

18 β -glycyrrhetic acid의 *S. mutans*의 바이오필름 형성에 대한 억제효과

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Inhibitory effect of 18 β -glycyrrhetic acid on the biofilm formation of *Streptococcus mutans*

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Objectives: The present study aimed at investigating the potential of using 18 β -glycyrrhetic acid against the cariogenic characteristics of *Streptococcus mutans* UA159.

Methods: The effects of 18 β -glycyrrhetic acid on biofilm formation and acid production were evaluated; the latter are indicators of cariogenicity of *S. mutans*. Biofilm architecture was also analyzed by scanning electron microscopy (SEM), and changes in gene expression related to biofilm formation were studied by quantitative RT-PCR.

Results: Treatment with 18 β -glycyrrhetic acid at a concentration of 20 μ g/ml inhibited biofilm formation by 95% in the absence of sucrose and 60% in its presence, reduced acid production by 88.8%, and significantly suppressed the gene expression of *comDE*, *gbpB*, *gtfC* and *vicR*, which are thought to be involved in the virulence of *S. mutans*.

Conclusions: These results suggest that 18 β -glycyrrhetic acid could be used as a complementary or alternative agent for preventing dental caries by interfering with the virulence properties of *S. mutans* without affecting the viability of the bacterial population.

Key Words: 18 β -glycyrrhetic acid, Biofilm inhibition, *S. mutans*, Dental caries

Introduction

Dental caries is one of the infectious diseases that results in tooth decay. Although other oral bacteria may cause dental caries, *Streptococcus mutans* is considered to be a major pathogen because of initiating the cariogenic biofilm formation¹⁾.

As a primary causative agent of dental caries, the mechanisms for *S. mutans* to form pathogenic biofilm are important

targets for anti-cariogenicity. Adhesion to dental surface is the first step in biofilm formation, by sucrose-independent and/or dependent pathways. The sucrose-independent mechanism, mediated by surface adhesions, promotes microbial colonization by supplying binding sites for bacteria²⁾. This process is thought to be profoundly influenced by antigen I/II, a bacterial surface protein³⁾. Sucrose-dependent mechanism, mediated by extracellular enzymes (glucosyltransferases and fructosyltransferases) and glucan binding proteins, has virulent roles of

*S. mutans*⁴⁾. Glucosyltransferases (GTFs) play the major role in sucrose-dependent mechanism. The GTFs split sucrose into glucose and fructose and synthesize both water-soluble and insoluble glucans, considered to promote bacterial colonization and dental caries.

Following initial adhesion, *S. mutans* in the biofilm has distinct phenotypic characteristics different from those in planktonic state. There are significant changes in gene expressions to adapt to the biofilm lifestyle⁵⁾. Those genes are involved in quorum sensing signaling systems, adhesion and carbohydrate metabolism.

To prevent dental caries, many antimicrobial agents have been used in many oral care products. Applying antimicrobials, however, has several limitations or demerits. First, they attenuate oral immunity by killing not only harmful bacteria but also harmless, even beneficial bacteria. Second, they could not easily penetrate into the biofilm matrix. Third, antimicrobial resistances could be induced by antimicrobial agents^{6,7)}. Therefore, it is necessary to develop alternative agents for prevention of dental caries.

Glycyrrhiza species have been frequently used as traditional medicine all over the world. Many studies have reported that *Glycyrrhiza glabra* extracts have antitumor, anti-inflammatory, antiviral and antimicrobial effects⁸⁾. Especially, various studies have been proved that extracts of *Glycyrrhiza glabra* inhibit various species of bacteria. The ethanolic extract of *Glycyrrhiza glabra* has the inhibitory activity against oral bacteria such as *Streptococcus mutans*, *S. mitis*, *S. sanguis* and *Lactobacillus acidophilus*⁹⁾. 18 β -glycyrrhetic acid, one of the main components of *Glycyrrhiza glabra* L., has been demonstrated for its pharmacological activities, such as healing gastric ulcers and relief of rheumatic pain^{10,11)}.

In spite of many pharmacologic properties, the activity of 18 β -glycyrrhetic acid against *S. mutans* has not been studied in detail. In this study, we evaluated the inhibitory activities of 18 β -glycyrrhetic acid on biofilm forming properties of *S. mutans*.

Materials and Methods

1. Chemicals and bacterial strain

18 β -glycyrrhetic acid (purity $\geq 97\%$) was provided by Shaanxi Fujie Pharmaceutical Company in China. *S. mutans* strain UA159 (ATCC) was used in this study. *S. mutans* UA159 was grown aerobically in Brain Heart Infusion (BHI) broth (Difco Laboratories, MD, USA) at 37°C.

2. Growth curve assay

The effect of 18 β -glycyrrhetic acid on *S. mutans* growth was tested. Briefly *S. mutans* was grown in BHI broth at 37°C until the optical density at 600 nm (OD₆₀₀) reached 0.5 and adjusted to concentration of 1×10^6 CFU/ml. 0.2 ml of culture (2×10^5 CFU) was seeded into a 96-well microtiter plate (Falcon, USA). The culture contained with or without 20 μ g/ml 18 β -glycyrrhetic acid. The growth rate was monitored using spectrophotometer (BioTek, USA). The absorbance at 600 nm was recorded every hour for 24 hr incubation. All determinations were performed in triplicates.

3. Biofilm formation assay

S. mutans was cultured in fresh BHI broth at 37°C until OD₆₀₀ reached 0.5. It was then diluted 1:100 in BHI broth medium containing 18 β -glycyrrhetic acid at various concentrations with or without 1% sucrose and 0.2 ml of each diluent was transferred into the wells of 96-well microtitre plates. After incubation overnight at 37°C, the culture was blotted out and the wells were washed three times with phosphate buffered saline (PBS) to remove planktonic cells. Quantification of the biofilms formed was determined by alamar blue assay^{12,13)}. Three independent experiments were performed and cultures without 18 β -glycyrrhetic acid treatment served as control.

4. Effect on acid production

Effect of 18 β -glycyrrhetic acid on acid production was tested according to the earlier method with modifications¹⁴⁾. Briefly, *S. mutans* was cultured in BHI broth containing 1% sucrose and varying concentrations of 18 β -glycyrrhetic acid at 37°C overnight and acid production was evaluated by measuring the pH of bacterial broth. The bacterial culture without 18 β -glycyrrhetic acid treatment served as control. All experiments were performed as triplicates.

5. Scanning Electron Microscopy (SEM)

Sucrose-independent biofilms of *S. mutans* for scanning electron microscopy were generated on glass coverslips in 24-well plates (Falcon, USA). Overnight culture was transferred to fresh BHI medium and grown to the mid-exponential phase (OD₆₀₀=0.5). The culture was then diluted 1:100 in fresh medium with or without 20 μ g/ml 18 β -glycyrrhetic acid, as described above. Untreated BHI medium was used as a negative control. Following overnight growth at 37°C, each coverslips were washed three times in PBS and then fixed with 2% formaldehyde and 2.5% glutaraldehyde in PBS overnight. After that biofilms were dehydrated in graded ethanol series.

Table 1. Nucleotide sequences of primers used in this study

| Gene [†] | Description | Primer sequence (5'→3') ¹⁵⁾ | |
|-------------------|---------------------------------|--|-------------------------------|
| | | Forward | Reverse |
| <i>comDE</i> | Competence-stimulating peptide | ACAATTCCTTGAGTTCCATCCAAG | TGGTCTGCTGCCTGTTGC |
| <i>gbpB</i> | Glucan-binding proteins (GBPs) | ATGCGCGTTATGGACACGTT | TTTGGCCACCTTGAACACCT |
| <i>gtfC</i> | Glucan production | CTCAACCAACCGCCACTGTT | GGTTTAACGTCAAAATTAGCTGTATTAGC |
| <i>vicR</i> | Two-component regulatory system | CGTCTAGTTCTGGTAACATTAAGTCCAATA | TGACACGATTACAGCCTTTGATG |
| <i>16S rRNA</i> | Normalizing internal standard | CTTACCAGGTCTTGACATCCCG | ACCCAACATCTCACGACACGAG |

[†]Based on the National Center for Biotechnology Information (NCBI) *S. mutans* genome database.

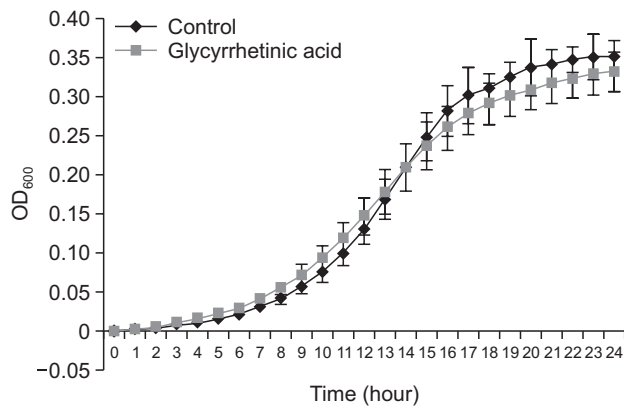


Fig. 1. Effect of 18 β -glycyrrhetic acid on growth of *S. mutans*. *S. mutans* incubated with present and absent of 20 μ g/ml 18 β -glycyrrhetic acid in a 96well plate at 37°C.

And samples were completely dried, coated with gold and analyzed by SEM (Hitachi, Japan). The assays were performed with three replicates per treatment and representative pictures were shown.

6. RNA extraction and real-time quantitative polymerase chain reaction (RT-PCR)

Quantitative real-time polymerase chain reaction (RT-PCR) analysis was performed to trace the changes in expression levels of 4 genes related to biofilm formation, *comDE*, *gbpB*, *gbpC* and *vicR*. Biofilms that formed in BHI supplemented with or without 20 μ g/ml 18 β -glycyrrhetic acid for overnight were collected by centrifugation. Total RNA was extracted using RNeasy Protect Bacteria Mini Kit (Qiagen, MD, USA) following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using the cDNA synthesis kit (Philekorea Technology, Korea). RT-PCR was performed by using the Step One Plus real-time PCR system (Applied Biosystems, Foster City, CA) with SYBR Green PCR Master Mix (Applied Biosystems). PCR conditions included an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing and extension 60°C

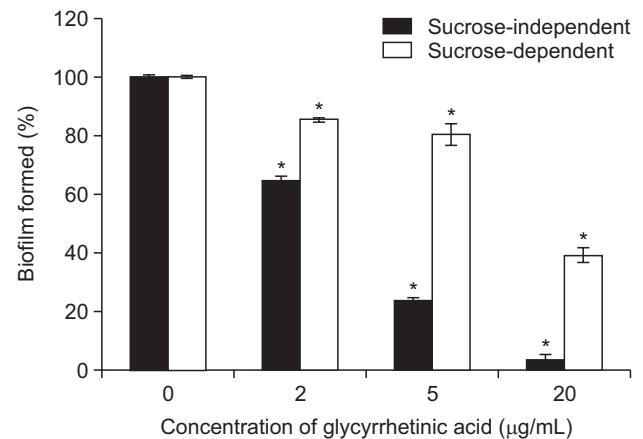


Fig. 2. Inhibitory effect of 18 β -glycyrrhetic acid on the biofilm formation by *S. mutans* in the absence (sucrose-independent) and presence of 1% sucrose (sucrose-dependent). *Significant inhibition compared with untreated control ($P < 0.05$).

for 1 min. The expression levels of all targeted genes (Table 1) were normalized using the 16s rRNA gene of *S. mutans* as an endogenous control.

7. Statistical analysis

All assays were performed in triplicates. For each result, data was expressed as mean \pm standard deviation (SD). Statistical analysis was performed by ANOVA using SPSS 21.0 (SPSS Inc., Chicago, IL, USA). A P value of < 0.05 was considered statistically significant.

Results

1. Effect on growth

The inhibitory effect of 18 β -glycyrrhetic acid on *S. mutans* growth was shown in Fig. 1. Exposure of *S. mutans* to 20 μ g/ml 18 β -glycyrrhetic acid revealed that there was no significant difference in growth rates compared with untreated cells.

2. Effects on sucrose-independent and dependent biofilm formation

The inhibitory effects of 18 β -glycyrrhetic acid on biofilm formation of *S. mutans* were shown in Fig. 2. The 18 β -glycyrrhetic acid significantly inhibited both sucrose-independent and dependent biofilm formation in concentration-dependent manners as compared to non-treated control cultures ($P<0.05$). The inhibition in sucrose-independent biofilm formation was more pronounced. Treatment of 20 μ g/ml 18 β -glycyrrhetic acid reduced the biofilm formation by 95% in the absence of sucrose and 60% in the presence of sucrose. Decrease in biofilm formation by 18 β -glycyrrhetic acid did not relate to bacterial growth itself because the treatment of 20 μ g/ml 18 β -glycyrrhetic acid did not affect the growth of *S. mutans* (Fig. 1).

3. Effect on the pH of *S. mutans*

Acid production by *S. mutans* plays a pivotal role in the pathogenesis of dental caries so we evaluated the effect of 18 β -glycyrrhetic acid. Treatment of 18 β -glycyrrhetic acid increased the pH in a concentration dependent manner (Table

2). Treatment of 18 β -glycyrrhetic acid at a concentration of 20 μ g/ml significantly increased the pH of overnight culture from 5.3 to 6.2, compared to non-treated control ($P<0.05$). In respect of proton concentration, this change in pH represents 88.8% reduction in acid production.

4. Changes of biofilm architecture visualized by scanning electron microscopy

The biofilm generated by *S. mutans* with or without 18 β -glycyrrhetic acid was characterized. The scanning electron microscopy depicted the effect of 18 β -glycyrrhetic acid at 20 μ g/ml on the biofilm accumulation (Fig. 3). In the absence of 18 β -glycyrrhetic acid, the bacteria were apt to form large aggregates in the generated biofilm. In the presence of 18 β -glycyrrhetic acid, however, the image showed the reduction of biofilm accumulation without affecting the viability of *S. mutans*. We performed this assay in triplicate and similar results were obtained.

5. Effects on gene expression related to biofilm formation

To investigate the differences in gene expression between cells grown with or without 18 β -glycyrrhetic acid, we performed RT-PCR analysis. The expression levels of biofilm-related genes were determined on cells in biofilm untreated and treated with 18 β -glycyrrhetic acid. It was found that all the tested genes (Table 1) were significantly down-regulated in the treated cells up to 75% ($P<0.05$) (Fig. 4). The most affected gene was *vicR*, decreased by 75% compared with cells in untreated biofilm. The expression of other genes including *comDE*, *gpbB* and *gftC* was reduced approximately by 70%.

Table 2. Effect of 18 β -glycyrrhetic acid on the acid production of *S. mutans*

| Concentration of Glycyrrhetic acid (μ g/ml) | pH (mean \pm SD) | |
|--|--------------------|-----------------|
| | 0 hr | After overnight |
| 0 | 7.1 \pm 0.15 | 5.3 \pm 0.07 |
| 5 | 7.1 \pm 0.00 | 5.4 \pm 0.07 |
| 10 | 7.1 \pm 0.07 | 5.6 \pm 0.00 |
| 20 | 7.1 \pm 0.07 | 6.2 \pm 0.07* |

*Significant different compared with untreated control ($P<0.05$).

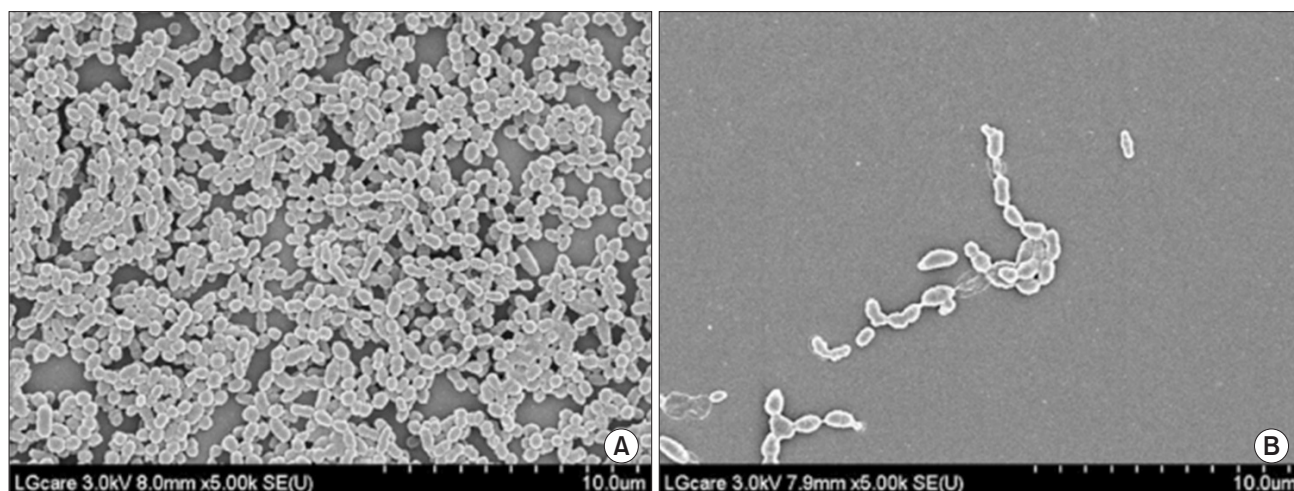


Fig. 3. SEM images of *S. mutans* biofilm formed in the presence and absence of the 18 β -glycyrrhetic acid after at 37°C overnight incubation. (A) control, (B) 18 β -glycyrrhetic acid.

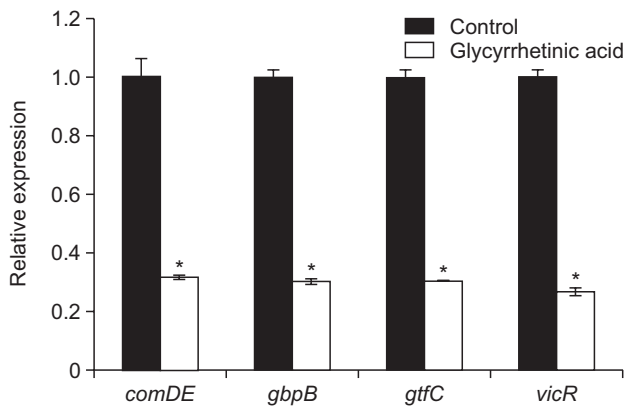


Fig. 4. Effect of 18 β -glycyrrhetic acid on the biofilm related genes expression of *S. mutans*. *Significant inhibition compared with untreated control ($P < 0.05$).

Discussion

There are many bacterial pathogenesis related to biofilm formation. Dental caries is one of the most well-known biofilm-related diseases which originate with certain bacteria, especially *S. mutans*¹⁶. *S. mutans* initiates the cariogenic process by biofilm formation and has many other virulence factors associated with cariogenicity such as adhesion, acidogenicity, synthesis of exopolysaccharides (glucan), and cell to cell signaling (quorum sensing)¹⁷. Therefore, it is necessary to focus on the virulence traits of *S. mutans* to control dental caries.

It has been found that some natural products, for example, *Emblica officinalis* and curcumin have inhibitory effects on these characteristics¹⁸. It has not been studied, however, in *S. mutans* for 18 β -glycyrrhetic acid's activities on inhibiting biofilm formation. In this report we firstly show that 18 β -glycyrrhetic acid has outstanding activities on the virulence factors of *S. mutans* without inhibiting the growth of *S. mutans* unlike antibacterial agents.

Since 18 β -glycyrrhetic acid treatment up to 20 μ g/ml did not inhibit the growth of *S. mutans* (Fig. 1), 18 β -glycyrrhetic acid inhibited the virulence properties of *S. mutans* without affecting the cell viability. It could be a preferable approach that 18 β -glycyrrhetic acid interferes with microbes' physiological pathways without killing *S. mutans*, thus avoiding resistance development.

Biofilm formation of *S. mutans* is considered one of the important factors of caries pathogenesis and its inhibition reduces the virulence of *S. mutans*¹⁹. The results showed that the biofilm formation was markedly inhibited by treatment of 20 μ g/ml 18 β -glycyrrhetic acid as compared to non-treated

control (Fig. 2). The sucrose-independent biofilm formation was more greatly reduced than sucrose-dependent process. These results propose that the decrease in biofilm formation by 18 β -glycyrrhetic acid is not associated with an bactericidal effect.

The acidogenicity is one of the main physiological factors associated with dental caries³. *S. mutans* has a glycolytic pathway and produces fermentation products such as lactate. Acidogenicity of *S. mutans* changes the ecological environment in the plaque flora and reduces plaque pH²⁰. Plaque pH below 5.4 results in cariogenic flora, causing the demineralization of tooth enamel²¹. Therefore the change of pH was used as an indicator for determining the potential of anti-cariogenicity. Acid production was significantly decreased by 20 μ g/ml of 18 β -glycyrrhetic acid treatment (Table 2). Reducing the acid production could result in inhibiting dental caries.

Scanning electron microscopy revealed the effect of 18 β -glycyrrhetic acid on biofilm integrity. SEM images showed changes in the morphology of biofilm. Cells in untreated biofilm were dense on the surface and had longer adherent chains like the general biofilm. Cells grown in 20 μ g/ml of 18 β -glycyrrhetic acid, on the other hand, were sparse on the surface and had short scattered chains (Fig. 3). This showed the consistent result that 18 β -glycyrrhetic acid significantly reduced biofilm formation of *S. mutans*.

Because 18 β -glycyrrhetic acid could effectively inhibit biofilm formation by *S. mutans*, the expression of several genes was examined to understand its action mechanisms in biofilm formation process. The levels of biofilm-related gene expression were compared in cultures untreated and treated with 18 β -glycyrrhetic acid (Fig. 4). Those genes are related to intercellular and environmental communication systems, adhesion function, and carbohydrate metabolism. The treatment of 18 β -glycyrrhetic acid resulted in decreased expression of *comDE*. *comDE*, a gene reported to play a role in cell-density dependent Com system, regulates the genetic competence development¹⁴. *gbpB* which encodes a GBP (glucan-binding protein) was also down-regulated by the addition of 18 β -glycyrrhetic acid. Glucan is able to bind both bacteria and salivary pellicle, facilitating the adhesion of *S. mutans*. Thus glucan-binding proteins of *S. mutans* possibly play important roles in promoting plaque formation²². *gtfC* which encodes GTFC (glucosyltransferases) enzyme and cleaves sucrose to form extracellular glucan polysaccharides was down-regulated in the presence of 18 β -glycyrrhetic acid²³. Interaction between glucans and surface associated

glucan-binding proteins (GBPs) results in oral bacterial aggregation, thereby promoting biofilm formation²⁴. *vicR*, a gene for response regulator in two component regulatory system, was most profoundly down-regulated about 75% among the tested target genes. Our data suggest that *vicR* gene products seem important to modulate biofilm formation process in *S. mutans*. It is reported that *vicR* products directly regulate genes encoding critical surface proteins for adherence to tooth surface, including *gtfC* and *gbpB*²⁵. Therefore, the reduced expression in these genes may lead to disruption of *S. mutans* biofilm formation and reflect the caries inhibitory potential of 18 β -glycyrrhetic acid.

Our results showed that 18 β -glycyrrhetic acid had no antibacterial effect on *S. mutans*, whereas it played an important role in the inhibition abilities of biofilm formation and acid production of *S. mutans*. It might be possible to modulate the physiological activity of cariogenic biofilms by reducing the transcription level of biofilm-associated genes. Therefore this study provides the possibility that 18 β -glycyrrhetic acid could be an outstanding natural anti-cariogenicity agent for oral care products.

Our study was used the *S. mutans* biofilm model because it could provide benefits for reducing variances and reproducibility of data. However, Oral cavity is composed of complex microbial community *in vivo*. This study model doesn't exactly mimic the dental biofilms in oral cavity. Further studies on effects of 18 β -glycyrrhetic acid in multispecies model are necessary.

Conclusions

We demonstrated that 18 β -glycyrrhetic acid inhibited biofilm formation both in sucrose-dependent and independent pathways by suppressing the expression of genes related to virulence of *S. mutans*, without affecting the viability of bacteria. We also found that 18 β -glycyrrhetic acid decreased the acid production by *S. mutans*, which is thought to be the main causative agent for tooth decay. Our data clearly suggest that 18 β -glycyrrhetic acid could be used as a safe agent in oral care products for prevention of dental caries.

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