Adsorption of Salivary Proteins on Titanium Implants

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I. Introduction

Saliva has several important functions in the oral cavity including modulation of oral microflora by favoring certain microorganisms[1], and protecting oral tissues from acids and degradative enzymes [2,3].

Salivary pellicles are thin, continuous cuticle of which components play an important role in bacterial adhesion to host surfaces. An early pellicle which formed within 2 hours contains mucins, α-amylase, sIgA, lysozyme, cystatins, and proline-rich proteins (PRPs)[4-6]. In addition to components originating from salivary glands, pellicles also contain non-salivary gland origin molecules including albumin[4,8], and bacterial products such as the glycosyltransferase of S. mutans[10].

Mucins are high-molecular-weight glycoproteins that contain greater than 40% carbohydrates. Two chemically distinct mucins have been identified in human submandibular-sublingual saliva, designated mucin-glycoprotein 1(MG1) and mucinglycoprotein 2(MG2)[11]. Mucins play a role in lubrication, tissue coating, digestion, and microbial interactions with bacteria, fungi, and viruses[12].

Amylase, one of the most prevalent enzymes in the saliva, is generally considered to be a reliable marker of serous cell function. In addition to enzyme activity amylase coats the oral tissues, binds to streptococci, has anti-microbial activity and is involved in the selective clearance and adherence of microorganisms[4,13].

sIgA is thought to participate in the local disposal of environmental antigens by providing an immunologic "first line of defense" in the oral cavity. The Local sIgA antibody can play a role in viral neutralization, attenuation of viral growth and replication within oral tissues and effect neutralization and disposal of toxins and food antigens[13].

PRPs are comprised of acidic and basic phosphoproteins and basic glycoproteins that are characterized by an amino acid content containing 75 to 80% proline, glutamine, and glycine[14]. These proteins can bind calcium and have a high affinity for hydroxyapatite[15]. While there is ample evidence for a role of PRPs as pellicle receptors for microbial adhesion, other, more subtle colonization mecha-

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nisms may also involve these molecules.

Ti implants may provide a unique microenvironment in the oral cavity for the colonization of bacteria, and this microenvironment differs from that provided by natural teeth. Clinical studies suggested that a peri-implant disease may represent a different disease process from that of natural teeth in the composition of pathogenic microorganisms as well as the contributions of these microbial pathogens to the bone[16,17].

To the best of our knowledge, there was not a report of the salivary pellicle adsorption on the commercially available plasma sprayed Ti implant compared to that of the enamel. The purpose of this study was to characterize the pellicle that forms on enamel and plasma sprayed Ti implants *in vivo*.

II. Materials and Methods

1. Subject

A healthy male volunteer aged 36 was chosen for this *in vivo* experiment. There were neither untreated carious lesions, nor insufficient restorations. He was in good systemic condition and oral health with fully dentulous state. He did not receive any medication for the last 6 months. At the outset of the tests, the subject received a professional tooth cleaning.

2. Material samples

1) Enamel slabs preparation

Teeth without clinically detectable carious lesions were obtained. The Enamel slabs were prepared by sectioning the crowns with a diamond drill (Pfingst & Company, USA.) and low speed saw (Buehler Isomet, USA). Prepared enamel slabs were autoclaved and embedded in orthodontic acrylic. Six templates containing 28 enamel slabs were prepared and the average enamel surface area of a template was measured. The total surface area of 28 exposed enamel slabs was 846mm^2. The average surface area of the enamel slab was approximately 30.2mm^2.

2) Implant fixtures

The used implant fixtures were obtained from ITI (Straumann Co., Switzerland), which were composed of a machined surface in the neck area and rough surface body and generated by a Ti plasma spray coating. Eight implant fixtures were used for this study.

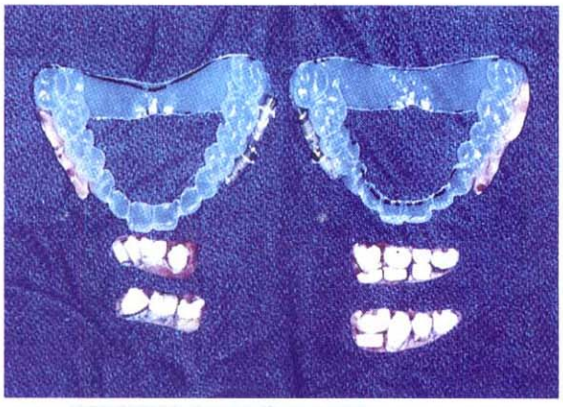

Figure 1. Splint with enamel slab and implant fixture

3) Preparation of splint

Splints were fabricated by using vacuum forming machine(STA-VAC, Buffalo Dental Mfg Co, USA) with 0.060 inch thickness clear sheet resin. Prepared enamel slab template and implant fixtures were attached to either buccal side (Figure 1).

3. Pellicle Collection

Saliva collection was performed from 9:30 A.M. to 11:30 A.M. to minimize the effects of diurnal variability in salivary composition. Before wearing, enamel slabs were cleaned with a tooth brush with paste and polished with fine pumice. The splints were held in the mouth for 2 hours and the salivary pellicle was then allowed to be laid down. During the experiment, no food or drinks, except water were allowed. At the end of 2 hours, the acrylic templates were separated from the splint, rinsed with distilled water, air dried and kept on ice.

The pellicles were collected as described by Sonju and Rolla.[59] Briefly, the exposed enamel surfaces were scraped with a Gracey curette and pellicles were collected on a glass-wool plug adapted into a disposable Pasteur pipette connected to an aspirator of a dental unit, The glass-wool plugs containing the pellicle were suspended in 1.0 ml of distilled water, vortexed for 60 seconds and centrifuged at 225g for 20 min at room temperature. The clear supernatant was transferred to a 15ml Pyrex centrifuge tube and lyophilized. Ti implants were separated from the splint and washed to remove unbound salivary components. The implants were put into 4ml of distilled water and vortexed for 1 min. Distilled water with pellicles from enamels and Ti implants was transferred to a flask and frozen and then lyophilized(Figure 2).

Figure 2. Lyophilized pellicle in flask

4. Saliva Collection

Stimulated saliva samples were collected from the same donor using 2% citric acid. Human parotid saliva (HPS) was collected using a modified Carlson-Crittenden cup, placed directly over Stensen's duct orifice. Human submandibular-sublingual saliva (HSMSL) was collected using a custom-fitted device consisting of a universal saliva collector held in place by a silicone stent (Kerr). Whole saliva was collected by direct expectoration into a tube. Each saliva sample was collected into chilled centrifuge tubes and centrifuged at 10,000 × g for 1 min at 4℃ and stored at -50℃ until used.

5. Characterization of the pellicle components

1) Polyacrylamide Gel Electrophoresis (PAGE)

Each pellicle components were separated on the basis of their molecular weight and charged using 12.5% SDS-PAGE. 20 ㎕ of each of the samples were poured onto the gel and electrophoresis was done at 200V for 45 min. The proteins were stained with Coomassie staining, followed by destaining with 7% acetic acid solution and 10% methanol at room temperature.

2) Western Blot Analysis

Following the separation of the protein on SDS-PAGE, proteins were further analyzed by immunoblotting. Proteins were transferred onto a nitrocellulose membrane by an electrophoretic transfer as previously described[18]. Western transfer was performed at 0.4 mA/cm^2 for 3 hours. Subsequently, the blot was treated with a blocking buffer for 2 hours and kept overnight in fresh blocking buffer containing proper dilution of primary antibody. Unbound antibodies were removed by washing with blocking buffer containing 1.0 M NaCl. Antigen/antibody complexes were incubated with a 1:2,500 dilution of alkaline phosphatase conjugated affinity purified goat anti-rabbit IgG (Bio-Rad Laboratories, USA). Antiserum used in this study were rabbit anti-human submandibular-sublingual saliva, rabbit anti-human salivary amylase, rabbit anti-human sIgA, rabbit anti-human lactoferrin and generously donated from Dr. Al-Hashimi (Baylor College of Dentistry, USA).

6. Scanning Electron Microscopy

Pellicle samples were fixed in 10% buffered formalin for 24 hours. Then they were rinsed with the buffer and dehydrated in a graded series of ascending concentrations of ethanol (50%, 70%, 90%, and 100%). Once dehydrated, the samples were critical point dried (Sorvall critical point drying system, USA), mounted on aluminum stubs with silver paste, and sputter coated with 20 nm of a gold/palladium mixture using a Denton DV-502 Sputter Coater. The morphological structures of the pellicle samples were analyzed at 15 kV and micrographs were taken using a scanning electron microscope (JEOL JSM 35-CF, USA).

III. Results

1. SDS-PAGE

On 12.5% SDS-PAGE stained with Coomassie brilliant blue showed selective nature of protein adsorption according to the sources(Figure 3). Human whole saliva(HWS)revealed thin bands at 49 kDa, 27.5 kDa and another thin one below 18.5 kDa(lane 2). In lane 3, human submandibular-sub-

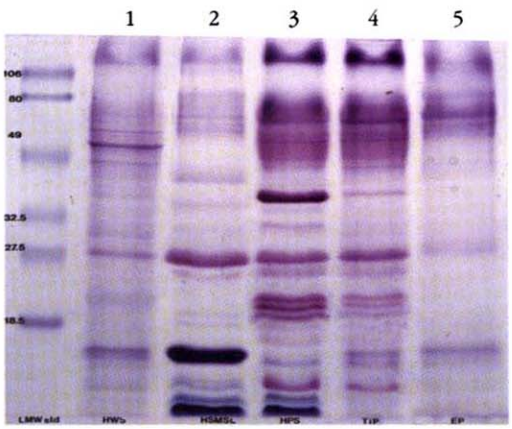

Figure 3. 12.5% SDS-PAGE stained with Coomassie briliant blue, (Lane 1 : Low molecular weight standard(LMWstd), Lane 2 : Human whole saliva (HWS), Lane 3 : Human submandibular-sublingual saliva (HSMSL), Lane 4 : Human parotid saliva (HPS), Lane 5 : Ti pellicle (TiP), Lane 6 : Enamel pellicle(EP))

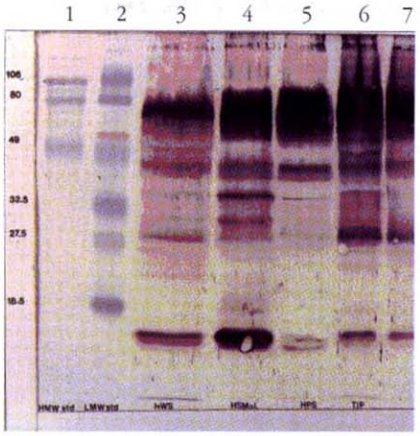

Figure 4. Western blot analysis of pellicle using rabbit anti-HSMSL Antiserum (Lane 1 : High molecular weight standard(HMW std), Lane 2 : LMW std, Lane 3 : HWS, Lane 4 : HSMSL, Lane 5 : HPS, Lane 6 : TiP, Lane 7 : EP)

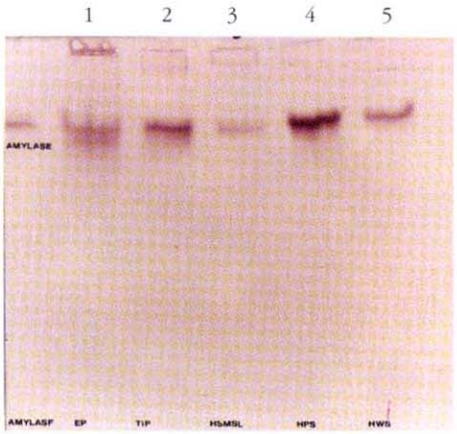

Figure 5. SDS-PAGE/Western transfer anaylsis of pellicle using rabbit anti-salivary amylase. (Lane 1 : Amylase,Lane 2 : EP, Lane 3 : TiP, Lane 4 : HSMSL, Lane 5 : HPS, Lane 6 : HWS)

lingual saliva(HSMSL) showed different band density from HWS at the same M.W. but lack of 49kDa band. Human parotid saliva(HPS) has a unique thick band between 32.5 to 49 kDa and triplets around 18.5 kDa(lane 4). In Ti pellicle(TiP), all protein components had similar patterns to HPS with lesser density(lane 5). Enamel pellicle(EP) has a thin band around 80 kDa and no triplets' band around 18.5 kDa. Though the existence of salivary protein adsorption was successfully detected with SDS-PAGE, adsorbed with Coomassie brilliant blue, however, it was necessary to analyze immunochemically to give a more exact identification of proteins.

2. Identification of the salivary components by Western blotting

Western blot with anti-salivary amylase showed

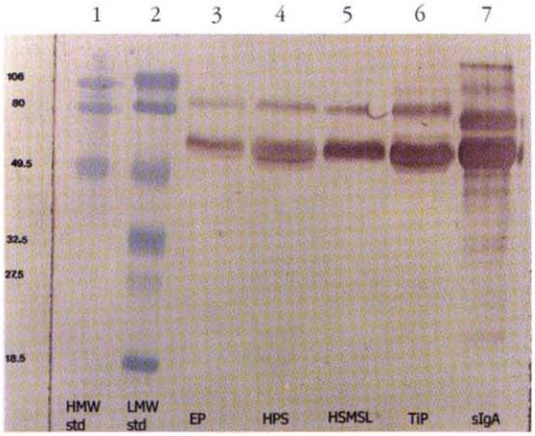

Figure 6. SDS-PAGE/Wester transfer anaylsis of pellicle using rabbit anti-sIgA sera. (Lane 1 : High molecular weight standard(HMWstd), Lane 2 : LMWstd, Lane 3 : HWS, Lane 4 : HPS, Lane 5 : HSMSL,Lane 6 : TiP, Lane 7 : sIgA)

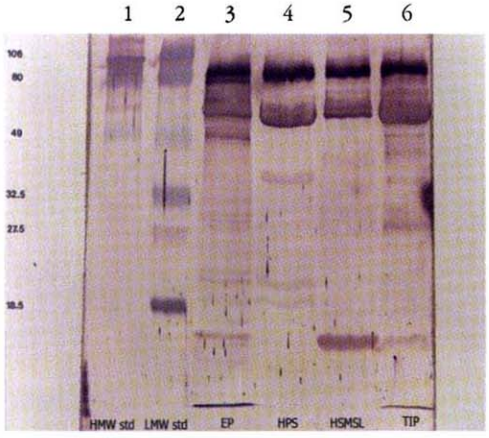

Figure 7. SDS-PAGE/Western transfer anaylsis of pellicle using rabbit anti-latoferrin sera. (Lane 1 : HMWstd, Lane 2 : LMWstd, Lane 3 : HWS, Lane 4 : HPS,Lane 5 : HSMSL, Lane 6 : TiP)

that each of the samples had different amylase contents. HPS had most distinct affinity following TiP and HWS. EP and HSMSL had a similar affinity and there were quite distinct patterns between TiP and EP(Figure 4) Adsorption affinity of amylase revealed that HPS had the highest density following TiP and HWS. EP and HSMSL had similar density and there were quite distinct patterns between TiP and EP(Figure 5) Using anti-sIgA antibody, all samples reacted as distinct bands, however, TiP and HSMSL had a similar affinity with higher density than those of EP and HPS(Figure 6). Adsorption affinity of lactoferrin turned out in all test samples around 80 kDa M.W. and there could not be seen any different affinity among them(Figure 7).

3. SEM

Figure 8 showed the enamel slabs before and after the 2 hour experiment. There were distinct scratches on the enamel surface which occurred

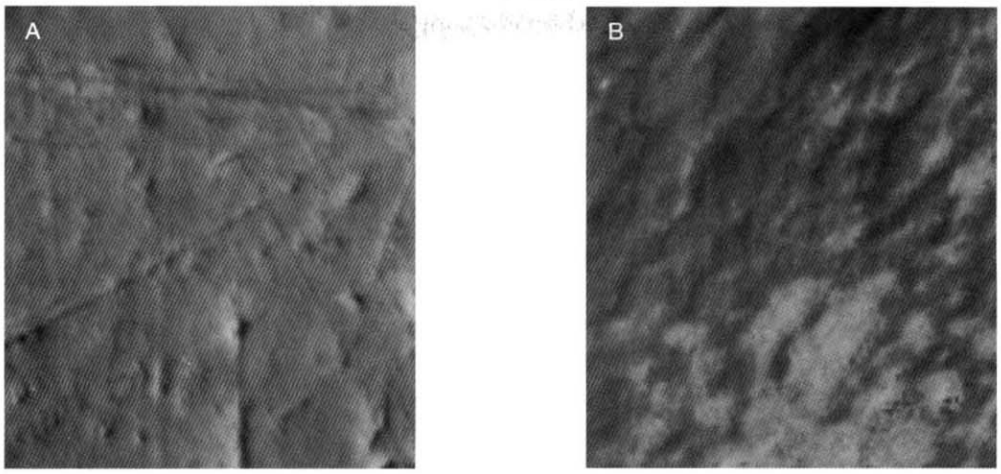

Figure 8. Scanning electron micrographs of enamel slab. Enamel surface with distinct scratch which occurred during pellicle collecting procedure with curette was seen(A). After 2 hour, whole enamel surface was covered with newly formed homogenous pellicle(B).

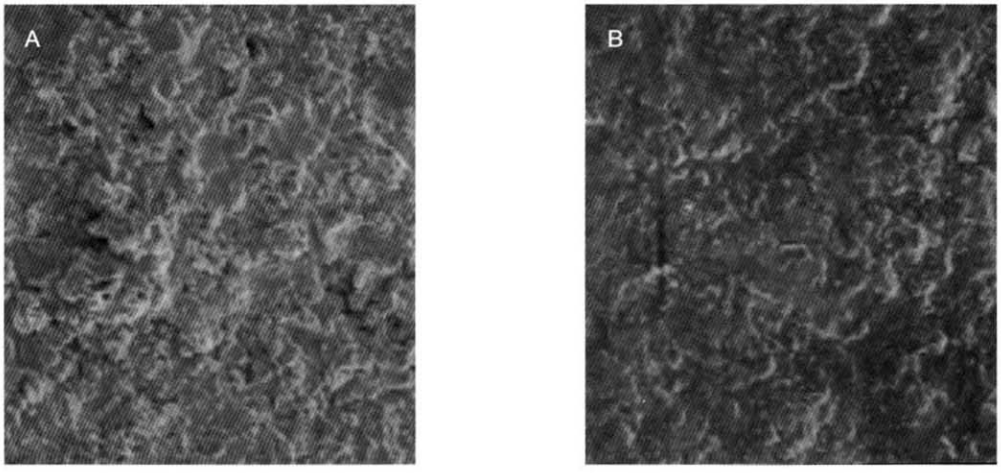

Figure 9. Scanning electron micrographs of Ti implant. , Adsorbed pellicle overlaid the plasma sprayed irregular surface after 2 hour(A: before experiment, B: after 2 hour experiment).

during the pellicle collecting procedure with curette(A). After 2 hours, the whole enamel surface was covered with a newly formed pellicle(B). In the Ti implant specimen, the 2 hour adsorbed pellicle overlaid the plasma sprayed irregular surface(Figure 9). Neither specimen revealed distinct bacterial accumulation, if any, some cocci and rod on enamel slabs only(data are not shown).

IV. Discussion

Alterations in pellicle surface lead to changes in early bacterial attachment at the surface, and in the relationship between the bacteria constituting the plaque. Recently, Kohavi and colleagues found that the amount of salivary proteins adsorbed onto the Ti was less than the amount adsorbed onto the enamel

using the machined Ti and enamel disc *in vivo* [19]. To the best of our knowledge, there was no report of the salivary pellicle adsorption on the commercially available plasma sprayed Ti implant comparing with that of the enamel. Plasma spray-coating is one of the most common methods for surface modification for enhancing osseointegration[20], however, when exposed to the oral cavity, bacterial accumulation can occur easily which results in ailing or failing implants.

We compared the protein component of human saliva and pellicles adsorbed on Ti and enamel using 12.5% SDS-PAGE, and found that selective nature of protein adsorption according to the sources(Figure 3). In Ti pellicle(TiP), all protein components had similar patterns to HPS with lesser density(lane 5). Enamel pellicle(EP) has a thin band around 80 kDa and no triplets' band around 18.5 kDa compared to human parotid saliva. Though the existence of salivary protein adsorption was successfully detected with the SDS-PAGE, adsorbed with Coomassie brilliant blue, however, it was necessary to analyze immunochemically to identify proteins more exactly.

Jenkins had suggested that the vivo enamel pellicle was derived early, primarily from HSMSL[21]. Therefore, we used Western transfer analysis with rabbit anti-HSMSL to compare the salivary components of *in vivo*. Our data also showed that the blotting pattern of TiP and EP had closer similarity to HSMSL than HPS.

Amylase exists in saliva with high concentration and it binds not only to hydroxyapatite but also promotes the adherence of pathogenic microorganisms[12]. Although the enzymatic activity of amylase undoubtedly plays a role in carbohydrate digestion, the digestive role of salivary amylase is thought to be minor at best, because of the relatively short time that food actually remains within the oral cavity. Amylases bound to bacteria in plaque facilitate dietary starch hydrolysis to provide additional glucose for metabolism by plaque microorganisms in close proximity to the tooth surfaces. The resulting lactic acid produced may be added to the poll of acid in plaque to contribute to dental plaque and caries formation. In our experiment, each of the samples had different amylase contents. HPS had the highest density following TiP and HWS. EP and HSMSL had similar density and there were quite distinct patterns between TiP and EP(Figure 5).

Secretary IgA, which provides a first line of defense via immunological means[22], showed outstanding variation of pellicle adsorption patterns between HSMSL and HSP samples in our qualitative analysis. In our experiment using anti-sIgA antibody, all samples reacted as distinct bands, however, TiP and HSMSL had higher density than those of EP and HPS(Figure 6). Lee and colleagues found that the salivary pellicle adsorbed on orthodontic materials in quite different patterns and showed the similar Western blotting configuration to our data[23].

Lactoferrin is a protein found naturally within biological fluids, such as milk and saliva, at mucosal surfaces and within white blood cells. Lactoferrin binds iron, which is an important nutrient factor for many microbial species[24]. It also displays bacteriostatic and/or bactericidal activity towards several microbial species[25,26]. In our experiment, the absorption affinity of lactoferrin turned out in a similar pattern of all test samples around 80 kDa M.W.(Figure 7).

In order to identify the changes of surface configuration of Ti and enamel after a 2 hour saliva adsorption, the SEM study was performed. We found a few cocci and rods forming bacteria on some enamel specimen (data are not shown), however, none were present on the Ti implant and the other enamel surface. As the early colonizer can be

found on the enamel surface only after 4 hour, we could not see them for the 2 hour experiment[27]. The adsorbed pellicle laid over the surface with relatively homogenous appearances in the Ti and enamel specimen.

Taken together, within the limit of our experiment, our data underlies that the Ti pellicle had unique salivary protein components compared to the EP, and possesses more antibacterial component in the pellicle such as amylase, sIgA and lactoferrin, which dictates the sequence of bacterial adherence in the developing plaque.

V. Conclusions

In order to understand the nature of pellicles adsorbed onto the plasma sprayed Ti implant *in vivo*, we compared the salivary components with the pellicle components on the enamel by SDS-PAGE and Western blot analyses. The SEM assessment was also conducted to identify the surface configuration changes. The results can be summarized as follows:

1. The Ti implant pellicle exhibited a unique protein banding pattern which shares similarities with HWS, HPS and HSMSL.
2. Salivary proteins exhibited unique selectivity in their adsorption to enamel and Ti surfaces.
3. While amylase, sIgA and lactoferrin existed both in TiP and EP, the blotting intensity was different.
4. A pellicle absorbed for two hours laid homogenously onto the Ti implant surface and there could not be seen a prominent bacterial accumulation.

Within the limit of our experiment, the results from the present study lead to the conclusion that experimental pellicles formed on plasma sprayed titanium surfaces differ qualitatively from those reported on the enamel *in vivo*.

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-국문초록-

타이태늄 임플란트 표면에 형성된 타액성 단백질에 관한 생체연구

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치과용 임플란트 실패의 주요 원인은 임플란트 표면에 부착되는 세균의 침착의 결과로 생기는 임플란트 주위염이다. 구강 내에서 세균성 치태의 침착은 치태가 부착하는 기질 표면의 물리적 성상과 타액성 피막의 성분에 영향을 받으며 형성된 피막의 유기질 성분의 차이가 치태의 성분과 병원성에 영향을 미친다. 최근 연구에 의하면 생체재료의 표면에 침착되는 치태세균은 사용되는 재료에 따라 특이한 세균 침착을 보이며 이는 초기 타액성 피막의 차이에 의한 것으로 알려져 있다. 이 연구의 목적은 플라즈마분사법으로 표면 처리된 타이태늄 임플란트에 흡착되는 타액성 단백질 피막의 특성을 정성적인 방법으로 분석하는 데 있었다.

법랑질 조각과 플라즈마분사법으로 표면 처리된 타이태늄 임플란트를 스프린트에 치실을 이용하여 연결한 장치를 구강 내 장착하여 2시간 동안 피막이 침착되게 한 후 피막을 분리 추출하여 냉동 건조시켰다. 재수화 과정을 거치고 나서 전기영동법과 Western transfer 분석을 통해 단백질 성분에 관한 분석을 시행하였다. 사람의 총 타액과 이하선 타액 및 악하선-설하선 타액을 수집기를 이용하여 채취하고 같은 방법으로 처리한 후 성분분석을 실시하였다. 피막 흡착 전후의 표면변화를 주사전자현미경을 이용하여 관찰하였다.

실험결과 타이태늄 임플란트에 흡착된 피막은 법랑질 표면의 피막과는 다른 단백질 성분을 가지고 있었으며, 주로 악하선-설하선 타액에서 유래하였다. 임플란트와 법랑질 표면 모두에서 흡착된 피막에는 아밀라제, 분비성 면역 글로불린A 및 락토페린이 존재함을 알 수 있었으나 법랑질의 경우는 blotting이 약하게 나타났다. 주사전자현미경 관찰결과 시편의 표면에 균질한 피막이 덮고 있었으며 세균의 부착은 거의 관찰되지 않았다.

이상의 실험 결과들을 통하여 플라즈마분사법으로 표면 처리된 타이태늄 임플란트 표면에 부착된 타액성 단백질 성분은 법랑질과는 차이가 있음을 알 수 있었으며, 이러한 차이는 치태세균의 종류 및 병원성에 영향을 미칠 것으로 생각된다. 법랑질과 타이태늄 임플란트는 기질과 표면구조가 다르므로 표면에 형성 되는 치태성분도 다르다는 사실과 본 연구 결과를 종합하여 볼 때, 타이태늄 임플란트 표면에 흡착되는 초기 타액성 단백질의 성분이 타이태늄 표면에 침착되는 미생물 군의 조절에 중요한 역할을 가지고 있으며, 임플란트 치료 시에 올바른 치태 관리법의 교육을 통하여 환자 스스로 적절한 관리를 하도록 함으로써 임플란트 치료의 성공률을 높일 수 있을 것으로 생각된다.

주요어 : implant, protein, saliva, salivary pellicle