18β-glycyrrhetinic acid의 S. mutans의 바이오필름 형성에 대한 억제효과
유정현, 전정훈, 이상화
(주)LG 생활건강 기술연구원

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

Jungheon Yu, Jeonghoon Jeon, Sanghwa Lee
LG Household & Health Care Ltd., Daejeon, Korea

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\textsuperscript{6}. 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 lifestyle\textsuperscript{5}. 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\textsuperscript{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 effects\textsuperscript{8}. 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\textsuperscript{9}. 18β-glycyrrhetinic 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\textsuperscript{10,11}.

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

**Materials and Methods**

1. Chemicals and bacterial strain

18β-glycyrrhetinic acid (purity ≥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 18β-glycyrrhetinic 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 (OD\textsubscript{600}) reached 0.5 and adjusted to concentration of 1×10\textsuperscript{6} CFU/ml. 0.2 ml of culture (2×10\textsuperscript{5} CFU) was seeded into a 96-well microtiter plate (Falcon, USA). The culture contained with or without 20 μg/ml 18β-glycyrrhetinic 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 OD\textsubscript{600} reached 0.5. It was then diluted 1:100 in BHI broth medium containing 18β-glycyrrhetinic acid at various concentrations with or without 1% sucrose and 0.2 ml of each diluent was transferred into the wells of 96-well microtiter 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\textsuperscript{12,13}. Three independent experiments were performed and cultures without 18β-glycyrrhetinic acid treatment served as control.

4. Effect on acid production

Effect of 18β-glycyrrhetinic acid on acid production was tested according to the earlier method with modifications\textsuperscript{14}. Briefly, S. mutans was cultured in BHI broth containing 1% sucrose and varying concentrations of 18β-glycyrrhetinic acid at 37°C overnight and acid production was evaluated by measuring the pH of bacterial broth. The bacterial culture without 18β-glycyrrhetinic 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 (OD\textsubscript{600}=0.5). The culture was then diluted 1:100 in fresh medium with or without 20 μg/ml 18β-glycyrrhetinic 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.
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 μg/ml 18β-glycyrrhetinic 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 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±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 18β-glycyrrhetinic acid on S. mutans growth was shown in Fig. 1. Exposure of S. mutans to 20 μg/ml 18β-glycyrrhetinic acid revealed that there was no significant difference in growth rates compared with untreated cells.

---

Table 1. Nucleotide sequences of primers used in this study

| Gene | Description | Primer sequence (5’→3’) | Forward | Reverse |
|------|-------------|-------------------------|---------|---------|
| comDE | Competence-stimulating peptide | ACAATTCTGAGTTCCATCCAAG TGGTCTGCGTGTGTCG | TGGTCTGCGTGTGTCG | |
| gbpB | Glucan-binding proteins (GBPs) | ATGGCGGTTATGGACACGTT TTTGGCCACCTTGAACACCT | TTGGGCCACCTTGAACACCT | |
| gfpC | Glucan production | CTCAACCAACGCACCTTGTG | CTTGGGCCACCTTGAACACCT | |
| vicR | Two-component regulatory system | CGTCAGATGCTGTAACATATTAGC TCGAGAATACGCCGTTGG | TCGAGAATACGCCGTTGG | |
| 16S rRNA | Normalizing internal standard | CTTACCAGGTCTTGACATCGG | ACCCAACATTCACAGACACCG | |

†Based on the National Center for Biotechnology Information (NCBI) S. mutans genome database.
2. Effects on sucrose-independent and dependent biofilm formation

The inhibitory effects of 18β-glycyrrhetinic acid on biofilm formation of S. mutans were shown in Fig. 2. The 18β-glycyrrhetinic 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 μg/ml 18β-glycyrrhetinic acid reduced the biofilm formation by 95% in the absence of sucrose and 60% in the presence of sucrose. Decrease in biofilm formation by 18β-glycyrrhetinic acid did not related to bacterial growth itself because the treatment of 20 μg/ml 18β-glycyrrhetinic 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 18β-glycyrrhetinic acid. Treatment of 18β-glycyrrhetinic acid increased the pH in a concentration dependent manner (Table 2). Treatment of 18β-glycyrrhetinic acid at a concentration of 20 μg/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 18β-glycyrrhetinic acid was characterized. The scanning electron microscopy depicted the effect of 18β-glycyrrhetinic acid at 20 μg/ml on the biofilm accumulation (Fig. 3). In the absence of 18β-glycyrrhetinic acid, the bacteria were apt to form large aggregates in the generated biofilm. In the presence of 18β-glycyrrhetinic 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 18β-glycyrrhetinic acid, we performed RT-PCR analysis. The expression levels of biofilm-related genes were determined on cells in biofilm untreated and treated with 18β-glycyrrhetinic 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, gbpB and gftC was reduced approximately by 70%.

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

| Concentration of Glycyrrhetinic acid (μg/ml) | pH (mean±SD)               |
|--------------------------------------------|----------------------------|
|                                            | 0 hr                       | After overnight            |
| 0                                         | 7.1±0.15                   | 5.3±0.07                   |
| 5                                         | 7.1±0.00                   | 5.4±0.07                   |
| 10                                        | 7.1±0.07                   | 5.6±0.00                   |
| 20                                        | 7.1±0.07                   | 6.2±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 18β-glycyrrhetinic acid after at 37°C overnight incubation. (A) control, (B) 18β-glycyrrhetinic acid.
Biofilm formation of S. mutans is considered one of the important factors of caries pathogenesis and its inhibition reduces the virulence of S. mutans. The results showed that the biofilm formation was markedly inhibited by treatment of 20 μg/ml 18β-glycyrrhetinic 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 18β-glycyrrhetinic acid is not associated with an bactericidal effect.

The acidogenicity is one of the main physiological factors associated with dental caries. 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 pH. Plaque pH below 5.4 results in cariogenic flora, causing the demineralization of tooth enamel. Therefore the change of pH was used as an indicator for determining the potential of anti-cariogenicity. Acid production was significantly decreased by 20 μg/ml of 18β-glycyrrhetinic acid treatment (Table 2). Reducing the acid production could result in inhibiting dental caries.

Since 18β-glycyrrhetinic acid treatment up to 20 μg/ml did not inhibit the growth of S. mutans (Fig. 1), 18β-glycyrrhetinic acid inhibited the virulence properties of S. mutans without affecting the cell viability. It could be a preferable approach that 18β-glycyrrhetinic 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. The results showed that the biofilm formation was markedly inhibited by treatment of 20 μg/ml 18β-glycyrrhetinic 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 18β-glycyrrhetinic acid is not associated with an bactericidal effect.

The acidogenicity is one of the main physiological factors associated with dental caries. 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 pH. Plaque pH below 5.4 results in cariogenic flora, causing the demineralization of tooth enamel. Therefore the change of pH was used as an indicator for determining the potential of anti-cariogenicity. Acid production was significantly decreased by 20 μg/ml of 18β-glycyrrhetinic acid treatment (Table 2). Reducing the acid production could result in inhibiting dental caries.

Scanning electron microscopy revealed the effect of 18β-glycyrrhetinic 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 μg/ml of 18β-glycyrrhetinic acid, on the other hand, were sparse on the surface and had short scattered chains (Fig. 3). This showed the consistent result that 18β-glycyrrhetinic acid significantly reduced biofilm formation of S. mutans.

Because 18β-glycyrrhetinic 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 18β-glycyrrhetinic acid (Fig. 4). Those genes are related to intercellular and environmental communication systems, adhesion function, and carbohydrate metabolism. The treatment of 18β-glycyrrhetinic 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 development. gbpB which encodes a GBPB (glucan-binding protein) was also down-regulated by the addition of 18β-glycyrrhetinic 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 formation.

gtfC which encodes GTFC (glucosyltransferases) enzyme and cleaves sucrose to form extracellular glucan polysaccharides was down-regulated in the presence of 18β-glycyrrhetinic acid. Interaction between glucans and surface associated
glucan-binding proteins (GBPs) results in oral bacterial aggregation, thereby promoting biofilm formation. 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. Therefore, the reduced expression in these genes may lead to disruption of S. mutans biofilm formation and reflect the caries inhibitory potential of 18β-glycyrrhetinic acid.

Our results showed that 18β-glycyrrhetinic 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 18β-glycyrrhetinic 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 18β-glycyrrhetinic acid in multispecies model are necessary.

Conclusions

We demonstrated that 18β-glycyrrhetinic 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 18β-glycyrrhetinic 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 18β-glycyrrhetinic acid could be used as a safe agent in oral care products for prevention of dental caries.

References

1. Chau NP, Pandit S, Jung JE, Jeon JG. Evaluation of Streptococcus mutans adhesion to fluoride varnishes and subsequent change in biofilm accumulation and acidogenicity. J Dent 2014;42(6):726-734.
2. Mitchell TJ. The pathogenesis of streptococcal infections: from tooth decay to meningitis. Nat Rev Microbiol 2003;1(3):219-230.
3. Pecharki D, Petersen FC, Assev S, Scheie AA. Involvement of antigen 1/II surface proteins in Streptococcus mutans and Streptococcus intermedius biofilm formation. Oral Microbiol Immunol 2005;20(5):366-371.
4. Bowen WH, Koo H. Biology of Streptococcus mutans-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. Caries Res 2011;45(1):69-86.
5. Zhiyan H, Qian W, Yuejian H, Jingping L, Yuntao J, Rui M, et al. Use of the quorum sensing inhibitor furanone C-50 to interfere with biofilm formation by Streptococcus mutans and its luxS mutant strain. Int J Antimicrob Agents 2012;40:30-35.
6. De Sousa DL, Lima RA, Zanin IC, Klein ML, Janal MN, Duarte S. Effect of twice-daily blue light treatment on matrix-rich biofilm development. PLoS One 2015;10(7):e0131941. DOI:10.1371/journal.pone.0131941 PMID: 26230333.
7. Matesanz-Perez P, Garcia-Gargallo M, Figueroo E, Bascones-Martinez A, Sanz M, Herrera D. A systematic review on the effects of local antimicrobials as adjuncts to subgingival debridement, compared with subgingival debridement alone, in the treatment of chronic periodontitis. J Clin Perodontol 2013;40:227-241.
8. Asl MN, Hosseinzadeh H. Review of pharmacological effects of Glycyrrhiza sp. and its bioactive compounds. Phytother Res 2008;22:709-724.
9. Geetha RV, Roy A. In-vitro evaluation of anti bacterial activity of Ethanolic root extract of Glycyrrhiza glabra on oral microbes. IJDDR 2012;4(4):161-165.
10. Fiore C, Eisenhut M, Ragazzi E, Zanchin G, Armanini D. A history of the therapeutic use of liquorice in Europe. J Ethnopharmacol 2005;99:317-324.
11. Tsukiyama RI, Kasura H, Tokuriki N, Kobayashi M. Anti-bacterial activity of licochalicone A against spore-forming bacteria. Antimicrob Agents and Chemother 2002;46:1226-1230.
12. Hamid R, Reisheeyn Y, Rabadi L, Parikh R, Bullock P. Comparison of alamar blue and MTT assays for high through-put screening. Toxicol In Vitro 2004:18:705-710.
13. Petrit RK, Weber CA, Kean NJ, Hoffmann H, Petrit GR, Tan R, et al. Microplate alamar blue assay for Staphylococcus epidermidis biofilm susceptibility testing. Antimicrob Agents Chemother 2005;49:2612-2617.
14. Ciardi JE, Rosenthal AB, Bowen WH. Rapid quantitative determination of the effect of antiplaque agents and antisera on the growth, acid production and adherence of Streptococcus mutans. J Dent Res 1981;60:756-762.
15. Hasan S, Daniushdinn M, Adil M, Singh K, Verma PK, Khan AU. Efficacy of E officinalis on the cariogenic properties of Streptococcus mutans: a novel and alternative approach to suppress Quorum-sensing mechanism. PLoS One 2012;7(7):e40319. DOI:10.1371/journal.pone.0040319.
16. Marsh PD. Plaque as a biofilm: pharmacological principles of drug delivery and action in the sub- and supragingival environment. Oral Dis 2003;9:16-22.
17. Banas JA. Virulence properties of Streptococcus mutans. Front Biosci 2004;9:1267-1277.
18. Islam B, Khan SN, Naem A, Sharma V, Khan AU. Novel effect of plant lectins on the inhibition of Streptococcus mutans biofilm formation on saliva-coated surface. J Appl Microbiol 2009;106:1682-1689.
19. Matsuzako M, Inamichi T, Sawada H, Sato M, Hamada S, Ooshima T. Inhibitory effects of oolong tea extract on caries inducing properties of mutans streptococci. Caries Res 1999;33:441-445.
20. Aldi D, McShan WM, McLaughlin RE, Savić G, Chang J, Carson MB,
유정헌 외 | 18β-glycyrrhetinic acid의 S. mutans의 바이오필름 형성에 대한 억제효과

et al. Genome sequence of Streptococcus mutans UA159, a cariogenic dental pathogen. Proc Natl Acad Sci USA 2002;99:14434-14439.

21. Oatmen TR. Preventive dentistry techniques in the treatment of dental caries and biofilm control: A Review. Honors Projects 2011:88.

22. Matsumi Y, Fujita K, Takashima Y, Yanagida K, Morikawa Y, Matsunoto M. Contribution of glucan-binding protein A to firm and stable biofilm formation by Streptococcus mutans. Mol Oral Microbiol 2015;30:217-226.

23. Kim D, Hwang G, Liu Y, Wang Y, Singh AP, Vorsa N, et al. Cranberry flavonoids modulate cariogenic properties of mixed-species biofilm through exopolysaccharides-matrix disruption. PLoS One 2015;10(12):e0145844. DOI:10.1371/journal.pone.0145844 PMID:26715488.

24. Kuramitsu HK. Virulence properties of oral bacterial: impact of molecular biology. Curr Issues Mol Biol 2001;3:35-36.

25. Senadheera D, Guguenheim B, Spatafora GA, Huang YC, Choi J, Hung DC, et al. A VcrRK signal transduction system in Streptococcus mutans affects gtfBCD, gbpB, and fif expression, biofilm formation, and genetic competence development. J Bacteriol 2005;187:4064-4076.