Microbial Colonization in Orthodontic Mini-Implants

Amanda Osório Ayres de FREITAS[1]
Celuta Sales ALVIANO[2]
Daniela Sales ALVIANO[2]
José Freitas SIQUEIRA Jr[3]
Lincoln Issamu NOJIMA[1]
Matilde da Cunha Gonçalves NOJIMA[1]

[1]*Department of Pediatric Dentistry and Orthodontics, Dental School,*
UFRJ - Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil
[2]*Prof. Paulo de Góes Microbiology Institute, Health Science Center,*
UFRJ - Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil
[3]*Department of Endodontics, Dental School, Estácio de Sá University, Rio de Janeiro, RJ, Brazil*

Peri-implant inflammation contributes for loss of secondary stability of orthodontic mini-implants. The investigation of microbial colonization in this area would benefit its control, and consequently favor the long-term success of mini-implants. Therefore, the aim of this study was to determine the establishment and the evolution of microbial colonization process in orthodontic mini-implants for 3 months, since the time of their installation. One-hundred and fifty samples collected from 15 mini-implants were investigated from baseline up to 3 months. The biological material was obtained from peri-implant area using paper points. Nonspecific, *Streptococcus* spp*, Lactobacillus casei* and *Candida* spp colonizations were analyzed by cell growth methods. *Porphyromonas gingivalis* colonization was observed by 16S rDNA-directed polymerase chain reaction. Data from cell growth were submitted to the Wilcoxon sign rank test and results from molecular analysis were presented in a descriptive way. There was no significant difference in the microbial colonization among the examined time intervals, except for *Streptococcus* spp, between baseline and 24 h, which characterized the initial colonization in this time interval. *Lactobacillus casei* and *Candida* spp colonizations were insignificant. No *Porphyromonas gingivalis* was detected among the analyzed samples. The microbial colonization of mini-implants did not significantly change during the study. However, it should be monitored by orthodontists, since it is an important factor for mini-implants success.

Key Words: orthodontic anchorage procedures, microbiological colonization, microbial colony count, polymerase chain reaction.

INTRODUCTION

Orthodontic anchorage can be defined as resistance to undesirable tooth movement. The increasing demand for treatment by adult patients requires the development of new mechanical strategies without the disadvantages of a complicated design and the need for patient's cooperation (1-8). With the introduction of mini-screws, which are temporary devices for intraoral skeletal anchorage, special orthodontic anchorage problems could be better controlled (2-4,6,7,9-12).

There are many types of commercially available mini-screws, classified as either self-tapping or self-drilling (7,13). They consist of three parts: the screw head, the transmucosal neck and the infrabony portion. Selection of the proper implant site is an essential factor for its success. Mini-screws can be implanted in the maxillary alveolar bone, palate region, zygomatic buttress, as well as in the mandibular buccal alveolar bone, retromolar region and symphysis (2,7,14,15). The temporary anchorage devices should be implanted in attached gingiva, clear of the frenulum, in order to promote no damage to adjacent tissues (3,7,9,14,16).

The long-term success of a mini-implant depends on some factors, including its design, diameter, length and primary stability, as well as the host's bone thickness,

Correspondence: Profa. Dra. Matilde da Cunha Gonçalves Nojima, Departamento de Odontopediatria e Ortodontia, Faculdade de Odontologia, Universidade Federal do Rio de Janeiro, UFRJ, Avenida Professor Rodolpho Paulo Rocco, 325, Ilha do Fundão, 21941-617 Rio de Janeiro, RJ, Brasil. Tel: +55-21-2590-2727. e-mail: matildenojima@uol.com.br

oral hygiene and orthodontic mechanics (14,16). Home care and oral hygiene are considered critical factors for mini-implant success. Chronic inflammation caused by plaque retention leads to mobility and loss of the orthodontic mini-screw (1-3,6,9,11,13-15).

Considering the difficulty of maintaining peri-implant hygiene, it is important to monitor the microbiological colonization in this area. Then, the authors aimed to determine the establishment and the evolution of the microbial colonization process in orthodontic mini-implants for 3 months since their installation time. The knowledge of the quantitative and qualitative aspects of these microorganisms should help reducing inflammation, improving oral hygiene and consequently increasing the long-term success of mini-implants.

MATERIAL AND METHODS

Sample Selection and Clinical Procedures

The study was conducted in a population of adolescents and young adults aged 15 to 23 years (5 females and 3 males), under treatment at the Post-graduation Orthodontics Clinic at the School of Dentistry of the Federal University of Rio de Janeiro, Brazil. Approval was granted by the Research Ethics Committee of IESC/ UFRJ (Protocol #06/2009). All patients were asked to sign an informed consent form and answered a questionnaire about their general health condition, in order to identify factors that could influence oral bacterial colonization or the healing process of oral tissues (1). Selected subjects were not affected by any systemic disease neither had smoking habits. They were required not to take antibiotics during the three months preceding each microbiological examination, mouthwash rinses or gel one month before or during the study time, except for the recommended oral hygiene instructions.

The individuals had fixed orthodontic appliances, upper and lower 0.019" x 0.026" stainless-steel arch wires installed according to the Edgewise technique and had indication for skeletal anchorage with orthodontic mini-screws during incisors retraction.

A total of 15 mini-screws were inserted in the attached gingiva in the alveolar bone area of the maxilla and mandible. Self-drilling $Ti_6 Al_4 V$ alloy mini-implants (SIN, São Paulo, SP, Brazil), 8.0 mm long, 1.4 mm diameter, and 2.0 mm transmucosal neck were used. Patients received oral hygiene instructions

and followed a special protocol for the mini-implant region. This protocol recommended mechanical oral hygiene, mouthrinse with 0.12% chlorhexidine for a week after insertion of the mini-screw and continued peri-implant hygiene with a toothbrush soaked with 0.12% chlorhexidine, until the mini-screw was removed.

During a 90-day period, a total of 150 microbial collections were performed in the sulcus formed between the transmucosal neck of the temporary anchorage device and the attached gingiva. Two samples of material from mini-screws were taken at each collection time for cell culture and molecular analysis. Samples were collected with #35 paper points (Endo Points, Manacapuru, AM, Brazil) and stored in 500 µL of sterile saline (0.85% sodium hypochlorite and 1% sodium thioglycolate) for subsequent cell culture (Fig. 1). The paper points with material for molecular analysis were stored in 1 mL of TE (10 mM Tris, 1 mM EDTA and distilled water). The latter samples were immediately frozen at -20°C. Collection times were as follows: 1 h after surgery (T_{1h}), 24 h after surgery (T_{24h}), 7 days after surgery (T_{7d}), 14 days after surgery (T_{14d}) and 3 months after surgery (T_{3m}). The interval between collection and transportation of material for cell culture was less than 24 h.

Microbiological Analysis/Cell Growth

Solid culture media were used for nonspecific bacterial, fungal, as well as for specific analysis to detect *Streptococcus* spp, *Lactobacillus casei* and *Candida* spp.

The biological material was inoculated onto Petri plates containing nonspecific culture media (Brain Heart Infusion - BHI) and specific culture media for *Streptococcus* spp (Mitis Salivarius) and *L. casei* (Rogosa). *Streptococcus* spp incubation was performed in an anaerobic jar and maintained in a heater at 37°C for 24 h. However, nonspecific microorganism and *L. casei* were cultured without oxygen restriction. The fungal analysis was realized by specific quantification and identification of *Candida* spp, in Chromagar medium, after 48 h at 37°C in a heater.

Identification and counting of colonies was done macroscopically. Numbers of viable microorganisms were calculated from the numbers of colony forming units (CFU).

16S rDNA-Directed Polymerase Chain Reaction (PCR)

DNA was extracted from the samples and

analyzed for the presence of *Porphyromonas gingivalis* using PCR. Samples in TE were thawed at room temperature and vortexed for 30 s. Microbial suspensions were washed three times with 100 µL of Milliq water by centrifugation for 5 min at 2,500 x g. Pellets were then resuspended in 100 µL of Milliq water, boiled for 10 min and chilled on ice. Samples were then centrifuged for 10 s at 10,000 x g at 4°C. After this, 80 µL of the supernatant was transferred to a new tube and used as template in PCR analysis. Reference DNA from *P. gingivalis* (ATCC 33277) was also extracted for positive control (17).

PCR identification was carried out using ubiquitous 16S rDNA gene primers, to check for the presence of bacteria in the samples and 16S rDNA gene-based specific primers for *P. gingivalis*. Primers were as described by Ashimoto et al. (*P. gingivalis* specific primer pair 5'-3' AGG CAG CTT GCC ATA CTG CG; ACT GTT AGC AAC TAC CGA TGT; amplicon length 404 bp) (17). Aliquots of 5 µL of supernatant from clinical samples and 5 µL of reference strain nucleic acid were amplified. The PCR reaction to assess both the occurrence of bacteria using ubiquitous primers and *P. gingivalis* specific primers was performed in 50 µL of reaction mixture containing 1 µL of primer (40 pmol), 5 µL of 10 x PCR buffer (Invitrogen, Carlsbad, CA, USA), 1.25 U *Taq* DNA polymerase (Invitrogen), and 0.2 mM of each desoxyribonucleoside triphosphate (dATP, dCTP, dGTP, dTTp) (Invitrogen), and 2 mM $MgCl_2$ (17). PCR temperature profile included an initial denaturation step at 95°C for 2 min, followed by 36 cycles of a denaturation step at 94°C for 30 s, a primer annealing step at 60°C for 1 min, an extension step at 72°C for 2 min and a final step at 72°C for 10 min (17).

PCR cycling was performed in a DNA thermal cycler (PTC-100; MJ Research, Inc., Watertown, MA, U.S.A.). Amplicons were analyzed by 1.5% agarose gel electrophoresis performed at 4 V/cm in Tris-borate EDTA buffer. The gel was stained with 0.5 µg/mL ethidium bromide and visualized on an ultraviolet transilluminator. As size marker, 100 bp DNA ladder digest was used.

Data Analysis

Data were analyzed using the Wilcoxon sign rank test to determine microbial colonization in mini-implants during sampling times at 5% significance level. Findings from genetic analysis were presented during description of the results.

RESULTS

Cell Growth

There was no significant difference in the nonspecific colonization among the different time intervals. Specific microbial colonization of *Streptococcus* spp showed difference between T_{1h} and T_{24h}. Data can be better seen in the Tables 1 and 2, which represent the p values after comparisons between results from collection
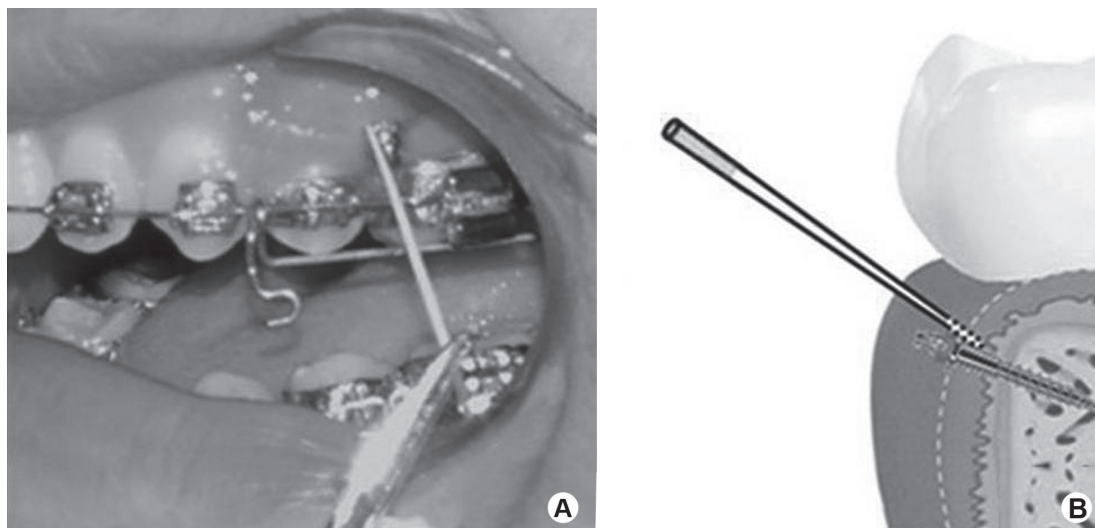

Figure 1. A. Fluid collection from attached gingival sulcus and the transmucosal neck of the mini-implant; B. Schematic design of the collection procedure.

times, according to the therapeutic strategy. From T_{1h} to T_{7d}, patients used 0.12% chlorhexidine mouthrinse, and from T_{14d} to T_{3m}, they performed local application of the same solution. The results from *L. casei* and *Candida* spp were unexpressive.

PCR Identification

There was no amplification for *P. gingivalis* among the analyzed samples. All samples were positive for the ubiquitous primers, indicating that bacterial DNA was present and that the PCR reaction was performed without significant inhibition by components of clinical samples.

DISCUSSION

The mere presence of fixed orthodontic appliances makes oral hygiene difficult and can cause environmental changes in the oral cavity, altering the nature of bacterial plaque in orthodontic patients (18-20).

Early colonizer lineages for complex biofilm formation and subsequent colonization of periodontopathic bacteria (*Streptococcus* spp, *L. casei* and fungal species of *Candida*) were selected for investigation because they are part of the normal oral microbiota (1,19,21). *P. gingivalis* was also included in this study, because it is a member of the red complex of subgingival plaque, which represents the most virulent species in the development of periodontal disease and loss of periodontal support (21-25).

All individuals participating in this study were periodontally healthy subjects and did not present any clinical sign of gingival inflammation (4). They received post-surgical oral hygiene instructions to reduce the possibility of peri-implant inflammation, and consequent loss of stability and failure of the device (5,11,12). The medication selected for microbial control was 0.12% chlorhexidine (4,5). Chlorhexidine properties, especially the cationic aspect, provides its substantivity, or persistent adherence to the dental surface and soft tissues, promoting extended bactericidal and bacteriostatic effects (5). At every appointment, a clinical exam was performed in order to evaluate whether the anchorage device was still stable, with no clinical sign of inflammation or mobility, since the objective of the study was to determine the microbial colonization in orthodontic mini-implants under normal conditions.

The anchorage devices were installed in the attached gingiva associated to the alveolar bone of maxilla or mandible. Although the maxillary and mandibular bone present distinct anatomic characteristics, such as bone density and cortical thickness (3,16), they are more important for primary stability of mini-implants. The amount of and the quality of keratinized mucosa around the anchorage device is determinant for primary and secondary stability (3,7,9,14,16). Independent of maxillary or mandibular arch, the mini-implants included in this study were placed in favorable keratinized mucosa areas. As mobility of the devices and the associated inflammation were considered failure factors (6,14), every implant that presented these characteristics during the study time was excluded. The placement of mini-implant in the maxilla or mandible did not affect the obtained results. This fact was confirmed by the absence of statistically significant difference among the observed unspecific colonization data.

After mini-implant insertion, a new site for microbial colonization is created. It can be defined as a gingival sulcus between the attached gingiva and the transmucosal neck of the mini-implant (2). This peri-implant area remains in tight contact with adjacent tissues, with restricted clinical access and, consequently, is difficult to clean (2). Then, the sulcus would be associated with the microbial colonization and plaque development and that could compromise the longevity

Table 1. Evolution of nonspecific microbial colonization across sampling times.

Comparison	p value
T_{1h} x T_{24h}	0.128
T_{24h} x T_{7d}	0.917
T_{24h} x T_{3m}	0.889
T_{14d} x T_{3m}	0.237

Significance level: p≤0.05.

Table 2. Evolution of *Streptococcus* spp colonization across sampling times.

Comparison	p value
T_i x T_{24h}	0.018*
T_{24h} x T_{7d}	0.917
T_{24h} x T_{3m}	0.889
T_{14d} x T_{3m}	0.237

Significance level: p≤0.05. *Significant p value.

of the anchorage device in the oral cavity (1,2,7,8,14).

In this study, mini-implants were immediately loaded and were able to remain healthy during the whole experiment time. It has been shown that a layer of fibrous tissue is formed between the contact surface of mini-implants and the alveolar bone (4). The presence of a fibrous layer avoids osseointegration and could compromise the secondary stability, although the mechanical retention seems to be enough for sustain the orthodontic forces applied, and maintain the clinical stability (4).

It is important to emphasize that peri-implant inflammation can occur dissociated from infection, unless significant microbial colonization is observed. Some authors described that peri-implant inflammation can be determined by the device's mobility, swelling, redness of adjacent mucosa and loss of supporting bone mediated by the host's immune and inflammatory responses (1-3,5,6,9,11). Histologically, formation of granulation tissue can be observed if inflammation becomes chronic (6). These characteristics associated with poor mini-implant hygiene can lead to peri-implant infection, as a consequence of increased microbial levels due to the favorable environmental conditions (1-3,6,9,14). Healthy peri-implant tissue is an important biologic barrier to bacteria (5,14). Sato et al. (1) suggested that an anaerobic environment in crevices around titanium orthodontic anchor plates are favorable to growth of anaerobic bacteria, which may initiate mucosa inflammation around the plates by the presence of these microorganisms and their toxins. Thus, plaque control around anchorage devices that form sulcus between titanium surface and adjacent mucosa is also an outstanding strategy to prevent inflammation (3,4,11,16).

Results expressing no significant increase in the colonization of the mini-implant sulcus after the total period of investigation were expected, since these devices are manufactured with biocompatible titanium alloy and their transmucosal neck surfaces had a special polishing to avoid the adherence of microorganisms (1). In addition, the results were probably influenced by the used therapy. Subjects were submitted to this therapy because this medication has been used at our institution as a protocol for peri-implant hygiene control, since it is very effective for plaque inhibition (5).

Bacteria possibly associated with the development of peri-implant inflammation are related to periodontal disease, including *P. gingivalis* (2). This bacterium is generally found in periodontal patients, although it can also be detected in healthy patients in unexpressive quantity (25). Patients in this study had no signs of periodontal inflammation and had stable temporary anchorage devices. Thus, negative results for *P. gingivalis* in the present research are reasonable.

Apel et al. (2) investigated the microflora associated with failed and successful mini-implants by real-time quantitative PCR complemented with microarray-based identification of 20 oral species. They described that species of oral origin associated with peri-implantitis, including *P. gingivalis,* were absent in healthy implants. *Streptococcus* spp (*S. gordonii,* as well as *S. mitis*) were found in all successful devices. These findings corroborate with the results of the present study. Apel et al. (2) also revealed that there was no significant difference in the total amount or species of bacteria, comparing control (successful) and failed mini-implants.

The findings of the present study confirmed that the establishment of microbial colonization occurred in the first 24 h after exposure of mini-implants to the oral cavity, and the development of colonization during the 3 months of the investigation was balanced, without predominance of any of the studied microorganisms.

RESUMO

A inflamação peri-implantar contribui para a perda da estabilidade secundária dos mini-implantes ortodônticos. A investigação da colonização microbiana desta área beneficiaria o seu controle e, consequentemente, favoreceria o sucesso dos mini-implantes a longo prazo. Portanto, o objetivo dos autores foi determinar o estabelecimento e evolução do processo de colonização microbiana em mini-implantes ortodônticos por três meses desde a instalação. Cento e cinquenta amostras coletadas de 15 mini-implantes foram investigadas desde o tempo inicial até 3 meses. O material biológico foi obtido da área peri-implantar com auxílio de cones de papel absorvente. As colonizações inespecíficas de *Streptococcus spp, Lactobacillus casei* e *Candida spp* foram analisadas por métodos de crescimento celular. A colonização por *Porphyromonas gingivalis* foi observada por meio da reação em cadeia da polimerase 16S rDNA. Os dados do crescimento celular foram submetidos ao teste de Wilcoxon *sign rank* e os resultados da biologia molecular foram apresentados de modo descritivo. Não houve diferença estatisticamente significante da colonização microbiana entre os intervalos de tempo avaliados, exceto para *Streptococcus spp* entre os tempos inicial e 24 h, o que caracterizou o início da colonização neste intervalo de tempo. As colonizações por *Lactobacillus casei* e *Candida spp* foram insignificantes. Não foi detectada a presença de *Porphyromonas gingivalis* nas amostras analisadas. A colonização microbiana nos mini-implantes não se alterou significativamente durante o estudo. No entanto, deve ser monitorada por ortodontistas, uma vez que é um fator importante para o sucesso dos mini-implantes.

ACKNOWLEDGEMENTS

REFERENCES

1. Sato R, Sato T, Takahashi I, Sugawara I, Takahashi N. Profiling of bacterial flora in crevices around titanium orthodontic anchor plates. Clin Oral Impl Res 2007;18:21-26.

2. Apel S, Apel C, Morea C, Tortamano A, Dominguez GC, Conrad G. Microflora associated with successful and failed orthodontic mini-implants. Clin Oral Impl Res 2009;20:1186-1190.

3. Chen YJ, Chang HH, Lin HY, Lai EHH, Hung HC. Stability of miniplates and miniscrews used for orthodontic anchorage: experience with 492 temporary anchorage devices. Clin Oral Impl Res 2008;19:1188-1196.

4. Justens E, De Bruyn H. Clinical outcome of mini-screws used as orthodontic anchorage. Clin Implant Dent Relat Res 2008;10:174-180.

5. Kravitz ND, Kusnoto B. Risks and complications of orthodontic miniscrews. Am J Orthod Dentofacial Orthop 2007;131:S42-S51.

6. Takaki T, Tamura N, Yamamoto M, Takano N, Shibahara T, Yasumura T, et al.. Clinical study of temporary anchorage devices for orthodontic treatment. Stability of micro, mini-screws and mini-plates. Experience with 455 cases. Bull Tokyo Dent Coll 2010;51:151-163.

7. Topouzelis N, Tsaousoglou P. Clinical factors correlated with the success rate of miniscrews in orthodontic treatment. Int J Oral Sci 2012;4:38-44.

8. Wu TY, Kuang SH, Wu CH. Factors associated with the stability of mini-implants for orthodontic anchorage: a study of 414 samples in Taiwan. J Oral Maxillofac Surg 2009;67:1595-1599.

9. Park HS, Jeong SH, Kwon OW. Factors affecting the clinical success of screw implants used as orthodontic anchorage. Am J Orthod Dentofacial Orthop 2006;130:18-25.

10. Kanomi R. Mini-implant for orthodontic anchorage. J Clin Orthod 1997;31:763-767.

11. Miyawaki S, Koyama I, Inoue M, Mishima K, Sugahara T, Takano Yamamoto T. Factors associated with the stability of the titanium screws placed in the posterior region for orthodontic anchorage. Am J Orthod Dentofacial Orthop 2003;124:373-378.

12. Wilmes B, Rademacher C, Olthoff G, Drescher D. Parameters affecting primary stability of orthodontic mini-implants. J Orofac Orthop 2006;3:162-174.

13. Carano A, Velo S, Leone P, Siciliani G. Clinical aplications of the miniscrew anchorage system. J Clin Orthod 2005;39:9-24.

14. Cheng SJ, Tseng IY, Lee JJ, Kok SH. A prospective study of the risk factors associated with failure of mini-implants used for orthodontic anchorage. Int J Oral Maxillofac Implants 2004;19:100-106.

15. Melsen B. Mini-implants: where are we? J Clin Orthod 2005;39:539-547.

16. Chen YJ, Chang HH, Huang CY, Hung HC, Lai EHH, Yao CCJ. A retrospective analysis of the failure rate of three different orthodontic skeletal anchorage systems. Clin Oral Impl Res 2007;18:768-775.

17. Roças IN, Siqueira Jr JF, Andrade AFB, Uzeda M. Identification of selected putative oral pathogens in primary root canal infections associated with symptoms. Anaerobe 2002;8:200-208.

18. Balenseifen JW, Madonia JV. Study of dental plaque in orthodontic patients. J Dent Res 1970;36:320-324.

19. Hagg U, Kaveewatcharanont P, Samaranayake YH, Samaranayake LP. The effect of fixed orthodontic appliances on the oral carriage of Candida species and enterobacteriaceae. Eur J Orthod 2004;26:623-629.

20. Sukontapatipark W, El-Agroudi MA, Selliseth NJ, Thunold K, Selving KA. Bacterial colonization associated with fixed orthodontic appliances. A scanning electron microscopy Study. Eur J Orthod 2001;23:475-484.

21. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol 1998;25:134-144.

22. Bodet C, Chandad F, Grenier D. Potentiel pathogénique de *Porphyromonas gingivalis*, *Treponema denticola* et *Tannarella forsythia*, le complexe bacterèrien rouge associé à la parodontite. Pathologie Biologie 2007;55:154-162.

23. Heuer W, Elter C, Demling A, Neumann A, Suerbaum S, Hannig M, et al.. Analysis of early biofilm formation on oral implants in man. J Oral Rehab 2007;34:377-382.

24. Pier-Francesco A, Adams RJ, Waters MGJ, Wiliams DW. Titanium surface modification and its effect on the adherence of *Porphyromonas gingivalis*: an *in vitro* study. Clin Oral Implants Res 2006;17:633-637.

25. Yoshinari M, Oda Y, Kato T, Okuda K, Hirayama A. Influence of surface modifications to titanium on oral bacterial adhesion *in vitro*. J Biomed Mat Res 2000;52:388-394.