치의과학박사 학위논문

Effects of Surface Roughness on Composition of Biofilm Formed on Composite Resins

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치의과학과 치과교정학 전공

박 지 원

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이 논문을 치의과학박사 학위논문으로 제출함

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- ABSTRACT -

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표면거칠기가 복합레진에 형성된 바이오필름 조성에 미치는 영향에 대한 연구

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Objective: The aim of this study was to investigate the effects of surface roughness (SR) on composition of multi-species biofilm formed on composite resins.

Materials and Methods: Composite resin disks were prepared using a polytetrafluoroethylene mold with glass slide and were randomly assigned

to SR180, SR400, SR1500, and SRGlass. Disks in SR180, SR400, and SR1500 were roughened with 180-, 400-, 1500-grit silicon carbide papers, respectively. Disks in SRGlass had intact surfaces without surface roughening. SR was analyzed using a confocal laser scanning and scanning electron microscopy. After multi-species biofilms were grown on composite resin surfaces, adhesion of *Streptococcus mutans* (Sm), *Streptococcus sobrinus* (Ss), *Aggregatibacter actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg), and total bacteria were determined at day 1 (T1) and day 4 (T2). Differences in SR among the four groups were tested using one-way analysis of variance and Tukey HSD multiple comparisons. Multifactorial analysis of variance was used to determine time-related changes in bacterial composition with respect to SR.

Results: The order of SR, from highest to lowest, was SR180 (1.45 ± 0.11 μm), SR400 (0.62 ± 0.05 μm), SR1500 (0.35 ± 0.02 μm), and SRGlass (0.15 ± 0.01 μm) (SR180 > SR400 > SR1500 > SRGlass, $p < 0.05$). Interestingly, increased SR was not proportional to bacterial adhesion. The significant differences in the adhesion of total bacteria was only found between SRGlass and SR180 (SR180 > SRGlass, $p < 0.05$). The adhesion of Sm and Ss to SR180 and SR400 was higher than to SRGlass (SR180 = SR400 > SRGlass, $p < 0.05$). However, adhesion of Aa and Pg to composite resins

was not significantly influenced by SR. Adhesion of total bacteria, Sm and Ss increased from T1 to T2 (T1 < T2, $p < 0.05$), whereas the adhesion of periodontopathogens (Ag and Pg) decreased from T1 to T2 (T1 > T2, $p < 0.05$).

Conclusion: Considering that decreased adhesion of cariogenic streptococci (Sm and Ss) and total bacteria was observed at values around 0.15 μm of SR, a periodic smoothening to around 0.15 μm of SR should be considered to minimize adhesion of cariogenic streptococci to surfaces of composite resins.

Keywords : Multi-species biofilm, Surface roughness, Composite resin

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I. INTRODUCTION

Enamel demineralization and gingival inflammation are the commonly recognized side effects of fixed orthodontic treatment.[1,2] Remaining orthodontic adhesive around the brackets and complex design of orthodontic brackets increase biofilm formation, because they provide retention site for oral bacteria and make it difficult to clean the tooth surfaces around appliances.[3,4]

When fixed orthodontic appliances are placed on the tooth surface, it is common for the tooth surface, particularly the boundary between the tooth surfaces and the orthodontic bracket, to be covered with an orthodontic adhesive. The surface of the remaining orthodontic adhesive made of composite resin becomes roughened with aging due to a mechanical force, such as tooth brushing. The roughed surface of composite resins may facilitate biofilm formation.[5]

Biofilms are structured communities of bacterial cells.[6] Oral biofilms in the mouth, dental plaques, are associated with caries and periodontal disease according to their bacterial compositions.[7] In particular, mutans streptococci (MS), such as *Streptococcus mutans* and *Streptococcus sobrinus,* are commonly present in oral biofilms and responsible for caries.[8]

A combination among five major periodontal pathogens - *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Treponema denticola*, and *Tannerella forsythia* in oral biofilms showed the strongest association with periodontal disease.[9] Among them, *A. actinomycetemcomitans* and *P. gingivalis* were commonly used as target bacteria for periodontal research, because of their prevalence, influences on pathogenicity, and their convenience of experiment.[10]

Bacterial adhesion and biofilm formation have been investigated using orthodontic materials.[10-12] Most of them have evaluated surface properties, such as surface roughness (SR) and surface free energy (SFE) as contributing factors for biofilm formation,[10-12] because they significantly affect adhesion and retention of oral microorganisms.[13] Surface with a high SFE thermodynamically promote bacterial adhesion.[13] SR influences the initial bacterial adhesion and stagnation,[13] because a rough surface plays a protective role against shear force, increases the area available for biofilm formation,[13] and promote rapid regrowth by interfering cleaning process.[14]

However, it has not been fully investigated exactly which of SR or SFE plays a more important role in bacterial adhesion and biofilm formation. Some studies suggest that the adhesion of bacteria is significantly influenced by SFE rather than SR,[11,12] but the recent studies using multi-

species biofilm model propose that SR plays a major role exceeding the importance of SFE.[5,10] This is because previous studies investigated the effects of orthodontic materials on bacterial adhesion, without controlling interaction between surface properties.[10-12]

Although many studies have evaluated the effect of SR on biofilm formation,[15,16,17] SR ranges have not covered the clinical SR ranges of the composite resins.[15] In addition, different materials with various SFE were used in previous studies.[16,17] Furthermore, most studies only investigated the adhesion of single bacterial species to composite resins, mainly *S. mutans*.[16-18] However, the single-species method could not represent interaction and complex functions of microorganisms in oral biofilm.[14]

The purpose of this study was to investigate the effects of SR on the composition of multi-species biofilm formed on composite resins. For this purpose, we used a 13-species biofilm model and the same composite resins within clinical SR ranges and similar SFE. The null hypothesis was that there would be no significant differences in biofilm composition with respect to SR.

II. REVIEW OF LITERATURE

1. Effects of surface characteristics on biofilm formation

1.1 Surface roughness

SR has been reported to play a prominent role in biofilm formation of oral bacteria.[13] The effects of SR on biofilm formation can be explained by the fact that a rough surface can function as shelter against shear force and can increase the area available for biofilm formation.[13] Park et al.[18] studied the effects of SR of composite resins on biofilm formation of *S. mutans* in the presence of saliva. Three groups of composite resins disks were prepared by curing composite against 400-grit silicon carbide paper, 800-grit silicon carbide paper, or a glass slide. Biofilm formation significantly increased on high SR group. Mei et al.[19] made three SR group of orthodontic, light-cured composite resins by polishing and grinding: smooth (SR 0.02 μm), moderately rough (SR 0.15 μm), and rough (SR 0.35 μm). Adhesion forces between *S. mutans* and the surfaces of composite resins were measured using atomic force microscopy. They found that streptococcal adhesion forces to orthodontic composite resins increase with increasing roughness

of the surfaces of composite resins. Ikeda et al.[20] evaluated the adherence of biofilms to the surfaces of two composite resins. Disks were prepared from composite resins, and then either ground with 800-grit silicon carbide paper or polished with diamond pastes up to 1 μm. Artificial biofilms of *S. mutans* were grown on the composite resins slabs in an artificial mouth system for 20 hours. Results of this study indicated that a lower SR had less bacterial adhesion compared with a rougher specimen surface.

On the contrary, other studies have reported insignificant relationships between the SR of dental materials and the amount of biofilm formation. Cazzaniga et al.[21] studied *in vitro* biofilm formation on composite resins after different finishing and polishing procedures. Four composite resins (microhybrid, nanohybrid, nanofilled, and bulk-filled) and six finishing and polishing procedures (open-air light-curing, light-curing against Mylar strip, aluminum oxide disks, one-step rubber point, diamond bur, and multi-blade carbide bur) were evaluated. The tested finishing and polishing procedures significantly influenced SR but did not significantly affect *S. mutans* biofilm formation. Dezelic et al.[22] assessed the influence of SR on the formation of a multi-species biofilm on dental materials (adhesive patch, composite resins, amalgam, and enamel). Rough and smooth specimens of each material were prepared, and the mean SR was assessed

profilometrically. The biofilms were then allowed to grow either for 15 min or 15 hours respectively on saliva preconditioned specimens of each material, and colony-forming units on blood agar were counted. This study concluded that SR may influence the initial biofilm adherence, but the effect of different SR vanished following growth and maturation.

These contradictions mainly may be due to the limitation of the use of a single species *S. mutans*.[18-20] In addition, the surfaces were roughened using various polishing techniques with rotating burs, resulting in irregular SR with large standard deviation.[19,21,22]

1.2 Surface free energy

SFE describes the whole energy of a solid surface as an equivalent to the surface tension of a fluid. It is defined as "the work required to increase the area of a substance by 1 cm^2" and represents an important factor determining the reactivity of a surface.[11] Several different approaches can be used to determine the SFE by measuring the contact angle formed by a range of liquids differing in hydrophobicity on a given surface.

SFE is usually associated with initial microbial adhesion to solid

surfaces rather than prolonged biofilm formation.[11] Ionescu et al.[23] also supported this assumption and found less *S. mutans* biofilm formation occurred on composite resins surfaces with low SFE, even after 96 hours of incubation *in vitro*. Ahn et el.[11] evaluated the influence of orthodontic materials with different surface characteristics on microbial biofilm formation suggested that SFE significantly influenced microbial adhesion to these materials, even more than SR. However, Flausino et al.[24] concluded differently at the *in vitro* study evaluating biofilm formation on restorative materials used to restore non-carious cervical lesions in relation to SR and SFE parameters. It was demonstrated that materials with similar SFE values showed different levels of biofilm formation suggesting no strong correlation between microbial adhesion and SFE but a stronger influence of the SR parameter. Tanner et al.[25] led similar conclusion that SR seemed to be more important than SFE in influencing biofilm formation on dental materials.

2. Previous studies on bacterial adhesion to orthodontic materials

Many studies have evaluated bacterial adhesion to various orthodontic

materials. Blunden at al.[26] examined the adhesion of a strain of *S. mutans* to the surface of disks of selected orthodontic bonding composite resins and a glass ionomer cement. Colonization by the bacterium was assessed by weight gain and scanning electron microscopy. Initial colonization occurred after about 3 days, and the specimens were usually completely covered by the fourth day. There were statistically significant differences between materials for weight gain, indicating a strong correlation between weight gain and SFE of the materials.

Lee et al.[12] investigated the adhesion of bacteria to nine different materials (four orthodontic adhesives, three bracket raw materials, hydroxyapatite blocks, and bovine incisors) using confocal laser scanning microscopy and sessile drop method. Four orthodontic adhesives included fluoride-releasing composite resins, nonfluoride-releasing composite resins, polyacid-modified composite resins, and resin-modified glass ionomer (RMGI) cement. Three bracket raw materials were stainless steel metal, polycrystalline alumina, and monocrystalline sapphire. Orthodontic adhesives had higher SFE characteristics and lower SR than bracket materials. Orthodontic adhesives showed a higher MS retaining capacity than bracket materials, and MS adhesion to RMGI and hydroxyapatite was highest. Extended incubation time increased MS adhesion, while saliva

coating did not significantly influence MS adhesion. SFE was positively correlated with MS adhesion, irrespective of saliva coating.

Ahn et al.[11] analyzed the SR and SFE characteristics of various orthodontic adhesives and their effects on the adhesion of MS. Three nonfluoride-releasing composite resins, 1 fluoride-releasing composite resin, 1 polyacid-modified composite resin, and 2 RMGIs were analyzed with confocal laser scanning microscopy and the sessile drop method. Adhesion assays were then performed by incubating each material with tritium-labeled cariogenic streptococci. SFE were significantly different among the adhesives, despite relatively uniform SR. This study suggests that initial MS adhesion is significantly influenced by SFE characteristics of adhesives rather than SR or saliva coating.

An et al.[10] investigated multi-species biofilm formation on various orthodontic adhesives using 13 species biofilms. Multi-species biofilms were grown on the surfaces of composite resin, compomer, and RMGI. The adhesion of *S. mutans*, *S. sobrinus,* and *P. gingivalis* was higher to RMGI which showed a rougher surface relative to composite resin or compomer. This study suggested that the SR is the cause of greater adhesion of bacteria, considering the greater differences in SR than SFE among the adhesives.

3. Debonding methods of residual orthodontic adhesives

After active orthodontic treatment, brackets are mechanically debonded and residual adhesive must be mechanically removed, since composite resins remnants accumulate dental plaque and might discolor.[27] Currently, no technique allows removal of the remnants of composite resins without any damage of the enamel surface. The underlying reasons are acid etching resulting in infiltration of composite resins into the enamel.[28] Efforts are made to minimize the loss of the enamel external layer, because it is the richest in fluoride and hardest.[29] In addition, the enamel surface should be left as smooth as possible after debonding, since deep scratching is not polished through the years by tooth brushing.[30] Although it was not possible to smooth out the debonding surfaces, tungsten carbide burs in either a low-speed or a high-speed hand piece have been the method of choice for removing remnants of adhesive resins.[31] Diamond burs were extremely destructive, stainless steel burs were inefficient and tungsten carbide burs were efficient, but left unsatisfactory enamel surface.[32] Polishing disks produced surfaces, which could be readily restored satisfactory after receiving a final polishing, but were slow in adhesive resin removal.[32] Using different burs in conjunction with rubber cups and pumice paste seems to be appropriate for adhesive resin removal.[31] Other studies used laser

energy for removal of adhesive resin, since it degrades the adhesive resin and reduces the force needed to remove orthodontic attachments. However, the Er:YAG laser also has been shown to cause irreversible enamel damage.[33]

4. Finishing and polishing methods of composite resins

Finishing refers to the contouring of the restoration to obtain the desired anatomy and complete any necessary occlusal adjustments, whereas polishing refers to the reduction of surface irregularities created by the finishing instruments.[34] A wide variety of finishing and polishing devices is available for the clinician today. Multi-fluted carbide finishing burs, hard-bonded/surface-coated ceramic diamond rotary instruments, impregnated rubber or silicon disks and wheels, and silicon carbide-coated or aluminum oxide-coated abrasive disks are among the most common devices used to finish dental restoratives.[35]

The longevity and esthetic appearance of tooth-colored dental restorative materials greatly depend on the quality of the finishing and polishing techniques employed.[36] High quality finishing and polishing improve both the esthetics and the longevity of composite restorations,

whereas rough, poorly polished surfaces contribute to staining, biofilm formation, gingival irritation, recurrent caries, and discoloration of the restoration.[37]

It is important to investigate biofilm formation of composite resins after finishing and polishing, because the formation of oral biofilms on the surface of it attenuates the longevity of restorations.[36] Yuan et al.[38] investigated the effects of the surface properties of composite resins after different polishing on early adhesion of *S. mutans in vitro*. They found that early adhesion of *S. mutans* on composite resins was mainly affected by SR. Cazzaniga et al.[21] evaluated *in vitro* biofilm formation on four composite resins after six different finishing and polishing procedures. Finishing and polishing procedures significantly influenced SR and gloss. However, finishing and polishing procedures did not significantly influence *S. mutans* biofilm formation. As found on these studies,[21,38] it is controversial that the effect of finishing and polishing on biofilm formation. In addition, the multiple changes of SR, SFE, and surface composition on various composite resins made it difficult to understand the effect of each change.

5. Multi-species biofilm model using CDC Biofilm reactor

Biofilms are structured communities of bacterial cells.[6] There is an increasing trend of laboratory studies focusing on microbial communities rather than single species populations. This is an important and highly relevant shift in focus because most biological systems in nature are multi-species communities rather than single species populations.[39] Christensen et al.[40] investigated cooperative interactions by which cross-feeding enables communities to digest hydrocarbons and other recalcitrant compounds that individual populations cannot. Biofilm communities also play a significant role during infection where different species can cooperate to enhance their virulence, increase tolerance to the host immune response or antimicrobials, alter the niche of infection and modify the host immune response.[41] There are many different approaches and methods that can be used for studying biofilm formation and development. A defined and engineered biofilm model system is highly reproducible, ease of manipulation, time and cost effective.[39]

A CDC biofilm reactor is often used to provide reproducible biofilm samples for the evaluation of antimicrobial agents and surface materials.[42] Biofilms develop upon coupons held within eight polypropylene holders (each housing three coupons of 12.7-mm diameter) suspended from the lid

and immersed in growth medium.[42] These are arranged such that they surround a centrally located stirring vane, which is operated magnetically. Sampling is achieved by removing coupon holders from the lid that can then be replaced or substituted with a stopper. Coupons made from various materials are commercially available.

Because each rod can be removed intermittently, the reactor allows evaluation of time-related microbial changes. In addition, CDC biofilm reactor provides dynamic condition rather than static one. As the fluid is pumped in and out with the same rate and mixed continuously, steady state condition without concentration gradient can be assumed.[43] Furthermore, controllable shearing force from stirring simulates fluid shear in the oral cavity.[42,43]

III. MATERIALS AND METHODS

1. Specimen preparation

The microhybrid composite resin, DenFil™ (Vericom, Anyang, Gyeonggido, Korea) was used to make specimens for this study. The composite resin was directly injected into a polytetrafluoroethylene disk shaped mold (3 mm thick and 10 mm in diameter) and packed carefully to evenly fill the mold before glass slide was put on the top of the mold. After extra material was carefully removed, the specimen was light cured for 20 seconds using light curing unit (B&Lite, B&L biotech, Ansan, Gyeonggido, Korea) from the top and bottom, respectively. The surface against glass slide was used as the experimental surface in this study. Eighty-four disks were prepared and randomly assigned to four groups: SR180, SR400, SR1500, and SRGlass. Disks in SR180, SR400, and SR1500 were manually roughened with 180-, 400-, 1500-grit wetted silicon carbide (SiC) papers (Daesung, Chungnam, Korea), respectively. Only one side of each disk was grinded 50 times against a wet SiC paper in constant straight distance (23 cm, the height of SiC papers) by a single investigator. Disks in SRGlass had an intact surface without surface roughening. In 84 disks, 80 disks (20 disks per each group)

were used for both SR measurements and biofilm experiments and 4 for scanning electron microscopy (SEM).

2. Surface analysis

The SR was analyzed using a confocal laser scanning microscopy (LSM 5 Pascal, Carl Zeiss MicroImaging GmbH, Göttingen, Germany). This allows for calculation of the arithmetic mean SR from a mean plane within the sampling area (450 × 450 × 50 μm). The measurements were performed on the three random points of each disk. SR was evaluated from experimental surfaces of all disks before biofilm experiments.

To examine the surface texture of each group (SR180, SR400, SR1500, and SRGlass), SEM analysis was used. Each surface was observed with a magnification set at both 200x and 3000x using a S-4700 microscope (Hitachi, Tokyo, Japan).

3. Bacterial preparation

Thirteen-species bacterial consortium containing *S. mutans* ATCC 700610, *S. sobrinus* ATCC 27607, *Streptococcus sanguinis* CCUG 17826,

Streptococcus salivarius CCUG 50207, *Streptococcus oralis* ATCC 9811, *Actinomyces naeslundii* KCOM 1472, *Lactobacillus rhamnosus* ATCC 7469, *Veillonella dispar* KCOM 1864, *Neisseria subflava* ATCC 49275. *Fusobacterium nucleatum* ATCC 10953, *Prevotella nigrescens* ATCC 33563, *P. gingivalis* KCOM 2797, and *A. actinomycetemcomitans* ATCC 43718 was used because of their prevalence, metabolic properties, influences on pathogenicity, and their convenience of experiment as previously described.[10] These bacteria reflects process of oral biofilm formation and intercommunication with bacteria, which starts with the early colonizers including *Streptococcus* and *Actinomyces* spp., followed by early-colonizing *Veillonellae* spp., middle-colonizing *Porphyromonads* spp. and *Fusobacteria* spp., and late-colonizing Gram-negative anaerobes (*P. nigrescens*, *P. gingivalis*, and *A. actinomycetemcomitans*).[44]

Each strain was individually grown to mid-exponential phase according to their properties.[10] *S. mutans, S. sobrinus, S. sanguinis, S. salivarius, S. oralis, A. naeslundii, L. rhamnosus, V. dispar*, and *N. subflava* were cultured in a brain heart infusion (BHI, Becton Dickinson, Sparks, MD, USA) medium at 37°C with 5% CO_2. *F. nucleatum, P. nigrescens*, and *P. gingivalis* were anaerobically grown in a tryptic soy agar (Becton Dickinson) medium supplemented with 10 μg/mL vitamin K, 5 μg/mL hemin, and 5%

sheep blood at 37°C for 7 days. These species were subcultured in BHI medium supplemented with 10 μg/mL vitamin K and 5 μg /mL hemin, and then grown to mid-exponential phase anaerobically at 37°C. *A. actinomycetemcomitans* was grown in a BHI medium at 37°C in an anaerobic atmosphere.

4. Multi-species biofilm formation

Modified McBain medium (MBM) containing 2.5 g/L porcine gastric mucin, 2 g/L peptone, 2.5 g/L KCl, 1 g/L yeast extract, 1 g/L trypticase peptone, 0.1 g/L cysteine hydrochloride, 0.001 g/L hemin, 10 mM glucose, and 10 mM urea was used to supply nutrition sources and to simulate saliva as previously described.[45]

A CDC biofilm reactor (BioSurface Technologies, Bozeman, MT, USA) which reproduces oral environment, was used for cultivating multi-species biofilms.[42] There were shear force and the constant flow of fresh medium in this biofilm reactor to simulate dynamic oral environment. Each disk was put into a polytetrafluoroethylene cylinder to only exposure the experimental surfaces and to mask the opposite surfaces. Each disk in the polytetrafluoroethylene cylinder was randomly inserted into each rod. After

sterilization of the rods with disks, the equipment, and MBM, the reactor was set on a hot stir plate set at 37°C with a rotation speed of 60 rpm as previously descried.[10] The consortium of the bacteria mixture listed above was inserted to the biofilm reactor (3.5 mL, 1% of the reactor volume) and a constant flow of MBM was then flushed through the reactor at a rate of 100 mL/hour during the experiment.

5. Microbial analysis

To compare time-related biofilm composition, eight disks (two sets of four different composite resin groups) were collected from the reactor at two time points: days 1 (T1) and 4 (T2). Because mature biofilms formed in the CDC biofilm reactor after 72 hours in the previous study,[46] biofilms at T1 and T2 reflects early and mature biofilms, respectively.

Each disk was transferred into a round tube and washed two times with 1.0 mL phosphate-buffered saline (PBS, pH = 7.4) to remove unbound bacteria. The biofilm was then detached from each disk by sonication with three 30-second pulses and 30-second intermittent cooling stages in a chilled ice box. After removing the disk, the bacterial cell suspension was centrifuged at 13,000 rpm for 10 minutes and washed twice with 1.0 mL

PBS.

Bacterial chromosomal DNA was extracted using a CellEase Bacteria II Genomic DNA Extraction kit (Biocosm, Osaka, Japan) according to the manufacturer's instruction. A NanoVue spectrophotometer (General Electric Healthcare Life Sciences, Pittsburgh, PA, USA) was used to assess the quality of the extracted DNA after preparation.

Known specific polymerase chain reaction (PCR) primers that amplify the dextranase genes of *S. mutans* and *S. sobrinus* were designed from the *gtfB* and *gtfU* genes, respectively.[47] The PCR primers for *P. gingivalis* were designed based on the 16S rRNA gene, and the primers for *A. actinomycetemcomitans* were based on the *rpoB* gene of RNA polymerase β subunit gene. A conserved sequence in the 16S rRNA gene was selected to quantify the numbers of total bacteria.[47] All primers were commercially synthesized (Bioneer, Daejeon, Korea).

DNA was extracted from *S. mutans* ATCC 700610, *S. sobrinus* ATCC 27607, *P. gingivalis* KCOM 2797, and *A. actinomycetemcomitans* ATCC 43718 using a G-spin Genomic DNA Extraction kit (iNtRON Biotechnology, Seongnam, Korea) according to the manufacturer's instructions. The DNA standard curve was obtained from known amounts of purified PCR product. PCR products were isolated from agarose gels using a QIAquick Gel

Extraction kit (Qiagen, Düsseldorf, Germany). DNA concentration was estimated by absorbance at 260 nm and a series of 10-fold dilutions ranging from 10 to 10^8 copies was prepared for standard curves as previously described.[48] The amount of bacterial DNA in the samples was estimated from the standard curve.

Real-time PCR was performed using the iQ5 system (Bio-Rad, Hercules, CA, USA). The reaction mixtures contained 2 μL purified DNA from the disk samples, 100 pM primer, and 10 μL 2x iQ SYBR Green Supermix (Bio-Rad). Distilled water was added to a final volume of 20 μL. Thermal cycling conditions were presented in Table 1. PCR data were analyzed using iQ5 Optical System Software (BioRad). All the experiments for quantifying bacterial levels were performed in duplicate and independently repeated five times.

6. Statistical analysis

Differences in SR among the four groups were tested using one-way analysis of variance and Tukey HSD multiple comparisons. Multifactorial analysis of variance using the Bonferroni correction was used to determine the time-related differences in the bacterial amounts with respect to SR. For all analyses, $p < 0.05$ was considered statistically significant.

IV. RESULTS

1. Surface roughness

There were significant differences in SR among the four groups (Table 2). The order of SR, from highest to lowest, was SR180 (1.45 ± 0.11 μm), SR400 (0.62 ± 0.05 μm), SR1500 (0.35 ± 0.02 μm), and SRGlass (0.15 ± 0.01 μm) (SR180 > SR400 > SR1500 > SRGlass, $p < 0.05$) (Table 2). The mean variation of SR in each group was relatively small (less than 0.11 μm), compared to previous studies,[18,49] indicating uniform SR of experimental surfaces within the same group.

2. Surface texture

SEM images at a 200x magnification showed that the roughed surface had a noticeable increase in narrower grooves with increased SR (Figures 1A, 1B, and 1C), compared to the smooth surface of SRGlass (Figure 1D). At a magnification of 3000x, surface irregularities gradually increased as SR increased from SR1500 to SR 180 (Figures 2A, 2B, and 2C).

3. Biofilm composition with respect to surface roughness

Table 3 shows the differences in biofilm composition with respect to SR and incubation time. The results showed that both SR and incubation time had significant effects on biofilm compositions, without significant interaction effects. There was a significant difference in incubation time among the four SR groups. The number of total bacteria, *S. mutans*, and *S. sobrinus* increased from T1 to T2 ($T1 < T2$, $p < 0.05$), while *P. gingivalis* and *A. actinomycetemcomitans* significantly decreased from T1 to T2 ($T1 > T2$, $p < 0.05$).

SR also significantly affected the biofilm composition, but there was a significant difference in adhesion patterns between bacterial species. The significant differences in the adhesion of total bacteria were only found between SRGlass and SR180 (SR180 > SRGlass, $p < 0.05$). The adhesion of *S. mutans* and *S. sobrinus* to SR180 and SR400 was higher than to that of SRGlass (SR180 = SR400 > SRGlass, $p < 0.05$) (Table 3). However, there was no significant difference in the adhesion of *A. actinomycetemcomitans* and *P. gingivalis* to composite resins with respect to SR.

V. DISCUSSION

Biofilm formation is significantly affected by surface properties of underlying dental biomaterials.[13] Surface properties that influence biofilm formation on composite resins consist of SR, SFE, and surface chemistry.[5] Many studies have evaluated the differences in bacterial adhesion to composite resins with various surface properties.[17,49] In those studies, the effects of SR on biofilm formation have not been fully explained, because each surface property had not been properly controlled. Although a few studies have investigated the effect of SR on biofilm formation on composite resins after controlling surface properties,[17,18] they only used a single-species biofilm model, which is quite different from oral ecology. In this study, we used a multi-species biofilm model to evaluate the effects of SR on biofilm composition by controlling other surface properties.

The SFE of polymers influences bacterial adhesion.[13] The SFE is determined by measuring the contact angle of the drop of a probe liquid after visualizing the shape of the drop formed on underlying materials.[10] Changes of more than 0.1 μm of SR significantly influence the contact angle when measured on a smooth surface,[13] but SR changes do not significantly influence SFE when the contact angle of an underlying material is between

60° and 86°.[50] When we measured the water contact angle of the composite resins in our pilot study, there was no significant difference among the four SR groups; SR180 (66.24 ± 1.28°), SR400 (65.54 ± 0.88°), SR1500 (65.48 ± 1.42°) and SRGlass (66.32 ± 0.50°), which is consistent with previous studies.[50] This suggests that the SFE of the composite resins disks may not be significantly influenced by SR changes in the present study. This might be due to the use of a single composite resin as a specimen. The filler/matrix ratio and the type and amount of composite resin matrix monomers, significantly influence surface chemistry as well as SFE. In addition, different roughening procedures can affect surface properties. We used a single composite resin and the same manual roughening procedure to control the effects of other surface properties, except SR. Therefore, based on this approach, only SR could significantly influence biofilm composition in this study.

Table 2 shows significant differences in SR among the groups. Polishing and/or finishing using an aluminum oxide-coated abrasive disks, a white stone, a carbide bur, or a diamond bur roughens surfaces of composite resins to a range of 0.11 to 1.47 μm.[51,52] In this study, SRs of SR180, SR400, SR1500, and SRGlass groups (0.15 to 1.45 μm) represent those achieved by clinical finishing and/or polishing techniques (Table 2);

SR180 reproduces polishing with a diamond bur at high speed (average 1.47 μm),[51] SR400 with white stone (average 0.70 μm),[52] SR1500 with carbide bur (30 blades, average 0.31 μm),[35] and SRGlass with aluminum oxide-coated abrasive disks (average 0.15 μm),[35] respectively.

There were significant differences in biofilm composition formed on composite resins with respect to SR. However, the adhesion profiles differed between bacterial species. Surface roughening significantly promoted adhesion of total bacteria and cariogenic bacteria (*S. mutans* and *S. sobrinus*), but did not significantly influence adhesion of *A. actinomycetemcomitans* and *P. gingivalis* to composite resins (Table 3). This indicates that SR can have a more significant influence on the adhesion of early colonizers (*S. mutans* and *S. sobrinus*) than on that of the late colonizers (*A. actinomycetemcomitans* and *P. gingivalis*).[13,14] This might be because the secondary colonizers (e.g. *P. intermedia*, *Prevotella loescheii*, *Capnocytophaga spp.*, *F. nucleatum*, and *P. gingivalis*) do not initially colonize on tooth surfaces, but adhere to early colonizers that have already adhered to tooth surfaces.[14] Adhesion of total bacteria is significantly influenced by early colonizers rather than later colonizers,[53] which might be why adhesion of total bacteria to composite resins was significantly increased after surface roughening.

Although surface roughening significantly influenced bacterial composition formed on composite resins, an increase in SR was not directly proportional to bacterial adhesion. In this study, the adhesion of *S. mutans* and *S. sobrinus* to SR180 and SR400 was higher than to SRGlass (SR180 = SR400 > SRGlass, $p < 0.05$), while there was no significant difference in adhesion of *S. mutans* and *S. sobrinus* between SR180 or SR400 and SR1500 and between SR1500 and SRGlass. In other words, adhesion of *S. mutans* and *S. sobrinus* was significantly increased in composite resins with average 0.62 μm of SR (SR400), but there were no significant differences in bacterial adhesion between SR400 (0.62 μm) and SR180 (1.45 μm). This indicates that an SR over a certain level might not significantly influence biofilm composition on composite resins. Saliva that coats the rough surfaces could be one of the reasons for this nonlinear proportion between SR and bacterial adhesion. In this study, we used MBM containing porcine gastric mucin that coats the surface, similar to salivary pellicles.[45] The formation of mucin coating on the roughened surface would mask the physiochemical surface properties of dental materials and alter SR effects.[54] Multi-species biofilm models might be another reason for these nonlinear proportions. A previous study that used a six-species biofilm model revealed that the roughness of the substrate had no effect on the biofilm mass after 15 hours of incubation time.[22] The formation of multi-species biofilms is part of a complex process

and has inter-species interactions. Biofilm formation is not only governed by initial adherence to the surface of dental biomaterials, but also by growth conditions.[22] During growth, middle and/or late colonizers adhere to the already present biofilm and not the surface of materials. Although there was a significant difference in SR among the groups, SEM images showed that SR180, SR400, and SR1500 had similar surface irregularity patterns (Figures 1 and 2). This could also reduce the differences in biofilm composition with respect to SR change.

Incubation time also significantly influenced the composition of biofilms (Table 3). The amount of *S. mutans* and *S. sobrinus* in biofilms significantly increased (T1 < T2), while that of *P. gingivalis* and *A. actinomycetemcomitans* was significantly decreased with extended incubation time (T1 > T2). As biofilm matures, the microenvironment changes from aerobic to anaerobic, and anaerobic bacteria become prevalent.[55] In this experiment, however, we simulated the supragingival oral environment with aerobic conditions, continuous shear force, and a constant flow of fresh medium. Therefore, this could cause a continuous oxygen supply in the reactor, which can decease anaerobic bacteria (*P. gingivalis* and *A. actinomycetemcomitans*) and increase facultative bacteria (*S. mutans* and *S. sobrinus*) with extended incubation time. This result is

consistent with a previous study.[10]

These results indicated that the threshold SR value for increased adhesion of *S. mutans* and *S. sobrinus* to the composite resin lies between 0.35 μm (SR1500) and 0.62 μm (SR400). When SRs of the orthodontic materials measured in the previous study were compared with those of the present study, SRs of four orthodontic adhesives were smaller than 0.62 μm (SR400); fluoride-releasing composite resins (SR 0.43 ± 0.01 μm), nonfluoride-releasing composite resins (SR 0.38 ± 0.02 μm), compomer (SR 0.42 ± 0.01 μm), and RMGI (SR 0.39 ± 0.02 μm),[12] indicating that SRs of the orthodontic adhesives may not significantly influence on the adhesion of MS. However, SRs of two bracket materials were larger than the 0.62 μm (SR400); polycrystalline alumina (SR 0.76 ± 0.03 μm) and stainless-steel metal (SR 0.62 ± 0.03 μm),[12] indicating that SRs of bracket materials may significantly facilitate the adhesion of MS to them.

After active orthodontic treatment, brackets are mechanically debonded and residual adhesive must be removed, since adhesive resin remnants accumulate dental plaque and might discolor.[27] Tungsten carbide burs in either a low-speed or a high-speed hand piece have been the method of choice for removing adhesive resin remnants.[31] Adhesive resin removal by tungsten carbide bur increased SR of the enamel by about 0.02

μm, which may not be clinically relevant.[33] However, when the brackets are debonded from the enamel surfaces restored with composite resins, tungsten carbide burs may significantly increase SR of the composite resin surfaces by up to 1.51 μm depending on the type of composite resins and burs.[51,52]

In addition, daily tooth brushing and aging process also increases the SR of composite resin by about 0.34 μm,[56,57] which may worsen the durability of the restoration by inducing increased adhesion of cariogenic streptococci. Highly-polished surfaces can be easily achieved by reducing filler size[58] and periodic polishing systems with several techniques (for example, aluminum oxide-coated disks) may smoothen composite resins below 0.15 μm of SR.[35] This study suggests that using the combination of composites with smaller filler sizes and a periodic repolishing with a specific device are recommended to minimize cariogenic biofilm development around composite surfaces, which may increase longevity of restoration and oral health.

There were some limitations in this study. The specimens prepared in the laboratory were different from those prepared in the clinical environment. In addition, over 500 species are present in the oral cavity,[59] which is also different from our experimental condition. Further study will

be required to investigate the effects of SR on biofilm composition in *in vivo* clinical situations.

VII. CONCLUSIONS

The purpose of this study was to investigate the effects of SR on multi-species biofilm composition of composite resins. The results showed that surface roughening significantly increased the adhesion of total bacteria, *S. mutans*, and *S. sobrinus* to composite resins. However, an increase in SR was not directly proportional to bacterial adhesion when the SR was over 0.62 μm. As a result, there was no significant difference in the adhesion of *S. mutans*, and *S. sobrinus* to composite resins with an SR greater than 0.62 μm. Late colonizers, such as *A. actinomycetemcomitans* and *P. gingivalis*, were not significantly influenced by SR changes. Incubation time also significantly influenced the composition of biofilms. Adhesion of total bacteria and cariogenic streptococci (*S. mutans* and *S. sobrinus*) increased, but adhesion of periodontopathogens (*A. actinomycetemcomitans* and *P. gingivalis*) decreased with extended incubation time. A periodic smoothening to around 0.15 μm of SR is an effective to minimize cariogenic biofilm formation through controlling composite surface SRs, because decreased adhesion of cariogenic streptococci was observed at values around 0.15 μm of SR.

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TABLES

Table 1. Primers and cycling conditions used

Primer	Sequence(5'-to-3')	Size of amplification (base pairs)	Initial denaturation	Denaturation	Annealing	Extension	Cycles
Universal	Forward:TGGAGCATGTGGTTAATTCGA	160	94℃	95℃	60℃	60℃	40
	Reverse:TGCGGACTTAACCCAACA		30 seconds	20 seconds	45 seconds	10 seconds	
Streptococcus mutans	Forward:CTACACTTTCGGGTGGCTTG	261	94℃	95℃	60℃	60℃	40
	Reverse:GAAGCTTTTCACCATTAGAAGCTG		30 seconds	20 seconds	45 seconds	10 seconds	
Streptococcus sobrinus	Forward:AAAACATTGGGTTACGATTGCG	156	94℃	95℃	60℃	60℃	40
	Reverse:CGTCATTGGTAGTAGCCTGA		30 seconds	20 seconds	45 seconds	10 seconds	
Porphyromonas gingivalis	Forward:TGCAACTTGCCTTACAGAGGG	344	95℃	95℃	61℃	72℃	40
	Reverse:ACTCGTATCGCCCGTTATTC		60 seconds	5 seconds	15 seconds	33 seconds	
Aggregatibacter actinomycetemcomitans	Forward:GGCGAGCCTGTATTTGATGTGCG	113	95℃	95℃	72℃		40
	Reverse:GTGCCCGGTGGTGCGTCTTTG		10 minutes	10 seconds	30 seconds		

Table 2. The surface roughness difference according to the surface treatment conditions.

	Surface treatment				Multiple comparisons
	SR180[a]	SR400[b]	SR1500[c]	SRGlass[d]	
Surface roughness (μm)	1.45 ± 0.11	0.62 ± 0.05	0.35 ± 0.02	0.15 ± 0.01	SR180 > SR400 > SR1500 > SRGlass ($p < 0.001$)

[a] The composite surface roughened with 180-grit silicon carbide paper.

[b] The composite surface roughened with 400-grit silicon carbide paper.

[c] The composite surface roughened with 1500-grit silicon carbide paper.

[d] The composite surface prepared with a glass slide.

One-way analysis of variance followed by the post-hoc Tukey HSD multiple comparison test were performed to analyze the intergroup difference at the level of $\alpha = 0.05$.

Table 3. Biofilm composition with respect to surface roughness

	Day 1 (T1)	Day 4 (T2)	Multiple comparisons	
			Time	Surface roughness
Total bacteria (Log_{10}/unit area)				
SR180[a]	7.25 ± 0.19	7.80 ± 0.38		
SR400[b]	7.20 ± 0.24	7.77 ± 0.33	T1 < T2	SR180 > SRGlass
SR1500[c]	7.10 ± 0.21	7.70 ± 0.36	(p < 0.001)	(p = 0.029)
SRGlass[d]	7.06 ± 0.24	7.62 ± 0.39		
Streptococcus mutans (Log_{10}/unit area)				
SR180[a]	3.87 ± 0.42	4.24 ± 0.60		
SR400[b]	3.81 ± 0.46	4.12 ± 0.48	T1 < T2	SR180 = SR400 > SRGlass
SR1500[c]	3.76 ± 0.42	4.06 ± 0.27	(p < 0.001)	(p = 0.003)
SRGlass[d]	3.60 ± 0.36	3.81 ± 0.33		
Streptococcus sobrinus (Log_{10}/unit area)				
SR180[a]	4.05 ± 0.20	4.97 ± 0.61		
SR400[b]	4.05 ± 0.37	4.75 ± 0.62	T1 < T2	SR180 = SR400 > SRGlass
SR1500[c]	3.94 ± 0.38	4.80 ± 0.58	(p < 0.001)	(p = 0.002)
SRGlass[d]	3.78 ± 0.22	4.44 ± 0.61		
Aggregatibacter actinomycetemcomitans (Log_{10}/unit area)				
SR180[a]	4.31 ± 0.50	3.93 ± 0.38		
SR400[b]	4.17 ± 0.27	3.88 ± 0.29	T1 > T2	SR180 = SR400 = SR1500 = SRGlass
SR1500[c]	4.12 ± 0.20	3.91 ± 0.27	(p < 0.001)	(p = 0.155)
SRGlass[d]	4.02 ± 0.23	3.87 ± 0.46		
Porphyromonas gingivalis (Log_{10}/unit area)				
SR180[a]	2.15 ± 0.98	1.96 ± 0.67		
SR400[b]	2.02 ± 1.16	1.69 ± 0.91	T1 > T2	SR180 = SR400 = SR1500 = SRGlass
SR1500[c]	2.08 ± 0.88	1.71 ± 0.86	(p = 0.013)	(p = 0.745)
SRGlass[d]	2.21 ± 0.61	1.74 ± 0.70		

[a] The composite surface roughened with 180-grit silicon carbide paper.

[b] The composite surface roughened with 400-grit silicon carbide paper.

[c] The composite surface roughened with 1500-grit silicon carbide paper.

[d] The composite surface prepared with a glass slide.

Multifactorial analysis of variance was used to determine significant differences between the two time points using the Bonferroni corrections.

FIGURES

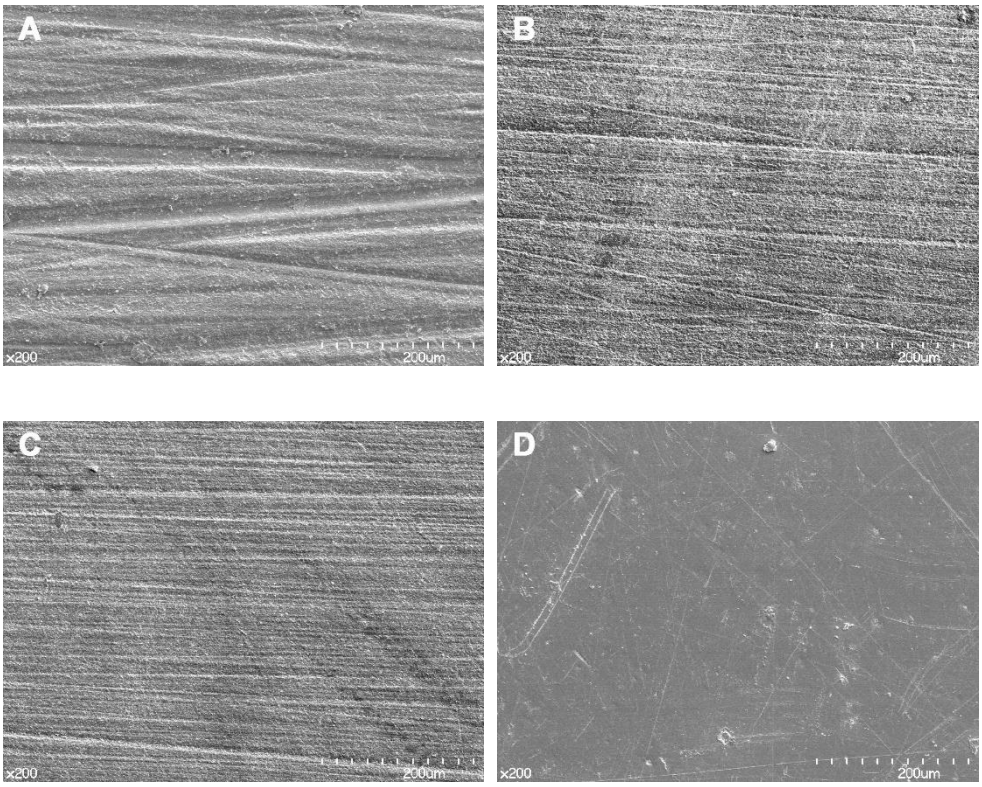

Figure 1. Scanning electron microscopic images of composite surfaces at 200x. (A) Composite surface prepared against 180-grit silicon carbide paper at 200x. (B) Composite surface prepared against 400-grit silicon carbide paper at 200x. (C) Composite surface prepared against 1500-grit silicon carbide paper at 200x. (D) Composite surface prepared with a glass slide at 200x.

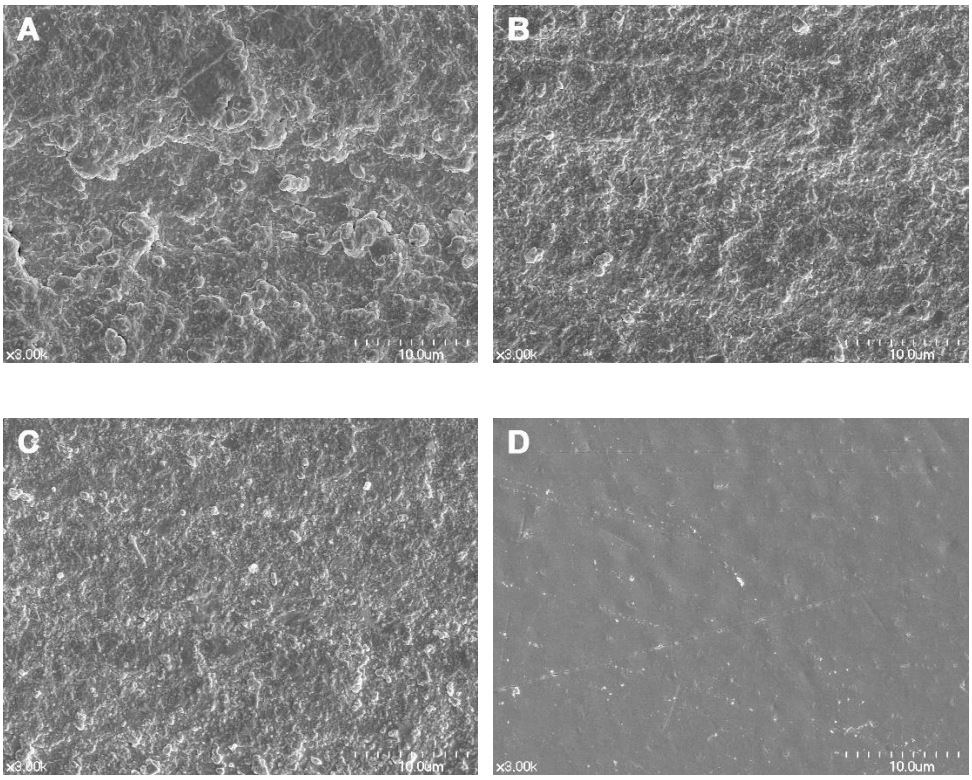

Figure 2. Scanning electron microscopic images of composite surfaces at 3000x. (A) Composite surface prepared against 180-grit silicon carbide paper at 3000x. (B) Composite surface prepared against 400-grit silicon carbide paper at 3000x. (C) Composite surface prepared against 1500-grit silicon carbide paper at 3000x. (D) Composite surface prepared with a glass slide at 3000x.

국문 초록

표면거칠기가 복합레진에 형성된 바이오필름 조성에 미치는 영향에 대한 연구

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목적: 본 연구는 표면거칠기가 복합레진 표면에 형성된 다세균종 바이오필름의 조성에 미치는 영향을 평가하기 위해 시행되었다.

방법: Polytetrafluoroethylene 주형과 유리판을 이용하여 복합레진 시편을 준비하고 SR180, SR400, SR1500, SRGlass 그룹에 무작위로 배정하였다. SR180, SR400, SR1500에 배정된 시편들은 180-, 400-, 1500-grit의 사포를 이용하여 표면을 연마하였고, SRGlass 시편은 표면을 연마하지 않은

대조군으로 사용하였다. 복합레진 표면에 다세균종 바이오필름을 형성시킨 후 병원성 세균인 *Streptococcus mutans* (Sm), *Streptococcus sobrinus* (Ss), *Aggregatibacter actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg) 및 total bacteria의 부착량을 실험 1일차(T1) 와 4일차(T2)에 평가하였다. 표면거칠기가 바이오필름 조성에 미치는 영향을 평가하기 위해 공초점레이저주사현미경과 주사전자현미경을 이용하여 표면거칠기와 표면형태를 각각 분석하였다. 각 군 사이 표면거칠기의 차이는 일원분산분석 및 Tukey HSD 다중비교를 이용하여 평가하고, 다요인 분산분석을 이용하여 표면거칠기 및 시간에 따른 세균 조성의 변화를 평가한 후 다음과 같은 결과를 얻었다.

결과: 표면거칠기는 네 군 사이에 통계적으로 유의한 차이를 나타내었고, 표면거칠기의 크기 차이는 SR180 (1.45 ± 0.11 μm), SR400 (0.62 ± 0.05 μm), SR1500 (0.35 ± 0.02 μm), SRGlass (0.15 ± 0.01 μm) (SR180 > SR400 > SR1500 > SRGlass, $p < 0.05$)였다. 표면 거칠기의 증가는 세균 부착과 비례하지 않았다. Total bacteria 부착은 SRGlass와 SR180 사이에서 유의한 차이가 나타난 반면 (SR180 > SRGlass, $p < 0.05$), Sm과 Ss의 부착은 SRGlass에 비해 SR180과 SR400에서 유의한 증가를 보였다 (SR180 = SR400 > SRGlass, $p < 0.05$). 이 결과는 표면거칠기가 약 0.15 μm 이하에서 우식 원인균 (Sm 와 Ss)과 total bacteria의 부착이 감소한 것을 의미한다. 복합레진에 대한 Aa와 Pg의 부착은 표면거칠기에 따라 통계적으로 유의한

차이가 나타나지 않았다. Total bacteria, Sm, Ss의 부착은 T1에서 T2사이에서 유의하게 증가한 반면 ($T1 < T2$, $p < 0.05$), 치주병원균들의 부착은 T1에서 T2사이에서 유의한 감소를 보였다 ($T1 > T2$, $p < 0.05$).

결론: 이상의 결과를 고려할 때 본 연구는 복합레진 표면의 우식원인균 부착을 억제하기 위해서 표면거칠기를 0.15 μm 이하로 유지하도록 복합레진의 주기적인 표면 연마가 필요함을 제시한다.

주요어 : 다세균종 바이오필름, 표면거칠기, 복합레진

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