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In vivo early plaque formation on pure titanium and ceramic abutments: a comparative microbiological and SEM analysis

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A lack of information exists about the influence of different implant abutment materials on bacterial colonization and its role in the development of perimplantar infections. In order to study these aspects, removable acrylic devices, harboring samples of titanium and novel ceramic abutments (Nobel Biocare) were adapted to the molar–premolar region in 2 mandibular quadrants of 4 volunteers. Samples of each material were collected at 6 and 24 h, 7 and 14 days. Samples were observed by scanning electron microscopy and bacterial counts were made by means of ATP detection and direct plate count. The electron micrographs demonstrated that the bacteria colonization was already present after 6 h of presence in the oral cavity. After 24 h, both the materials were covered by several layers of bacterial cells. No differences in microbial colonization were observed between titanium and ceramic samples. The microbiological analysis confirmed the presence of relevant amounts of microbial cells on the tested samples. The maximum of colonization was achieved after 24 h in the oral cavity and the bacterial counts remained constant over the 14 day period. No significant differences were observed between the two materials analyzed in this study. In addition, ATP-bioluminescence technology was demonstrated to be a suitable system to evaluate bacterial colonization in the oral cavity.

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Key words: bacterial adhesion – implant abutments – surface characteristics – *in vivo* study – early plaque formation – microbiology – SEM – bacterial count – ATP and bioluminescence technology

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The Brånemark implant technique was originally developed for the treatment of total edentulisms both in the upper and in the lower jaws. Subsequently, the long-term successful results (Adell et al. 1981) of the technique have encouraged clinicians to utilize osseointegrated dental implants for the treatment of partially edentulous patients, and several reports (Linquist et al. 1987; Zarb & Symington 1982) have been published demonstrating successful results in this new application. The use of osseointegrated implants for single tooth replacement and for the rehabilitation of partially edentulous ridges in the anterior areas has increased the esthetic requirements of the final implant supported prosthetic restoration. As a consequence, different new titanium abutments have been introduced in the market to reduce the height of the transmucosal abutment cylinder, thus allowing the porcelain restoration to emerge through the gingival tissues. More recently, a sintered allumine abutment cylinder has been proposed (Prestipino & Ingber 1993a, 1993b) to further improve the esthetic results. This abutment, named CerAdapt™ (Nobel Biocare AB, Goteburg, Sweden), is connected directly to the implant head and prepared like a natural tooth. The provisional and final prosthetic restoration is retained with provisional cement in order to allow overstructure retrievability. The advantages of this abutment are the high biocompatibility and the brightness of the material, the customization of the abutment preparation according to the tooth anatomy and to the gingival contours, and the elimination

of the screw holes through the crown surface. In spite of successful long-term results that have been widely documented, peri-implant bone loss may, occasionally, occur during the implant maintenance phase, resulting in the exposure of implant surfaces or threads previously embedded in bone. Peri-implant tissue progressive destruction has been termed "peri-implantitis" because of clinical, microbiologic, and histologic similarities to periodontitis (Becker et al. 1990; Lindhe et al. 1992; Mombelli et al. 1988; Quirynen & Listgarten 1990).

Although osseointegrated dental implants play an important role in restorative dentistry, there is the risk of failure associated to this technique due to bacterial infections (Becker et al. 1990; Jovanovic 1994; Mombelli et al. 1988; Tonetti & Schmid 1994). Several studies have shown that the presence of oral microflora and plaque formation may cause periodontal and perimplantar diseases (Jovanovic 1994; Mombelli et al. 1987; Quirynen & Listgarten 1990; Tonetti & Schmid 1994). Adhesion of bacterial cells on titanium has been already described both *in vivo* (McCollum et al. 1992; Mombelli et al. 1987; Quirynen & Listgarten 1990; Siegrist et al. 1991) and *in vitro* (Lentz & Uzodinma 1989; Oga et al. 1993; Rasperini et al. 1995). The rapid accumulation of bacterial cells on titanium surfaces was demonstrated by McCollum et al. (1992) and Rasperini et al. (1995). Siegrist et al. (1991) have demontrated the formation of plaque *in vivo* on different supporting materials, including ceramic, during a 24 h period in the oral cavity. These experiments on early plaque formation showed that the degree of colonization was related more to surface roughness than supporting material, while plaque formation was qualitatively similar among the tested substances. The influence of abutment material and surface roughness on the plaque accumulation was studied by Bollen et al. (1996) in 1-year *in vivo* experiments. These authors, testing titanium and highly polished ceramic abutment types, demonstrated that rougher abutments were colonized by a microbial community composed by a higher proportion of Gram negative bacteria, but no differences were detected in the amount of potentially pathogenic micro-organisms.

A lack of specific information exists about the influence of different implant abutment materials on bacterial colonization and plaque formation after 24 h in the oral cavity.

Recently new methods for the microbiological analysis of samples have been developed; among them the ATP(adenosine triphosphate)-bioluminescence assay is considered one of the most reliable methods for the rapid analysis of microbial count (Chen & Cushion 1994; Selan et al. 1992). This analytical indirect method uses the biological phenomenon of bioluminescence: when the enzyme complex luciferine–luciferase comes into contact with ATP it results in an emission of light which can be measured by a luminometer. The quantity of emitted light is related to the molar amount of ATP.

The aim of the present study was to investigate and compare the colonization process of titanium and ceramic abutments by oral microflora in an *in vivo* system during the first 14 days by means of microbiological methods, both direct count plate and ATP analysis (Blackburn et al. 1989; Miller et al. 1992), and scanning electron microscopy (SEM). An additional goal was to evaluate the ATP analysis technique as a method for estimating the plaque formation process in the oral cavity.

Materials and methods

Patient selection

Four healthy males, non-smokers, aged 28 to 35 years (mean age=30.2) selected on the basis of excellent periodontal health and absence of mouth breathing, participated in the study. The subjects were thoroughly informed and accepted to participate in the experiment.

Prior to insertion of the experimental devices, the subjects underwent professional tooth cleaning with a rubber cup and pumice. At the start of the experiment, the gingival index of Löe & Silness (1963) of the individuals was close to zero. None of them used mouth rinses or took antibiotics 6 months prior to or during the study.

In vivo experiment

A removable acrylic device was adapted to the lingual lower jaw of the 4 volunteers, in order to give the volunteers the possibility to practice normal daily oral hygiene. The devices were removed twice a day to allow proper oral hygiene procedures; no brushing was applied to the devices. Every day each volunteer was questioned by the supervisor to ensure the previous day's procedures had been performed as scheduled. The study, as outlined above, was approved by the Human Research Ethics Committee at the University of Milan.

Experimental design

Eight samples of titanium and 8 samples of highly polished ceramic were fixed to each removable device. The samples tested were from the standard titanium and novel ceramic CerAdapt™ abutments (Nobel Biocare, Gothenburg, Sweden). The samples, rectangular in shape with a dimension of 4 mm in height, 3 mm in width, and 1 mm in thickness were supplied by Nobel Biocare (Gothenburg, Swe-

den). In this study bacterial colonization of the 2 materials was compared within the same patients at the same time. Two samples of each material were collected at 6 and 24 h, 7 and 14 days. The abutment fragments were recovered with sterile forceps and transferred into sterile 1.5 ml tubes. The samples for microbiological analysis were immediately frozen after the collection, while samples for scanning electron microscopy were conserved in 75% ethanol.

Scanning electron microscope observations

The samples were dehydrated in ethanol series (75, 80, 85, 95 and 100% for 15 min each). After the 15 min critical point-drying, the specimens were mounted on SEM disks, coated with gold and observed with a scanning electron microscope (Hitachi, mod. S2300, Japan).

Microbiological analysis

The colonization of titanium and ceramic abutments was followed by counting the bacterial cells adhered to abutment surfaces. Two methods for testing bacteria counts were used: all the samples were tested with the Luciferase assay (LUMAC Microbial Kit, Perstorp Analytical Company, USA), which calculated the amount of ATP released from bacterial cells. The tests were performed following the instructions of the manufacturer. In order to create a calibration curve for the indirect count method (LUMAC Microbial Kit), one sample for each collection time was serially diluted in saline solution and plated onto Brain Hearth Infusion agar (Oxoid, Basingstoke, UK). The plates were incubated for 48 h at 37°C. The data from the plate count and bioluminescent assay, plotted on a graph, allowed us to design the calibration curve used to transform the data from luminometer into the number of bacteria.

The microbiological data are expressed as the number of bacteria present on the entire surface of each sample.

Results

Microbiological and SEM analyses

The results obtained from microbiogical analyses of the titanium and ceramic abutments are reported in Fig. 1. The values were calculated as described in Materials and methods.

The 6 h observation

After 6 h of growth the number of bacterial cells was close to 10^3 for both titanium and ceramic mate-

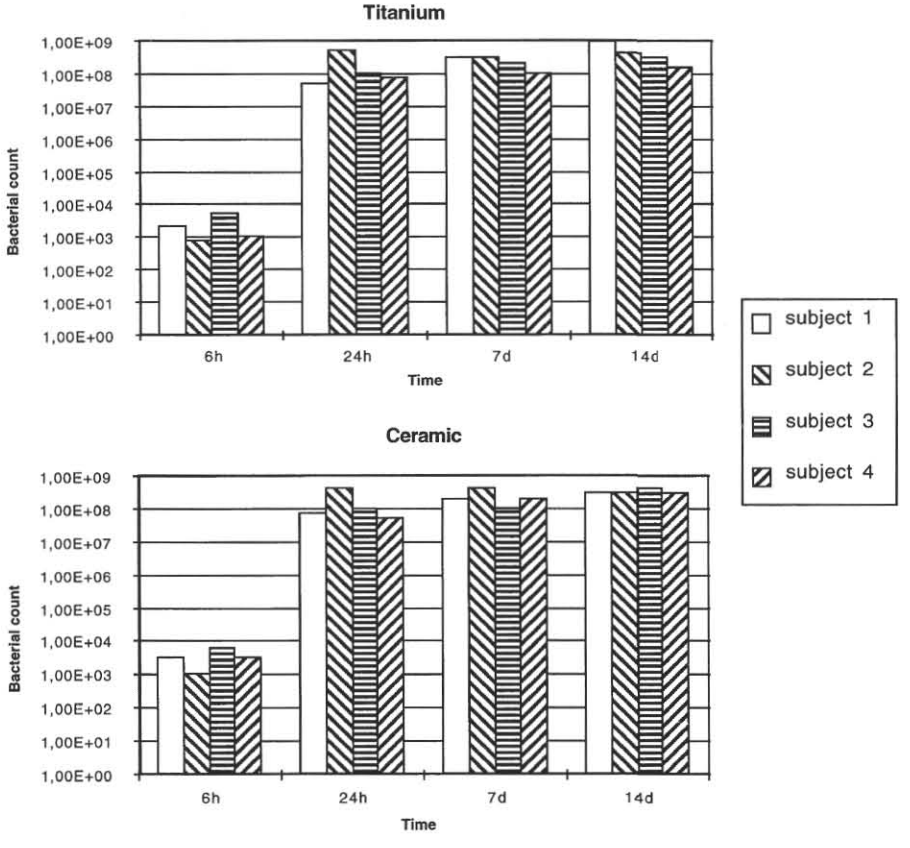
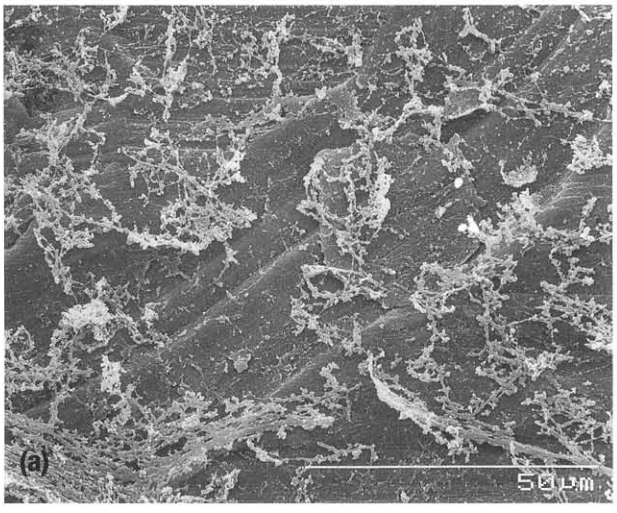


Fig. 1. Results of microbiological analysis on titatnium and ceramic abutments after different time periods in the oral cavity. The values, calculated by means of ATP analysis, were expressed as bacterial cell number using a calibration curve obtained analyzing abutments of subject 1 with both ATP system and direct plate count method.



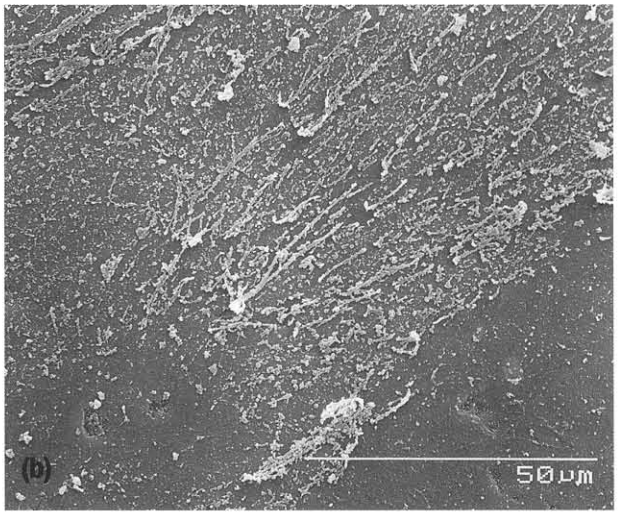
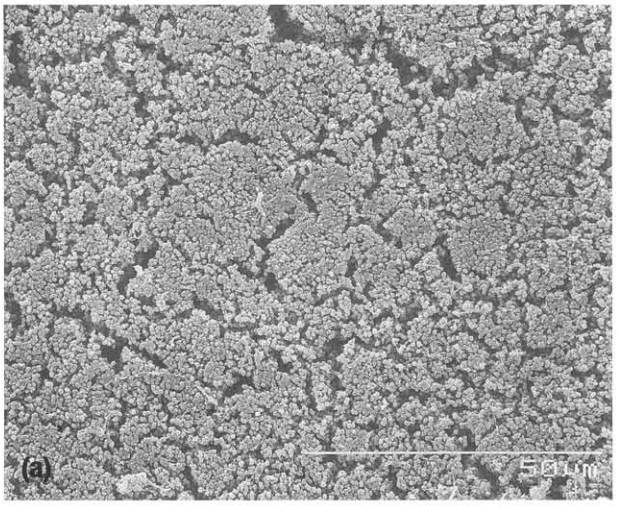


Fig. 2. (a) SEM observation of titanium abutment fragment after 6 h in the oral cavity. Bacterial cell aggregates are present on the surface. Original magnification 1000×. (b) Microbial cells adhered onto a ceramic fragment after 6 h in the oral cavity. Original magnification 1000×.



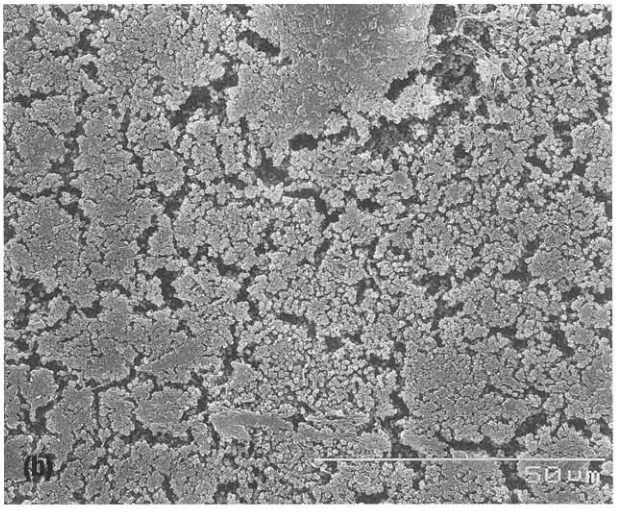


Fig. 3. (a) Thick layer of coccal cells cover the abutment fragments of titanium (a) and ceramic (b) after 24 h. No significant differences can be detected between the two materials. Original magnification 1000×.

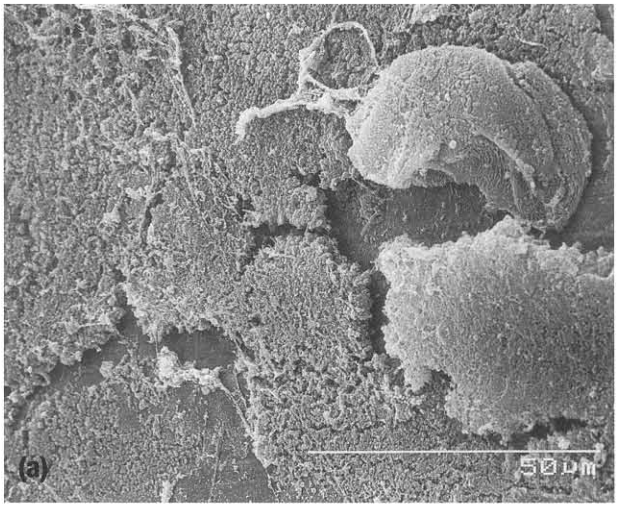
rials (Fig. 1). These data are confirmed by the presence on the surface of the analyzed samples of few aggregates of bacterial cells in a matrix of organic material, probably salivary proteins and desquamated epithelial cells, as shown in Fig. 2. No significant differences were observed between the 2 materials tested, as shown in Fig. 2 (a and b), and among the 4 subjects that harboured the acrylic removable devices.

The 24 h observation

After 24 h, bacterial colonizations on the abutment fragments were present. As shown in Fig. 1, the number of bacteria reached 10^8 for both materials analyzed. SEM observations revealed the presence of a thick layer of cells on both the surface of titanium (Fig. 3a) and ceramic (Fig. 3b) fragments. The plaque covering the abutments was mainly composed by coccal shaped cells.

The 7 day observation

After 7 days in the oral cavity, the maximum level of bacterial colonization was achieved. On all the samples tested the bacterial counts were over 10^8 cells per fragment and no significant differences were found among the different subject and the 2



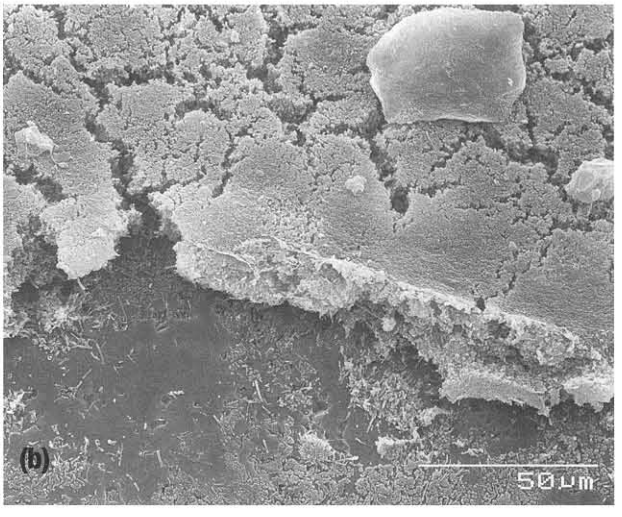
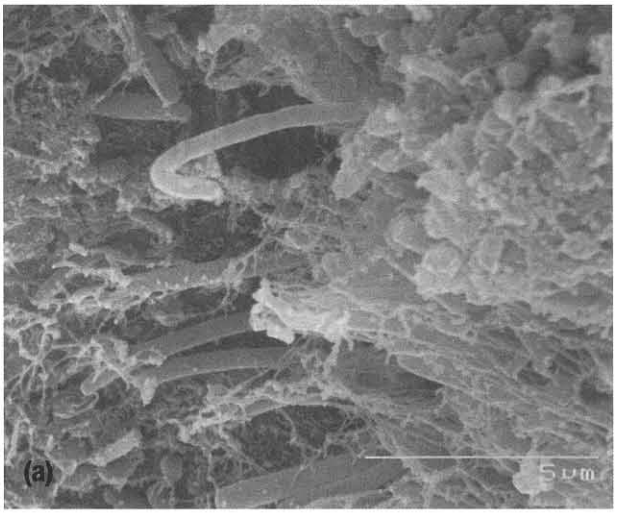


Fig. 4. SEM analysis after 7 days of incubation in the oral cavity. In both titanium (a) and ceramic (b) abutment fragments can be observed a significant colonization of bacterial cells. Original magnifications: 1000× (a) and 600× (b).



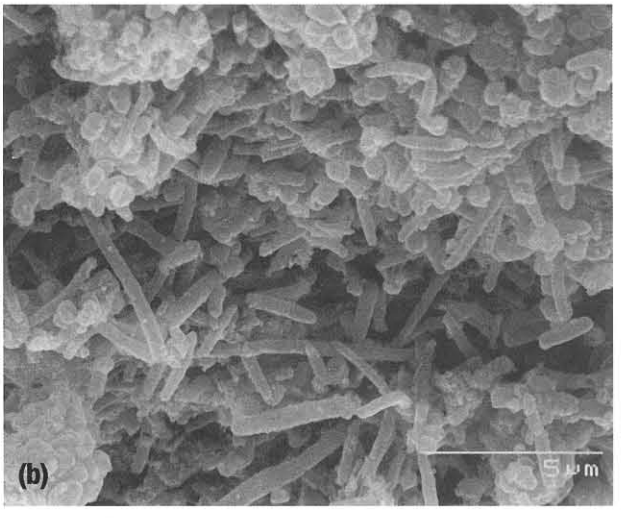


Fig. 5. The microbial community colonizing titanium (a) and ceramic (b) abutment fragments after 14 days. The thick layer of bacterial cells is composed of a mixed population of rods and cocci. Extracellular matrix, covering bacteria is present. Original magnifications: 8000× (a) and 6000× (b).

analyzed materials. The morphology of plaque accumulation formed on abutments is shown in Fig. 4. Bacilli and cocci were present in the plaque matrix. Desquamated epithelial cells were adhered over the layer of bacterial cells (Fig. 4a, b).

The 14 day observation

The same values of bacterial counts achieved after 7 days were maintained after 14 days in the oral cavity, since the counts for all the samples were near to 5×10^8 and no relevant differences were found among the different subjects and samples analyzed. Moreover, the scanning electron microscope observation at low magnification revealed a plaque layer very similar to the condition observed at 7 days. SEM higher magnifications allowed to identify in the plaque bacterial aggregates composed of rods and cocci. No differences in plaque composition were observed between titanium (Fig. 5a) and ceramic (Fig. 5b).

Discussion

In this study the *in vivo* process of bacterial colonization and plaque formation of titanium and new developed ceramic abutments was studied and analyzed with microbiological methods and scanning electron microscopy. Comparative analysis of the 2 materials tested revealed the lack of significant differences in the rate of bacterial colonization. After

24 h, most of the colonization was already present on both of the materials considered, as shown in Fig. 3, and the maximum level was achieved after 7 days. This study demonstrated that standard titanium and novel ceramic abutments show very similar properties in terms of colonization by oral microflora. Most of the bacterial colonization took place during the first 24 h in the oral cavity, when the bacterial counts reached almost 10^8 cells per fragment analyzed. The timing of the colonization process on both materials corroborated the observations of Siegrist et al. (1991), indicating that the experimental device was properly studied to simulate the plaque accumulation process. The maximum level obtained after 7 days was maintained for an additional 7 days, without significant variations. The differences in the bacterial counts among the 4 volunteers were not significant as observed in Fig. 1, indicating that the plaque formation process on abutment fragments is independent of the subject analyzed. In addition, the use of a rapid method for indirect bacterial count, based on the ATP analysis, was demonstrated to be an efficient and reliable system for counting oral microflora and for following the plaque formation in the oral cavity. Most of the studies of bacterial adherence to abutments were performed estimating the degree of microbial colonization by means of direct plate agar count and SEM analysis (Quirynen & Listgarten 1990; Rasperini et al. 1995; Siegrist et al. 1991). Recently, alternative procedures of bacterial count based on indirect estimation tests have been developed. Among them, the ATP method, based on the release of light after reaction with the luciferne–luciferase complex have been demonstrated to be a fast and reliable system for calculating the degree of bacterial contamination (Blackburn et al. 1989; Chen & Cushion 1994; Connolly et al. 1993; Miller et al. 1992; Selan et al. 1992). The advantages of this method, applied to this study of oral microbiology, are the rapidity of this technique and, more important, the ability of this system to calculate the amount of microbial cells in an independent way to media and culture conditions. Thus the ATP Luciferase Test allows to estimate all the microbial forms, culturable and non-culturable, aerobic and anaerobic in one step.

Although the microbiological methods used in this work do not allow any identification of bacterial groups involved in the colonization and adhesion processes, the electron microscopy showed a dominance of coccal shaped cells during the early plaque formation. After 7 days, higher morphological variability was observed on both titanium and ceramic, presenting a microbial community composed by bacilli and cocci. In all the analyzed samples, the bacterial aggregates were covered by a matrix, probably salivary proteins and bacterial extracellular compounds, involved in the adhesion of micro-organisms onto the titanium and ceramic as shown by Edgerton et al. (1996).

The experimental device was designed to be removed from the oral cavity prior to tooth brushing, in order to avoid interference with the plaque formation process on the titanium and ceramic fragments. In this study, lingually rather than buccally positioned devices were used (Simion et al. 1994). This choice was taken to improve the comfort for the volunteers, but could have resulted in a lingual scraping on the material surfaces. However, this scraping phenomenon would have been present also by positioning the device on the buccal side. The scraping effect of the tongue was probably responsible for the detachment of cell layers from the materials, as shown in Fig. 3.

The gingival index (Loe & Silness 1963) in all of the 4 subjects at the end of the experiment was close to zero, indicating the absence of inflammation due to the experimental device. This *in vivo* study, which had dealt with early plaque formation, indicates that the CerAdapt™ abutments show the same properties of titanium in terms of bacterial adhesion, colonization and plaque formation. As already reported by Bollen et al. (1996), one of the main factors affecting the bacterial colonization of abutments is the surface roughness of the material used. The mean R_a value for the ceramic abutment used in this study was 0.7, very close to the standard machined titanium value (0.6), as indicated by the supplier of the abutment materials.

Although this study was performed *in vivo* it represents a model for bacterial colonization on abutments rather than a true *in vivo* study. Thus, the abutment fragments analyze in this study were not positioned within the gingival sulcus, the usual clinical position of the abutments. Additional studies will be required to analyze the long-term behaviour of new materials in the oral cavity, to develop specific hygiene and maintenance protocols, and to study the colonization process into the sulcus.

Acknowledgements

The authors acknowledge Nicola Maglione for the technical assistance provided in the preparation of the experimental device and Iginio Rossi for the electron microscope technical assistance.

Résumé

Peu d'information existe quant à l'influence de différents matériaux du pilier sur implant sur la colonisation bactérienne et son rôle dans le développement des infections paroïmplantaires. Des supports en acrylique amovibles portant des échantillons de piliers en titane et en une céramique nouvelle (Nobel Biocare) one été adaptés dans la région prémolaires-molaires

des deux quadrants mandibulaires chez quatre volontaires. Les échantillons de chaque matériel ont été collectés après six et vingt-quatre heures, et sept et quatorze jours. Des échantillons ont été observés au microscope électronique à balayage et des comptages bactériens ont été effectués à l'aide de la détection d'ATP et par le comptage direct sur la boîte. L'analyse au microscope électronique à balayage a mis en évidence une colonisation bactérienne six heures après le début de la formation de la plaque dentaire *in situ*. Après vingt-quatre heures les deux matériaux étaient recouverts de plusieurs couches de cellules bactériennes. Aucune différence dans la colonisation microbienne n'a été observée entre le titane et la céramique. L'analyse microbiologique confirme la présence de quantités importantes de cellules microbiennes sur les échantillons testés. Le maximum de colonisation a été atteint après vingt-quatre heures *in situ* et les comptages bactériens restaient constants pendant quatorze jours. Aucune différence significative n'a été observée entre les deux types de matériaux utilisés. De plus la technologie de bioluminescence à l'ATP s'est avérée être un système valable pour étudier la colonisation bactérienne dans la cavité buccale.

Zusammenfassung

Es herrscht ein grosses Informationsdefizit bezüglich Einfluss von verschiedenen Sekundärteilmaterialen auf die bakterielle Kolonisation und ihrer Rolle bei der Entwicklung einer periimplantären Infektion. Um diese Aspekte zu studieren wurden in der Molaren- und Prämolarenregion von Unterkieferquadranten bei 4 Freiwilligen wieder entfernbare Acryschienen mit Titanproben und einem neuen Keramiksekundärteil (Nobel Biocare) eingesetzt. Von jedem Material wurden nach 6 und 24 Stunden, sowie nach 7 und 14 Tagen Plaqueproben entnommen. Diese Proben untersuchte man mit einem Rasterelektronenmikroskop und Bakterienzählung wurde sowohl mittles ATP-Registration als auch direkter Plattenauszählung durchgeführt. Die Elektronenmikrographien zeigten, dass die bakterielle Kolonisation schon sechs Stunden nach dem Einbringen in die Mundhöhle vorhanden war. Nach 24 Stunden ware beide Materialien mit diversen Schichten von Bakterienzellen belegt. Man stellte keinen Unterschied der mikrobiellen Kolonisation zwischen den Titan- und Keramikproben fest. Die mikrobiologischen Analysen bestätigten aber die Präsenz einer relevanten Mengen von mikrobiellen Zellen auf den getesteten Proben. Die maximale Kolonisation wurde nach 24 Stunden Präsenz in der Mundhöhle erreicht und die Werte der Bakterienzählungen blieben während der 14-tägigen Periode konstant. Zwischen den zwei in dieser Studie analysierten Materialien konnte man keine signifikanten Unterschiede feststellen. Zusätzlich erwies sich die ATP-Bioluminiszenz-Technik als probates System zur Analyse der bakteriellen Kolonisation der Mundhöhle.

Resumen

Existe una carencia de información sobre la influencia de diferentes materiales de pilares para implantes en la colonización bacteriana y su papel en el desarrollo de infecciones periimplantarias. En orden a estudiar estos aspectos, se adaptaron dispositivos acrílicos removibles, que llevaban muestras de pilares de titanio y cerámica novel (Nobel Biocare) a la región molar-premolar en dos cuadrantes mandibulares de cuatro voluntarios. Se recogieron muestras de cada material a las 6 y 24 horas, 7 y 14 días. Las muestras se observaron por microscopio electrónico de barrido y se realizó recuento bacteriano por medio de detección de ATP y recuento directo en la placa. La microscopía electrónica demostró que la colonización bacteriana ya estaba presente después de 6 horas de estancia en la cavidad oral. Después de 24 horas ambos materiales estaban cubiertos por varias capas de células bacterianas. No se observaron diferencias en la colonización microbiana entre las muestras de titanio y cerámica. El análisis microbiológico confirmó la presencia de cantidades relevantes de células microbianas en las muestras probadas. La colonización máxima se alcanzó a las 24 horas en la cavidad oral y el recuento bacteriano se mantuvo constante durante el periodo de 14 días. No se observaron diferencias significativas entre los dos materiales analizados en este estudio. Además, la tecnología de ATP bioluminiscente demostró ser un sistema adecuado para evaluar la colonización bacteriana en la cavidad oral.

要約

細菌のコロニー化とインプラント周囲の感染発症における役割に対して、異なるインプラント・アバットメントの材料が及ぼす影響に関する情報が不足している。これらの側面を研究するために、４名のボランティアの下顎片顎２カ所の大臼歯－小臼歯領域に可撤式アクリル製装置を装着して、チタン製及び新セラミック製アバットメント（Novel Biocare）の細菌標本を収容した。各材料の細菌標本は、６時間、２４時間、７日、１４日後に採取した。標本は走査電子顕微鏡で観察し、細菌数をATP検出法及び直接プレート・カウント法によって数えた。電子顕微鏡像は細菌のコロニーが口腔内に入れた６時間後に既に存在していることを示した。２４時間後には両材料は、数層の細菌細胞によって被覆されていた。

細菌のコロニー化について、チタン及びセラミックの標本の間に有意差は観察されなかった。細菌学的分析は、試験した標本上に妥当な数の細菌細胞が存在している事を確認した。口腔内で２４時間後にコロニー化は最大に達し、その後１４日間細菌数は一定を保った。本研究で分析した二つの材料の間に有意差は観察されなかった。さらにATP生物発光テクノロジーは、口腔内細菌のコロニー化を評価するための適切なシステムであることが示された。

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