *A. naeslundii* adherence between HA and dental implant abutments materials. In addition, the presence of proline-rich proteins (PRPs), which is known to interact with type 1 fimbriae of actinomyces (Whittaker et al. 1996), was not analyzed in this study. Clearly, studies on the interaction of *A. naeslundii* with the experimental pellicles from dental implant abutments are worthy of additional exploration.

In conclusion, titanium and zirconia ceramic displayed similar biological properties in terms of protein adsorption and bacterial adherence. However, the dental implant materials showed subtle, but significant differences on the bacterial binding pattern when compared to HA (surrogate tooth enamel), especially for *A. naeslundii.* Zirconia ceramic may be considered as a suitable material for manufacturing implant abutments with biological properties similar to titanium, a commonly used dental implant material. We are currently investigating the ability of these microorganisms to form biofilms on the surface of Ti and Zi.

Acknowledgements

This manuscript was based on a thesis submitted by the first author to Faculty of Dentistry of Piracicaba, UNICAMP, SP, Brazil as a partial fulfillment of the requirements of the PhD program in Clinical Dentistry (Prosthodontics). During this program she was a scholarship from CNPq and part of the study was performed at University of Rochester as a sandwich PhD program supported by CAPES (Coordination of Training of Higher Education Graduate). The authors would like to express their gratitude to the technician of the Center of Oral Biology of the University of Rochester Kathleen Scott-Anne for the technical assistance and to Conexão (Conexão, SP, Brazil) for providing the titanium discs and to Nobel Biocare (Nobel Biocare, Goteborg, Sweden) for providing the zirconia ceramic discs used in this study. We also thank the Center for Oral Biology, University of Rochester for providing the monoclonal antibodies.

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Table 1. Salivary/serum components found in the experimental pellicles of Hydroxyapatite (HA), Titanium (Ti) and Zirconia (Zi) .

Antiserum	HA sal	Ti sal	Zi sal	HA sal+ser	Ti sal + ser	Zi sal + ser	HA ser	Ti ser	Zi ser
IgA	+	+	+	+	+	+	+	+	+
GTFB	+	+	+	+	+	+	+	+	+
IgG	+	+	+	+	+	+	+	+	+
Fibronectin	+	+	+	+	+	+	+	+	+
Fibrinogen	+	+	+	+	+	+	+	+	+
Albumin	-	-	-	+	+	+	+	+	+
Amylase	+	+	+	+	+	+	-	-	-
Cystatin S	-	-	-	-	-	-	-	-	-
Cystatin SN	-	-	-	-	-	-	-	-	-

(+) Presence (-) absence of a protein

Sal (saliva coating); Sal+ser (saliva+serum coating); Ser (serum coating)

Table 2. *S. mutans* cells (10^4) adherence according to non-coated and pellicle-coated materials (Hydroxyapatie (HA), Titanium (Ti) and Zirconia (Zi) (Mean ± SD; n= 6).

	S. mutans (x 10^4 cells)			
Materials	Non-coated	Saliva coated	Saliva + Serum coated	Serum coated
HA	2.70 ± 0.39^{aA}	1.60 ± 0.33^{aB}	1.65 ± 0.35^{aB}	1.58 ± 0.42^{aB}
Ti	4.23 ± 1.22^{bA}	1.62 ± 0.19^{aB}	2.65 ± 0.80^{aB}	4.30 ± 1.11^{bA}
Zi	4.07 ± 0.59^{bA}	1.55 ± 0.39^{aB}	2.65 ± 0.76^{aC}	3.08 ± 0.60^{bC}

Distinct letters shows statistically significant differences ($P<0.05$). Lower case letters show differences among materials and upper case letters show differences among conditions (non-coated, saliva coated, saliva+serum coated and serum-coated).

Table 3. *A. naeslundii* cells (10^4) adherence according to the non-coated and pellicle-coated materials (Hydroxyapatie (HA), Titanium (Ti) and Zirconia (Zi) (Mean ± SD; n= 6).

	A. naeslundii (x 10^4 cells)			
Materials	Non-coated	Saliva coated	Saliva + Serum coated	Serum coated
HA	2.62 ± 0.38^{aA}	2.65 ± 0.29^{aA}	2.88 ± 0.26^{aA}	3.05 ± 0.74^{aA}
Ti	1.43 ± 0.40^{bA}	1.73 ± 0.41^{bA}	2.00 ± 0.75^{bA}	1.85 ± 0.54^{bA}
Zi	1.97 ± 0.27^{bA}	2.07 ± 0.64^{abA}	2.22 ± 0.58^{abA}	2.10 ± 0.45^{bA}

Distinct letters shows statistically significant differences ($P<0.05$). Lower case letters show differences among materials and upper case letters show differences among conditions (non-coated, saliva coated, saliva+serum coated and serum-coated).

4. CONCLUSÃO GERAL

Os implantes e materiais dentários restauradores com características de superfícies diferentes daquelas dos dentes podem afetar a formação da película e a habilidade da bactéria colonizar a cavidade oral. De acordo com os resultados obtidos no primeiro estudo pode-se verificar que os materiais avaliados (Ti-6Al-4V e Tilite) apresentaram diferenças nas características de superfícies, exceto para dureza de superfície. No segundo estudo foi possível observar que o titânio e a cerâmica zircônia apresentaram propriedades biológicas similares em termos de adsorção de proteínas e aderência bacteriana. Entretanto, esses materiais apresentaram diferenças na adesão bacteriana quando comparados com a hidroxiapatita, principalmente para *A. naeslundii*. Assim, constatou-se que a cerâmica a base de zircônia pode ser considerada um material adequado para confecção de abutments com baixo potencial para colonização bacteriana semelhante ao titânio já comumente utilizado. No entanto, pouco tem sido relatado sobre a aderência bacteriana nas superfícies deste material e sobre a liga alternativa (Tilite). Desse modo, outros estudos devem ser feitos para elucidar essas questões.

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* De acordo com a norma utilizada na FOP/Unicamp, baseada no modelo Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

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Anexo 1 - Figuras Capítulo 2

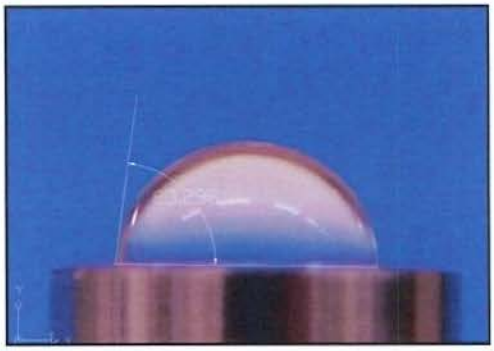


Figura 1 - Mensuração do ângulo de contato entre a superfície dos materiais e gota séssil de água bi-destilada e deionizada.

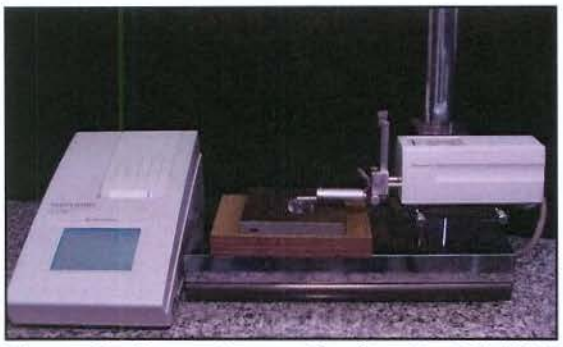


Figura 2 - Rugosímetro Surfcorder SE 1700

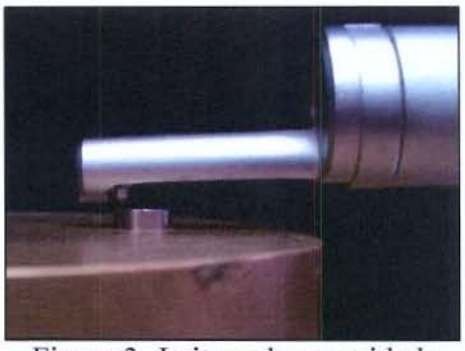


Figura 3- Leitura da rugosidade superficial Percurso de medição - 2,0mm, comp. de onda - 0,25mm, vel. - 0,5mm/s.

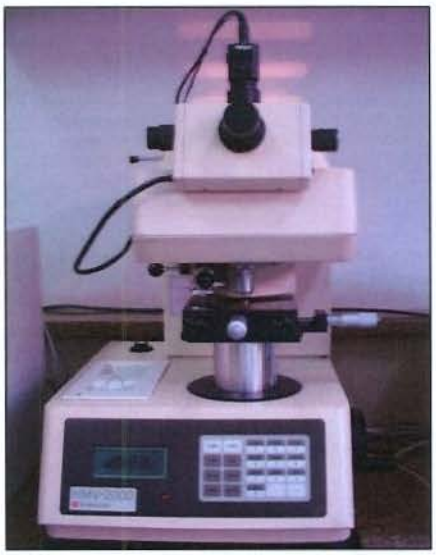


Figura 4 - Microdurometro Shimadzu model HMV 2000, Kyoto, Japan.

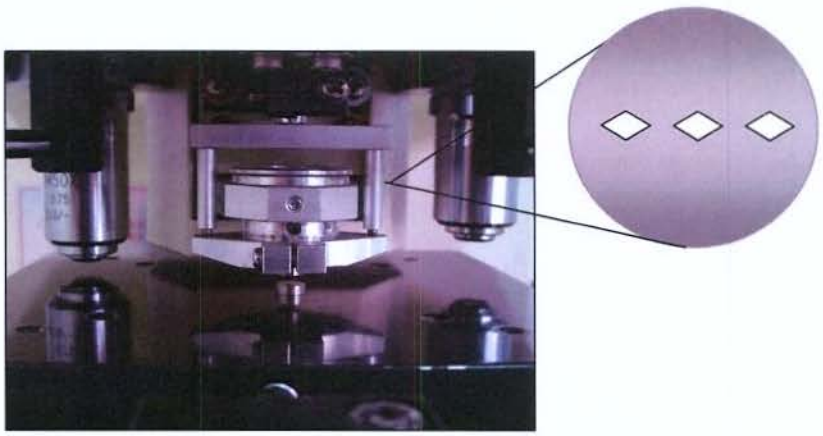


Figura 5 - Mensuração da dureza Vickers - média de três endentações (carga=50 g; t=10 s).

Anexo 2 – Submissão do artigo à revista Brazilian Oral Research

Brazilian Oral Research

Of. POB 182 Web/2006.

Prezado (a) Senhor (a)

Recebemos em **26/04/2006** através do site da SBPqO, o trabalho abaixo relacionado:

Título: EVALUATION OF SURFACE CHARACTERISTICS OF TI-6AL-4V AND TILITE ALLOYS.

Autor(a) principal: - EMILENA MARIA CASTOR XISTO LIMA - Wander José da Silva - Juliana Silva Moura - Fernanda Faot - Altair Antoninha Del Bel Cury, para publicação na revista *Brazilian Oral Research*, a ser analisado pela Comissão de Publicação, recebendo o **número 182 Web**, **que deverá ser utilizado para futuros contatos**.

Anexo 3 – Figuras Capítulo 3.

Figura 1 (A)

Figura 2 (B)

Figura 3 (C)

Figuras 1, 2 e 3. Materiais utilizados no estudo: (A) hidroxiapatita, (B) titânio e (C) cerâmica zircônia.

Figura 4 e 5. Espécimes de cada material incubados com saliva, saliva + soro ou soro em placas de 24 poços.

Figura 6. Espécimes incubados durante 1 hora a 37°C, em agitação.

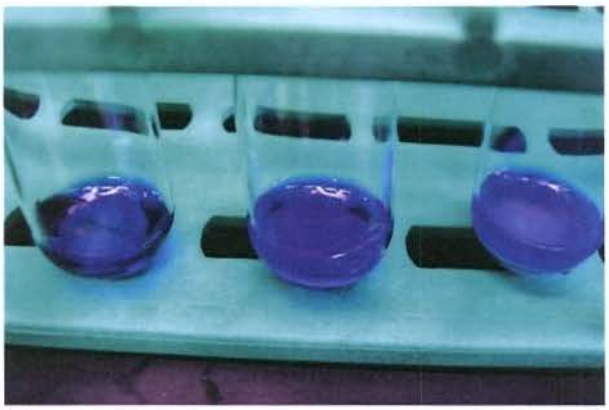
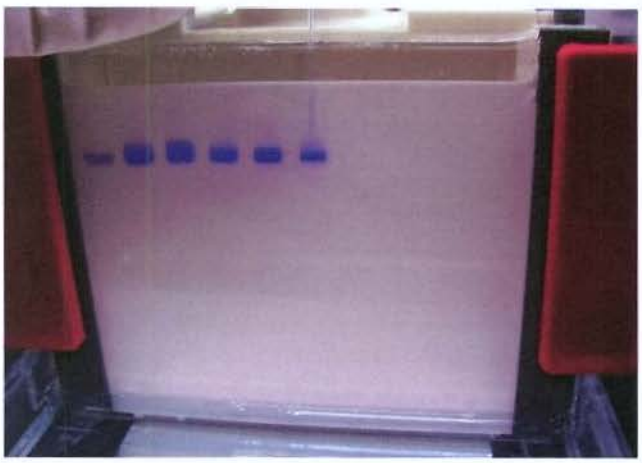


Figura 7. Adição do tampão SDS - sulfato dodecil sódio para remoção das películas experimentais adsorvidas nos discos.



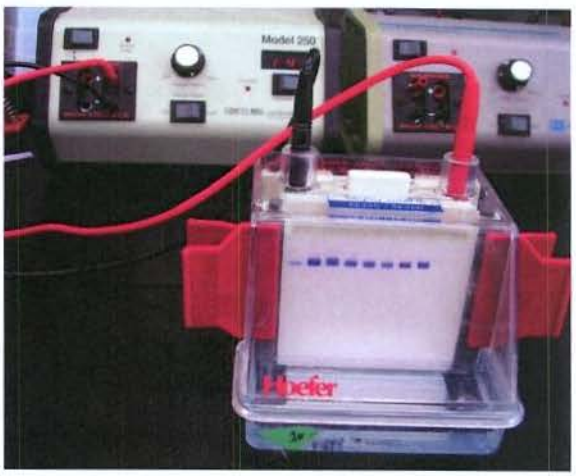
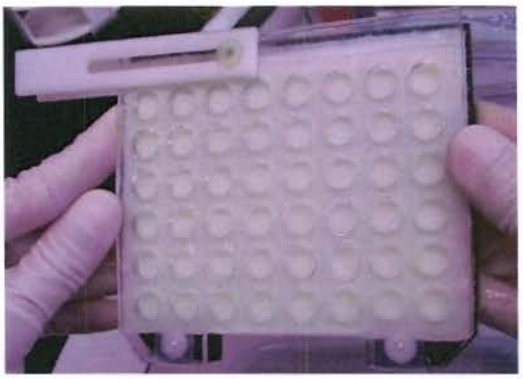


Figura 8 e 9. Amostras das películas experimentais sendo submetidas a SDS-PAGE - eletroforese do gel de poliacrilamida-sulfato dodecil sódio.



Figuras 10 e 11. Transferência do gel para membrana de PVDF.

Figura 12 e 13. Western Blot para identificação das proteínas adsorvidas nos discos.

Figura 14. Membranas incubadas com anticorpos apropriados durante 1 hora em mesa agitadora (Rocker - Reliable Scientific (USA.)

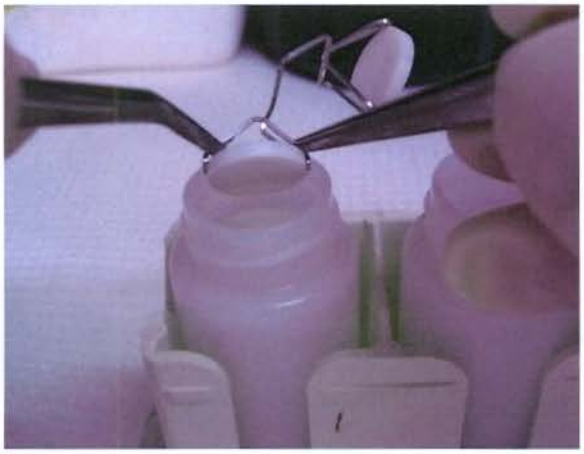


Figura 15. Transferência dos discos para os vials.

Figura 16. Colocação do liquido de cintilação.

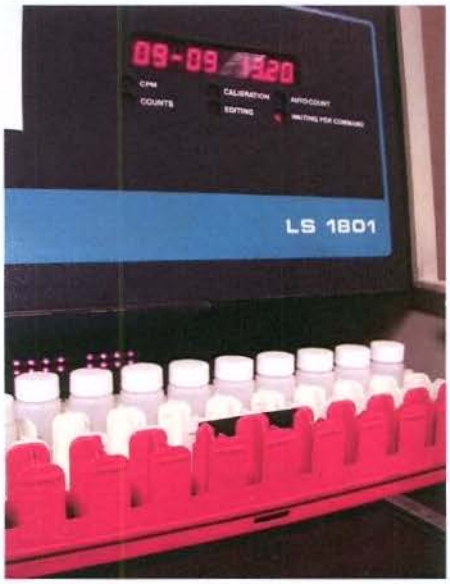


Figura 17. Vials posicionados no cintilador.

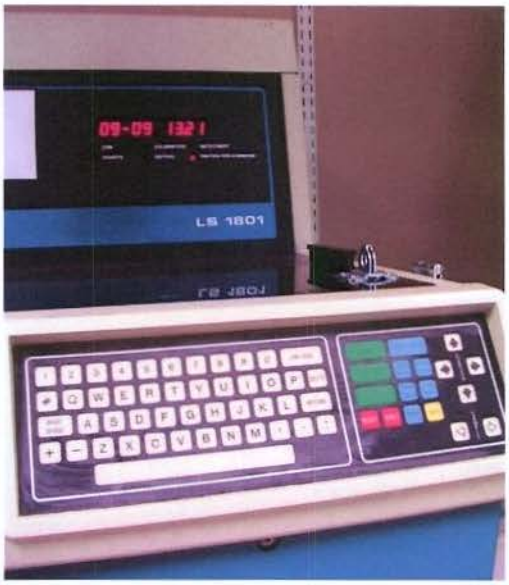


Figura 18. Cintilador (Scintilation Counter Beckman LS 1801, Fullerton, CA, USA) utilizado para contagem do numero de células aderidas nos discos.

Anexo 4 – Certificado de aprovação do Comitê de Ética em Pesquisa – FOP-UNICAMP

UNICAMP

COMITÊ DE ÉTICA EM PESQUISA

UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ODONTOLOGIA DE PIRACICABA

CERTIFICADO

Certificamos que o Projeto de pesquisa intitulado "Influência das características de superfície dos abutments de titânio e de cerâmica na adsorção de proteínas salivares e aderência bacteriana", sob o protocolo nº **144/2003**, da Pesquisadora ***Emilena Maria Castor Xisto Lima***, sob a responsabilidade da Profa. Dra. **Altair Antoninha Del Bel Cury**, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde/MS, de 10/10/96, tendo sido aprovado pelo Comitê de Ética em Pesquisa – FOP.

Piracicaba, 05 de novembro de 2003

We certify that the research project with title "iIfluence of the surface characteristics of titanium and ceramic abutments on adsorption of salivary proteins and bacterial adhesion", protocol nº **144/2003**, by Researcher ***Emilena Maria Castor Xisto Lima***, responsibility by Prof. Dr. **Altair Antoninha Del Bel Cury**, is in agreement with the Resolution 196/96 from National Committee of Health/Health Department (BR) and was approved by the Ethical Committee in Research at the Piracicaba Dentistry School/UNICAMP (State University of Campinas).

Piracicaba, SP, Brazil, November 05 2003

Prof. Dr. Antonio Bento Alves de Moraes

Coordenador

CEP/FOP/UNICAMP

Anexo 5 – Carta de aprovação do Comitê de Ética em Pesquisa – Universidade de Rochester

Letter of Approval

RSRB: RSRB00011691 **Principal Investigator**: Hyun Koo

Study Title: Bacterial Adherence on Saliva and/or Blood Serum-Coated Dental Materials

Initial Approval: 5/17/05

Study Approval Expires: 5/16/06

Review Level: Expedited

Additional Remarks:
Protocol: last modified date 4/5/05

This approval is contingent upon the investigation being conducted in compliance with the approved study protocol including all requirements and/or determinations of the RSRB. Unless a Waiver of Consent is specified above, consent must be obtained and documented in the manner approved by the RSRB. Please note all remarks and/or attachments. Only consent forms bearing a current 'RSRB Approved' Watermark may be used. Only the most recently approved version of any consent or recruitment document may be used when obtaining consent. **Consent forms/recruitment letters must be printed on department letterhead.**

As the Principal Investigator, you are responsible for the following activities:

- Timely submission of continuing review progress reports. Federal Regulations require that the RSRB conduct continuing review of research. You will receive Progress Report forms from the RSRB.
- Requesting any proposed changes in the above research activity. All subject recruitment materials must be approved prior to use. Changes may not be initiated without RSRB approval except when necessary to eliminate apparent immediate hazards to the subject(s) and then a report must be submitted along with the amendment request
- Maintaining all approved study documents in your study file

- Maintaining signed consent forms for at least three years after the research is completed or for a longer term if required by FDA regulations
- Reporting any unexpected serious problems involving risks to subjects or others (including unexpected deaths, hospitalizations or serious injuries) in accordance with the RSRB Adverse Event guidelines
- Submitting a final progress report to the RSRB upon completion of this study

Robert DiCenzo, RSRB Chair 5/17/05

601 Elmwood Avenue, Box 315
Rochester, New York 14642
(585) 275-2398

Anexo 6 – Resultados Capítulo 2

Frequencies

	Material	N
Rugosidade	Tilite	25
	Titânio	25
	Total	50
Angulação	Tilite	25
	Titânio	25
	Total	50
Energia	Tilite	25
	Titânio	25
	Total	50
Dureza	Tilite	9
	Titânio	9
	Total	18

ANOVA Table[a,b,c]

			F	Sig.
Rugosidade * Material	Between Groups	(Combined)	12,814	,001
	Within Groups			
	Total			
Angulação * Material	Between Groups	(Combined)	25,064	,000
	Within Groups			
	Total			
Dureza * Material	Between Groups	(Combined)	,015	,903
	Within Groups			
	Total			

a. With fewer than three groups, linearity measures for Rugosidade * Material cannot be computed.

b. With fewer than three groups, linearity measures for Angulação * Material cannot be computed.

c. With fewer than three groups, linearity measures for Dureza * Material cannot be computed.

T-Test

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Material	1,500	50	,5051	,0714
	Rugosidade	,179252	50	,0441093	,0062380
Pair 2	Material	1,500	50	,5051	,0714
	Angulação	80,66690	50	7,126618	1,007856
Pair 3	Material	1,500	18	,5145	,1213
	Dureza	324,66111	18	12,339088	2,908351

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	Material & Rugosidade	50	,459	,001
Pair 2	Material & Angulação	50	-,586	,000
Pair 3	Material & Dureza	18	,031	,903

ANOVA

				F	Sig.
Rugosidade	Between Groups	(Combined)		12.814	.001
		Linear Term	Contrast	12.814	.001
	Within Groups				
	Total				
Angulação	Between Groups	(Combined)		25.064	.000
		Linear Term	Contrast	25.064	.000
	Within Groups				
	Total				
Energia	Between Groups	(Combined)		25.140	.000
		Linear Term	Contrast	25.140	.000
	Within Groups				
	Total				
Dureza	Between Groups	(Combined)		.015	.903
		Linear Term	Contrast	.015	.903
	Within Groups				
	Total				

Robust Tests of Equality of Means

		Statistic[a]	df1	df2	Sig.
Rugosidade	Welch	12.814	1	42.766	.001
	Brown-Forsythe	12.814	1	42.766	.001
Angulação	Welch	25.064	1	47.397	.000
	Brown-Forsythe	25.064	1	47.397	.000
Energia	Welch	25.140	1	47.344	.000
	Brown-Forsythe	25.140	1	47.344	.000
Dureza	Welch	.015	1	14.044	.903
	Brown-Forsythe	.015	1	14.044	.903

Correlations

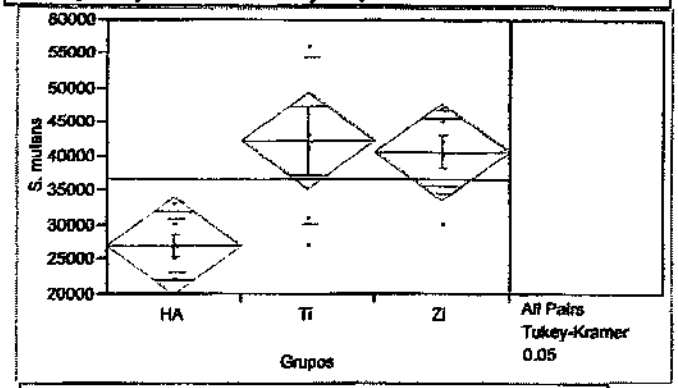
		Rugosidade	Angulação	Energia	Dureza
Rugosidade	Pearson Correlation	1	-.237	.238	.136
	Sig. (2-tailed)		.098	.096	.590
	Sum of Squares and Cross-products	.095	-3.646	2.343	1.379
	Covariance	.002	-.074	.048	.081
	N	50	50	50	18
Angulação	Pearson Correlation	-.237	1	-1.000**	-.308
	Sig. (2-tailed)	.098		.000	.214
	Sum of Squares and Cross-products	-3.646	2488.645	-1590.473	-566.946
	Covariance	-.074	50.769	-32.459	-33.350
	N	50	50	50	18
Energia	Pearson Correlation	.238	-1.000**	1	.308
	Sig. (2-tailed)	.096	.000		.214
	Sum of Squares and Cross-products	2.343	-1590.473	1016.480	362.059
	Covariance	.048	-32.459	20.744	21.298
	N	50	50	50	18
Dureza	Pearson Correlation	.136	-.308	.308	1
	Sig. (2-tailed)	.590	.214	.214	
	Sum of Squares and Cross-products	1.379	-566.946	362.059	2588.303
	Covariance	.081	-33.350	21.298	152.253
	N	18	18	18	18

**. Correlation is significant at the 0.01 level (2-tailed).

Anexo 7 – Resultados Capítulo 3

Smutansstat: Oneway

Oneway Analysis of S. mutans By Grupos



Oneway Anova

Summary of Fit

Rsquare	0.461093
Adj Rsquare	0.389229
Root Mean Square Error	8134.973
Mean of Response	38666.67
Observations (or Sum Wgts)	18

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Grupos	2	849333333	424666667	6.4171	0.0097
Error	15	992666667	66177778		
C. Total	17	1842000000			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
HA	6	27000.0	3321.1	19921	34079
Ti	6	42333.3	3321.1	35255	49412
Zi	6	40666.7	3321.1	33588	47745

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
HA	6	27000.0	3949.7	1612.5	23563	30437
Ti	6	42333.3	12160.0	4964.3	31752	52915
Zi	6	40666.7	5921.7	2417.5	35514	45820

Means Comparisons

Dif=Mean[i]-Mean[j]

	Ti	Zi	HA
Ti	0.0	1666.7	15333.3
Zi	-1666.7	0.0	13666.7
HA	-15333.3	-13666.7	0.0

Alpha=0.05

Comparisons for all pairs using Tukey-Kramer HSD

q*

2.59747

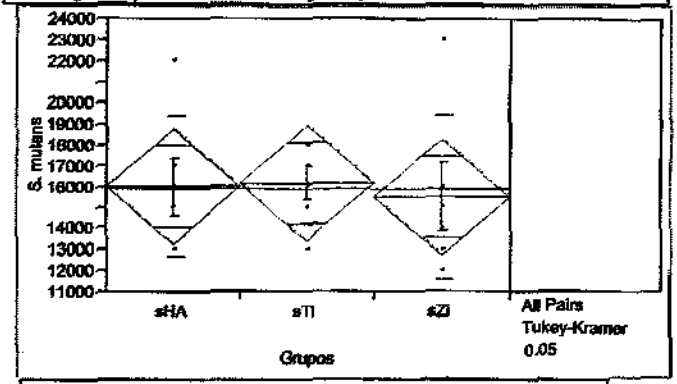
Abs(Dif)-LSD

	Ti	Zi	HA
Ti	-12199.6	-10532.9	3133.7
Zi	-10532.9	-12199.6	1467.1
HA	3133.7	1467.1	-12199.6

Positive values show pairs of means that are significantly different.

Smutansstat: Oneway

Oneway Analysis of S. mutans By Grupos



Oneway Anova

Summary of Fit

Rsquare	0.009393
Adj Rsquare	-0.12289
Root Mean Square Error	3186.778
Mean of Response	15888.89
Observations (or Sum Wgts)	18

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Grupos	2	1444444	722222.22	0.0711	0.9317
Error	15	152333333	10155556		
C. Total	17	153777778			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
sHA	6	16000.0	1301.0	13227	18773
sTi	6	16166.7	1301.0	13394	18940
sZi	6	15500.0	1301.0	12727	18273

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
sHA	6	16000.0	3346.64	1366.3	13088	18912
sTi	6	16166.7	1940.79	792.3	14478	17856
sZi	6	15500.0	3937.00	1607.3	12074	18926

Means Comparisons

Dif=Mean[i]-Mean[j]

	sTi	sHA	sZi
sTi	0.000	166.667	666.667
sHA	-166.667	0.000	500.000
sZi	-666.667	-500.000	0.000

Alpha=0.05

Comparisons for all pairs using Tukey-Kramer HSD

q*

2.59747

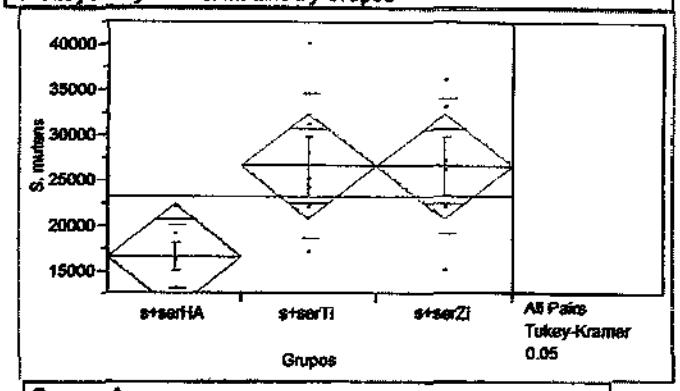
Abs(Dif)-LSD

	sTi	sHA	sZi
sTi	-4779.05	-4612.39	-4112.39
sHA	-4612.39	-4779.05	-4279.05
sZi	-4112.39	-4279.05	-4779.05

Positive values show pairs of means that are significantly different.

Smutansstat: Oneway

Oneway Analysis of S. mutans By Grupos



Oneway Anova

Summary of Fit

Rsquare	0.374357
Adj Rsquare	0.290937
Root Mean Square Error	6675.827
Mean of Response	23166.67
Observations (or Sum Wgts)	18

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Grupos	2	400000000	200000000	4.4877	0.0297
Error	15	668500000	44566667		
C. Total	17	1068500000			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
s+serHA	6	16500.0	2725.4	10691	22309
s+serTi	6	26500.0	2725.4	20691	32309
s+serZi	6	26500.0	2725.4	20691	32309

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
s+serHA	6	16500.0	3507.14	1431.8	13448	19552
s+serTi	6	26500.0	6018.73	3273.6	19522	33478
s+serZi	6	26500.0	7556.45	3084.9	19925	33075

Means Comparisons

Dif=Mean[i]-Mean[j]

	s+serTi	s+serZi	s+serHA
s+serTi	0.0	0.0	10000.0
s+serZi	0.0	0.0	10000.0
s+serHA	-10000.0	-10000.0	0.0

Alpha= 0.05

Comparisons for all pairs using Tukey-Kramer HSD

q*

2.59747

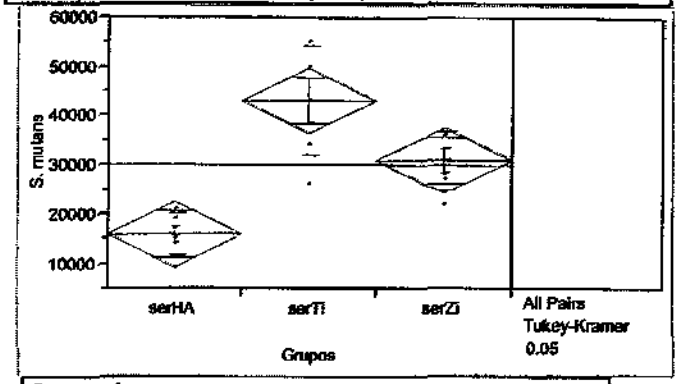
Abs(Dif)-LSD

	s+serTi	s+serZi	s+serHA
s+serTi	-10011.4	-10011.4	-11.4
s+serZi	-10011.4	-10011.4	-11.4
s+serHA	-11.4	-11.4	-10011.4

Positive values show pairs of means that are significantly different.

Smutansstat: Oneway

Oneway Analysis of S. mutans By Grupos



Oneway Anova

Summary of Fit

Rsquare	0.715938
Adj Rsquare	0.678063
Root Mean Square Error	7666.667
Mean of Response	29888.89
Observations (or Sum Wgts)	18

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Grupos	2	2222111111	1.11106e9	18.9026	<.0001
Error	15	881666667	58777778		
C. Total	17	3103777778			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
serHA	6	15833.3	3129.9	9162	22505
serTi	6	43000.0	3129.9	36329	49671
serZi	6	30833.3	3129.9	24162	37505

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
serHA	6	15833.3	4215.1	1720.8	12166	19501
serTi	6	43000.0	11063.5	4516.6	33373	52627
serZi	6	30833.3	6013.9	2455.2	25600	36066

Means Comparisons

Dif=Mean[i]-Mean[j]

	serTi	serZi	serHA
serTi	0.0	12166.7	27166.7
serZi	-12166.7	0.0	15000.0
serHA	-27166.7	-15000.0	0.0

Alpha=0.05

Comparisons for all pairs using Tukey-Kramer HSD

q*

2.59747

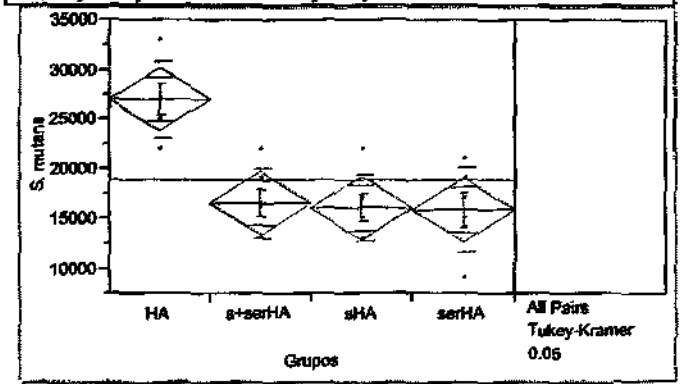
Abs(Dif)-LSD

	serTi	serZi	serHA
serTi	-11497.3	669.3	15669.3
serZi	669.3	-11497.3	3502.7
serHA	15669.3	3502.7	-11497.3

Positive values show pairs of means that are significantly different.

Smutanssiat: Oneway

Oneway Analysis of S. mutans By Grupos



Oneway Anova

Summary of Fit

Rsquare	0.65297
Adj Rsquare	0.600915
Root Mean Square Error	3770.6
Mean of Response	18833.33
Observations (or Sum Wgts)	24

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Grupos	3	535000000	178333333	12.5440	<.0001
Error	20	284333333	14216667		
C. Total	23	819333333			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
HA	6	27000,0	1539,3	23789	30211
s+serHA	6	16500,0	1539,3	13289	19711
sHA	6	16000,0	1539,3	12789	19211
serHA	6	15833,3	1539,3	12622	19044

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
HA	6	27000,0	3949,68	1612,5	23536	30364
s+serHA	6	16500,0	3507,14	1431,8	13513	19487
sHA	6	16000,0	3346,64	1366,3	13150	18850
serHA	6	15833,3	4215,05	1720,8	12244	19423

Means Comparisons

Dif=Mean[i]-Mean[j]

	HA	s+serHA	sHA	serHA
HA	0,0	10500,0	11000,0	11166,7
s+serHA	-10500,0	0,0	500,0	666,7
sHA	-11000,0	-500,0	0,0	166,7
serHA	-11166,7	-666,7	-166,7	0,0

Alpha=0,05

Comparisons for all pairs using Tukey-Kramer HSD

q*
2,79894

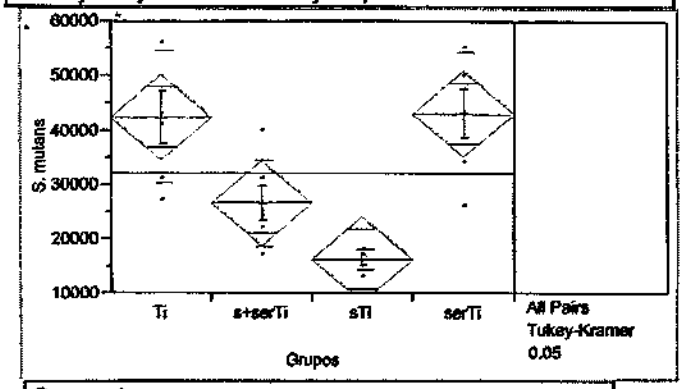
Abs(Dif)-LSD

	HA	s+serHA	sHA	serHA
HA	-6093,00	4407,00	4907,00	5073,66
s+serHA	4407,00	-6093,00	-5593,00	-5426,34
sHA	4907,00	-5593,00	-6093,00	-5926,34
serHA	5073,66	-5426,34	-5926,34	-6093,00

Positive values show pairs of means that are significantly different.

Smutanastat: Oneway

Oneway Analysis of S. mutans By Grupos



Oneway Anova

Summary of Fit

Rsquare	0.643409
Adj Rsquare	0.589921
Root Mean Square Error	9196.92
Mean of Response	32000
Observations (or Sum Wgts)	24

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Grupos	3	3052333333	1.01744e9	12.0269	0.0001
Error	20	1691666667	84583333		
C. Total	23	4744000000			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Ti	6	42333.3	3754.6	34501	50165
s+serTi	6	26500.0	3754.6	18668	34332
sTi	6	16166.7	3754.6	8335	23999
serTi	6	43000.0	3754.6	35168	50832

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
Ti	6	42333.3	12160.0	4964.3	31978	52689
s+serTi	6	26500.0	8018.7	3273.6	19671	33329
sTi	6	16166.7	1940.8	792.3	14514	17819
serTi	6	43000.0	11063.5	4516.6	33578	52422

Means Comparisons

Dif=Mean[i]-Mean[j]

	serTi	Ti	s+serTi	sTi
serTi	0.0	666.7	16500.0	26833.3
Ti	-666.7	0.0	15833.3	26166.7
s+serTi	-16500.0	-15833.3	0.0	10333.3
sTi	-26833.3	-26166.7	-10333.3	0.0

Alpha=0.05

Comparisons for all pairs using Tukey-Kramer HSD

q*

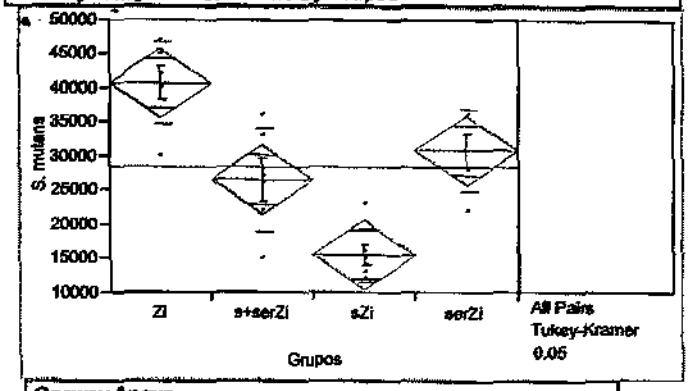
2.79894

Abs(Dif)-LSD

	serTi	Ti	s+serTi	sTi
serTi	-14861.9	-14195.3	1638.1	11971.4
Ti	-14195.3	-14861.9	971.4	11304.7
s+serTi	1638.1	971.4	-14861.9	-4528.6
sTi	11971.4	11304.7	-4528.6	-14861.9

Positive values show pairs of means that are significantly different.

Oneway Analysis of S. mutans By Grupos



Oneway Anova

Summary of Fit

Rsquare	0.731416
Adj Rsquare	0.691129
Root Mean Square Error	5896.527
Mean of Response	28375
Observations (or Sum Wgts)	24

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Grupos	3	1958458333	652819444	18.1549	<.0001
Error	20	719166667	35958333		
C. Total	23	2677625000			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Zi	6	40666.7	2448.1	35560	45773
s+serZi	6	26500.0	2448.1	21393	31607
sZi	6	15500.0	2448.1	10393	20607
serZi	6	30833.3	2448.1	25727	35940

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
Zi	6	40666.7	5921.71	2417.5	35624	45710
s+serZi	6	26500.0	7556.45	3084.9	20085	32935
sZi	6	15500.0	3937.00	1607.3	12147	18853
serZi	6	30833.3	6013.87	2455.2	25712	36955

Means Comparisons

Dif=Mean[i]-Mean[j]

	Zi	serZi	s+serZi	sZi
Zi	0.0	9833.3	14166.7	25166.7
serZi	-9833.3	0.0	4333.3	15333.3
s+serZi	-14166.7	-4333.3	0.0	11000.0
sZi	-25166.7	-15333.3	-11000.0	0.0

Alpha=0.05

Comparisons for all pairs using Tukey-Kramer HSD

q*
2.79894

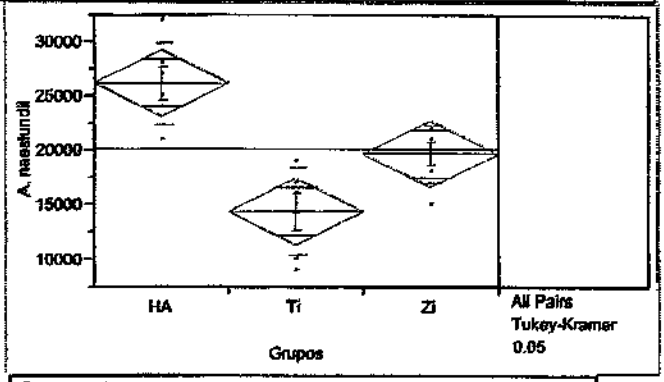
Abs(Dif)-LSD

	Zi	serZi	s+serZi	sZi
Zi	-9890.2	143.1	4476.5	15476.5
serZi	143.1	-9890.2	-5356.9	5643.1
s+serZi	4476.5	-5356.9	-9890.2	1309.8
sZi	15476.5	5643.1	1309.8	-9890.2

Positive values show pairs of means that are significantly different.

Anaeslunstat: Oneway

Oneway Analysis of A. naeslundii By Grupos



Oneway Anova

Summary of Fit

Rsquare	0.89209
Adj Rsquare	0.851035
Root Mean Square Error	3535.534
Mean of Response	20055.56
Observations (or Sum Wgts)	18

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Grupos	2	421444444	210722222	16.8578	0.0001
Error	15	187500000	12500000		
C. Total	17	608944444			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
HA	6	26166.7	1443.4	23090	29243
Ti	6	14333.3	1443.4	11257	17410
Zi	6	19666.7	1443.4	16590	22743

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
HA	6	26166.7	3763.86	1536.6	22892	29442
Ti	6	14333.3	3983.30	1626.2	10867	17799
Zi	6	19666.7	2732.52	1115.5	17289	22044

Means Comparisons

Dif=Mean[i]-Mean[j]

	HA	Zi	Ti
HA	0.0	6500.0	11833.3
Zi	-6500.0	0.0	5333.3
Ti	-11833.3	-5333.3	0.0

Alpha= 0.05

Comparisons for all pairs using Tukey-Kramer HSD

q*

2.59747

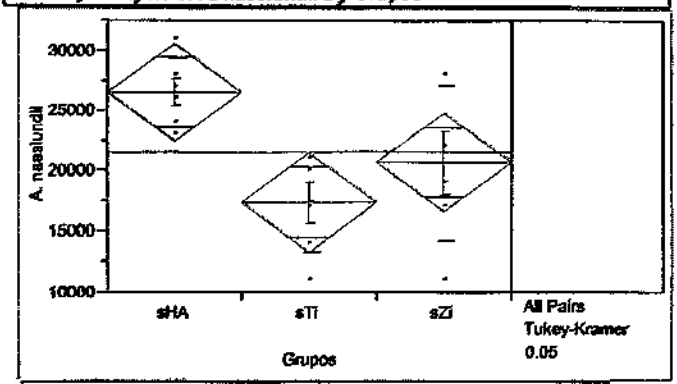
Abs(Dif)-LSD

	HA	Zi	Ti
HA	-5302.06	1197.94	6531.27
Zi	1197.94	-5302.06	31.27
Ti	6531.27	31.27	-5302.06

Positive values show pairs of means that are significantly different.

Anaeslunstat: Oneway

Oneway Analysis of A. naeslundii By Grupos



Oneway Anova

Summary of Fit

Rsquare	0.437462
Adj Rsquare	0.36248
Root Mean Square Error	4705.788
Mean of Response	21500
Observations (or Sum Wgts)	18

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Grupos	2	258333333	129166667	5.8329	0.0134
Error	15	332166667	22144444		
C. Total	17	590500000			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
sHA	6	26500.0	1921.1	22405	30595
sTi	6	17333.3	1921.1	13239	21428
sZi	6	20666.7	1921.1	16572	24761

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
sHA	6	26500.0	2690.97	1176.2	23993	29007
sTi	6	17333.3	4131.18	1686.5	13739	20928
sZi	6	20666.7	6408.33	2616.2	15090	26243

Means Comparisons

Dif=Mean[i]-Mean[j]

	sHA	sZi	sTi
sHA	0.00	5833.33	9166.67
sZi	-5833.33	0.00	3333.33
sTi	-9166.67	-3333.33	0.00

Alpha=0.05

Comparisons for all pairs using Tukey-Kramer HSD

q*

2.59747

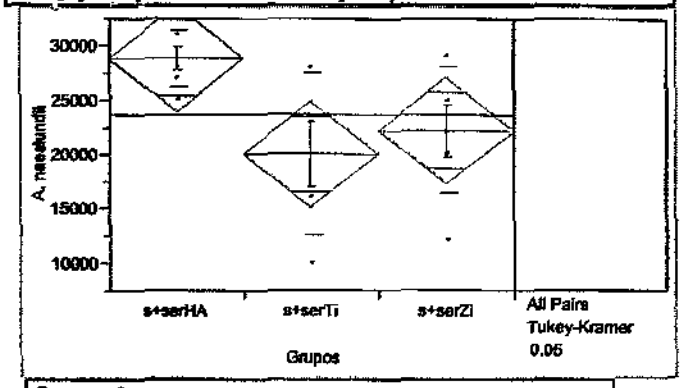
Abs(Dif)-LSD

	sHA	sZi	sTi
sHA	-7057.04	-1223.70	2109.63
sZi	-1223.70	-7057.04	-3723.70
sTi	2109.63	-3723.70	-7057.04

Positive values show pairs of means that are significantly different.

Anaeslunstat: Oneway

Oneway Analysis of A. naeslundii By Grupos



Oneway Anova

Summary of Fit

Rsquare	0.345582
Adj Rsquare	0.258303
Root Mean Square Error	5666.667
Mean of Response	23666.67
Observations (or Sum Wgts)	18

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Grupos	2	254333333	127166667	3.9602	0.0416
Error	15	481666667	32111111		
C. Total	17	736000000			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
s+serHA	6	28833.3	2313.4	23902	33764
s+serTi	6	20000.0	2313.4	15069	24931
s+serZi	6	22166.7	2313.4	17236	27098

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
s+serHA	6	28833.3	2562.55	1046.2	26604	31063
s+serTi	6	20000.0	7456.54	3044.1	13512	26488
s+serZi	6	22166.7	5845.23	2386.3	17060	27253

Means Comparisons

Dif=Mean[i]-Mean[j]

	s+serHA	s+serZi	s+serTi
s+serHA	0.00	6666.67	8833.33
s+serZi	-6666.67	0.00	2166.67
s+serTi	-8833.33	-2166.67	0.00

Alpha=0.05

Comparisons for all pairs using Tukey-Kramer HSD

q*

2.59747

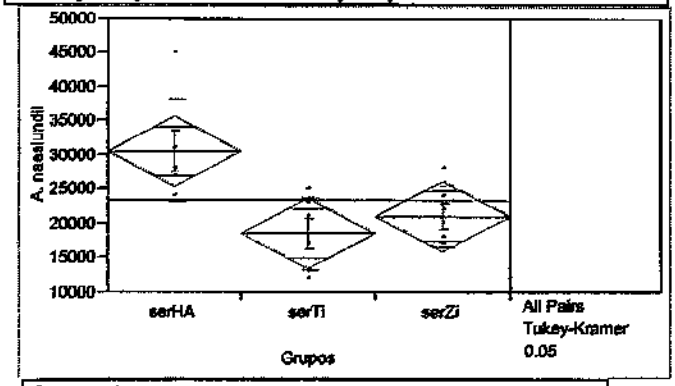
Abs(Dif)-LSD

	s+serHA	s+serZi	s+serTi
s+serHA	-8498.02	-1831.35	335.32
s+serZi	-1831.35	-8498.02	-6331.35
s+serTi	335.32	-6331.35	-8498.02

Positive values show pairs of means that are significantly different.

Anaeslumstat: Oneway

Oneway Analysis of A. naeslundii By Grupos



Oneway Anova

Summary of Fit

Rsquare	0.48004
Adj Rsquare	0.410712
Root Mean Square Error	5893.499
Mean of Response	23333.33
Observations (or Sum Wgts)	18

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Grupos	2	481000000	240500000	6.9242	0.0074
Error	15	521000000	34733333		
C. Total	17	1002000000			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
serHA	6	30500.0	2406.0	25372	35628
serTi	6	18500.0	2406.0	13372	23628
serZi	6	21000.0	2406.0	15872	26128

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
serHA	6	30500.0	7449.83	3041.4	24017	36983
serTi	6	18500.0	5357.24	2187.1	13838	23162
serZi	6	21000.0	4472.14	1825.7	17109	24891

Means Comparisons

Dif=Mean[i]-Mean[j]

	serHA	serZi	serTi
serHA	0.0	9500.0	12000.0
serZi	-9500.0	0.0	2500.0
serTi	-12000.0	-2500.0	0.0

Alpha=0.05

Comparisons for all pairs using Tukey-Kramer HSD

q*

2.59747

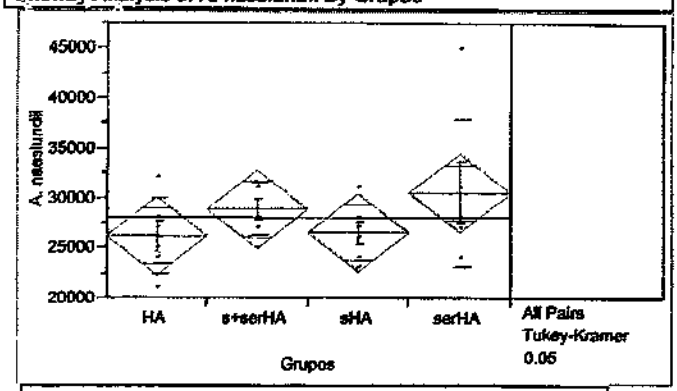
Abs(Dif)-LSD

	serHA	serZi	serTi
serHA	-6838.19	661.81	3161.81
serZi	661.81	-6838.19	-6338.19
serTi	3161.81	-6338.19	-6838.19

Positive values show pairs of means that are significantly different.

Anaeslunstat Oneway

Oneway Analysis of A. naeslundii By Grupos



Oneway Anova

Summary of Fit

Rsquare	0.151272
Adj Rsquare	0.023963
Root Mean Square Error	4597.101
Mean of Response	28000
Observations (or Sum Wgts)	24

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Grupos	3	75333333	25111111	1.1882	0.3394
Error	20	422666667	21133333		
C. Total	23	498000000			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
HA	6	26166.7	1876.8	22252	30082
s+serHA	6	28833.3	1876.8	24918	32748
sHA	6	26500.0	1876.8	22585	30415
serHA	6	30500.0	1876.8	26585	34415

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
HA	6	26166.7	3763.88	1536.6	22961	29372
s+serHA	6	28833.3	2582.55	1046.2	26651	31016
sHA	6	26500.0	2880.97	1176.2	24047	28953
serHA	6	30500.0	7449.83	3041.4	24156	36844

Means Comparisons

Dif=Mean[i]-Mean[j]

	serHA	s+serHA	sHA	HA
serHA	0.00	1666.67	4000.00	4333.33
s+serHA	-1666.67	0.00	2333.33	2666.67
sHA	-4000.00	-2333.33	0.00	333.33
HA	-4333.33	-2666.67	-333.33	0.00

Alpha=0.05

Comparisons for all pairs using Tukey-Kramer HSD

q*

2.79894

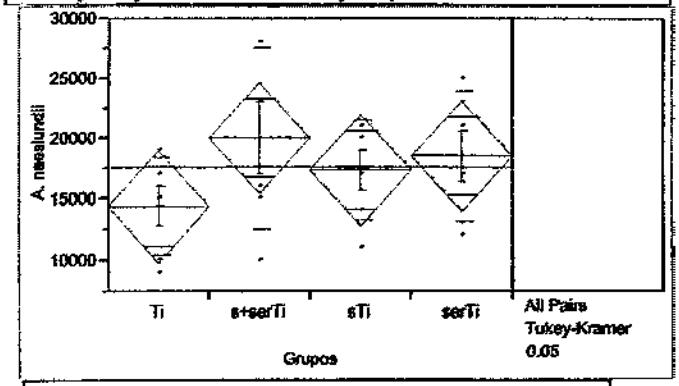
Abs(Dif)-LSD

	serHA	s+serHA	sHA	HA
serHA	-7428.76	-5762.10	-3428.76	-3095.43
s+serHA	-5762.10	-7428.76	-5095.43	-4762.10
sHA	-3428.76	-5095.43	-7428.76	-7095.43
HA	-3095.43	-4762.10	-7095.43	-7428.76

Positive values show pairs of means that are significantly different.

Anaestunstat: Oneway

Oneway Analysis of A. naestundii By Grupos



Oneway Anova

Summary of Fit

Rsquare	0.150432
Adj Rsquare	0.022997
Root Mean Square Error	5413.717
Mean of Response	17541.67
Observations (or Sum Wgts)	24

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Grupos	3	103791667	34597222	1.1805	0.3422
Error	20	586166667	29308333		
C. Total	23	689958333			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Ti	6	14333.3	2210.1	9723	18944
s+serTi	6	20000.0	2210.1	15390	24610
sTi	6	17333.3	2210.1	12723	21944
serTi	6	18500.0	2210.1	13890	23110

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
Ti	6	14333.3	3963.30	1628.2	10941	17725
s+serTi	6	20000.0	7456.54	3044.1	13650	26350
sTi	6	17333.3	4131.16	1686.5	13815	20851
serTi	6	18500.0	5357.24	2187.1	13938	23062

Means Comparisons

Dif=Mean[i]-Mean[j]

	s+serTi	serTi	sTi	Ti
s+serTi	0.00	1500.00	2666.67	5666.67
serTi	-1500.00	0.00	1166.67	4166.67
sTi	-2666.67	-1166.67	0.00	3000.00
Ti	-5666.67	-4166.67	-3000.00	0.00

Alpha=0.05

Comparisons for all pairs using Tukey-Kramer HSD

q*

2.79894

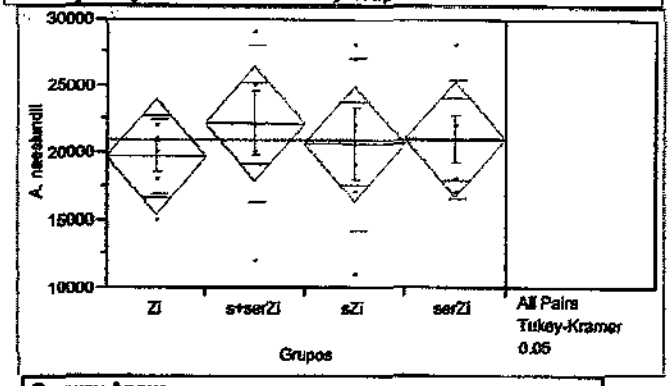
Abs(Dif)-LSD

	s+serTi	serTi	sTi	Ti
s+serTi	-6748.39	-7248.39	-6081.72	-3081.72
serTi	-7248.39	-6748.39	-7581.72	-4581.72
sTi	-6081.72	-7581.72	-6748.39	-5748.39
Ti	-3081.72	-4581.72	-5748.39	-6748.39

Positive values show pairs of means that are significantly different.

Anaeslunstat: Oneway

Oneway Analysis of A. naeslundii By Grupos



Oneway Anova

Summary of Fit

Rsquare	0.035907
Adj Rsquare	-0.10871
Root Mean Square Error	5067.05
Mean of Response	20875
Observations (or Sum Wgts)	24

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Grupos	3	19125000	6375000	0.2483	0.8616
Error	20	513500000	25675000		
C. Total	23	532625000			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Zi	6	19666.7	2068.6	15352	23982
s+serZi	6	22166.7	2068.6	17852	26482
sZi	6	20666.7	2068.6	16352	24982
serZi	6	21000.0	2068.6	16685	25315

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
Zi	6	19666.7	2732.52	1115.5	17340	21994
s+serZi	6	22166.7	5845.23	2386.3	17189	27144
sZi	6	20666.7	6406.33	2616.2	15209	26124
serZi	6	21000.0	4472.14	1825.7	17192	24808

Means Comparisons

Dif=Mean[i]-Mean[j]

	s+serZi	serZi	sZi	Zi
s+serZi	0.00	1166.67	1500.00	2500.00
serZi	-1166.67	0.00	333.33	1333.33
sZi	-1500.00	-333.33	0.00	1000.00
Zi	-2500.00	-1333.33	-1000.00	0.00

Alpha=0.05

Comparisons for all pairs using Tukey-Kramer HSD

q*

2.79864

Abs(Dif)-LSD

	s+serZi	serZi	sZi	Zi
s+serZi	-8168.19	-7021.52	-6668.19	-5668.19
serZi	-7021.52	-8168.19	-7854.85	-6854.85
sZi	-6668.19	-7854.85	-8168.19	-7168.19
Zi	-5668.19	-6854.85	-7168.19	-8168.19

Positive values show pairs of means that are significantly different.