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ETIOLOGIA, PROGRESSÃO E TRATAMENTO DAS PERI-IMPLANTITES

Tese apresentada à Faculdade de Odontologia de Araraquara, Universidade Estadual Paulista "Júlio de Mesquita Filho", como parte dos requisitos para a obtenção do título de Doutor do Curso de Pós-Graduação em Odontologia, Nível de Doutorado, Área de Periodontia.
Orientador: *Prof. Dr. Elcio Marcantonio Junior*

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1. PREFÁCIO

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I . Shibli JA, Martins MC, Lotufo RFM, Marcantonio Jr. E. Microbiologic and radiographic analysis of ligature-induced peri-implantitis with different dental implant surfaces *(Parcialmente aceito para publicação na International Journal of Oral and Maxillofacial Implants)*

II. Shibli JA, Martins MC, Jordan SF, Araujo MB, Haraszthy VI, Zambon JJ, Marcantonio Jr. E. Detection of periodontal pathogens in ligature-induced peri-implantitis. An experimental study in dogs *(Submetido ao Journal of Periodontology)*

III. Shibli JA, Martins MC, Jordan SF, Haraszthy VI, Zambon JJ, Marcantonio Jr. E. Progression of experimental chronic peri-implantitis. Clinical and microbiological evaluation in a canine model *(Finalizado para o envio ao Clinical Oral Implants Research)*

IV. Shibli JA, Martins MC, Theodoro LH, Lotufo RF, Garcia VG, Marcantonio Jr. E. Lethal photosensitization in microbiological treatment of ligature-induced peri-implantitis: a preliminary study in dogs. *Journal of Oral Science 2003, (in press)*

V. Shibli JA, Martins MC, Nociti Jr. FH, Garcia VG, Marcantonio Jr. E. Treatment of ligature-induced peri-implantitis by lethal photosensitization and guided bone regeneration: a preliminary histologic study in dogs. *J Periodontol* 2003, 74; 338-345.

VI. Shibli JA, Martins MC, Nociti Jr. FH, Marcantonio Jr. E. Guided bone regeneration and lethal photosensitization in treatment of ligature-induced peri-implantitis in different dental implants surfaces. A histomorphometrical study in dogs. (*Finalizado para submissão ao Journal of Periodontology*)

VII. Shibli JA, Jordan SF, Haraszthy VI, Zambon JJ, Marcantonio Jr. E. Host response and microbiological evaluation of peri-implantitis in patients with periodontal diseases *(Submetido ao Journal of Periodontology)*

VIII. Shibli JA, Silverio KG, Martins MC, Marcantonio Jr. E., Rossa Jr. C. Effect of air-powder system in titanium surface on fibroblast adhesion and morphology *Implant Dent* 2003, 12 (in press)

IX. Shibli JA, Marcantonio E, d'Avila S, Guastaldi AC, Marcantonio Jr. E. Analysis of failed dental implant surfaces. (*Finalizado para submissão - International Journal of Oral and Maxillofacial Implants*)

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INTRODUÇÃO E JUSTIFICATIVA

A necessidade de um tecido saudável ao redor dos implantes osseointegrados é essencial para obtenção de sucesso a longo prazo. (El Askary et al.[15] 1999; Shibli et al.[72] 2003). Vários autores têm estudado o papel do biofilme bacteriano no desenvolvimento da peri-implantite em humanos (Mombelli et al.[44] 1987; Mombelli et al.[45] 1988; Mombelli et al.[47] 1995; Lee et al.[39] 1999; Listgarten & Lai,[41] 1999) e também em modelos animais por meio da utilização de ligaduras, juntamente com a cessação dos procedimentos de higienização bucal (Akagawa et al.[4]1993; Lang et al.[37] 1993; Shou et al.[69] 1996; Hanisch et al.[28] 1997; Eke et al.[14] 1998; Tillmanns et al.[77] 1998).

O papel do biofilme bacteriano na falência dos implantes osseointegrados sob função tem atraído a atenção de muitos pesquisadores. No entanto algumas controvérsias em relação às condições que propiciam um maior risco para a peri-implantite, tais como a influência da macroestrutura e microestrutura, tipos de superfícies, pacientes tratados periodontalmente, pacientes com doença periodontal crônica, microbiota, histofisiologia do tecido peri-implantar e oclusão, ainda não estão totalmente esclarecidos (Quirynen et al.[63] 2002, van Steenberghe et al.[79] 1999; Esposito et al.[18] 1998, Mombelli[51] 2002, Mombelli & Lang,[50] 1998, Shibli & Marcantonio[73] 2002).

Segundo Ellen[16] (1998), os modelos de periodontite e peri-implantite induzidos por ligadura têm sido usados por diversas razões: confirmar a seqüência de alterações na composição bacteriana (aeróbios Gram-positivos) antes da ocorrência de lesões destrutivas (anaeróbia Gram-negativa); confirmar

o efeito exacerbado da *Porphyromonas gingivalis* quando super-infectante na microbiota periodontal ou peri-implantar; testar a importância da infecção experimental e trauma oclusal (Isidor,[32] 1997; Hurzeler et al.[30] 1998) na progressão das lesões periodontais e peri-implantares e prover lesões reprodutíveis que possam ser tratadas de acordo com vários protocolos: debridamento das lesões utilizando jatos abrasivos e/ou curetas de teflon (Hurzeler et al.,[29] 1997; Wetzel et al.[82] 1999) associados a antimicrobianos e antibióticos (Ericsson et al.[17] 1996); cirúrgicos, RTG, lasers de baixa intensidade (Haas et al.[25] 1997, Haas et al.[26] 2000) e associações. (Persson et al.[59] 1996; Grunder et al.[23] 1993; Jovanovic et al.[34] 1993)

No entanto, os tratamentos desses defeitos peri-implantares, por serem de origem bacteriana, são de difícil prognóstico, já que pesquisas buscando otimizar o processo de osseointegração (Buser et al.[8] 1991; Buser et al.[9] 1998), têm desenvolvido micro e ultraestruturas (plasma *spray* de titânio, superfícies modificadas por meio de ácidos e superfícies jateadas com óxidos), que dificultam o debridamento e detoxificação tanto da superfície do implante quanto da superfície peri-implantar. No entanto, ainda são escassos os estudos que visam esclarecer qual tipo de superfície de implante osseointegrado é mais ou menos favorável à progressão e tratamento da peri-implantite (Esposito et al.[18] 1998; Esposito et al.[19] 1998; Shibli et al.[71] 2003; Shibli et al.[72] 2003).

Algumas importantes diferenças entre os diversos tipos de superfícies que recobrem os implantes osseointegrados parecem influenciar a adsorção e colonização bacteriana (Nakazato et al.[52] 1989; Gatewood et al.[20] 1993;

Quirynen et al.[62] 1993; Bollen et al.[7] 1996; Rimondini et al. 1997; Ichikawa et al.[31] 1998; Rasperini et al.[64] 1998; Steinberg et al.[76] 1998). Sabe-se, também, que com o aumento da rugosidade da superfície do implante há uma maior dificuldade para os procedimentos de higienização/manutenção da saúde dos tecidos peri-implantares assim como no tratamento dos defeitos ósseos peri-implanteres (Persson et al.[60] 2001; Persson et al.[61] 2001). A presença de porosidades e irregularidades, inerentes a superfícies de implantes tratadas com ácidos ou jateadas com titânio plasma *spray* e hidroxiapatita, funcionariam como nichos perfeitos para a proliferação bacteriana, desde que expostas ao meio bucal, tendo assim uma rota direta para a infecção do tecido ósseo peri-implantar (Siegrist et al.[74] 1991; Gatewood et al.[20] 1993; Bollen et al.[7] 1996; Ichikawa et al.[31] 1998), enquanto uma diminuição da rugosidade da superfície dos implantes abaixo de R_a de 0,2µm poderia retardar a maturação do biofilme supra e subgengival (Bollen et al.[7]1995).

De forma semelhante à doença periodontal, a peri-implantite é resultante do desequilíbrio hospedeiro-microrganismo que pode manifestar por meio de uma série de mudanças inflamatórias levando a duas síndromes distintas: mucosite peri-implantar que é uma lesão confinada aos tecidos moles peri-implantares e peri-implantite que envolve, além dos tecidos moles, o tecido ósseo adjacente ao implante osseointegrado (Mombelli & Lang,[49] 1998; Tonetti & Schmid,[80] 1994; Lie et al.[39] 1995).

Enquanto, não há um consenso sobre a microbiota presente na doença peri-implantar, na doença periodontal, microrganismos, atuando isolados ou em

combinações, como *Actinobacillus actinomycetemcomitans, Bacteroides forsythus, Porphyromonas gingivalis, Prevotella intermedia, Campylobacter rectus, Fusobacterium nucleatum, Prevotella nigrescens, Peptostreptococcus micros, Eubacterium nodatum e Treponema* spp. são freqüentemente associadas a doença (Zambon,[84] 1996; Socransky & Haffajee,[75] 2002); enquanto *Porphyromonas gingivalis* e *Prevotella intermedia* estão diretamente associadas à indução e progessão das doenças periodontais. (Renvert et al.[65] 1996; Zambon[84] 1996; Socransky & Haffajee,[75] 2002)

Uma clara relação entre acúmulo do biofilme dental e mudanças inflamatórias superficiais (mucosite peri-implantar) tem sido demonstrada em estudos envolvendo modelos animais (Abrahamsson et al.[2] 1988; Berglundh et al.[6] 1992, Schou et al.[70] 2002) e humanos (Bollen et al.[7] 1996).

Alguns autores têm utilizado modelos animais induzindo a peri-implantite por meio de ligaduras ao redor dos implantes (Akagawa et al.[4]1993; Lang et al.[37] 1993, Schou et al.[69] 1996; Hanisch et al.[28] 1997; Eke et al.[14]1998; Tillmanns et al.[77] 1998; Nociti et al.[53] 2001, Schou et al.[70] 2002) demonstrando a importância do biofilme bacteriano no processo de falência desses implantes. Tonetti & Schmid[80] (1994) chegaram a afirmar que esses estudos indicam, inequivocamente, que a peri-implantite induzida por ligadura é resultado da ação de uma microbiota patogênica. Alguns trabalhos (Abrahamsson et al.[2]1998; Berglundh et al.[6] 1992) evidenciaram tal afirmação, no qual o acúmulo de biofilme sem a presença de ligaduras não induziu à peri-implantite, mas a uma mucosite peri-implantar, já que as ligaduras poderiam levar à uma modificação

tecidual ou a um trauma mecânico (Esposito et al.[19] 1998), sem no entanto haver um desequilíbrio "verdadeiro" entre a relação hospedeiro-parasita.

Neste contexto, ainda não está bem demonstrado que a presença de periodontopatógenos necessariamente tem como conseqüência a destruição do tecido peri-implantar (Mombelli et al.[47] 1995, Zambon[84] 1996), entretanto a detecção de periodontopatógenos poderia evidenciar um aumento no risco para a progressão das doenças peri-implantares.

A correlação entre a profundidade da bolsa peri-implantar e a presença de espiroquetas e bastonetes móveis Gram-negativos (Papaioannou et al.[57] 1995) sugerem que a profundidade da bolsa peri-implantar, que depende também da espessura e qualidade da mucosa peri-implantar (Warrer et al.[81] 1995, Toljanic et al.[78] 2001; Schou et al.[70] 2002), proporciona um ambiente adequado para o crescimento bacteriano.

Outra evidência indireta sobre o importante papel dos microrganismos na falência dos implantes pode ser observado nos estudos que analisaram a natureza do tecido peri-implantar (Sanz et al.[66] 1991) ou a composição do fluido crevicular peri-implantar (Apse et al.[5] 1986). Jepsen et al.[33](1996) sugeriram, ainda, que as enzimas proteóliticas de origem bacteriana seria um indicador importante no diagnóstico e monitoramento dos implantes ao longo da fase de manutenção.

Os achados microbiológicos sugerem que a infeção peri-implantar é, possivelmente, a causa da perda tardia dos implantes, ou seja, após estarem sob função mastigatória. Os microrganismos mais associados com a doença

peri-implantar são as espiroquetas, bastonetes, organismos Gram-negativos facultativos ou anaeróbios estritos, tais como *Porphyromonas gingivalis*, *Prevotella intermedia* e *Actinobacillus actinomycetemcomitans*. Estas bactérias podem lesar os tecidos peri-implantares de diferentes maneiras: invasão e destruição direta dos tecidos peri-implantares por meio da liberação de enzimas, subprodutos e fatores de reabsorção óssea, evasão das defesas do hospedeiro, indução de uma reação inflamatória mediada pelo sistema imune do hospedeiro ou ainda uma combinação dos fatores citados. Similaridades nos componentes encontrados nos fluidos gengival e peri-implantar demonstraram que existe um mecanismo análogo que controla a resposta imune e inflamatória ao redor de dentes e implantes (Adonogianaki et al.[3]1995).

Bactérias como a *Capnocytophaga* spp. estão associadas ao desenvolvimento de gengivites e podem participar da transição entre gengivite e periodontite, sendo menos importante no estabelecimento da doença periodontal e até nas doenças peri-implantares (Mombelli & Mericske-Stern,[46] 1990). *Porphyromonas gingivalis* e *Prevotella intermedia* parecem estar associadas à progressão e indução da doença periodontal (Renvert et al.[65] 1996). *Treponema denticola*, uma das principais representantes do grupo das espiroquetas, o *Fusobacterium nucleatum* e *Campylobacter rectus* foram também associados às doenças periodontal e peri-implantar. *Eikenella corrodens* tem papel pouco esclarecido sobre a sua participação na patogênese da peri-implantite (Papaioannou et al.[58] 1996). *Bacteroides forsythus* tem sido relatado em poucos estudos já que, somente técnicas de biologia molecular tais como a reação de

polimerase em cadeia (PCR) e a hibridização de sondas de DNA parecem detectar sua presença, pois a cultura falha na grande maioria das vezes (Danser et al.[10] 1997).

Os diferentes métodos utilizados para detecção dos microrganismos presentes na peri-implantite (cultura, PCR e sondas de DNA), poderiam justificar os diferentes resultados encontrados na literatura.

A resposta local do hospedeiro frente à infecção bacteriana levanta algumas questões que necessitam ser discernidas e respondidas. Será que a perda de inserção e perda óssea ocorrem com maior velocidade no implante de superfície lisa quando compara ao implante de superfície tratada? A diferente histofisiologia do tecido peri-implantar aumenta as condições de risco frente o acúmulo do biofilme bacteriano? Existe uma correlação entre obtenção de re-osseointegração e superfície de implante? Esta superfície apresenta alguma característica especial em implantes falidos? Está associado ao biofilme bacteriano?

PROPOSIÇÃO GERAL

O objetivo geral deste trabalho é avaliar o modelo de estudo animal, etiologia e tratamento das peri-implantites.

Objetivos Específicos

- Analisar clínica e microbiologicamente a peri-implantite induzida por ligadura em diferentes superfícies de implantes osseointegrados (*Artigos I, II e III*)
- Avaliar, microbiologicamente, o efeito da utilização do laser de baixa intensidade associado a agente fotossensibilizador no tratamento das peri-implantites (*Artigo IV*)
- Analisar o potencial de re-osseointegração de defeitos peri-implantares após a utilização de laser de baixa intensidade associado a agente fotossensibilizador e Regeneração Óssea Guiada (ROG) (*Artigos V e VI*)
- Comparar a resposta do hospedeiro frente as deonças periodontal e peri-implantar (*Artigo VII*)
- Verificar a biocompatibilidade da superfície de titânio após a utilização de jato de bicarbonato por meio de cultura de células assim como a presença de contaminantes na superfície de implantes falidos removidos precocemente ou após estarem sob função (*Artigos VIII e IX*)

CAPITULO 5 - MICROBIOLOGIC AND RADIOGRAPHIC ANALYSIS OF LIGATURE-INDUCED PERI-IMPLANTITIS WITH DIFFERENT DENTAL IMPLANT SURFACES

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MICROBIOLOGIC AND RADIOGRAPHIC ANALYSIS OF LIGATURE-INDUCED PERI-IMPLANTITIS IN DIFFERENT DENTAL IMPLANT SURFACES

ABSTRACT

PURPOSE: The goal of this study was to evaluate microbiota and radiographic peri-implant bone loss associated with ligature-induced peri-implantitis.

METHOD AND MATERIALS: In 6 dogs, 36 dental implants with four different surfaces (9 CPTi, 9 TPS, 9 HA, and 9 acid-etched) were placed in the edentulous mandibles. After 3 months with optimal plaque control, abutment connection was performed. On day 0, 20, 40, and 60 after cotton ligature placement, both microbiologic samples and periapical radiographs were obtained. The presence of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia/nigrescens*, *Campylobacter* spp., *Capnocytophaga* spp., *Fusobacterium* sp., beta-hemolytic *Streptococcus* and *Candida* spp. was evaluated culturally.

RESULTS: *P. intermedia/nigrescens* was detected in 13.89% at baseline and 100% of implants at other periods. *P. gingivalis* was not detected at baseline; but after 20 and 40 days it was detected in 33.34% and 60 days, 29.03% of dental implants. *Fusobacterium* spp. was detected in all periods. Streptococci were detected in 16.67% at baseline and 83.34; 72.22 and 77.42% of implants respectively at 20, 40, and 60 days. *Campylobacter* spp. and *Candida* spp. were detected in low proportions. The total viable count analysis showed no significant differences among surfaces (P=0.831), although significant difference was observed after ligature placement ($P \leq 0.0014$). However, there was no significant qualitative difference, in spite of the difference among the periods. The peri-implant bone loss was not significant among the dental implant surfaces (P=0.908).

CONCLUSIONS: These data suggest that with ligature-induced peri-implantitis both time period and periodontal pathogens affect all surfaces equally after 60 days.

KEY WORDS: Dental implants; ligature-induced/peri-implantitis; microbiology/periodontal pathogens; periapical radiograph; dogs; dental implants/surfaces.

INTRODUCTION

Healthy soft and hard peri-implant tissue around dental implants is essential for long term success.[1,2] The relationship between different dental implant surfaces and bacterial biofilm in peri-implantitis development has not been completely studied. Cross-sectional microbiologic studies of dental implants with clinically healthy marginal peri-implant tissues in humans[3-8] and animals[9,10] have demonstrated a scattered submucosal microbiota dominated by facultative Gram-positive cocci and rods. In contrast, failing dental implants have been associated with periodontal pathogens such as fusobacteria, spirochets, *Actinobacillus actinomycetemcomitans*, the black-pigmented species *Porphyromonas gingivalis* and *Prevotella intermedia* and *Campylobacter* rectus.[3,11-13] These bacterial shifts have been reported to be caused by peri-implant bone loss resulting in osseointegration failure.[10,14]

The importance of microbiologic factors for the development and progression of pathologic conditions in the tissues supporting dental implants is controversial. In addition, studies seeking to determine which implant surface (microstructure) or coating is more favorable for the progression of the peri-implantitis are scarce.

Therefore, the aims of this study were: (1) to identify, by culture tests, the presence of periodontal pathogens and (2) to evaluate peri-implant bone loss by standardized radiography in ligature-induced peri-implantitis in dogs with endosseous implants having different surfaces.

MATERIAL AND METHODS

Animals and Anesthesia

Six adult, systemically healthy, male mongrel dogs were used. Dogs were 2-years old with an average weight of 18Kg. Animal selection, management, and surgical protocol routines were approved by the Dental School at Araraquara Institutional Animal Care and Use Committee. All surgical and clinical procedures, as well as the removal of microbial samples, were performed under general anesthesia accomplished by 0.05mg/Kg of subcutaneous preanesthesia sedation (atropine sulphate) and intravenous injection of chlorpromazine and thiopental.

Oral prophylaxis was performed up to 2 weeks before teeth extraction. All mandibular premolars were then extracted creating an edentulous ridge and both the mandibular quadrants and the alveoli were allowed to heal for a period of 3 months. The maxillar premolars were extracted to avoid occlusion trauma interference. Plaque control was instituted during the healing period by scrubbing daily with 0.12% chlorhexidine, scaling and root planing once a month, until ligature placement (Fig. 1).

Implant Design

Thirty-six dental implants with four different surfaces involving three different implant systems were used in this study. Nine commercially pure titanium implants (CPTi, 3*i*® Implants Innovations, Palm Beach Gardens, FL, USA), nine titanium plasma sprayed implants (TPS, *Esthetic plus* ITI®, Straumann AG, Waldenburg, Switzerland), nine hydroxyapatite (HA, Calcitek®, Sulzer Medica, Carlsbad, CA, USA) and nine hybrid surface–machined titanium in the three first threads and acid-etched in other threads (Acid, *Osseotite*®-3*i*® Implants Innovations, Palm Beach

Gardens, FL, USA) were used. All implants had lengths of 10mm and diameters of 3.75mm (except TPS which had a diameter of 4.1mm) (Fig.2).

Surgical Procedures

Under aseptic surgical conditions, the dental implants were placed after preparation of a full thickness flap. The recipient sites were prepared using original instruments for each dental implant surface, according to the surgical techniques indicated by each implant manufacturer. The implants were randomly distributed among the dogs so that each dental implant surface was represented at least once in each animal (TABLE 1). The implants were placed at the bone level and a cover screw was screwed onto the implant, including the TPS dental implant which had been modified in technique placement as indicated by the manufacturer. The flaps were sutured with single interrupted sutures to submerge all implants. Antibiotic coverage with potassium and sodium benzyl penicillin was given once a week, for 2 weeks, to avoid post-surgical infection. Paracetamol was given for pain control medication, and the sutures were removed after 10 days. A soft diet was instituted post-surgically.

After a healing period of 3 months, healing abutment connections were made, according to the indication of each dental implant system. After 45 days and healing of the soft tissue, cotton floss ligatures were placed in a submarginal position around the dental implants and sutured in peri-implant mucosa to hold the ligatures in position. The positions of the ligatures were checked twice a week. Peri-implant bone loss was accelerated by tying further ligatures at 20-day intervals for a period of 60 days, or until the implants had a loss of about 40% of radiographic bone height.[15]

Microbial Samples

Peri-implant microbial samples were obtained with paper points immediately before the ligature placement and 20, 40 and 60 days after ligature placement from the mesio-distal sites of all dental implants, as described by Slots et al.[16]

Supramucosal debridement at the sample site was initially performed with a sterile plastic curette and dry gauze after isolation from saliva using cotton tips/wool and suction. Four sterile paper points were subsequently inserted into the peri-implant sulci, as far apical as possible, for a period of 20 seconds, at the baseline and immediately after removal of the ligature at 20, 40 and 60 days. The paper points and cotton floss ligatures were removed and placed into to a 3mL vials containing VMGA III anaerobic transport medium.[17] All samples were collected by the same operator and coded by an assistant for blind identification. The microbiologic procedures were initiated within 24 hours.

The samples were centrifuged for 60s and serially diluted 10-fold in peptonated water to between 10^{-1} and 10^{-6} for quantitative evaluation of CFU/ml and to obtain isolated colonies for qualitative identification. Aliquots of 0.1 ml of the dilutions were plated onto Enriched Tryptic Soy Agar (ETSA)[18] and Tryptic Soy-Serum-Bacitracin-Vancomycin agar (TSBV)[19] in a standardized manner. ETSA plates were incubated in anaerobic jars containing an atmosphere with mixed gas (85% N_2, 10% H_2, 5%CO_2) at 37^{o}C for 7 to 10 days. TSBV agar plates were incubated in a 5% CO_2 atmosphere for 5 days at 37^{o}C. The bacterial species were identified from anaerobic cultures based on gram-stain, aerotolerance, colony morphology, esculin hydrolysis,[20] [alpha]-glucosidase and N-benzoyl-DL-arginine-2-naphthylamide (BANA) hydrolysis,[21] oxidase and catalase activities. Total viable count (TVC) and cultivable microbiota, including *Porphyromonas gingivalis,*

Prevotella intermedia/nigrescens, *Fusobacterium* spp., *Capnocytophaga* spp., beta-hemolytic *Streptococcus*, *Campylobacter* spp. and *Actinobacillus actinomycetemcomitans*, detection were performed based on colony morphology and positive catalase tests.[19] *Candida* spp. identification was also performed.

Radiographs

Baseline periapical radiographs were taken at the time of ligature placement, 20, 40 and 60 days after ligature-induced peri-implantitis to evaluate changes in bone levels. The standardized radiographs were obtained with a customized occlusal index fabricated from a film holder by affixing a silicone bite block made of polyvinyl siloxane putty impression material.

A dental x-ray machine equipped with a 35-cm-long cone was used to expose the periapical intraoral film (Agfa Dentus, Size 0, Agfa Gevaert, Mortsel, Belgium). Exposure parameters were 70 kilivolt (peak), 15 mA, and ¼ second at a focus-to-sensor distance of 37cm. The linear distance between a fixed point in the abutment and the first visible bone-to-implant contact was determined at the mesial and distal of each implant digital image. The mesial and distal values were averaged to obtain a mean implant value. Relative peri-implant bone loss was measured to avoid interference by the different dental implant macrostructures used in this study.

All measurements were made independently by 2 of the authors. If discrepancies were of 0.5mm or less, the mean value of the 2 measurements was used. In situations with greater discrepancies, the images were analyzed again and discussed until consensus was reached.

Data Analysis

The TVC were transformed into colony forming units per mL (CFU/mL) using predetermined conversion factors to account for dilution and the size of the

evaluated surface on the plate. Data were then analyzed for dental implant surface and time of ligature placement and relative bone loss via nonparametric analysis of variance (Kruskal-Wallis test) with alpha 0.05. Differences between groups were assessed by the Dunn test. Microorganism analysis was performed after logarithmic transformation. All test were stratified according to dog (unit of analysis), i.e., n=6.

RESULTS

Microbiologic Analysis

Microbiological data were available for analysis from 36 sites/implants in 6 dogs (6 sites per animal). Five implants (2CPTi, 1HA and 2 acid-etched) did not receive ligatures within 40 days of ligature-induction since they demonstrated 40% bone loss, therefore at 60 days, just 31 implants were analyzed. Therefore, hundred thirty-nine microbiological samples were analyzed over the experimental period.

TABLE 2 summarizes the positive samples for each dental implant surface at all times for each microorganism. *A. actinomycetemcomitans* and *Capnocytophaga* spp. could not be detected for any of the dental implants in this study.

In the TVC, there were no statistically significant differences between the dental implant surfaces (P=0.813). However, after the ligature placement, statistically significance was observed among the periods ($P \leq 0.0014$) (Fig. 3a). The measurements taken following ligature breakdown increased. The TPS and acid surface were observed, on average, to be less colonized. In relation to time, the baseline was statistically significantly different in relation to the other times.

Porphyromonas gingivalis was not detected at baseline. At other times low colonization was detected: 12 dental implants (4CPTi, 2TPS, 4HA, and 2 acid-etched) were colonized at days 20 and 40. At day 60, the number of positive implants decreased, 2CPTi and 1HA did not receive ligatures because there was a

40% peri-implant bone loss. There was no significant quantitative difference among the dental implant surfaces (P=0.704), neither between days 0 and 20, 40, and 60 (P>0.05) (Fig. 3b).

Prevotella intermedia/nigrescens was detected at baseline on 5 dental implants (2CPTi, 2HA, and 1 acid-etched). At days 20, 40, and 60 all implants were colonized. Quantitative difference was not significant among implant surfaces (P=0.877); significance was observed between day 0 and days 20, 40, and 60 ($P \leq 0.0033$) (Fig. 3c).

Fusobacterium spp. was identified on 4 dental implants (2CPTi and 2HA) at baseline. At days 20, 40, and 60, respectively, 24 (6CPTi, 7TPS, 6HA, and 5 acid-etched); 27(6CPTi; 8TPS; 7HA, and 6 acid-etched), and 25 (5CPTi, 7TPS, 7HA, and 6 acid-etched) dental implants were colonized by *Fusobacterium* spp. Significant difference was observed between day 0 and days 20, 40, and 60 ($P \leq 0.047$), except for CPTi surface (P=0.143). There was no significant difference between the different dental implant surfaces (P=0.375) (Fig.3d).

Seven dental implants (3CPTi, 1TPS, and 3HA) were beta-hemolytic *Streptococcus* positive at baseline. At the other times, 30 (7CPTi, 8TPS, 7HA, and 8 acid-etched), 26 (6CPTi, 6TPS, 6HA, and 8 acid-etched), and 24 (6CPTi, 6TPS, 6HA, and 6 acid-etched) dental implants were colonized at days 20, 40, and 60, respectively. Differences among the dental implant surfaces were not observed (P=0.993), although significant difference between times was demonstrated for CPTi and acid surfaces ($P \leq 0.0284$) (Fig.3e).

Campylobacter spp. was not identified at baseline. However, it was detected at days 20, 40, and 60, in 4 (2CPTi, 1TPS, and 1 acid-etched), 6(4CPTi, 1TPS, and 1 acid-etched), and 3 (1CPTi, 1TPS, and 1 acid-etched) dental implants,

respectively. Therefore significant difference was not observed among dental implant surfaces (P=0.425), so periods either (P>0.05) (Fig.3f).

Candida spp. was isolated at only 6 dental implants (2CPTi, 1TPS, and 1 acid-etched) at day 20.

Radiographic Analysis

At the start of ligature-induced peri-implantitis, the linear distance between the fixed point and first relative peri-implant bone loss was measured to avoid interference from the different macrostructures of the dental implants utilized in this study. The radiographically measured mean bone loss was observed at days 20, 40, and 60 (Figs 4a and 4b). No dental implant exhibited peri-implant radiolucencies at baseline.

Significant difference was not found between surfaces (P=0.908), despite the fact that the relative means of the TPS (1.79$\pm$1.52mm) and the acid-etched surface (1.62$\pm$1.32mm) were lower than those of the HA (1.94$\pm$1.59mm) and CPTi (2.09$\pm$1.70mm) implants among the periods (Fig. 5). Significant differences ($P\leq$0.005) were found between baseline and the other time points.

DISCUSSION

In this study, it was observed that ligature-enhanced bacterial biofilm accumulation around different dental implant surfaces resulted in rapid peri-implant tissue breakdown. Radiographically significant peri-implant bone loss was established within 60 days.

Tissue breakdown around different dental implant surfaces was accomplished by bacterial shift in a relatively short period (20 days), in agreement with Schou et al[9] and Nociti et al.[22] Other reports evaluated just the microbiota for longer periods: Hanisch et al[10] for 10 months and Tillmanns et al[14] for 3 months.

However, all reports found similar microbiota before and after ligature placement. The increase in radiographic bone loss takes place between days 0 and 60 in dogs, not because of mechanical trauma fom the ligature, but as result of peri-implant microbiota. These data are in accordance with those of Zappa & Polson;[23] Schou et al.[24] However,Tonetti[25] disagreed with this statement, and further studies could answer this question. Persson et al[26] reported that 6 weeks after ligature placement, about 20% bone loss was observed. Ligature-placement was (8 weeks) able to rapidly induce significant peri-implant bone loss, comparable with the studies of Hanisch et al[10], Tillmanns et al[14], Lang et al[27], and Hurzeler et al.[28]

Some important differences between the types of surfaces that affect dental implant microstructure and ultrastructure seem to influence the adsorption and the bacterial colonization. Statistical difference was not observed for TVC, although lower counts for TPS and acid-etched could be observed. Several studies[29,30] have shown that the presence and density of periodontal pathogens were influenced more by oral status than by the implant surface characteristics. Although this study used ligatures to facilitate bacterial colonization, TVC means were significantly higher in CPTi than in the TPS surface. It is speculated that the smooth surface present on the neck of TPS dental implants could explain these data. In the case of an acid-etched surface, which has a machined surface on the first three threads and a treated surface on the other threads, the difference in results compared with CPTi surface could be explained by oxide present after acid treatment. The presence of different oxides could influence the affinity of LPS (bacterial lipopolissacaride) for these components.[31] In addition, the sample size and the short period evaluated in present study could be explain these microbiologic and radiographic data.

The *P. gingivalis* and *P. Intermedia/nigrescens*, in this study, were associated with the induction and progression of the peri-implantitis, as well as with periodontal diseases.[32] *Fusobacterium* spp. and *Campylobacter* spp. which were also identified on some dental implant surfaces have also been associated with peri-implant diseases, according to Papaioannou et al[33], Macuch & Tanner[34], and Mombelli et al.[35] The greatest increase in bone loss was accomplished by the detection of these microorganisms. These microbiologic results confirm those of Mombelli et al[3], Mombelli et al[35], Mombelli et al[36], Shou et al[9], Hanisch et al[10], Tillmanns et al[14], Lee et al[7], and Listgarten & Lai[37].

The presence of beta-hemolytic *Streptococcus* agrees with the results of Hanisch et al[10], although this bacteria was not found in the same proportion. This bacterium was detected on five implants (62.5%) at 10 months in the study by Hanisch et al[10] in comparison to 30 implants (83.3%) at 20 days after ligature placement in the present study. The absence of this microorganism in the buccal cavity or at low frequency[38] indicates the existence of a low pH resulting from the induced peri-implantitis[39]. Leonhardt et al[40] reported the presence of *Candida* sp. in association with failing implants, in accordance with the present data.

The absence of *A. Actinomycetemcomitans* and *Capnocytophaga* sp. is not in accordance with the reports of Renvert et al[41], Shou et al[9], Hanisch et al[10] and Tillmanns et al[14]. The difference between the results of this study and the aforementioned studies is possibly related to diet, time of evaluation, marginal inflammation, ligature materials, use of clorhexidine and antibiotics, and different microbiologic methods (culture media, PCR and DNA probes).

In the present investigation, the association between increased viable counts of periodontal pathogens and peri-implant bone loss was evident. Thus, within the

limits of this study, it can be concluded that: (1) there was no quantitative significant statistical difference, considering the TVC on the different implant surfaces, without qualitative difference; (2) there was a bacterial shift at 20 days after the ligature placement, and (3) these data suggest that coating dental implant surfaces were as susceptible as smooth surfaces to ligature-induced peri-implantitis in 60 days.

ACKNOWLEDGMENTS

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ILLUSTRATIONS

Fig. 1

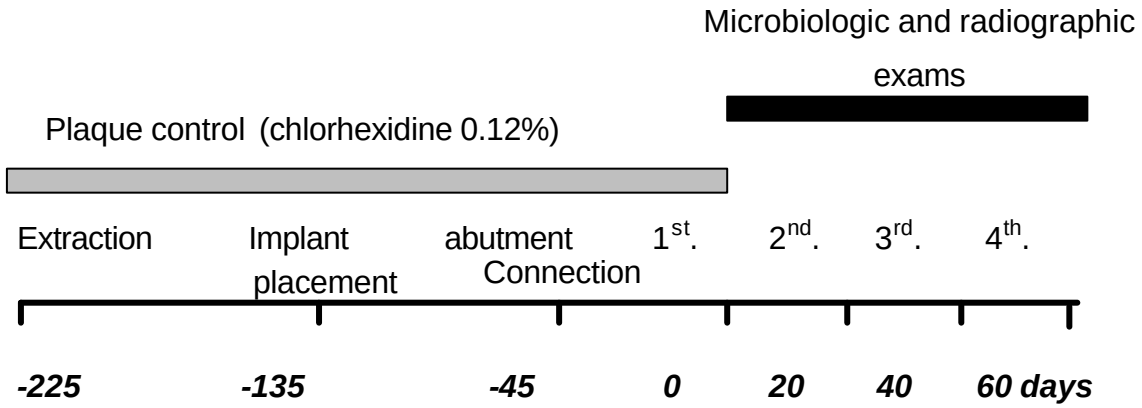
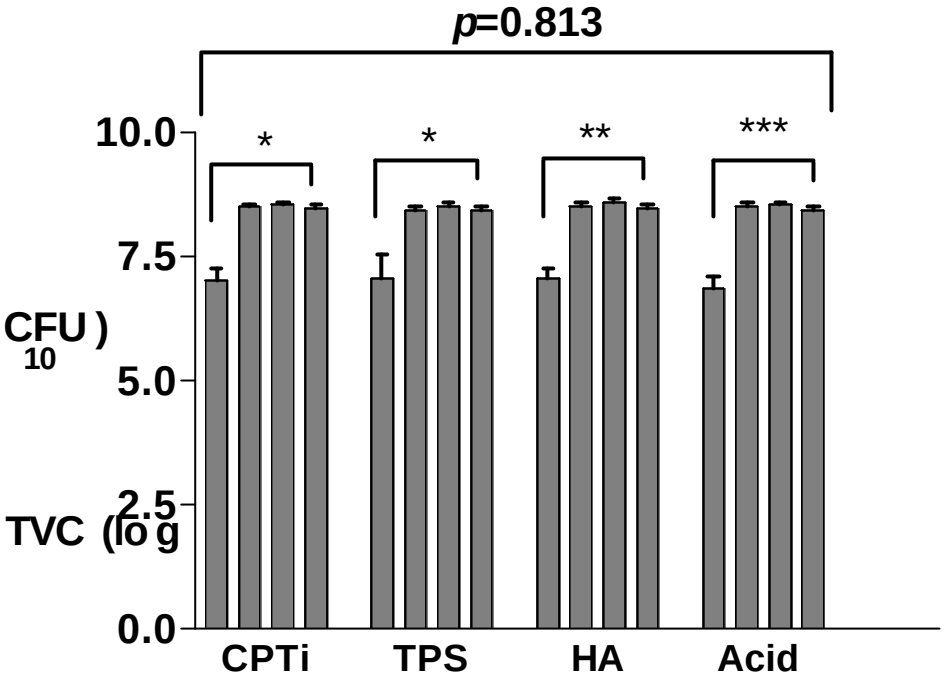


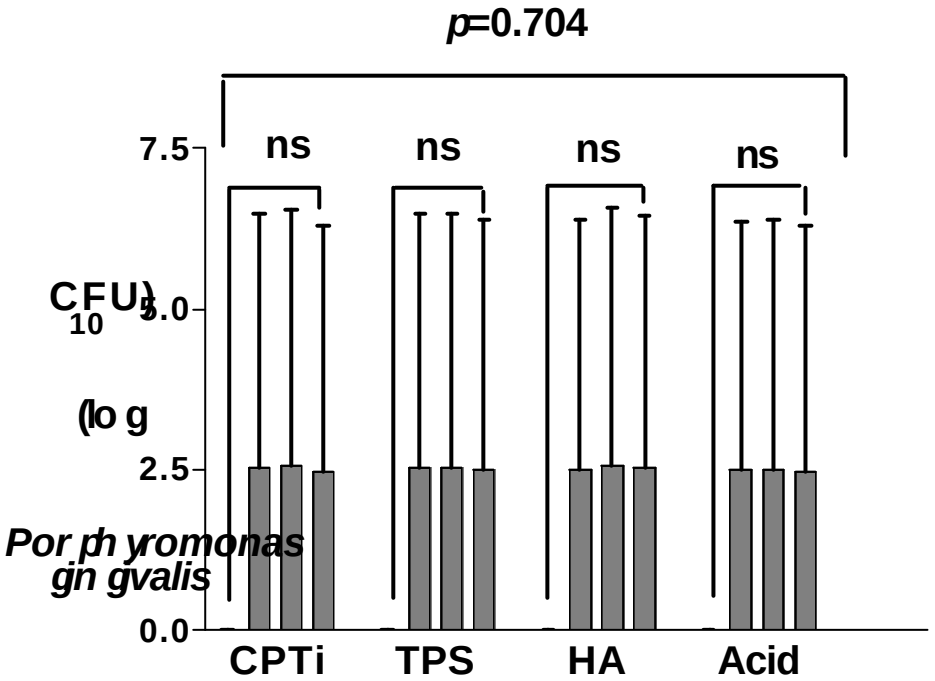
Figure 2

Fig. 3a



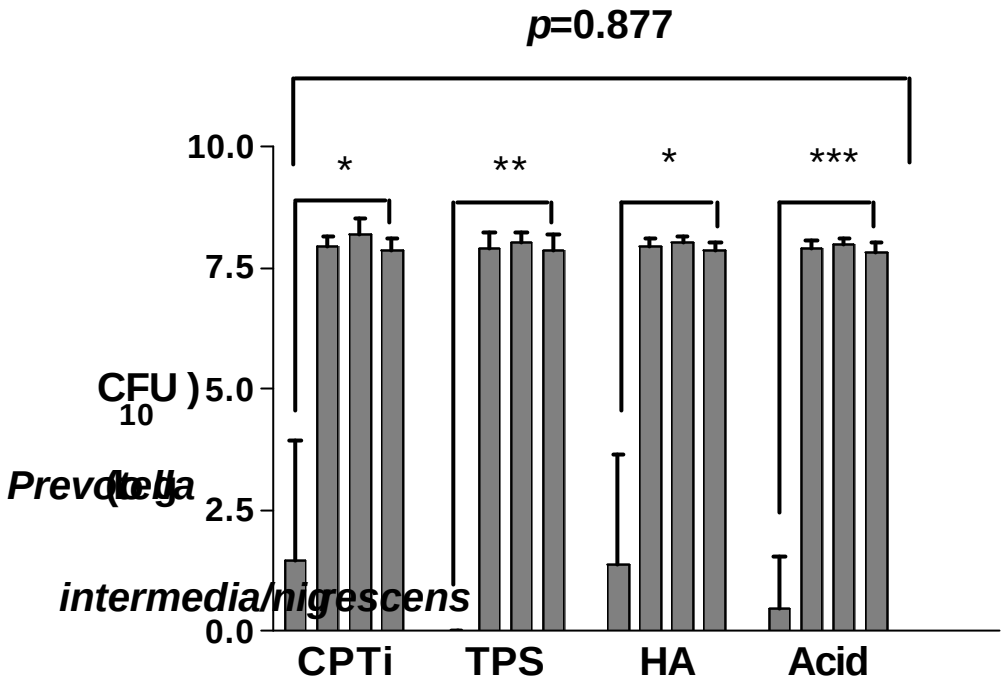
*p=0.0015; **p=0.0014; ***p=0.0024

Fig. 3b



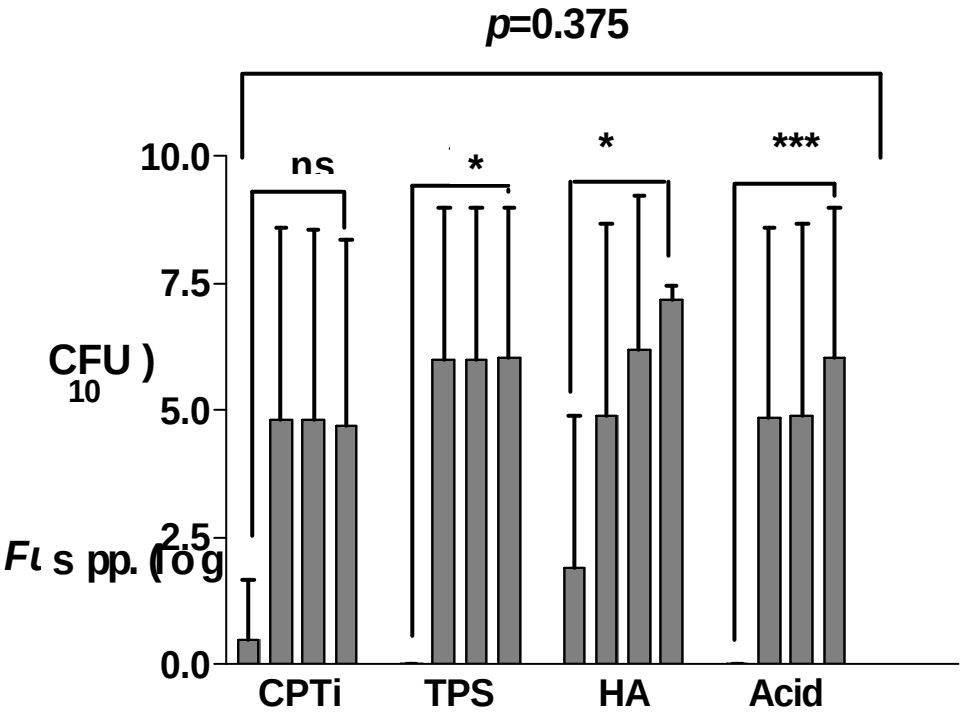
ns=Non-significant p>0.05

Fig. 3c



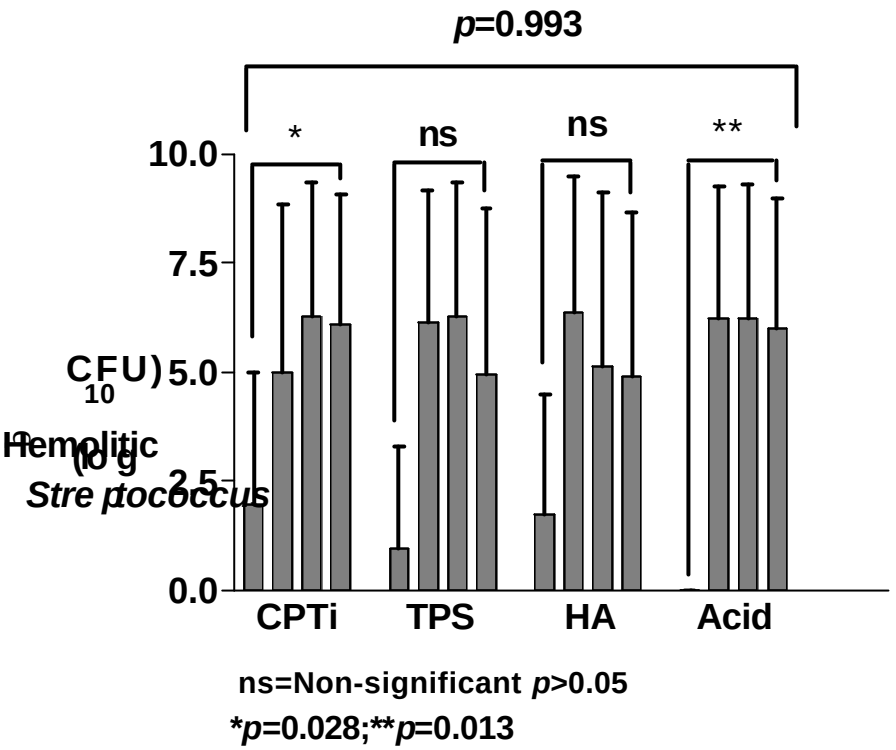
p=0.002; **p=0.003;p=0.0008**

Fig. 3d



ns=Non significant p>0.05; *p=0.018; **p=0.028; ***p=0.047

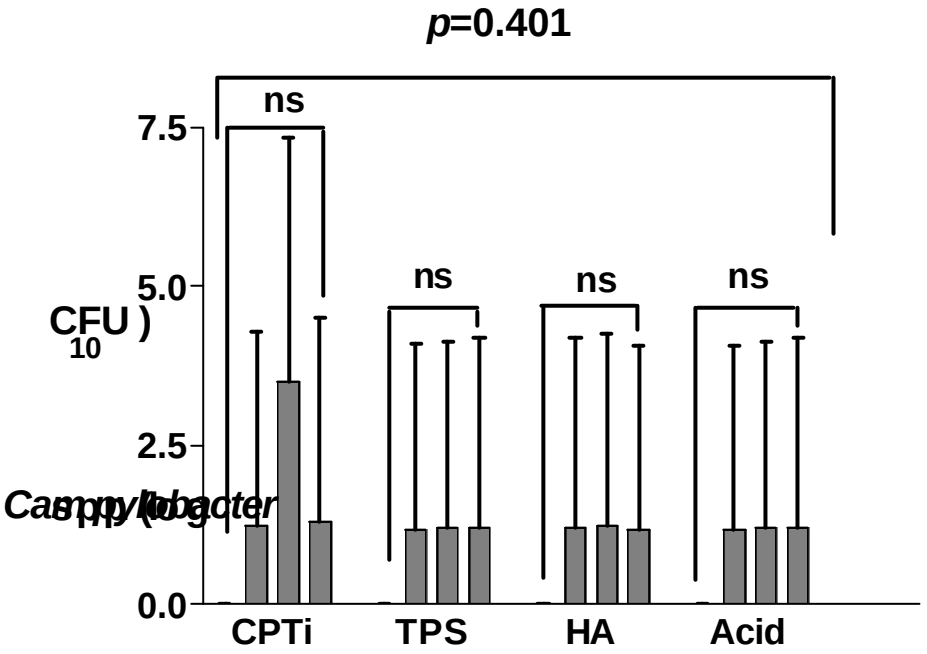
Fig. 3e



ns=Non-significant $p>0.05$

*$p=0.028$;**$p=0.013$

Fig. 3f



ns=Non-significant $p>0.05$

Fig. 4a

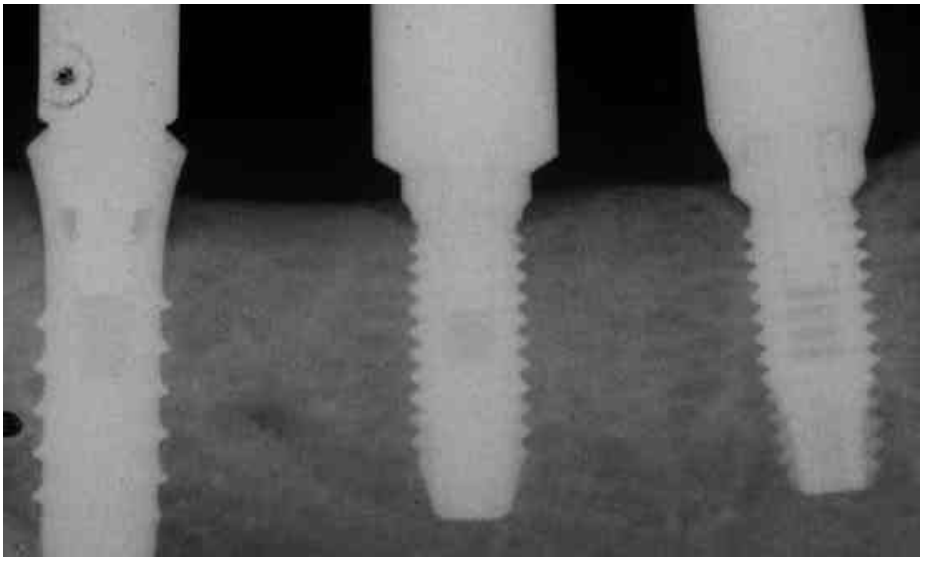


Fig. 4b

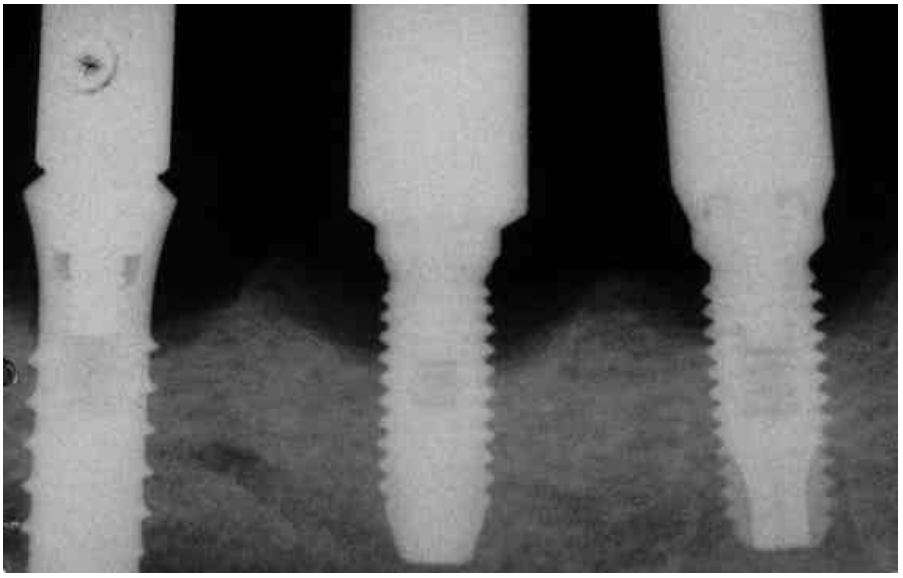
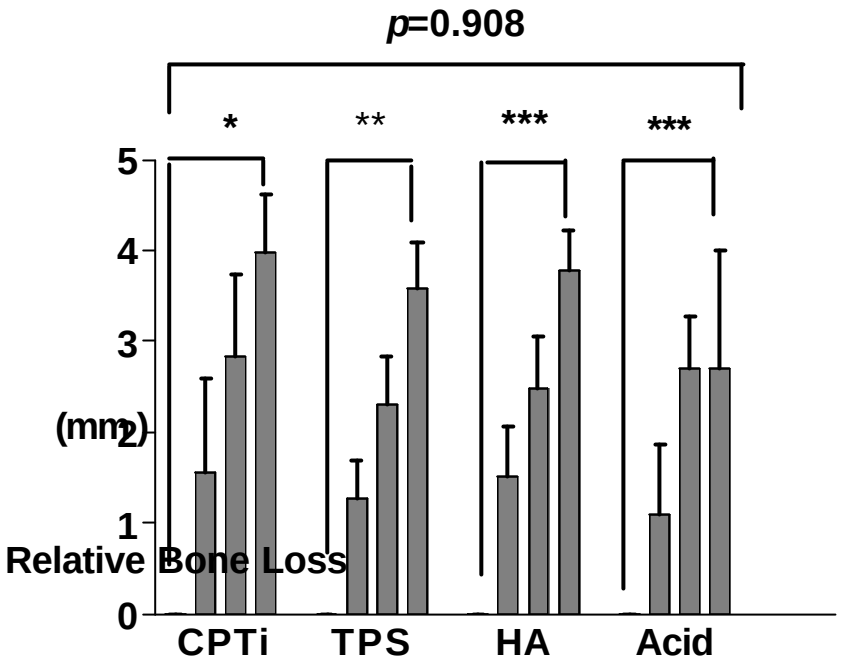


Fig. 5



***p=0.005; **p<0.0001; ***p=0.0001**

Legends

Fig. 1. Outline of the experiment. Ligatures were placed around the implants on day 0, and were changed every 20 days, when microbiologic and radiographic procedures were performed. Animals *n*=6; implants *n*=36.

Fig. 2. Dental implant surfaces used in this experiment, from left to right: titanium-plasma sprayed (TPS); hydroxyapatite (HA); acid-etched surface in three first threads and machined surface in others threads (acid); commercially pure titanium (CPTi).

Fig. 3a. Mean and standard deviation of Total Viable Count (TVC) of different dental implant surfaces at baseline and 20, 40, and 60 day-period.

Fig. 3b. Mean and standard deviation of *P. gingivalis* of different dental implant surfaces at baseline and 20, 40, and 60 day-period.

Fig. 3c. Mean and standard deviation of *P.intermedia/nigrescens* of different dental implant surfaces at baseline and 20, 40, and 60 day-period.

Fig. 3d Mean and standard deviation of *Fusobacterium* spp. of different dental implant surfaces at baseline and 20, 40, and 60 day-period.

Fig. 3e Mean and standard deviation of beta-hemolytic *Streptococcus* of different dental implant surfaces at baseline and 20, 40, and 60 day-period.

Fig. 3f. Mean and standard deviation of *Campylobacter* spp. of different dental implant surfaces at baseline and 20, 40, and 60 day-period.

Fig.4a. Periapical radiograph taken at baseline.

Fig. 4b. Periapical radiograph taken at 60 days after ligature-placement.

Fig.5. Mean and standard deviation of radiographic bone loss of different dental implant surfaces at 20, 40, and 60 day-period.

Table 1- Distribuition of dental implants with different surfaces in 6 dogs.

Animal	Right jaw side			Left jaw side		
	PM2	**PM3**	**PM4**	**PM2**	**PM3**	**PM4**
1	CPTi	Acid	TPS	TPS	HA	Acid
2	CPTi	TPS	HA	HA	Acid	CPTi
3	HA	Acid	CPTi	CPTi	TPS	HA
4	TPS	HA	Acid	Acid	CPTi	TPS
5	HA	Acid	CPTi	CPTi	TPS	HA
6	TPS	HA	Acid	Acid	CPTi	TPS

CPTi- Comercially pure titanium**; TPS –** titanium plasma sprayed**; HA –** hydroxyapatite**; Acid –** acid-etched surface

PM2, PM3, PM4 – Mandibular premolars

Table 2 - Microorganism detected during the experiment.

MICROORGANISM	TIME (DAYS)															
	0				20				40				60			
	CPTi	TPS	HA	Acid	CPTi	TPS	HA	Acid	CPTi	TPS	HA	Acid	CPTi[1]	TPS	HA[2]	Acid[3]
P. gingivalis	0	0	0	0	4	2	4	2	4	2	4	2	2	2	3	2
P.intermedia/ nigrescens	2	0	2	1	9	9	9	9	9	9	9	9	7	9	8	7
***Campylobacter* spp.**	0	0	0	0	2	1	0	1	4	1	0	1	1	1	0	1
***Fusobacterium* spp.**	2	0	3	0	6	7	6	5	6	8	7	6	5	7	7	6
beta-hemolític *Streptococcus*	3	1	3	0	7	8	7	8	6	6	6	8	6	6	6	6
***Candida* spp.**	0	0	0	0	2	1	2	1	0	0	0	0	0	0	0	0

([1]*n*=7; [2]*n*=8; [3]*n*=7 – Dental implants excluded because of 40% radiographic bone loss at period 40 day)

CAPITULO 5 - DETECTION OF PERIODONTAL PATHOGENS IN LIGATURE-INDUCED PERI-IMPLANTITIS. AN EXPERIMENTAL STUDY IN DOGS.

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Running Title: Periodontal pathogens in ligature-induce peri-implantitis

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DETECTION OF PERIODONTAL PATHOGENS IN LIGATURE-INDUCED PERI-IMPLANTITIS. AN EXPERIMENTAL STUDY IN DOGS.

ABSTRACT

Background: The purpose of this study was to evaluate the attachment loss around different dental implant surfaces by means of microbiological, clinical and radiographic analysis.

Methods: In 6 male mongrel dogs, a total of 36 dental implants of four different surface coating (9 hydroxyapatite-HA, 9 titanium plasma-sprayed-TPS; 9 acid-etched surface; 9 commercially pure titanium surface-CPTi) were inserted after 3 months healing period of mandibular premolars extraction. After 3 months with optimal plaque control, abutment connection was performed. Microbiological samples were taken at 0, 20, 40, and 60 days after cotton ligature placement. The presence of *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Bacteroides forsythus, Prevotella intermedia, Fusobacterium nucleatum, Fusobacterium nucleatum* ss *vicentii, Campylobacter rectus*, *Eikenella corrodens, Neisseria* spp., *Treponema* spp. and spirochetes were assessed by DNA probes in a checkerboard assay and analysis of amplified 16S rDNA by means polymerase chain reaction.

Results: No differences among the four dental implants surfaces were observed for microbiological features except for the higher detection of *Neisseria* spp. at acid surface (p=0.003). Clinical attachment levels and radiographic bone loss were not statistically significant among dental implant surfaces (p>0.05), however all surfaces were suceptible to ligature induced peri-implatitis over time (p<0.001).

Correlation analysis revealed a statistical significance among *P. gingivalis, P. intermedia* and *B. forsythus* with CAL to acid, CPTi and TPS surface ($p<0.05$). *P. gingivalis* and *B. forsythus* presented significant correlation for CPTi surface with bone loss.

Conclusions: The detection of periodontal pathogens was associated to peri-implant tissue breakdown. In addition, it can be concluded that different dental implants surfaces presented similar rate of attachment loss after bacterial shift induced by cotton floss ligatures at 60 days.

Key Words: Dental implants/microbiology; peri-implantitis/biofilm; peri-implantitis/etiology; dental implants/microstructure; dogs.

INTRODUCTION

The potential cause of osseointegration failure is the biofilm accumulation.[1-3] Several animal studies have shown that the peri-implantitis is a condition characterized by soft tissue inflammation, bleeding and suppuration, presented both rapid clinical attachment loss and vertical/horizontal bone loss.[4-7] These studies compare the clinical, histological and microbiological changes around dental implants, after plaque accumulation by means of ligature placement. The placement of ligature nearly always resulted in a marginal bone loss destruction.

The peri-implant bone loss generally is associated with the presence of periodontal pathogens such as *Porphyromonas gingivalis, Prevotella intermedia, Prevoltella nigrescens, Bacteroides forsythus, Campylobacter rectus* and *Actinobacillus actinomycetemcomitans.*[8-11] Special attention has been focused on a presence of periodontal pathogens in diseased dental implants sites without considering the microstructure of dental implant. However, there is scarce the information about what microorganism are more suitable for each different dental implant surface.

Recently,numerous studies have investigated the osseointegration around different dental implant coatings such as titanium plasma-sprayed (TPS), hydroxyapatite (HA), and commercially pure titanium surface treated with acids[12-16]. These studies seeking the better surface for osseointegration and consequently, a higher long-term favorable prognosis as well as utilize these implants to immediate loading[17,18].

Therefore, the purpose of the present study was to identify the periodontal pathogens associated with ligature-induced peri-implantitis in dogs, at different dental implant surfaces.

MATERIAL AND METHODS

Dental implant surfaces

In this study, thirty-six dental implants with four different surfaces were used. Nine hydroxyapatite-HA[¶], nine titanium plasma sprayed implants-TPS[#], nine hybrid surface–machined titanium in the three first screws and acid-etched in other screws-Acid[**], and nine commercially pure titanium implants-CPTi[††], were used. All implants had lengths of 10mm and diameters of 3.75mm (except TPS which had a diameter of 4.1mm).

Animals

Institute of Animal Care and Use Committee of the Dental School of Araraquara, approved this protocol. Six adult, male mongrel dogs were used. At the beginning of the study, the dogs were 2-years old with an average weight of 18Kg.

Surgeries

Extraction

Extractions were carried out in an operatory room under general anesthesia and sterile conditions utilizing 0.05mg/Kg of subcutaneous pre-anesthesia sedation (atropine sulphate) and intravenous injection of chlorpromazine (0.2mL/Kg body weight) and 4% thiopental-Na solution (0.5mL/Kg body weight). The surgical site was disinfected with 0.2% chlorhexidine and subsequently, 2% lidocaine HCl with epinifrine 1:100,000 was given as local anesthesia, and all 4 mandibular premolars were extracted creating an edentulous ridge and both the mandibular quadrants

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[¶] Calcitek®, Sulzer Medica, Carlsbad, CA
[#] *Esthetic plus* ITI®, Straumann AG, Waldenburg, Switzerland
[**] *Osseotite*®-3*i*® Implants Innovations, Palm Beach Gardens, FL
[††] 3*i*® Implants Innovations, Palm Beach Gardens, FL

and the alveoli were allowed to heal for a period of 3 months. The maxillary premolars were also extracted to avoid occlusion trauma interference.

Oral prophylaxis was performed up to 2 weeks before teeth extraction. Plaque control was instituted during the healing period by scrubbing daily with 0.12% chlorhexidine, scaling and root planning once a month, until ligature placement (Fig. 1).

Dental implant placement

Under aseptic surgical conditions, all dental implants were placed over the full thickness flap. Three implant sites were prepared per mandibular quadrant using original instruments for each dental implant system, according to the surgical techniques indicated by each implant manufacturer. A distance of approximately 10mm between dental implant centers was maintained to avoid comunication among the further bone defects.

The implants were randomly distributed among the dogs so that each dental implant surface was represented at least once in each animal (Table 1). The implants were placed at the bone level and a cover screw was screwed onto the implant, including the TPS dental implant surface due to a modification in technique insertion indicated by the manufacturer. The flaps were sutured with single interrupted sutures to submerge all implants. Antibiotic therapy with potassic and sodic benzilpenicilin (24.000UI/Kg) was startedand continued once a week, for 2 weeks, to avoid post-surgical infection. Paracetamol was given for pain control medication, and the sutures were removed after 10 days.

Experimental Peri-implantitis

After a healing period of 3 months, healing abutment connections were installed, according to the indication of each dental implant system. After soft tissue healing of 2-months, cotton floss ligatures were placed in a submarginal position around dental implants and sutured in peri-implant mucosa to hold the ligatures in position. The positions of the ligatures were checked twice a week. Tying further ligatures at 20-day intervals for a period of 60 days accelerated peri-implant bone loss.

Microbiological Procedures

Peri-implant microbial samples were taken from the mesio-distal site with paper points immediately before the ligature placement and 20, 40 and 60 days after ligature placement. Supra-mucosal debridment at the sample site was initially performed with a sterile plastic curette and dry gauze after isolation from saliva using cotton rolls. The area was carefully dried and the bacterial sample was collected with 4 sterile paper points gently inserted into the peri-implant sulcus, as far apical as possible and left for 20 seconds. The paper points were placed in sterile transport vials and storaged at freezer until to be processed. All samples were collected by the same examiner and coded by an assistant to keep the blindness of the study.

Periodontal Pathogens Detection

Analysis of Amplified 16S rDNA

The PCR-amplified 16S rDNA using digoxigenin-labed species-specific oligonucleotide probes[19,20] were tested for *Actinobacillus actinomycetemcomitans* (CACTTAAAGGTCCGCCTACGTGCC), *Porphyromonas gingivalis* (GCAGTTTCA

ACGGCAGGGCTGAACG), *Prevotella intermedia* (GGTCCTTATTCGAAGGGTAA ATGC), *Bacteroides forsythus* (CGTATCTCATTTTATTCCCCTGTA), *Campylobacter rectus* (CAAGCTACTTAATCTCCGTTCGAC), *Fusobacterium nucleatum* (GGTTTCCCCGAAGGGACATGAAAC); *Fusobacterium nucleatum* ss *vincetii* (ACTTCACAGCTTTGCGACTCTCTGTTC), *Eikenella corrodens* (ACC GTCAGCAAAAAGTGGTATTAGCAC), *Treponema* spp.(GGCAGTAGGGGTTGC GCTCGTT), *Neisseria* spp. (CCTCTGTACCGACCATTGTATGAC) and for spirochetes (CGACTTTGCATGSTTAARAC). The oligonucleotide probes were 3'-end labeled with digoxigenin-11-ddUTP[§§]. The DNA was tranferred overnight onto membranes using 10x standart salt phosphate EDTA buffer (SSPE). Hybridization with digoxigenin-labeled oligonucleotide probes was performed at 45^{o}C. The hybrydized membranes were washed twice with hight salt solution (2xSCC[0.15M NaCl plus 0.015 trisodium citrate, pH 7.0] in 0.1% sodium dodecyl sulphate) at room temperature and then twice with a low salt solution (0.1xSCC in 0.1% sodium dodecyl sulphate) at 45^{o}C. The membranes were reacted with anti-digoxigenin alkaline phosphatase conjugate. The color reaction was produced with 5-bromo-4chloro-3-indolyl phosphate and nitroblue tetrazolium salts.

Polymerase Chain Reaction of 16S rDNA

In addition to DNA probes in checkerboard assay, the PCR-amplification of conserved region of 16S ribossomal DNA were also tested for periodontal pathogens including *A. actinomycetemcomitans* (primer 1-ATTGGGGTTTAGCCC TGGTG and Rev16s-ACGTCATCCCCACCTTCCTC), *P. gingivalis* (primer1-TG

[§§] Genius 5, Boehringer Mannheim, Indianapolis,IN.

TAGATGACTGATGGTGAAAACC and Rev16s-ACGTCATCCCCACCTTCCTC) and *B. forsythus* (primer 1-TACAGGGGAATAAAATGAGATACG and Rev 16s-ACGTCATCCCCACCTTCCTC). All these PCR primers were obtained commercially[||||]. Between 30 to 100ng of genomic DNA was added to the PCR mixture wich contained 1µmol/L of the primers, 2.5U of *Taq* polymerase[¶¶] in 1x buffer and 0.2mmol/L of dCTP, dGTP, dATP, and dTTP[¶¶] in a total volume of 50µL. The amplification was performed for 30 cycles of 30 seconds at 95^{o}C, 30 seconds at 55^{o}C and 30 seconds at 72^{o}C in thermocycler[##]. Positive and negative controls were included with each set. The negative control includes all the PCR reagents except for the sample DNA. The positive control contained all the PCR reagents together with positive controls for *A. actinomycetemconitans*; *P.gingivalis* and *B. forsythus*. Twenty µL of each PCR reaction mixture was electroforesed in 1.5% (*A. actinomycetemconitans* and *B. forsythus*) and in 2% (for *P.gingivalis*) agarose gel in TBE buffer and the amplification products were visualized under 302nm ultraviolet light, on ethidium bromide-stained gels.

Clinical examination

After microbiological sampling, the clinical attachment level was recorded at baseline and 20, 40, and 60 days after ligature tissue-breakdown. A single pre-calibrated examiner carried out the clinical exams. The attachment levels were

[||||] Gibco BRL, Grand Island, NY

[¶¶] Perkin-Elmer,Norwalk,CT

[##] GeneAmp PCR System 9600, Perkin-Elmer, Norwalk,CT.

registered using a force-controlled calibrated periodontal probe[***] with a constant probing force of 0.20N and a probe-tip diameter of 0.4mm. The distobuccal, midbuccal, mesiobuccal, mesiolingual, midlingual, and distolingual were measured. Probing depth (PD) and the distance between gingival margin (GM) and the fixed point in the abutment surface (FP) was recorded. CAL was then calculated according the formula: PD- (GM-FP).

Radiographs

Intraoral radiographs were taken at the time of ligature placement, 20, 40 and 60 days after ligature-induced peri-implantitis to evaluate changes in bone levels. The standardized radiographs were taken with a customized occlusal index fabricated from a sensor holder by placing a silicone bite block made of polyvinyl siloxane impression putty material.

A dental x-ray machine equipped with a 35-cm-long cone was used to expose the periapical intra-oral sensor[†††]. Exposure parameters were 70 kilivolt (peak), 15 mA, and 1/4 second at a focus-to-sensor distance of 37cm. The linear distance between the fixed point in abutment and the first visible bone-to-implant contact was determined mesial and distal of each implant digital image. The mesial and distal values were averaged to obtain a mean implant value. Relative peri-implant bone loss was measured to avoid interference by the different dental implant macrostructures used in this study.

[***] Florida Probe, Computerized Probe Inc, Gainesville, FL

[†††] CDR- Computed Dental Radiography – Schink Tecnologis Inc.,USA

All measurements were made independently by 2 examiners. If discrepancies were of 0.5mm or less, the mean value of the 2 measurements was used. In situations with greater discrepancies, the images were analyzed again until desired measurements were reached.

Data Analysis

Data management and calculation were done using statistical software[‡‡‡]. Analysis of variance, using comparison of several proportions (contingency table) was used to compared the distribuition of different bacteria for each type of implant, and also to compare bacterial colonization at different points.[21]

The clinical attachment loss and Radiographical bone loss were compared by means paired *t*-test (2-tailed). To determine the correlations of microbiological features on CAL and vertical bone loss, a Pearson Correlation was determined. All test were stratified according to dog (unit of analysis), i.e., *n*=6. Level of significance was set at 0.05.

RESULTS

Clinical and microbiological data were available for analysis from 36 sites/implants in 6 dogs (6 sites per animal). Hundred forty-four microbiological samples were analyzed over the experimental period. None implant was lost due to ligature-induced peri-implantitis.

[‡‡‡] SPSS-Statistical Package for the Social Sciences version 10.1, Chicago, IL.

Microbiological features

Figures 2 to 12 summarize the prevalence and incidence of target periodontal pathogens at each period. All target periodontal pathogens (Table 2) were found in all animals in varied proportions.

A. actinomycetemcomitans was not identified at baseline and 20 days. Although a higher prevalence have been detected to HA-coated surface, no statistically difference was observed among the surfaces ($p>0.05$).

P. gingivalis was not detected at baseline in HA-coated surface. The positive sites percentage range between 36,12% for HA-coated surface to 25% for CPTi. However statisticall significance was not achieved among the different dental implants surfaces ($p>0.05$).

B. forsythus was detected only in acid surface at baseline and 20 days. The higher detection over time was found in acid surface (13.88% of the samples). The same occurred to *C. rectus*: the higher percentages were observed in acid and HA-coated surface (22.23% of the samples). For both microorganisms significant difference was not observed.

F. nucleatum and *F. nucleatum* ss *vicentii* were detected in higher percentages at baseline and 20 days for all surfaces. At 40 and 60 days their detection was lower for all differente dental implant surfaces ($p>0.05$). *E. corrodens* and spirochetes were detected in lower porcentage at baseline, 20, 40 and 60 days. However *E. corredens* shown more positive sites to CPTi surface (11,12% of the samples), even though not significant ($p>0.05$).

Treponema spp. and *Neisseria* spp. were detected in all surfaces at baseline and 20 days. At 40 days both bacteria were not detected in TPS surface. A statistical significance was observed among the positive detection sites for *Neisseria* spp. at acid surfaces (p=0.003).

Clinical Attachment Level

After ligature-induced tissue breakdown, all dental implants were associated with a continuous increase of clinical attachment loss. Differences in clinical attachment loss among the different implant surfaces were not statistically significant (p>0.05). The lower attachment loss was presented by TPS surface (3.87$\pm$1.69) and the higher to CPTi (5.16$\pm$1.53) (Table 3). Over time, all surfaces exhibited statistically significant attachment loss when compared to baseline recordings (p<0.001 for HA, Acid and CPTi and p=0.02 for TPS).

Radiographic Analysis

At baseline, none implant surface exhibited peri-implant radiolucencies. The means of relative bone loss for all dental implants surfaces are presented in Table 4. The Ha-coated surface showed the higher bone loss measurement (4.20$\pm$0.47mm) and the lower for TPS surface (3.50$\pm$0.97mm). However statistical significance was no assessed for different dental implants surfaces (p>0.05). When the bone levels were compared between baseline and 60 days after ligature placement, a statistically significant difference (p<0.0001) was found, demonstrating a clear effect of biofilm accumulation over time.

Correlations

Correlation analysis revealed a high correlation of *P. gingivalis* (r=0.991; p=0.032) and *B. forsythus* (r= 0.948; p=0.004) with CAL to Acid surface. *P. intermedia* was statistically correlated with CAL measurements to CPTi surface (r=0.853; p=0.031). *P. gingivalis* was also correlated with CAL in TPS surface (r=0.831; p=0.040) A consistent correlation was noted for CPTi surface to vertical bone loss measurements with the detection of *P. gingivalis* (r= 0.952; p=0.003) and *B. forsythus* (r=0.997; p=0.006).

DISCUSSION

The ligature-induced peri-implant tissue breakdown has been evaluated in canines,[6,7,22] microswine[23] and non-human primates.[4,5,24,25] The acute inflammatory tissue response to biolfim accumulation seemed to represent a localized lesion, comparable to that encountered in periodontal as well as in peri-implant diseases.

The objective of this study was to evaluate, by means microbiological and clinical analysis, the ligature-induced peri-implantitis around different dental implant surfaces. A significant clinical attachment loss associated to bacterial shift was observed after placing cotton floss ligatures around dental implants and ceasing plaque control procedures. A significant radiographic bone loss was also observed around all dental implants after short period (60 days). These features were in accordance with Schou et al.[26] and Nociti et al.[22] Other studies reported clinical and microbiological evaluations for longer periods: Hanisch et al.[24] for 10 months and Tillmanns et al.[6,7] for 3 and 6 months. However, all reports agreed on bacterial shift after ligature-placement resulting in attachment and bone loss.

The different coating surfaces could to influence the bacterial adsorption.[27,28] Physical and chemical factors can affect the attachment of biofilms to a hard surface. The roughness of the surface can increase surface area and hence increase the colonization[29]. Roughness also provides protection from shear forces and increases the difficulty of cleaning methods. Further, Quirynen and Bollen[29] have shown that supragingival plaque formation, after initial colonization has occurred, was faster on a roughened surface. The initial colonization of an intra-oral hard surface starts from surface irregularities such as cracks, grooves, or abrasion defects and subsequently spreads out from these areas as a relatively even monolayer of cells.[30] The roughness of different dental implant surfaces can work like grooves for initial pathogens adhesion. The chemical composition of dental implant surface also acts on a bacterial colonization since it may contain beneficial or detriment components.[31] Those events probably occurred after ligature-induced breakdown peri-implant tissues. This may explain the higher detection of *Neisseria* spp. at acid surfaces observed in our investigation. The smoother surface (CPTi) showed the lowest positive detection number for the target microorganisms at all periods (Table 2). However, the data of the present study did not show any statistical difference for microbiological features among the different dental implant surfaces in a 60 days time period. The non-statistical difference for detection frequencies for periodontal pathogens among the different surfaces may be explained by the small sample size used in this study.

In relation to clinical and radiographic data, no relevant differences among HA, TPS, Acid and CPTi surfaces were found, similar to microbiological findings. There

was not a agreement shown in previously published studies, where HA-coated surface and TPS implants were associated with substantially more failures resulting from peri-implantitis.[32,33,34] However, in two studies conducted by Tillmanns et al.[6,7], differences were not found among the HA, TPS and CPTi surfaces in clinical, microbiological and histological aspects. Probably the different results obtained for HA surface are due to the short period of time utilized in our methodology. In this study the most severe radiographic evidence of bone loss was observed for HA surface, although statistically significance was not observed ($p>0.05$).

The detection of *A. actinomycetemcomitans* was similar to that shown by Ong et al.[35]; Hanisch et al.[24]; Eke et al.[25]; Schou et al.[26]; Tillmanns et al.[7] However, only Tillmanns et al.[7] evaluated the bacterial shift in canine model. Nociti et al.[22], analyzed the clinical and microbiological status of periodontitis and peri-implantitis induced in dogs, but they did not find *A. actinomycetemcomitans* using polymerase chain reaction. This result may have occurred due to time ligature-induced breakdown tissues (30 days) used by Nociti et al.[22] when compared with our methodology. The *A. actinomycetemcomitans* detection in our study occurred after 40 days of ligature-induced peri-implantitis.

The detection of *P. gingivalis, P. intermedia and F. nucleatum* confirm previous studies that analyzed dental implants with peri-implant diseases in animals[7,22,24,25,26,36] and humans.[10,11,37-39] The canine peri-implantitis model used in this study found similar results about microbiological features at periodontal diseases[40,41] and peri-implant diseases[22,24,26] related in literature.

The detection of *F. nucleatum* ss *vicentii, C. rectus* and *E. corrodens,* has also been associated both failing and ailing implants.[11,39]

The spirochetes have been associated with failing implants.[37,42] However, its detection was lower in all surfaces. The use of chlorhexidine at plaque control phase may have reduced the potential sources from that spirochetes could colonize.[24]

The presence of *A. actinomycetemcomitans, P. gingivalis* and *B. forsythus* were strongly associated with periodontal disease status, disease progression and unsuccessful therapy.[43] A statistical correlation found between *P. gingivalis* and CAL/VBL in this investigation was in agreement with that statement. *F. nucleatum*, *C. rectus*, *P. intermedia* and various spirochetes have also been implicated in causing periodontal diseases, although the evidence for their causative role is less expressive.[44] The progression of clinical attachment loss and vertical bone loss observed in our study were statistically associated with target periodontal pathogens detection such as *P. gingivalis* and *P. intermedia,* after ligature placement.

This study applied DNA probes in a checkerboard assay and amplification of 16S rDNA by means of polymerase chain reaction, which unlike cultural methods that have been used, does not require anaerobic methods for sample viability and species characterization. The molecular biology techniques are be able to detect species that may be at low relative proportions, comparing to other species in samples, but are above the probe assay threshold. Some periodontal pathogens

such as *B. forsythus* are rarely reported in peri-implantitis due to its difficult to be cultivated[45].

In previous study conducted by our group, there was no identification of *A. actinomycetemcomitans* using culture media, probably due to low prevalence of this bacterium in samples from ligature-induced peri-implantitis in dogs. These techniques are more sensitive than culture, for which sensitivities are low as 50 bacteria for PCR, 10^3 for DNA probe and $2x10^5$ for culture.[46,47,48]

In this investigation, the microbiological samples positive for *A. actinomycetemcomitans*, *P. gingivalis* and *B. forsythus* were also assessed by PCR of 16S rDNA. Several investigations[49-52] have related the existence of high probability of false-positive results with the use of whole genomic DNA probes, principally for *A. actinomycetemcomitans*.

Dental implants with peri-implantitis thus reveal a complex microbiota encompassing conventional periodontal pathogens. These features confirm the bacterial shifts associated to attachment loss detected in this animal study after the induction of experimental peri-implantitis. Species such as *A. actinomycetemcomitans, Campylobacter rectus, Fusobacterium nucleatum* are often isolated from failing sites, but can also be detected around healthy peri-implant sites. These microorganisms are commonly associated with progressive periodontal diseases and virulence factors that could be important to peri-implantitis progression.

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FIGURE LEGENDS

1. Outline of the experiment. Animals *n*=6, dental implants *n*=36.

2. Frequency distribuition of *Actinobacillus actinomycetemcomitans* around different dental implant surfaces before ligature placement (baseline) and after 20, 40 and 60 days.

3. Frequency distribuition of *Porphyromonas gingivalis* around different dental implant surfaces before ligature placement (baseline) and after 20, 40 and 60 days.

4. Frequency distribuition of *Bacteroides forsythus* around different dental implant surfaces before ligature placement (baseline) and after 20, 40 and 60 days.

5. Frequency distribuition of *Prevotella intermedia* around different dental implant surfaces before ligature placement (baseline) and after 20, 40 and 60 days.

6. Frequency distribuition of *Campylobacter recuts* around different dental implant surfaces before ligature placement (baseline) and after 20, 40 and 60 days.

7. Frequency distribuition of *Fusobacterium nucleatum* around different dental implant surfaces before ligature placement (baseline) and after 20, 40 and 60 days.

8. Frequency distribuition of *Fusobacterium nucleatum* ss *vicentii* around different dental implant surfaces before ligature placement (baseline) and after 20, 40 and 60 days.

9. Frequency distribuition of *Eikenella corrodens* around different dental implant surfaces before ligature placement (baseline) and after 20, 40 and 60 days.

10. Frequency distribuition of *Treponema* spp. around different dental implant surfaces before ligature placement (baseline) and after 20, 40 and 60 days.

11. Frequency distribuition of *Neisseria* spp. around different dental implant surfaces before ligature placement (baseline) and after 20, 40 and 60 days.

12. Frequency distribuition of spiroquetes around different dental implant surfaces before ligature placement (baseline) and after 20, 40 and 60 days.

Table 1- Randow distribuition of different implant surfaces in six animals.

Animal	Right jaw side			Left jaw side		
	PM2	**PM3**	**PM4**	**PM2**	**PM3**	**PM4**
1	TPS	HA	Acid	Acid	CPTi	TPS
2	HA	Acid	CPTi	CPTi	TPS	HA
3	TPS	HA	Acid	Acid	CPTi	TPS
4	HA	Acid	CPTi	CPTi	TPS	HA
5	CPTI	TPS	HA	HA	Acid	CPTi
6	CPTi	Acid	TPS	TPS	HA	Acid

HA – hydroxyapatite**; TPS –** titanium plasma sprayed**; Acid –** acid-etched surface; **CPTi-** Comercially pure titanium

PM2, PM3, PM4 – Mandibular premolars

Table 2. Distribuition of implants being positive for indicated periodontal pathogen at baseline, 20, 40, and 60 days.

Periodontal Pathogens	TIME (DAYS)															
	0				20				40				60			
	HA	TPS	Acid	CPTi	HA	TPS	Acid	CPTi	HA	TPS	Acid	CPTi	HA	TPS	Acid	CPTi
A. actinomycetemcomitans	0	0	0	0	0	0	0	0	3	4	0	3	4	1	3	1
P. gingivalis	0	1	2	1	3	3	0	1	5	5	3	4	5	3	5	3
P. intermedia	0	0	1	0	0	0	0	0	1	1	1	0	2	1	2	1
B. forsythus	0	0	1	0	0	0	1	0	1	1	1	1	3	2	2	1
C. rectus	0	0	0	0	2	1	2	0	3	3	2	3	3	3	3	3
F. nucleatum	2	2	4	2	5	6	5	5	1	2	0	0	3	3	3	2
F. nucleatum ss *vicentii*	3	3	1	2	2	3	3	4	0	1	0	1	1	1	1	1
E. corrodens	1	2	1	0	2	1	1	2	0	0	0	1	0	1	0	1
Neisseria spp.	2	3	2	2	6	2	6	1	3	0	5	1	0	0	3	0
Treponema spp.	5	6	6	5	4	4	1	2	2	0	0	3	2	0	0	0
Spirochetes	0	0	0	0	1	1	1	2	1	0	0	0	0	1	2	0
Total of positive bacteria detection	13	17	18	12	25	21	20	17	20	17	12	17	22	16	24	13

Table 3 – Mean±SD of clinical attachment levels, measured in mm, at baseline and 20, 40 and 60 days after ligature placement.

Surface	Baseline	20 days	40 days	60 days	Clinical attachment loss
HA	7.47±0.48	10.25±0.84*	11.52±0.60*	12.11±0.60*	4.64±0.80‡
TPS	7.60±1.35	9.75±0.93*	9.78±0.93*	11.49±1.27†	3.87±1.69‡
Acid	8.08±0.53	11.03±0.90*	11.68±0.96*	12.75±0.98*	4.66±1.13‡
CPTi	8.24±0.80	11.37±1.06*	12.22±1.18*	13.40±1.20*	5.16±1.53‡

* Significantly different from baseline $p<0.0001$

† Significantly different from baseline $p=0.002$

‡ Not significant among the surfaces $p>0.05$

Table 4. Crestal bone levels at baseline and 20, 40 and 60 days after ligature placement and crestal bone loss measured in mm from implant healing abutment fixed point (mean±SD)

Surface	Baseline	20 days	40 days	60 days	Crestal bone loss
HA	2.01±0.46	3.62±0.29*	4.65±0.84*	6.22±0.50*	4.20±0.47†
TPS	2.50±0.61	3.85±0.95*	4.62±0.90*	6.00±0.70*	3.50±0.97†
Acid	2.36±0.54	3.64±0.17*	5.19±0.51*	6.06±0.27*	3.70±0.57†
CPTi	2.40±0.51	4.12±0.72*	5.20±0.71*	6.32±0.33*	3.92±0.61†

* Significantly different from baseline $p<0.0001$

†Not significant among the surfaces $p>0.05$

Figure 1

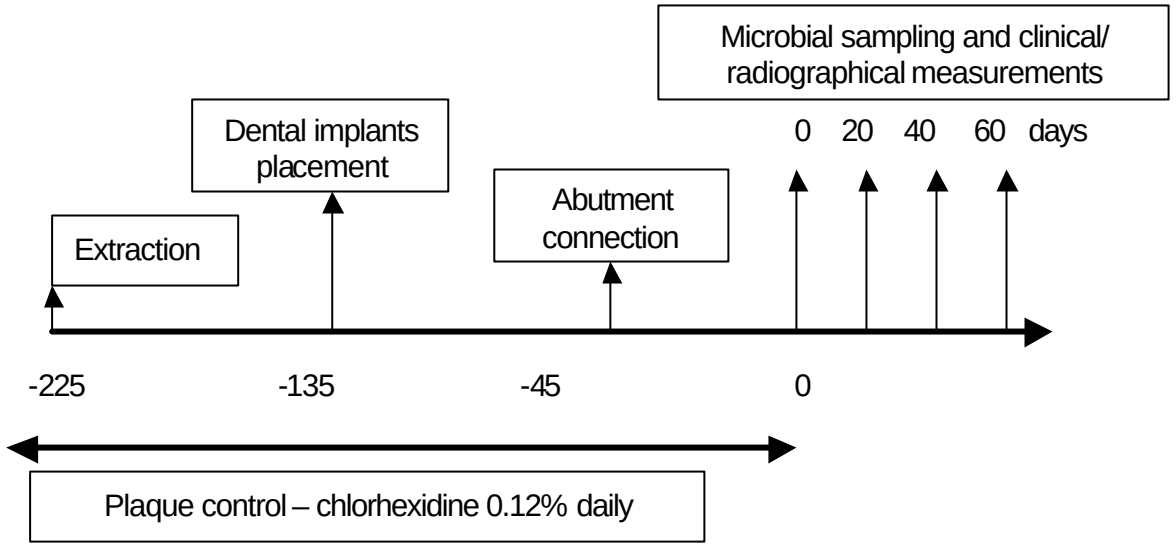

Microbial sampling and clinical/ radiographical measurements
Dental implants placement
0 20 40 60 days
Abutment connection
Extraction
-225
-135
-45
0
Plaque control – chlorhexidine 0.12% daily

Fig. 2

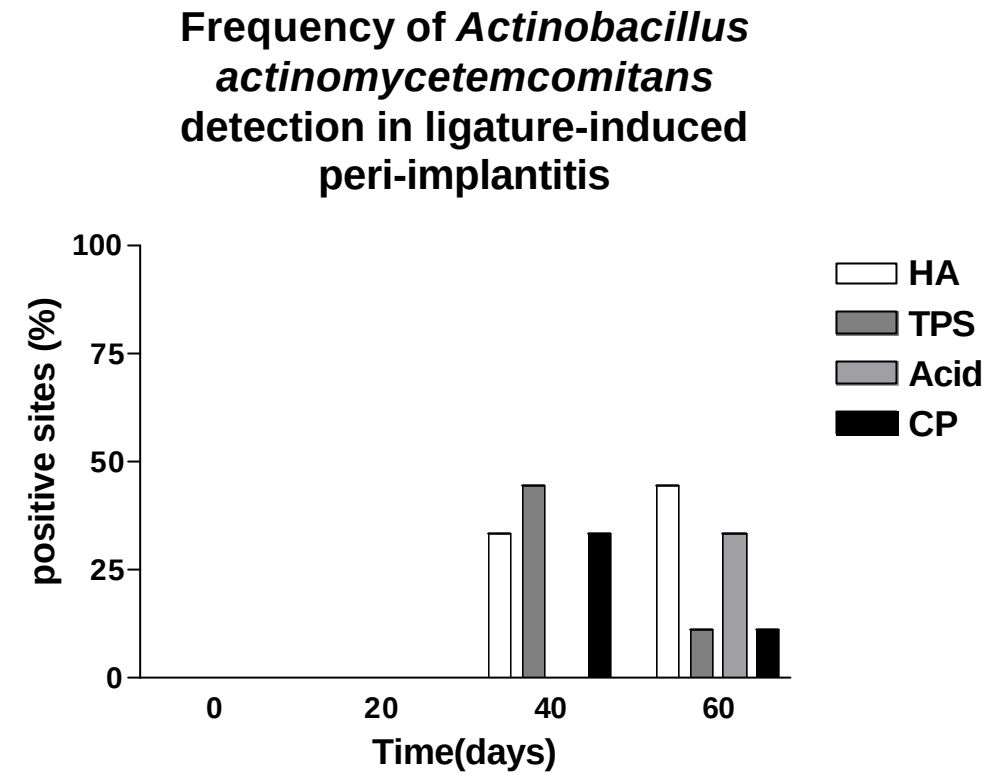

Frequency of Actinobacillus actinomycetemcomitans detection in ligature-induced peri-implantitis
positive sites (%)
100
75
50
25
0
0
20
40
60
Time(days)
HA
TPS
Acid
CP

Fig.3

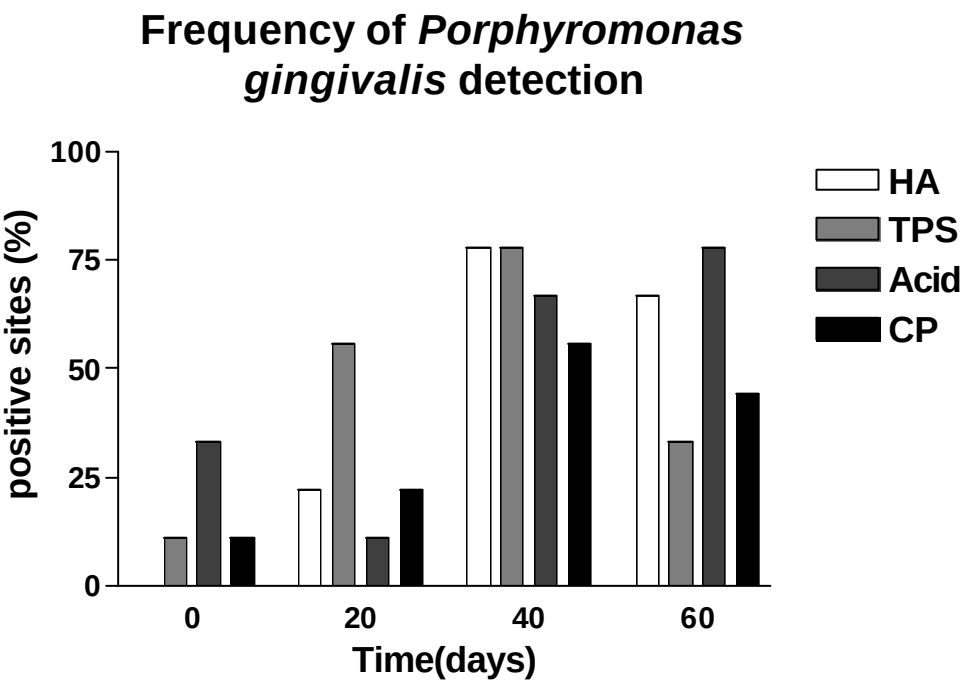

Frequency of Porphyromonas gingivalis detection
positive sites (%)
100
75
50
25
0
0
20
40
60
Time(days)
HA
TPS
Acid
CP

Fig.4

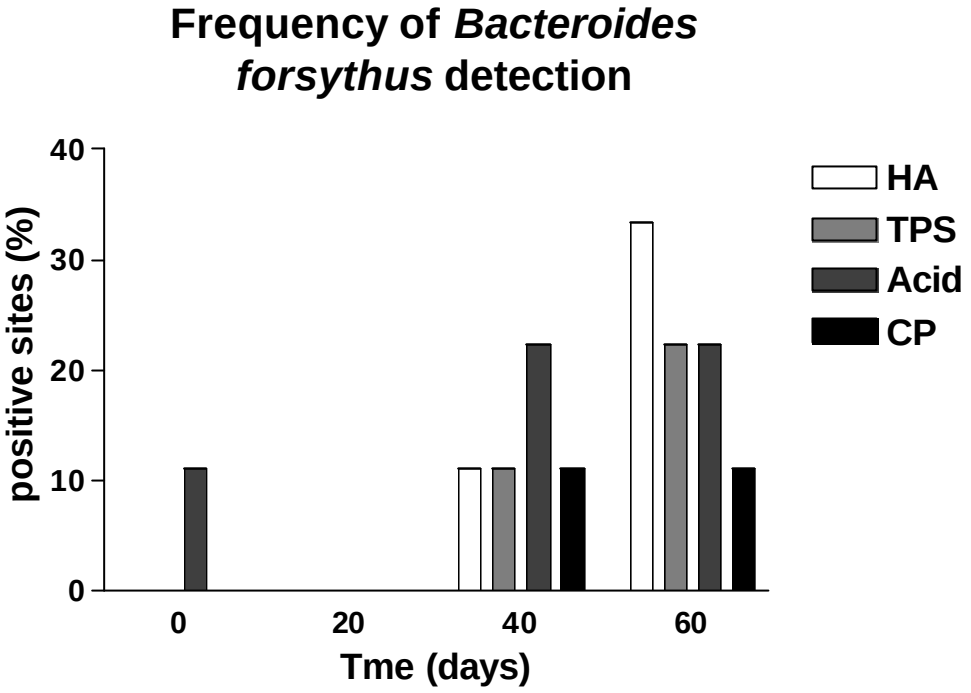

Frequency of Bacteroides forsythus detection
positive sites (%)
0
10
20
30
40
0
20
40
60
Tme (days)
HA
TPS
Acid
CP

Fig.5

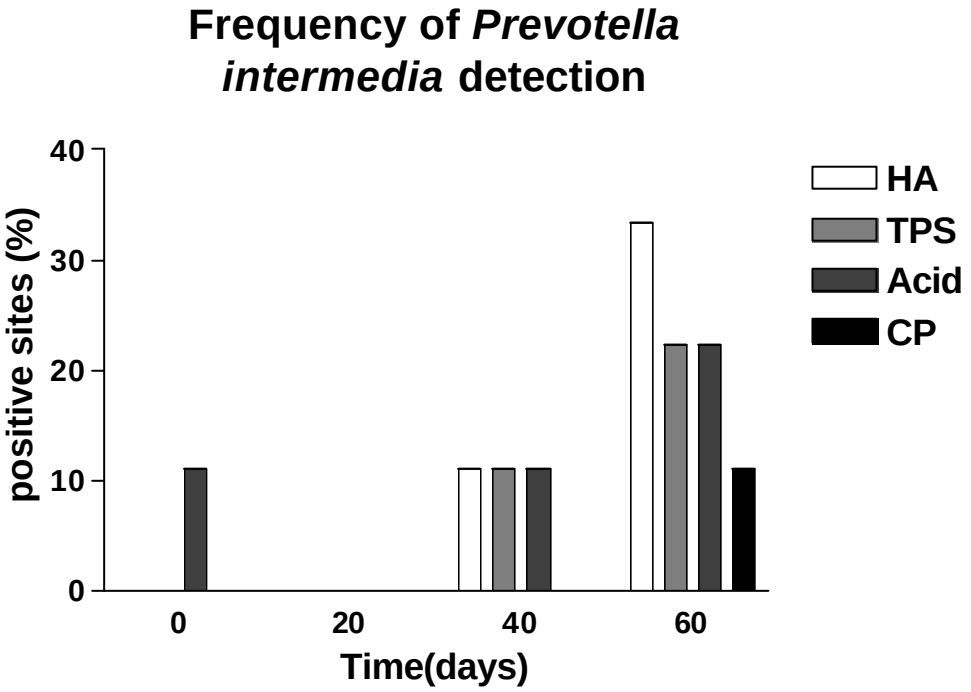

Frequency of Prevotella intermedia detection
positive sites (%)
0
10
20
30
40
0
20
40
60
Time(days)
HA
TPS
Acid
CP

Fig.6

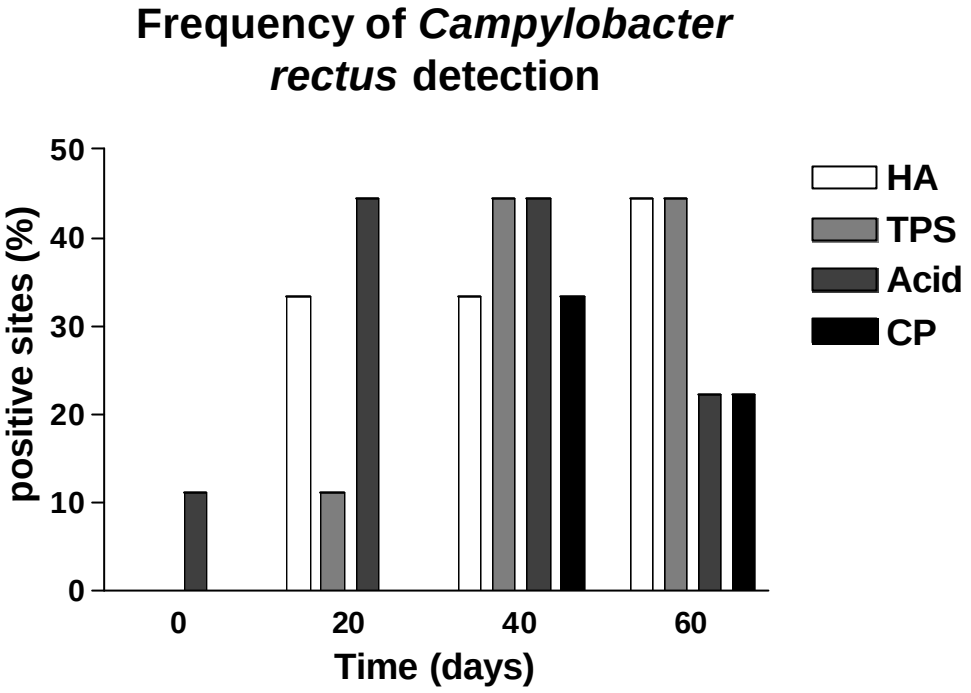

Frequency of *Campylobacter rectus* detection
positive sites (%)
50
40
30
20
10
0
0
20
40
60
Time (days)
HA
TPS
Acid
CP

Fig.7

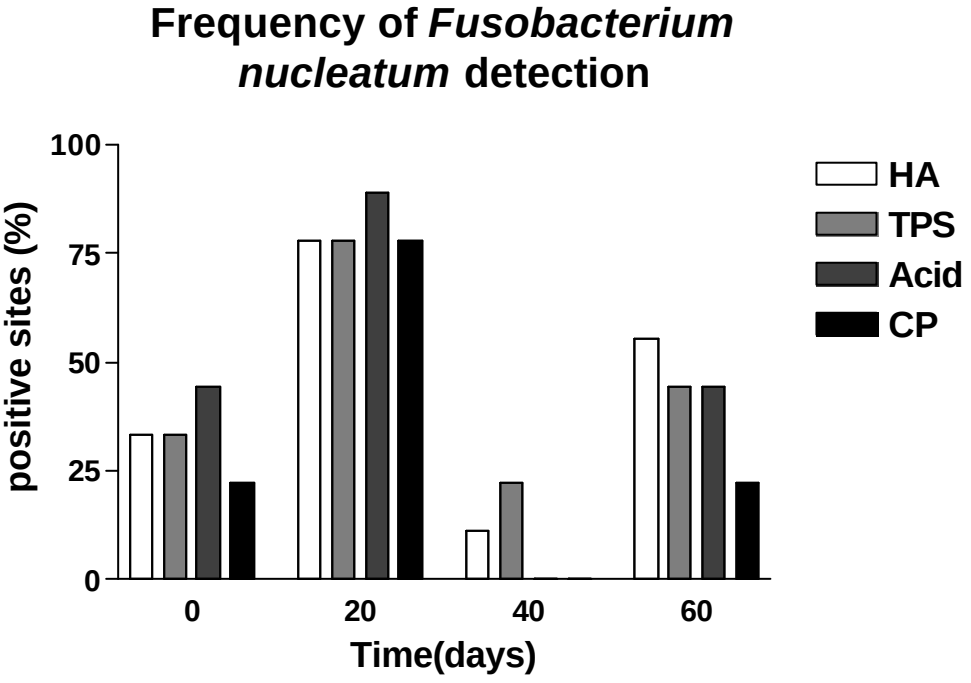

Frequency of *Fusobacterium nucleatum* detection
positive sites (%)
100
75
50
25
0
0
20
40
60
Time(days)
HA
TPS
Acid
CP

Fig. 8

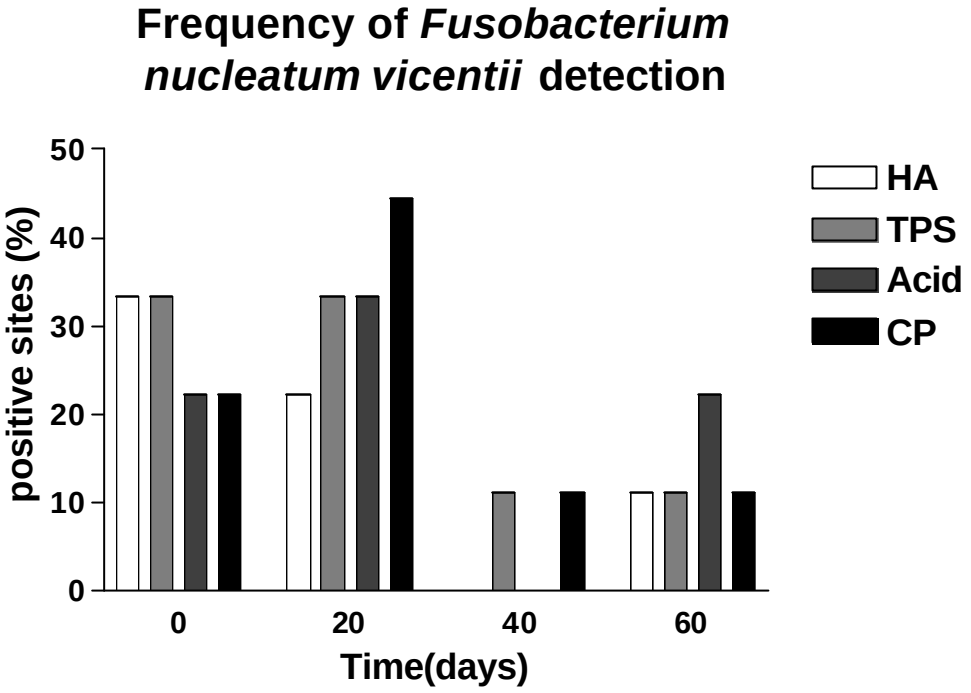

Frequency of *Fusobacterium nucleatum vicentii* detection
positive sites (%)
0
10
20
30
40
50
0
20
40
60
Time(days)
HA
TPS
Acid
CP

Fig. 9

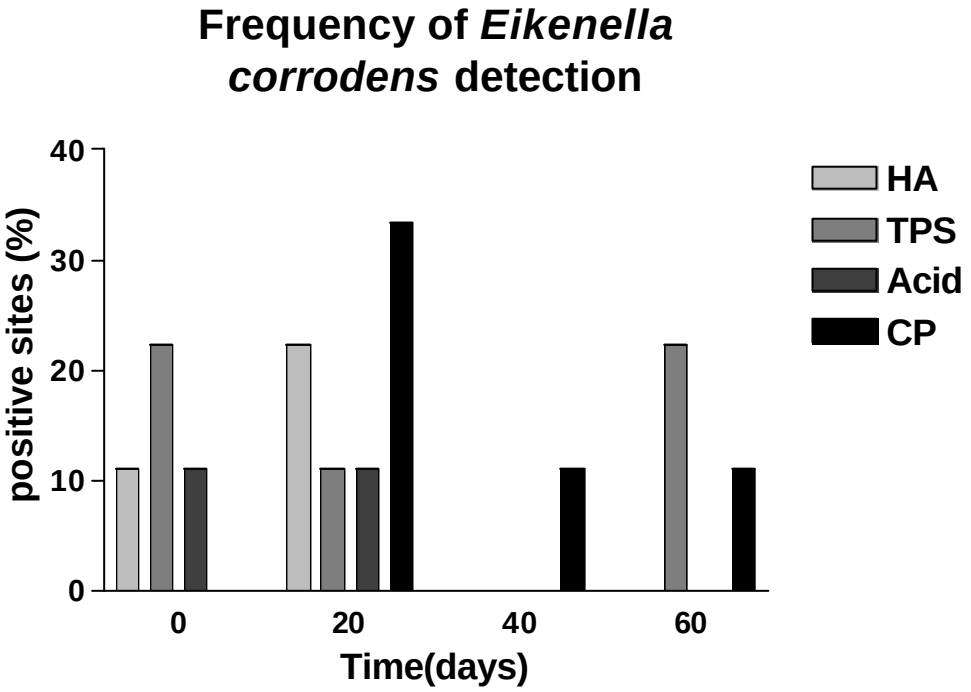

Frequency of *Eikenella corrodens* detection
positive sites (%)
0
10
20
30
40
0
20
40
60
Time(days)
HA
TPS
Acid
CP

Fig. 10

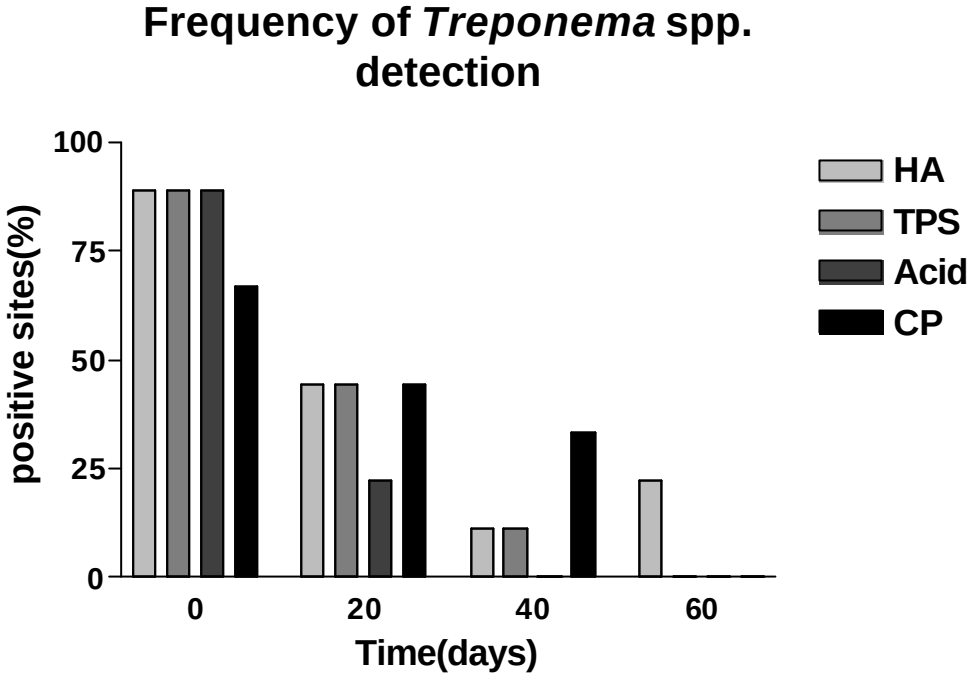

Frequency of *Treponema* spp. detection
positive sites(%)
100
75
50
25
0
0
20
40
60
Time(days)
HA
TPS
Acid
CP

Fig. 11

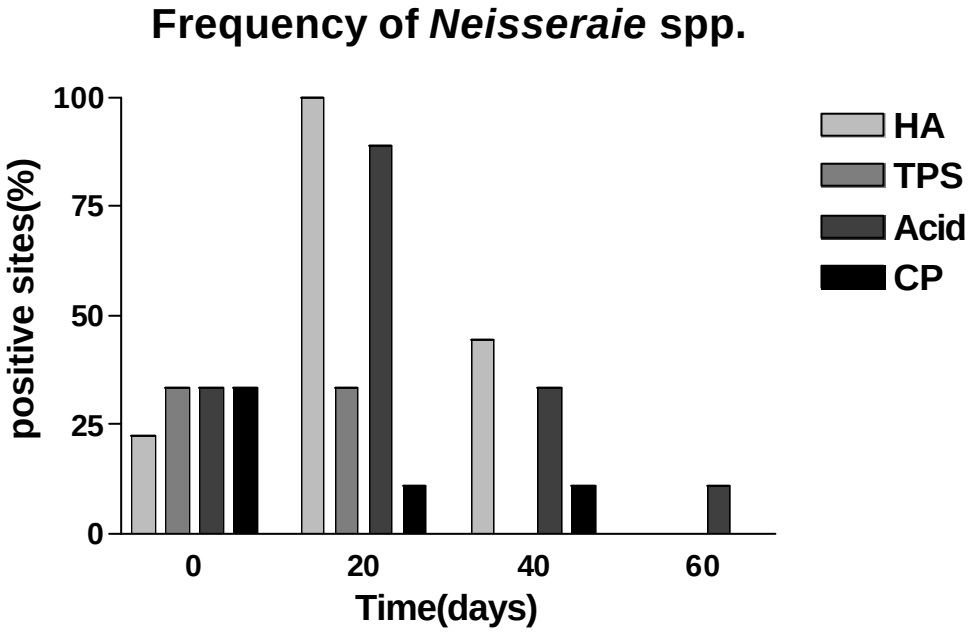
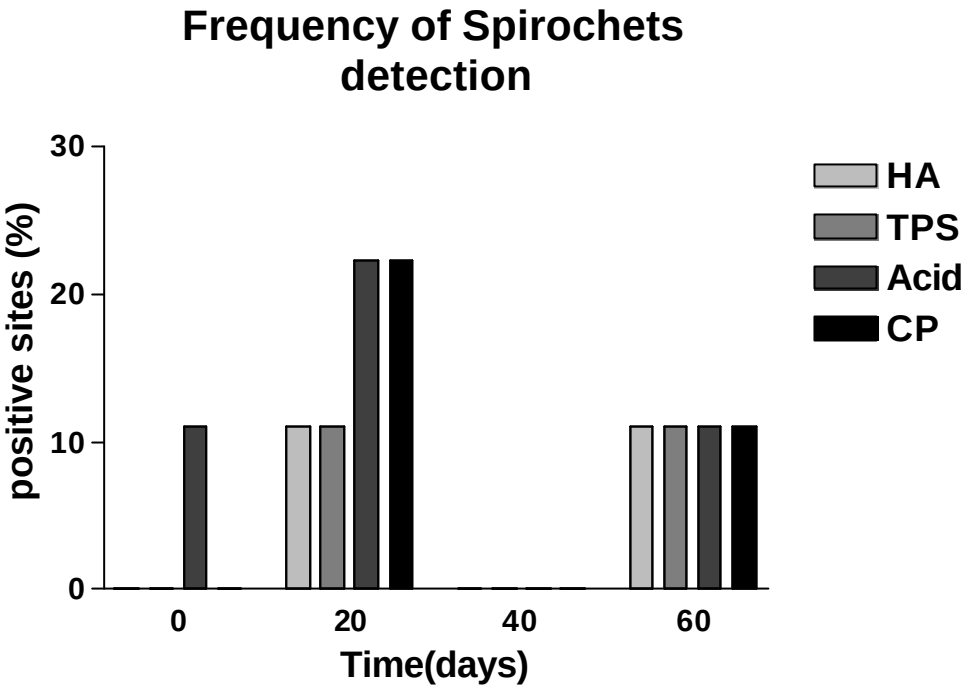

Frequency of *Neisseraie* spp.
positive sites(%)
100
75
50
25
0
0
20
40
60
Time(days)
HA
TPS
Acid
CP

Fig. 12


Frequency of Spirochets detection
positive sites (%)
30
20
10
0
0
20
40
60
Time(days)
HA
TPS
Acid
CP

CAPITULO-5 PROGRESSION OF EXPERIMENTAL CHRONIC PERI-IMPLANTITIS. CLINICAL AND MICROBIOLOGICAL EVALUATION IN A CANINE MODEL.

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Runing Title: Progression of chronic peri-implantitis

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Shibli JA, Martins MC, Jordan SF, Haraszthy VI, Zambon JJ, Marcantonio E. Jr. Progression of experimental chronic peri-implantitis. Clinical and microbiological evaluation in a canine model.

Abstract

The aim of this study was to evaluate progression of experimental peri-implantitis in dogs using implants with different surface coatings. Thirty-six dental implants with four different surface coatings (machined-CPTi; titanium plasma-sprayed-TPS; hydroxyapatite-HA; and acid-etched) were placed in 6 mongrel dogs. Five months after implantation, peri-implantitis was induced by cotton ligatures placement to facilitate plaque accumulation. The ligatures were changed at 20 day-intervals for 60 days. After 60 days the ligatures were removed and supragingival plaque control was initiated for 12-months. Clinical attachment level (CAL), bone height and microbiological samples were obtained at baseline (ligature placement), at 60 days and at 425 days. The microbiological samples were studied by polymearase chain reaction by amplifying the 16S rDNA and subsequently by the use of strain specific probe. CAL changed around all implant surfaces after ligature placement ($p<0.0001$). However, the means of CAL were not statistically significant among the different surfaces ($p>0.05$). The range of CAL variation, calculated between baseline and 60 days and between 60 and 425 days decreased. The HA surface showed the greatest bone loss measurement (4.78$\pm$0.38mm) and the TPS showed the smallest bone loss (4.27$\pm$0.62mm). However, statistical significance was not assessed for different coatings ($p>0.05$). Bone loss increased during the entire experiment ($p<0.0001$).

Actinobacillus actinomycetemcomitans, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Bacteroides forsythus*, *Campylobacter rectus*, *Fusobacterium nucleatum, Fusobacterium nucleatum* ss *vicentii*, *Eikenella corrodens*, *Neisseria* spp., *Treponema* spp. and spirochetes were detected around each implant surface both after 60 and 425 days. No statistically significant differences were observed around different implant surfaces concerning these bacteria. Experimental peri-implantitis in dogs may be a useful model to evaluate the progression of peri-implantitis. The clinical data observed at initial phase showed rapid and severe peri-implant tissue breakdown associated with bacterial shift toward gram-negative flora. After ligature removal, the progression of peri-implantitis was observed. While there was no significant difference observed between different implant surfaces, HA-coated surfaces appeared to be more susceptible to peri-implantitis in this experimental dog model.

Key words: Dental implants/microstructure; peri-implantitis/etiology; peri-implantitis/progression; biofilm/microbiology; dogs.

INTRODUCTION

Several longitudinal studies have reported high survival and success rates for dental implants (for review see van Steenberghe et al. 1999). Nevertheless, dental implant failures due to peri-implant infection have also been reported (for review see Quirynen et al. 2002). Animal studies have shown that peri-implantitis is a condition characterized by soft tissue inflammation, bleeding and suppuration, presented both rapid clinical attachment loss and vertical/horizontal bone loss (Lindhe et al. 1992; Lang et al. 1993; Shou et al. 1996). These studies compared the clinical, histological and microbiological changes around dental implants, after plaque accumulation by means of ligature placement. The placement of biofilm-retentive ligatures in animals leads to shifts in the composition of the microflora and peri-implantitis (Lindhe et al. 1992; Lang et al. 1993; Tillmanns et al. 1998). This condition is often characterized as acute inflammatory process that does not reproduce the real peri-implant lesion (Tonetti & Schimdt, 1994).

Despite the evidence that the primary etiologic agent in both periodontitis and in peri-implantitis is periodontal bacteria, the host response to the bacterial infection also seems to play a major role in the pathogenesis of periodontal diseases (Page 1991; Ebersole 1990). Those animal investigations reported that a bacterial shift was correlated with clinical attachment loss and peri-implant bone loss. Tillmanns et al. (1997, 1998) evaluated the peri-implantitis around different coated-implant surfaces and concluded that all implants were equally susceptible to peri-implant breakdown. Hanish et al. (1997) monitored the peri-

implant tissue breakdown in hydroxyapatite (HA) coated-implants and reported 2.7mm of attachement loss. The average measured to attachment loss in commercially pure titanium (CPTi) was 3.7mm (Nociti et al. 2001).

Although the increase of probing depth and clinical attachment loss often was associated with the detection of *Porphyromonas gingivalis*, *Prevotella intermedia*, *Bacteroides forsythus*, *Fusobacterium nucleatum* and, less frequently, *Actinobacillus actinomycetemcomitans* (Nociti et al. 2001), the relationship between different dental implant surfaces and bacterial biofilm in peri-implantitis development is unclear.

The different coating surfaces could influence the bacterial adsorption (Nakazato et al. 1989). Physical and chemical factors can affect the attachment of biofilms to a hard surface. The roughness of the surface can increase surface area and hence increase the colonization. Roughness also provides protection from shear forces and increases the difficulty of cleaning methods. Further, Quirynen & Bollen (1995) have shown that supragingival plaque formation, after initial colonization has occurred, was faster on a rough surface. The initial colonization of an intra-oral hard surface starts from surface irregularities such as cracks, grooves, or abrasion defects and subsequently spreads out from these areas as a relatively even monolayer of cells. The roughness of different dental implant surfaces can work like grooves for initial periodontal pathogen adhesion.

Therefore, the purpose of this investigation was to induce chronic peri-implantitis around different dental implant surfaces in dogs and evaluate the clinical and microbiological changes over time.

MATERIAL AND METHODS

Animals

The outline of the study is presented in Fig. 1. Six adult, systemically healthy, male mongrel dogs, 2 years of age and an average weight of 18Kg were used. Animal selection, management, and surgical protocol followed routines approved for this study by Araraquara Dental School Institutional Animal Care and Use Committee.

All surgical and clinical procedures as well as the microbiological sampling were performed under general anesthesia accomplished by 0.05mg/Kg of subcutaneous preanesthesia sedation (atropine sulphate) and intravenous injection of chlorpromazine and thiopental. Oral prophylaxis was performed within 2 weeks before teeth extraction. After that, all mandibular premolars were extracted creating an edentulous ridge. Both the mandibular quadrants and the alveoli were allowed to heal for a period of 3 months. The upper premolars were also extracted to avoid occlusion trauma interference and to avoid altering the experimental design proposed by this study. During the healing period, bacterial biofilm control was instituted by means of scrubbing 0.12% chlorhexidine daily, scaling and root planing once a month, until the placement of cotton ligatures.

Implant Surfaces

Thirty-six dental implants with four different surfaces of three different implant systems were inserted in canine jaws. Each implant possessed one of four different dental implant surfaces as follows: nine commercially pure titanium implants– CPTi (3*i*® Implant Innovations, Palm Beach, Fl, USA); nine titanium

plasma sprayed – TPS (Esthetic plus, ITI® Dental Implant System, Straumann AG, Waldenburg, Switzerland); nine hydroxyapatite – HA (Calcitek®, Sulzer Medica, Carsbad, CA, USA), and nine hybrid surfaces – machined titanium in the three first threads and acid-etched in others threads – acid (Osseotite®, 3*i* Implant Innovations, Palm Beach, Fl, USA). All implants measure 10mm in length and 3.75mm in diameter (except TPS, with 4.1mm of diameter).

Implant Surgery

The dental implants were placed after a full thickness flap under aseptic surgical conditions. The recipient sites were prepared using original instruments for each dental implant system, according to the surgical techniques advocated by each implant manufacturer. The implants were randomly distributed among the six animals so that each dental implant surface was represented at least once in each dog. The implants were placed at bone level and a cover screw was screwed onto the implant, including the TPS dental implant surface due to a modification in technique insertion as indicated by the manufacturer. The flaps were sutured with single interrupted sutures, submerging all dental implants.

To avoid post-surgical infection, an antibiotic coverage with potassic and sodic benzilpenicilin was given once a week for 2 weeks. Paracetamol was given as pain control medication, and the sutures were removed after 10 days.

Experimental Chronic Peri-implantitis

Three months after dental implant insertion, healing abutment connections were installed, according to the instructions of each dental implant system. After two months of a plaque control program and the healing of the soft tissue, cotton

floss ligatures were placed around the dental implants and sutured in the peri-implant mucosa, not only to facilitate plaque accumulation, but also to hold the ligatures in position. Tying further ligatures at 20 day-intervals for a period of 60 days accelerated acute peri-implant bone loss. At 60 days, when approximately 40% of the initial bone support was lost, the ligatures were finally removed. A 12-month experimental chronic peri-implantitits was initiated with a supragingival plaque control by means of scrubbing daily with 0.12% chlorhexidine and scaling the abutment surface once a month.

Clinical evaluation

Clinical parameters were recorded at baseline and 20, 40, 60, and 425 days after ligature tissue-breakdown. A single pre-calibrated examiner carried out the clinical exams. The probing depth (PD) and clinical attachment loss (CAL) were registered using a force-controlled calibrated periodontal probe (Florida Probe, Computerized Probe Inc, Gainesville, Fl,USA) with a constant probing force of 0.20N and a probe-tip diameter of 0.4mm (Fig. 4). These data were recorded at distobuccal, midbuccal, mesiobuccal, mesiolingual, midlingual, and distolingual aspects of each dental implant. PD and the distance between the gingival margin (GM) and the fixed point in the abutment surface (FP) were recorded. CAL was then calculated according the formula: PD- (GM-FP). All measurements were perfomed in same position and same place with the aid of a dot marked in the abutment at baseline.

Mobility

Implant mobility was evaluated with the Periotest® (Siemens, Bensheim, Germany) device by means of the Periotest scores (PTV). The implants were tapped with the Periotest® rod perpendicular to the longitudinal axis of the implants. The Periotest handpiece was held parallel to the floor at a distance of about 2.0mm from the abutment surface. The spot chosen for tapping was at the buccal aspect of the abutment, and the spot was marked with a dot, so the measurement was always performed at the same place. The same Periotest® device was used during all experiment. The Periotest® was calibrated before every measurement and all measurements were performed by the same investigator. The PTV mean variations were assessed intra-surfaces to avoid variation among the different implants systems used in this study.

Radiographic analysis

Standardized periapical radiographs were taken with a digital image system – CDR® (Computed Dental Radiography, Schink Tecnologies Inc., USA) in order to measure the relative vertical peri-implant bone loss (VBL) and horizontal bone loss (HBL). A film holder system was affixed to a silicone bite block made of polivinyl siloxane putty impression material used to standardize the placement of the CDR sensor in relationship to the implants and the x-ray source. Images were obtained at baseline and 20, 40, 60, and 425 days after ligature placement.

A dental x-ray machined equipped with a 35-cm-long cone was used to expose the periapical intra-oral sensor. Exposure parameters were 70 kilivolt

(peak), 15 mA, and 1/4 second at a focus-to-sensor distance of 37cm.The linear distance between the fixed point in abutment and the first visible bone-to-implant contact was determined mesially and distally using digital images of implant to determine VBL. The HBL was measured between a fixed point in implant shoulder and the crestal bone margins in horizontal aspect. The mesial and distal values were averaged to obtain a mean implant value for both radiographic recordings. Two examiners made all measurements independently. If there was a discrepancy of 0.5mm or less, the mean value of the 2 measurements was used. In situations with greater discrepancies, the images were analysed again and discussed until a consensus was reached.

Microbiological Procedures

Peri-implant microbial samples were taken from the mesio-distal site with paper points immediately before the ligature placement and 20, 40, 60 and 425 days after ligature placement. Supra-mucosal debridment at the sample site was initially performed with a sterile plastic curette and dry gauze after isolation from saliva using cotton rolls. The area was carefully dried and the bacterial sample was collected with four sterile paper points gently inserted into the peri-implant sulcus, as far apically as possible, and left for 20 seconds. The paper points were placed in sterile transport vials and stored in a freezer pending analysis. All samples were collected by the same examiner and coded by an assistant to keep the blindness of the study.

Analysis of Amplified 16S rDNA

The PCR-amplified 16S rDNA samples using digoxigenin-labed species-specific oligonucleotide probes (Dix et al. 1990; Dewhirst & Paster 1991) were tested for *Actinobacillus actinomycetemcomitans* (CACTTAAAGG TCCGCCTACGTGCC), *Porphyromonas gingivalis* (GCAGTTTCAACGGCAGG GCTGAACG), *Prevotella intermedia* (GGTCCTTATTCGAAGGGTAAATGC), *Bacteroides forsythus* (CGTATCTCATTTTATTCCCCTGTA), *Campylobacter rectus* (CAAGCTACTTAATCTCCGTTCGAC), *Fusobacterium nucleatum* (GGTTTCCCCGAAGGGACATGAAAC); *F. nucleatum* ss *vincetii* (ACTTCACA GCTTTGCGACTCTCTGTTC), *Eikenella corrodens* (ACCGTCAGCAAAAAG TGGTATTAGCAC), *Treponema* spp.(GGCAGTAGGGGTTGCGCTCGTT), *Neisseria* spp. (CCTCTGTACCGACCATTGTATGAC) and spirochetes (CGAC TTTGCATGSTTAARAC). The oligonucleotide probes were 3'-end labeled with digoxigenin-11-ddUTP (Genius 5, Boehringer Mannheim, Indianapolis, In, USA). The DNA was tranferred overnight onto membranes using 10x standard salt phosphate EDTA buffer (SSPE). Hybridization with digoxigenin-labeled oligonucleotide probes was performed at 45^{o}C. The hybrydized membranes were washed twice with a high salt solution (2xSCC[0.15M NaCl plus 0.015 trisodium citrate, pH 7.0] in 0.1% sodium dodecyl sulphate) at room temperature and then twice with a low salt solution (0.1xSCC in 0.1% sodium dodecyl sulphate) at 45^{o}C. The membranes were reacted with anti-digoxigenin alkaline phosphatase conjugate. The color reaction was produced with 5-bromo-4chloro-3-indolyl phosphate and nitroblue tetrazolium salts.

Polymerase Chain Reaction of 16S rDNA

In addition to DNA probing by checkerboard assay, the PCR-amplification of the conserved region of 16S ribossomal DNA were also tested for periodontal pathogens including *A. actinomycetemcomitans* (primer 1-ATTGGGG TTTAGCCCTGGTG and Rev16s-ACGTCATCCCCACCTTCCTC), *P. gingivalis* (primer1-TGTAGATGACTGATGGTGAAAACC and Rev16s-ACGTCATCCCCAC CTTCCTC) and *B. forsythus* (primer 1-TACAGGGGAATAAAATGAGATACG and Rev 16s-ACGTCATCCCCACCTTCCTC). All these PCR primers were obtained commercially (Gibco BRL, Grand Island, NY, USA). Between 30 and 100ng of genomic DNA was added to the PCR mixture, which contained 1µmol/L of the primers, 2.5U of *Taq* polymerase (Perkin-Elmer, Norwalk, CT, USA) in 1x buffer and 0.2mmol/L of dCTP, dGTP, dATP, and dTTP in a total volume of 50µL. The amplification was performed for 30 cycles of 30 seconds at 95^{o}C, 30 seconds at 55^{o}C and 30 seconds at 72^{o}C in thermocycler.

Positive and negative controls were included with each set. The negative control consisted of all the PCR reagents except for the sample DNA. The positive control contained all the PCR reagents together with positive controls for *A. actinomycetemconitans*; *P.gingivalis* and *B. forsythus*. Twenty µL of each PCR reaction mixture was electrophorese in 1.5% (*A. actinomycetemconitans* and *B. forsythus*) and in 2% (for *P.gingivalis*) agarose gel in TBE buffer and the amplification products were visualized under 302nm ultraviolet light, on ethidium bromide-stained gels.

Statistical Analysis

Analysis of variance, using comparison of several proportions (contingency table) was used to compare the distribution of different bacteria for each type of implant, and to compare bacterial colonization at different points (Armitage P et al. 2002). The clinical and radiographical data were compared by mean paired *t*-test (2-tailed). The differences between buccal and approximal sites were assessed by unpaired *t*-test (2-tailed). All tests were stratified according to dog (unit of analysis, n=6). Level of significance was set at 0.05.

RESULTS

Of the 36 implants, none were lost during the acute phase of experimental peri-implantitis (initial 60 days). At 425 days, 17 dental implants were lost due to either great bone loss or mobility. The distribution of implants per dog after chronic peri-implantitis phase was as follows: four dogs retained at least one osseointegrated implant CPTi, five dogs presented six implants with a TPS surface, three dogs presented the three remaining HA-coated surface and, finally, five dogs showed five implants with an acid surface. The HA-coated lost implants show evidencies of HA-coated resorption (Fig. 2).

Probing depth

The buccal PD at baseline ranged between 1.25$\pm$0.41mm to acid surface and 1.47$\pm$0.80mm to CPTi (Table 1). After ligature placement, the means of both buccal and approximal sites increased statistically for all surfaces (p<0.05). At 425 days, the buccal PD means for CPTi and TPS surface were lower as

compared to measurements at 60 days, despite no significant difference was assessed (Fig. 3).

The means of approximal PD are presented in Table 2. The higher means at baseline were observed in HA (2.27±0.83mm) and acid surface (2.27±0.97mm) respectivelly. At the end of the chronic peri-implantitis period, the CPTi surface showed a decrease in approximal PD (Fig. 4). Although statistical differences were not assessed among the implant surfaces, in either buccal or approximal sites in regard to the coated surface ($p>0.05$), significant intra-group differences were observed between approximal and buccal sites.

The CPTi surface showed a difference between the means of buccal and approximal PD at 20 days ($p=0.046$). TPS and acid surface show also differences between approximal and buccal PD measurements at baseline ($p\leq0.043$). The HA-coated surface depicted differences for both PD measurements at baseline, 20, and 60 days ($p\leq0.044$).

Clinical Attachment Level

Clinical attachment at buccal and approximal sites level changed around all dental implants surfaces not only after ligature-induced tissue breakdown but also ligature removal-chronic phase-($p<0.0001$) (Figs.5 and 6). The CAL at buccal sites is presented in Table 3. The TPS surface shows the highest CAL at buccal sites over time (7.68±0.63mm). The CAL at approximal sites varied, in general, between 5.87mm to acid surface and 6.22mm to HA-coated surface (Table 4). The means of CAL at buccal sites were always higher than CAL at approximal sites for CPTi ($p=0.012$), TPS ($p<0.0001$), HA ($p=0.002$), and acid

surface (p<0.0001). However, the means of buccal and approximal CAL were not statistically significant among the different implant surfaces (p>0.05).

When the range of CAL progression was calculated between baseline and 60 days (acute phase) and between 60 and 425 days (chronic phase), a decrease in the means of CAL was observed for both buccal and approximal sites (Figs. 7 and 8). The mean of CAL progression at buccal sites for CPTi surface decreased from 4.30±0.44mm to 2.47±1.08mm (p=0.036). A statistical difference of p=0.019 between acute (4.31±0.37mm) and chronic phase (1.55±0.97mm) were also observed at approximal sites.

TPS surface did not exhibit statistical differences for either buccal (p=0.167) or approximal sites (p=0.149). At buccal sites the CAL progression decreased from 4.46±0.37mm to 2.72±0.92mm. The progressions of CAL at approximal sites for acute and chronic phases were 4.07±1.14mm and 2.20±1.17mm respectively.

The progression of CAL in HA-coated surface at buccal sites was of 4.29±0.92mm at acute phase and 2.90±0.52mm at chronic phase (p=0.232). A significant difference between acute (4.78±1.09mm) and chronic phase (2.06±0.35mm) (p=0.039) was observed for CAL progression at approximal sites.

Acid surfaces presented statistical difference of CAL progression at both sites. The mean of CAL progression at buccal sites decreased from 5.31±0.51mm at acute phase to 2.37±0.38mm at chronic phase (p=0.001). The approximal sites exhibited a decrease of CAL progression from 4.59±1.10mm to 1.46±0.89mm (p=0.034).

Radiographic Bone Loss

At baseline, no implant surface exhibited peri-implant radiolucencies. The means of VBL and HBL for all dental implant surfaces are presented in tables 5 and 6, respectively. The Ha-coated surface showed the greatest bone loss measurement (4.78±0.38mm) and the TPS surface showed the smallest bone loss measurement (4.27±0.62mm). However, statistical significance was not assessed for different dental implants surfaces ($p>0.05$). The VBL increased statistically not only the acute pahse but also during the ligature removal (chronic phase) ($p<0.0001$) (Fig. 9).

The progression of vertical bone loss between acute and chronic phase decreased significantly for all implant surfaces (Fig. 10). The CPTi surface showed the highest decrease in VBL progression. At acute and chronic phase, the mean of VBL progression was 3.85±0.66mm and 0.66±0.26mm respectively ($p=0.0014$). The VBL progression for TPS surface ranged between 3.74±0.86mm for acute phase and 1.18±0.78mm at the end of the experiment ($p=0.010$).

Although the number of HA-coated surfaces had been elevated, the VBL progression was similar to means observed for TPS and acid surfaces. The VBL progression for HA surfaces decreased from 3.88±041mm to 1.10±0.51mm ($p=0.033$). The acid surface presented a VBL progression of 3.50±0.62mm at acute phase and 1.08±0.66mm at chronic phase ($p=0.026$).

The HBL increased during the first 60 days relative to ligature placement (Fig. 11). At 425 days, the means of HBL decreased for all surfaces, however a

statistical significance was observed over time ($p<0.05$). In addition, the different dental implant surface was not found to be significant ($p>0.05$).

Mobility assessment

The abutments were checked before each evaluation in order to avoid false results with regard to mobility. None of the implants exhibited mobility on manual examination.

The Periotest measurements produced PTV scores ranging from -2.66±2.48 for HA and –1.50±2.34 for CPTi surface at baseline (Table 7). Figure 12 shows the time course of PTV scores. After ligature-induced tissue breakdown, these scores increase over time ($p<0.05$), although of differences among the surfaces were not observed ($p>0.05$).

Microbiological results

All dogs were positive for all periodontal pathogens over time. A statistically significant relation between a positive detection of a target bacteria and dental implant surface was not observed. *A. actinomycetemcomitans* was not detected at baseline and at 20 days for all dental implants. At 425 days, no HA-coated surface showed a positive sample for *A.actinomycetemcomitans* (Fig.13). *P. gingivalis* was detected in 100% of samples at 425 days (Fig. 14). The frequency of detection of *P. intermedia* increased after 20 days except, for CPTi surface, on which *P. intermedia* was detected at 60 days (Fig. 15). A statistical significance was observed for HA-coated surface and *P. intermedia* detection ($p=0.027$). *B. forsythus* was not detected at 20 days in any sample, although its frequency of detection increased over time principally, for CPTi and

acid surface. Despite this, statististical significance was not observed (p=0.123) (Fig. 16). The detection of *C. rectus* ranged between 33.34% for CPTi and HA surface and 46.76% for TPS and acid surface (Fig. 17). At 425 days the lowest *C. rectus* detection was observed in the HA surface, although a statistical difference was not observed.

The fusobacterium group presented lower detections at 20 and 40 days for all groups (Figs. 18 and 19). *E. corrodens* was detected statistically more in HA sites at 425 days (p=0.002) (Fig.20). *Neisseria* spp., *Treponema* spp. and spirochetes have lower detection at 40 days (Figs. 21 to 23).

DISCUSSION

The aim of this study was to evaluate the chronic peri-implant breakdown around four different implant surfaces in a canine model. Several studies evaluated the experimental peri-implantitis around machine, TPS and HA-coated surfaces using ligatures for plaque accumulation (Lang et al. 1993, Hanisch et al. 1997, Nociti et al. 2001). In addition, placement of cotton (Hanisch et al. 1997; Tilmmanns et al. 1997,1998; Nociti et al. 2001) or silk ligatures (Schou et al. 1996,1993, Hickey et al. 1991) could induce a foreign body reaction which is different from peri-implant diseases. The trauma due to ligature placement has been implicated as a cause of periodontal breakdown in experimental periodontitis (Rovin et al. 1966, Jansen et al. 1992; Groisman & Klinge 1990).

In this investigation, tying further ligatures at 20 day-intervals for a period of 60 days accelerated the loss of peri-implant apparatus. A significant peri-implant bone loss was accomplishing of bacterial shift in a relative short period

(acute phase). After that, the ligatures were removed and a supragingival plaque control was performed during 12 months (chronic phase). However, the removal of ligatures did not convert the acute destructive peri-implant phase in a non-aggressive lesion as reported by Hanish et al. (1997), Marinello et al. (1995) and Gotfredesn et al. 2002.

More than 66% of HA-coated implants were lost after chronic peri-implantitis, in contrast to other surfaces used in this study. Firstly, HA resorption is most likely caused by low pH (Maxian et al.1993; Daculsi et al.1989) and phagocythosis mediated by osteoclast-like cells, monocytes, and fibroblasts (Overgaard et al.1998). Secondly, HA coatings have been shown that the resorption depends on characteristics such as composition, porosity, crystallinity and thickness (Frayssinet et al.1995; Gineste et al.1999). Ginneste et al. (1999) reported a resorption rate of more than 50% of HA coatings in dogs after a 1-year implantation period. We can speculate that the HA resorption must be higher with the presence of periodontal pathogens (Mukherjee et al.2000). The higher mean of vertical bone loss, in conjunction with the increase in mobility (micromotion) observed for HA-surface in this study could be additional factors for HA dissolution (Overgaard et al. 1996).

The course of PD at acute phase was characterized by a constant increase in both buccal and approximal sites. However, at chronic phase, different characteristics were observed among the surfaces. Overall, the CPTi surface showed a decrease of probing depth followed by TPS at buccal sites. HA-coated and acid surfaces showed an increase of probing pocket depth, but

not in the same proportion that was observed at acute phase. After conducting a detailed analysis of each surface, many observations can be made. The CAL was always higher at buccal sites than approximal sites. These observations were similar to results achieved by Schou et al (2002). In this study, it was observed that increase of probing depth contributed to initial attachment loss, while peri-implant mucosal recession was responsible for continued attachment loss, in agreement with Lang et al. (1994) and Tillmanns et al. (1997). Although our study design (peri-implantitis model) does not permit a direct conclusion, the different marginal connective tissue fiber configurations associated with bone availability (approximal sites versus buccal/lingual sites) may explain the higher range of approximal CAL. Previous studies have demonstrated many features in common for initial peri-implant/periodontal breakdown (Berglundh et al. 1992; Pontoriero et al 1994; Schou et al. 2002), despite their inconclusive results (Schou et al. 2002). In addition, Schou et al. (2002) reported that the differences among teeth and implants may occur not only due to different configurations of tissues but also due to the necessity of ligature removal. In this way, the different macrostructures and microstructures used in this canine model could have over or underestimated the clinical measurements.

The bone loss increased over time, but at chronic phase the proportion of VBL decreased statistically when compared with initial phase (Fig. 10). The vertical bone loss was initially lowest to HA surface (baseline) and highest at end of experiment. These results can verify the loss of HA-coated implants over time. In contrast to VBL, the horizontal bone loss decreased over time and the bone

defect became horizontal instead of the characteristic saucerization. Tarnow et al. (1999) in a human study showed that there was a lateral component of bone loss once the biological width has formed. In addition, the parallel colagen fiber orientation observed around dental implants can influence this bone resorption mechanism in the horizontal aspect.

Implant surface characteristics may influence the bone response during the healing and long-term period (Buser et al. 1991). The TPS showed the lowest range of VBL over time, followed by CPTi and acid surface. However, a statistical difference was not observed for dental implants surface. The observed radiographic data reflected the association of factors such as bone remodeling on the coronal aspect, bone loss due to ligature trauma, and finally, bone loss induced by bacterial products. The CPTi exhibited presented the lowest VBL progression at chronic phase. The stylus profilometry of TPS and HA surface (undercuts) and acid surface (micropits) could have retained more bacterial biofilm and consequently more periodontal pathogens, suggesting that CPTi surface may behave better during maintenance after peri-implantitis infection.

However, we cannot understand clearly the features observed for acid surface. The acid surface used in this study is hybrid, showed CPTi surface in the first three screws, and acid treatment in the others screws with HCl/H_2SO_4 (manufacter information). After the acid treatment, the oxide formation differs from that shown in CPTi surface, and alters the surface affinity for LPS (Nelson et al.1997). This aspect could explain not only the degree of bone loss but also the predilection of *P. gingivalis* and *P. intermedia* at baseline in acid surface.

Both periodontal pathogens were associated with the induction and progression of the peri-implantitis, as well as the periodontal diseases. (Eke et al. 1998).

There was an increase of detection to *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia* and *B. forsthus* at 425 days. *P. gingivalis* and *B. forsythus* were also associated with attachment loss (Nociti et al. 2001). The role of *F. nucleatum* in coaggregation has been observed by several studies (Bradshaw et al. 1998; Kolenbrander et al. 1989) and can explain the high levels at baseline and 20 days for all surfaces. In addition, *C. rectus* and *F. nucleatum* have also been associated with peri-implant diseases, according to Papaioannou et al. (1995), Macuch & Tanner (2000), and Mombelli et al. (2001).

The detection of *F. nucleatum* ss *vicentii* and *E. corrodens,* has been associated with failing implants (Salcetti et al. 1997; Mombelli et al. 2001). Spirochetes have also been associated with failing implants (Rosemberg et al. 1991; Mombelli et al. 1987). However, detection of spirochetes was lowest in all surfaces. The use of chlorhexidine at plaque control phase may have reduced the potential sources of spirochetes colonization at baseline (Hanisch et al. 1997), however, spirochete detection has increased at 425 days. After ligature removal, the periodontal pathogens identified at acute phase remained and could be detected at chronic phase. The microbiological data showed that this canine model could reproduce the microbiota associated with peri-implantitis in humans and non-human studies.

The detection of *A. actinomycetemcomitans* was with accordance to human studies conducted by Mombelli et al. (2001), Mombelli et al. (1995),

Leonhardt et al. (1999) as well as in animal studies (Schou et al. 1996; Tillmans et al.1998). In our canine model the identification of *A. actinomycetemcomitans* disagreed with the features of Nociti et al. (2001) who failed to detect it using polymerase chain reaction. Tillmanns et al. (1998) detected *A. actinomycetemcomitans* in dogs using DNA probes. In this investigation, the microbiological samples were scored as positive for *A. actinomycetemcomitans*, *P. gingivalis* and *B. forsythus* when the microbiological samples were positive using both DNA probes and PCR. Several investigations have related the existence of high probability of false-positive results with the use of whole genomic DNA probes, principally for *A. actinomycetemcomitans* (Strzempko et al. 1987; Ali et al. 1996, 1997; van Steenberghen 1996).

The PTV variation was lowest to TPS surface, however the mobility as assessed by Periotest device did not show a statistical difference among the surfaces. The microstructure and greather diameter of implant used in this study (4.1mm for TPS surface vs. 3.75mm for other surfaces) can explain and validate these data.

In conclusion, this study indicates that experimental chronic peri-implantitis in dogs may be a useful model to evaluate peri-implant diseases. The clinical data observed at initial phase showed a rapid and severe peri-implant tissue breakdown associated with bacterial shift. After ligature removal, progressive peri-implantitis occurred and the HA-coated surface appeared to be more susceptible to tissue breakdown in this experimental model. However,

these results should be considered with caution due to the sample size evaluated, and further investigations must be conducted.

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Figure Legends

1. Outline of the experiment. Animals *n*=6, dental implants *n*=36. Ligatures were placed at 0 day (+Lig.) and removed at 60 days (-Lig.). Supragingival prophylaxis was performed during 12 months after ligature removal (day 425).
2. HA coated-implant surface retrieved due to mobility. Compare the macroscopic aspect of resorption of HA with a unused HA implant (right).
3. Time course of probing depth at buccal sites in millimeters at acute and chronic phase.
4. Time course of probing depth at approximal sites in millimeters at acute and chronic phase.
5. Time course of clinical attachment level at buccal sites in millimeters at acute and chronic phase.
6. Time course of clinical attachment at approximal sites in millimeters at acute and chronic phase.
7. Mean and standard deviation of Clinical Attachment Loss (CAL) progression at buccal sites at acute and chronic phase.
8. Mean and standard deviation of Clinical Attachment Loss (CAL) progression at approximal sites at acute and chronic phase.
9. Time course of vertical bone loss in millimeters at acute and chronic phase.
10. Mean and standard deviation of Vertical Bone Loss (VBL) progression at buccal sites at acute and chronic phase.

11. Time course of horizontal bone loss in millimeters at acute and chronic phase.

12. Time course of periotest value (PTV) at acute and chronic phase.

13. Frequency of distribution of samples positives of *A. actinomycetemcomitans*

14. Frequency of distribution of samples positives of *P. gingivalis*

15. Frequency of distribution of samples positives of *P. intermedia*

16. Frequency of distribution of samples positives of *B. forsythus*

17. Frequency of distribution of samples positives of *C. rectus*

18. Frequency of distribution of samples positives of *F. nucleatum*

19. Frequency of distribution of samples positives of *F. nucleatum* ss *vicentii*

20. Frequency of distribution of samples positives of *E. corrodens*

21. Frequency of distribution of samples positives of *Neisseria* spp.

22. Frequency of distribution of samples positives of *Treponema* spp.

23. Frequency of distribution of samples positives of spirochetes

Table 1: Mean±SD of probing depth at buccal faces (mm).

Surface	Baseline	20 days	40 days	60 days	425 days	PD over time
CPTi	1.47±0.80	3.43±0.80*	4.84±1.37*	4.85±0.82*	3.80±1.30*	2.32±0.63^{ns}
TPS	1.33±0.30	3.14±0.61*	3.42±0.61*	4.58±0.86*	4.40±1.14*	3.06±0.48^{ns}
HA	1.41±0.37	3.62±0.48*	4.70±0.39*	5.16±0.37*	5.66±0.76*	4.25±0.36^{ns}
Acid	1.25±0.41	3.87±0.62*	4.99±0.55*	5.45±0.67*	5.75±1.50*	4.50±0.63^{ns}

* Statistically different from baseline $p<0.05$

^{ns} Non significant regarding to different surfaces

Table 2: Mean±SD of probing depth at approximal faces (mm).

Surface	Baseline	20 days	40 days	60 days	425 days	PD over time
CPTi	2.18±0.87	4.53±0.86*	5.32±0.98*	5.87±0.97*	4.30±1.03*	2.11±0.57^{ns}
TPS	2.03±0.68	3.58±0.94*	3.95±0.61*	5.01±0.47*	5.06±2.70*	3.56±1.13^{ns}
HA	2.27±0.83	4.77±0.98*	5.25±0.57*	5.66±0.20*	5.66±0.57*	3.39±0.54^{ns}
Acid	2.27±0.97	4.77±0.77*	5.56±0.32*	5.68±0.79*	6.25±0.64*	3.98±0.55^{ns}

* Statistically different from baseline $p<0.05$

^{ns} Non significant regarding to different surfaces

Table 3: Mean±SD of clinical attachment loss at buccal faces (mm).

Surface	Baseline	20 days	40 days	60 days	425 days	CAL over time
CPTi	8.18±0.74	11.29±0.80*	12.18±0.93*	12.75±0.82*	14.9±1.04*	6.71±0.55^{ns}
TPS	7.68±0.95	9.82±0.70*	10.67±0.98*	11.93±0.77*	14.75±1.04*	7.68±0.63^{ns}
HA	7.81±0.62	10.16±1.06*	11.14±0.92*	11.95±0.95*	15.06±0.11*	7.25±0.37^{ns}
Acid	8.08±0.68	10.95±0.52*	12.16±0.73*	13.04±0.91*	15.50±0.38*	7.41±0.38^{ns}

* Statistically different from baseline $p<0.0001$

^{ns} Non significant regarding to different surfaces

Table 4: Mean±SD of clinical attachment levels at approximal faces (mm).

Surface	Baseline	20 days	40 days	60 days	425 days	CAL over time
CPTi	8.37±0.54*	11.35±1.10*	11.97±0.93*	13.25±1.19*	14.3±1.43*	5.92±0.63^{ns}
TPS	7.77±0.88*	9.66±0.68*	10.18±0.40*	11.57±0.42*	13.75±1.44*	5.97±0.72^{ns}
HA	7.64±0.61*	10.48±0.68*	11.81±0.39*	12.55±0.45*	13.86±1.02*	6.22±0.53^{ns}
Acid	8.39±1.00*	11.42±0.63*	11.70±0.62*	12.66±0.54*	14.27±0.53*	5.87±0.55^{ns}

* Statistically different from baseline $p<0.0001$

^{ns} Non significant regarding to different surfaces

Table 5: Mean±SD of vertical bone levels (mm) at baseline, 20, 40, 60 and 425 days.

Surface	Baseline	20 days	40 days	60 days	425 days	VBL
CPTi	2.32±0.53	4.12±0.72*	5.20±0.71*	6.32±0.33*	6.92±0.22*	4.52±0.25^{ns}
TPS	2.50±0.61	3.85±0.95*	4.61±0.90*	6.00±0.70*	6.77±1.38*	4.27±0.62^{ns}
HA	2.01±0.46	3.62±0.29*	4.65±0.84*	6.22±0.50*	7.07±0.72*	4.78±0.38^{ns}
Acid	2.36±0.54	3.64±0.17*	5.19±0.51*	6.06±0.27*	6.80±0.80*	4.70±0.42^{ns}

* Statistically different from baseline $p<0.0001$

^{ns} Non significant regarding to different surfaces

Table 6: Mean±SD of horizontal bone loss (mm) at baseline, 20, 40, 60 and 425 days.

Surface	Baseline	20 days	40 days	60 days	425 days	HBL
CPTi	0.66±0.41	2.03±0.77*	2.86±0.64*	3.31±0.44*	2.52±1.75*	1.85±0.73^{ns}
TPS	0.68±0.34	1.60±0.53*	2.98±0.84*	3.53±0.58*	2.47±1.82*	1.79±0.74^{ns}
HA	0.63±0.36	2.18±0.68*	3.20±0.61*	3.90±0.27*	1.63±2.82*	1.00±1.09^{ns}
Acid	0.57±0.37	1.61±0.69*	2.76±0.68*	3.85±1.00*	3.12±1.00*	2.50±0.43^{ns}

* Statistically different from baseline $p<0.05$

^{ns} Non significant regarding to different surfaces

Table 7: Mean±SD of periotest values (PTV-score) at baseline, 20, 40, 60 and 425 days after experimental peri-implantitis

Surface	Baseline	20 days	40 days	60 days	425 days	PTV Variation
CPTi	-1.50±2.34	-0.5±1.22*	1.50±3.39*	2.33±2.25*	5.80±1.92*	7.30±1.31^{ns}
TPS	-2.16±2.48	-1.66±2.25*	-2.16±2.22*	-0.50±2.50*	4.00±1.41*	5.60±1.20^{ns}
HA	-2.66±2.25	-1.16±1.83*	-0.50±1.64*	1.66±2.06*	6.00±2.64*	8.66±1.67^{ns}
Acid	-1.66±2.65	-1.00±1.78*	1.00±1.78*	2.33±1.63*	6.80±2.48*	8.46±1.56^{ns}

* Statistically different from baseline $p<0.05$

^{ns} Non significant regarding to different surfaces

Fig. 1

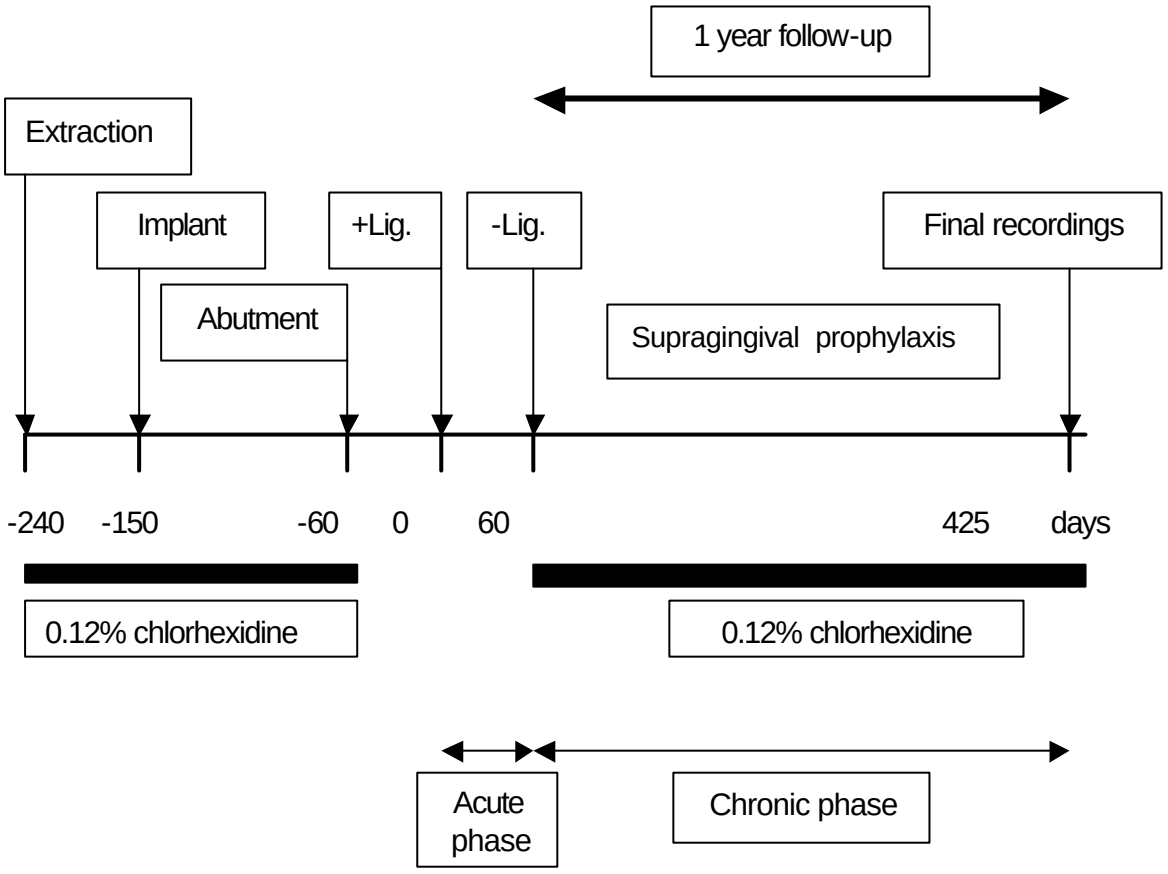

1 year follow-up
Extraction
Implant
+Lig.
-Lig.
Final recordings
Abutment
Supragingival prophylaxis
-240
-150
-60
0
60
425
days
0.12% chlorhexidine
0.12% chlorhexidine
Acute phase
Chronic phase

Fig.2

Fig. 3

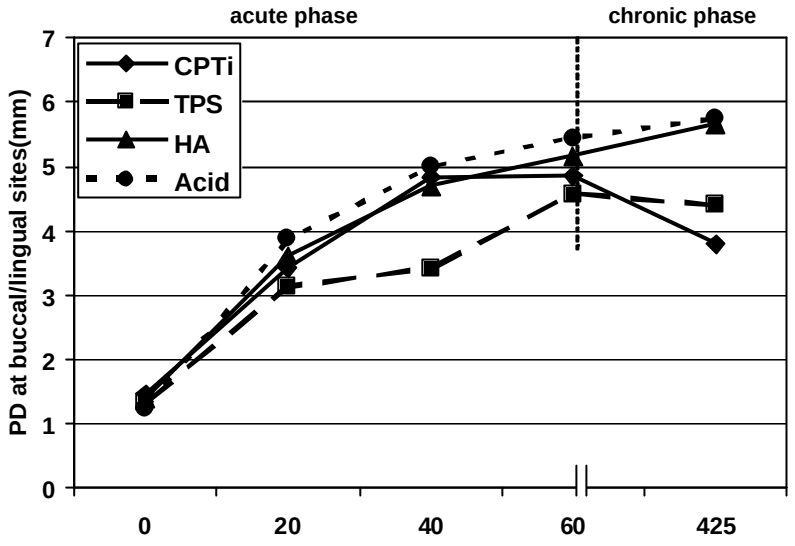

acute phase
chronic phase
CPTi
TPS
HA
Acid
PD at buccal/lingual sites(mm)
0
1
2
3
4
5
6
7
0
20
40
60
425

Fig.4

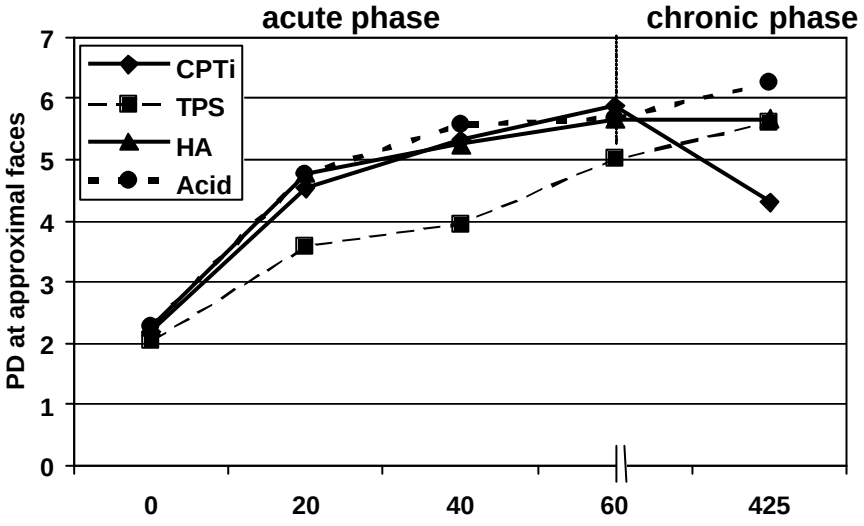

acute phase
chronic phase
CPTi
TPS
HA
Acid
PD at approximal faces
0
1
2
3
4
5
6
7
0
20
40
60
425

Fig.5

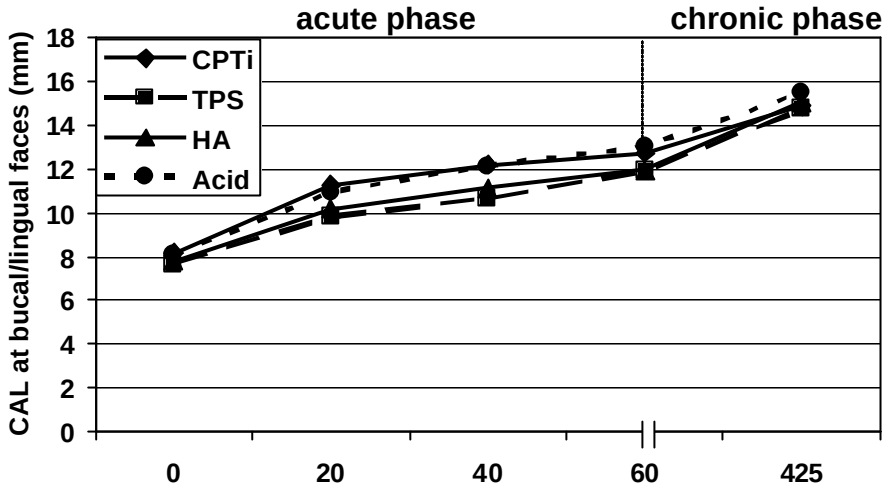

acute phase
chronic phase
CAL at bucal/lingual faces (mm)
CPTi
TPS
HA
Acid
0
2
4
6
8
10
12
14
16
18
0
20
40
60
425

Fig.6

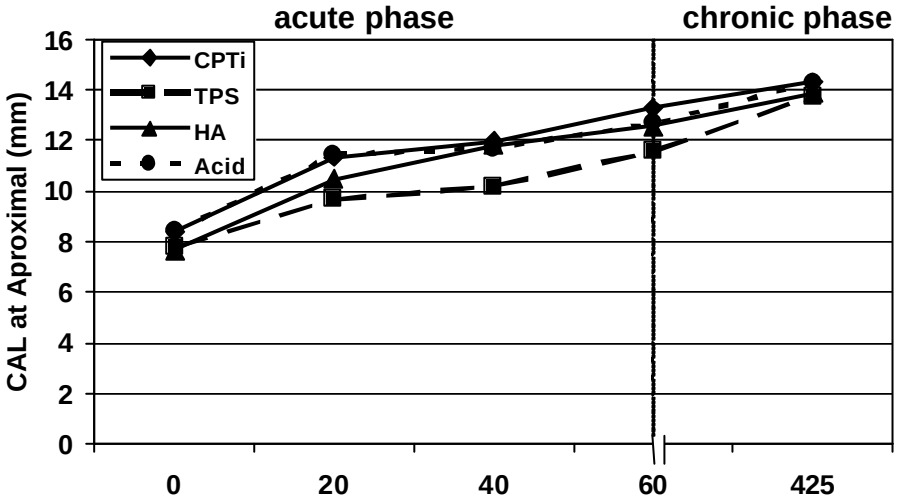

acute phase
chronic phase
CAL at Aproximal (mm)
CPTi
TPS
HA
Acid
0
2
4
6
8
10
12
14
16
0
20
40
60
425

Fig.7

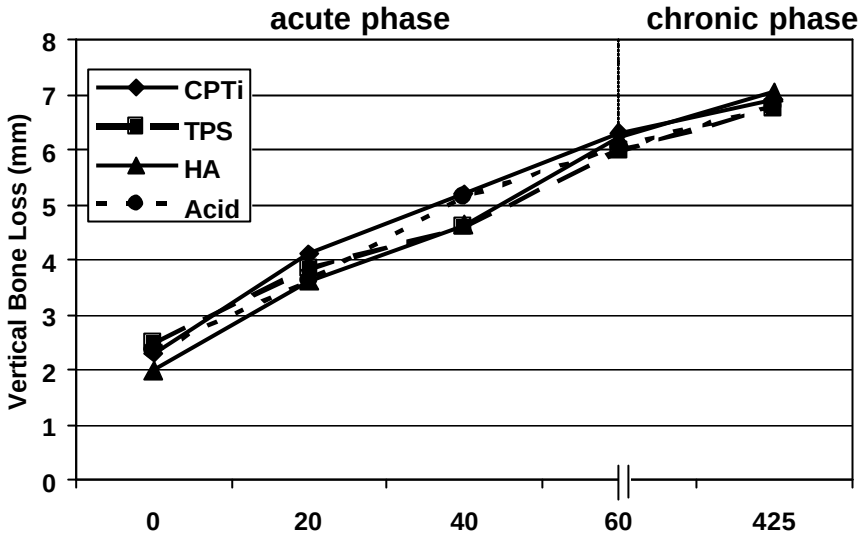

acute phase
chronic phase
Vertical Bone Loss (mm)
CPTi
TPS
HA
Acid
0
1
2
3
4
5
6
7
8
0
20
40
60
425

Fig.8

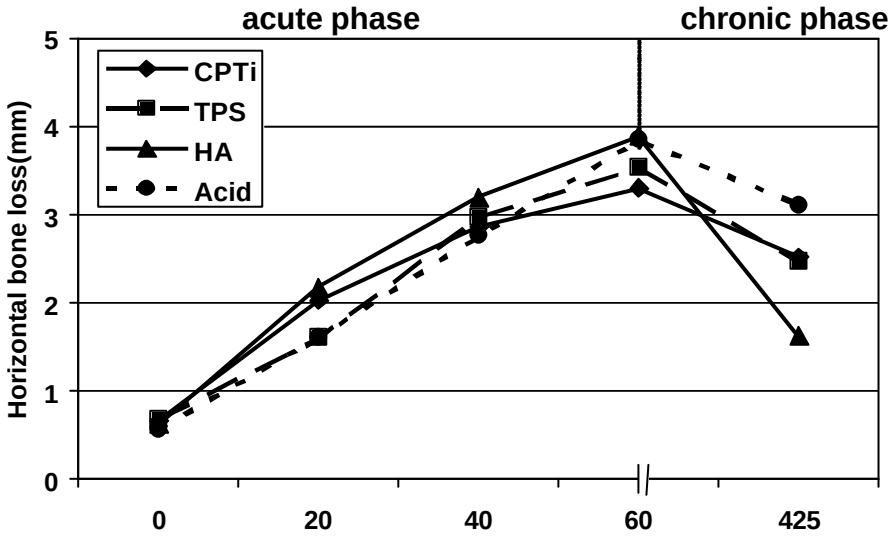

acute phase
chronic phase
Horizontal bone loss(mm)
CPTi
TPS
HA
Acid
0
1
2
3
4
5
0
20
40
60
425

Fig. 9

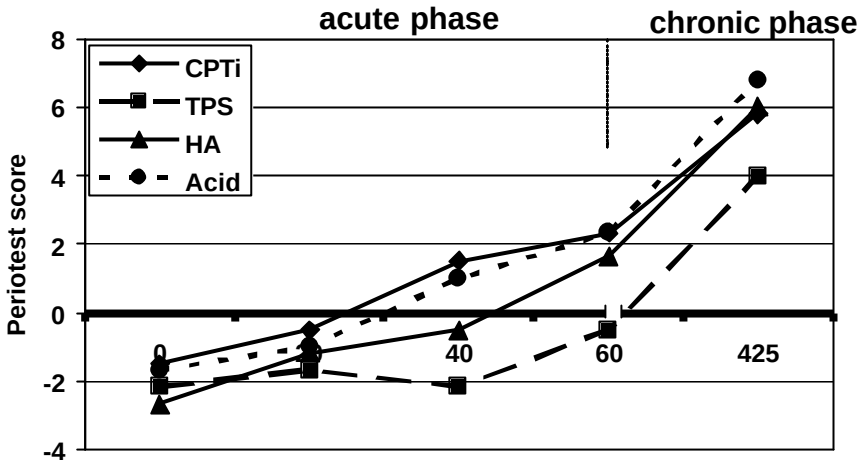

acute phase
chronic phase
Periotest score
8
6
4
2
0
-2
-4
0
40
60
425
CPTi
TPS
HA
Acid

Fig. 10

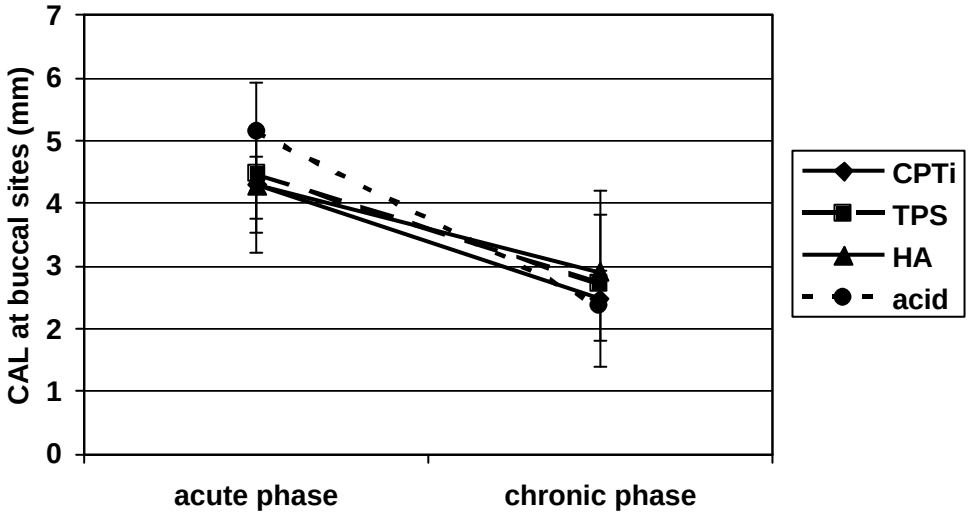

CAL at buccal sites (mm)
7
6
5
4
3
2
1
0
acute phase
chronic phase
CPTi
TPS
HA
acid

Fig. 11

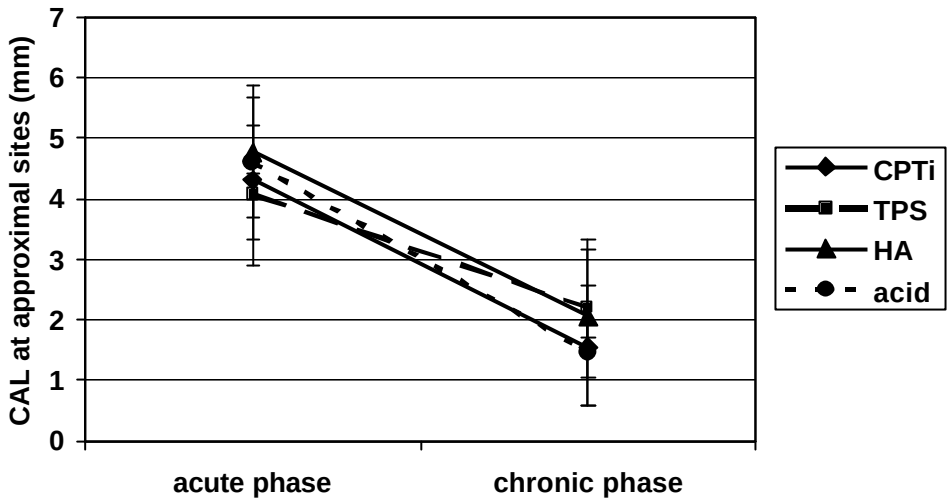

CAL at approximal sites (mm)
7
6
5
4
3
2
1
0
acute phase
chronic phase
CPTi
TPS
HA
acid

Fig.12

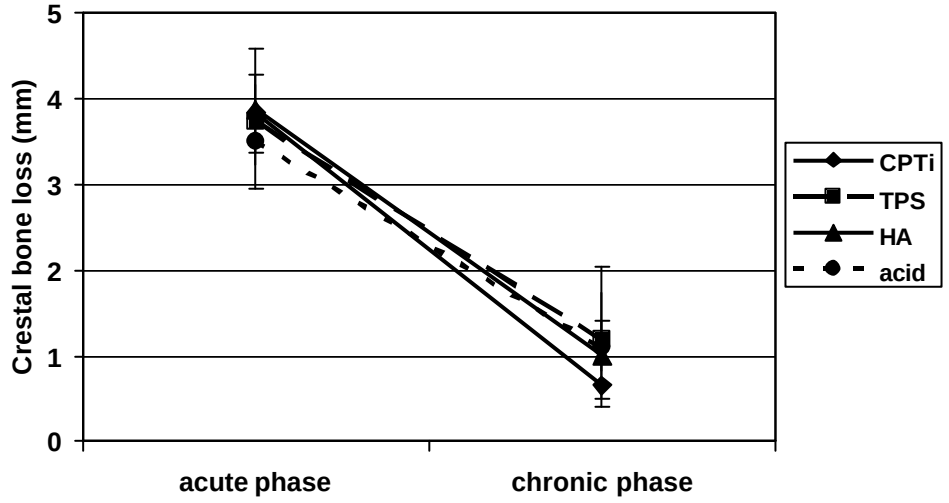

Crestal bone loss (mm)
5
4
3
2
1
0
acute phase
chronic phase
CPTi
TPS
HA
acid

Fig. 13

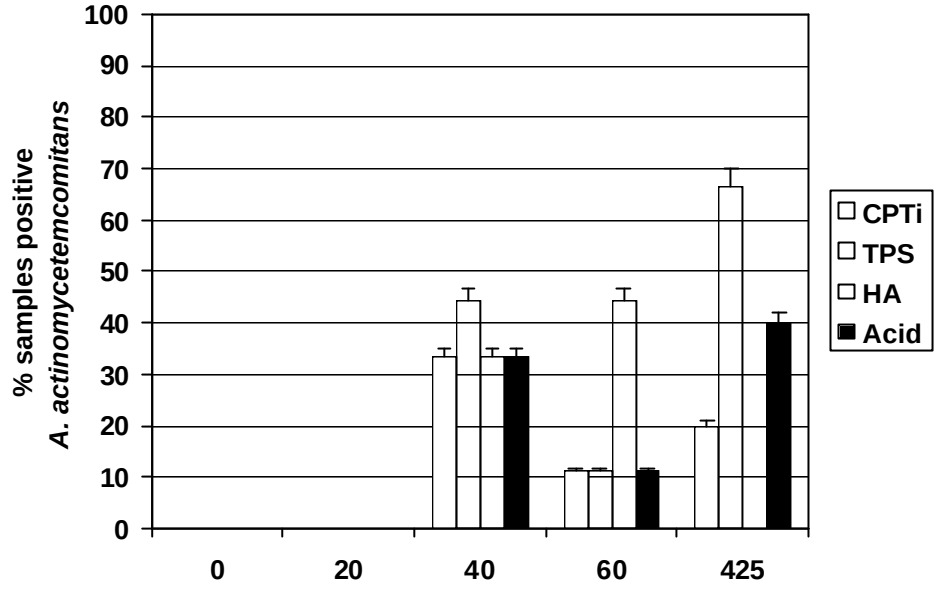

100
90
80
70
60
50
40
30
20
10
0
% samples positive
A. actinomycetemcomitans
0
20
40
60
425
CPTi
TPS
HA
Acid

Fig.14

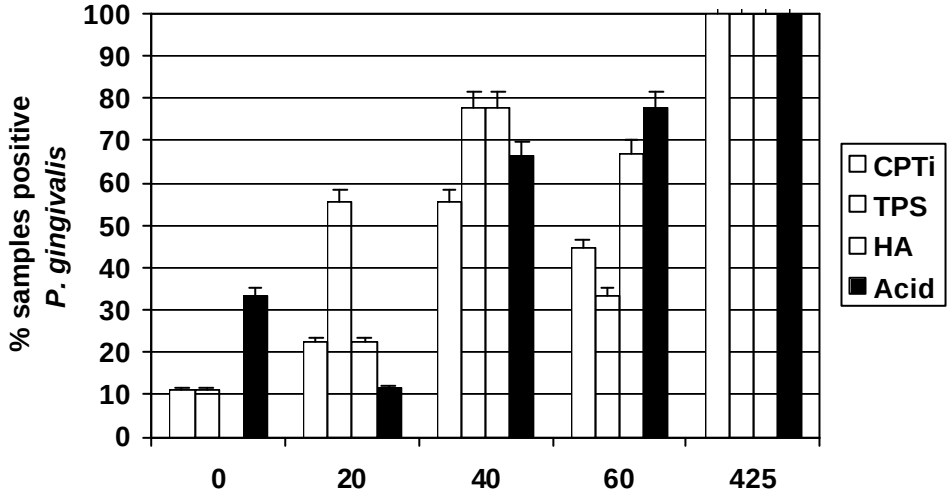

100
90
80
70
60
50
40
30
20
10
0
% samples positive
P. gingivalis
0
20
40
60
425
CPTi
TPS
HA
Acid

Fig. 15

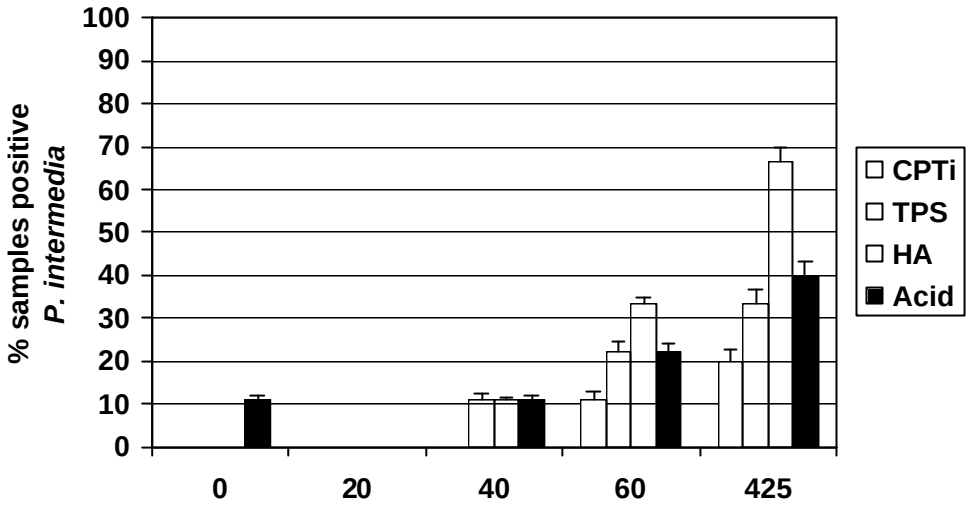

% samples positive
P. intermedia
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
425
CPTi
TPS
HA
Acid

Fig.16

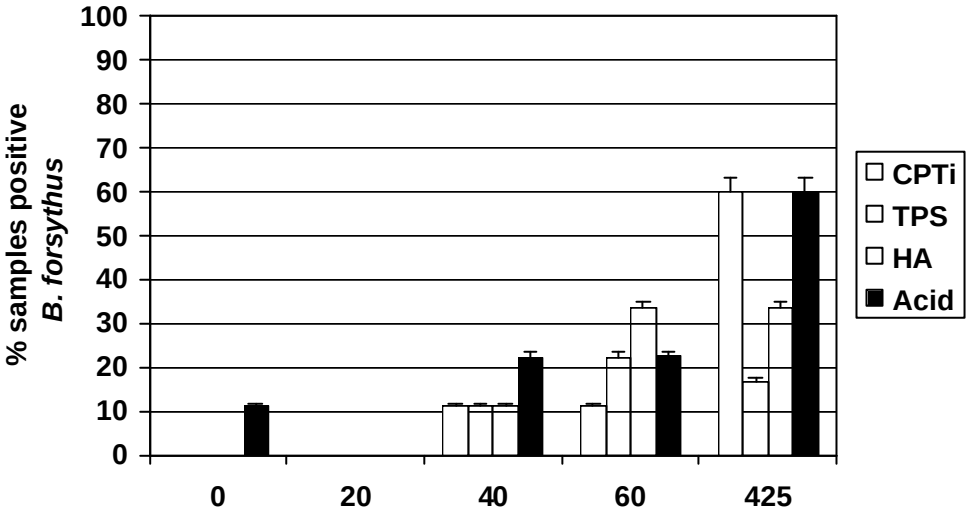

% samples positive
B. forsythus
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
425
CPTi
TPS
HA
Acid

Fig.17

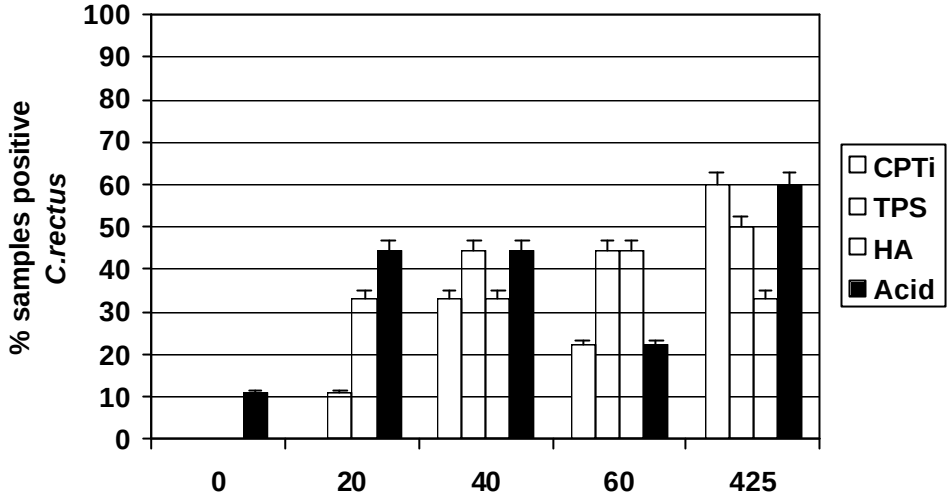
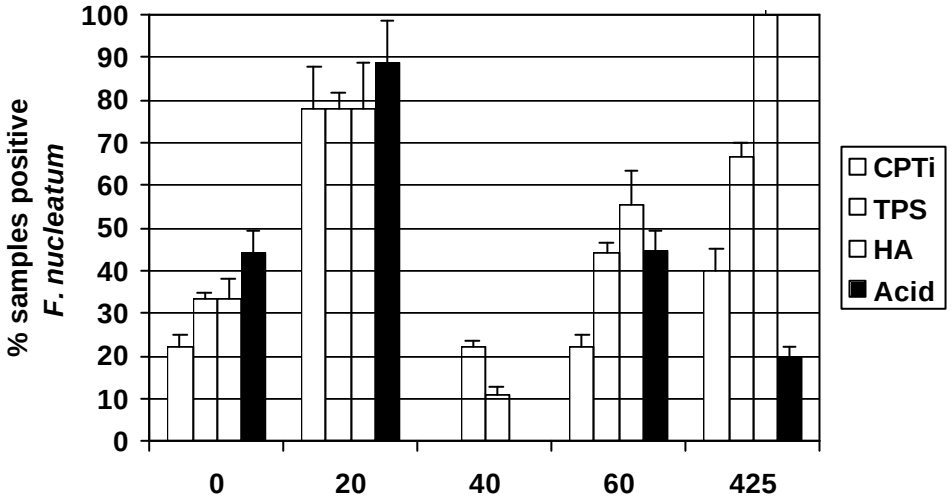

% samples positive
C.rectus
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
425
CPTi
TPS
HA
Acid

Fig.18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F. nucleatum
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
425
CPTi
TPS
HA
Acid

Fig.19

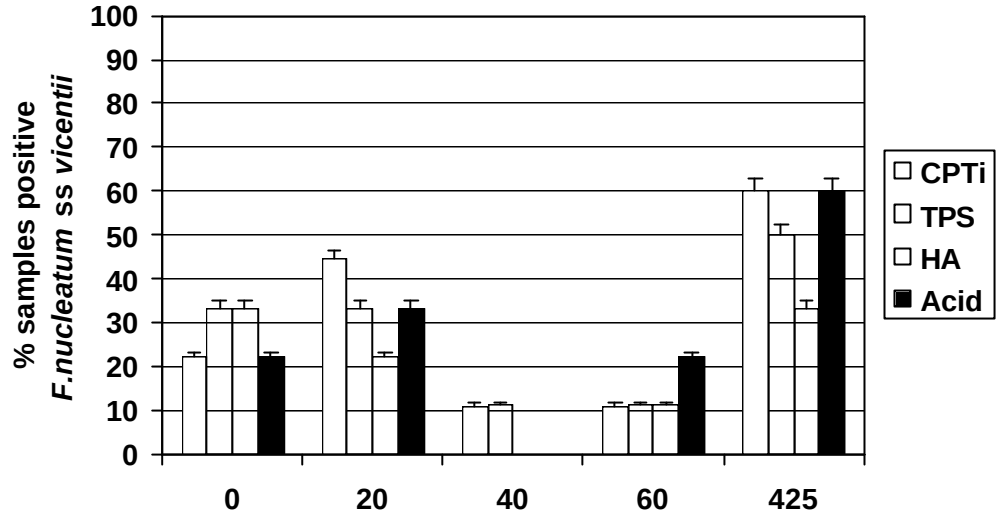

% samples positive
F.nucleatum ss vicentii
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
425
CPTi
TPS
HA
Acid

Fig.20

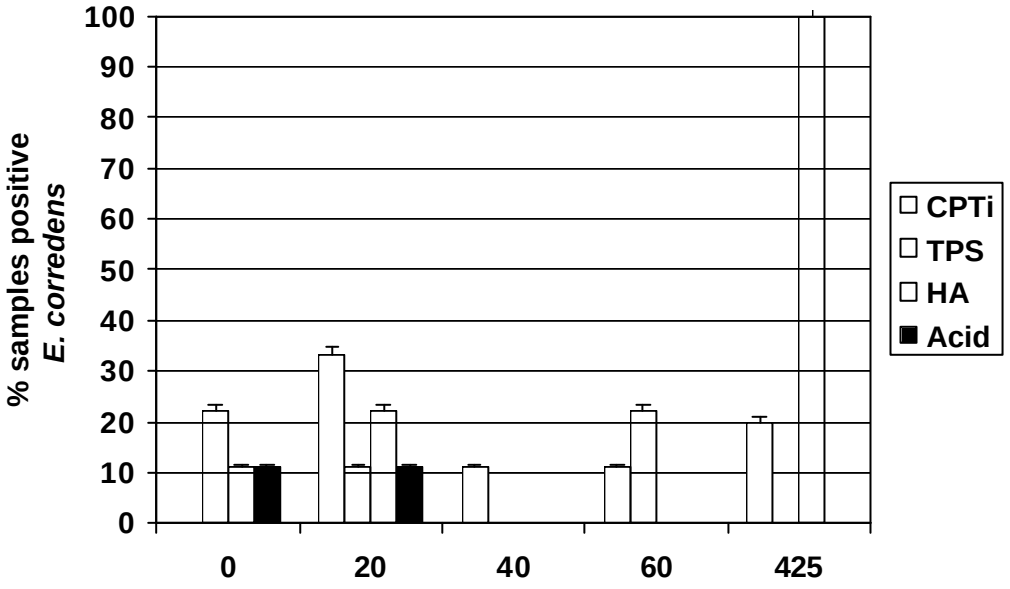

% samples positive
E. corredens
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
425
CPTi
TPS
HA
Acid

Fig.21

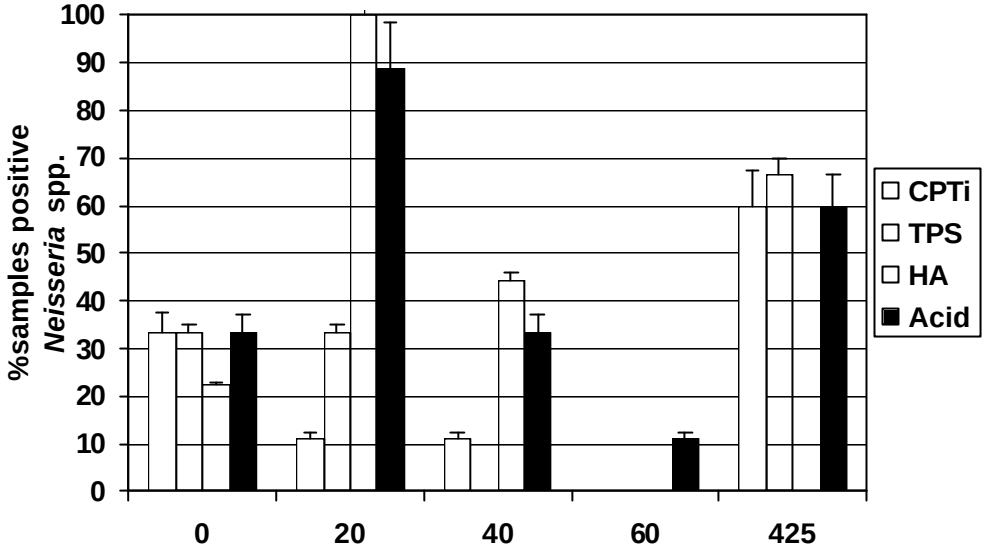

%samples positive
Neisseria spp.
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
425
CPTi
TPS
HA
Acid

Fig.22

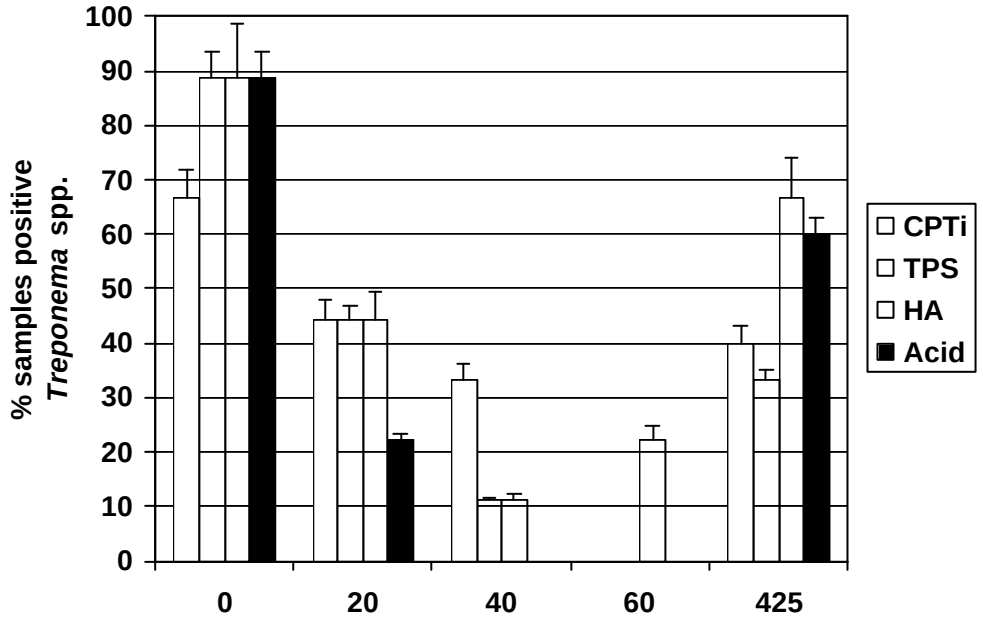
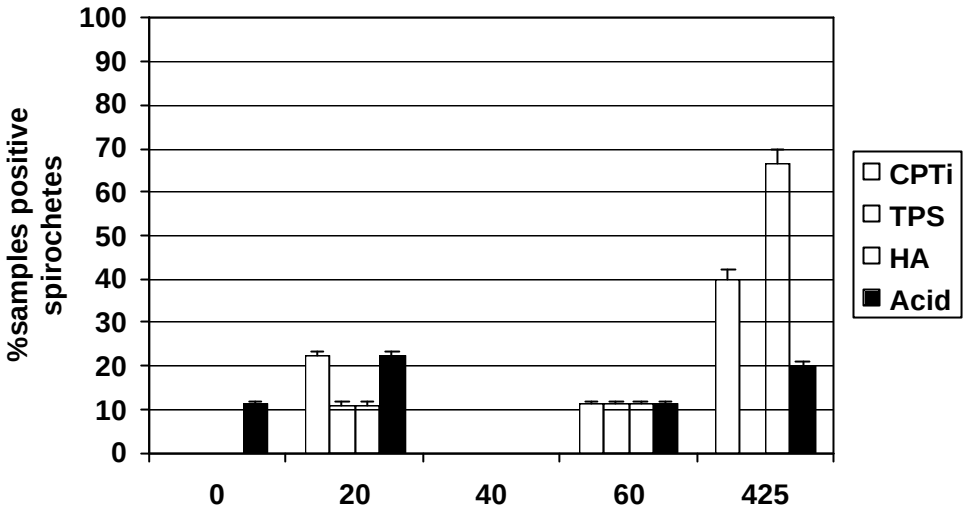

% samples positive
Treponema spp.
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
425
CPTi
TPS
HA
Acid

Fig.23


%samples positive spirochetes
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
425
CPTi
TPS
HA
Acid

CAPITULO - 5 Lethal photosensitization in microbiological treatment of ligature-induced peri-implantitis: a preliminary study in dogs

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Abstract

This pilot study evaluated, by culture testing, the effectiveness of lethal photosensitization for the microbiological treatment of peri-implantitis in dogs. Experimental peri-implantitis was induced by ligature placement for 2 months. Following ligature removal, plaque control was instituted by scrubbing with 0.12% chlorhexidine daily for 12 months. Subsequently, mucoperiosteal flaps were elevated for scaling the implant surface. Microbial samples were obtained with paper points before and after treatment of implant surfaces by means of 100 μg/ml toluidine blue O (TBO,) and were exposed, for 80 s, to light with a wavelength of 685 nm from a 50 mW GaAlAs diode laser. The mean initial and final bacterial counts were 7.22±0.20 and 6.84±0.44 CFU/ml, respectively for TVC (*P*<0.0001); 6.19±0.45 and 3.14±3.29 CFU/ml for *P. intermedia/nigrescens* (*P*=0.0010); 5.98±0.38 and 1.69±2.90 CFU/ml for *Fusobacterium* spp. (*P*<0.0010); and 6.07±0.22 to 1.69±2.94 CFU/ml for beta-hemolytic *Streptococcus* (*P*=0.0039). It may be concluded that lethal photosensitization resulted in a reduction of the bacterial count. Complete elimination of bacteria was achieved in some samples.

Key words: Dental implants, laser therapy, osseointegration, periodontal pathogens, peri-implantitis, photodynamic antimicrobial chemotherapy.

Introduction

Early studies have documented the excellent long-term prognosis of osseointegration(1). However, several different etiologic factors are associated with dental implant failures, such as poor surgical management, failure to achieve osseointegration, premature loading, biomechanical overload (2), and main peri-implant infection, due to colonization by bacteria such as *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia, Fusobacterium nucleatum*, and *Bacteroides forsythus* (3). Dental implant failure, or peri-implantitis, is defined as an inflammatory process affecting the tissues around a in function dental implant, resulting in loss of supporting bone (4).

Several therapeutic strategies can be applied for the treatment of peri-implantitis (5-7). In some of these *in vivo* studies, clinical, radiographical, and histological evaluations were performed without considering the microbiological features before and after contamination of the implant surface.

Discontamination by mechanical (7), chemical (5) and physical (8, 9) methods have been used.

The physical method, utilizing a low-power laser following the application of a photosensitizing substance, such as toluidine blue O (TBO) (10), has been used to treat periodontal diseases (11-14) and peri-implantitis (8, 9). The mechanism by which TBO plus laser irradiation kills microorganisms such as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Actinobacillus actinomycetemcomitans,* and *Fusobacterium nucleatum* has not yet been established. However, it is believed that lethal photosensitization of these microorganisms may involve changes in their membranes and/or plasma membrane proteins, as well as DNA damage mediated by singlet oxygen (13).

The purpose of this study, therefore, was to evaluate, by culture testing, the viability of microorganisms, following lethal photosensitization, during the microbiological treatment of ligature-induced peri-implantitis in dogs.

Materials and Methods

Animals and implants

The outline of the experiment is presented in Fig. 1. A total of six consecutively treated mongrel dogs (24-months old with an average weight of 18 Kg) with ligature-induced peri-implantitis around 19 dental implants were treated in this study. Animal selection, management, and surgical protocol followed routines approved by the Dental School of Araraquara Institutional Animal Care and Use Committee.

All mandibular premolars were extracted to create space for dental implants. After three months of healing, 36 dental implants with four different surfaces and three different implant systems: commercially pure titanium implants-CP, 3*i*® Implants Innovations, Palm Beach Gardens, FL, USA; titanium plasma sprayed-TPS, *Esthetic plus* ITI®, Straumann AG, Waldenburg, Switzerland; hydroxyapatite-HA, Calcitek®, Sulzer Medica, Carlsbad, CA, USA; hybrid surface–machined titanium in the three screws and acid-etched-acid, *Osseotite*®-3*i*® Implants Innovations, Palm Beach Gardens, FL, USA were installed in each quadrant of the mandible.

Three months after fixture installation, healing abutment connections were installed, according to the instructions of each dental implant system. After a plaque control program for two months and healing of the soft tissue, cotton floss ligatures were placed around the dental implants and sutured in the peri-implant mucosa, not only to facilitate plaque accumulation, but also to hold the ligatures in position. Tying further ligatures at 20 day-intervals for a period of 60 days accelerated peri-implant bone loss. At 60 days, when approximately 40% of the initial bone support was lost, the ligatures were finally removed.

A 12-month plaque control program was initiated by means of scrubbing daily with 0.12% chlorhexidine and scaling the abutment surface once a month. At the

end of this period, natural peri-implantitis progression was observed, and only 19 dental implants (5 CP, 6 TPS, 3 HA, and 5 acid) remained osseointegrated.

Treatment

A crestal incision was made through the mucosa and buccal and lingual full-thickness flaps were elevated. The abutments were removed and the granulation tissue present in the bone craters around the dental implants was curetted with a plastic curette (Fig. 2). The implant surface was then rinsed with physiological saline solution and the first peri-implant microbial sample was taken. TBO (100 μm/ml) was then injected into the peri-implant defect as far as the bony border with a thin needle; the TBO was left in place for 1 minute and then carefully drawn off again. The stained area was subsequently irradiated with a GaAlAs diode laser (IR 500-Laser Beam, Brazil) with a measured power output of 50mW (Fig. 3). This laser emits radiation in collimated beams (2mm^2) with a wavelength of 685nm, for 80 s and a total energy of 4 J (energy density, 200 J/cm^2). The diode laser was focalized in contact with the mesial, distal, buccal and lingual surfaces by a scanning method for 20 s on each face, making a total of 80s. The second microbial sample was then obtained.

Microbial Samples

The paper points were removed and placed into 3-ml vials containing VMGAIII anaerobic transport medium (15). All samples were collected by the same operator and coded by an assistant to provide blind identification. The microbiological procedures were initiated within 24 hours.

The samples were centrifuged for 60 s and were serially diluted 10-fold in peptonated water to between 10^{-1} and 10^{-6} for quantitative evaluation of CFU/ml and

to obtain isolated colonies for qualitative identification. Aliquots of 0.1 ml of the dilutions were plated onto Enriched Tryptic Soy Agar (ETSA) (16) and Tryptic Soy-Serum-Bacitracin-Vancomycin agar (TSBV) (17) in a standard manner. ETSA plates were incubated in anaerobic jars containing a mixed gas atmosphere (85% N_2, 10% H_2, 5%CO_2) at 37^{o}C for 7 to 10 days. TSBV agar plates were incubated in a 5% CO_2 atmosphere for 5 days at 37^{o}C. The bacterial species were identified from anaerobic cultures based on gram-stain, aerotolerance, colony morphology, esculin hydrolysis (18), nitrate reduction, indole production, [alpha]-glucosidase and N-benzoyl-DL-arginine-2-naphthylamide (BANA) hydrolysis (19), oxidase and catalase activities. Total viable count (TVC) and cultivable microbiota, including *Porphyromonas gingivalis, Prevotella intermedia/nigrescens*, *Fusobacterium* spp., beta-hemolytic *Streptococcus*. *Actinobacillus actinomycetemcomitans* detection was performed based on colony morphology and positive catalase tests (20).

Data Analysis

The TVC were transformed into colony-forming units per ml (CFU/ml) using predetermined conversion factors to account for dilution and the size of the evaluated surface on the plate.

Data were then analyzed for each dental implant. Differences between groups and bacterial species were assessed by Wilcoxon's signed rank-test (P<0.05). Microorganism analysis was performed after logarithmic transformation of TVC for each periodontal pathogen.

Results

P. gingivalis and *A. actinomycetemcomitans* were not detected in any peri-implant microbial sample.

The mean initial bacterial count ranged around 7.22±0.20CFU/ml for TVC, 6.19±0.45CFU/ml for *P. intermedia/nigrescens*, 5.98±0.38CFU/ml for *Fusobacterium* spp., and 6.07±0.22CFU/ml for beta-hemolytic *Streptococcus*.

The means measured following lethal photosensitization were even lower; 6.84±0.44CFU/ml for TVC (Fig.4), 3.14±3.29CFU/ml for *P. intermedia/nigrescens* (Fig.5), 1.69±2.90CFU/ml for *Fusobacterium* spp.(Fig.6), and 5.01±1.90CFU/ml for beta-hemolytic *Streptococcus* (Fig.7). Significant decreases in counts following lethal photosensitization were observed for TVC ($P<0.0001$), *P. intermedia/nigrescens* ($P=0.001$), *Fusobacterium* spp. ($P=0.001$) and to beta-hemolytic *Streptococcus* ($P=0.0039$).

Complete bacterial elimination was achieved in *P. intermedia/nigrescens* (6 out of 12 samples), *Fusobacterium* spp. (6 in 11 samples), and beta-hemolytic *Streptococcus* (1 in 9 samples) (Table 1).

Discussion

Several studies have demonstrated the bactericidal effect of high-power laser on contaminated dental implant surfaces (21- 23). This energy is specifically absorbed by water molecules, which cause the water-rich tissue to be preferentially vaporized, in bacterial cytoplasm this effect causes cell lyses.

In addition, studies (8, 9, 24) have shown the effectiveness of lethal photosensitization in decreasing the viable count of periodontal pathogens in peri-implantitis lesions without damage to the dental implant surface.

This preliminary study attempted to examine whether lethal photosensitization, which has been shown to be effective in eliminating periodontal pathogens (*in vitro*) on contaminated titanium implants surfaces (8), is also effective *in vivo* (9, 24). The results of this preliminary study indicate that photosensitization with TBO plus irradiation with a GaAlAs diode laser (200J/cm^2) result in a statistical reduction of periodontal pathogens of up to a maximum of 1 log step.

These results confirm those obtained by other studies that use various photosensitizers in combination with a low-power laser to induce bacterial reduction (25, 26, 27). Periodontal pathogens, such as *Fusobacterium nucleatum*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* were killed after photodynamic therapy (28). Furthermore, the dye/laser combination was demonstrated for the first time, to be able to reduce the beta-hemolytic *Streptococcus* mean count. However these data should be considered with caution due the sample size utilized in this pilot study and also due to absence of control group. In addition, other possibility that reduction of bacterial density in a fluid that filled the ligature-induced peri-implant defect can also caused by physical removal of the microorganisms prior to the second sample collection by stream of the TBO solution while staining and suctioning

since it is well know that bacteria attach to the infragingival surfaces quite loosely.

Many factors may interfere in the laser irradiation, these include the capacity for light absorption by the microorganism, the wavelength of the laser, the physiological state of the bacteria, the emission form of the laser, the time of exposition to the laser, the pH of the medium, the staining of the area to be irradiated, water content, thermal conductivity and the organic matrix (29). In our study we could conclude that biofilm present on dental implants surfaces was susceptible to photodynamic treatment and their variables.

Various drugs have been used over the last few years in association with low-power lasers to promote their bactericidal effects, since the low-intensity lasers by themselves do not have the capacity to cause significant reductions in the microorganisms as they induce a photochemical reaction rather than a thermal reaction. However, the association of these lasers with photosensitizers causes an alteration in the viabilities of different microorganisms (26, 29, 30), confirmed by our data. Several authors conclude that neither low intensity laser irradiation nor photosensitizers alone can kill these bacteria. Haas et al. (8) evaluated, by microbiologic examinations, the effectiveness of lethal photosensitization in different implant surfaces. They conclude that the group that utilized TBO plus laser was effective while the others groups (TBO alone, Laser alone, saline solution) were not effective. These results agree with the studies conducted by Bhatti et al. (31) and Bhatti et al. (8). In these works the authors evaluated not only the distribution of TBO but also the dosimetric effect and physiologic factors on lethal photosensitization of *P. gingivalis*. We can also cite Sarkar and Wilson (12), and Dortbudak et al. (9), which agree with statement, that lethal photosensitization is effective when the photosensitizer was used plus laser irradiation.

Although the mechanism by which the low-power lasers cause bacterial reduction when associated with photosensitizers is not totally clarified, some authors believe that when the laser is strongly absorbed locally, due to the sensitization of the bacterial photoreceptors by the stains, an oxygen molecule with a high degree of atomic excitation (oxygen triplet= O_3) is produced, but only has a bactericidal effect upon the production of free radicals or singlet oxygen (29). The reason why an oxygen molecule affects only the bacteria is because the light dose required to kill bacteria treated with TBO is far lower than that causing toxicity in cultured human keratinocytes and fibroblasts (32). In addition to this important effect is the presence of the selectivity of the laser by the use of specific stains for each wavelength, due to the different degrees of absorption by the stains (33).

As well as being non-toxic to man, the ideal photosensitizer must be able to absorb strongly at the wavelength of light used, have high excitation efficiency (a high probability of triplet state formation per photon absorbed) and a relatively long (several microseconds) triplet state (29). Wilson et al. (33) screened a number of compounds for their ability to sensitize oral bacteria to killer by low power laser. These compounds include TBO, methylene blue, aluminum disulphonated phthalocyanine, thionin, crystal violet and dihaematoporphyrin ester (29).

This study utilized sensitization to TBO, since other studies (25, 31, 34) have demonstrated the effectiveness of this stain in association with red spectrum-wavelength lasers on bacterial viability, probably due to the fact that TBO strongly absorbs visible spectrum wavelengths (red). This finding agrees with the theory that the bactericidal effect of lasers depends upon their wavelength (29). The wavelength used for kill periodontal pathogens in ligature-induce peri-implantitis in this study shown be effective, however additional investigations must be conducted.

In addition, this approach to killing periodontal pathogens offers some advantages over the use of conventional antimicrobials: avoids development of resistance among target organisms to the photochemically-generated free radicals thought to responsible for bacterial killing and, unlike antiseptics and antibiotics, there would be no need to maintain high concentrations of the TBO in the peri-implant defects for long periods.

Within the limits of this pilot study, it may be concluded that microbiological peri-implantitis treatment by lethal photosensitization reduced and eliminated, in some samples, the periodontal pathogens involved in this pathological condition. Further studies will be necessary to evaluate the amount of re-osseointegration after lethal photosensitization.

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Table 1- CFU/ml after logarithmic transformation, before and after lethal photosensitization. (Legend: *(-) Species were not identified in this site)*

Implant Surface	TVC		*P.intermedia/nigrescens*		*Fusobacterium* sp.		Streptococci	
	Before	After	Before	After	Before	After	Before	After
	7.18	7.00	6.40	6.40	6.18	0.00	5.70	5.40

	7.40	7.35	6.18	0.00	5.87	0.00	6.30	6.00
	7.30	6.40	6.40	6.30	6.30	0.00	6.08	5.70
	7.30	6.09	-	-	-	-	-	-
	7.40	7.35	-	-	-	-	-	-
TPS	7.18	6.40	6.07	0.00	6.30	0.00	6.18	5.40
	7.47	7.30	6.40	6.09	6.40	6.30	6.40	6.18
	7.40	7.10	6.70	6.57	6.00	0.00	6.18	5.69
	6.87	6.57	6.18	6.00	-	-	5.87	5.40
	7.47	7.40	-	-	-	-	-	-
	7.18	7.18	-	-	-	-	-	-
HA	7.00	6.70	6.10	0.00	5.70	0.00	5.87	0.00
	7.18	7.10	5.40	0.00	-	-	-	-
	7.00	6.70	-	-	-	-	-	-
Acid	7.10	6.40	6.30	0.00	5.30	0.00	6.10	5.40
	7.30	6.70	6.87	6.40	5.70	0.00	-	-
	6.87	6.70	5.30	0.00	6.40	6.17	-	-
	7.57	7.44	-	-	5.55	0.00	-	-
	7.04	6.10	-	-	-	-	-	-

Fig.1

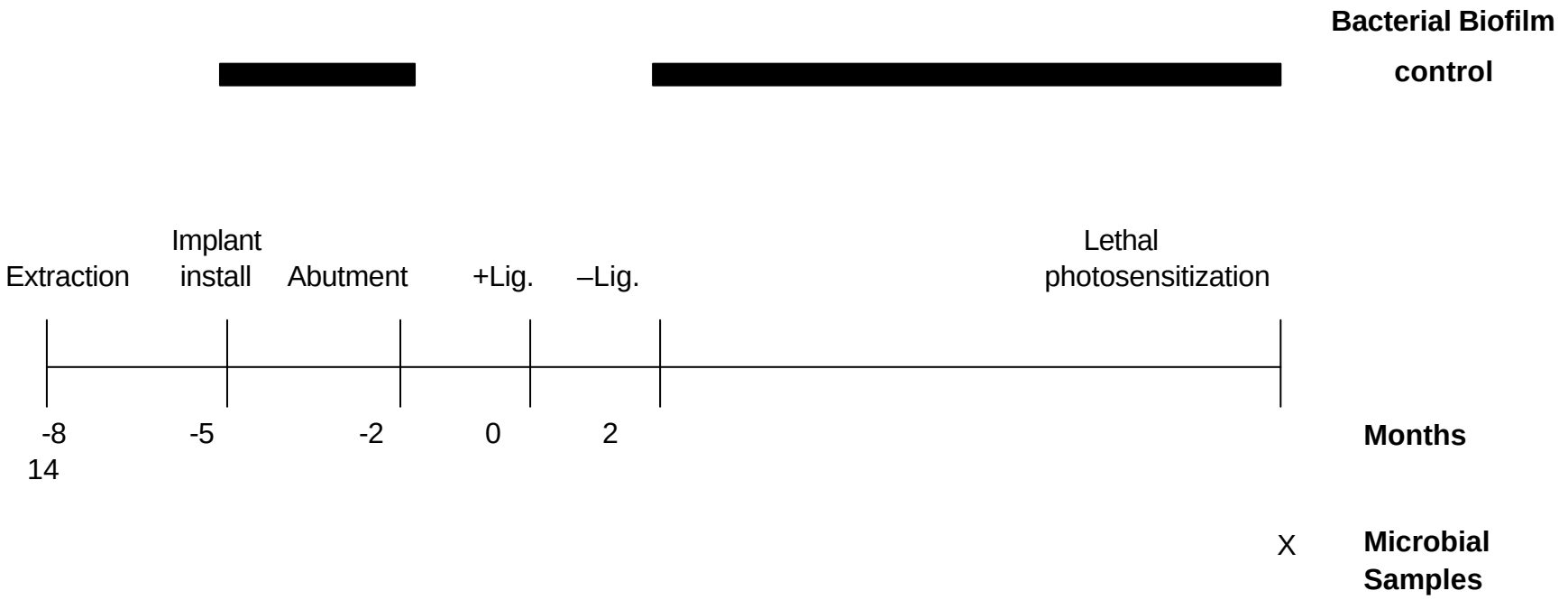
Bacterial Biofilm
control
Implant
Extraction
install
Abutment
+Lig.
–Lig.
Lethal
photosensitization
-8
14
-5
-2
0
2
Months
X
Microbial
Samples

Fig.4

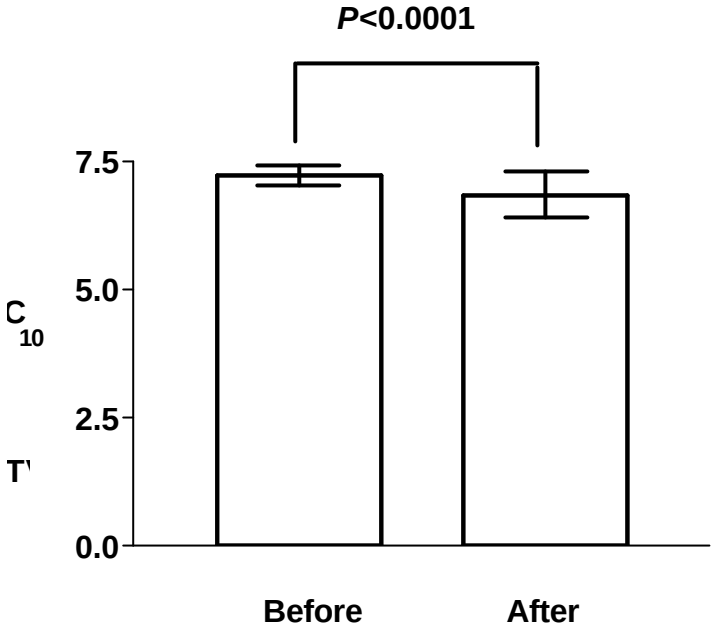

FIG.5

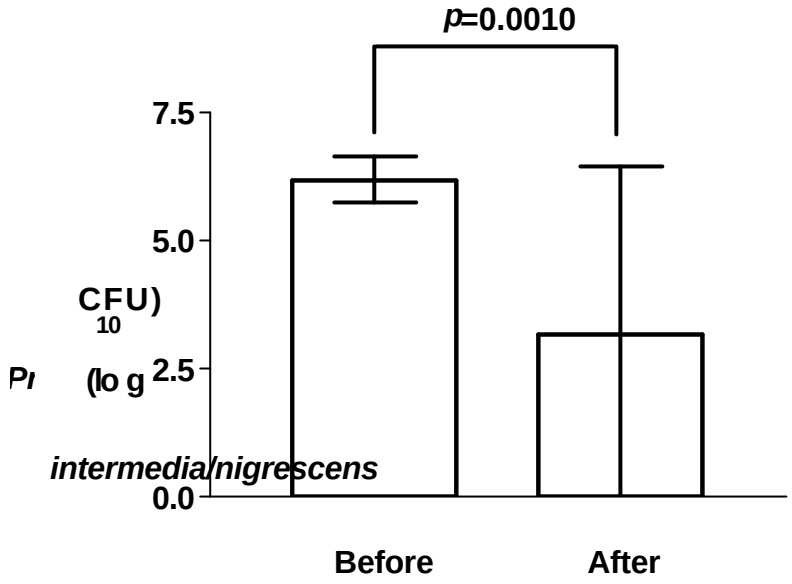
p=0.0010
7.5
5.0
CFU)
10
(log
2.5
0.0
intermedia/nigrescens
Before
After

FIG.6

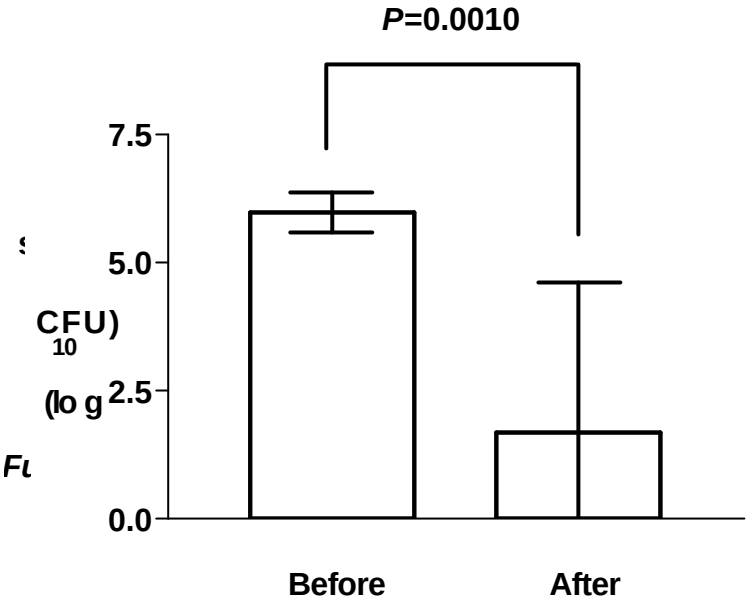

FIG.7

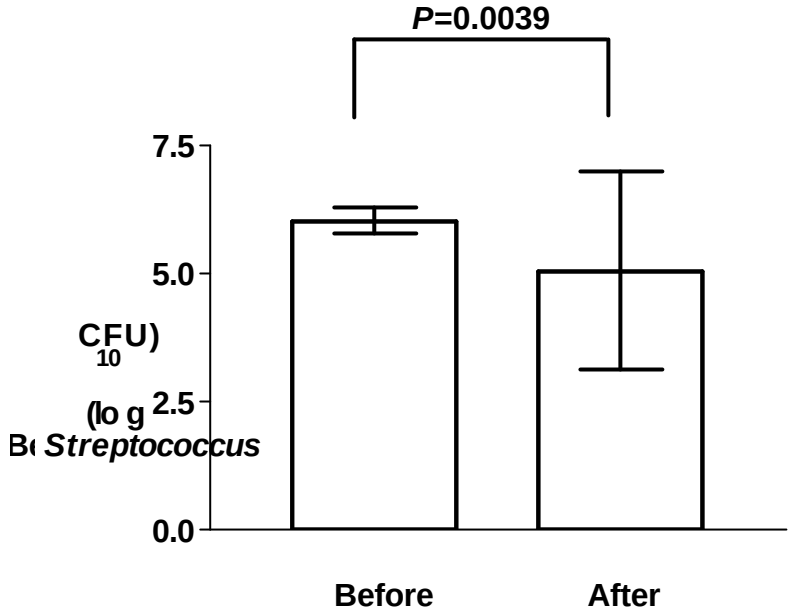

FIGURE LEGENDS

Fig. 1. Outline of the experiment. Ligatures were placed around the dental implants at 0 months (+Lig.) and removed at 2 months (-Lig.). Treatment of peri-implantitis was performed at 14 months by photodynamic therapy.

Fig.2. Clinical view of peri-implant defects after debridment

Fig.3. Clinical view of peri-implant defect and dental implant surface after stained by TBO.

Fig. 4. Mean and standard deviation of the effect of lethal photosensitization on the viability of bacteria (TVC) in peri-implant microbial samples.

Fig. 5. Mean and standard deviation of the effect of lethal photosensitization on viability in all samples positive for *Prevotella intermedia/nigrescens* .

Fig. 6. Mean and standard deviation of the effect of lethal photosensitization on viability in all samples positive for *Fusobacterium* spp .

Fig. 7. Mean and standard deviation of the effect of lethal photosensitization on viability in all samples positive for beta-hemolytic *Streptococcus*.

CAPITULO-5 TREATMENT OF LIGATURE-INDUCED PERI-IMPLANTITIS BY LETHAL PHOTOSENSITIZATION AND GUIDED BONE REGENERATION: A PRELIMINARY HISTOLOGIC STUDY IN DOGS.

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Running Title: Treatment of peri-implantitis by lethal photosensitization

TREATMENT OF LIGATURE-INDUCED PERI-IMPLANTITIS BY LETHAL PHOTOSENSITIZATION AND GUIDED BONE REGENERATION: A PRELIMINARY HISTOLOGIC STUDY IN DOGS.

ABSTRACT:

Background: The purpose of this pilot study was to evaluate the healing potential and re-osseointegration in ligature-induced peri-implantitis defects adjacent to various dental implants surfaces following lethal photosensitization.

Methods: A total of 36 dental implants with four different surface coatings (9 commercially pure titanium surface-CPTi; 9 titanium plasma-sprayed-TPS;9 hydroxyapatite-HA ; 9 acid-etched surface) were inserted in 6 male mongrel dogs, after 3 months healing period of mandibular premolar extractions. After a 2 months period ligature-induced peri-implantitis and natural peri-implantitis progression period of additionally 12 months of natural peri-implantitis progression, only 19 dental implants remained. The dogs underwent surgical debridment of the remaining dental implant sites and lethal photosensitization by combination of toluidine blue O (100µg/mL) and irradiation with diode laser. All exposed dental implant surfaces and bone craters were meticulously cleaned by mechanical means, submitted to photodynamic therapy, and guided bone regeneration (GBR) using e-PTFE membranes. Five months later, biopsies of the implant sites were dissected and prepared for ground sectioning and analysis.

Results: The bone fill amounted to 48.28$\pm$15.00; 39.54$\pm$12,34; 26.88$\pm$22.16; 26.70$\pm$16.50% to HA, TPS, Acid, and CPTi respectively. The re-osseointegration was

achieved in 25.25±11.96% for the TPS surface, 24.91±17.78% for the CPTi surface, 17.30±15.41% for the Acid surface, and 15.83±9.64% for the HA surface.

Conclusions: The data of the present study suggest that the lethal photosensitization may have potential in treatment of peri-implantitis.

Key Words: Dental implants/peri-implant diseases; Histometry; Peri-implantitis/treatment; Photodynamic Therapy/ Photosensitizers; Re-osseointegration; Guided Bone Regeneration.

INTRODUCTION

Several animal experiments have shown that bacterial biofilm accumulation around dental implants promoted by ligature-placement can develop a peri-implant tissue breakdown or peri-implantitis[1-4]. Although the literature reports the difficulties of attempting re-osseointegration on dental implant surfaces after contamination for periodontal pathogens such as *Actinobacillus actinomycetemcomitans; Prevotella intermedia, Porphyromonas gingivalis, Bacteroides forsythus, Fusobacterium nucleatum*, several therapeutic strategies can be applied for the treatment of peri-implantitis[2,5-8].

Decontamination with mechanical[3,4,8], chemical[2,5], and physical[6,9] methods have been used.

The physical method utilizes a low-power laser following application of a photosensitizing substance. Toluidine blue O (TBO) (for review see Wainwright[10]) has been utilized in periodontal diseases[11-16] and peri-implant diseases[6,17]. The mechanism by which TBO causes the microorganism killer such as *P. gingivalis*, *P. intermedia*, *A. actinomycetemcomitans*, and *F. nucleatum* has not yet been established, but it is believed that lethal photosensitization of these microorganisms may involve changes in the membranes and/or plasma membrane proteins and DNA damage mediated by singlet oxygen[15,18,19].

The objective of this pilot study was to report the results of a prospective study on lethal photosensitization within ligature-induced peri-implantitis in dogs with different dental implant surfaces.

MATERIAL AND METHODS

Animals

The outline of the experiment is presented in Fig. 1. Six adult, systemically healthy, male mongrel dogs, 2 years of age in an average weight of 18Kg were used. Animal selection, management, and surgical protocol followed routines approved for this study by Dental School of Araraquara Institutional Animal Care and Use Committee.

All surgical and clinical procedures as well as the laser irradiation were performed under general anesthesia accomplished by 0.05mg/Kg of subcutaneous preanesthesia sedation (atropine sulphate[||]) and intravenous injection of chlorpromazine[¶] and thiopental[#]. Oral prophylaxis was performed within 2 weeks before teeth extraction. After that, all mandibular premolars were extracted creating an edentulous ridge. Both the mandibular quadrants and the alveoli were allowed to heal for 3 months. The upper premolars were also extracted to avoid occlusion trauma interference. During the healing period, bacterial biofilm control was instituted by means of scrubbing 0.12% chlorhexidine[**] daily, scaling and root planning once a month, until cotton ligatures placement.

[||] Atropine sulphate - 0.5mg, Ariston Inds. Química e Farms. LTDA, São Paulo, Brasil
[¶] Amplictil 25mg, Rhodia Farma LTDA, São Paulo, Brasil
[#] Tiopental - ABBOTT Laboratórios do Brasil Ltda, São Paulo, Brasil
[**] Pharmacy School -State University of Sao Paulo (UNESP), Araraquara, SP, Brazil.

Implant Design and Surfaces

Initially, 36 dental implants with four different surfaces of three different implant system were used, as follows: Nine commercially pure titanium implants[††]– CPTi; nine titanium plasma sprayed[‡‡] – TPS; nine hydroxyapatite[§§] – HA, and nine hybrid surfaces – machined titanium in the three first screws and acid-etched in others screws[||||] – acid. All implants have length 10mm and a diameter 3.75mm (except TPS with 4.1mm of diameter).

Implant Surgery

The dental implants were placed after the full thickness flap under aseptic surgical conditions. The recipient sites were prepared using original instruments for each dental implant surface, according to the surgical techniques indicated by each implant manufacturer. The implants were randomly distributed among the dogs so that each dental implant surface was represented at least once in each animal. The implants were placed at bone level and a cover screw was screwed onto the implant, including the TPS dental implant surface due to a modification in technique insertion indicated by the manufacturer. The flaps were sutured with single interrupted sutures, submerging all implants.

[††] 3*i*® Implants Innovations, Palm Beach Gardens, FL, USA
[‡‡] Es*thetic plus* ITI®, Straumann AG, Waldenburg, Switzerland
[§§] Calcitek®, Sulzer Medica, Carlsbad, CA, USA
[||||] *Osseotite*®-3*i*® Implants Innovations, Palm Beach Gardens, FL, USA

An antibiotic coverage with potassic and sodic benzilpenicilin[¶¶] was given once a week for 2 weeks, in order to avoid post-surgical infection. Paracetamol[##] was given for pain control medication, and the sutures were removed after 10 days.

Experimental Peri-implantitis

Three months after dental implant installation, healing abutment connections were installed, according to the instructions of each dental implant system. After two months of a plaque control program and the healing of the soft tissue, cotton floss ligatures were placed around the dental implants and sutured in the peri-implant mucosa, not only to facilitate plaque accumulation, but also to hold the ligatures in position. Tying further ligatures at 20 day-intervals for a period of 60 days accelerated peri-implant bone loss. At 60 days, when approximately 40% of the initial bone support was lost, the ligatures were finally removed.

A 12-month plaque control program was initiated by means of scrubbing daily with 0.12% chlorhexidine and scaling the abutment surface once a month. At the end of this period, natural peri-implantitis progression was observed and only 19 dental implants (6 TPS; 5CPTi; 5 acid-etched surface; 3HA), out of the 36 implants placed were able to withstand to peri-implantitis progression. The others 17 dental implants presented mobility due to greater peri-implant bone loss and were excluded of our sample.

All dogs were subjected to (1) surgical debridment of dental implant surface and bone craters, (2) lethal photosensitization, and (3) guided bone regeneration-GBR of the implant sites.

[¶¶] Fort Dodge Saúde Animal LTDA, Campinas, SP, Brasil
[##] ABBOTT Laboratórios do Brasil Ltda, São Paulo, Brazil

Lethal Photosensitization and Guided Bone Regeneration

A crestal incision was made through the mucosa, and buccal and lingual full thickness flaps were elevated (Fig. 2A). The abutments were removed and the granulation tissue present in bone craters around the dental implants was curetted with a plastic curette (Fig.2B).

The implant surface was then rinsed with physiological saline solution. Following, TBO[***] (100μg/ml)[6,9,17] was injected into the peri-implant defect as far as the bony border, with a thin needle. TBO was left in place for 1 minute and then carefully drawn off again. The stained area was subsequently irradiated with a GaAlAs diode laser[†††] with a measured power output of 50mW. This laser emits radiation in collimated beams ($2mm^2$) with a wavelength of 685nm, for 80s and a total energy of 4J (energy density, $200J/cm^2$). The diode laser was focalized in contact with the mesial, distal, buccal and lingual surfaces by a scanning method for 20s on each face, making a total of 80s.

At all the sites, an e-PTFE membrane[‡‡‡] was placed to over the implants.

The e-PTFE membranes extended circumferentially 3 to 5mm over the adjacent alveolar bone to exclude ingrowths of soft connective tissue. The membranes were stabilized not only by CPTi tacks[§§§] on the buccal and lingual aspects but also by cover screws (Fig.2C). After completion of clot positioning and membrane placement, periosteal releasing incisions were made buccally and lingually to allow tension-free

[***] Sigma LTDA, Poole, UK
[†††] IR 500-Laser Beam, Brazil
[‡‡‡] G-TAM, W.L. Gore & Associates, Flagstaff, AZ, USA
[§§§] Sitema INP, Implantes Nacionais e de Proteses Comercio Ltda, SP, Brazil

flap apposition and closure. Primary wound closure was achieved with horizontal mattress alternated with interrupted e-PTFE sutures[||||||].

Each animal received anti-inflammatory medication (2 mg betamethasone[¶¶¶] 2 times a day) and appropriate analgesia (paracetamol) for 3 days following surgery, in order to reduce postoperative swelling and pain. Sutures were removed 2 weeks after surgical treatment. Oral prophylaxis was performed with 0.12% chlorhexidine daily for 5 months. Observations of the sites with respect to gingival health, maintenance of suture line closure, material exposure or infection were made daily.

A fluorochrome[###] (25mg/Kg body weight) was injected 19 weeks after lethal photosensitization. Five months after surgery, the animals were sacrificed by induction of deep anesthesia followed by intravenous sodium pentobarbital euthanasia.

Histological Procedures

The mandibles were removed and block biopsies of each implant site were dissected. The biopsies were fixed in 4% neutral formalin for 48 hours. The biopsies were prepared for ground sectioning according to methods previously described[20]. The specimens were cut into a mesio-distal plane using a cutting-grinding unit[****]. From each implant site, one central section was prepared and reduced to a final thickness of about 50 to 70µm by micro-grinding and polishing using a micro-grinding unit[††††]. Before staining, each section was evaluated regarding the location of the fluorchrome

[||||||] Gore-Tex Sutures, W.L. Gore, Flagstaff, AZ, USA
[¶¶¶] Celestone, Schering-Plough S/A, Rio de Janeiro, RJ, Brazil
[###] Oxytetracycline, Pfizer do Brasil, Sao Paulo, SP, Brazil
[****] Exact® Cutting, System, Apparatebau, Gmbh, Hamburg, Germany
[††††] Exact® , System, Apparatebau, Gmbh, Hamburg, Germany

marker. The analysis was carried out in a microscopy[‡‡‡‡] equipped with an image system[§§§§]. In the unstained sections, fluorescence light and a filter cube compatible to the fluorochrome were used to check the osseointegration in the 1/3 apical area of the implant that was not affected by peri-implant infection as well as to assess the bone remodelation after 24 months of insertion (Fig.1).

Following, the sections were stained in toluidine blue in order to assess the histometric parameters: (1) Distance from the original bottom of the defect - identified by the difference in coloration after staining a) to the most coronal point of the newly formed bone with intimate contact to the implant surface, b) (=re-osseointegation); (2) Area of a) to the most apical border of the newly formed bone, c) to implant shoulder, d)(=bone fill); (3) Percentage of osseointegration (mineralized bone contact with the implant surface) and (4) Bone area within the limits of the implant threads at the portion of the implant, apical of the peri-implant defect were peri-implantitis did not occur (Fig. 3). The data was obtained in pixels and pixels^2 and transformed into percentage to avoid the influence of the different macrostructure among the dental implants.

RESULTS

Clinical observations

The 19 remaining dental implants successfully integrated and survived the subsequent period of the treatment, including mechanical debridment, lethal photosensitization and the principle of the GBR. Clinically, the three HA dental implant surfaces appeared resorbed (Fig.4A). Heavy calculus deposits were also observed in some dental

[‡‡‡‡] Leitz DM-RBE microscopy, Leica, Germany
[§§§§] Qwin, Leica, Germany

implants (Fig. 4B). After peri-implantitis treatment, none of the membranes placed during the treatment of the peri-implantitis defects had to be removed.

Histological examinations and measurements

The peri-implant soft and hard tissues generally appeared healthy. The alveolar bone was apical to the connective tissue and the implants were embedded to a noticeable variable height. The old bone was mostly lamellar and compact, and numerous osteocytes were presented in their lacunae (Fig. 5A). The newly formed bone exhibited different stages of maturation and remodelation. When observed under fluorescence light, presence of bone remodelation adjacent to dental implant screws was present at 19-months period (Fig. 5B).

The defects created by 2-month period of the ligature-induced plaque accumulation and 12 months under supragingival plaque control, histometrically amounted to 5.86±2.24mm for the TPS surface, 5.52±0.73mm for the HA surface, 4.68±1.39mm for the Acid surface, and 3.26±1.58mm for the CPTi surface.

The percentage is depicted in Fig. 7. The highest proportion of mineralized bone contact with the dental implant surface was seen with the HA (75.69%±12.94), TPS (58.95%±2.43), Acid (62.40%±9.62), and CPTi (52.73%±4.47). The mean and standard deviation of the bone area within the limits of the implant threads showed the highest percentage to HA (79.29%+5.35), followed by TPS (75.87%+16.32), Acid (51.61%±12.65), and CPTi (48.40%±11.39).

The Figs. 6A and 6B are characterizing the bone fill amounted to 48.28%±15.00; 39.54%±12,34; 26.88%±22.16; 26.70%±16.50% to HA, TPS, Acid, and CPTi respectively. In some specimens, the lateral aspect of the coronal part of the dental

implant, i.e. the previously contamined portion, was covered by a dense connective tissue capsule that separated the newly formed bone from the dental implant surface (Fig. 7)

The re-osseointegration (Figs. 8A and 8B) was achieved in 25.25±11.96% for the TPS surface, 24.91%±17.78 for the CPTi surface, 17.30%±15.41 for the Acid surface, and 15.83%±9.64 for the HA surface. However, in just one specimen (acid surface), there was no observation of the new bone in contact with the previously contamined implant surface. (Fig. 9) This data for acid-surface as well as for the other surfaces, demonstrate all implants remained in place. Therefore we took into account all sites, even thought one specimen did not show re-osseointegration.

DISCUSSION

The difficulties in obtaining re-osseointegration after treatment of peri-implantitis have been documented in several animal studies[2,3,21-23]. Most of those studies utilized systemic antibiotics associated with air-powder abrasive[8,24,25] or mechanical debridement[4,23,26]. To our knowledge, this pilot study was the first studies to histometrically evaluate the treatment of chronic-induced peri-implantitis using lethal photosensitization associated to guided bone regeneration.

Recently, several studies have demonstrated the bactericidal effect of high power laser on contaminated dental implant surfaces[27-29]. This energy is specifically absorbed by water molecules, which causes the water-rich tissue to be preferentially vaporized. In bacterial cytoplasm, this effect causes cell lyses and variable degrees of damage to the dental implant surface.

In addition, studies such as those by Hass et al.[6], Dortbudak et al.[9] and Hass et al.[17], have shown the effectiveness of lethal photosensitization in decreasing the viable count of periodontal pathogens in peri-implantitis lesions without damage to the dental implant surface.

The histometric analysis depicts an amount of new bone formation with variable degree, in all dental implant surfaces. Although the percentage of bone fill observed in studies such as Wetzel et al.[23] and Persson et al.[26] have been higher, our results ranged to 48.28% for HA surface to 26.70% for Acid surface, in agreement to Persson et al.[3], Persson et al.[4], although the data of the last two references cited utilized CPTi surfaces.

The re-osseointegration was achieved in all dental implant surfaces principally at the base of the angular bony defect, in agreement to Persson et al.[7], Jovanovic et al.[24], Singh et al.[30] The higher percentage observed in this investigation was 25.15±11.96 to TPS surface and the lower to HA surface (15.83±9.64) being this range in accordance to Hanisch et al.[22], Wetzel et al.[23] Despite controversy on the amount of re-osseointegration, [3,4,7,8,24-26] these different results can be attributed to different experimental designs and variables such as ligature-induced peri-implantitis period, microstructure utilized, clean methods of contaminated implant surface and their efficiency, bony defect shape, and combination of graft materials and GBR. The different dental implant surfaces, their chemical compositions (CPTi, HA, TPS, acid) and their different surface-free energies, did seem to be relevant for amount of histometrical variables.

On the other hand, Persson et al.[26] found 83.7±8.6% of re-osseointegration in sandblasted large grit acid-etched surface (SLA) and 21.8±16.7% for turned surface. The authors speculated that SLA surface could provide a better condition for coagulum stability, facilitating the bone regeneration process. In addition, it has also been suggested that single monolayers from the environment or bulk material can invalidate or difficultate re-osseointegration.[31,32] However these results are not totally understood. The other important observation realized in this study was the dissolution of HA coating after peri-implant infection. Factors such as thickness of coating and crystallinity of the coating may be altered due to periodontal pathogens exposure.[33] Consequently, this factor could difficult the peri-implantitis treatment. It can be speculated that the worst results would be achieved to dental implants with HA coatings. However, these results should be analyzed with caution due to a small sample size used in this study. A dense connective tissue capsule that separated the newly formed bone from the dental implant surface was observed in some specimens in agreement to Persson et al.[3], Persson et al.[4], and Wetzel et al.[23]. In these studies, the treatment utilized was the association between systemic antibiotic treatment and chemical means: delmopinol or chlorhexidine. These substances showed to form a dense, stable film with thickness of 7 to 10nm, on the oxide layer of the dental implant surface[32]. According to Persson et al.[7] this film may prevent the bone fill and re-osseointegration. In our study, the utilization of lethal photosensitization presented similar results. However we could not conclude that either TBO alone or TBO plus diode laser would form the same film.

In addition, the use of lethal photosensitization in order to kill periodontal pathogens offers some advantages over the use of conventional antimicrobials: (1) it avoids development of resistance among target organisms to the photochemically-generated free radicals thought to be responsible for bacterial killing and; (2) unlike antiseptics and antibiotics, there would be no need to maintain high concentrations of the TBO in the peri-implant defects for long periods.

In conclusion, data from the present pilot study suggests that the treatment of chronic peri-implantitis by means of lethal photosensitization may obtain significant bone fill associated to re-osseointegration. However, these results should be considered with caution and further investigations must be conducted.

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Figure Legends

Fig.1: Outline of the experiment. Animals *n*=6, dental implants *n*=19. Ligatures were placed at 0 month (+Lig.) and removed at 2 months (-Lig.). Treatment of dental implants was performed at 14 months and the animals sacrificed at 19 months.

Fig.2A: Clinical view of peri-implant defects before surgical debridment.

Fig.2B: Peri-implant defects adjacent to HA surface after surgical debridment with plastic curettes

Fig.2C: e-PTFE membranes placed over the dental implants fixed with cover screws and CPTi tacks.

Fig.3: Schematic drawing illustrating the landmarks used for the histometric measurements.

Fig.4A: Clinical view of partially resorbed Ha-coating after peri-implantitis infection.

Fig.4B: Presence of calculus (arrow) after 14-months period after experimental peri-implantitis in acid implant surface.

Fig.5A: Ground section of acid implant surface were peri-implantitis did not occur (Toluidine blue, magnification 80X)

Fig.5B: Mesio-distal ground section of one CPTi surface. Fluorescence light (original magnification x100). The fluorochrome marks shows the bone remodelation at 19-months period.

Fig.6A: Mesio-distal ground section of one HA surface. (Toluidine blue staining, original magnification x40) showing the borderline between the “old” original bone and the newly formed bone.

Fig. 6B: Mesio-distal ground section of one TPS surface. (Toluidine blue staining, original magnification x40) showing the borderline between the “old” original bone and the newly formed bone in apical portion of the peri-implant defect.

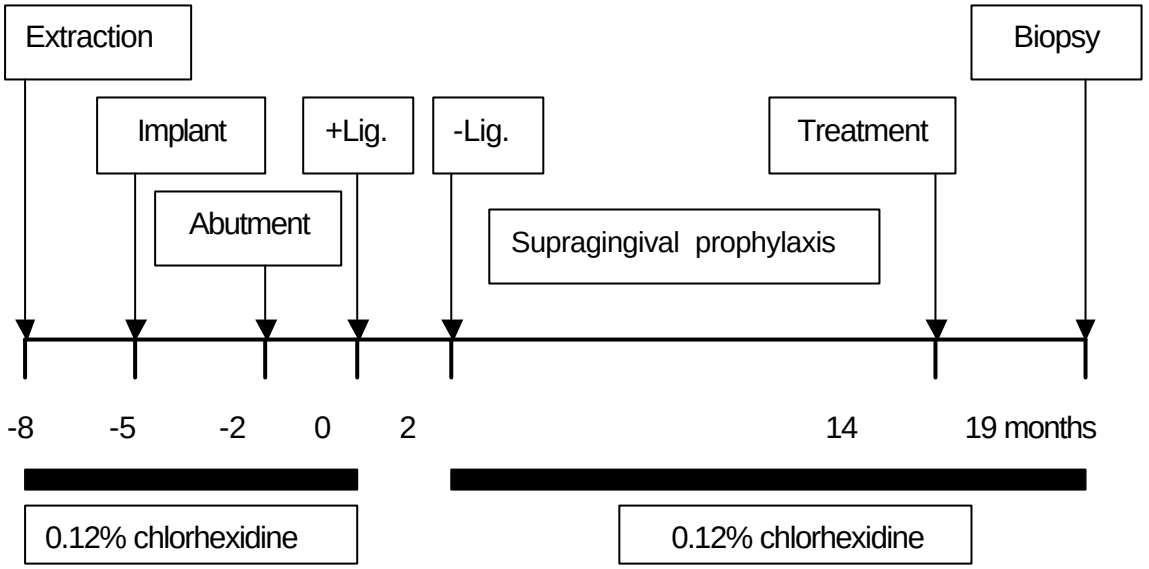
Fig. 7: Histologic section showing dense connective tissue at the implant/new bone interface (polarized light, original magnification 40X)

Fig. 8A: Mesio-distal ground section of one TPS surface. (Toluidine blue staining, original magnification x100) showing the borderline between the “old” original bone and the newly formed bone in direct contact with dental implant surface previously contamined (re-osseointegration)

Fig. 8B: Detail depicting re-osseointegration in CPTi surface. Note that direct contact was established between the newly formed bone and the previously contamined portion of dental implant surface (Toluidine blue staining, original magnification 100x).

Fig. 9: Higher magnification of ground section of acid surface showing the absence of direct contact between the newly formed bone and the dental implant surface (Toluidine blue staining, original magnification 100x)

Fig. 1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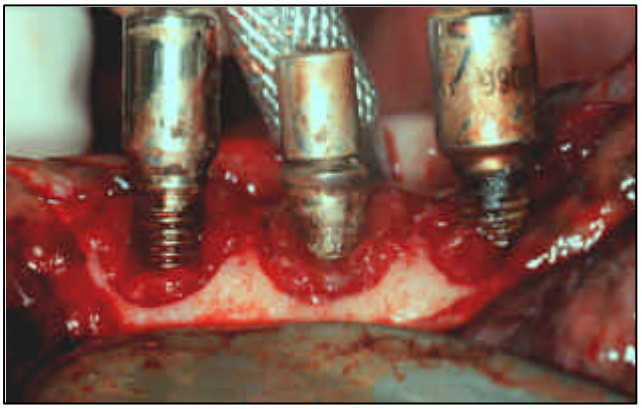

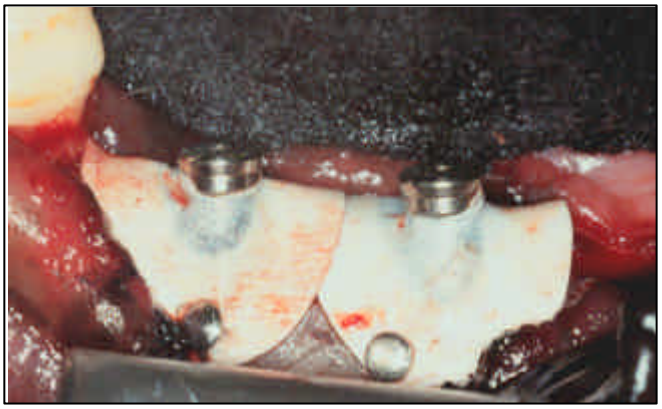

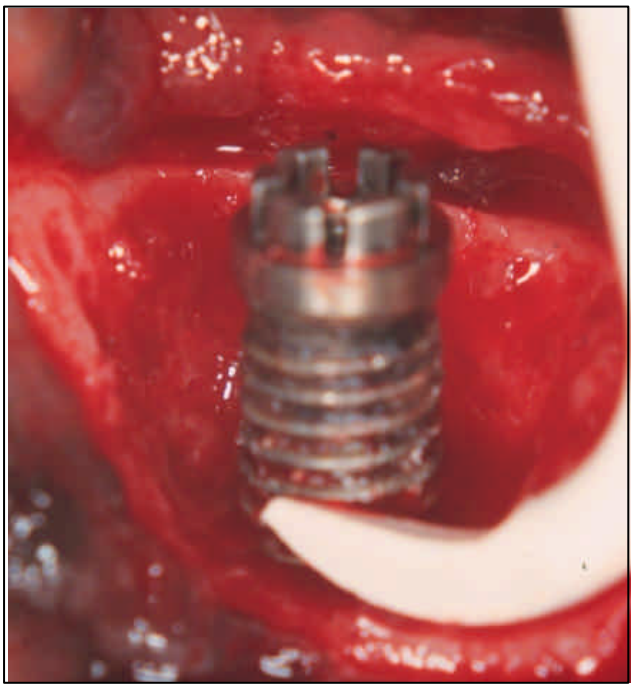

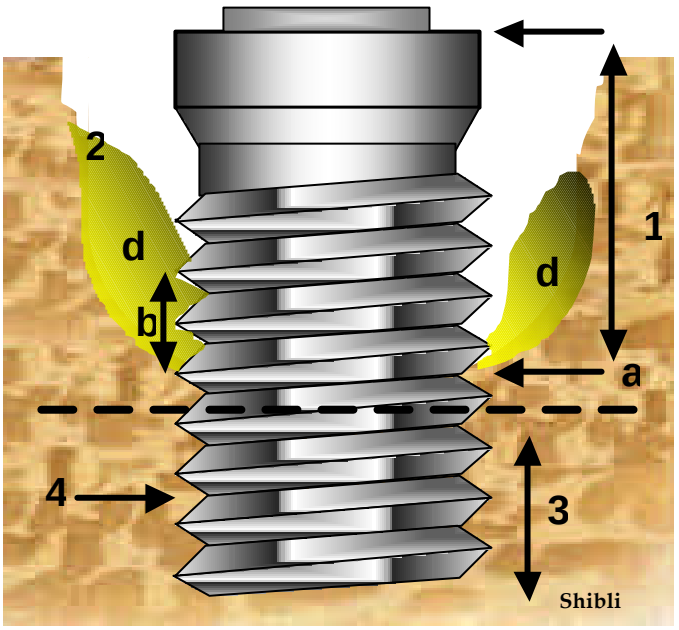
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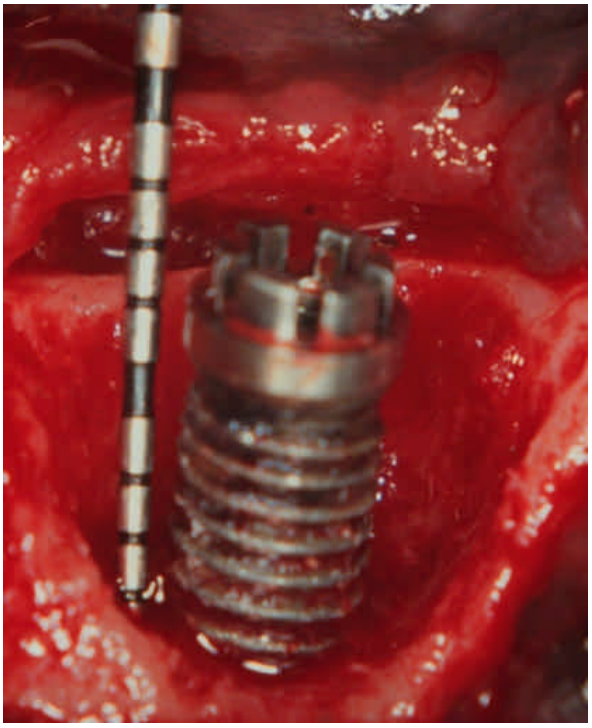

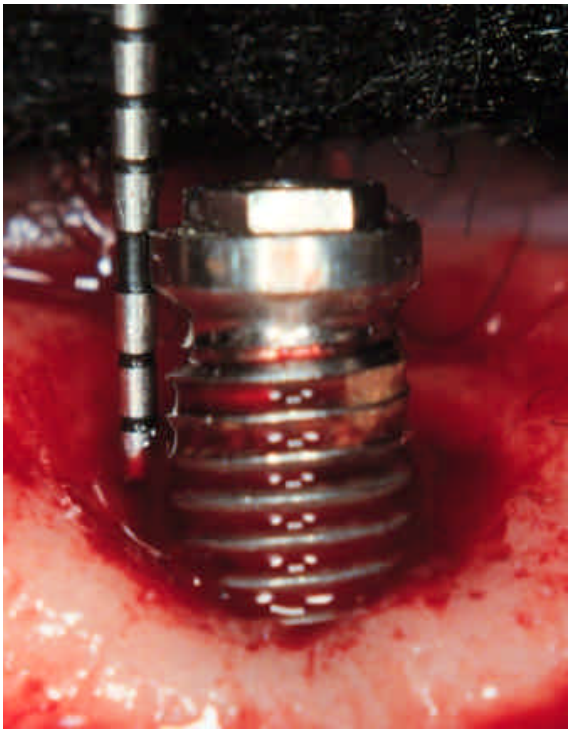

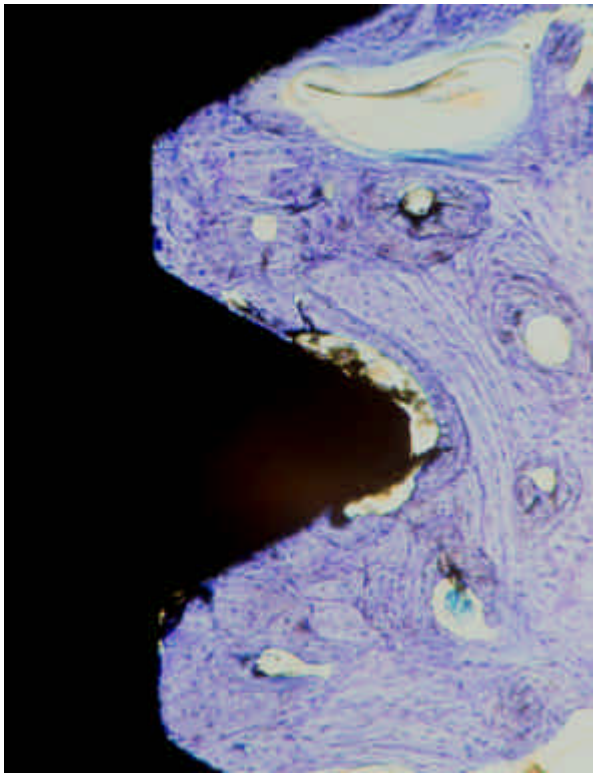

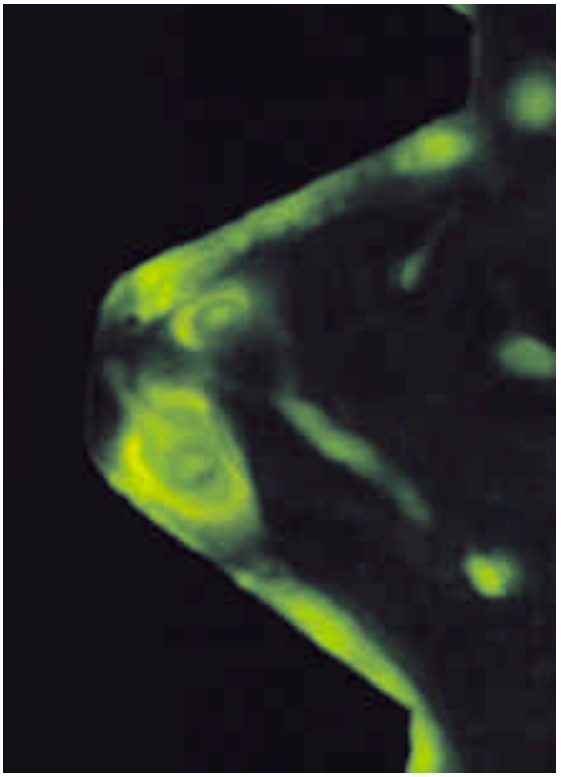

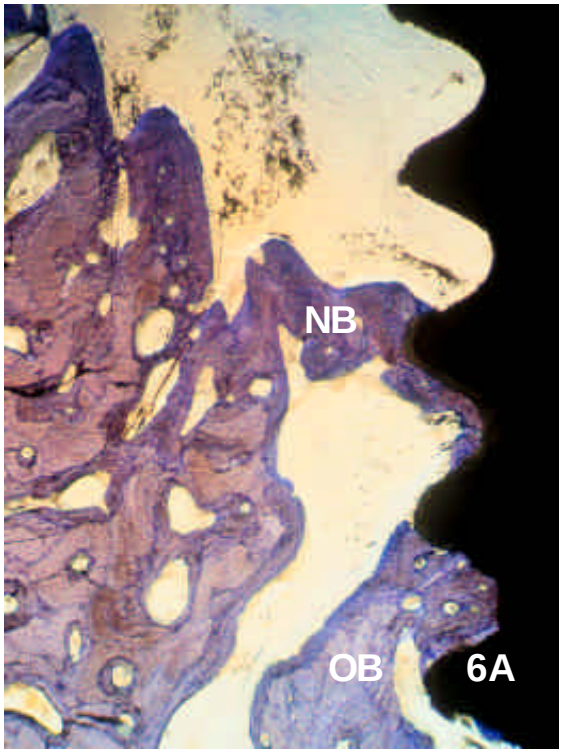
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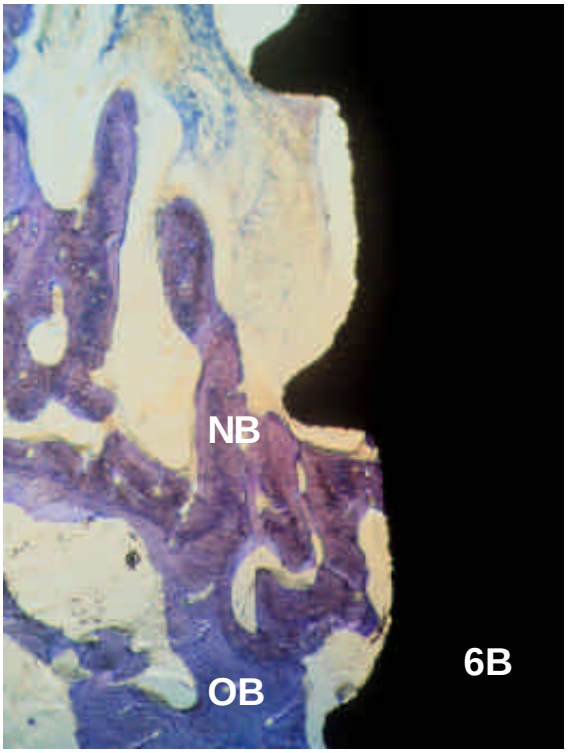
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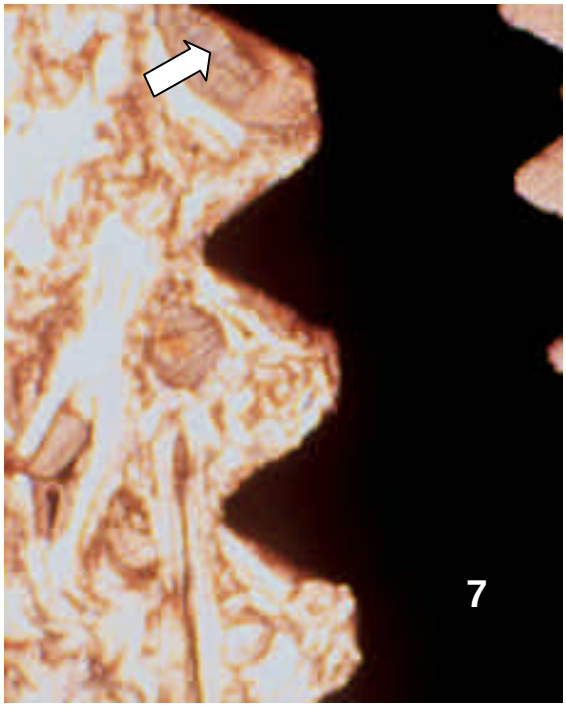
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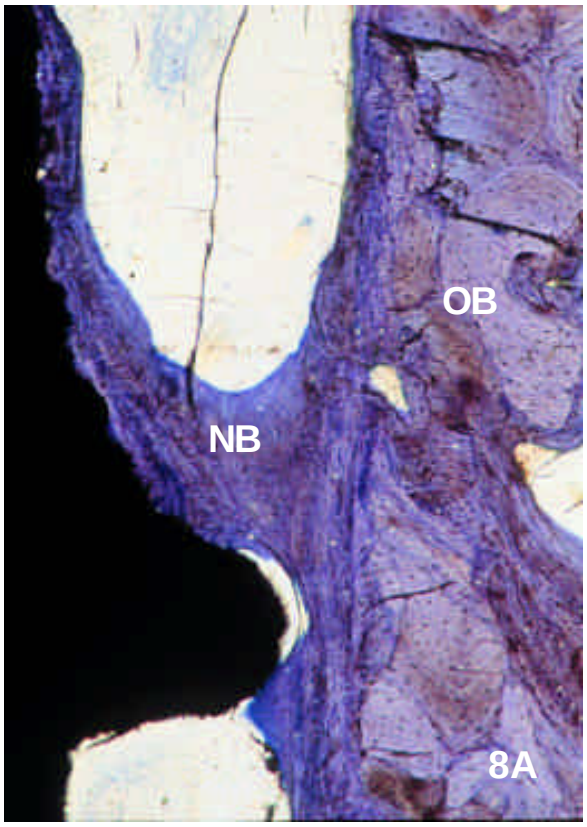
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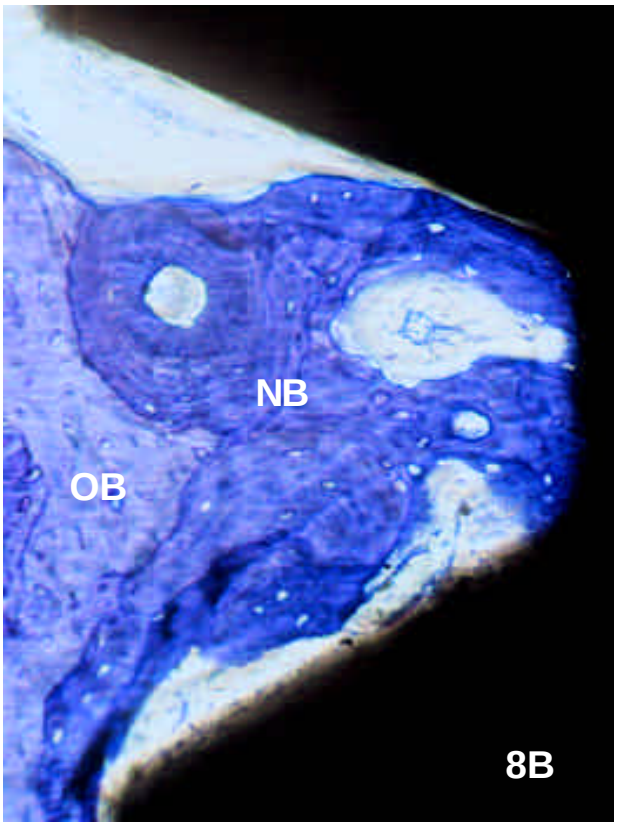
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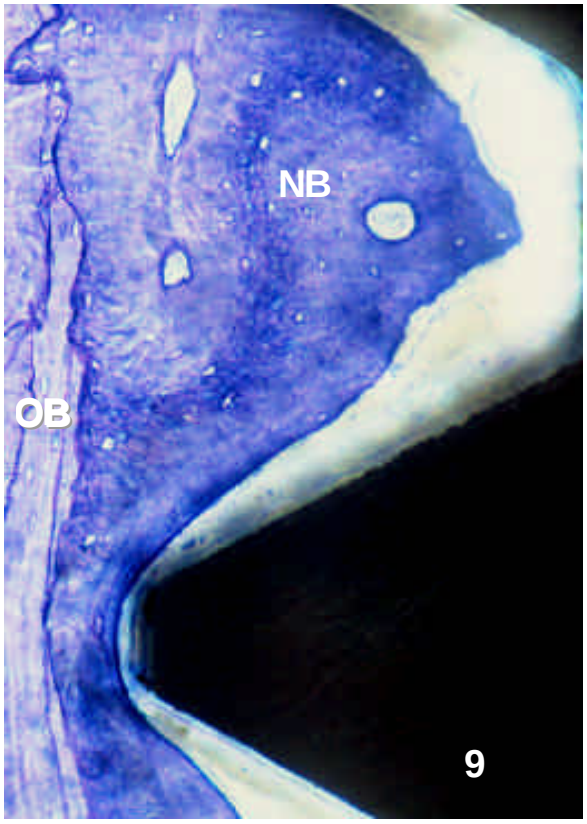
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CAPITULO-5 GUIDED BONE REGENERATION AND LETHAL PHOTOSENSITIZATION IN TREATMENT OF PERI-IMPLANTITIS IN DIFFERENT IMPLANT SURFACES. A HISTOMORPHOMETRICAL STUDY IN DOGS

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Running Title: Lethal photosensitization in treatment of peri-implantitis

GUIDED BONE REGENERATION AND LETHAL PHOTOSENSITIZATION IN TREATMENT OF PERI-IMPLANTITIS IN DIFFERENT IMPLANT SURFACES. A HISTOMORPHOMETRICAL STUDY IN DOGS

ABSTRACT:

Background: The purpose of this study was to evaluate the effect of lethal photosensitization and guided bone regeneration in the treatment of experimentally induced peri-implantitis lesions adjacent to various dental implant surfaces.

Methods: Five mongrel dogs, about 2-year old, were included in the study. In order to establish bilateral recipient sites for implants, all mandibular molars and pre-molars were removed. A total of 40 dental implants with four different surface coatings (10 commercially pure titanium surface-CPTi; 10 titanium plasma-sprayed-TPS; 10 acid-etched surface-AE; 10 sandblasted surface with alumina oxide-SAO) were inserted. After a 2 months period of ligature-induced peri-implantitis with cotton floss, the dogs were subjected to surgical debridment and guided bone regeneration (control side) and surgical debridement, guided bone regeneration, and lethal photosensitization by combination of toluidine blue O (100μg/mL) and irradiation with diode laser GaAlAs with a wavelength of 685nm and power output of 50mW for 80s, and a total energy of 4J (energy density of 200J/cm^2). After 5 months of treatment block biopsies including one implant with adjacent hard and soft tissue were harvested and prepared for ground sectioning and analysis.

Results: The bone fill was statistically higher to photosensitization group ($p \leq 0.0132$) with average between 46.67±32.00 to acid surface and 58.38±23.67% for TPS. The re-osseointegration was achieved in 45.14±28.00% for the CPTi surface and the lower

mean to TPS surface (33.60±12.20%) in spite of the control group depicted lower means ($p \leq 0.0097$).

Conclusions: The lethal photosensitization associated with guided bone regeneration improved statistically better condition to obtain re-osseointegration adjacent to several implant surfaces.

Key Words: Dental implants/peri-implant diseases; Implant surfaces/microstructure; Peri-implantitis/treatment; Photodynamic Therapy/ Photosensitizers; Re-osseointegration; Guided Bone Regeneration.

INTRODUCTION

Plaque-induced peri-implantitis has been implicated as one etiological factor associated with long-term failure of dental implants. Several animal experiments have shown that bacterial biofilm accumulation around dental implants promoted by ligature-placement can develop a peri-implant tissue breakdown or peri-implantitis[1-4]. Although the literature reports the difficulties of attempting re-osseointegration on dental implant surfaces after contamination for periodontal pathogens, several therapeutic strategies can be applied for the treatment of peri-implantitis[2,5-8].

Recent evaluations have been questioned the importance of implant surface cleanliness after peri-implantitis treatment.[9-10] It has been hypothesized that surface contaminants may be released from contaminated implant surface, enhancing and perpetuating the inflammatory response, thus altering the healing process and possibly provoking the dissolution of titanium.[11-14] In addition, the alterations of this oxide layer surface may difficult the re-osseointegration.

In an earlier pilot study, Shibli et al[10] shown the potential use of photodynamic therapy associated to guided bone regeneration in treatment of peri-implant lesions. The average of re-osseointegration was 25.15% being similar with previous studies that used mechanical treatment associated with antibiotics.[7-9] The photodynamic therapy used a low-power laser following application of a photosensitizing substance, as toluidine blue O (TBO). The mechanism by which TBO causes the microorganism killer such as *P. gingivalis*, *P. intermedia*, *A. actinomycetemcomitans*, and *F. nucleatum* has not yet been established, but it is believed that lethal photosensitization of these

microorganisms may involve changes in the membranes and/or plasma membrane proteins and DNA damage mediated by singlet oxygen.[15-22]

The objective of this study was to evaluate the efficacy of lethal photosensitization associated with guided bone regeneration in treatment of ligature-induced peri-implantitis in dogs with different dental implant coated-surfaces.

MATERIAL AND METHODS

Animals

The outline of the experiment is presented in Fig. 1. Five adult, systemically healthy, male mongrel dogs, 2 years of age in an average weight of 18Kg were used. Animal selection, management, and surgical protocol followed routines approved for this study by Dental School of Araraquara Institutional Animal Care and Use Committee.

All surgical and clinical procedures as well as the laser irradiation were performed under general anesthesia accomplished by 0.05mg/Kg of subcutaneous preanesthesia sedation (atropine sulphate[||]) and intravenous injection of chlorpromazine[¶] and thiopental[#]. Oral prophylaxis was performed within 2 weeks before teeth extraction. After that, all mandibular premolars and first molars were extracted creating an edentulous ridge. Both the mandibular quadrants and the alveoli were allowed to heal for a period of 3 months. The upper premolars were also extracted to avoid occlusion trauma interference. During the healing period, bacterial biofilm control was instituted by means of scrubbing 0.12% chlorhexidine** daily, scaling and root planing once a month, until cotton ligatures placement.

[||] Atropine sulphate - 0.5mg, Ariston Inds. Química e Farms. LTDA, São Paulo, Brasil

[¶]Amplictil 25mg, Rhodia Farma LTDA, São Paulo, Brasil

[#]Tiopental - ABBOTT Laboratórios do Brasil Ltda, São Paulo, Brasil

****** Pharmacy School -State University of Sao Paulo (UNESP), Araraquara, SP, Brazil.

Implant Design and Surfaces

Forty dental implants with four different surfaces of three different implant systems were used, as follows: ten commercially pure titanium implants[††]– CPTi; ten titanium plasma sprayed[‡‡] – TPS; ten hybrid surfaces – machined titanium in the three first screws and acid-etched in others screws[IIII]– acid, and ten sandblasted with alumina oxide[§§] – oxid,. All implants have length 10mm and a diameter 3.75mm (Fig. 2)

Implant Surgery

The dental implants were placed after the full thickness flap under aseptic surgical conditions. The recipient sites were prepared using original instruments for each dental implant surface, according to the surgical techniques indicated by each implant manufacturer. The implants were randomly distributed among the dogs so that each dental implant surface was represented in each mandibular side. The flaps were sutured with single interrupted sutures, submerging all implants.

An antibiotic coverage with potassic and sodic benzilpenicilin[¶¶] was given once a week for 2 weeks, in order to avoid post-surgical infection. Paracetamol[##] was given for pain control medication, and the sutures were removed after 14 days.

[††] CPTi- Sterngold, Implamed, Attleboro, MA,

[‡‡]TPS Sterngold, Implamed, Attleboro, MA,

[§§] *Osseotite*®-3*i*® Implants Innovations, Palm Beach Gardens, FL

[IIII] Porous, Conexão Implantes, SP, Brazil

[¶¶]Fort Dodge Saúde Animal LTDA, Campinas, SP, Brasil

[##] ABBOTT Laboratórios do Brasil Ltda, São Paulo, Brazil

Experimental Peri-implantitis

Three months after dental implant installation, healing abutment connections were installed, according to the instructions of each dental implant system. After two months of a plaque control program and the healing of the soft tissue, cotton floss ligatures were placed around the dental implants and sutured in the peri-implant mucosa, not only to facilitate plaque accumulation, but also to hold the ligatures in position. Tying further ligatures at 20 day-intervals for a period of 60 days accelerated peri-implant bone loss. At 60 days, when approximately 40% of the initial bone support was lost, the ligatures were finally removed.

A 2-month plaque control program was initiated by means of scrubbing daily with 0.12% chlorhexidine and scaling the abutment surface once a month.

The mandibular quadrants of the dogs were randomly divided so that a split mouth design could be designed: control side-mechanical debridment and GBR, and test side-mechanical debridment, lethal photosensitization and GBR.

Lethal Photosensitization and Guided Bone Regeneration

A crestal incision was made through the mucosa, and buccal and lingual full thickness flaps were elevated (Figs. 3A and 3B). The abutments were removed and the granulation tissue present in bone craters around the dental implants was curetted with a plastic curette.

The implant surfaces of the test side were then rinsed with physiological saline solution. Following, TBO[***](100µg/ml)[10,15,23,24] was injected into the peri-implant defect as far as the bony border, with a thin needle.

[***] Sigma LTDA, Poole, UK

TBO was left in place for 1 minute and then carefully drawn off again. The stained area was subsequently irradiated with a GaAlAs diode laser[†††] with a measured power output of 50mW. This laser emits radiation in collimated beams (2mm^2) with a wavelength of 685nm, for 80s and a total energy of 4J (energy density, 200J/cm^2). The diode laser was focalized in contact with the mesial, distal, buccal and lingual surfaces by a scanning method for 20s on each face, making a total of 80s.[10,24]

Follow, all the sites, including control sides, received an e-PTFE membrane[‡‡‡] that it was placed to over the implants.

The e-PTFE membranes extended circumferentially 3 to 5mm over the adjacent alveolar bone to exclude ingrowths of soft connective tissue. The membranes were stabilized not only by CPTi tacks[§§§] on the buccal and lingual aspects but also by cover screws. After completion of clot positioning and membrane placement, periosteal releasing incisions were made buccally and lingually to allow tension-free flap apposition and closure. Primary wound closure was achieved with horizontal mattress alternated with interrupted sutures[ǁǁǁ].

Each animal received anti-inflammatory medication (2 mg betamethasone[¶¶¶] 2 times a day) and appropriate analgesia (paracetamol) for 3 days following surgery, in order to reduce postoperative swelling and pain. Two weeks after local therapy, the sutures were removed and a fluorochrome[###] (25mg/Kg body weight) was injected i.v.

[†††] IR 500-Laser Beam, Brazil

[‡‡‡] Tef-Gen, USA

[§§§] Sitema INP, Implantes Nacionais e de Proteses Comercio Ltda, SP, Brazil

[ǁǁǁ] Gore-Tex Sutures, W.L. Gore, Flagstaff, AZ, USA

[¶¶¶] Celestone, Schering-Plough S/A, Rio de Janeiro, RJ, Brazil

[###] Oxytetracycline, Pfizer do Brasil, Sao Paulo, SP, Brazil

Oral prophylaxis was performed with 0.12% chlorhexidine daily for 5 months. Observations of the sites with respect to gingival health, maintenance of suture line closure, material exposure or infection were made daily.

Four months after treatment, the e-PTFE membranes were surgically removed and the implants were allowed to heal for one month. Four days before the sacrificed, a second fluorochrome (alizarine red-18mg/Kg body weight) was injected i.v. with the purpose of to observe the remodelation of peri-implant bone tissue at 5-month after treatment

Five months after treatment, the animals were sacrificed by induction of deep anesthesia followed by intravenous sodium pentobarbital euthanasia.

Histological Procedures

The mandibles were removed and block biopsies of each implant site were dissected. The biopsies were fixed in 4% neutral formalin for 48 hours. The biopsies were prepared for ground sectioning according to methods previously described[25]. The specimens were cut into a mesio-distal plane using a cutting-grinding unit[****]. From each implant site, one central section was prepared and reduced to a final thickness of about 50 to 70μm by micro-grinding and polishing using a micro-grinding unit[††††]. Before staining, each section was evaluated regarding the location of the fluorchrome marker. The analysis was carried out in a microscopy[‡‡‡‡] equipped with an image system[§§§§]. In the unstained sections, fluorescence light and a filter cube compatible to the fluorochrome were used not only to assess the bone defect border but also the remodelation of the new bone.

[****]Exact® Cutting, System, Apparatebau, Gmbh, Hamburg, Germany
[††††]Exact® , System, Apparatebau, Gmbh, Hamburg, Germany
[‡‡‡‡]Leitz DM-RBE microscopy, Leica, Germany
[§§§§]Qwin, Leica, Germany

Following, the sections were stained in toluidine blue in order to assess the histometric parameters[10]: (1) Distance from the original bottom of the defect - identified by the difference in coloration after staining a) to the most coronal point of the newly formed bone with intimate contact to the implant surface, b) (=re-osseointegation); (2) Area of a) to the most apical border of the newly formed bone, c) to implant shoulder, d)(=bone fill); (3) Percentage of osseointegration (mineralized bone contact with the implant surface) and (4) Bone area within the limits of the implant threads at the portion of the implant, apical of the peri-implant defect were peri-implantitis did not occur (Fig. 4). The data was obtained in pixels and pixels^2 and transformed into percentage to avoid the influence of the different macrostructure among the dental implants. Non parametric analysis of variance and paired t-test were performed to assess the differences between test and control group and among the several implant surfaces (a=5%)..

RESULTS

Clinical observations

The dental implants of test group successfully integrated and survived the subsequent period of the treatment, including mechanical debridment, lethal photosensitization and the principle of the GBR. Clinically, some dental implant surfaces from test group appeared covered by newly bone (Figs.5A and 5B). After peri-implantitis treatment, all the membranes placed during the treatment of the peri-implantitis defects adjacent to implants had to be surgically removed. However, all the control sides depicted earlier exposition of the membranes when compared with test sides.

Histological examinations and measurements

The peri-implant soft and hard tissues generally appeared healthy. The alveolar bone was apical to the connective tissue and the implants were embedded to a noticeable variable height for both groups. The old bone was mostly lamellar and compact, and numerous osteocytes were presented in their lacunae. The newly formed bone exhibited different stages of maturation and remodelation, principally for the test group. When observed under fluorescence light, a band of oxytetracycline was found in the bone tissue at a level about 7mm apical of the implant shoulder. The bone marker projected in a lateral-coronal direction and separated a triangular shaped portion of newly formed regenerated bone (NB) from the old bone (OB) (Figs. 6A to 6C).

The defects created by 2-month period of the ligature-induced plaque accumulation and 2 months under supragingival plaque control, histometrically ranged between 3.35±0.50mm for the acid surface and 4.25±1.00mm for the oxide surface. (Fig.7). However, statistical difference was not observed among the different implant surfaces (p=0.17).

The percentage of mineralized bone contact with the dental implant surface is depicted in Fig. 8. The highest proportion was seen with the TPS surface (76.58%±7.31), and the lower to acid surface (61.48%±11.66%). The mean and standard deviation of the bone area within the limits of the implant threads showed the highest percentage to oxide, followed by TPS, CPTi, and acid surface (Fig. 9).

The Figs. 10A and 10B are characterizing the bone fill amounted to 58.38%±23.67 and 3.38%±4.70 to TPS surface for test and control group respectively (Fig. 11). Significant differences were observed between test and control group (p≤0.0132)

except for acid surface that shown a higher standard deviation (p=0.0625). In some specimens, the lateral aspect of the coronal part of the dental implant, i.e. the previously contamined portion, was covered by a dense connective tissue capsule that separated the newly formed bone from the dental implant surface (Fig. 12).

The re-osseointegration (Figs. 13A and 13B) was achieved in 45.14%$\pm$28.00 and 3.64%$\pm$0.73 for the CPTi surface in the test and control group respectively. The test group depicted a better performance than control group for this histometrical variable ($p \leq 0.0097$) (Fig. 14). However, in some specimens of implants from control group, there was no observation of the new bone in contact with the previously contamined implant surface. (Fig. 15) Statistical differences were not observed for any histometrical measurements among the four dental implant coated-surfaces (p>0.05).

DISCUSSION

The results of the present investigation support the use o guided bone regeneration associated with antimicrobial therapy for the treatment of peri-implant bony defects experimentally induced by ligatures-placement. In addition, the use of photodynamic therapy as local treatment may be useful alternative to antibiotics for the treatment of local infection such as mucositis/gingivitis and peri-implantitis/periodontitis by eradication of target cells using oxygen species produced by interaction between a photosensitizing agent and light of an appropriate wavelength.[26,27] The difficulties in obtaining re-osseointegration after treatment of peri-implantitis have been documented in several animal studies[2,3,28-30]. Most of those studies utilized systemic antibiotics associated with air-powder abrasive[8,31,32] or mechanical debridement[4,32,33].

Recently, several studies have demonstrated the bactericidal effect of high power laser on contaminated dental implant surfaces[34-36]. This energy is specifically absorbed by water molecules, which causes the water-rich tissue to be preferentially vaporized. In bacterial cytoplasm, this effect causes cell lyses and variable degrees of damage to the dental implant surface.

In addition, studies such as those by Hass et al.[6], Dortbudak et al.[15] and Hass et al.[23], Shibli et al.[24], have shown the effectiveness of lethal photosensitization in decreasing the viable count of periodontal pathogens in peri-implantitis lesions without damage to the dental implant surface. The complications with the barrier from control groups can support the findings that the presence of periodontal pathogens at the dental implant fixture altered the cells responses together with enhanced protease activities and foreign body reactions decreasing the efficacy of guided bone regeneration. [37,38]

The histometric analysis depicts an amount of new bone formation statistically higher to test group. Although the percentage of bone fill observed in studies such as Wetzel et al.[30] and Persson et al.[33] have been higher, our results ranged to 46.67% for acid surface to 58.38% for TPS surface, in agreement to Persson et al.[3], Persson et al.[4], although the data of the last two references cited utilized machined surfaces.

The re-osseointegration was achieved in all dental implant surfaces principally at the base of the angular bony defect, in agreement to Persson et al.[7], Jovanovic et al.[31], Singh et al.[39], and Shibli et al.[10] The higher percentage observed in our investigation was 45.14±28.00 to CPTi surface and the lower to TPS surface (33.60±12.20) being this range in accordance to Hanisch et al.[29] and higher when compared with results presented by Wetzel et al.[30] Despite controversy on the amount of re-osseointegration,

[3,4,7,8,31-33] these different results can be attributed to different experimental designs and variables such as ligature-induced peri-implantitis period, microstructure utilized, clean methods of contaminated implant surface and their efficiency, bony defect shape, and combination of graft materials and GBR. In this study, the different dental implant surfaces, their chemical compositions and their different surface-free energies, did not seem to be statistically relevant for amount of histometrical variables.

On the other hand, Persson et al.[33] found 83.7±8.6% of re-osseointegration in sandblasted large grit acid-etched surface (SLA) and 21.8±16.7% for turned surface. The authors speculated that SLA surface could provide a better condition for coagulum stability, facilitating the bone regeneration process. However, the different surfaces evaluated in our study showed lower means of re-osseointegration than CPTi, contrarians the hypothesis of that study.[33]

A dense connective tissue capsule that separated the newly formed bone from the dental implant surface was observed in some specimens in agreement to Persson et al.[3], Persson et al.[4], and Wetzel et al.[30]. In these studies, the treatment utilized was the association between systemic antibiotic treatment and chemical means: delmopinol or chlorhexidine. These substances showed to form a dense, stable film with thickness of 7 to 10nm, on the oxide layer of the dental implant surface[40]. According to Persson et al.[7] this film may prevent the bone fill and re-osseointegration. In our study, the utilization of lethal photosensitization presented similar results.

In conclusion, data from the present study presented that the treatment of ligature-induced peri-implantitis by means of lethal photosensitization associated with guided bone regeneration can obtain significant bone fill associated to re-osseointegration.

However, these results should be considered with caution and further investigations must be conducted.

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Figure Legends

Fig.1: Outline of the experiment. Animals *n*=5, dental implants *n*=40. Ligatures were placed at 0 month (+Lig.) and removed at 2 months (-Lig.).

Fig.2: Dental implants used in this study. Left from right: CPTi, acid, oxide and TPS

Fig.3A: Clinical view of the implants at abutment connection.

Fig.3B: Clinical view of peri-implant defects after experimental peri-implant tissue breakdown.

Fig.4: Schematic drawing illustrating the landmarks used for the histometric measurements.

Fig.5A: Peri-implant defect adjacent to CPTi (left) and acid surface (right) after mechanical debridment.

Fig.5B: Clinical view of the same defect 4 months after lethal photosensitizationan GBR. Note the contour of the newly formed bone (arrows) covered the peri-implant defect.

Fig.6A: Ground section of acid implant surface from test group. Fluorescence light (original magnification x200). The fluorochrome marks show the border of the bone defect (yellow) and the newly bone formation (red)

Fig.6B: Mesio-distal ground section of the same area, (Toluidine blue staining, original magnification x200) showing the re-osseointegration with dental implant surface previously contamined.

Fig.6C: Fluorescence light (original magnification x200). The fluorochrome marks show the border of the bone defect (yellow) and the newly bone formation (red) in oxide surface.

Fig.7: Mean and standard deviation of the distance of the cover screw/fixture junction to bottom of the defect (mm).

Fig.8: Mean and standard deviation of percentage of direct bone-to-implant contact of the implant surface part that was not infected.

Fig.9: Mean and standard deviation of percentage of the bone area within the limits of the implant threads of the implant surface part that was not infected

Fig. 10A: Mesio-distal ground section of acid surface implant (test group) showed in Fig. 5B. (Toluidine blue staining, original magnification x25) showing the borderline (arrows) between the "old" original bone (OB) and the newly formed bone(NB) .

Fig. 10B: Mesio-distal ground section of one oxide surface from control group. (Toluidine blue staining, original magnification x40) showing the borderline between the "old" original bone (OB) and the newly formed bone (NB) in apical portion of the peri-implant defect.

Fig. 11: Mean and standard deviation of percentage of bone fill.

Fig. 12: Mesio-distal ground section of one oxide surface. (Toluidine blue staining, original magnification x100) showing the borderline between the "old" original bone and the newly formed bone in direct contact with dental implant surface previously contamined (re-osseointegration). Note a dense connective tissue (CT) between the implant surface and newly formed bone.

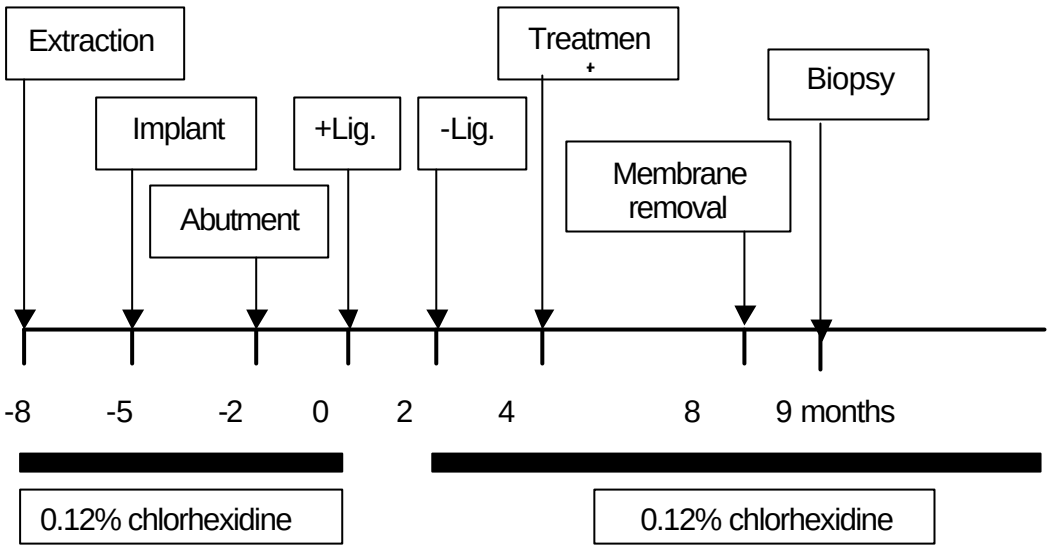
Fig. 13A: Detail depicting re-osseointegration in TPS surface. Note that direct contact was established between the newly formed bone (NB) and the previously contamined portion of dental implant surface (Toluidine blue staining, original magnification 100x).

Fig.13B: Ground section depicting re-osseointegration in oxide surface. Note that direct contact was established between the newly formed bone (NB) and the previously contamined portion of dental implant surface (Toluidine blue staining, original magnification 200x).

Fig. 14. Mean and standard deviation of re-osseointegration in percent of the previously contamined surface

Fig. 15: Higher magnification of ground section of CPTi surface showing the absence of direct contact between the newly formed bone (NB) and the dental implant surface (Toluidine blue staining, original magnification 100x)

Fig. 1


Extraction
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0.12% chlorhexidine

Fig.2

Figs.3A and 3B

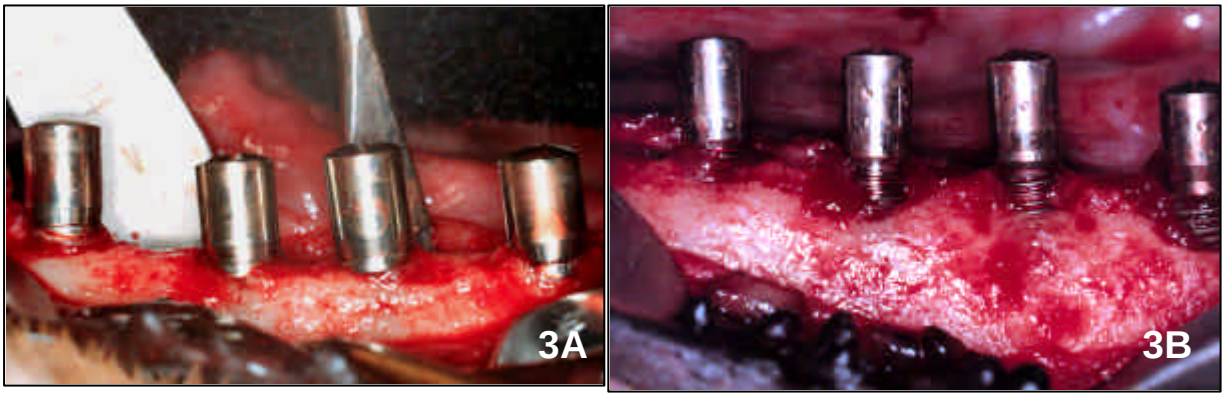
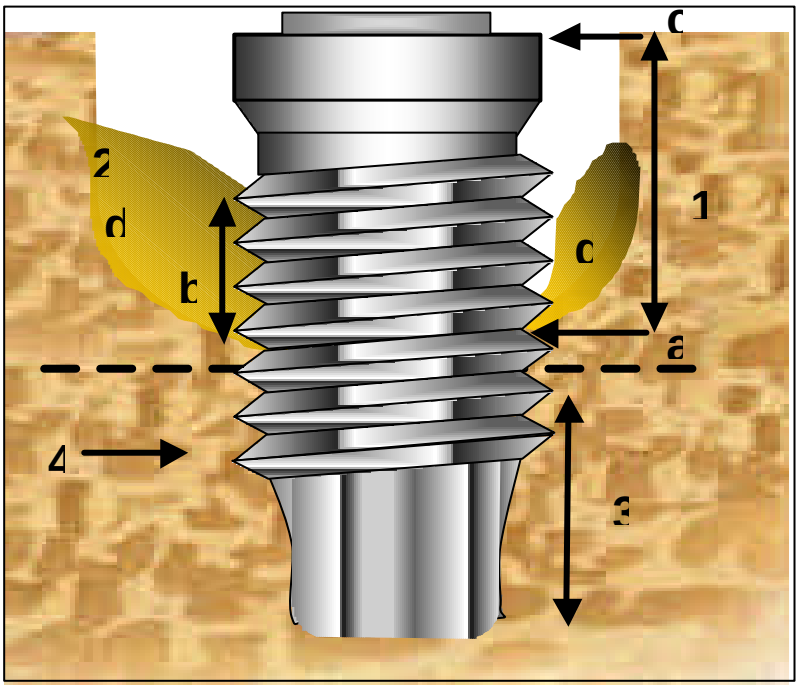
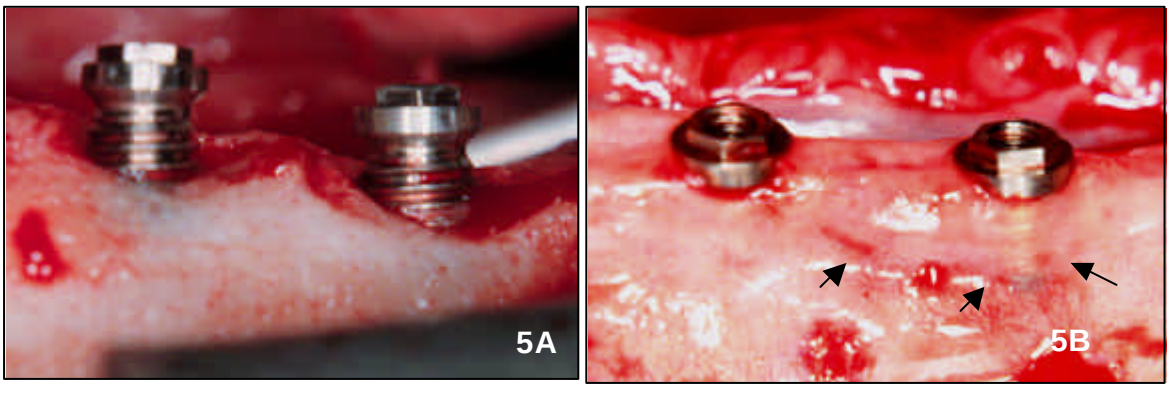


Fig. 4



Figs. 5A and 5B



Figs 6A, 6B, and 6C

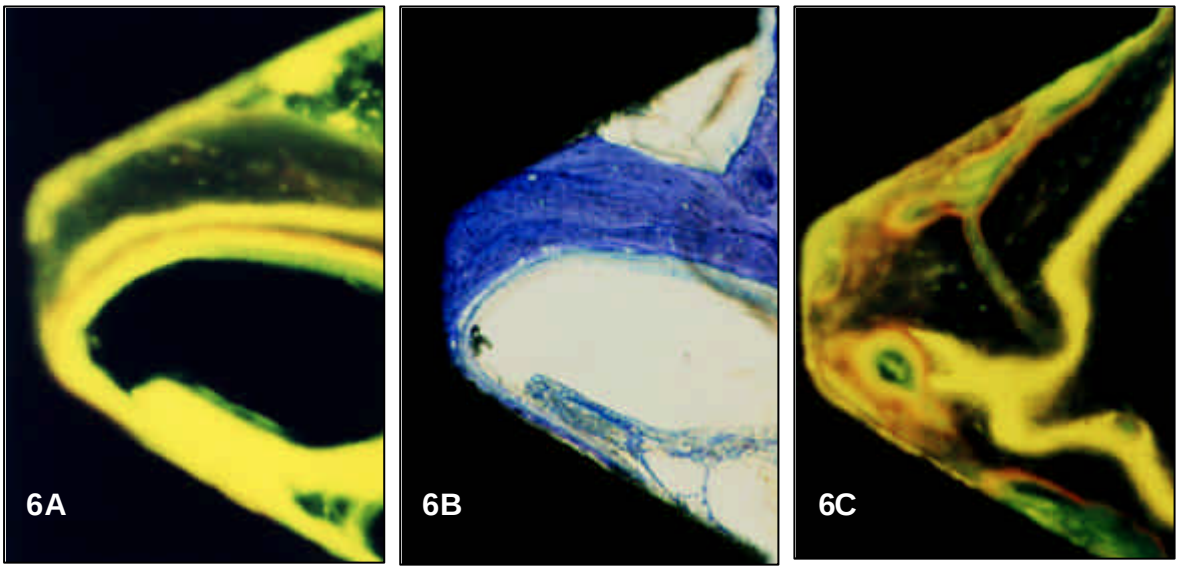


Fig 7.

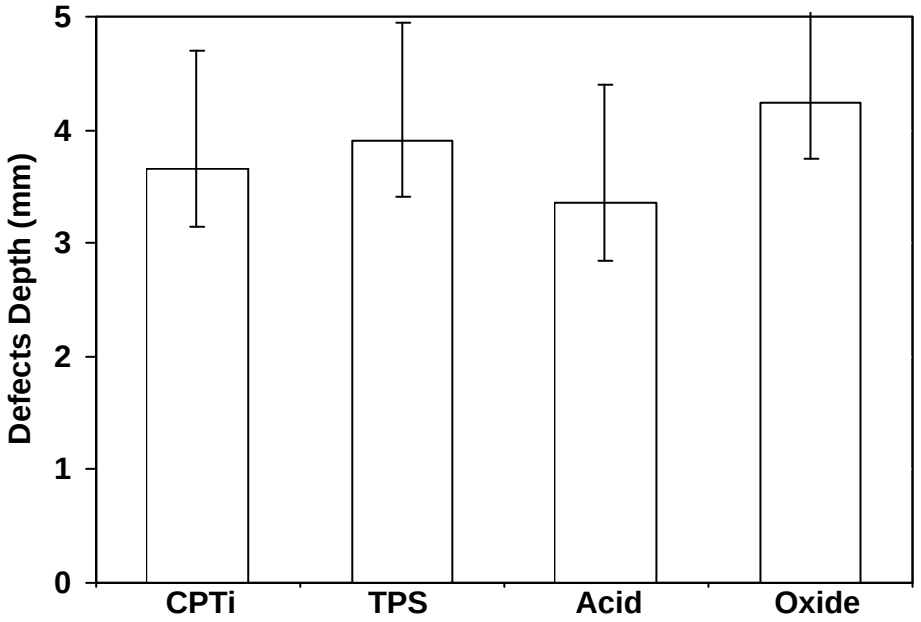


Fig. 8

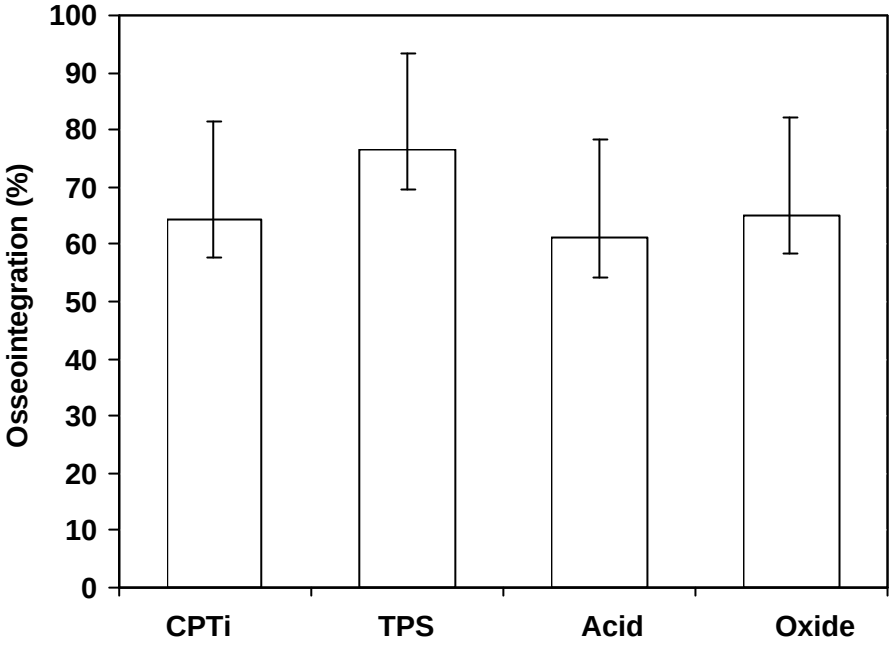
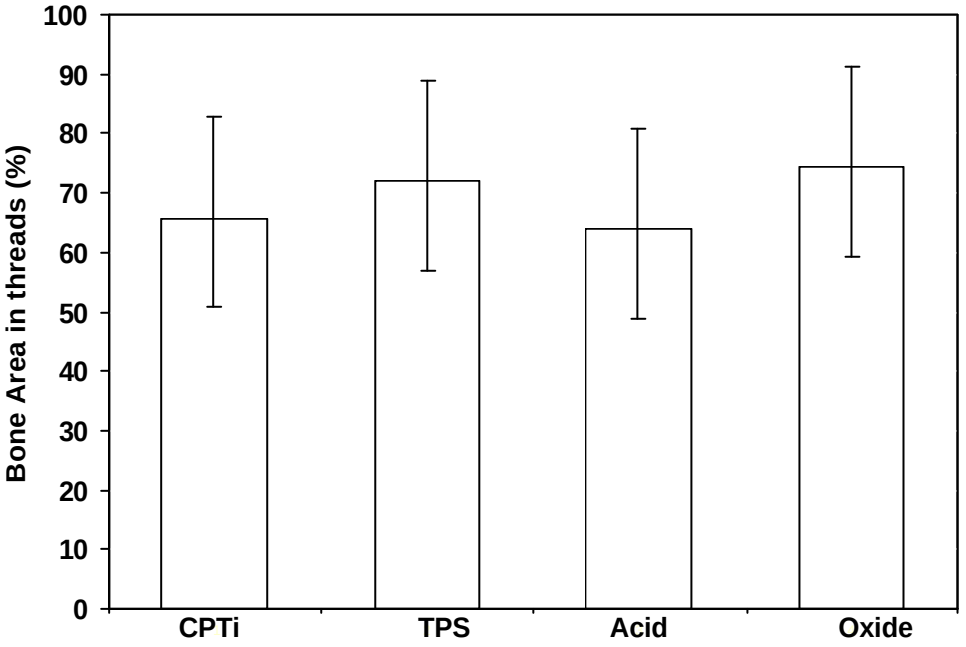

Osseointegration (%)
100
90
80
70
60
50
40
30
20
10
0
CPTi
TPS
Acid
Oxide

Fig. 9


Bone Area in threads (%)
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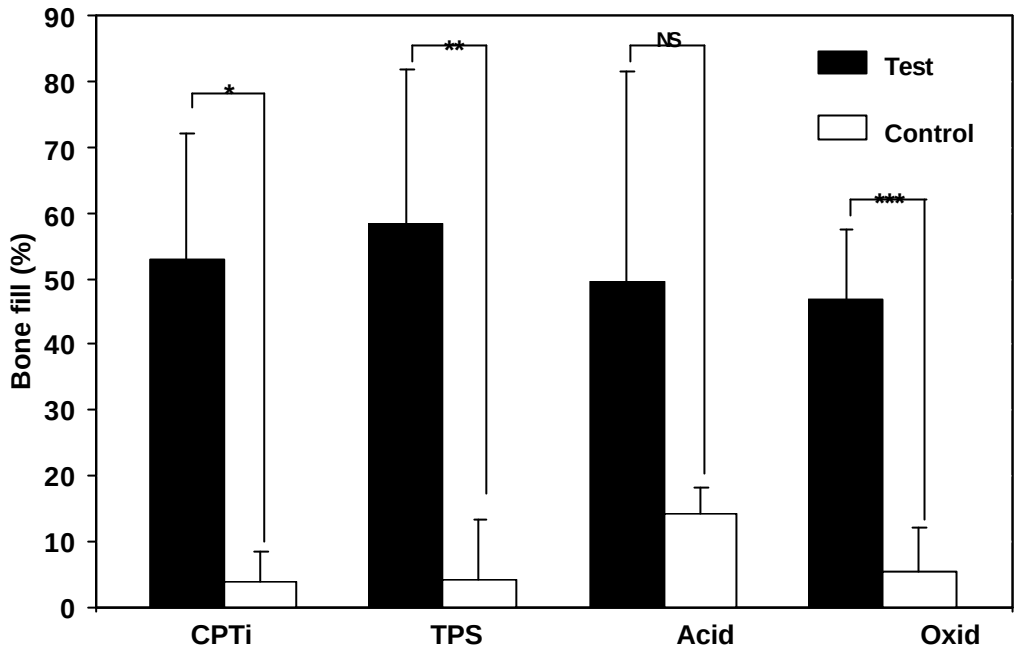
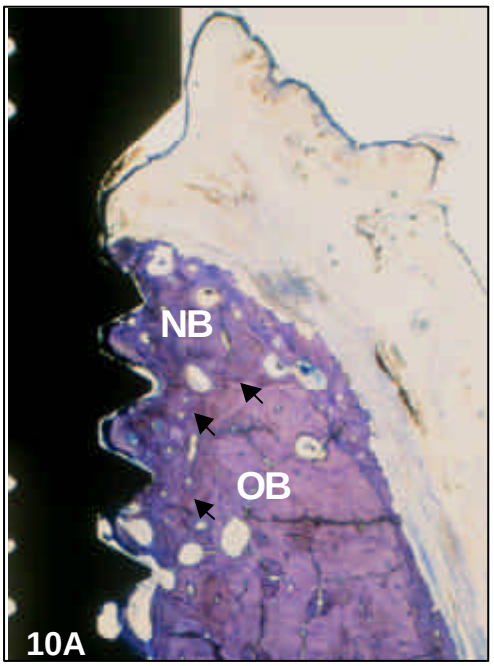
80
70
60
50
40
30
20
10
0
CPTi
TPS
Acid
Oxide

Fig.11



NS=Non significant, * p= 0.0046; **p=0.0132, ***p=0.0039

Figs. 10A and 10B



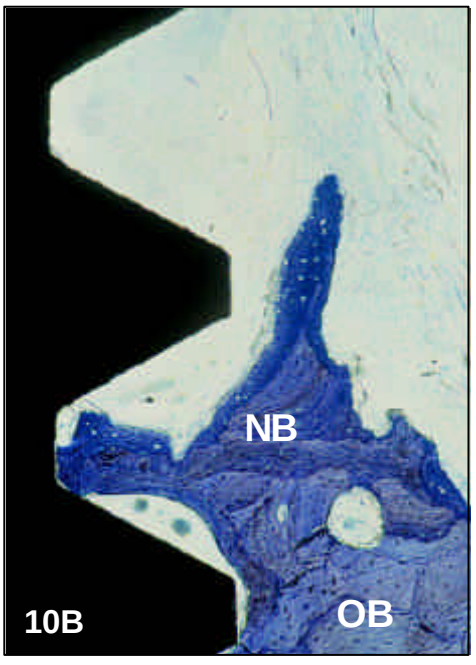
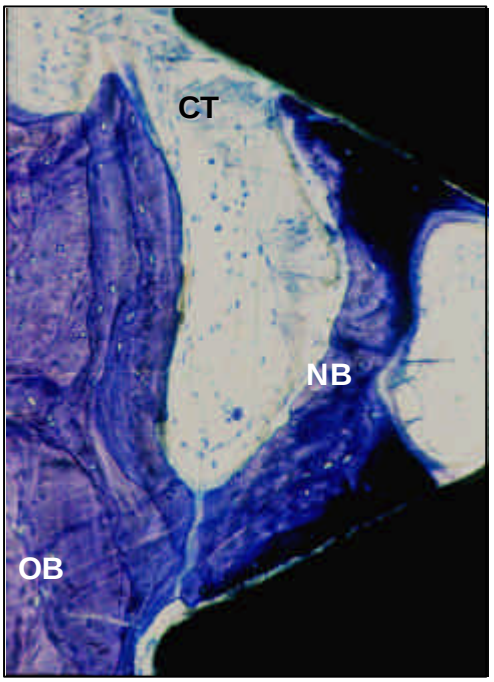
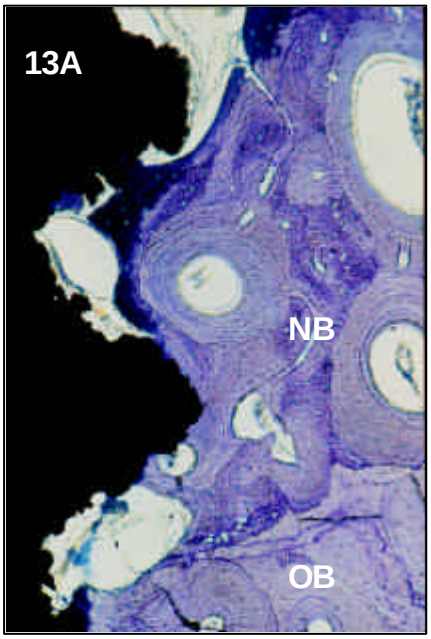


Fig.12





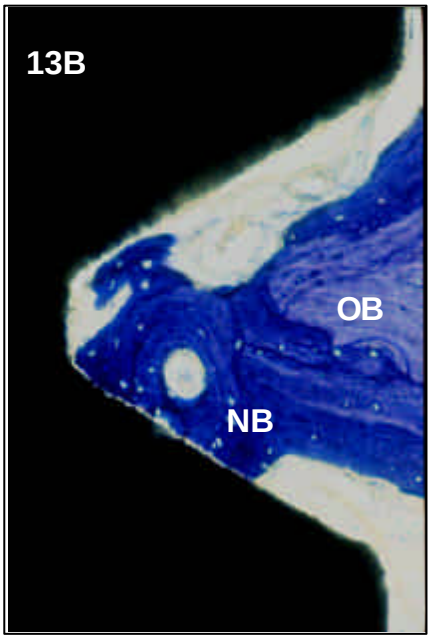


Fig. 15

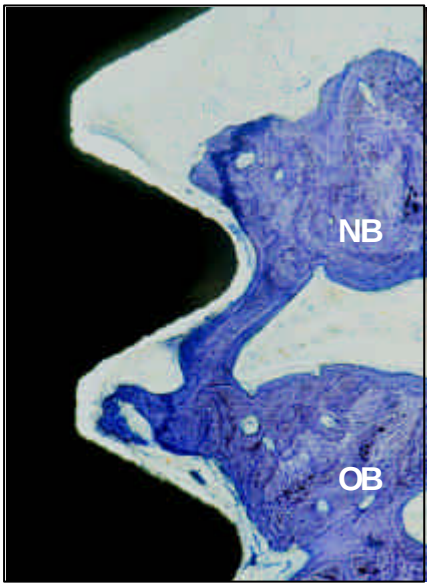
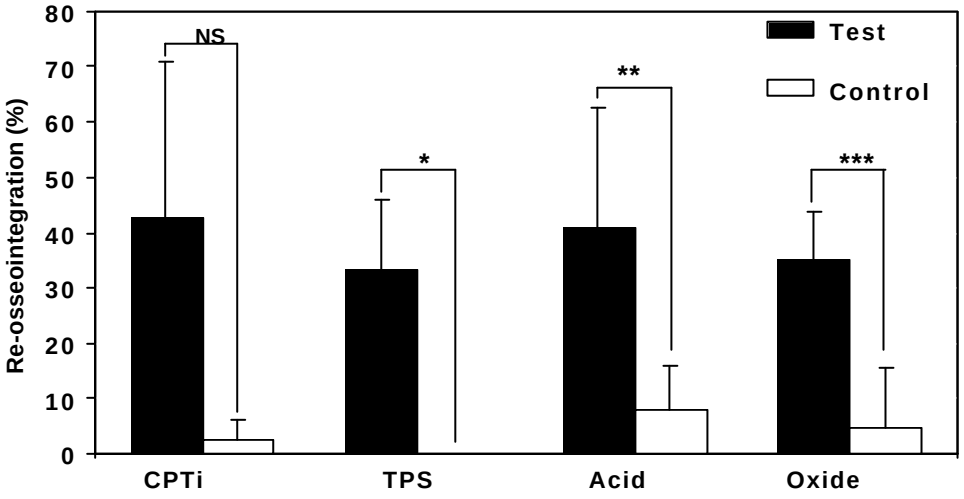


Fig. 14



NS=Non significant, * p= 0.0035; **p=0.0077, ***p=0.0097

CAPITULO-6 HOST RESPONSE AND MICROBIOLOGICAL EVALUATION OF PERI-IMPLANTITIS IN PATIENTS WITH PERIODONTAL DISEASES

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Running Title: Peri-implantitis in periodontal patients

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HOST RESPONSE AND MICROBIOLOGICAL EVALUATION OF PERI-IMPLANTITIS IN PATIENTS WITH PERIODONTAL DISEASES

ABSTRACT

BACKGROUND: The aim of this cross-sectional investigation was to evaluate the clinical, microbiological and immunological characterisitics of peri-implantitis in patients with moderate chronic periodontitis.

METHODS: Sixty-seven dental implants with peri-implantitis in twenty-seven partially edentulous patients (mean age of 51.5 years) were compared to their natural teeth with periodontal diseases. Clinical measurements including % of sites with plaque, gingival redness, bleeding on probing (BOP), calculus, probing pocket depth (PPD), probing attachment level (PAL), width of keratinized mucosa and vertical bone loss (VBL) were taken. Microbial samples, peri-implant sulcular fluid (PISF) and gingival crevicular fluid (GCF) were collected at mesial and distal sites both implants and teeth. The periodontal site with the deepest probing pocket was selected for sampling. The microbiota of samples was evaluated by checkerboard assays using 16S rDNA probes specific for 11 periodontal pathogens. The checkerboard assays were confirmed by polymerase chain reaction (PCR) assays using species-specific primers from conserved regions of 16S rDNA. The immunological parameters were assessed by enzymeimmunoassay (EIA) for prostaglandin E_2 (PGE_2) and enzyme-linked immunosorbent assay (ELISA) for interleukin-1β (IL-1β).

RESULTS: Means of PAL for implants (4.00±0.39mm) and teeth (6.09±0.35mm) were statistically different (p=0.0003). The mean of percentage of presence of

calculus was higher for teeth than implants (p=0.023). Significant correlation was found between BOP and width of keratinized tissue lower than 2mm in peri-implant sites (r=-0.242; p=0.046). All target bacteria were detected in implants and teeth. The frequencies of detection of *Bacteroides forsythus* and *Fusobacterium nucleatum* ss *vicentii* were always higher for teeth (p<0.05). The concetrations of PGE_2 and IL-1β levels were not statistically different between diseased implants and teeth (p>0.05).

CONCLUSIONS: The findings of this investigation demostrated that peri-implantitis have similar clinical, microbiological and immunological periodontal characteristics in patients with moderate chronic periodontitis.

KEY WORDS: Peri-implantitis/microbiology; peri-implantitis/etiology; periodontal diseases/microbiology; prostaglandin E_2; interleukin-1β; peri-implant sulcular fluid/gingival crevicular fluid.

INTRODUCTION

The concept that specific bacteria play an etiologic role in periodontal diseases resulting in clinical attachment loss and alveolar bone loss is well established and accepted.[1] The peri-implant diseases have been also correlated with plaque accumullation and progressive bone loss.[2] At the present moment, little information is available to make a definitive statement regarding the microbiological and immunological aspects present in peri-implantitis.[3]

Several studies have been reported the microbiota similarity found in periodontal diseases and peri-implant diseases.[4-6] Cross-sectional microbiological studies of dental implants with clinically healthy marginal peri-implant tissues in humans[6-8] and animals[9,10] have demonstrated a scattered submucosal microbiota dominated by facultative Gram-positive cocci and rods. In contrast, failing dental implants have been associated with periodontal pathogens such as fusobacteria, spirochetes, *Actinobacillus actinomycetemcomitans*, the black-pigmented species *Porphyromonas gingivalis* and *Prevotella intermedia*, and *Campylobacter rectus*.[11-13]

However, the microbiota present in diseased dental implants in edentulous subjects differs from that found in partially dentate subjects,[14,15] suggesting that the periodontal pocket may serve as a reservoir of periodontal pathogens for further colonization of dental implants.[8,16-18]

In adition, the gingival crevicular fluid (GCF) and peri-implant sulcular fluid (PISF) have frequently been used for the assessment of periodontal and peri-implant diseases respectivelly with aim of identifying potential host markers, which may

permit diagnosis of disease activity and prognosis of early disease.[19, 20] The GCF and PISF serve yet as indicators of infiltration of Gram bacteria or bacteria products in periodontal/peri-implant tissues. This reaction active macrophages that release cytokines, and some individuals respond to microbial challenge with an abnormally high delivery of such inflammatory mediators such as prostaglandin E_2 (PGE_2), interleukin-1α (IL-1α), interleukin-1β (IL-1β), and tumoral necrosis factor-α (TNF-α). These mediators initiate pathways that stimulate osteoclastic bone resorption and clinical attachment loss.[21]

Several authors have been correlated the clinical, microbial and immunological features around healthy and diseased implants. Salcetti et al.[22] evaluted the microbial and host response characteristic of failing implants and concluded that *Prevotella nigrescens* and *Peptostreptococcus micros* were correlated with concentrations of PGE_2. Kao et al.[23] compared IL-1β levels between diseased and healthy dental implants in twelve patients. The levels of IL-1β were higher at failing implants suggesting that this cytokine may be useful for monitoring disease activity around dental implants. These data were in according to Aboyoussef et al.[24] In additon, this authors observed that PGE_2 was not correlated with peri-implant diseases.

These features about microbiological and immunological conditions in periodontitis suggested that a history of periodontal diseases had a greather impact on the peri-implant site. However it is scarce in literature data about peri-implantitis at the same time at patients with active periodontal infection.

The objective of this cross-sectional study was therefore to analyze and compare the clinical, microbiological and immunological parameters between diseased implants and teeth in partially edentulous patients with moderate chronic periodontitis.

MATERIALS AND METHODS

Patient Selection

The population of this cross-sectional study consited of 27 partially edentulous patients, 11 males and 16 females, between 21 to 74 years of age (mean age 51 years) selected from a group of patients treated with dental implants at Dental School of Araraquara. All patients that presented either 1 or more commercially pure titanium dental implants with clinical/radiographic signs of peri-implantitis[25] and diagnosis of chronic periodontitis[26] were included in this study. In brief, peri-implantitis was characterized as peri-implant sites with radiographical defects with a saucer shape, bleending on probing and inflammation of soft tissues. Any mobile dental implant was considered failed dental implant and it was not considered in the study.

The follow criteria were used for patient's exclusion: implant with coated surface; implant supported prostheses with mobile abutments and/or screws, smokers; patient had taken antibiotic or any steroidal/nonsteroidal anti-inflammatory drugs within 6 months prior the clinical examination; patient had undergone any periodontal or peri-implant therapy within 1 year; patient has had history of chronic diseases, such as cardiovascular, hepatic, rheumatic fever, asthma, diabetes, immune disorder and bleeding disorder; women who take pregnant or

nursing. All subjects had given informed consent, which was approved by the Committee on Research Involving Human Subjects at the Dental School of Araraquara, State University of Sao Paulo, Brazil.

Clinical Protocol

Sixty-nine failing dental implants and 27 teeth (1 tooth per patient as control site) were evaluated. The periodontal measurements were taken from teeth with the deepest periodontal pockets in each subject. Measurements were recorded to nearest millimeter by a single calibrated examiner with a force-controlled calibrated periodontal probe[||] with a constant probing force of 0.20N and a probe-tip diameter of 0.4mm. The probing pocket depth (PPD) was determined at teeth and implants by measuring the distance from the gingival margin to the bottom of the pocket to the nearest 0.1mm at 6 sites (disto-buccal, dito-lingual, buccal, mesio-buccal, mesio-lingual, lingual). The PPD measurements in peri-implant sites were done without removing the prosthetic restoration. The probing attachment level (PAL) was measured for implants sites from a fixed point on the prosthetic restoration to the apical penetration of the probe for implants and cemento-enamel junction (CEJ) for teeth. The presence of plaque, gingival redness, bleeding on probing, suppuration and calculus were registered dichotomously. The width of keratinized mucosa was measured using periodontal probing[¶] at buccal and lingual faces.

Intra-oral periapical radiographs were taken with long-cone technique for dental implants and teeth, using a direction indicator in order to have the X-rays

[||] Florida Probe, Computerised Probe Inc, Gainesville, Fl

[¶] PCPUNC 15 Hu-friedy Mfg Co Inc. Chigago IL

perpendicular onto the radiographic film. The radiographs were taken and examined by the same examiner. The distance from the top of the dental implant to the bone-implant contact level as well as the distance from CEJ to bone level was measured mesially and distally. One bone loss value per implant and tooth was calculated as the mean of the mesial and distal value.

PISF and GCF collection

PISF and GCF were colected mesially and distally to each implant and tooth after assessing the presence or absence of plaque, and before registration of any other clinical parameters. The site was isolated with cotton rolls and dried gently with air. After three minutes, two sterile paper points were inserted into the pockets until slight resistance was felt and left in place for 30s. Paper points contamined by bleeding or saliva were discarded. The paper points were then immediately transferred to plastic 1,5mL tube with elution fluid sterile[27] and frozen until analysis. In briefly, the elution fluid consisted of phosphate buffered saline (PBS) with bovine serum albumin (BSA, 1mg/mL) and protease inhibitor cocktail consiting of antipain (1µg/mL), aprotinin (1µg/mL), leupeptin (1µg/mL), N-ethylmaleimide (1mM), and Zwittergent 3-12 (50µg/mL).

PGE_2 and IL-1b Analysis

The tubes were allowed to stand at room temperature for 30 minutes, with vortexing every 5 minutes to facilitate extraction of the sample from the paper points. Aliquots 50µL of the extracted sample were then used in the assay, which was performed immediately after the extraction. The concentrations of PGE_2 in the samples were determined using a commercially available

enzymeimmunoassay (EIA) system[#]. The levels of IL-1β were determined by enzime-linked immunosorbent assay (ELISA) system.[#] These assays detect levels as low as 1pg/mL. According to the manufacturer, the assays do not show any significant cross-reactivity to a wide spectrum of different related cytokines. All assays runs included extraction eficiency and quality control standards. The results were obtained using a microplate reader at 405nm and expressed as means of duplicated samples assayed independently for each mediator.

Microbiological Sampling

The peri-implant sites and periodontal sites were isolated with cotton rolls and the supragingival plaque was removed with sterile cotton pellets. The microbiological samples were taken using 2 sterile paper points. The paper points were inserted to the depth of the mesio/distal aspect and kept in position for 30s. The paper points were placed in tube containing 1.5mL of sterile buffer phosphate saline (BPS) and keeping froozen until to be analysed.

Periodontal Pathogens Detection

Analysis of Amplified 16S rDNA

The polymerase chain reaction (PCR) amplified 16S rDNA using digoxigenin-labed species-specific oligonucleotide probes[28,29] were tested for *A. actinomycetemcomitans* (CACTTAAAGGTCCGCCTACGTGCC), *P. gingivalis* (GCAGTTTCAACGGCAGGGCTGAACG), *P. intermedia* (GGTCCTTATTCGA AGGGTAAATGC), *B. forsythus* (CGTATCTCATTTTATTCCCCTGTA), *C. rectus* (CAAGCTACTTAATCTCCGTTCGAC), *Fusobacterium nucleatum* (GGTTT

[#] Amershan Pharmacia Biotech Inc, Piscataway, NJ

CCCCGAAGGGACATGAAAC), *F. nucleatum* ss *vincetii* (ACTTCACA GCTTTGCGACTCTCTGTTC), *Eikenella corrodens* (ACCGTCAGCAAA AAGTGGTATTAGCAC), *Treponema* spp. (GGCAGTAGGGGTTGCG CTCGTT), *Neisseria* spp. (CCTCTGTACCGACCATTGTATGAC) and for spirochetes (CGACTTTGCATGSTTAARAC). The oligonucleotide probes were 3'-end labeled with digoxigenin-11-ddUTP[**]. The DNA was tranferred overnight onto nylon membranes positively charged using 10x standart salt phosphate EDTA buffer (SSPE). Hybridization with digoxigenin-labeled oligonucleotide probes was performed at 45^{o}C. The hybrydized membranes were washed twice with hight salt solution (2xSCC[0.15M NaCl plus 0.015 trisodium citrate, pH 7.0] in 0.1% sodium dodecyl sulphate) at room temperature and then twice with a low salt solution (0.1xSCC in 0.1% sodium dodecyl sulphate) at 45^{o}C. The membranes were reacted with anti-digoxigenin alkaline phosphatase conjugate. The color reaction was produced with 5-bromo-4chloro-3-indolyl phosphate and nitroblue tetrazolium salts.

Polymerase Chain Reaction of 16S Ribossomal DNA

The PCR-amplification of conserved region of 16S rDNA was tested to confirm the presence of periodontal pathogens including *A. actinomycetemcomitans* (primer AaF ATTGGGGTTTAGCCCTGGTG and primer Rev16s-ACGTCATCC CCACCTTCCTC), *P. gingivalis* (primer PgF - TGTAGATGACTGATGGTGA AAACC and primer Rev16s-ACGTCATCCCCACCTTCCTC) and *B. forsythus* (primer BfF-TACAGGGGAATAAAATGAGATACG and primer Rev 16s-ACGTC

[**] Genius 5, Boehringer Mannheim, Indianapolis,IN.

ATCCCCACCTTCCTC). All these PCR primers were obtained commercially.[††] Between 30 to 100ng of genomic DNA was added to the PCR mixture wich contained 1µmol/L of the primers, 2.5U of *Taq* polymerase[‡‡] in 1x buffer and 0.2mmol/L of dCTP, dGTP, dATP, and dTTP[‡‡] in a total volume of 50µL. The amplification was performed for 30 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 30 seconds at 72°C in thermocycler[##]. Positive and negative controls were included with each set. The negative control includes all the PCR reagents except for the sample DNA. The positive control contained all the PCR reagents together with positive controls for *A. actinomycetemconitans*; *P.gingivalis* and *B. forsythus*. Twenty µL of each PCR reaction mixture was electroforesed in 1.5% (*A. actinomycetemconitans* and *B. forsythus*) and in 2% (for *P.gingivalis*) agarose gel in TBE buffer and the amplification products were visualized under 302nm ultraviolet light, on ethidium bromide-stained gels.

Data analysis

The data were assessed per subject and per site. Fisher's exact test was used to calculted the different detected proportions of target bacteria bertween implants and teeth. The clinical and immunological data were assessed by unpaired *t*-test (2-tailed). To determine the correlations among clinical, microbiological and immunological features a Person Correlation coeficient was determined. Level of significance was set at 0.05.

[††] Gibco BRL, Grand Island, NY.

[‡‡] Perkin-Elmer,Norwalk,CT

[##] GeneAmp PCR System 9600, Perkin-Elmer, Norwalk,CT.

RESULTS

Clinical Features

The clinical parameters (per site) are presented in Table 1. The mean±SD of missing teeth for this population was 10.65±7.23.

In the present study, 11.12% (3) of patients contribuited with six diseased implants each; 11.12% (3) presented four implants each, 18.51% (5) patients contribuited with three diseased implants, 22.22% (6) contribuited with two dental implants each and 37.03% (10) patients presented single implants.

The peri-implant sites had a PPD mean by patient of 3.30±0.31mm and 3.62±0.25mm for periodontal sites. The PPD measurements show sligh difference between implant and teeth, even though statisticall significance did not observerd (p=0.45). On average 26.20%±33.87 of peri-implants sites presented PPD between 0-1mm. Only 10.03%±18.72 of peri-implant sites show PPD higher than 5mm (Fig.2). In contrast, 50.34%±35.04 of periodontal sites presented PPD range 2 to 3mm and just 3.52%±8.87 with PPD over that 5mm. However these features were not statistically significant (p>0.05).

The PAL ranged between 4.00±0.39mm for implants and 6.09±0.35mm for teeth been this difference statistically significant (p=0.0003). Although the presence of plaque had been higher in teeth, 61.24%±7.39 of periodontal sites against 46.66%±7.32 at peri-implant sites, significant difference was not observed (p=0.16).

The amount of VBL was 3.76±0.24mm for failing implants and 4.63±0.46mm for teeth with periodontitis. This difference, however, was not significant (p=0.10).

The gingival redness was present in 40.91%±6.74 and 55.64%±6.36 in peri-implant and periodontal tissues respectivelly being this difference not statistically significant (p=0.11). The percentage of sites with BOP was observed in equal proportion both implants and teeth (p=0.99).

The percentage of calculus in peri-implant sites evaluated in this study was very lower (5.30%±14.88). In contrast, the percentage of periodontal sites with calculus was 5 times higher than peri-implant sites (p=0.021).

Forthy-nine peri-implant sites (73.13%) presented less than 2mm of keratinized mucosa in average of buccal and lingual faces. A negative correlation was found between % of sites with BOP and peri-implant sites >2mm of keratinized tissue (r=-0.242; p=0.046). No correlations were assessed for periodontal sites.

Microbiological Analysis

The Figure 3 presents the means of all target periodontal pathogens. *A. actinomycetemcomitans* were detected in 22.83% and 26.92% in implant and teeth respectivelly.

The *P. gingivalis* and *F. nucleatum* were the most frequently detected in peri-implant pockets. *P. gingivalis* was detected in 65% of samples from implants and teeth. *P. intermedia* was detected in only 25.37% of peri-implant samples. *B. forsyhtus* was detected in statistically higher frequency in teeth (57.69%) than dental implants (31.34%) (p=0.031). *F. nucleatum* ss *vicentii* was also statistically associated to periodontal samples (p=0.048). *F. nucleatum* and *E. corrodens* were detected more often in peri-implant samples than periodontal samples respectivelly, even though any significant difference was assessed (p>0.05). The

remaining bacteria were found in higher proportion in periodontal samples when compared to peri-implant samples, however statistical differences were not present ($p>0.05$). The Table 4 show the number of positive sites for each target periodontal pathogen and the number of patients that had both sites positives for determined bacterium.

Immunological analysis

The amount of PGE_2 in the GCF samples was 441.14±32.22pg/site and 440.77±19.10pg/site in the PISF (Fig. 4). The means levels of IL-1β were 368.79±40.04 pg/µL in PISF and 377.03±48.02 pg/µL in GCF (FIG5). Both immunological parameters were not statistically significant ($p>0.05$) when compared between them.

DISCUSSION

The findings of this study demostrated that peri-implantitis and moderate chronic periodontitis have similar clinical, microbiological and immunological responses. The clinical variables such as presence of plaque, gingival redness, bleeding on probing and probing depth were similar to reported in periodontal diseases. The presence of calculus at periodontal sites was five times higher than peri-implant site ($p=0.023$).

Some studies suggested that the calculus depositions were associated with increased loss attachment over time.[30,31] Although gingival redness and bleeding are recognized as significant clinically visible signs of a host inflammatory response to the presence of bacterial plaque and calculus, the presence of

bacterial plaque and calculus, the presence of gingival inflammation has not been reliable in predicting disease progression.

The PAL measurements observed in this cross-sectional report were statistically different between teeth and implants (p=0.0003). The amount of PAL ranged between 4.0 and 6.0mm for implants and teeth respectively. The higher attachment level observed for teeth may be explained by previous periodontal treatment that these patients were submitted. It is concluded that the attachment level was altered due to large range for gingival retraction, instead that means of PPD were similar for both groups. In a recent investigation, Schou et al.[32] compared the probing depth between teeth and implants in cynomolgus monkeys among healthy, mild and severe mucositis/gingivitis and peri-implantitis/periodontitis. The authors observed that the probing depth was different and the mild inflammation was associated with with deeper probe penetration around implants in comparison to teeth. Although these features may be explained by different marginal connective tissue fibre configuration between teeth and implants,[33] the PPD measurements observed in our study did not show significant difference between peri-implant and periodontal sites.

Several studies[33-35] suggested tha the peri-implant soft tissues were at greather risk for plaque-induced inflammatory changes as compared to the gingiva, but our data do not find any difference in regarding the clinical variables. This data is in agreement with Pontoriero et al.[36] that evaluated marginal tissues reactions around dental implants and teeth. The authors reaffirm the similar association

between bacterial biofilm accumulation and the development of peri-implant mucositis, as established for periodontal tissues (gingivitis) in similar models.

Histologically, the peri-implant mucosa consists of a dense connective tissue covered by a stratified squamous epithelium, which must play as in the normal periodontal tissue, an important role in the defense against periodontal pathogens invasion. Each dental implant is surronded by an outer implant epithelium, which is mostly keratinized, and an inner, non-kerainized epithelium terminating in a junctional epithelium only a few layers thick at the apex. BOP has been reported as a typical sign of peri-implant infection (Mombelli et al. 1987). A negative correlation was found between the widht of keratinized tissue $\geq$2mm and BOP. This correlation suggests that the presence of keratinized mucosa surronding dental implants may have significant effect on the healthy conditions of the peri-implant soft tissue.[37] Although, in periodontal diseases the BOP parameter failed to be a useful predictor of periodontal diseases activity (Baderstein et al. 1985, Lang et al. 1986), the ausence of bleeding on probing was useful clinical indicator of periodontal stability (Lang et al. 1990).

Studies have shown that submerged dental implants had 0.9mm to 1.6mm marginal bone loss from the first thread by the end of the first year in function, while only 0.05mm to 0.13mm bone of loss occurred at second year.[38] The dental implants evaluated in this study presented a range of loading of approximately 4 years and the mean of bone loss was 3.76mm for failing dental implants. So far, the bone loss observed in this study could be a sum of “biological remodelation” associated with peri-implant tissue breakdown induced by bacterial biofilm. In

contrast, the mean of periodontal bone loss was higher (4.63mm), even though not difference between them was observed. The difference in collagen fiber direction and amount of vascular structure present in peri-implant tissues may explain the faster pattern tissue destruction in peri-implant tissues than periodontal tissues.[33,34,39]

The detection frequencies of *P. gingivalis* and *F. nucleatum* were equally higher for implants and teeth ($p>0.05$). *A. actinomycetemconitans* were also detected both in peri-implant sites and periodontal sites. *B. forsythus* was detected in more frequently in teeth than implants ($p=0.031$) in agreement with Listgarten & Lai.[5] In that study the authors compared the microbiota between failing implants and periodontally diseased teeth. However these authors analised microbial samples from different patients. Previous studies have demonstred that the dental microflora can be an important source of bacteria colonizing dental implant surfaces.[8,12,17] However, the presence of putative periodontal pathogens around teeth does not necessarilly lead to periodontal diseases, although the presence of *A. actinomycetemcomitans*, *P. gingivalis*, and *B. forsythus* confers an increases risk for microbiologically induced periodontal breakdown tissue.[1,40,41] This also probable occurs in dental implants and peri-implant diseases confering the same risk for infection to the patients rehabilited with dental implants. *F. nucleatum* ss *vicentii* were also detected in higher levels in periodontal sites ($p=0.048$). In this investigation, the frequency of detection of *F. nucleatum ss. vicentii* in peri-implant sites was 38.8% similar with the 39.1% observed by Salcetti et al.[22]

The detection of *P. gingivalis, P. intermedia and F. nucleatum* confirm previous studies that analyzed dental implants with peri-implant diseases in animals[9,10,42,43] and humans.[6,7,13,22,44] Dental implants with peri-implantitis thus reveal a complex microbiota encompassing conventional periodontal pathogens. These features confirm the bacterial shifts associated to attachment loss detected in this animal study after the induction of experimental peri-implantitis. In our microbiological data, *F. nucleatum* and *E. corrodens* were the only two species that the detection of frequencies were higher in implants than teeth, although this difference was not significant. These bacteria not only were directly envolved with the coaggregation in bacterial biofilm in periodontal disease but also play a possible role for these organisms in the protective effect.[45,46]

Species such as *A. actinomycetemcomitans, C. rectus, F. nucleatum* are often isolated from failing sites, but can also be detected around healthy peri-implant sites.[18] These microorganisms are commonly associated with progressive periodontal diseases and virulence factors that could be important to peri-implantitis progression and treatment.

The host response to the bacterial infection and its products also seems to play a major role in the pathogenesis of periodontitis.[47] IL-1β and PGE_2 are important mediators of inflammation. IL-1β can induce neutrophil degranulation and mesenchymall-cell-derived MMP-8 synthesis, release, and activation.[48] IL-1β can also induce connective tissue destruction and osteoclastic bone resorption, and may work synergistically with the other cytokines, such as tumor necrosis factor, to cause bone destruction.[49] Elevated PGE_2 levels are potent stimulators of bone

resorption[50] and can inhibit the growth of fibroblasts.[51] IL-1β and PGE_2 are present at eleved levels in the GCF of patients with active periodontal diseases.[52] When we compared the levels of these cytokines between implants with peri-implantitis and teeth with chronic periodontitis, no significant difference was assessed, suggested that patients with peri-implantitis presented the same immunological characteristics than active periodontal diseases. Lipopolysaccharides (LPS), cell wall constituents and secretory products derived from periodontal pathogens potently stimulate IL-1α and IL-1β production within the periodontium. In addtion, *P. gingivalis* LPS can active osteoclasts directly and causes the release of PGE_2 and IL-1β from macrophages, monocytes, and fibroblasts.[53] IL-1β does not play only a pivotal role in the inflammatory cascade in reponse both bacteria and LPS but also prostaglandin biosynthesis.

When IL-1β levels were compared in patients with healthy and diseased implants, the cytokine levels were approximately four times higher at diseased sites.[23] Panagakos et al. 1996 show that elevated IL-1β levels were associated with failing implants when compared with healthy implants. The proportion of IL-1β level presented by Kao et al.[23] (385.95±209 pg/μL) was very close with the mean observed by us (368.79±48.02 pg/μL). However, the absence of any correlation with clinical parametrs of inflammation may be explained by the nature of clinical scores and several mechanisms intrinsic to IL-1β induction, as a down regulation of the production of IL-1β by PGE_2.[54]

Abououssef et al.[24] observed no differences PGE_2 levels between early peri-implantitis and healthy dental implants. However the criteria for failing implant used by those authors might explain these features.

The use of host markers as predictor of early disease using immunological parameters many times present different data from literature. First, the CGF and PISF collection and elution of its components varied among the studies, as did measurements assays and kits, difficulting the comparison among the several studies.

Second, The IL-1β and PGE_2 measurements may offer a convenient method of detection of gingival and peri-implant mucosal inflammation, however, the ELISA and EIA methods detect both biologically active and inactive material and cannot distinguish between them. Neverthless, while evidence suggests an important role for IL-1β in the pathophysiology of chronic periodontitis, clinical studies thus far have not defined how these levels can be used as diagnostically.[55]

In summary, this investigations suggested that peri-implantitis has the same biological reaction in patients with periodontal diseases. In addition, the microbiological data suggest that prevalence of the target pathogens around failing implants was similar to that found around teeth with periodontal diseases in the patients oral cavity. This is consistent with intra-oral dissemination of these pathogens but our data do not permit conclud conclusion as to the direction.

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FIGURE LEGENDS

Fig.1- Mean and standard deviation of percentage of frequencies of PPD for implants and teeth. (*n*=27)

Fig. 2- Frequencies and distribution of PPD around implants and teeth**.**

Fig. 3- Mean and standard deviation of percentage of frequencies of PAL for implants and teeth. (*n*=27)

Fig. 4- Mean and standard deviation of percentage of frequencies of presence of plaque for implants and teeth. (*n*=27)

Fig. 5- Mean and standard deviation of percentage of frequencies of VBL for implants and teeth. (*n*=27)

Fig. 6 – Mean and standard deviation of percentage of frequencies of presence of gingival redness for implants and teeth. (*n*=27)

Fig. 7 – Mean and standard deviation of percentage of frequencies of BOP for implants and teeth. (*n*=27)

Fig 8 – Mean and standard deviation of percentage of frequencies of presence of calculus for implants and teeth. (*n*=27)

Fig. 9 – Frequencies and distribution of microbiological findings around implants and teeth**.**

Fig. 10-Mean and SD of PGE_2 in implants and teeth

Fig 11- Mean and SD of IL-1ß in implants and teeth

Table 1:Mean (±SD) clinical parameters of peri-implantitis and periodontitis per site.

CLINICAL PARAMETERS	Peri-implant diseases (*n*=67)	Periodontal diseases (*n*=27)	*p* value
Probing pocket depth (mm)	3.30±1.76	3.62±1.29	0.273*
Attachment level (mm)	3.86±2.11	6.09±1.83	<0.0001†
Bone Loss (mm)	4.00±1.74	4.63±0.46	0.294*
% of sites with			
Plaque	45.72±39.69	61.24±37.68	0.077*
Gingival redness	40.17±36.42	55.64±32.36	0.046†
Bleeding on probing	68.86±29.64	68.63±30.11	0.961*
Calculus	5.30±14.88	22.62±33.90	0.025†

*** Non statistical significance**

† Statistical significance

Table 2: Profile of dental implants evaluated.

Mean±SD of time (months)		Location of implants		Type of prosthetic restoration			
Place:	74±29.46	***Maxilla:***	39	***Screwed:***	29	***Ceramic:***	40
Loading:	49.93±27.80	***Mandibula:***	28	***Cimented:***	38	***Resin***:	27

Table 3. Frequency of detection of periodontal pathogens in peri-implant site and periodontal site.

Periodontal pathogens	Peri-implant site (*n*=67)	Periodontal site (*n*=27)	Patients positives for both sites
A.actinomycetemcomitans	15	7	4 (14.81%)
P. gingivalis	44	17	14 (51.85%)
P. intermedia	17	10	8 (29.62%)
B. forsythus	21	15	10 (37.03%)
C. rectus	33	14	13 (48.14%)
F. nucleatum	43	15	14 (51.85%)
F. nucleatum* ss *vicentii	26	16	12 (44.45%)
E. corrodens	22	6	5 (18.51%)
***Treponema* spp.**	32	13	11 (40.74%)
***Neisseria* spp.**	24	12	8 (29.62%)
spirochetes	16	9	3 (11.12%)

Fig.1

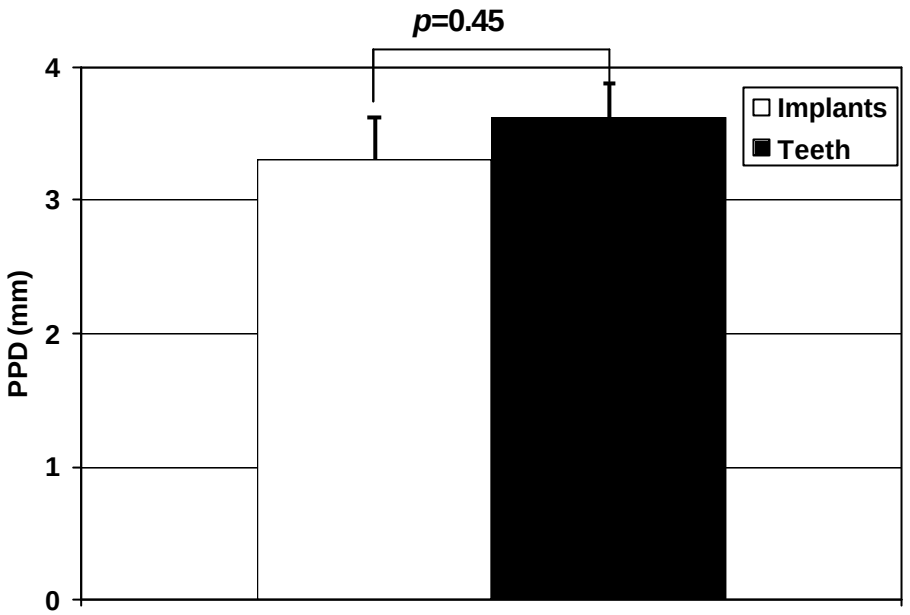

p=0.45
Implants
Teeth
PPD (mm)
0
1
2
3
4

Fig.2

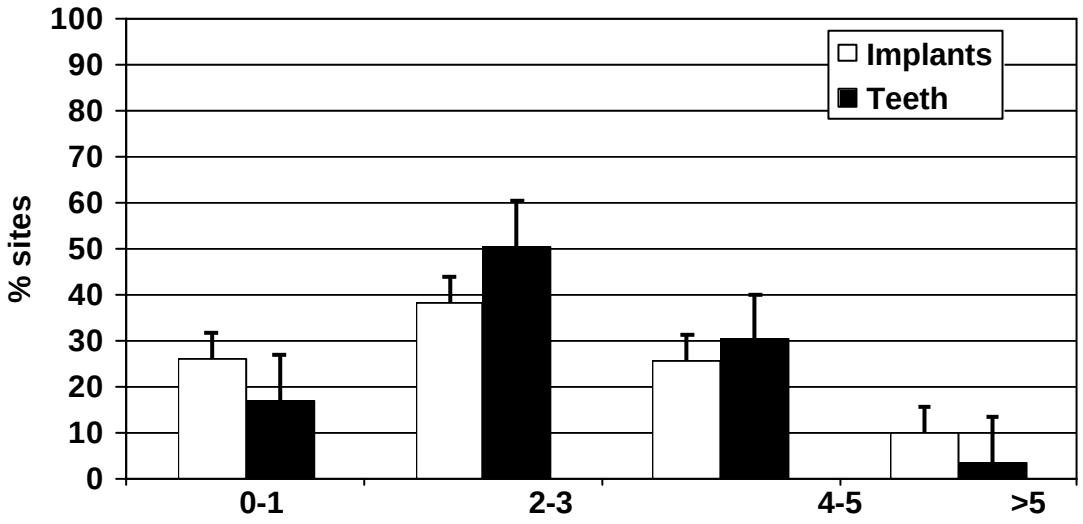

Implants
Teeth
% sites
0
10
20
30
40
50
60
70
80
90
100
0-1
2-3
4-5
>5

Fig.3

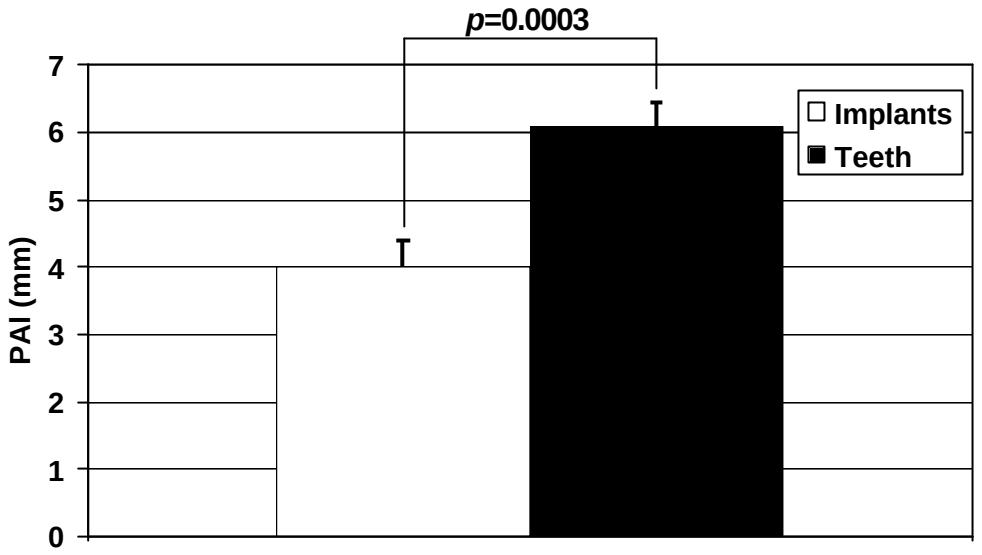

p=0.0003
PAI (mm)
0
1
2
3
4
5
6
7
Implants
Teeth

Fig. 4

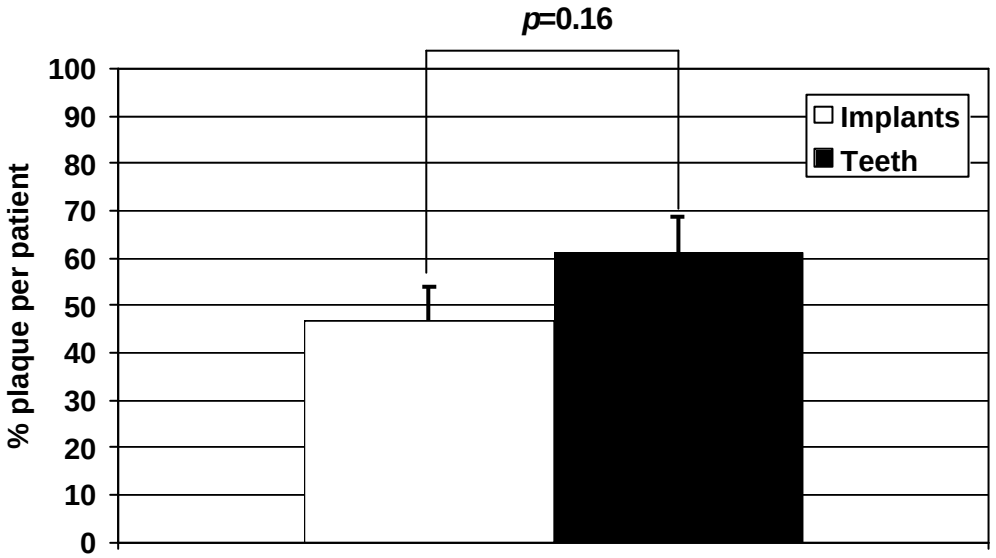

p=0.16
% plaque per patient
0
10
20
30
40
50
60
70
80
90
100
Implants
Teeth

Fig. 5

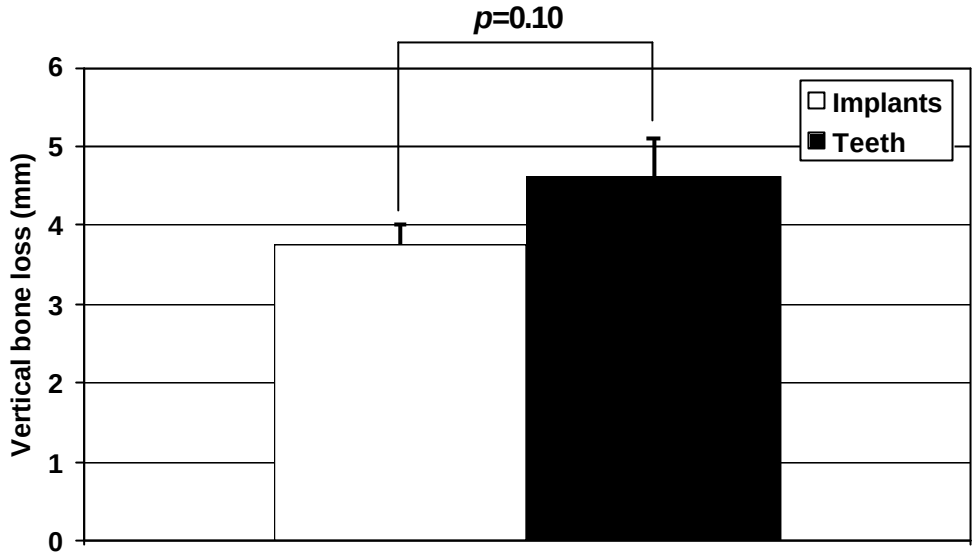

p=0.10
Vertical bone loss (mm)
0
1
2
3
4
5
6
Implants
Teeth

Fig. 6

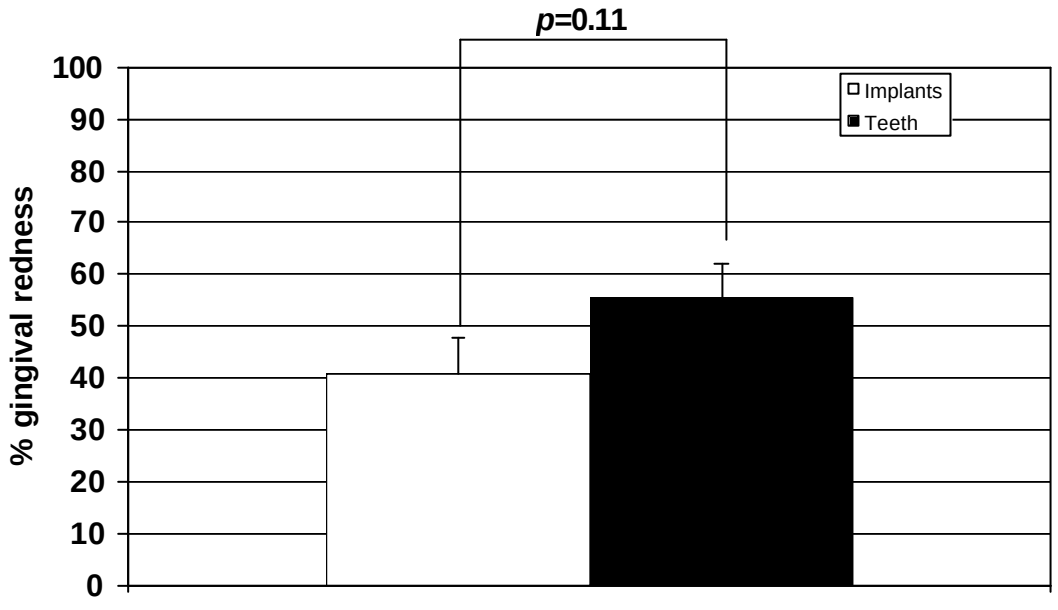

p=0.11
% gingival redness
0
10
20
30
40
50
60
70
80
90
100
Implants
Teeth

Fig. 7

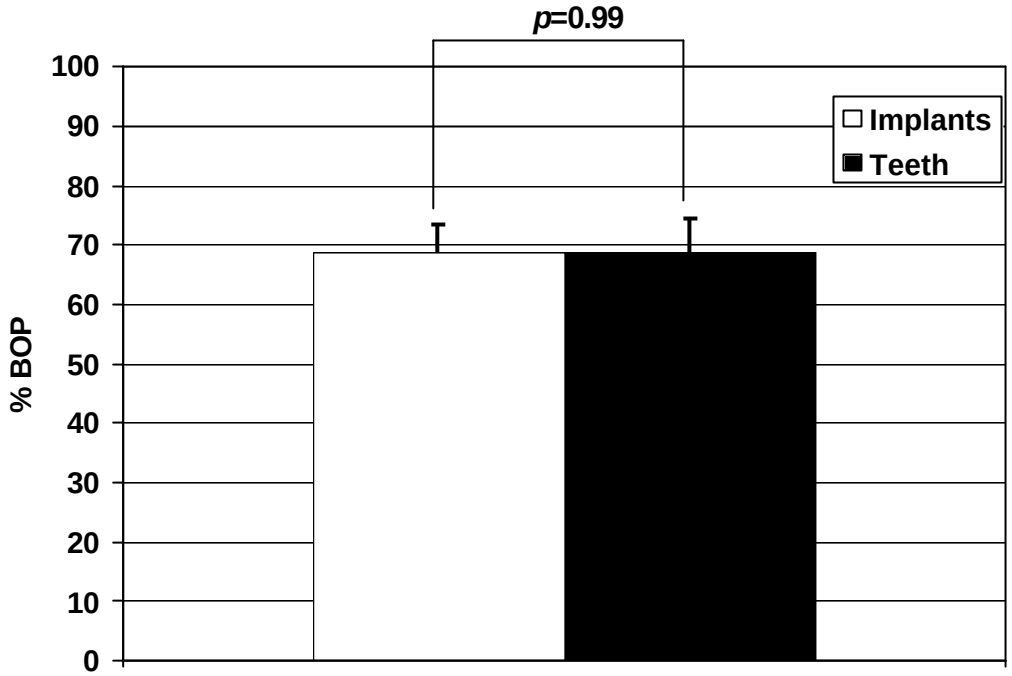

p=0.99
100
90
80
70
60
50
40
30
20
10
0
% BOP
Implants
Teeth

Fig. 8

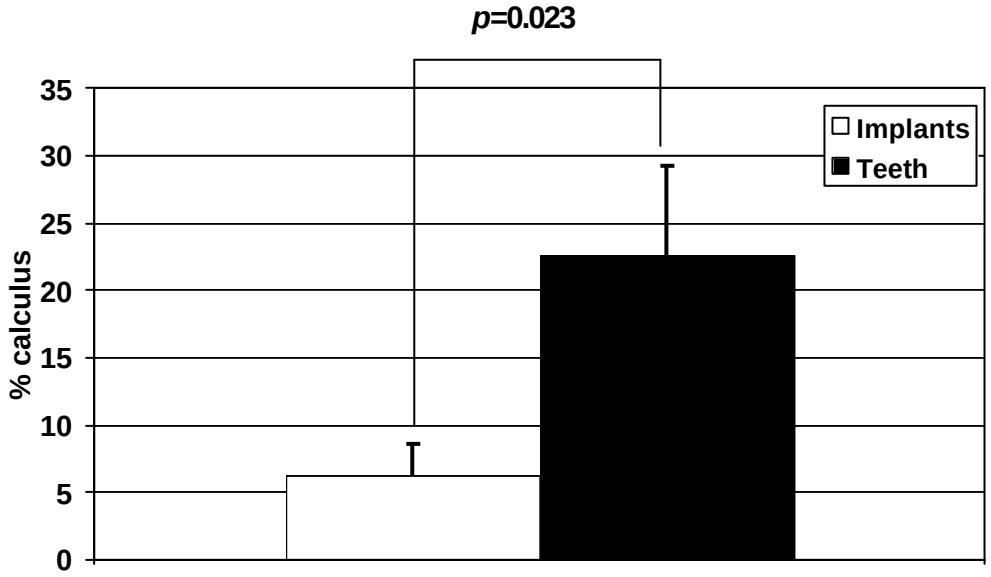
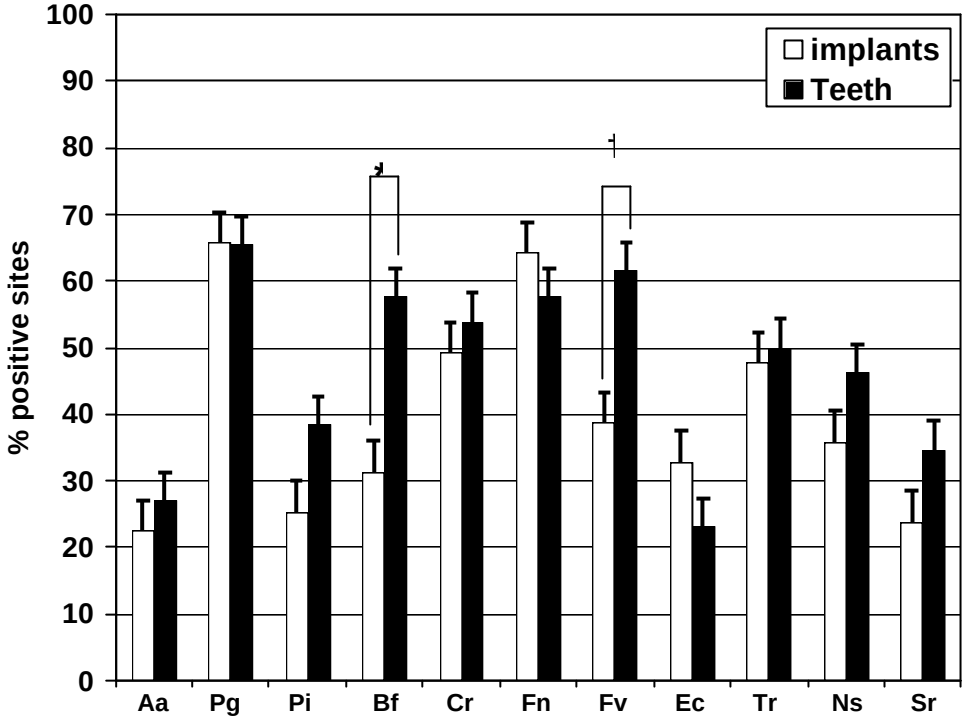

p=0.023
35
30
25
20
15
10
5
0
% calculus
Implants
Teeth

Fig. 9



*** *p*=0.031; † *p*=0.048**

Fig. 10

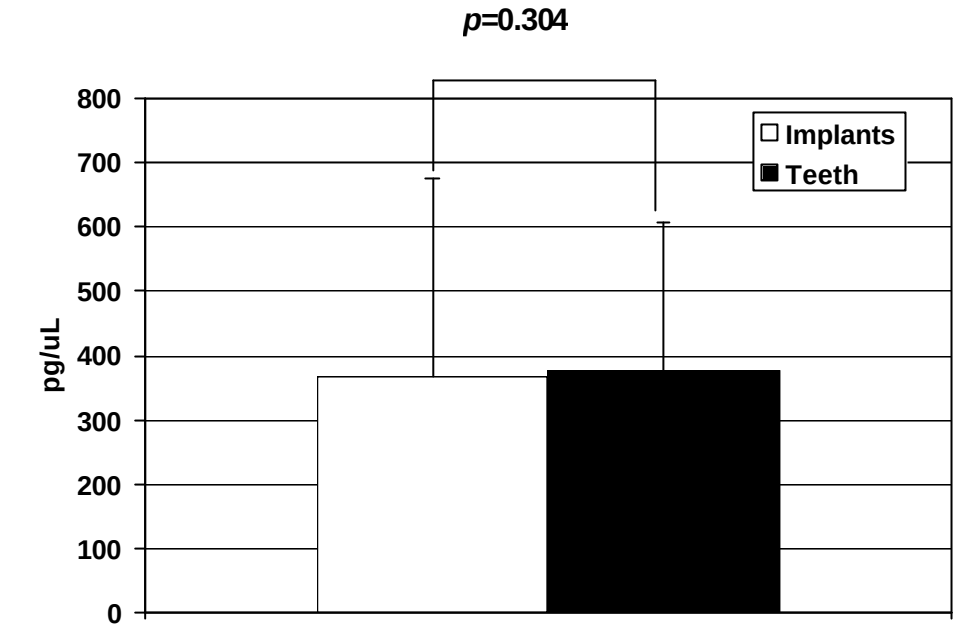
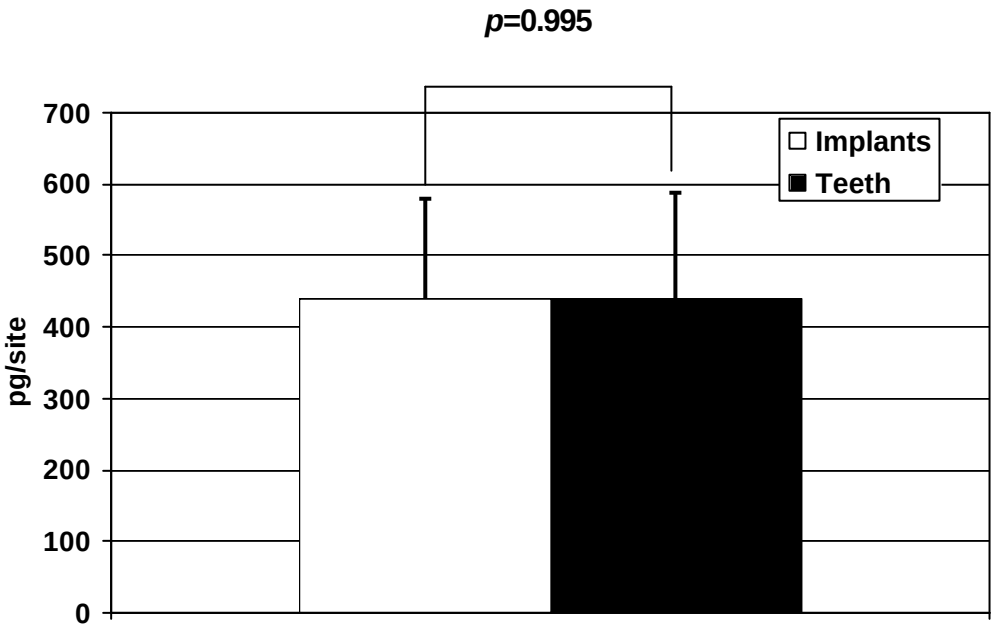

p=0.304
800
700
600
500
400
300
200
100
0
pg/uL
Implants
Teeth

Fig. 11


p=0.995
700
600
500
400
300
200
100
0
pg/site
Implants
Teeth

CAPITULO - 7 EFFECT OF AIR-POWDER SYSTEM IN TITANIUM SURFACE ON FIBROBLAST ADHESION AND MORPHOLOGY

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***ABSTRACT*:** The purpose of this study was to evaluate the number and morphology of fibroblasts grown on machined titanium healing abutments treated with an air-powder system. Twenty-six machined titanium abutments were assigned to 2 experimental groups: Control (no treatment) and treated – exposed to the Prophy-Jet for 30 seconds. The samples were incubated for 24h in multiwell plates containing fibroblastic cells, followed by routine laboratory processing for SEM analysis. The samples were photographed at 350X and the cell number was counted on an area of approximately 200 um^2. No significant differences were found on morphology between the groups ($p>0.05$), however the control group presented a significantly greater amount of cells (71.44±31.93, mean±s.d.) in comparison to treated group (35.31±28.14), as indicated by a non-paired t test ($p=0,001$). The use of air-abrasive prophylaxis system on the surface of titanium healing abutments reduced the cells proliferation, but did not influence cell morphology.

KEY WORDS: Dental implants, cell culture, titanium, fibroblasts, maintenance, scanning electron microscopy, peri-implantitis.

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INTRODUCTION

A direct contact between the dental implant surface and surrounding bone is preferred for the long-term success of dental implants. Nevertheless, in spite of a satisfactory osseointegration, this clinical success of dental implants can be guaranteed only if the integrity of peri-implant mucosa is maintained by an attachment by hemidesmosomal connection of soft-tissue to the transmucosal implant surfaces.[1,2]

On the other hand, it must be emphasized that clinical studies of the interface between gingival tissues and dental implants in humans are hampered by many difficulties, including ethical considerations. Many uncontrollable factors in the oral environment, as well as technical problems on sample preparations, impair experimental studies on soft tissue behavior. In vitro experiments appear to circumvent most of these difficulties; and thus can provide useful information on this subject.[3]

Common clinical procedures such as professional maintenance performed with stainless steel and plastic curettes, and/or abrasive pumice or air-powder abrasive system could to lead to alterations on the surface of titanium abutment, impairing, for instances adhesion of fibroblasts to this surface. Comparative experiments on the attachment and growth of human gingival fibroblasts and epithelial cells on titanium with different surface textures were carried out.[4,5] These studies showed that epithelial cells present more extensive migration on rough surfaces. However, gingival fibroblasts showed a more marked and oriented development on porous surfaces which was also observed by other authors.[6,7]

Even though, rough surfaces could enhance fibroblast responses, they can also be considered rather disadvantageous due to the possibility of promoting growth and organization of bacterial biofilms; thus facilitating peri-implant tissue infections such as mucositis and peri-implantitis.[8]

The purpose of this in vitro study was to evaluate the effect of using an air-powder abrasive system on titanium abutments on adhesion and morphology of fibroblasts.

MATERIALS AND METHODS

Cell lineage

A continuous cell lineage of fibroblastic morphology (McCoy) from the Adolfo Lutz Institute, Sao Paulo, Brazil, was used. These cells were cultured in 25 cm^2 flasks with Minimum Essential Media (MEM) supplemented with 7.5% of fetal bovine serum and 40 µg/mL of gentamicin. The cells were maintained in an incubator at 37^0C and 98% humidity atmosphere.

Treatment of samples

Twenty-six new commercially pure titanium healing abutment surface (Sterngold, Implamed, Attleboro, MA, USA, 4mm x 8mm) were used in this study. These abutments were removed from the original packing, cleaned on an ultrasonic device for 10 minutes and then sterilized by steam heat (autoclave). Care was taken not to contact the abutment cylinder surface with any foreign object other than the test instruments and materials.

Two titanium abutments were designed as negative control (no treatment with air abrasive system and no cells) and two positive controls (air-powder system treatment and no cells). The remaining 22 specimens were assigned to two experimental groups: Control Group – no air-powder treatment, and Test Group – air-powder system(Prophy-Ceramic II, Dabi Atlante, Ribeirão Preto, SP,Brazil) for 30s on a 45 degrees incidence. The air-powder system was performed with sodium bicarbonate. Immediately after treatment, the samples were coded and individually placed in 24 well plates. To each well, 2 mL of supplemented cell culture media and 1 mL of a cell suspension containing

$2x10^5$ cells/mL were added. These plates were incubated for 24 hours at 37°C and 98% humidity.

Preparation of samples for SEM

After the incubation period the culture medium was removed by aspiration from the wells and the titanium healing abutments were immersed on 2.5% glutaraldehyde for 15 minutes to fix the cells. Following fixation, the samples were dehydrated in increasing concentrations of ethanol (10, 30, 50, 70, 90 and 100%) and placed in a vacuum dissecator where they remained during 5 days. The healing abutments were then mounted on metallic stubs and coated with 20 nm of gold in order to be observed and photographed in the SEM at 500 and 1000x, for density and morphology evaluations, respectively. The assistant microscopy technician, who was unaware of the coding that identified the experimental groups randomly, determined the photographic fields.

Cell morphology assessment

The photomicrographs were submitted to 3 independent and previously calibrated examiners (examiner 1 x 2: kappa: 1.00; examiner 1x 3: kappa 0.84; examiner 2 x 3: kappa 0.84) who evaluated cell morphology according to an index system proposed by Gamal et al.[9] modified by us[10]. Briefly, score 0: no cells present; score 1: only flattened cells, score 2: only rounded cells and score 3: presence of both rounded and flattened cells.

Cell counting was performed on all photomicrographs using a black paper mask in which a window of 3 cm^2 (corresponding to a 'real' area of approximately 200 um^2) was cut. This mask was superimposed on the photomicrographs and triplicate counts of the number of

the cells adherent to the titanium abutments were made for each group. A single examiner, blind to experimental groups coding, performed these counts.

Data analysis

Experimental groups were considered independent and data related to the number of cells, although discrete in nature, were considered to present an approximately normal distribution. Comparison between groups was performed using a non-paired t-test. Since cell morphology was assessed by an index system, the non-parametric Mann-Whitney U-test was used for between group comparisons. Significance level was always set to 95%.

RESULTS

The distribution of morphology scores (see Fig. 1) according to experimental groups Mann-Whitney test did not indicate significant differences between groups ($p>0.05$), suggesting that cell morphology was not affected by treating the healing abutments with the air abrasive system.

The non-paired t-test indicated that the number of fibroblasts was significantly different ($p=0.001$) between groups. Control group (Figs. 2 and 3) presented a significantly greater amount of cells (71.44 ± 31.93) in comparison to test group (Figs. 4 and 5) (35.31 ± 28.14) (Fig.6).

DISCUSSION

Acquisition and maintenance of an effective attachment around the cervical portion of a dental implant is essential to establish a favorable prognosis. The peri-implant seal provides a biological barrier between the oral environment and peri-implant bone tissue. The disruption of this seal by inflammatory peri-implant disease can permit increased

accessibility of biofilm-derived substances into the connective tissues. Several studies have tested various measures for cleaning smooth implant surfaces.[8,11,12,13] Surface cleaning with an air-powder abrasive system has been suggested.[14, 15, 16]

The results of this in vitro study showed that proliferation and migration fibroblasts are possible on titanium surface in accordance with Gould et al.[17] (1981), whose in vitro results indicated a hemidesmosomal connection between epithelial cells and titanium surfaces. It was also shown that even the orientation fibroblasts could be influenced by titanium surface characteristics.[4, 19]

Using an air-powder abrasive system with sodium bicarbonate for 30s on a 45 degrees incidence device on commercially pure titanium did not alter the morphology of fibroblasts. In agreement with the literature,[20,21] morphology of these cells was predominantly elongated or flattened (bipolar or multipolar) which was considered a sign of adhesion to the substrate. This indicates that surface roughness and the presence of particles of bicarbonate were not able to alter this phenotypic expression of fibroblasts. However, what consequences this type of surface instrumentation can have on the attachment of peri-implant soft tissues in the long-term remains unclear.

On the other hand, on titanium surfaces treated with an air-powder abrasive system, a significant decrease in the number of fibroblasts was observed, in comparison to non-treated control titanium surfaces. Another study has documented similar results after surface treatment with stainless steel curettes.[22] There are some in vitro data suggesting that smooth surfaces are superior in promoting fibroblast proliferation as well as on the number of cells attaching to the surface.[23] These results can be attributed to the release of toxic ions from the titanium alloy[24] or to the presence of powder particles on instrumented surfaces, which can disturb cellular adhesion.

Results obtained in these studies show that the nature and surface geometry of the implant surfaces may influence gingival fibroblasts attachment in vivo in agreement with our data. However, one has to bear in mind the limitation of the methods used in this study when considering these results. In this study, a continuous lineage of fibroblastic cells was used. The advantages of this type of culture are the rapid proliferation of cells (reducing the probability of contamination), infinite life-span of cells allowing for many repetitions of experiments, apart from the fact these cells are also easier to grow and maintain. Considering the purpose of this study, which was to perform an initial evaluation of adhesion and proliferation of fibroblasts on titanium surfaces after treatment with an air-powder system, cells from continuous lineages are considered adequate.[25]

The appropriate care was taken to minimize possible sources of variation on the assay. This care included preparation of cell suspensions at the exponential growth phase as well as obtaining these cells (always) from the same 75cm^2 cell culture flask to avoid possible differences on cell behavior due to variations in culture conditions. In this sense, the same supplemented culture medium batch was used throughout the study.

CONCLUSION

Adhesion of fibroblasts to titanium surfaces previously submitted to air-powder treatment was shown. This procedure can be useful in both peri-implant mucositis and maintenance treatment. Furthermore, this pre-treatment of titanium surfaces with the air abrasive system did not alter cell morphology, even though the number of cells attached to these treated surfaces was significantly reduced.

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LEGENDS

Fig. 1 Frequency distribution of the percentage of scores for cell morphology according to the experimental groups.

Fig. 2 Scanning electron microphotograph of negative control titanium abutment (Original Magnification X 1000)

Fig. 3 Scanning electron microphotograph of control group. (Original Magnification X 1000)

Fig. 4 Scanning electron microphotograph of control titanium abutment after air-powder treatment (Original Magnification X 500)

Fig. 5 Scanning electron microphotograph of control group abutment presenting fibroblasts on its surface (Original magnification X 1000).

Fig. 6 Mean and standard deviation of number of cells according to experimental groups.

ILLUSTRATIONS

Fig. 1

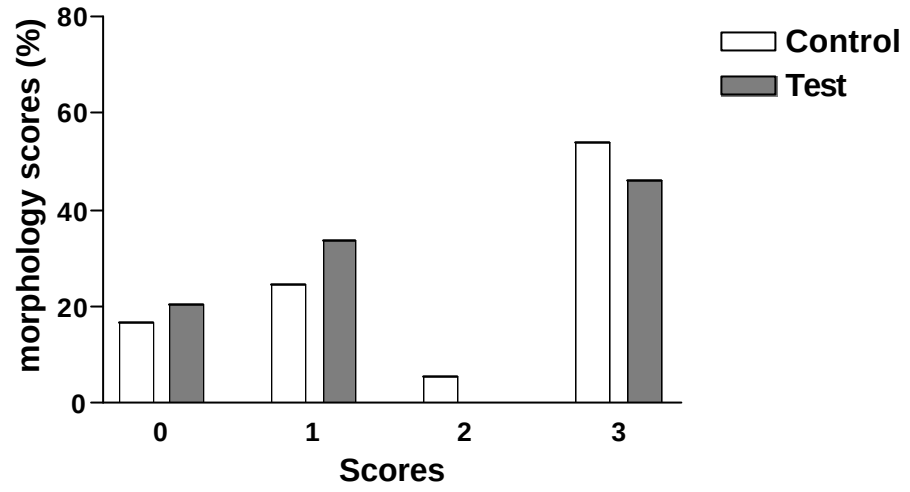
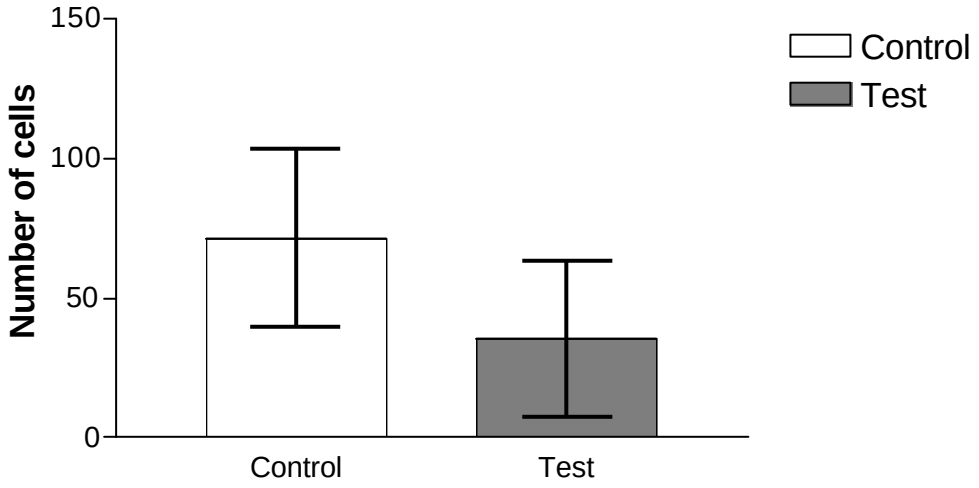
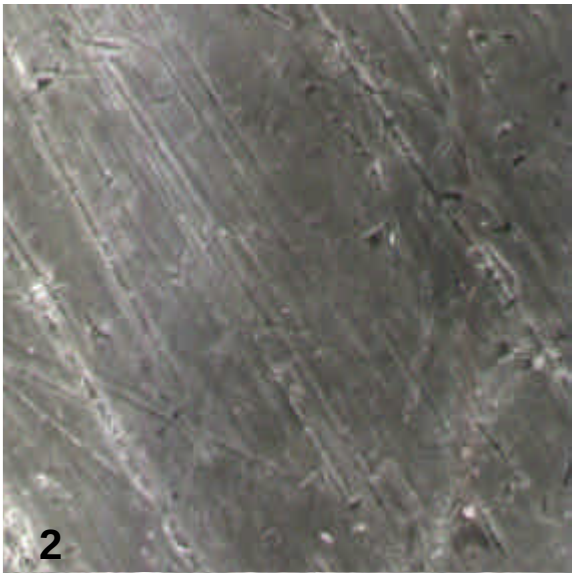


Fig. 6

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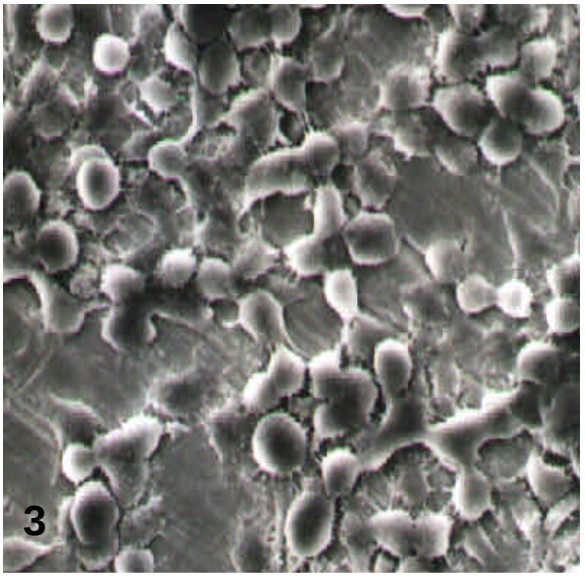
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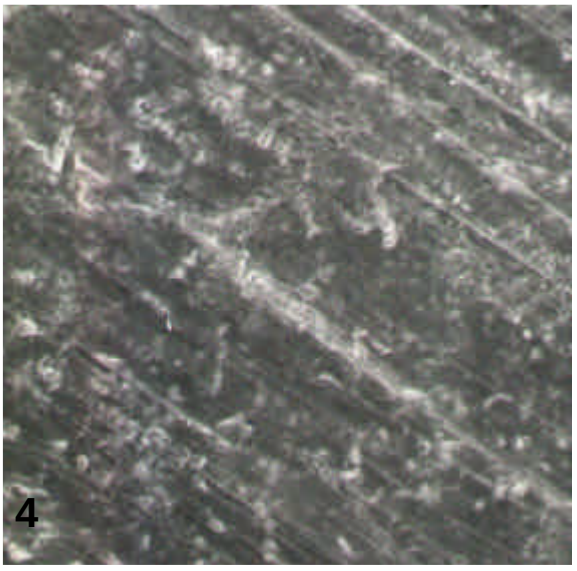
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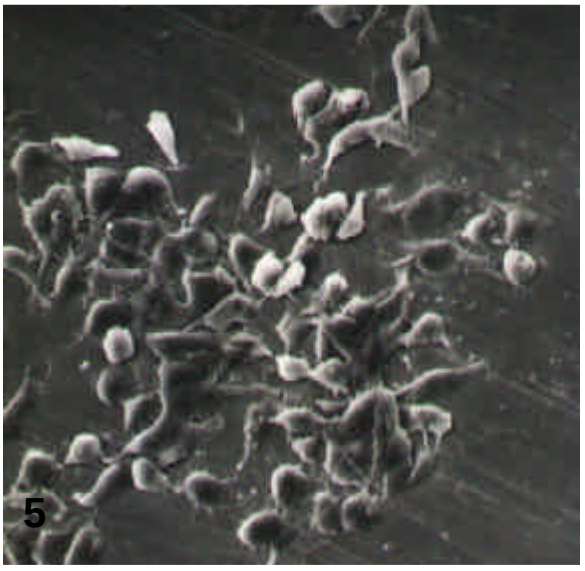
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CAPITULO - 7 ANALYSIS OF FAILED DENTAL IMPLANT SURFACES.

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Running title: Surface properties of failed dental implants

ANALYSIS OF FAILED DENTAL IMPLANT SURFACES.

ABSTRACT

PURPOSE: Several longitudinal studies have reported high survival and success rates for dental implants. Nevertheless, dental implant failures due to peri-implant infection have also been reported. The aim of this investigation was to analyze the surface topography and composition of failed titanium dental implants in order to determine possible causes of failure.

MATERIALS AND METHODS: Twenty-three failed commercially pure titanium dental implants were retrieved from 18 patients (mean age of 50.33±11.81 years). Sixteen dental implants were retrieved before loading (early failures), 6 after loading (late failures), and 1 because of mandibular canal damage. The failure criterion was lack of osseointegration characterized as dental implant mobility. The late failures were clinical and radiographically characterized as peri-implantitis, i.e., bleeding upon probing, suppuration, and alveolar bone loss. Three unused dental implants were used as control group. All dental implant surfaces were examined by scanning electron microscopy (SEM) and energy-dispersive spectrometer x-ray (EDX) to element analysis. Evaluations were performed at different locations of each implant.

RESULTS: SEM showed that the surface of all retrieved dental implants consisted of different degrees of organic residues, appearing mainly as dark stains. The surface topography presented as grooves and ridges along the machined surface similar to control group. Overall, foreign elements such as carbon, oxygen, sodium, calcium, silicon and aluminum were detected in failed implants. The dental implants from control group presented no macroscopic contamination and clear signs of titanium.

CONCLUSIONS: These results do not suggest any material-related cause for dental implant failures, although different element composition was assessed between failed implants and control implants (unused implants).

KEY WORDS: Dental implants/titanium, failure/peri-implantitis, surface analysis, scanning electron microscopy, energy dispersive spectroscopy.

INTRODUCTION

The use of dental implants in oral rehabilitation has gained importance in daily clinical practice. Nevertheless, dental implant failures have also been reported.[1,2] These failures can be classified on the basis of both chronologic (early or late) and etiologic aspects. Early implants failures have been attributed to surgical trauma, inadequate bone quality and quantity, lack of primary stability and bacterial contamination of the recipient site.[3]

Late implant failures are commonly associated with the occurrence of peri-implantitis. Peri-implantitis is described such as been a destructive inflammatory process affecting the soft and hard tissues around osseointegrated implants, leading to the formation of a peri-implant pocket and loss of supporting bone.[4,5]

The integration of titanium dental implants in alveolar bone has been partly ascribed to the biocompatibility of the surface oxide layer.[6] Several authors described the titanium oxide layer as a thin of 2-6nm of TiO_2 covered by a carbon-dominated contamination layer and trace of nitrogenous (N), calcium (Ca), phosphorous (P), chlorine (Cl), sulfur (S), sodium (Na) and silicon (Si).[7-10] However, the cellular alterations to ions in salt solutions *in vitro* depend on the concentration of ions exposed to the cells.[10]

Recent evaluations have been questioned the importance of implant surface cleanliness after peri-implantitis treatment.[11-13] It has been hypothesized that surface contaminants may be released from contaminated implant surface, enhancing and perpetuating the inflammatory response, thus altering the healing process and possibly provoking the dissolution of titanium.[7,14-16] In addition, the alterations of this oxide layer surface may difficult the re-osseointegration.

Therefore, the aim of this study was to evaluate using scanning electron microscopy (SEM) and energy dispersive x-ray spectrometer (EDX) the surface properties of consecutively retrieved dental implants in order to determine the possible role of surface contamination for the failure of titanium dental implants.

MATERIAL AND METHODS

Patients and implants

Twenty-three dental implants were retrieved from 18 patients (9 female; mean age of 50.33±11.81 years) who did show any medical or dental contraindication for implant placement. All dental implants (except implant #3) were inserted at Department of Periodontology, Dental School at Araraquara, Brazil, to support fixed prostheses in partially and totally edentulous patients. The dental implants evaluated in this study were from four different manufactures: Conexão Implants, São Paulo, SP, Brazil; Branemark System, Nobel Biocare AB, Götemborg, Sweden; 3*i* Implant Innovations, Palm Beach, Fl,USA; Serson Implants, São Paulo,SP, Brazil (Table 1).

Eleven dental implants have been placed in maxilla posterior (bone type 4-poor density)[17] and five in resorbed maxillas (type E). The procedures of bone grafting were performed when available bone height and volume were insufficient. Fifteen dental implants were retrieved from grafted areas (7 maxillary sinus lift and 8 onlay grafts). The mean of loading of the failed implants because overload/peri-implantitis was 44.83±18.53 months. Overall, the mean of implantation time was 18.43±22.48 months.

Immediately after the dental implant insertion, no complications or infection was noted for any patient (dental implant #3).

Dental implant retrieval and processing

The criterion for dental implant removal was lack and/or loss of osseointegration. Exception for the dental implant 3 that was retrieved by means a trephine bur because of mandibular canal damage (Table 1). Some aspects of this specimen were osseointegrated and the reverse screwed was not enough to remove the implant.

The lack of osseointegration was recorded as the slightest mobility tested by rotating and moving every dental implant back and forward using instruments or Periotest®(Siemens, Bensheim, Germany). All the retrieved dental implants were mobile and in such cases were surrounded by a radiolucent line on radiographs using a standardized method with the parallel, long cone technique. The failed dental implants were retrieved after an insertion time ranging from 2 months up to 5 years. The sample was composed for different lengths and diameters (Table 1).

The dental implants were retrieved under local anesthesia by gently unscrewing them with stainless steel forceps, which were carefully positioned on the healing abutment or on the abutment/prosthetic restoration in order to avoid any possible contamination of the dental implant surface. The implants were rinsed with saline solution and immersed in 4% formaline[18] and storage in sterile plastic or glass vials (Table 2).

The failed dental implants were inspected for macroscopic soft tissue remnants, which were removed using titanium tweezers (3*i* Implant Innovations,Palm Beach,Fl).

Scanning Electron Microscopy and Energy Dispersive Spectroscopy

After dehydration in a graded series of alcohol, the failed implants were dried and mounted on metallic stubs using double side tape. In addition, three unused dental implants were used as control: two CPTi implant surface from different manufactures (Conexão Implantes, São Paulo, SP, Brazil; Branemark System, Nobel Biocare AB, Götemborg, Sweden) and one sandblasted surface (Porous, Conexão Implantes, São Paulo, SP, Brazil) (Table 3). These control implants were mounted with no pretreatment. All samples were introduced into the vacuum chamber of a scanning electron microscopy (SEM) JEOL JSM-T330A (JEOL Ltd, Tokyo, Japan). Thereafter, the implant surfaces observed by means SEM were submitted to an element analysis. The regions of interest[7] (Fig. 1) and the element detection were done simultaneously by verification of electron beam-induced x-ray radiation at two different regions. An energy-dispersive spectrometer x-ray (EDX – Noran Instruments, Inc., USA) was coupled to the JEOL JSM-T330A SEM.

RESULTS

Different degrees of organic residues (Table 2) were detected on most of retrieved samples (Figs.2 and 3). These residues appeared mainly as dark area (Fig. 4). The dental implant surface aspect was dominated by grooves and ridges along the machining direction appeared essentially unchanged on the retrieved samples, as compared to the controls (Figs. 5 and 6).

The EDX analysis showed that all failed dental implant surfaces consisted of Ti oxide, with varying amounts of contaminants. The dental implants used as control depicted signs of Ti (Table 3), while for the failed implants weak Ti signs were detected at the outmost surface. In most of cases, carbon was the dominant element detected. O, N, Na, Ca, and P were also detected. Si was detected in the organic overlayers of some

samples (Table 2). The exception was the specimen 10, which displayed unusual bismuth (Bi).

Discussion

The present study evaluated the surface composition and presence of contaminants in failed dental implants surfaces. Although the dental implants evaluated in the present study were from different manufactures and origin, the surface composition appear to be the same in all retrieved implants. However, from the materials surface science point of view, the CPTi dental implants brands probably differ significantly from each other, because the companies that produce them apply their proprietary preparation procedures and/or sterilization. One may suspect differences in some aspects: origin and purity of titanium metal, oxide film formation and oxide film crystallographic structure, surface roughness, and oxide film thickness.[10]

EDX surface analysis showed the incorporation of some contaminants into both in failed and control dental implant surfaces. There are many possible critical events that may happen and possibly trigger dental implant incompatibility. The inorganic contaminants such as C, Ca, Na, and P, present in failed implants, were probably relevant to the absorption mechanism of solvated ions that naturally occur in the body fluids. In addition, the control of the surface characteristics of CPTi dental implants is regarded as one important factor in order to achieve an optimal tissue response during healing of bone and soft tissues.[19] The degree of contamination of the titanium surface can determine the mechanical stability and osseoinduction/osseointegration qualities.[20]

Kasemo and Lausmaa[21] noted that there is usually a large C signal, a smaller N signal, and traces of Cl, S, and Ca present. They attribute the C-, N-, S-, and Cl-containing molecules to adsorption during preparation procedures. They also reported

that the Ca usually persisted throughout the oxide layer and may have been the result of surface segregation of minute Ca quantities in the commercially pure Ti stock. The ASTM F-67 standard specifies that nitrogen content should be less than 0.05 wt% in unalloyed material. However, only the Ca and Na were found in dental implant used as control in this study. The Bi detection in both samples from patient 10 may be result of the use by patient of gastric protector due to duodenal diseases reported in anamneses.

In part of the implant, the intensity of the oxygen signal should not be considered as representative of true composition. Indeed, Ti is highly reactive metal and even in an ultrahigh vacuum environment because of SEM. The detection of these is not related to impurities in CPTi. Olefjord & Hansson[16] suggest that inorganic contaminants should be avoided because these species can possibly provoke dissolution of the titanium. Si and P probably traces of the finishing step in the Ti preparation. The presence of Ca and Na probably come from body fluids. In contrast, Si being one of the major constituents of glass, the observed Si contamination was mainly attributed to ion dissolution from the glass vials in agreement with Esposito et al.[7] In addition, sources of the contamination by silicon and carbon could also be the residues left by rubber gloves.[22]

Some contamination may have originated from several sources, such as fabrication process, result of cleaning and sterilization procedures such as Na detection, from the environment during handling and storage (glass vials), at implant insertion, or during retrieval and analysis preparation procedures.[7,8,10,15,22]

The influence of contaminants on the dissolution rate of Ti in body fluids has not been evaluated. It is suggested that, in principle, organic compounds lower the dissolution rate of titanium because they block the sites for the oxygen cathodic reaction. Foreign

ions on the titanium oxide surface may catalyze the oxygen reaction and thereby promote the dissolution of titanium. For example, ions of Fe, Cr, and Mo are added to reducing acids as sulphuric to enhance the electrochemical reactions so that passivation can occur. It has been reported[23] that the corrosion products formed on the surface of Ti during its exposure to $NaNO_3$ and NaCl containing aqueous solution catalyze the reduction process of NO^{3-} and thereby increase the dissolution rate of Ti. Zinc in both solid and liquid form may cause embitterment of Ti.[24] It is not known whether the Ca^{2+} and Si^{4+} found on the surface in this study have the effect of promoting dissolution of Ti and thus "poison" the tissue or cause stress-corrosion cracking of the implant.

The contact time of dental implant with air prior to EDX analysis could be critical for the titanium levels which seemed to be the result of absorption of carbon.[16] The bioactivity of a biomaterial is associated with the surface energy of the material, is an important criterion for the determination of bioacceptability and is influenced by purity of the material. Contamination of the biomaterial surface with hydrocarbons and other molecules and elements can reduce the surface energy and thereby also the potential bioacceptability of dental implant surface. [26]

Some studies [7,10,27,28] evaluated the failed implant surface using X-ray photoelectron spectroscopy (XPS) and Auger electron spectroscopy (AES). These equipments represent a much higher surface sensitivity than EDX used in our study, which is usually available on scanning electron microscopes (SEM). AES is capable of determining the chemical composition of the uppermost 5-30 Å of the surface, corresponding to 2-10 atomic layers. In EDX equipment, the element composition evaluation was derived from analysis of the characteristic x-ray emission caused by excitation of atoms in the dental implants by the impinging electrons. The latter have a

relatively high energy (>10 keV) and penetrate relatively deep into the sample (~ 1 µm), and the escape depth of the emitted x-rays is also large. The compositional information is therefore averaged over a depth of 1 µm. In XPS and AES equipment, the important information is carried out from the implants by electrons whose energies are typically in the range of 0.1 to 1 keV, and whose mean of escape depth is approximately 1 nm. These characteristics and differences among the equipments may explain the different elements detected in our sample data.

There is evidence that the oxide continues to grow in vivo. Implants retrieved after 6 and 8 years of function have demonstrated oxide layers of 200 nm.[29,30] These oxide layers all contained P, Ca, and S in addition to Ti, O, and C. The authors also concluded that TiO as a passivating insulator was essentially "useless" in the long term. Contrary to the expected behavior of decreased growth and eventual cessation, the oxide layer continues as an expanding biologically active entity in living bone. Titanium compounds have been identified in the tissues approximating implants.[30,31] Above-normal compound levels have also been documented in rabbit spleen and lung tissue following implant insertion.[32] It is therefore prudent to focus careful attention on any oxide contaminants since these could be leached out over time.

In conclusion, data from the present study suggests that the EDX analysis do not show any material-related to cause either early or late dental implant failure. However, these results should be considered with caution and further investigations must be conducted.

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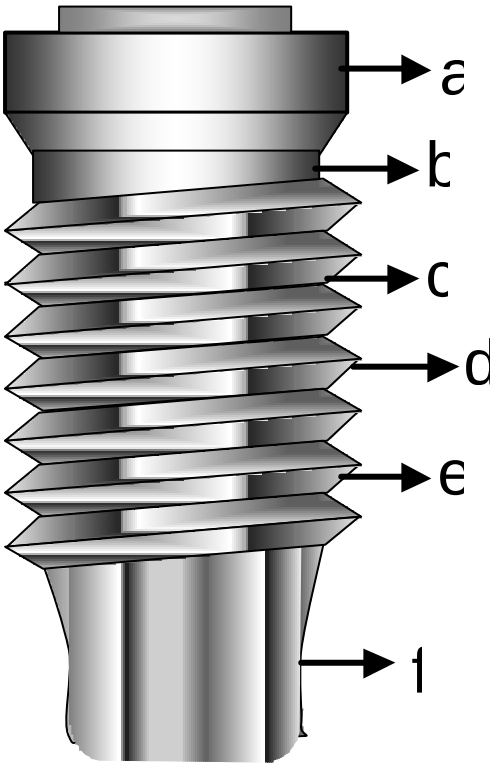
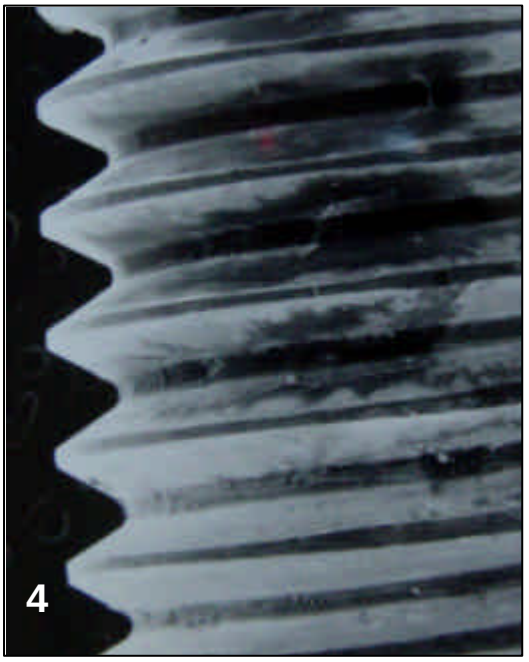
Fig.1



Figs. 2 and 3

Fig. 4



Figs. 5 and 6

LEGENDS

Fig.1. Schematic drawing illustrating the different analysis locations on the dental implant. a: cylinder; b: neck; c: bottom of thread; d: tip of thread; e: flank; f: cone.

Fig.2. Scanning electron microphotograph of retrieved dental implant showing organic debris (Original Magnification X75)

Fig.3. Scanning electron microphotograph of retrieved dental implant showing organic debris on cone area(Original Magnification X35)

Fig. 4. Scanning electron microphotograph showing residues as dark areas (Original Magnification X35)

Fig. 5. Scanning electron microphotograph of dental implant used as control (Branemark Dental Implant) (Original Magnification X35)

Fig. 6. Scanning electron microphotograph showing grooves and ridge along the machined direction on retrieved implant (Original Magnification X35)

Table 1. Clinical data of patients and their respective failed implants

Implant Type	Patient/ Implant Sample[1]	Age (years), gender	Implant position and Primary Stability[2]	Dental Implant Length and Diameter	Implantation time (months)	Loading time (months)	Patient's notes[3]	Possible reason for Failure and Symptoms
***3*i Implant**	1A	56f	Maxilla anterior, good	13x4.0mm	60	53	Smoker, overdenture	Overload/pain at percussion
	1B		Maxilla anterior, optimal	10x3.75mm	60	53	------	Overload/pain at percussion
	2	46m	Maxilla posterior good	10x3.75mm	36	27	Sinus lifting,cemented	Bacterial Infection/suppuration
Branemark	3	69f	Mandible posterior, good	13x3.75mm	24	---	No loading	Damage in inferior alveolar nerve
Serson	4	62m	Maxilla posterior, good	7x3.75mm	60	54	Cemented	Overload/pain at percussion
	5	69m	Maxilla posterior, optimal	10x3.75mm	72	65	Sinus lifting, Screwed	Bacterial infection/suppuration

Conexão	6	41m	Mandible posterior, optimal	13x3.75mm	5	---	------	Impaired healing
	7	51m	Maxilla posterior, good	10x3.75mm	24	17	Smoker, cemented	Overload/pain at percussion
	8	37f	Maxilla posterior, bad	10x3.75mm	6	----	Sinus lifting	Impaired healing
	9	45m	Mandible posterior, optimal	10x4mm	5	---	Onlay bone graft	Impaired healing
	10	33m	Maxilla posterior , bad	10x3.75	4	---	Osteotome technique	Impaired healing
	11	39f	Maxilla anterior, good	15x4mm	6	---	Onlay bone graft	Impaired healing
	12	40f	Maxilla anterior, optimal	8x5mm	6	---	Onlay bone graft	Impaired healing
	13A	58f	Maxilla anterior, good	15x3.75mm	6	---	Onlay bone graft	Impaired healing

	13B		Maxilla posterior, good	10x3.75mm	6	---	Sinus lifting	Impaired healing
	13C		Maxilla posterior, bad	13x3.75mm	6	---	Sinus lifting	Impaired healing
	14A	49m	Mandible posterior,good	13x3.75mm	6	---	Onlay bone graft	Impaired healing
	14B		Mandible posterior,good	10x3.75mm	6	---	Onlay bone graft	Impaired healing
	15A	62m	Mandible posterior, good	11.5x4mm	6	---	Onlay bone graft	Impaired healing
	15B		Mandible posterior, bad	8x5mm	6	---	Onlay bone graft	Impaired healing
	16	67f	Maxilla posterior, good	13x4mm	2	---	Sinus lifting	Exfoliation
Conexão Porous	17	43f	Maxilla posterior, good	13x3.75mm	7	---	Sinus lifting	Impaired healing
	18	39f	Maxilla posterior, bad	10x3.75mm	5	---	------	Impaired healing

[1] **Implant sample**: When the patient present more than 1 failed implant, letter was used, [2]**Stability:** Verified at moment of implant insertion, [3] **Patient's notes:** Procedures of bone graft, cemented or screwed restoration

Table 2. Surface composition and contaminants of failed implants in different areas.

Implant Type	Implant Sample	Packaging Material	Implant Area evaluated	Elements detected	SEM Observations
***3*i Implant**	1A	Plastic	cylinder flank	C,Na,O,N,P,Ti C,Na,O,N,P,Ti	Organic film
	1B	Plastic	flank botton of treated	C,Na,O,N,P,Ti C, N,O,P,Ti	Dark area
	2	Glass	cone neck	C,Na,O,Al,Ti C,Na,O,Al,Ti,Si	No macroscopic contamination
Branemark	3	Plastic	neck Tip of thread	C,Ca,O,Ti C,Ca,P,O,Ti	Bone debris
Serson	4	Plastic	Botton of thread cone	C,Ca,Al,Ti C,Ca,Al,Ti	Soft tissue
	5	Glass	neck flank	C,Ca,Al,P,Si C,Na,Ca,Al,P,Si	Soft tissue
	6	Plastic	Tip of thread Cylinder	C,Ca,O,P,Ti C,Ca,O,P,Ti	Dark area
	7	Glass	cylinder botton of thread	C,Na,Al,O,N,P,Ti Si C,Na,Al,O,N,Ti,Si	Soft tissue and dark area
	8	Plastic	Cylinder cone	C, Na,O, P, Ti C, O, P, Ti	No macroscopic contamination
	9	Plastic	Tip of thread cone	C,Na,Ca, P,Ti C,Na,Ca,O,P,Ti	No macroscopic contamination
	10	Plastic	neck flank	C,Na,Ca,P,Ti,Bi C,Na,Ca,O,Ti,Bi	Organic film

Conexão	11	Plastic	Neck cone	C,Na,Ca,P,Ti C,Na,Ca,P,Ti	Organic film
	12	Plastic	neck flank	C,O,Na,Al,Ti C, Na,O,Al,Ti	Soft tissue
	13A	Plastic	Neck Botton of thread	C,Na,P,Al,Ti C,O,Na,Al,Ti	Soft tissue
	13B	Plastic	flank Cone	C, Na,Ca,P,Ti C, Ca,P,Ti	Soft tissue
	13C	Plastic	Cylinder cone	C,Na,Al,Ti C,Na,Al,Ti	Soft tissue
	14A	Plastic	Tip of thread Flank	C,Na,P,Ti C,Na,Al,Ti	No macroscopic contamination
	14B	Plastic	Botton of thread Tip of thread	C,Na,P,Ti C,Na,Ti	No macroscopic contamination
	15A	Plastic	cylinder Botton of thread	C,O,Al,Ti C,O,Al,Ti	Soft tissue
	15B	Plastic	Flank Tip of thread	C,O,Al,Ti C,O,Al,Ti	Soft tissue and dark area
	16	Plastic	Flank cone	C,Ca,P,Ti C,Ca,P,Ti	Soft tissue and some debris
Conexão PorousÒ	17	Plastic	Botton of thread cone	C,Na,P,Al,Ti C,Na,P,Al,Ti	No macroscopic contamination
	18	Plastic	Tip of thread flank	C,O,Al,Ti C,Na,O,Al,Ti	Soft tissue

Table 3. Surface composition of dental implants used as control.

Implant Type	Implant Area evaluated	Elements detected	SEM Observations
Branemark	cylinder flank	O,Ti,Ca Ti,O	No macroscopic contamination
Conexão	botton of treated flank	Ti,Al,Na Ti,O	No macroscopic contamination
PorousÒ	cone neck	Ti,O,Al Ti,O,Al,Ca	No macroscopic contamination

8. DISCUSSÃO GERAL

A utilização de implantes osseointegrados na reabilitação de pacientes parcialmente ou totalmente desdentados tem sido empregada com freqüência na clínica odontológica. Entretanto, em vista da natureza crônica da doença periodontal/peri-implantar, nosso conhecimento em relação ao índice de sucesso e manutenção a longo prazo das restaurações implanto-suportadas ainda é limitado. Neste estudo, alguns aspectos biológicos como a presença do biofilme bacteriano, resposta imunológica do hospedeiro, presença de contaminantes na superfície de implantes falidos, assim como os tratamentos das doenças peri-implantares foram abordados.

ETIOLOGIA DAS PERI-IMPLANTITES

Nos *artigos I* e *II* a microbiota encontrada antes da indução da peri-implantite foi composta basicamente por gram positivos facultativos. Entretanto, após a indução da peri-implantite, observou-se uma predominância de periodontopatógenos tais como *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum* e *Campylobacter rectus* semelhante aos achados de Schou et al.[69] 1996; Nociti et al.[53] 2001, Hanisch et al.[27] 1997, Tillmanns et al.[77] 1998; Eke et al.[14] 1998. *Actinobacillus actinomycetemcomitans* e *Bacteroides forsythus* também foram detectados, no entanto só o foram no *artigo II*, provavelmente devido ao uso de técnicas de biologia molecular. A presenças destes patógenos é ratificada pelos achados de Hanisch et al.[27] 1997, Eke et al.[14] 1998, Schou et al.[69]1996, Tillmanns et al.[77] 1998. Cumpre salientar que

Nociti et al.[53] 2001 também utilizaram biología molecular, no caso, reação de polimerase em cadeia (PCR), entretanto não detectaram a presença de *A. actinomycetemcomitans* na peri-implantite em cães.

O shift bacteriano ocorrido após a indução da peri-implantite foi acompanhado de perda óssea alveolar e perda clínica de inserção, sem, no entanto, haver diferença estatística entre as várias superfícies de implantes osseointegrados. Estes achados suportam a idéia de que as superfícies utilizadas neste estudo (titânio comercialmente puro, plasma spray de titânio e hidroxiapatita) foram igualmente susceptíveis a peri-implantite induzida por ligadura. A similaridade na resposta da osseointegração frente à doença peri-implantar ao redor dos implantes osseointegrados de diferentes superfícies pode ser explicado de duas maneiras: (1) o período de avaliação relativamente curto (60 dias) para a completa maturação do biofilme bacteriano presente nos implantes, e (2) o tamanho da amostra utilizada.

As perdas clínica de inserção e óssea peri-implantar podem ter sido ocasionadas tanto pelo trauma mecânico da colocação dos fios de algodão, como pela destruição direta dos tecidos peri-implantares por meio da liberação de enzimas, subprodutos e fatores de reabsorção óssea, evasão das defesas do hospedeiro, indução de uma reação inflamatória mediada pelo sistema imune do hospedeiro ou ainda uma combinação destes fatores (*artigos III e VII*).

No *artigo VII* avaliou-se a microbiota presente na peri-implantite em pacientes portadores de doença periodontal crônica moderada, assim como a resposta do hospedeiro junto aos sítios peri-implantar e periodontal. A perda

clínica de inserção foi estatisticamente maior nos sítios periodontais, provavelmente devido à recessão gengival decorrente de tratamentos periodontais prévios a que os pacientes foram submetidos, já que não foi detectada diferença entre as médias de profundidade de sondagem entre os sítios avaliados. Entretanto, a perda óssea radiográfica foi similar para ambos os sítios.

Outro fator importante a ser considerado é a diferente configuração e orientação do tecido peri-implantar frente ao tecido periodontal. Vários estudos (Berglundh et al.[6] 1992; Lindhe et al.[40] 1992; Toljanic et al.[78] 2001; Schou et al.[70] 2002) têm sugerido que os tecidos peri-implantares são mais susceptíveis ao acúmulo de biofilme bacteriano. Nesta tese (*Artigo VII*), a presença de uma faixa de mucosa ceratinizada $\geq$2mm correlacionou-se negativamente à presença de sangramento à sondagem, sugerindo que a presença de uma faixa de mucosa ceratinizada ao redor dos implantes osseointegrados poderia ter um efeito significativo na manutenção da saúde peri-implantar.

Avaliando-se a microbiota presente ao redor de sítios com periodontite e peri-implantite, *B. forsythus* e *F. nucleatum* ss *vicentii* foram detectados com maior freqüência nos sítios peri-implantares ($p \leq 0.048$). *A. actinomycetemconitans*, *P. gingivalis* e *F. nucleatum* foram detectados em ambos os sítios, sem, no entanto, haver diferença estatística. A detecção destes patógenos periodontais é corroborada pelos estudos de Mombelli et al.[47] 1995, Mombelli et al.[44] 1987, Mombelli et al.[50] 2001. Estes dados ainda ratificam os

achados dos *Artigos I e II* além de validar o modelo canino utilizado para avaliação microbiológica da peri-implantite.

Os achados do *Artigo VII* sugerem ainda, mesmo que indiretamente, que os dentes acometidos pela doença periodontal podem servir de reservatórios de microrganismo patogênicos que futuramente podem colonizar o sulco peri-implantar (Papaioannou et al.[58] 1996, Apse et al.[5] 1989, Mombelli et al.[47] 1995).

A resposta do hospedeiro frente à infecção bacteriana e seus produtos parece ser o maior precursor da patogênese da doença periodontal (Page[56] 1991). Analisando os fluidos crevicular gengival e peri-implantar, a detecção e quantificação de interleucina 1ß (IL-1 ß) e da prostaglandina E_2 (PGE_2), foram similares para ambos os sítios. Paganos et al.[55] (1996), Aboyoussef et al.[1] (1998) e Kao et al.[35] (1995) verificaram a associação de elevados níveis de IL-1 ß em implantes falidos. Offenbacher et al.[54] (1993) concluíram que os pacientes portadores de doença periodontal ativa apresentam altos níveis de IL-1 ß e PGE_2. Kao et al.[35] (1995) ainda sugerem que estas citocinas poderiam ser utilizadas no monitoramento de pacientes portadores de próteses implanto-suportadas para ínicio/progressão da doença peri-implantar.

No *artigo IX* analisou-se a presença de contaminantes na superfície de implantes falidos utilizando microscopia eletrônica de varredura associada a um espectrofotômetro (EDX). A análise mostrou que os implantes falidos, independentes do tipo de falência (precoce ou tardia/peri-implantite), apresentaram na sua grande maioria uma quantidade de carbono e cálcio provavelmente devido ao filme orgânico. Embora elementos como cloro,

nitrogênio, silício e sódio estivessem presentes na camada de óxido destes implantes, nenhum destes elementos pode ser associado como fator etiológico para perda de osseointegração e conseqüentemente falência do implante. Os implantes novos usados como controle apresentaram fortes sinais de titânio, e a presença de pouquíssimos contaminantes como o sódio e o alumínio, sendo o último associado ao processo de jateamento com óxido de alumina.

PROGRESSÃO DAS PERI-IMPLANTITES

A progressão da peri-implantite crônica em um modelo experimental canino foi avaliada no Artigo *V*. Após indução da doença peri-implantar por meio de ligaduras durante um período de 60 dias (fase aguda), os cães foram mantidos com o controle químico e mecânico supragengival sem a presença das ligaduras (fase crônica). Observou-se a progressão desta peri-implantite experimental contradizendo os estudos de Hanish et al.[28] 1997, Marinello et al.[43] 1995 e Godfredsen et al.[21] 2002. Achado importante foi à perda de mais de 65% dos implantes revestidos com hidroxiapatita, sem, no entanto haver diferença estatística entre as diferentes superfícies. As taxas de progressão da profundidade de sondagem e da perda clínica de inserção, tanto para as faces livres quanto para as faces proximais, foram menores na fase crônica (sem ligaduras). Entretanto as médias de progressão das faces livres foram sempre maiores quando comparadas as proximais.

A perda óssea radiográfica foi aumentando progressivamente ao longo do estudo (*Artigos I e II*), entretanto a perda óssea vertical foi estatisticamente

menor no período crônico do estudo. Com base nestes dados pode-se especular que a peri-implantite experimental apresenta duas fases distintas: a inicial oriunda do trauma mecânico dos fios da ligadura associada ao shift bacteriano e, a segunda fase, a doença peri-implantar, esta se perpetua devido a microbiota patogênica presente nestes sítios. Entretanto, o desenho experimental deste estudo obteve dados contrários aos obtidos por Lindhe et al.[40] (1992) Marinello et al.[43] (1995) e Gottfresen et al.[21] (2002) após indução da peri-implante por meio de fios de algodão.

A mobilidade dos implantes, aferida por meio do Periotest, foi menor para os implantes revestidos com plasma spray de titânio, embora não houvesse diferença estatística entre as superfícies avaliadas. A análise microbiológica mostrou a presença e permanência de periodontopatógenos identificados na fase aguda da peri-implanite (*artigos I e II*).

TRATAMENTO DAS PERI-IMPLANTITES

O tratamento das peri-implantites visa a remoção de contaminantes bacterianos sem no entanto alterar a superfície do implante para comportar uma futura re-osseointegração. O objetivo do *Artigo VIII* foi avaliar, *in vitro*, a biocompatibilidade da superfície implantar após tratamento com jato de bicarbonato de sódio, utilizando fibroblastos de linhagem contínua. O tratamento proposto não alterou a morfologia nem a expressão fenotípica dos fibroblastos ratificando os achados de Gould et al.[22] (1981); Guy et al.[24] (1993); Sauberlich et al.[68] (1999). Entretanto houve uma diminuição do número de fibroblastos nas

superfícies tratadas quanto comparadas com as superfícies controle (não tratadas), resultado este confirmado por Dmytryk et al.[11] (1990), Kononen et al.[36] (1992) e Eisenbarth et al.[13] (1996). A diferença entre a proliferação e adesão dos fibroblastos à superfície previamente tratada podem ser atribuídas provavelmente às liberações de íons tóxicos ou ainda pela presença de partículas do bicarbonato de sódio

Já nos *Artigos IV,V e VI* avaliou-se a efetividade microbiana da fotossensibilização sobre superfícies contaminadas por periodontopatógenos. O total de unidades formadoras de colônias de *Fusobacterium* spp., *Prevotella intermedia/nigrescens* e estreptococos foi estatisticamente reduzido após a utilização do laser de baixa intensidade associado a substância fotossensibilizadora, semelhante aos resultados de Haas et al.[25] (1997); Dörtbudak et al.[12] (2001), Sarkar et al.[67] (1993), Haas et al.[26] (2000) e Wilson et al.[83] (1995). A utilização do laser de baixa intensidade associado ao fotossensibilizador além de não danificar a superfície do implante, age especificamente nos microrganismos patogênicos, sem, no entanto prejudicar as células do hospedeiro.

Neste ínterim, os tratamentos mecânicos, associados aos meios físicos apresentam algumas vantagens: (1) não danificam as superfícies dos implantes, uma vez que a utilização de jatos abrasivos pode diminuir a biocompatibilidade da superfície (*Artigo VIII*); (2) associado ao debridamento peri-implantar, a fotossensibilização pode facilitar os procedimentos regenerativos por meio de barreiras/membranas diminuindo ou até eliminando a presença de patógenos

periodontais freqüentemente encontrados em membranas prematuramente expostas na cavidade bucal diminuindo assim o ganho clínico de inserção (Machtei et al.[42] 1995); (3) diminui o processo de resistência microbiana frente a antibióticos associados geralmente nos processos de regeneração tecidual/óssea guiada (Zambon[84] 1996).

O percentual de re-osseointegração foi estatisticamente diferente entre os grupos fotossensibilização associado ao regeneração óssea guiada quando comparados ao grupo controle. Entretanto não houve diferença significante entre as várias superfícies avaliadas.

9. CONCLUSÕES

Substanciado nos presentes resultados, pode-se afirmar que:

1- A microbiota detectada na peri-implantite experimental era constituída, principalmente, por *A. actinomycetemconitans*, *P. gingivalis*, *B. forsythus*, *P. intermedia* e *F. nucleatum*

2- A peri-implantite induzida por ligadura tende a progredir com maior severidade na superfície revestida por hidroxiapatita

3- A fotossensibilização pode reduzir o número de periodontopatógenos

4- A fotossensibilização associada a ROG resultou em re-osseointegração e preenchimento ósseo.

5- Nenhum dos contaminantes detectados na superfície dos implantes falidos pode ser associado como fator causal pela perda do implante.

6- O modelo animal utilizado neste estudo pode reproduzir clínica e microbiologicamente as condições de doença peri-implantar

10. PERSPECTIVAS FUTURAS

Além do fator microbiano, fatores como sobrecarga oclusal, tipo de restauração, presença de mucosa ceratinizada, qualidade óssea e tipo de superfície devem ser avaliados quanto à possibilidade de exercerem riscos inerentes aos processos de falência de implantes e peri-implantites. Entretanto, assim como na doença periodontal, a interação destes fatores junto ao hospedeiro deve ser mais bem compreendida.

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