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Effects of orthodontic bonding procedures on multi-species biofilm formation

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전 다 미

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전 다 미

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- ABSTRACT-

Effects of orthodontic bonding procedures on multi-species biofilm formation

Da-Mi Jeon, DDS, MSD

Department of Orthodontics, Graduate School,
Seoul National University
(Directed by Professor Sug-Joon Ahn, DDS, MSD, PhD)

Objectives: To investigate the effects of orthodontic bonding on multi-species biofilm formation.

Materials and methods: Four specimens were prepared with bovine incisors and bonding adhesive: untreated enamel surface (BI), enamel surface etched with 37% phosphoric acid (ET), primed enamel surface after etching (PR), and adhesive surface (AD). Surface roughness (SR), surface wettability (SW), and surface morphology were evaluated. A multi-species biofilm was formed on each surface and adhesion amounts of *Streptococcus mutans*, *Porphyromonas gingivalis*, and total bacteria

were determined at days 1 and 4 using real-time polymerase chain reaction. After determining the differences in biofilm formation, SR, and SW between the four surfaces, relationships between bacteria levels and surface properties were analyzed.

Results: The order of SR was AD < PR < BI < ET, because BI and ET showed more irregular surface morphology than PR and AD. For SW, ET had the greatest value followed by PR, BI, and AD. *S. mutans*, *P. gingivalis*, and total bacteria adhered more to BI and ET with rougher and more wettable surfaces than to AD with smoother and less wettable surfaces. The adhesion of total bacteria and *S. mutans* significantly increased from day 1 to 4, but the amount of *P. gingivalis* decreased over time. The adhesion amounts of all bacteria were positively correlated with SR and SW, irrespective of incubation time.

Conclusions: Changes in SR and SW during orthodontic bonding had significant effects on biofilm formation and composition of *S. mutans* and *P. gingivalis*.

Key words: Orthodontic bonding, Surface roughness, Surface wettability, Surface morphology, Biofilm, Composition

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Da-Mi Jeon, DDS, MSD

Department of Orthodontics, Graduate school, Seoul National University

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(지도교수 : 안 석 준)

전 다 미

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I. INTRODUCTION

Oral biofilm is a highly complex and structured microbial system, and interactions between biofilm microbes are critical to its virulence.[1,2] Microbial interactions and specific microorganisms are both associated with infectious oral diseases including dental caries and periodontal disease. In particular, a high prevalence of *Streptococcus mutans* in biofilm is associated with enamel demineralization and dental caries, and *Porphyromonas gingivalis* plays a key role in progression of gingival and periodontal inflammation.[3]

Patients undergoing orthodontic treatments with fixed appliances have difficulty in performing proper oral hygiene, which contributes to extensive formation of oral biofilm.[4] Substantial biofilm formation around fixed orthodontic appliances can induce enamel demineralization and gingivitis during orthodontic treatment and prevention of these complications is a continuous challenge faced by orthodontists.[5]

A common orthodontic bonding procedure includes etching the tooth surface, priming the tooth surface, applying a bracket with a bonding adhesive to the tooth surface, and curing the adhesive between the tooth and bracket. The purpose of this procedure is to change the tooth surface properties to increase the bond strength: the etched surface

provides increased surface area and hydrophilic properties; priming protects the etched enamel surface and enhances the bond with the adhesive[6]; and the bonding adhesive provides adequate physical strength between the bracket base and primed enamel surface, resists displacement by oral function, and transfers requisite orthodontic force to the tooth.[7]

Because surface roughness (SR) and surface wettability (SW) of biomaterials affect biofilm formation,[3,8-12] the changes in surface properties associated with the orthodontic bonding procedure may significantly influence biofilm development around orthodontic appliances. Many *in vitro* biofilm studies have investigated adhesion and/or biofilm formation using a single species, which has limitations in simulating actual interactions between various bacteria in the oral environment.[10-12] In order to understand biofilm-related diseases, it is necessary to study a multi-species model under dynamic conditions rather than a mono-species model under static conditions. In this study, we analyzed effects of the orthodontic bonding procedures on biofilm formation and compositional changes in oral pathogens using a multi-species biofilm model with dynamic culture conditions. The null hypothesis was that orthodontic bonding would have no significant effect on biofilm composition.

II. REVIEW OF LITERATURE

1. Multi-species biofilm and orthodontic treatment

1.1. The community of oral biofilm

Biofilms are highly dynamic and complex communities of microorganism.[1,2] Interactions between microbes are crucial for initial colonization and biofilm maturation on the tooth surface.[2] Without maintaining the attachment on the surface, the microbes may be easily removed by environmental stress, such as swallowing. Through firm adhesion and retention on the tooth surface, the microbes can successfully organize polymicrobial communities.

The development of the oral biofilm involves cooperation and competition among early colonizers (Streptococci, *Actinomyces* spp., *Prevotella* spp., *Propionibacterium* spp., and *Veillonella* spp.) and late colonizers (*Treponema denticola, P. gingivalis, Actinobacillus actinomycetemcomitans, Eubacterium* spp.).[1,2] The acquired pellicle formed by a host derived molecules coats the enamel surface and is recognized by early colonizers in oral biofilm.[2] Streptococci account for 60 to 90% of the bacteria that colonize the tooth surface shortly after professional cleaning.[13] *Streptococcus* among early colonizers is the unique species that have both intrageneric coaggregation and

intergeneric coaggregation, which lead to have an advantage in establishing early oral biofilm.[14]

Fusobacterium nucleatum, representative middle colonizer, plays a role as a bridge between early and late colonizers and are numerous in both healthy and diseased site.[15] Especially, *F. nucleatum* is always found with *T. denticola* and *P. gingivalis*, late colonizers, which suggest that it predate the two late colonizers.[16] Most late colonizers make a co-adhesion with *F. nucleatum*, but they do not form a co-adhesion with each other except for co-adhesion between *T. denticola* and *P. gingivalis*. In addition, they cannot form a biofilm on the tooth surface without early and middle colonizers and are more virulent than the early colonizers.[1,2]

1.2. Complications of orthodontic treatment related to virulent biofilm.

Orthodontic patients have a difficult to maintain good oral hygiene due to complex orthodontic appliances, which lead to increase the risk of infectious oral diseases, such as enamel demineralization and gingival inflammation.[4,5] The bands, brackets, adhesives and elastomeric modules of orthodontic appliances provide additional surface area for bacterial adhesion and accelerate the plaque accumulation.[17] After application of fixed orthodontic bondings, the bacterial levels increase

five folds.[18] When orthodontic appliances are applied into the oral cavity, the microbial flora in biofilm changes toward acidogenic bacteria, most remarkably *S. mutans and Latobacilli* metabolizing sucrose to lactic acid.[3,19] Especially, *S. mutans* synthesize extracellular glucans from dietary sucrose and can rapidly modulate the formation of cariogenic biofilms.[20,21] These processes cause the enamel surface to be vulnerable to demineralization. Enamel demineralization is founded within 1 month of bracket bonding and commonly seen on the buccal and gingival tooth surface in orthodontic patients.[22,23]

Oral biofilm is the important factor in progression of gingival inflammation and periodontal disease.[24] Gingivitis is characterized by swelling, redness, and bleeding of gingival tissue and does not involve the periodontal tissue.[25] Periodontitis is the irreversible biofilm-induced inflammation of periodontal tissue, which lead to pathologic changes in the periodontal ligament and alveolar bone.[24,25] *P. gingivalis* cause chronic periodontitis by the disruption of the homeostasis in host tissue.[26] In mouse models, co-infection with *P. gingivalis* and *T. denticola* caused interproximal and horizontal alveolar bone loss.[27]

Orthodontic appliances result in increased biofilm formation and inflammation of the tissues, such as increase in bleeding on probing and pocket probing depth, and a shift from aerobes to anaerobes.[28] These changes are believed to be normalized after finishing orthodontic

treatment, and not to cause continuous deleterious influence on the periodontium.[29] However, other studies demonstrated that orthodontic treatment had minor, but statistically significant, deleterious effect to the periodontal tissues, such as gingival recession, periodontal pocket depth, and alveolar bone loss.[30]

2. The influence of surface properties on biofilm formation.

The physicochemical surface properties can affect the quality and quantity of bacterial adhesion.[3,8-10,31] Surface roughness (SR) and surface wettability (SW) are known to the most important surface properties associated with bacterial adhesion and biofilm formation.[3,8,9,31] Since bacterial adhesion to the tooth surface is crucial step of biofilm formation, it is important to understand surface properties and bacteria-surface interactions.

2.1. Surface roughness (SR)

Many studies reported a positive correlation between SR and bacterial adhesion.[3,10,31] In several studies, the quantity of bacterial adhesion to titanium and ceramic surfaces demonstrated a positive relation with SR.[31-33] Kim et al.[33] examined the SR and microstructure of four

ceramic materials, and analyzed the biofilm formation following adjustments and simulated intraoral polishing procedures. They found that SR values were greater in all materials after polishing procedures, leading to more biofilm accumulation. This result showed that the main cause of biofilm formation was SR. Lorenzo et al.[34] demonstrated that biofilm formed by single species is more easily affected by SR and surface morphology. An el al.[3] investigated the difference in multi-species biofilm formation on composite, compomer, and resin-modified glass ionomer. They observed that biofilm formation was highest in resin-modified glass ionomer showing most rough surface.

Rough surface provides a favorable environment for plaque retention.[35-37] Ionescue et al.[35] reported that 3D properties of surface morphology could explain the significant effect of SR on biofilm formation. The larger and deeper irregularities provide more attachable and favorable environment for bacterial adhesion, and play a shelter against shear forces, which lead to strong and irreversible adhesion on the surface.[36,37] Therefore, it is difficult to remove micro-colonies in the rough surfaces, leading to biofilm maturation.[38]

2.2. Surface wettability (SW)

SW is determined by measuring the contact angle of the surface with the given liquid using sessile drop technique.[39] Because contact angle

means hydrophobicity, wettability is related parameter to hydrophobicity, contact angle, and surface free energy.[40,41] Wettability increases as contact angle decreases and smaller contact angle means higher surface hydrophilicity.[38] The inverse relationship between hydrophobicity and bacterial adhesion can explain that hydrophilic and wettable surface induce more bacterial accumulation.[38,41]

Several studies indicate the significant influence of surface wettability on bacterial adhesion. Etxeberria el al.[9] examined the adhesion of *Staphylococcus aureus* and *Esherichia coli* on various implant surfaces and found that wettability is strongly correlated with microbial adhesion. Quirynen et al.[42] reported that positive correlation was observed between initial bacterial adhesion and hydrophilicity of substratum. They found that hydrophobic surfaces exhibited much less plaque accumulation than hydrophilic ones and less wettable surface showed a lower plaque retention capacity because plaque mass decreased between days 6 and 9. The adhesion properties of various bacteria are influenced by the hydrophilic and hydrophobic characteristics of the bacteria.[43-45] Adhesion experiments to enamel surface showed that hydrophilic bacteria such as *S. mutans* adhered in higher number to hydrophilic surfaces.[43,44] Drake el al.[45] reported that hydrophobic titanium surface showed higher colonization of *Streptococcus sanguinis* known to be hydrophobic bacteria than hydrophilic titanium surfaces.

This overview indicates that highly wettable and hydrophilic surface facilitate biofilm formation and maturation through promoting initial adhesion and retention capacity of bacteria, especially hydrophilic species.

3. Surface changes during orthodontic bonding procedures

One of the crucial factors influencing the bond strength between the orthodontic brackets and the enamel surface is the preparation of the tooth surface. Attachment of orthodontic bracket begins with acid etching to the enamel surface. When phosphoric acid is treated to the enamel surface, the hydroxyapatite crystals are selectively dissolved, which lead to microporosities throughout the surface.[46,47] During acid etching, the loss of enamel surface is estimated to 10 μm to 30 μm.[48] The microporosities increase the surface irregularity and the surface area, which is related to an increase in SR.[47] Acid etching also converts the enamel surface from hydrophobic surface to hydrophilic surface, which lead to increase in surface tension and wettability.[49] The application of primer followed by acid etching protects the etched enamel surface. Transbond XT primer is unfilled resin and mainly composed of two monomers, bisphenol A glycidyl methacrylate (bis-GMA) and triethylene glycol dimethacrylate (TEGDMA). These

monomer components infiltrate into the porous enamel surface, which improve micromechanical bond strength between adhesive and tooth surface.[6] Orthodontic bracket is bonded with adhesive to the primed enamel surface. Adhesive use the micromechanical retention of etched enamel surface and require the application of adequate primer in order to facilitate a bond between adhesive and enamel surface.[6,47] Adhesive provides proper mechanical strength between primed enamel surface and the bracket base. It resists detachment by actual oral function and delivers the forces by the arch wire and other appliances to the teeth.[7]

Several studies reported that the surface changes according to etching, priming, and application of adhesive may affect plaque formation in the oral cavity. Lehman et al.[50] reported that the etched enamel surface exposed in the oral cavity without being covered with adhesive made the accumulation of the plaque more easily. The monomer of primer such as bis-GMA can change the virulence of *S. mutans* and cause biofilm related oral disease at the interface between tooth and resin based materials.[21] Because the bacteria adhere to adhesive more than orthodontic bracket[51] and it is difficult to prepare smooth adhesive surface around the bracket in the clinical situation, the rough adhesive remnant around the bracket base can provide new place to facilitate biofilm formation.[52]

4. Multi-species biofilm model in dynamic system

4.1. Dynamic system model

Dynamic models are open-system producing continuous culture condition. Because they are capable of providing of nutrients to the microbes and removal of metabolites simultaneously, the concentration of microbes and metabolites maintains constant.[53,54] In addition, the biofilm can keep in both dynamic balance and stable state.[54] This system simulates oral conditions by continuously supplying nutrients and creating shear forces. The shear forces by circulated materials are used for identification of the chemical and physical characteristics of biofilm, and replenished nutrients enable the biofilm to persist during extended culture periods. Zhu et al.[55] cultivated *T. denticola*, *P. gingivalis, and Tannerella forsythia*, which all have significant effect on the progression of periodontal disease, under culture condition simulating the interaction of these pathogens in periodontal pocket. They observed that the pathogens showing low biofilm formation in a static condition formed mature biofilm under dynamic condition. Therefore, the dynamic models simulate the oral environment better than the closed static models and have an advantage in control the rate of biofilm growth and various factors.[3,56]

CDC biofilm reactor is useful device for conditioning dynamic system.[56] This reactor consists of eight separated polypropylene rods

suspended from lid. Each rod can accommodate three coupons which can be made from various materials such as ceramic, steel, plastics, or can be coated with biologically relevant materials. CDC biofilm reactor allows the simultaneous examination of different types of specimens and analysis of time related bacterial changes through intermittent removal of each rod.[3]

4.2. Multi-species biofilm model

In open system, laboratory microbial culture models simulate the oral microflora. Multi-species biofilm model have been constructed with defined-species biofilm consortium including major prevalent species in oral biofilm. The designs of multi-species biofilm model vary with the purpose of researches.

Bradshaw et al.[57] designed defined-bacterial consortium of 10 species: *S. mutans*, *Streptococcus oralis*, *Streptococcus gordonii*, *Lactobacillus casei, Neisseria subflava, Actinomyces naeslundii*, *Veillonella dispar*, *F. nucleatum, Prevotella nigrescens, P. gingivalis*. These species were selected for significance in oral health and disease and for wide range of metabolic activities, and for easy identification. This 10-species consortium has been widely adopted and modified for the purpose of each study. Shu et al.[58] effectively used the modified 10-species biofilm model for demonstrating that the role of urease

enzymes is critical for stabilizing diverse bacterial communities. Blanc et al.[59] used 6-species consortium and cultivated multi-species biofilm under anaerobic condition to analyze interactions between early, middle, and late colonizers during the progression of oral disease.

III. MATERIALS AND METHODS

Specimen preparation

Sound bovine incisors were extracted and cleaned with a pumice and rotary brush. They were stored in 1% aqueous chloramine-T solution (Junsei Chemical, Tokyo, Japan) at 4°C. After careful preparation to a uniform size (7.5 mm diameter and 2.6 mm thickness), the bovine incisor specimens were randomly classified into three groups by the surface treatment: No surface treatment control (BI), acid-etched surface (ET), and primed surface (PR). The ET specimens were etched with 37% phosphoric acid gel (3M, Monrovia, CA, USA) for 20 s, rinsed, and air-dried. After acid-etching, Transbond XT primer (3M) was applied to the acid-etched surface and light-cured with OrthoLuxLED (3M) for 30 s in the PR group. For the adhesive specimen group (AD), Transbond XT adhesive (3M) specimens were made in the same size as the bovine incisor specimens using a Teflon template. The template was placed on the top of a glass slide and filled with Transbond XT adhesive until the material was reached to the top of the plate. The next slide was placed on the top and pressed down to make a flat adhesive surface. They were then light cured for 20 s each from the top and bottom by the manufacturer's instructions. A total of 76 disk-shaped specimens

(19 specimens per group) were used in this study: 72 (18 per group) for surface analyses and biofilm formation, and 4 (one per group) for scanning electron microscopy (SEM) analysis.

Surface analysis

In order to determine surface properties, SR and SW were measured from all 72 specimens prior to the biofilm experiment. SR of each specimen was determined by a confocal laser scanning microscope (LSM 5 Pascal, Carl Zeiss MicroImaging GmbH, Göttingen, Germany) This calculate the arithmetic mean value of SR in the examined area (230 × 230 × 30 μm).

SW was determined by water contact angle, which was measured using a sessile drop method with distilled deionized water. Since the degree of wetting increases with decreasing contact angle, the contact angle is a useful inverse measurement of SW.[41] The contact angle was measured using a video camera with an image analyzer (Phoenix 300; Surface Electro Optics, Suwon, Korea). The right and left contact angles of each drop were averaged. All specimens were examined by the same operator.

Surface morphology of each specimen was examined using SEM. Each surface was observed under S-4700 microscope (Hitachi, Tokyo,

Japan). Representative images were taken at × 500 and × 3000 magnifications.

Bacterial preparation

Because of their major prevalence in oral biofilm and relevance to health, we used a bacterial consortium of 13 species as previously described[57]: *S. mutans* ATCC 700610, *S. oralis* ATCC 9811, *Streptococcus salivarius* CCUG 50207, *S. sanguinis* CCUG 17826, *Streptococcus sobrinus* ATCC 27607, *N. subflava* ATCC 49275, *V. dispar* KCOM 1864, *Lactobacillus rhamnosus* ATCC 7469, *A. naeslundii* KCOM 1472, *P. nigrescens* ATCC 33563, *F. nucleatum* ATCC 10953, *P. gingivalis* KCOM 2797, and *A. actinomycetemcomitans* ATCC 43718. Since these species have different optimal growth environments, each bacterium was grown to mid-exponential growth phase according to their growth nature (Table 1).

Multi-species biofilm formation

We cultivated the multi-species biofilm using a CDC biofilm reactor (BioSurface Technologies, Bozeman, MT, USA) with a modified basal mucin medium to provide nutrients and simulate saliva.[58] The medium contained 2.5 g/L porcine gastric mucin, 2 g/L proteose peptone, 1 g/L yeast extract, 1 g/L trypticase peptone, 2.5 g/L KCL, 0.1 g/L cysteine

hydrochloride, 0.001 g/L hemin, 10 mM urea, and 10 mM glucose. The reactor has a lid supporting eight rods which hold three individual specimens. Three specimens were randomly selected from each of the four groups and inserted into each rod using a Teflon template to expose only the front surface to the culture medium. The equipment, the rods with specimens, and basal medium mucin were then sterilized. After 3.5 mL of prepared mixed cell culture (1% of the reactor volume) was injected into the biofilm reactor, modified basal mucin medium was consistently flowed into and out of the reactor at a rate 100 mL/h. According to the previous description, the device was set on a hot stir plate at 37°C with rotating at 60 rpm.[56]

Quantitative analysis of bacteria

The four rods (12 specimens) were removed from the biofilm reactor at day 1 (T1) and 4 (T2). The specimens were transferred into round tubes, then washed using 1.0 mL phosphate-buffered saline (PBS) with a pH 7.4 for removing unattached bacteria. Through sonication under three 30-s pulses and 30-s intermittent ice chilling procedures, the biofilm was detached from the specimen surface. To isolate the bacterial cells from the suspension, the centrifugation was performed at 13,000 rpm for 10 min.

A CellEase Bacteria II Genomic DNA Extraction Kit (Biocosm, Osaka, Japan) was used for extraction of bacterial chromosomal DNA. A NanoVue spectrophotometer (General Electric Healthcare Life Sciences, Pittsburgh, PA, USA) was then used to estimate the quality of the isolated DNA. For quantifying *S. mutans*, *P. gingivalis*, and total bacteria in biofilm using real-time polymerase chain reaction (PCR), commercially synthesized PCR primers (Bioneer, Daejeon, Korea) were used to amplify the target DNA. The specific primers for *S. mutans* were based on two glucosyltransferase genes (*gtfB* and *gtfU*) and 16S rRNA gene was used to design the primers of *P. gingivalis*. The universal primers for quantifying total bacteria were based on a conserved sequence in the 16S rRNA

To obtain the standard curve for DNA quantification, DNA was isolated from *S. mutans* ATCC 700610 and *P. gingivalis* KCOM 2797 and amplified. The amplified products were purified from agarose gels by a QIAquick Gel Extraction Kit (Qiagen, Düsseldorf, Germany) and the concentration of DNA was determined from absorbance at 260 nm. A ten-fold serial dilution ranging from 10 to 10^7 copies was performed.

Bio-Rad iQ5 system (Bio-Rad, Hercules, CA, USA) was used for performing the real-time PCR. The final volume of reaction mixtures was prepared at 20 μL containing 2 μL purified DNA, 100 pM primer, 10 μL 2x iQ SYBR Green Supermix (Bio-Rad), and distilled water.

The cycling conditions for quantifying target bacteria are presented in Table 2. Bio-Rad iQ5 Optical System Software was used to analyze all data. The entire quantifying procedure was performed in duplicate and individually repeated five times.

Statistical analysis

The differences in SR and water contact angle according to surface treatment were determined by the Kruskal-Wallis test. The time-related differences in the bacterial levels were analyzed from two-way analysis of variance using the Bonferroni correction. Spearman rank correlation coefficient test was used to examine the association between surface properties and bacterial levels at each time point. All values were considered significant at $P < 0.05$.

IV. RESULTS

There were significant differences in SR among the surface types (Table 3). ET had the roughest surface, while AD had the smoothest surface. Multiple comparisons showed that the order of SR was AD < PR < BI < ET ($P < 0.05$). The results of SR were consistent with those from SEM images. BI and ET showed rougher surface morphology than PR and AD (Figure 1). BI had an irregular and uneven appearance due to grooves, ridges, and microfissures (Figures 1A1 and 1A2). Because of dissolution of the prism core, ET showed many microporosities throughout the surface (Figures 1B1 and 1B2). PR showed a uniformly wrinkled surface and rougher morphology (Figures 1C1 and 1C2) than AD with minor flaws (Figures 1D1 and 1D2).

Significant differences in water contact angle were found among the different surface types, but the order of water contact angle tended to be opposite that of SR. The order of water contact angle was ET < PR < BI < AD ($P < 0.05$) (Table 3). Because the contact angle is a useful inverse measurement of SW,[41] the order of SW can be interpreted as AD < BI < PR < ET.

Table 4 exhibits the differences in bacterial adhesion according to the surface type and incubation time. The surface type significantly influenced the adhesion of target bacteria. In all target bacteria, BI and ET demonstrated higher bacterial adhesion than AD, but there was no significant difference in adhesion level between BI and ET (AD < BI = ET). Bacterial adhesion to PR varied among bacterial species. There was no significant difference in adhesion amount of *S. mutans* between PR and the other three surfaces. Adhesion amount of *P. gingivalis* to PR was lower than to ET, but there were no significant differences in adhesion amount of *P. gingivalis* among BI, PR, and AD. Total bacteria exhibited lower adhesion to PR than to BI and ET, but no significant difference in adhesion was found between PR and AD.

There were also significant differences in bacterial adhesion between T1 and T2 (Table 4). The adhesion amounts of total bacteria and *S. mutans* increased (T1 < T2, $P < 0.05$), but that of *P. gingivalis* decreased with increased incubation time (T1 > T2, $P < 0.05$).

The Spearman rank correlation test demonstrated that the adhesion level of all target bacteria was significantly associated with both SR and water contact angle at each time point (Table 5). Bacterial adhesion was positively correlated with SR, but negatively correlated with water

contact angle, irrespective of bacterial species and incubation time. Considering that water contact angle is inverse measurement of SW,[41] these results indicate that both SR and SW are positively correlated with bacterial adhesion.

V. DISCUSSION

When bonding orthodontic appliances to the teeth, the enamel surface is subjected to many treatments including acid etching, priming, and application of adhesive to the primed surface. Because the changes in surface properties affect bacterial adhesion,[8,9] orthodontic bonding procedures may significantly influence biofilm formation and composition around orthodontic appliances. Many *in vitro* studies have evaluated the association between orthodontic bonding and biofilm formation, which is important to prevent common orthodontic complications, such as enamel demineralization and gingival inflammation.[3-5,8,11,60] However, most studies used a mono-species biofilm model under static conditions,[8,11,60] which does not simulate the intraoral environment and complex interactions of oral microflora because the oral cavity is a diverse and changeable environment.[1] In this study, a multi-species biofilm model was used under dynamic culture conditions to assess the effects of the orthodontic bonding procedure on biofilm formation and compositional changes in two main oral pathogens, *S. mutans* and *P. gingivalis*.

Bovine teeth were used to examine the effects of surface properties

on biofilm formation in this study because they are the useful alternative for human teeth in dental research. They are easy to get in good condition and have a relatively large flat surface. Although the physicochemical characteristics of bovine teeth are not completely identical to those of human teeth,[61] many studies have reported that there are no significant differences in micro-morphology, physical properties, and chemical composition.[62,63] In addition, there is no significant difference in biofilm composition between bovine and human teeth.[64]

SR and SW are two main surface properties that influence bacterial adhesion and biofilm formation.[3,8-12] A rough surface provides a favorable environment for bacterial adhesion and biofilm formation, because a rough surface acts as a protective role against shear force and increases the surface area for biofilm formation.[10,11] On the other hand, higher SW facilitates biofilm formation on dental materials,[9,12] because SW is a related parameter of surface free energy and hydrophilicity.[40]

SW is determined through the contact angle formed by droplet of a liquid on a surface.[41] Water is a common liquid for measurement of the contact angle because it does not dissolve the specimen surface and has

no chemical reaction with the underlying surface.[65] We measured the water contact angle of all the specimens to determine the SW prior to starting biofilm experiments, because other probe liquids with different hydrophobicity may affect the surface properties of the underlying material, react with primer or adhesive components, and influence biofilm experiments.

This study demonstrated that different surface treatments during orthodontic bonding significantly influence SR and water contact angle. There were significant differences in SR among the surface types (Table 3). The order of SR was AD < PR < BI < ET, which is partly consistent with the results of a previous study showing that etched hydroxyapatite surface is rougher and adhesive surface is smoother than other surfaces.[11] Higher SRs of BI and ET than PR and AD might be due to the presence of grooves and ridges on bovine enamel and increased surface irregularities by acid etching,[37] respectively (Figure 1). Although the wrinkled surface of PR showed a smoother morphology (Figures 1C1 and 1C2) than BI and ET, wrinkle structures may cause PR to be more irregular than AD, which shows minor flaws (Figures 1D1 and 1D2).

There were also significant differences in water contact angle among

the four surface types (Table 3). AD exhibited the greatest value followed by BI, PR, and ET (ET < PR < BI < AD). Because of the inverse relationship between water contact angle and SW,[41] the order of SW may be AD < BI < PR < ET. These findings indicate that both SR and SW have the highest value in ET and the lowest value in AD.

This study demonstrated higher adhesion of *S. mutans* to BI and ET than to AD (Table 4), which could be explained by the higher SR and SW for BI and ET than for AD (Table 3). Various bacteria are associated with the development of oral biofilm, which starts with early colonizers such as streptococci and *Actinomyces* spp., followed by middle-colonizers including *Fusobacteria* spp. and *Porphyromonas* spp., and late-colonizers with anaerobic Gram-negative bacteria.[1,2] Because *S. mutans* initially adheres to the underlying surface as an early colonizer, adhesion of *S. mutans* may be more significantly affected by surface properties. Previous microscopic examination of biofilms revealed that bacterial adhesion to the enamel surface starts from surface irregularities, such as grooves, perikymata, and cracks[37] (Figure 1), because rough surfaces can act as a buffer against shear forces, which induce the shift from reversible to irreversible bacterial adhesion more readily and increase the area available for initial

bacterial adhesion. In addition, hydrophilic and wettable surfaces tend to cause more biofilm formation by selecting specific bacteria.[43,44] Since hydrophilic bacteria preferentially adhere to a hydrophilic surface,[43] hydrophilic oral bacteria such as *S. mutans*, easily adhere to the hydrophilic and wettable surface.[44] In this regard, the rougher and wetter surfaces of BI and ET may provide more a favorable surface for adhesion of *S. mutans* than AD.

This study showed that *P. gingivalis* and total bacteria also adhered more to BI and ET than to AD (Table 4). After colonization by early colonizers, a combination of bacterial proliferation and recruitment leads to a bacterial mass increase during biofilm maturation.[2] Therefore, successful adhesion of early colonizers such as *S. mutans* leads to sequential co-adhesion and proliferation of middle and late colonizers and results in increase and maturation of biofilm, which may explain why the adhesion tendency of *P. gingivalis* and total bacteria is similar to that of *S. mutans*. This hypothesis is supported by the findings of this study demonstrating that SR was positively correlated and water contact angle was negatively correlated with adhesion of *P. gingivalis* and total bacteria as well as *S. mutans* (Table 5). Several studies have also demonstrated that SR has a positive correlation with bacterial

adhesion and biofilm formation[3,10,11] and the significant effects of SW on biofilm formation are widely accepted.[9,12] All these findings suggest that the changes in SR and SW during orthodontic bonding procedures may significantly affect bacterial adhesion and biofilm composition.

Biofilm formation may be influenced not only by changes in SR and SW, but also by other surface factors during orthodontic bonding procedures. Bacterial adhesion to ET was expected to be higher than to BI, because of its rougher and wetter properties. However, there was no significant difference in adhesion of all the bacteria between BI and ET (Table 4). Cytotoxicity of the phosphoric acid used for acid etching may influence bacterial adhesion. A previous study demonstrated that 37% phosphoric acid has antimicrobial activity by increasing the concentration of hydrogen ions in the microorganism.[66] During the experiment, remaining phosphoric acid on the irregular surface of the bovine tooth may have influenced the bacterial viability. Although the rougher and wetter surface caused by acid etching could be favorable for bacterial adhesion, the cytotoxic action of phosphoric acid may offset the surface properties. In addition, a previous study reported that an SR over a certain level (over 0.35 μm) might not significantly influence biofilm formation.[67] Although ET was rougher than BI, BI may

be rough enough (average 1.61 μm of SR, Table 3) to make no difference in bacterial adhesion between the two.

Bacterial adhesion to PR was different than that to other surfaces. Because PR was rougher and more wettable than AD, but smoother and less wettable than ET (Table 3), bacterial adhesion to PR was expected to be lower than to ET and higher than to AD. However, adhesion of the two oral pathogens and total bacteria to PR was not significantly different from that to ET or AD. This result may be due to chemical properties of the primer. The primer is present in a chemically unstable state in the oral environment because of its lower degree of conversion.[68] In particular, bisphenol A-glycidyl methacrylate (bis-GMA), one of the main components of Transbond XT primer, has two opposite characteristics that influence bacterial adhesion and biofilm formation. One is to facilitate biofilm formation of *S. mutans* by increased adhesion capacity, enhanced glucan synthesis, and promotion of sugar transport activity.[21] The other is a toxic effect on oral bacteria, such as inhibiting bacterial growth and decreasing cell viability.[21] The leachable component of the primer with these opposing characteristics may differently influence bacterial adhesion to PR.

This study showed that adhesion of *S. mutans* and total bacteria

significantly increased from T1 to T2 (T1 < T2, Table 4). Since streptococci are facultative anaerobes, they can successfully adhere to the surface and continue to proliferate well in our aerobic culture condition, which led to subsequent maturation of biofilm and eventually resulted in an increase in total bacteria over time. In contrast to *S. mutans* and total bacteria, the amount of *P. gingivalis* significantly decreased with increased incubation time (T1 > T2). *P. gingivalis* is a late colonizer and obligate anaerobe. *P. gingivalis* is sensitive to an oxidative aerobic environment, which may make it difficult to grow well over time. These results are consistent with a previous study that examined biofilm formation on orthodontic adhesive under similar culture conditions to our study.[3]

This *in vitro* study showed the lowest bacterial adhesion to AD. In particular, the two main oral pathogens adhered less to AD than to BI and ET. Considering that plaque accumulation and enamel demineralization mainly occur at the interface between tooth and adhesive in the clinical practice,[69] these findings indicate that when acid etching is wider than intended, covering the etched surface with adhesive may be helpful to reduce biofilm formation around orthodontic appliances. However, it is difficult to maintain a smooth adhesive

surface and the remaining adhesive remnant around orthodontic appliances may be problem to clean properly in the clinical situation. Therefore, clinicians should uniformly apply adhesive, carefully remove adhesive remnants, and perform periodic cleaning around orthodontic appliances to avoid enamel demineralization.

The present study has some limitations. This study showed that there was no significant difference in bacterial adhesion between BI and ET, even though ET had a rougher and more wettable surface than BI. However, the effects of acid etching on bacterial adhesion may be different between human and bovine teeth, because bovine teeth are more irregular and undulating than human teeth.[61] In addition, the multi-species biofilm model used in this study does not simulate the actual oral environment. Further study using an *in situ* model is needed to evaluate the effects of orthodontic bonding procedures on biofilm formation and to find approaches to minimize the risk of pathologic side effects in orthodontic patients.

VI. CONCLUSIONS

In this study, changes in surface properties during orthodontic bonding and the effects of orthodontic bonding on biofilm formation were investigated using a multi-species biofilm under dynamic conditions. This study demonstrated that different surface treatments during orthodontic bonding significantly influence SR and SW. Acid etching significantly increased SR and SW, while application of the adhesive significantly decreased SR and SW. The changes in SR and SW were significantly associated with biofilm formation and composition. In particular, the two main oral pathogens, *S. mutans* and *P. gingivalis* adhered more to BI and ET with rougher and more wettable surfaces than to AD with smoother and less wettable surfaces. This *in vitro* study suggests that changes in surface properties during the orthodontic bonding procedure may be significantly associated with biofilm formation and composition of *S. mutans* and *P. gingivalis*.

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TABLES

Table 1. Growth conditions of each bacterial species for the multi-species biofilm model.

Bacterial species		Growth condition
Early colonizer	*Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus salivarius*, *Streptococcus sanguinis*, *Streptococcus sobrinus*, *Neisseria subflava*, *Veillonella dispar*, *Lactobacillus rhamnosus*, *Actinomyces naeslundii*	Grown in a brain heart infusion (BHI) agar medium at 37°C with 5% CO_2 for 48 hours and then subcultured overnight in a BHI liquid medium at 37°C with 5% CO_2
Middle colonizer	*Prevotella nigrescens*, *Fusobacterium nucleatum*	Grown in a tryptic soy agar medium supplemented with 10 μg/mL vitamin K, 5 μg/mL hemin and 5% sheep blood at 37°C in anaerobic condition for 7 days and then subcultured in a BHI liquid medium with 10 μg/mL vitamin K, 5 μm/mL hemin at 37°C in anaerobic condition
Late colonizer	*Porphyromonas gingivalis*	
	Actinobacillus actinomycetemcomitans	Grown in a BHI agar medium at 37°C in an anaerobic condition for 48 hours and then subcultured overnight in a BHI liquid medium at 37°C in an anaerobic condition

Table 2. Polymerase chain reaction conditions with respect to bacterial species.

Bacterial primer	Cycling condition
Primers for *Streptococcus mutans,* Universal primers	Initial denaturation for 30 s at 94°C Forty cycles of denaturation for 20 s at 95°C Annealing for 45 s at 60°C Extension for 10 s at 60°C
Primers for *Porphyromonas gingivalis*	Initial denaturation for 1 min at 95°C Forty cycles of denaturation for 5 s at 95°C Annealing for 15 s at 61°C Extension for 33 s at 72°C Final extension for 10 min at 72°C.

Table 3. Surface roughness (SR) and water contact angle (WCA) with respect to surface type.

	Bovine incisor (BI)	Etching (ET)	Primer (PR)	Transbond XT (AD)	Multiple Comparisons
SR (μm)	1.61 ± 0.22	3.50 ± 0.30	0.32 ± 0.04	0.11 ± 0.00	AD < PR < BI < ET
WCA (degree)	56.61 ± 2.50	30.08 ± 2.94	46.59 ± 1.51	72.47 ± 1.62	ET < PR < BI < AD

The Kruskal-Wallis test was used to determine differences among the four groups and multiple comparisons were performed using the Mann-Whitney tests with the Bonferroni correction at a significant level of $\alpha < 0.05$.

Table 4. Time-related differences in the levels of bacteria with respect to surface type.

	Day 1 (T1)	Day 4 (T2)	Significance†		*P* value for interaction
			Treatment	Time	
Streptococcus mutans (Log_{10}/cm^2)					
BI[a]	4.29 ± 0.22	5.22 ± 0.53	AD < BI = ET	T1 < T2	0.388
ET[b]	4.47 ± 0.36	5.31 ± 0.39			
PR[c]	4.05 ± 0.44	5.01 ± 0.27			
AD[d]	3.96 ± 0.37	4.49 ± 0.59			
Porphyromonas gingivalis (Log_{10}/cm^2)					
BI[a]	3.23 ± 0.31	2.82 ± 0.30	AD < BI = ET	T1 > T2	0.905
ET[b]	3.48 ± 0.40	3.14 ± 0.44	PR < ET		
PR[c]	2.80 ± 0.28	2.52 ± 0.53			
AD[d]	2.57 ± 0.17	2.36 ± 0.63			
Total bacteria (Log_{10}/cm^2)					
BI[a]	7.06 ± 0.34	7.99 ± 0.24	AD = PR < BI = ET	T1 < T2	0.104
ET[b]	7.25 ± 0.36	8.11 ± 0.33			
PR[c]	6.90 ± 0.48	7.70 ± 0.24			
AD[d]	6.87 ± 0.26	7.34 ± 0.42			

†Two-way ANOVA was used to determine significant differences between the two time points using the Bonferroni correction at a significant level of $\alpha < 0.05$: [a], untreated bovine incisor; [b], etched bovine incisor; [c], primed bovine incisor; [d], Transbond XT adhesive.

Table 5. Spearman rank correlation coefficients for surface properties and bacterial levels.

Day	Bacteria	Surface roughness (n = 36)	Water contact angle (n = 36)
1	*Streptococcus mutans*	0.590***	−0.456**
	Porphyromonas gingivalis	0.863***	−0.635***
	Total bacteria	0.599***	−0.566***
4	*Streptococcus mutans*	0.458**	−0.351**
	Porphyromonas gingivalis	0.465**	−0.373**
	Total bacteria	0.844***	−0.573***

* P < 0.05; ** P < 0.01; *** P < 0.001

FIGURES

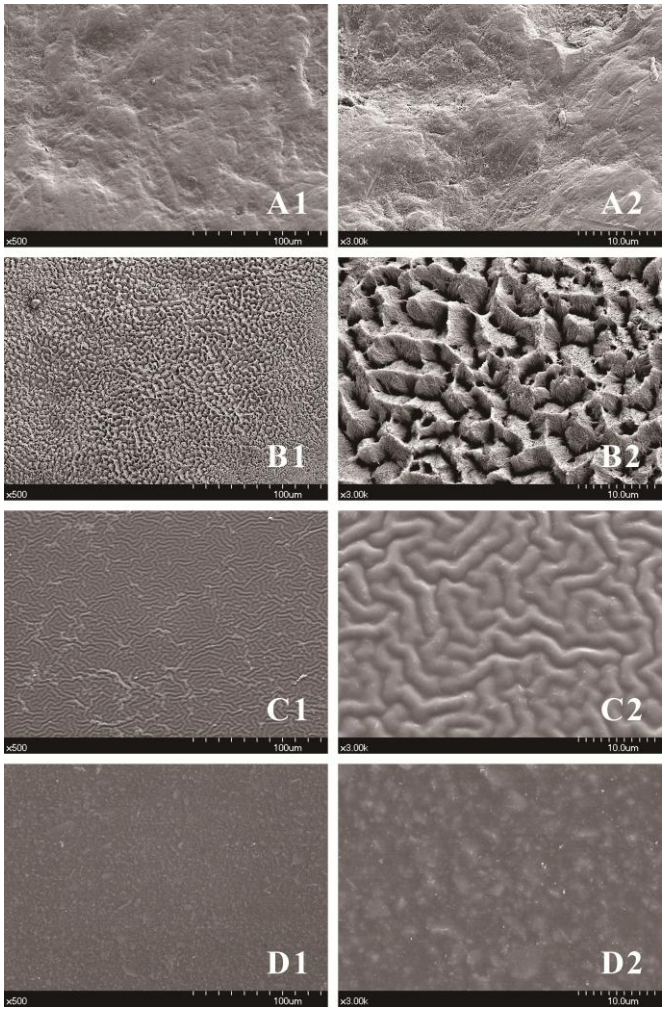

Figure 1. Scanning electron microscopy images with respect to surface type. Untreated bovine incisor: (A1) ×500 magnification, (A2) ×3000 magnification; Etched bovine incisor: (B1) ×500 magnification, (B2) × 3000 magnification; Primed bovine incisor: (C1) ×500 magnification, (C2) ×3000 magnification; Transbond XT adhesive: (D1) ×500 magnification, (D2) ×3000 magnification.

국문 초록

교정용 장치의 부착과정이 다세균종 바이오필름 형성에 미치는 영향에 대한 연구

전 다 미

서울대학교 대학원 치의과학과 치과교정학 전공

(지도교수: 안 석 준)

목적: 본 연구에서는 교정장치의 부착과정에 따른 치아 표면의 변화가 다세균종 바이오필름 형성에 미치는 영향을 평가하였다.

방법: 우치와 교정용 접착제를 이용하여 교정장치 부착과정에 따라 표면 처리된 네 가지 종류의 시편을 제작하였다: 표면 처리 되지 않은 우치 (BI), 37% 인산용액으로 산부식 처리된 우치 (ET), 산부식 후 프라이머를 처리한 우치 (PR), Transbond XT adhesive (AD). 모든 시편에서 표면 거칠기 (surface roughness, SR), 표면 젖음성 (surface wettability, SW) 및 표면 형상 (surface morphology)을 살핀 뒤, 각각의 표면에서 다세균종 바이오

필름을 형성하였다. 실시간 중합효소 연쇄반응 (real-time polymerase chain reaction)을 이용하여 배양 1일차 (T1)와 4일차 (T2)에 형성된 바이오필름에서 *Streptococcus mutans*, *Porphyromonas gingivalis* 및 전체 세균의 양을 측정하였다. 네 가지 표면에서 표면 종류에 따른 SR과 SW의 차이 및 바이오필름 형성의 차이를 확인한 후, 표면 특성과 세균 부착양 간의 상관관계를 분석하였다.

결과: SR은 AD, PR, BI, ET 순서로 증가하였으며, SW는 AD, BI, PR, ET 순서로 증가하였다. BI와 ET는 PR과 AD에 비해 더 불규칙하고 거친 표면을 나타냈다. *S. mutans*, *P. gingivalis* 그리고 전체 세균은 AD보다 더 거칠고 표면 젖음성이 높은 BI와 ET에서 더 많은 부착을 보였다. T1에서 T2 사이, *S. mutans*와 전체 세균의 부착양은 증가하였으나, *P. gingivalis*의 양은 감소하였다. 상관관계 분석을 통하여 배양시간과 관계없이, 모든 세균의 부착양이 SR과 SW에 대해 양의 상관관계를 가지는 것을 확인하였다.

결론: 교정장치의 부착과정에 따른 SR과 SW의 변화는 바이오필름 형성과 *S. mutans*, *P. gingivalis*의 조성에 유의한 영향을 미치는 것으로 보인다.

주요어: 교정장치부착, 표면 거칠기, 표면 젖음성, 표면 형상, 바이오필름, 조성
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