

Qualitative and quantitative assesment of initial bacterial plaque formation on different prosthetic restorative materials

Farklı protetik restorasyon materyalleri üzerinde başlangıç bakteriyel plak oluşumunun nitel ve nicel olarak değerlendirilmesi

Prof. Dr. Ali Murat Kökat
Okan University, Faculty of Dentistry,
Department of Prosthodontics, İstanbul
Orcid ID: 0000-0002-3752-4858

Prof. Dr. Nesrin Anil
Hacettepe University, Faculty of Dentistry,
Department of Prosthodontics, Ankara
Orcid ID: 000-0003-1708-7029

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Corresponding author:
Prof. Dr. Ali Murat Kökat
Aydıntepe Mahallesi, 34947 Tuzla/İstanbul
Phone: 444 9 863
E-mail: alimuratti@gmail.com

SUMMARY

Aim: The aim of this study was to determine the structure of bacterial plaque qualitatively and quantitatively and to evaluate the effect of restorative material variation on pellicle composition and bacterial adhesion.

Materials and Method: Titanium, Au-Ag-Pd alloy, Ni-Cr alloy, an all-ceramic system and a feldspathic porcelain system were used to evaluate bacterial adhesion and plaque composition characteristics. Acquired pellicle was analyzed by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Single colony isolated groups on samples were identified by using BBL-Crystal System. Statistical analysis was performed by using Kruskal Wallis Analysis of Variance (ANOVA).

Results: Acquired pellicle analysis by SDS-PAGE revealed amylase content for each group except feldspathic porcelain. Significant qualitative and quantitative differences were found among the materials for bacterial plaque content. Microorganism types varied most for feldspathic porcelain surface. The least variety of microorganisms were found on Cr-Ni. The lowest adhesion was observed on feldspathic porcelain.

Conclusion: Initial bacterial plaque shows material specific differences for compositional structure. Bacterial adhesion for each material shows internal compositional variations. Streptococci were found to be most revealed species for all materials studied.

Keywords: Bacterial colonization, prosthetic materials, SDS-PAGE, adsorption, dental plaque

ÖZET

Amaç: Bu çalışmanın amacı bakteriyel plak yapısını nitel ve nicel olarak değerlendirmek ve restoratif materyal çeşitliliğinin pelikül kompozisyonu ve bakteriyel adezyon üzerine etkisini incelemektir.

Gereç ve Yöntem: Titanyum, Au-Ag-Pt alaşımı, Ni-Cr alaşımı, feldspatik porselen ve bir tam seramik sistemi kullanılarak materyaller arası farklılıklar incelenmiştir. Bu amaçla dairesel olarak hazırlanmış örnekler oral kavitede 2 ve 48 saatlik sürelerle tutulmuştur 2 saat bekletilmiş örnekler üzerindeki kazanılmış pelikül SDS-PAGE yöntemi ile incelenmiştir. Bakteriyel plak analizi öncesi her bir örnek grubu steril edilmiştir. Örnekleri taşıyan alt tam protez 48 saat süreyle ağız içerisinde tutulduktan sonra örnekler 5 ml steril serum fizyolojik solüsyonu içeren tüplere yerleştirilmiş ve kültür elde edilmiştir. Görsel sayım ve mikroskopik ayırıştırma sonrası izole edilmiş olan tek koloni grupları BBL-Crystal sistemi ile tanımlanmıştır. İstatistiksel değerlendirme için Kruskal Wallis Varyans Analizi uygulanmıştır.

Bulgular: SDS-PAGE analizi feldspatik porselen hariç her grupta amilaz içeriği tespit etmiştir. Bakteriyel plak içeriği açısından materyaller arasında istatistiksel olarak belirgin farklılıklar görülmüştür. Mikroorganizma tipleri en büyük çeşitliliği feldspatik porselen üzerinde sergilemiştir. En az çeşitlilik ise Cr-Ni grubunda görülmüştür. En düşük miktarda adezyon da

feldspatik porselen üzerinde izlenmiştir.

Sonuç: Başlangıç bakteriyel plak içeriği materyale özgü farklılıklar sergilemektedir. Her bir materyal için bakteriyel adezyon yapısal çeşitlilikler göstermektedir. Tüm materyal gruplarında en çok görülen tür streptokoklardır.

Anahtar kelimeler: Bakteriyel kolonizasyon, protetik materyaller, SDS-PAGE, adsorpsiyon, dental plak

INTRODUCTION

Bacterial dental plaque is a deposit of microorganisms embedded in an organic intercellular matrix,¹ and the major etiologic factor in dental caries and periodontal diseases.² Many studies showed plaque carrying subgingival restorations happen to cause severe inflammation and loss of attachment.³⁻⁵

Plaque development begins with the adsorption of salivary glycoproteins onto tooth surfaces.⁶ This newly formed layer is called "acquired pellicle". Acquired pellicle formation presents the initial stage of plaque formation on exposed teeth and material surfaces. It consists of salivary proteins and acellular bacterial enzymes and acts as a medium for bacterial adherence.⁷ Microorganism adherence begins immediately after pellicle coating.^{8,9} It has been shown that composition of the plaque on natural teeth and restorations do not differ principally.¹⁰⁻¹² Adsorption of acquired pellicle depends on saliva composition and surface properties of adsorbant material. Salivary glycoproteins adsorb to chemically different surfaces selectively and microorganisms bind to specific proteins similarly.^{6,9} So various proteins can dictate which bacteria adhere first and which species develop the plaque. It was shown that qualitative and quantitative variations in acquired pellicle do affect plaque composition and its final pathogenesis.¹³⁻¹⁵ Bacterial plaque amount and composition demonstrates differences related to the restorative material.^{11,16-18}

The aim of this study was to determine the structure of bacterial plaque qualitatively and quantitatively and to evaluate the effect of restorative material type on pellicle composition and bacterial adhesion.

MATERIALS AND METHOD

1. Materials

Titanium (Goodfellow Cambridge Limited, Huntington, England), Au-Ag-Pd alloy (Argenco 58; Argen CO., San Diego, California, USA), Ni-Cr alloy (Remanium G soft; Dentaurum, Pforzheim, Deutschland), an all-ceramic system (IPS Empress 2; Williams/ Ivoclar, Amherst, NY, USA) and a feldspathic porcelain system (Ceramco; Ceramco INC, Burlington, NJ, USA) were used to evaluate bacterial adhesion and plaque composition of various dental restorative materials.

Ten samples of each material were made in disc form 5 mm in diameter and 1 mm thick. Titanium discs were obtained from manufacturer in same dimensions. Ceramic samples were produced at the same size according to the manufacturer's recommendations. IPS Empress, Ni-Cr and gold alloy samples were made by casting of disc shaped wax patterns. All samples were ground with 400-4000 grit silicone carbide papers (Microcut, Buehler, USA). Feldspathic porcelain and IPS Empress samples were glazed.

2. In-vivo pellicle formation and analysis

Acquired pellicle and bacterial plaque were formed in vivo for this study. An acrylic base plate with five round holes was prepared to be seated on mandibular arch of 34 years old, healthy, edentulous, male volunteer. Samples were placed on the trial denture to be contaminated on both sides (Figure 1).



Fig. 1. Samples were placed on the trial denture to be contaminated on both sides

Pellicle formation was achieved after 2 h insertion period during when no food or drinks were allowed. Five discs per one group were held in the mouth for one session and this procedure was repeated for each group. After 2 h discs were removed gently and placed in eppendorf tubes containing 2 ml of distilled water and afterwards discs were washed with double distilled water to eliminate non-adsorbed proteins. Discs were then stored in 0.2 M EDTA containing 0.05 M ammonium bicarbonate solution for 48 hours at 4°C to set all adsorbed proteins free efficiently. Supernatant were then dialysed against double-distilled water for 24 hours and aqueous samples lyophilised at -70°C for another 24 hours before suspending in 1 ml. PO4 buffered saline solution.

3. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were then evaluated by SDS-PAGE for determination of protein and/or amylase contents. 7.5 % separating gel and 4% loading gel were used. Loading volume for samples was 10 µl. Samples were diluted 1:1 with loading buffer prior applying to gel. Samples were denatura-

ted at 95°C for 5 minutes. Then applied to the grooves of gel by Hamilton tube. The samples were run at 120 V for 1 h until they passed the stacking gel. As bromphenol blue transversed the gel, process paused and gel inserted to appropriate solution for staining.

Protein bands formed after staining of the gel were compared with the standard's protein bands and were thoroughly examined. Bradford method was used to determine the bands with high protein content. Bovine serum albumine was used as standard.

4. In vivo bacterial plaque colonization and analysis

Ten samples were attached to the right and left buccal flanges of lower denture by using self-cured acrylic resin (Figure 2).



Fig. 2. Samples were attached to the right and left buccal flanges of lower denture by using self-cured acrylic resin.

Prior to the procedure each of the sample group were sterilized. Sample bearing denture was hold in the mouth for 48 hours. During this period the patient was told not to remove his denture, use any medication or perform any cleaning procedure.

Samples were gently removed after 48 hours by using a sterile curette and immediately placed into 5ml sterile saline containing tubes (Venoject, Terumo- Europe, Leuven, Belgium) separately. All 50 sample containing tubes were vortexed at 2200 rpm for 1 minute to achieve the releasing of bacterial content to the solution. Each tube content were diluated with phosphate buffered saline (PBS) 10 times and 100 times respectively.

100 µl sample was taken by a micropipette and injected into 900 µl PBS containing tubes for this process. Afterwards another 100 µl sample were taken from this diluated solution to be injected to 900 µl PBS containing tubes for 100 times diluation. 100 µl sample from this diluations were cultivated in Mitis-Salivarius (Difco laboratories, Detroit, Mich) and 5% blood agar (Merck AG, Darmstadt, Germany) mediums.

Colonies were visually defined and samples of each colony were studied microscopically after gram staining.

After quantitative assessment of visually and microscopically differentiated groups were subcultured and isolated.

5. Identification of bacteriae

Single colony isolated groups were identified by using BBL-Crystal System(BBL Crystal, ANR, GP, Rgp, ID. Becton, Dickinson and Co. Sparks, Shamon, Ireland). Scanning procedure was performed by appropriate software and computer and each of the colonies were identified with 99.8% accuracy.

Statistical Analysis

Statistical differences were determined by Kruskal-Wallis Analysis of Variance.

RESULTS

1. Biochemical results

SDS-PAGE analysis

One major Coomassie blue-stained protein band of α -amylase with corresponding molecular weight of 34-35 Kd was detected in all samples except feldspathic porcelain (Figure 3). The largest amount of α -amylase was determined on Cr-Ni alloy. Amylase deposition was found most for CrNi. Results were confirmed with Bradford Protein Assay (Table 1).

Table 1. Bradford method results of protein amounts

Sample	Protein amount (µg/ml extract)
III (CrNi)	15.9
IV (titanium)	9.63
V (AuAgPd)	7.22
VI (feldspat. Por.)	4.33
VII (IPS Empress)	9.5

2. Microbiological analysis

Results are shown on Table 2.

Table 2. Mean colonial distribution per material (x105)

	CrNi		AuAgPd		IPS Empress		Titanium		Feldspathic Porcelain	
	x	SD	x	SD	x	SD	x	SD	x	SD
S.salivarius	3,68	3,68	1,68	2,00	5,33	9,43	1,13	0,83	7,58	15,95
S.sanguinis	11,30	18,88	0,90	1,92	4,55	5,52	12,00	8,55	19,18	43,57
S.vestibul.	9,03	13,36	3,38	5,15	4,18	3,54	12,00	8,55	11,62	48,23
S.oralis	-	-	32,90	22,00	-	-	-	-	8,84	20,22
S.parasang.	-	-	2,75	2,87	1,80	2,92	-	-	5,76	10,32
L.pseudoms.	-	-	-	-	0,43	0,82	-	-	10,00	21,79
Staf.epiderm	-	-	9,35	11,51	-	-	-	-	10,00	43,59
Micrococcus	0,50	1,57	0,15	0,37	0,10	0,31	0,42	0,69	0,11	0,32
S.sanguis	6,75	11,48	1,55	2,06	13,75	18,00	2,79	2,49	-	-
S.intermedius	-	-	-	-	-	-	23,32	21,77	0,05	0,23
C.diphtheria	-	-	-	-	-	-	0,35	0,49	-	-
Staf.warnerii	-	-	-	-	0,30	0,47	-	-	-	-

Cr-Ni alloy

S.sanguinis showed significantly higher adhesion comparing other microorganisms. Micrococcus adhesion was found significantly lower than other microorganisms

($p < 0.001$). Other bacteriae showed similar adhesion onto Cr-Ni alloy surface.

Au-Ag-Pd alloy

S.oralis was found to adhere most and *Micrococcus* was found to be least. *S.oralis* showed significantly higher adhesion. The difference between *S.oralis* and *Staphylococcus epidermidis* was insignificant ($p < 0.001, X^2 = 59.026$).

IPS Empress

S.sanguis showed the highest adherence and *Micrococcus* the least. *S.sanguis* adherence was significantly higher than the other microorganisms ($p < 0.001, X^2 = 77.137$).

Titanium

S.intermedius showed the highest degree of adhesion, followed by *S.salivarius*, *S.sanguinis*, *Micrococcus*, and *S.sanguis*. *C.diphtheria* revealed the least adhesion ($p < 0.001, X^2 = 69.913$).

Feldspathic porcelain

S.sanguinis adhesion was found to be the most and *S.intermedius* was the least. *S.sanguinis* adhered to porcelain surface significantly higher than the other microorganisms found ($p < 0.001, X^2 = 70.255$).

DISCUSSION

First stage of bacterial plaque formation on restorative materials is adsorption of acquired pellicle on the material surface. Acquired pellicle is saliva originated and adsorption phenomenon depends on the composition of saliva and surface chemistry of adsorbant material.¹⁹ The biofilm formed on restorative material is structurally similar to biofilm formed on tooth surface. However, differences in plaque compositions for various restorative materials have not been clearly described. Importance of acquired pellicle is that it allows selective adherence of oral bacteria that are responsible for dental plaque formation.⁹

Initial bacterial adhesion to the acquired pellicle on tooth surfaces are affected from receptor-adhesin interactions based upon stereochemical specificity.^{9,18} First adherents of acquired pellicle are gram-positive cocci, especially *Streptococcus sanguis* and *Streptococcus mitior*.^{9,11} As plaque ages; gram-positive rods begin to dominate the flora. Bacterial population diversifies in 12 hours and *Actinomyces*, *Capnocytophaga*, *Haemophilium*, *Prevotella* and *Veillonella* species added to the early colonizers. These so called "late colonizers" are bound to early colonizers by *Fusobacterium* bridge.⁹

Results of this study are in accordance with previous studies proving the Streptococcal dominance in plaque composition. Also, results of this study demonstrating *Staphylococcus epidermidis* in plaque composition, are supported by previous literature.²⁰⁻²³

Steinberg and Eyal²³ demonstrated *S. sobrinus* content in the initial bacterial plaque formed on composite and

amalgam. Scannapieco et al.²² investigated *S. sanguis* adhesion related to α -amylase and showed *S.sanguis* existence in plaque. Schilling et al.²⁰ and Schilling & Bowen²¹ reported *S.mutans* and *S.sobrinus* adhesion on the glucan coated pellicle in situ. Gabriel et al.²⁴ showed *Staphylococcus epidermidis* adhesion onto Ti alloy surfaces.

In similar in vitro studies, it has been stated that effect of experimental pellicle was different from natural pellicle.^{16,25-27} The lack of enzymatic activity in vitro was found to be the reason for the difference, because this enzymatic activity plays an important role in pellicle adsorption and bacterial adhesion.^{1,16,28} In this study, the method described by Wise & Dykema²⁹ and Nakazato et al.³⁰ was used for pellicle and bacterial plaque formation. But in this study samples were placed on the mandibular denture not palate because saliva pooled in mouth floor contains all secretions from parotid, submandibular and sublingual salivary glands.^{3,31,32}

Different methods were introduced for determination of the molecular components of acquired pellicle. These methods are amino acid analysis (random arbitrarily primed PCR, transposane mutagenesis), immunological methods, gel infiltration ion exchange chromatography, histochemical staining, electrophoresis and blast search network. In this study protein content of pellicle was determined by using Laemmli's method³³ of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Bradford Protein Assay. SDS-PAGE was chosen for its accuracy and reliability.³⁴⁻³⁹

The greatest content of saliva is amylase. It was shown that, *Streptococci* have high affinity to amylase and bind specifically.^{22,40-42} It was found that developed dental plaque includes large quantities of amylase binding streptococci⁴³.

The interaction among bacterial adhesion-pellicle-material was investigated repeatedly.^{1,13,44-48} Besides that many authors have been studying the effect of hydrophobic interactions, zeta potential, porosity on bacterial accumulation for many years.^{3,49-53}

Bacterial plaque accumulation was considered from a different point of view in this study. Composition of bacterial plaque formed on various materials was evaluated from chemical and microbiological aspects in details.

The relation between material type and bacterial plaque accumulation was evaluated first by Clayton and Green in 1970. In the study gold, acrylic resin and glazed ceramics were used and insignificant differences in plaque retention amongst the above mentioned materials were found.⁵³

Ramberg et al.⁵⁴ evaluated bacterial colonization during de novo plaque formation and actinomyces species were shown to be the most dominant group (% 50) for day 0. For the following 4 days none of the actinomyces subgroups

increased significantly. However, it was found that some *streptococci* species like *capnocytophagae*, *campylobacteriae*, *fusobacteriae* and *A. actinomycetemcomitans sp.* increased quantitatively by time.

Wollinsky et al.⁵⁵ found that *S.sanguis* adhesion to saliva coated titanium is similar to the adhesion onto saliva coated enamel. However, *A.viscosus* adhesion on titanium surface was less than enamel. According to the results of this study, the adhesion of *A. viscosus* on the titanium surface was found less than the adhesion on the enamel surface, too.

Edgerton et al.⁵⁶ demonstrated increased *S.sanguis* and *S.gordonii challis* adhesion on the titanium coated with experimental pellicle. Data obtained from this study proves the bridging role of saliva pellicle components for adhesion of certain *streptococcus species*. Other researchers showed both of these bacterial species to have specific receptors for salivary amylase and proline-rich proteins.²²⁻³⁵ Edgerton et al.⁵⁷ showed proline rich proteins and salivary amylase as precursor components for pellicle on titanium in vitro and those molecules might be responsible for in vivo adhesion of same bacteriae.

In this study α -amylase was found on all of the surfaces except feldspathic porcelain. Additionally it was shown that *S.sanguis* adheres onto all of the surfaces except porcelain. This result gives an idea of a correlation between *S.sanguis* adhesion and α -amylase. More investigations are needed to explain this issue.

CONCLUSIONS

1.Acquired pellicle content showed similarity for Cr-Ni, Au-Ag-Pd, Ti alloys and IPS Empress, but SDS-PAGE revealed no α -amylase in the biofilm formed on feldspathic porcelain.

2.Composition of initial bacterial plaque showed material specific differences. Nine types of microorganisms were determined on the feldspathic porcelain, five types of microorganisms were determined on Cr-Ni alloy.

Microorganism species determined on the other material surfaces varied between feldspathic porcelain and Cr-Ni alloy.

3.Streptococci were determined on all of the materials tested. *L. Pseudomesenteroides*, *Staf. warnei*, *Staf. epidermidis* and *Corinobacteria* were found to be material specific.

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