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트리테르페노이드계 물질의 항바이오필름 효과에 관한 표면 형태 연구

Influence of surface topography on anti-biofilm activity of triterpenoid compound

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ABSTRACT

Influence of surface topography on anti-biofilm activity of triterpenoid compound

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OBJECTIVES: The purpose of this study was to investigate the effect of three-dimensional surface topography on the Streptococcus mutans UA159 biofilm formation in the presence of an antibacterial triterpenoid compound.

METHODS: Two types of composite resin disks, flat surface and surface with grooves, were used throughout this experiment. The biofilm-eradicating properties of ursolic acid (UA) and chlorhexidine (CHX) were evaluated according to various times (0, 15, 30, 45, 60 minutes) and sub-minimum biofilm inhibitory concentrations (0, 80, 160, 320, 640 μg/ml of UA and 0, 15.625, 31.25, 62.5, 125 μg/ml of CHX). To understand the role of surface topography on Streptococcus mutans biofilm formation, scanning electron microscopy (SEM) and crystal violet assay were used for qualitative and quantitative assessments, respectively.

RESULTS: After biofilm formation, treatment with antimicrobial agent led to no significant differences in the anti-biofilm activity of UA or CHX between the flat and
groove disks. When the disks were treated with antimicrobial before biofilm formation, there was more biofilm formation on groove composite resin disk compared to flat composite resin disk, while the control group without antimicrobial agent resulted in similar amount of biofilm formation for flat and groove disks.

CONCLUSIONS: This study suggests that antimicrobial can be used as an effective therapeutic regimen on pre-existing biofilm regardless of surface topography. However, when antimicrobial is administered as a preventive regimen, it will have limited effect in inhibiting biofilm formation on caries-prone surfaces of tooth. Therefore, surface topography plays a significant role in the biofilm formation of *Streptococcus mutans* UA159 in the presence of antimicrobial agent.

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Keywords: Surface topography, *Streptococcus mutans*, Biofilm, Triterpenoid compound

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Influence of surface topography on anti-biofilm activity of triterpenoid compound

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Contents

I. Introduction
II. Materials and Methods
III. Results
IV. Discussion
V. Conclusions

References

Korean Abstract
I. INTRODUCTION

The human oral cavity possesses inherent ability to hold various microorganisms through the existence of both shedding (soft tissue) and non-shedding (hard tissue) surfaces [1]. The pathogenicity of the microbes is manifested only when they adhere irreversibly to the non-shedding surfaces and accumulate into biofilm [2]. Specific regions of the oral cavity are susceptible to different types of bacterial species [3], and certain anatomical features, such as grooves, pits and fissures, and interproximal contacts, protect bacterial colonies from environmental fluctuations, making the host vulnerable to oral diseases [4]. This distinct anatomy of the tooth attracts *Streptococcus mutans*, the primary bacterial species responsible for dental caries, and serves as a reservoir, providing retentive sites for colonization and biofilm formation through the production of extracellular matrix [3]. As a result, these areas become more prone to the progression of carious lesions.

Mechanical oral hygiene practice is the most effective method in disrupting the oral biofilm [5]. For those areas difficult to access mechanically, an adjunctive use of antimicrobial agent is an excellent way of controlling pathogenic bacteria [5]. Currently, there are many antibacterial products used in dentistry known to inhibit and reduce cariogenic biofilm. The most potent and effective antiplaque agent among them is chlorhexidine [6, 7]. Nevertheless, due to its side effects, including extrinsic staining, calculus formation, and altered taste perception, long-term oral use of chlorhexidine is limited [8]. As a potential alternative, many naturally occurring antimicrobials have been investigated on cariogenic bacteria [8-12].

Two triterpenoids, ursolic acid (UA) and oleanolic acid (OA), are plant-derived
compounds that have been studied extensively due to their non-toxic and versatile effects, including anti-hepatotoxicity, anti-inflammation, tumor growth inhibition, and lipid reduction [13]. In addition, it has been documented that UA and OA possess anti-cariogenic properties, such as the glucosyltransferase inhibitory activities of *S. mutans* [14, 15]. However, the results from these *in vitro* experiments needed further validation with a biofilm model simulating the *in vivo* environment since the bacteria in the form of biofilm are primarily responsible for causing disease and more resistant to antimicrobial agents than the planktonic form [5].

An attempt has been made in the most recent study [9] to examine the antibacterial activity of UA and OA against cariogenic biofilms. Yet, the antibacterial effect of the agents on bacterial biofilm was assessed with the minimum inhibitory concentrations which were determined using planktonic cells. Even though it may be assumed that the antibacterial susceptibility against the biofilm will show higher resistance to the antimicrobials, verifying the inhibitory concentration of the drug against biofilm is fundamental for accurately interpreting the results of the associated biofilm experiments. Although the influence of UA and OA on the biofilm formation has already been evaluated by Zhou *et al.* on non-restored tooth surface [9], the effect of these antimicrobials on the biofilm formation on dental materials, such as composite resin, has not been published to the best of our knowledge. It has been reported that more dental biofilms inhabit the surfaces of composite resin, an esthetic restorative material that is being widely used to treat dental caries, compared to enamel and other restorations, resulting in deterioration of the material and recurrent caries [6, 7]. Also, the authors did not take into account the three-dimensional surface topographical features of teeth in their
study. Though it has been acknowledged that the surface topography characterizing the tooth anatomy makes the host more susceptible to dental diseases and may be of more importance than surface roughness in the S. mutans biofilm formation [16], no complete interpretation of its significance has been formulated. Therefore, the mechanism of biofilm control on the surface of restored tooth in the presence of antimicrobial agent is yet to be clarified.

The purpose of this study was to investigate the effect of surface topography on the elimination and prevention of *Streptococcus mutans* UA159 biofilm in relation to the anti-biofilm activity of UA and OA using a three-dimensional composite resin tooth model.
II. MATERIALS AND METHODS

Fabrication of composite resin disks

Resin-based composites (DenFil™, Vericom, Chuncheon, Korea) were used to prepare 2 types of disks, flat and groove, using a cylindrical Teflon mold (8.5 mm in diameter and 2.0 mm in thickness). The flat disk (Figure 1A) was formed by pressing 2 glass slides against the top and the bottom of the mold. Then both sides of the disk were light-cured for 20 seconds each, and a permanent marker was used to indicate the bottom of the disk. For the groove disk (Figure 1B), only one glass slide was used to form the base, and 2 indentations intersecting each other were made across the diameter of the mold with #15 scalpel blade to simulate the topographical characteristics of tooth. In order to replicate the groove disk, a mold was fabricated using light (Examixfine, GC Corporation, Tokyo, Japan) and heavy body vinyl polysiloxane (Delikit, HappiDen Corporation, Busan, Korea) and filled with composite resin. The disks were sterilized with ethylene oxide gas after polishing the edges with rubber points.

The surface areas of the flat and groove disks used in this study were 0.567 cm² and 0.665 cm², respectively. The resulting values from the biofilm assays were corrected by the difference in the surface areas, so it can be assumed that the surface area where biofilm formation took place was same for both disk types.
Surface roughness (Ra) of composite resin disks

Three samples from each disk group were randomly selected to measure the surface roughness (Ra) within the sampling area limited to 92.1 x 92.1 x 50 μm using confocal laser scanning microscopy (LSM 5 Pascal, Carl Zeiss, Jena, Germany). Ra values were obtained from 5 different regions of each sample. For the groove disk, a set of 5 readings were taken from each region outside and inside the groove.

Bacterial strain and antimicrobial preparation

Streptococcus mutans UA159 was streaked on brain heart infusion (BHI; Becton, Dickinson and Co., Sparks, MD, USA) agar plate and incubated for 48 hours at 37°C in 5% CO₂ atmosphere. Single bacterial colony was inoculated into 3 ml of BHI broth and grown overnight. Then the strain was kept at -80°C with 50% glycerol for further use.

Ursolic acid (UA, ≥90 %), oleanolic acid (OA, ≥97 %), and chlorhexidine digluconate (CHX, 20 % v/v) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and 102.4 mg of UA or OA were dissolved in 10 ml of dimethyl sulphoxide (DMSO; Sigma-Aldrich) to obtain 10.24 mg/ml stock solutions, as previously described [9]. The subsequent experiments were carried out with various concentrations of the antimicrobials diluted in
phosphate buffered saline (PBS, pH 7.4).

**Saliva collection**

Saliva was collected from healthy volunteers, who did not have any active dental caries or periodontal disease, in the morning at least 2 hours after brushing, drinking, and eating. The saliva sample was centrifuged at 4,500 rpm for 10 minutes at 4°C to remove any cellular debris, and the resulting supernatant was used after filter sterilization through a 0.22 μm Stericup & Steritop filter (Millipore, Billerica, MA, USA).

**Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)**

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined using a broth-dilution method in a 96-well sterile microtitre plate according to the National Committee for Clinical Laboratory Standards [17]. The overnight *S. mutans* culture was grown in BHI broth at 37°C in 5% CO₂ atmosphere until optical density (OD) of the subculture reached 0.5 at 600 nm, which is approximately 6.5 x 10⁷ colony forming unit/ml (CFU/ml). Ranges of UA (0.25 to 256 μg/ml), OA (0.25 to 256 μg/ml), and CHX (0.5 to 32 μg/ml) concentrations were obtained from two-fold serial dilutions using BHI broth. Then the bacterial culture was added to yield concentration of 1 x 10⁶ CFU/ml per well, resulting in a final DMSO concentration of less than 1% in each of the test cultures, and incubated overnight (37°C and 5% CO₂). The MIC was defined as the lowest concentration of the antimicrobial that inhibited bacterial growth, observed by a pellet of microorganisms in the bottom of the well and
confirmed with OD$_{595}$ readings. To determine the MBC, 50 μl of the bacterial culture from the MIC and above were spread onto BHI agar plates and incubated for 24 – 48 hours (37°C and 5% CO$_2$). The MBC was defined as the lowest concentration of the antimicrobial that killed 99.9% of the initial inoculum.

Minimum biofilm inhibitory concentration (MBIC)

The method described by Wei et al. [18] was modified to determine the minimum biofilm inhibitory concentration (MBIC) to test the susceptibility of $S. \text{mutans}$ UA159 biofilm formed on composite resin disks against UA, OA, and CHX. The overnight $S. \text{mutans}$ culture was grown in BHI broth at 37°C in 5% CO$_2$ atmosphere until optical density (OD) of the subculture reached 0.5 at 600 nm (approximately 6.5 x 10$^7$ CFU/ml), which was diluted 1:100 in a pre-warmed biofilm medium (BM) with 20 mM sucrose (BM-sucrose), as previously described [19] and inoculated on the flat and groove disks placed in a polystyrene 48-well cell culture plate. Then serial dilution method was performed to achieve the following concentration ranges of the antimicrobials: 160 to 2,560 μg/ml of UA, 160 to 2,560 μg/ml of OA, and 31.25 to 500 μg/ml of CHX. After biofilm formation for 24 hours at 37°C in 5% CO$_2$ atmosphere, the suspensions were carefully decanted, and the disks were washed twice with 500 μl of PBS and transferred to 14 ml round-bottom tubes containing 3 ml of PBS. The bacterial cells of the biofilm were detached by sonication using three 10-second pulses at 20 watts and kept on ice. To determine the MBIC, 50 μl of the bacterial suspensions were plated onto BHI agar and incubated for 24 – 48 hours at 37°C in 5% CO$_2$ atmosphere. The MBIC was defined as the lowest
concentration of antimicrobial that completely inhibited biofilm formation, shown by no colony forming unit (CFU).

**Time vs. concentration dependent killing assay**

The bactericidal effects of UA and CHX were evaluated on *S. mutans* UA159 biofilm grown on both flat and groove disks according to various incubation times (TIME) and concentrations (CONC) of the antimicrobials [20]. An overnight culture of *S. mutans* was prepared the same way as previously described in the biofilm susceptibility assay (MBIC).

Composite resin disks in a 48-well cell culture plate were coated with saliva for 2 hours in a shaking incubator (37°C and 120 rpm) and washed twice with PBS. Then 250 μl of the diluted subculture (1:100 in BM-sucrose) was inoculated in each well containing the disk, and the 48-well plate was incubated for 24 hours at 37°C in 5% CO₂ atmosphere. Again, the disks were washed twice with PBS.

For the CONC plate, serially diluted concentrations of UA (0 to 640 μg/ml) or CHX (0 to 125 μg/ml) were transferred to the wells, and the plate was placed in a shaking incubator (37°C and 5% CO₂) for 60 minutes. For the TIME plate, the sub-MBIC of antimicrobial agent, 320 μg/ml of UA or 62.5 μg/ml of CHX, was aliquoted in each well containing the disk and placed in a shaking incubator (37°C and 120 rpm) for 15, 30, 45, and 60 minutes. For 0 minutes, the antimicrobial was immediately removed before placing the plate in the shaking incubator, and the disks were washed twice and transferred to 14 ml round-bottom tubes containing 3 ml of PBS. This procedure was repeated for the rest of the wells of TIME and CONC plates after each designated
incubation time. Then the biofilms were disrupted by sonication using three 10-second pulses at 20 watts and kept on ice. The bacterial suspensions were serially diluted and plated onto BHI agar using a spiral method. After incubating (37°C and 5% CO₂) the plates for 48 hours, the CFUs were counted. Five independent experiments were performed in duplicate for each antimicrobial.

*Scanning electron microscopy*

In order to visually examine the effect of surface topography on *S. mutans* biofilm formation and any changes of the bacterial shape in the presence of UA and CHX, scanning electron microscopy (SEM) images were taken with a S-4700 microscope (Hitachi, Tokyo, Japan). The bacteria was prepared and inoculated in the wells containing composite resin disks as described in the previous biofilm assay.

For the investigation of influence of surface topography, the disks were treated with an appropriate amount of the antimicrobial to yield 320 μg/ml of UA and 62.5 μg/ml of CHX (the control disks were treated with PBS) and incubated (37°C and 5% CO₂) for 4 and 24 hours. The biofilm formations on the disks were analyzed at magnifications of x30, x200, and x10,000. For observing the morphological changes of the bacteria, biofilms were grown for 4 hours with 320, 640, and 1,280 μg/ml of UA and 62.5, 125, and 250 μg/ml of CHX and examined at a magnification of x50,000.

*Crystal violet assay*

To confirm the results from the SEM analysis, quantitative assessment was carried out
using crystal violet assay according to the method described by Djordjevic et al. with slight modifications [21]. Biofilms were grown for 4 and 24 hours as described previously with 320 μg/ml of UA and 62.5 μg/ml of CHX, including a positive control treated with PBS. Then the plates were allowed to air dry for 45 minutes after washing the disks twice with PBS, and 500 μl aliquots of 0.1% crystal violet solution in water were placed in the wells to stain the disks. After another 45 minutes, the disks were washed 5 times with PBS and transferred to a 24-well plate. One ml of 99% ethanol was added to each well, and the plate was gently shaken for 10 minutes. Then 100 μl from each well was transferred to a round-bottom 96-well plate to measure the absorbance (optical density) at 595 nm, and the values were subtracted by the optical density readings obtained from staining the composite resin disks (negative control). Five independent experiments were performed in triplicate.

Statistical analysis

Statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). The Kruskal-Wallis test was used to compare the Ra values of the disk samples. The anti-biofilm activities were analyzed by repeated measures analysis of variance (ANOVA) for incubation times and by factorial ANOVA for antimicrobial concentrations and types, followed by multiple comparison tests using the Bonferroni method. All values were considered statistically significant when $P < 0.05$. 

- 10 -
III. RESULTS

Surface roughness of composite resin disks

The surface roughness values (Ra; μm) of flat and groove composite resin disks are shown in Table 1. There were no significant differences in the surface roughness between the two disk types ($P = 0.365$). Therefore, all the samples were prepared with smooth surfaces, and the only differences between the disks were surface topography and surface area (flat = 0.567 cm²; groove = 0.665 cm²).

Table 1. Surface roughness (Ra; μm) of flat and groove composite resin disks (N = 15 per group)

<table>
<thead>
<tr>
<th></th>
<th>Flat</th>
<th>Outside the groove</th>
<th>Inside the groove</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ra (mean ± SD)</td>
<td>0.14 ± 0.02</td>
<td>0.16 ± 0.05</td>
<td>0.18 ± 0.05</td>
</tr>
</tbody>
</table>

Differences between the means were assessed by the Kruskal-Wallis test, $P = 0.365$.

Antimicrobial susceptibility of Streptococcus mutans UA159

The results of the MIC, MBC, and MBIC experiments to evaluate antimicrobial activities of UA, OA, and CHX against planktonic and biofilm cells of *Streptococcus mutans* UA159 are displayed in Table 2. The MICs of UA, OA, and CHX ranged from 4 to 8 μg/ml, 4 to 16 μg/ml, and 1 to 2 μg/ml, respectively. Both UA and OA had the same MBC and MBIC of 8 to 16 μg/ml and 2,560 μg/ml, respectively. For CHX, the MBC ranged from 1 to 4 μg/ml, and the MBIC was 500 μg/ml.

From higher MBICs compared to MICs, it was clear that *S. mutans* biofilm is more
resistant to the antimicrobials. Also, since UA and OA showed similar antimicrobial susceptibility to *S. mutans*, only the MBICs of UA and CHX were used in the subsequent experiments.

Table 2. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum biofilm inhibitory concentration (MBIC) of ursolic acid (UA), oleanolic acid (OA), and chlorhexidine (CHX) against *Streptococcus mutans* UA 159

<table>
<thead>
<tr>
<th></th>
<th>MIC (μg/ml)</th>
<th>MBC (μg/ml)</th>
<th>MBIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UA</td>
<td>4-8</td>
<td>8-16</td>
<td>2,560</td>
</tr>
<tr>
<td>OA</td>
<td>4-16</td>
<td>8-16</td>
<td>2,560</td>
</tr>
<tr>
<td>CHX</td>
<td>1-2</td>
<td>1-4</td>
<td>500</td>
</tr>
</tbody>
</table>

*Bactericidal effect of the antimicrobials with respect to time and concentration*

Bactericidal activities of UA and CHX were investigated on *S. mutans* UA159 biofilm formed on flat and groove composite resin disks. As shown in Figures 2A and 2C, killing of *S. mutans* was dependent on increase in incubation time, for both surface types (*P* < 0.05, repeated measures ANOVA). With UA treatment, decrease in CFU was statistically significant at 45 and 60 minutes (0 min > 45, 60 min; *P* < 0.05, Bonferroni) and at 15, 30, and 60 minutes with CHX treatment (0 min > 15, 30, 60 min; *P* < 0.05, Bonferroni). However, change in CFU was not statistically significant between the flat and groove disks for UA (*P* = 0.812, repeated measures ANOVA) and CHX (*P* = 0.187, repeated measures ANOVA), and no interaction was observed between surface type and incubation time (*P* = 0.578 for UA and *P* = 0.245 for CHX, repeated measures ANOVA).
The killing of *S. mutans* in both disk types was also dependent on increase in concentration, as shown in Figures 2B and 2D (*P* < 0.05, factorial ANOVA). Treatment with 80 and 320 μg/ml UA significantly inhibited *S. mutans* growth (*P* < 0.05, Bonferroni), and 62.5 and 125 μg/ml CHX significantly inhibited *S. mutans* compared to 0, 15.625, and 31.25 μg/ml CHX (*P* < 0.05, Bonferroni). A decrease in CFU was statistically significant between the flat and groove disks only when treated with CHX (*P* < 0.05, factorial ANOVA). However, there was no interaction between surface type and antimicrobial concentration (*P* = 0.817 for UA and *P* = 0.125 for CHX, factorial ANOVA).

**Qualitative analysis of biofilm formation using SEM**

Scanning electronic microscopy (SEM) images were taken to investigate morphological changes of *S. mutans* UA159 with respect to various UA and CHX concentrations and anti-biofilm activities of the antimicrobials on flat and groove composite resin disks.

The bacteria treated with PBS (Figures 3A and 3E) had spherical shape of a normal coccus cell with smooth surface. When UA or CHX was added, the shape of the bacteria was maintained, but there were changes in its surface topography. With 320 μg/ml of UA (Figure 3B) and 62.5 μg/ml of CHX (Figure 3F), the presence of small nodules gave rough appearances to the surfaces of the bacteria, with CHX group having greater number of more distinguished nodules. Increasing concentrations of CHX (Figures 3G and 3H) did not significantly change the surface topography of the bacteria in comparison to the group treated with 62.5 μg/ml of CHX (Figure 3F). However, considerable changes were seen in the surface topography when the concentrations of UA were increased to 640...
μg/ml (Figure 3C) and 1,280 μg/ml (Figure 3D). With 640 μg/ml of UA, the surface of the bacteria started to show wrinkled appearance through the presence of larger nodules, and concavities formed from higher concentration (1,280 μg/ml) of UA.

The SEM images from 4 hours of biofilm formation are presented in Figure 4A for the flat disk and Figure 4B for the groove disk. It was clearly observed in the control group at the lower magnifications (x30 and x200) that biofilms formed evenly on the surface of the flat disk, whereas biofilms were localized in the groove region for the groove disk after 4 hours of incubation. At higher magnification of x10.0k, the bacteria surrounded by glucans could be examined. However, when UA or CHX was added, there was less biofilm formation compared to the control group, and the presence of glucans was not as apparent as the control group. In Figures 4C and 4D, the results from 24 hours of biofilm formation on flat and groove disks, respectively, are displayed. After 24 hours of incubation, the entire surfaces of both disk types were uniformly covered with biofilm. At higher magnification (x10.0k), the structure of the biofilm was seen as a large meshwork composed of bacterial cells and glucans. For UA and CHX treatment, there was less biofilm formation as observed at the lower magnifications (x30 and x200) in the 24 hour biofilm, with CHX showing greater inhibition of biofilm formation. On the flat disk (Figure 4C), addition of UA resulted in aggregation of the bacterial cells, while there was even dispersion of bacteria along the surface with CHX treatment. For the groove disk (Figure 4D), almost no change was seen in the amount of biofilm formation compared to the 4 hour biofilm for the CHX group. However, in the UA group, less biofilm formation was observed in the groove region.
Quantitative analysis of biofilm formation using crystal violet assay

The results from the qualitative biofilm formation assay of *S. mutans* were analyzed quantitatively with crystal violet staining (Figure 5). At 4 hours (Figure 5A), change in CFU was not statistically significant according to the antimicrobial (control = UA = CHX; $P = 0.266$, factorial ANOVA), and no interaction effect was seen between antimicrobial and surface type ($P = 0.624$, factorial ANOVA). However, the difference in CFU was statistically significant between the two surface types (flat < groove; $P < 0.05$, factorial ANOVA).

At 24 hours (Figure 5B), on the contrary, change in CFU was statistically significant according to the antimicrobial ($P < 0.05$, factorial ANOVA). The inhibition of *S. mutans* biofilm formation was greatest for CHX and least for control (control > UA > CHX; $P < 0.05$, Bonferroni). Also, there was interaction between the effects of surface type and antimicrobial on biofilm formation ($P < 0.05$, factorial ANOVA). For control group, there was more biofilm formation on the flat disk compared to the groove disk, although there was not much difference between the two surface types. On the other hand, addition of UA or CHX resulted in more biofilm formation on the groove disk, and the difference in biofilm formation between the two surface types was greater when treated with UA.
IV. DISCUSSION

Biofilm is regulated by a combination of various environmental factors. Among them, the surface characteristics, such as surface roughness, surface free energy, and surface chemistry of the substrate to which the bacteria bind to have been evaluated in many studies. From these experiments, the influence of surface topography, which represents the three-dimensional feature of the substrate, on the biofilm formation has also received attention. However, there has been a continuous investigation for a new potential antibacterial agent despite the fact that the biofilm research in dentistry has not yet recognized the significance of topographical features of the oral cavity. In a search for a more accurate method of antimicrobial assessment, this study has attempted to investigate *Streptococcus mutans* UA159 biofilm formation with the incorporation of surface topography and explore the anti-biofilm activities of two naturally occurring triterpenoid compounds.

In a previous study that evaluated the antibacterial activities of UA and OA, *S. mutans* UA159 was found to be slightly less susceptible to OA compared to UA [9]. This is partly consistent with the present study which also showed that the bacterial strain was less susceptible to OA by a two-fold increase in MIC, but the biofilm susceptibility was same for both UA and OA (Table 2). The similar pharmacological effects of these two compounds mostly likely resulted from their comparable chemical structures [15]. Also, the minimum inhibitory concentrations of all the antimicrobials tested in this study were much higher for *S. mutans* biofilm compared to the planktonic bacterial cells, which validates the fact that the presence of biofilm makes the bacteria more resistant to the
drug [5]. This result also corresponds well with the ones found in the earlier experimental studies using different antimicrobial agents [18, 22]. There are several proposed explanations for the greater resistance of biofilm to antimicrobials. The penetration of an antimicrobial agent may be limited due to the complex structure of the biofilm; only the bacterial cells on the surface of the biofilm may be affected by the agent; or some antimicrobials may not show optimal mechanism of action in the inner structure of the biofilm [23]. In this experiment, however, the significantly high MBIC values may not have resulted solely from the structural integrity of biofilm on a surface. The surface topographical changes of composite resin resulting from biofilm promote faster growth of bacteria [7], which may lead to greater resistance to antimicrobials. Therefore, further investigation is required for a comprehensive understanding of the high MBIC values.

Overall, there was an indirect relationship between the amount of biofilm (CFU/cm²) and increase in time and concentration of both antimicrobials tested in this experiment, and CHX showed superior antibacterial activity against *S. mutans* biofilm. Significant decrease in the amount of biofilm after 15 minutes of CHX treatment coincides well with the already known substantive property of CHX that it readily penetrates into thick biofilms, adsorbs to the substrate, and exerts antibacterial action for a prolonged period of time [24, 25]. Also, it is in close agreement with a previous study by Brambilla *et al.* that CHX is an effective agent in reducing biofilm on composite resin surfaces [6]. On the contrary, the similar bactericidal activities between flat and groove disks at all incubation times and concentrations of UA may suggest that after the formation of biofilm, the antimicrobial produces comparable effect regardless of the surface type. This may be partly explained by the fact that the bacterial adhesion alters surface features and
promotes accumulation into biofilm, masking the substratum surface topography [26].

Figure 2. Bactericidal activity of UA and CHX against *Streptococcus mutans* UA 159 biofilm with respect to incubation time and concentration. After biofilm formation for 24 hours, 320 μg/ml of UA (A) and 62.5 μg/ml of CHX (C) were added and incubated for 0, 15, 30, 45, and 60 minutes at 37°C to investigate the time dependent bactericidal effects. Similarly, 0, 80, 160, 320, and 640 μg/ml of UA (B) and 0, 15.625, 31.25, 62.5, and 125 μg/ml of CHX (D) were added and incubated for 60 minutes at 37°C to observe the concentration dependent bactericidal effects. The viable bacterial cells were quantified via spreading the resulting suspensions on BHI agar plates after serial dilutions. All values expressed in CFU/cm² are from 5 independent experiments performed in duplicate, and the error bars represent standard deviations.

According to the SEM analysis, all concentrations of CHX resulted only in a change in the surface texture of *S. mutans*, whereas higher concentration of UA produced depressions, altering the morphology of the bacteria. This indicates that UA has excellent antibacterial activity and similar bactericidal mechanism to that of CHX, since it also kills bacteria through the disintegration of bacterial cell membrane [27].
Figure 3. Changes in physical appearance of *S. mutans* UA159 with increase in antimicrobial concentration. UA control treated with PBS (A); 320 μg/ml UA treatment (B); 640 μg/ml UA treatment (C); 1,280 μg/ml UA treatment (D); CHX control treated with PBS (E); 62.5 μg/ml CHX treatment (F); 125 μg/ml CHX treatment (G); and 250 μg/ml CHX treatment. SEM images were taken with 4 hour biofilms grown on flat composite resin disks and analyzed under x50.0k magnification.

The influence of surface topography was also examined through SEM images, which demonstrated that biofilm formation initially occurred in the grooves. Then the results of SEM analysis were quantified by crystal violet assay, confirming that surface topography promoted more biofilm formation. This is supported by the fact that the grooves and pits can act as shelter for the adherent bacterial cells from environmental fluctuations, represented by the PBS washing procedures, whereas the flat surface does not provide a retentive site for the bacteria to attach to [28]. Also, the results are consistent with the ones from previous studies where bacteria favored adherence to pits and edges of grooves, which prevented dislodgement of bacterial cells and promoted colonization and biofilm formation [29, 30].

- 19 -
Figure 4. SEM analysis of *S. mutans* UA159 biofilm formation on flat and groove composite resin disks in the presence of UA or CHX. Biofilms were grown for 4 hours on flat disks (A) and groove disks (B) with PBS (control), 320 μg/ml UA, or 62.5 μg/ml CHX and observed under x30, x200, and x10.0k magnifications.
Figure 4. SEM analysis of *S. mutans* UA159 biofilm formation on flat and groove composite resin disks in the presence of UA or CHX (cont’d). Biofilms were grown for 24 hours on flat disks (C) and groove disks (D) with PBS (control), 320 μg/ml UA, or 62.5 μg/ml CHX and observed under x30, x200, and x10.0k magnifications.
Figure 5. *Streptococcus mutans* UA159 biofilm formation on flat and groove composite resin disks in the presence of UA or CHX. Biofilms were grown (37°C and 5% CO₂) on flat and groove composite resin disks in a 48-well plate for 4 and 24 hours with PBS (control), 320 μg/ml UA, or 62.5 μg/ml CHX. The adherent bacterial cells were quantified via optical density measurements at 595 nm (OD₅₉₅) after 0.1% crystal violet staining. Five independent experiments were carried out in triplicate, and the error bars indicate standard deviations of the mean OD₅₉₅ values.

When the anti-biofilm activity was compared amongst the antimicrobials, treatment with PBS (control), UA, and CHX led to similar amount of biofilm formation at 4 hours, but there seemed to be more biofilm formation in the control group according to the SEM images. This phenomenon is seen due to the extracellular polysaccharide (EPS) matrix comprised of glucans [31]. The presence of glucans facilitates adhesion of bacteria and forms a sophisticated three-dimensional biofilm structure supported by EPS matrix, as shown in the control group at x10.0k magnification images of Figures 4C and 4D, whereas formation of glucans is inhibited in the presence of antimicrobials [14]. On the other hand, for 24 hours of biofilm formation, the results from crystal violet assay clearly reflect that of the SEM images. Because there is more biofilm formation after 24 hours of incubation, distinct difference could be seen between the antimicrobials. Regardless of surface topography, biofilm formation occurred from the greatest to least order of control,
UA, and CHX. When the disks were not treated with antimicrobial, there was similar amount of biofilm formation on the flat and groove disks. However, when antimicrobial was added, there was more biofilm formation on the groove disks compared to flat disks. In other words, the anti-biofilm activity of antimicrobial was greater on the flat disk. Since structural integrity of the biofilm is not as strong due to the inhibition of glucan formation by an antimicrobial [14], surface topography could play a significant role in the biofilm formation of \textit{S. mutans}. This may suggest that surface topography controls biofilm formation of \textit{S. mutans} in the presence of an antimicrobial, whereas the complex structure of glucan rather than surface topography promotes biofilm formation without prophylactic antimicrobial administration.
V. CONCLUSIONS

This study has investigated the relationship between surface topography and biofilm formation using minimum biofilm inhibitory concentrations of antimicrobials, which showed that triterpenoid compound exhibits excellent antibacterial property, yet inferior substantivity than CHX. In general, presence of grooves in the composite resin disk did not influence the anti-biofilm activity of antimicrobials after biofilm formation of *Streptococcus mutans* UA159, whereas the anti-biofilm activity of antimicrobials was greater on flat composite resin disk when the antimicrobials were added before biofilm formation. Therefore, an antimicrobial can be used as an effective therapeutic regimen on pre-existing plaque regardless of surface topography and not as a preventive regimen due to its limited effect in controlling biofilm formation in retentive areas of the tooth.
REFERENCES


연구목적: 본 연구의 목적인 삼차원 표면 형태가 항균성 물질인 트리테르페노이드 존재 하에 Streptococcus mutans UA159 바이오필름 형성에 미치는 영향을 조사하고자 하였다.

연구방법: 펴평한 표면을 가지고 있는 시편 (flat)과 치아 교합면과 유사한 표면 형태 (groove)를 가지고 있는 두 가지 유형의 복합러진 시편들을 제작하여 표면에 S. mutans 바이오필름을 형성하여 실험을 하였다. 다양한 배양시간 (0, 15, 30, 45, 60분)과 S. mutans 바이오필름의 최소억제농도 이하 (0, 80, 160, 320, 640 μg/ml UA와 0, 15.625, 31.25, 62.5, 125 μg/ml CHX)에서 트리테르페노이드계 화합물인 ursolic acid (UA)와 chlorhexidine (CHX)의 항바이오필름 활성도를 분석하였다. 그리고 표면 형태가 S. mutans 바이오필름 형성에 미치는 영향을 관찰하기 위해 주사전자현미경 (SEM)과 crystal violet assay로 각각 정성분석과 정량분석을 하였다.

결과: 바이오필름 형성 후 항균 물질을 첨가하였을 때, flat과 groove
시편 사이에서 UA 또는 CHX의 항바이오필름 활성도의 차이는 없었다. 하지만 바이오필름 형성 전 항균 물질을 첨가하였을 경우, flat 시편보다 groove 시편에서 더 많은 바이오필름 형성이 나타났고 항균 물질을 첨가하지 않은 대조군에서는 flat과 groove 시편에서 비슷한 양의 바이오필름 형성이 일어났다.

결론: 본 연구의 결과는 표면 형태와 상관없이 항균 물질을 기존에 형성되어 있는 바이오필름의 효과적인 치료 요법으로 제시하고 있다. 하지만 치아우식증에 취약한 치아 표면 형태에 항균 물질이 예방적인 차원에서 사용되었을 때는 바이오필름 형성을 억제하는 효과가 제한될 거라고 판단된다. 그러므로 표면 형태는 항균 물질 존재 하에 Streptococcus mutans UA159 바이오필름 형성에 중요한 역할을 한다고 볼 수 있다.

주요어: 표면 형태, Streptococcus mutans, 바이오필름, 트리테르페노이드
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