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Original Article

Streptococcus mutans biofilm model에서 오배자(*Galla Chinensis*) 추출물과 칼슘의 법랑질 재광화효과와 항균효과에 관한 연구

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Galla chinensis extracts and calcium induce remineralization and antibacterial effects of enamel in a *Streptococcus mutans* biofilm model

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Objectives: The purpose of this study was to investigate the combined effects of *Galla chinensis* extract (GCE) and calcium (CA) on enamel remineralization. The antibacterial effect of *G. chinensis* on *Streptococcus mutans* biofilm was also evaluated by examining the bacterial growth, acidogenesis, and morphology of the biofilm *in vitro*.

Methods: *S. mutans* biofilm was formed on bovine enamel specimens over a 72-h period and treated for 10 min with 1.0 mol CA, 4,000 ppm aqueous solution of GCE, or a combination of the two (GCE+CA). The enamel specimens were analyzed for enamel surface microhardness after remineralization. We tested the anti-cariogenic effects of GCE based on the inhibition of acid production, antibacterial activity, and morphological changes in *S. mutans*. The differences between the groups and antibacterial effects were analyzed using one-way analysis of variance.

Results: GCE+CA group showed the highest efficacy in enhancing remineralization. The GCE group showed the highest antibacterial activity against *S. mutans* biofilm. Although the GCE+CA group showed significant antibacterial activity, it was less than that of the GCE group ($P<0.05$). Both GCE and GCE+CA groups maintained a pH of approximately 7.0 for 1 h whereas the pH of the control group decreased rapidly from pH 7.3 to pH 6.1. SEM imaging revealed that *S. mutans* treated with GCE and GCE+CA showed irregular cell wall structure and showed fewer cells in the chain than the typical long chains observed in the control group.

Conclusions: This study found that natural *G. chinensis* significantly enhances enamel remineralization, and exerts synergistic effects with calcium. It also exerts strong bactericidal activity and inhibits acid production and changes in the microstructure of *S. mutans* biofilm.

Key Words: Anti-bacterial agents, Combined effect, *Galla chinensis*, Remineralization

Introduction

Enamel is highly mineralized and is the strongest biological hard tissue in the human body[1]. In contrast to other tissues, dental enamel cannot heal itself and must be re-hardening by a physiochemical process involving inorganic constituents from saliva or solutions[2]. Accordingly, high levels of mineral supplements, such as calcium, fluoride, and phosphates, have preventive effects on enamel mineral loss. However, some of these substances may have side effects in long-term use. For these reasons, there is growing interest in finding new compounds for long-term use[3]. Among them are several natural extracts that show the ability to have a better effect on balance tooth de-/remineralization of dental enamel[4,5].

Galla Chinensis (*G. Chinensis*), one of the traditional natural, non-toxic Chinese herb, has been used for the past 2,000 years. Previous studies have indicated that *G. Chinensis* had an ability to inhibiting cariogenic bacteria[6,7], enamel demineralization, and enhancing remineralization[8,9]. It is necessary to evaluate the effect of their chemical compounds on promoting remineralization of dental enamel. A previous study reported those co-operative effects of fluoride and the chemical compounds of *G. Chinensis* on enhancing remineralization of dental enamel[10], however, experiments have not been performed to investigate the co-operative effects of calcium and the *G. Chinensis* on enhancing the remineralization underneath a biofilm model. Moreover, some experiment also performed the potential rehardening effect of *G. Chinensis* under pH-cyclic conditions, but since this does not reflect the complex environment in the mouth, we performed this study to assess the effect of *G. Chinensis* on enamel by reproducing the oral ecological environment as much as possible using biofilm model. So far, no biofilm model has persuasively addressed the effectiveness of caries-preventive agents such as traditional herb on remineralization of dental hard tissue. Thus, it is of interest to study remineralization underneath a biofilm.

Therefore, this study tested the hypothesis that the combination of calcium and *G. Chinensis* would have a synergistic effect on the remineralization underneath a biofilm model. The purpose of this study was to investigate the effects of *G. Chinensis* with calcium on enhancing remineralization, and also the antibacterial effect of *G. Chinensis* underneath a *S. mutans* biofilm was evaluated by examining the bactericidal activity, acidogenesis and morphology *in vitro*.

Materials and Methods

1. Preparation of enamel specimens

Sound bovine incisors without cracks, infection or any lesions under Quantitative Light-Induced Fluorescence (QLF, QLF Pro®, Inspektor Research System BV, Amsterdam, Netherlands) were selected in this study. Cylindrical cores 5 mm in diameter were punched out at the top of the bovine enamel surface. Samples were placed in 1.2×1.0×0.8 cm molds and mounted in acrylic resin. The specimens were ground flat and polished using wetted silicon carbide paper (600-2,000 grid). Specimens were rinsed thoroughly with distilled water and stored in a 100% humidified atmosphere before use. A total of 84 specimens were used in the experiment. Only those specimens which enamel surface hardness raged from 300-330 VHN were selected. The selected specimens were treated with a pH 5.0 solution containing 0.2% Carbopol (#980, Noveon Inc, Cleveland, USA) with 0.1 M lactic acid containing 50% calcium hydroxide phosphate for 72 hours to form initial artificial caries enamel. The VHN of demineralized specimens was measured and 84 specimens having the surface hardness of the initial dental enamel with an average VHN of 35-55 were selected. For each group, GCE, GCE+CA, and CA groups were assigned to 24 and control to 12.

2. *G. Chinensis* extract (GCE)

The effective components of *G. Chinensis* (produced in the Gyeongbuk province of the Republic of Korea) were extracted as previously reported[11]. Briefly, it (1 kg) was dried in an oven at 60°C for 72 hours, finely powdered and added to 600 ml of distilled water. The mixture was stirred for 10 h at 60°C and then filtered. The extract was re-extracted with distilled water under the same conditions. Then, the extract was dissolved in 500 ml of ethanol (100%) for 48 h at 60°C and an agitator speed of 150 rpm. After filtration and evaporation of the ethanol, the remaining extract was lyophilized to provide a powder.

3. Bacteria strain, media, growth conditions

Streptococcus mutans ATCC 25175 was provided from Korean collection for Oral Microorganisms in Seoul National University and cultivated with a tryptic soy broth (TSB) at 37 C and 5% CO_2. The *S. mutans* genome sequence was determined using a shotgun high-throughput sequencing approach as described. The detailed methods are published as supporting information on the PNAS web site (www.pnas.org)[11]. Biofilms of *S. mutans* were formed on bovine specimens in a 50 ml

tube. Each specimen was transferred daily to fresh medium over a 3-day period[12]. The *S. mutans* biofilms on each specimen contained approximately 2×10^7 colony forming units per milliliter before experiment start.

4. Remineralization process

After exposing the biofilm to each solution (1.0 M calcium, a 4,000 ppm aqueous solution of GCE and a 4,000 ppm aqueous solution of GCE containing 1.0 M calcium) for 10 min, and then they were placed in a 50 ml tube containing a sterile saline solution. The specimens in the tube were ultra-sonicated at 50 W (Branson Sonic, USA) using 3×10 sec pulses with 2×5 sec intervals before measuring.

5. Measurement of bacterial viability

Using sterile bovine specimens that did not process anything, after exposing the biofilms to the solutions (1.0 M calcium, a 4,000 ppm GCE and a 4,000 ppm aqueous solution of GCE containing 1.0 M calcium) for 1, 5, 10 min and 1 hour, they were placed in 50 ml tube containing the sterile saline solution. The bovine specimens in the 50 ml tube were ultra-sonicated using 3×10 sec pulses with 2×5 sec intervals[12,13]. The suspension was diluted serially from 10^{-1} to 10^{-6}, and plated on tryptone soy agar. The plates were incubated in 5% CO_2 at 37°C for 48 h, and the CFU were determined by counting the number of colonies.

6. Measurement of acid production

The level of acid production from the *S. mutans* biofilms treated with the compounds was determined by measuring the pH[14]. The pH was measured using a pH electrode (Orion ROSS™, 8102BNUWP, Beverly, MA, USA) connected to a pH meter (Orion Star™, Beverly, MA, USA). After a 5, 10 min, and 1-hour treatment with the test compounds, the pH of the media was measured each time point. These assays were repeated at least three times.

7. SEM analysis

Scanning electron microscopy (SEM) S-4700 (Hitachi, Japan) was used to examine the changes in the *S. mutans* morphology. The bovine enamel specimens were fixed in 4% paraformaldehyde in 0.1 M PBS for 1 h at room temperature. The fixed samples were then washed 2 times with PBS and distilled water, and sputter-coated with platinum and observed by SEM.

8. Assessment of remineralization effect

The surface microhardness of enamel specimens was assessed using a Vickers microhardness tester (Shimadzu, HMV-2, Kyoto, Japan) at the beginning of the experiment, and after being immersed in a mineral and natural supplement. Indentations were measured for 10 s using diamonds at 9.807 N with a magnification of 40 X. The average microhardness was calculated.

9. Statistical analysis

The differences between the groups and antibacterial effects were analyzed using one-way analysis of variance (ANOVA) and a Tukey's post hoc honestly significant differences (HSD) test using the studentized range. The level of significance was $P<0.05$. The SPSS (Statistical Packages for Social Science, Ver. 19.0, Chicago, IL, USA) statistical program was used for all statistical analyzes.

Results

1. Enamel microhardness changes after the experimental procedure

Table 1 shows the mean enamel surface hardness after exposure to the remineralization solutions (GCE; *G. Chinensis* extract, GCE+CA; *G. Chinensis* extract + calcium, and CA; calcium). The GCE+CA groups showed the most enhanced remineralization; 44.2 ΔVHN; the lowest remineralization ef-

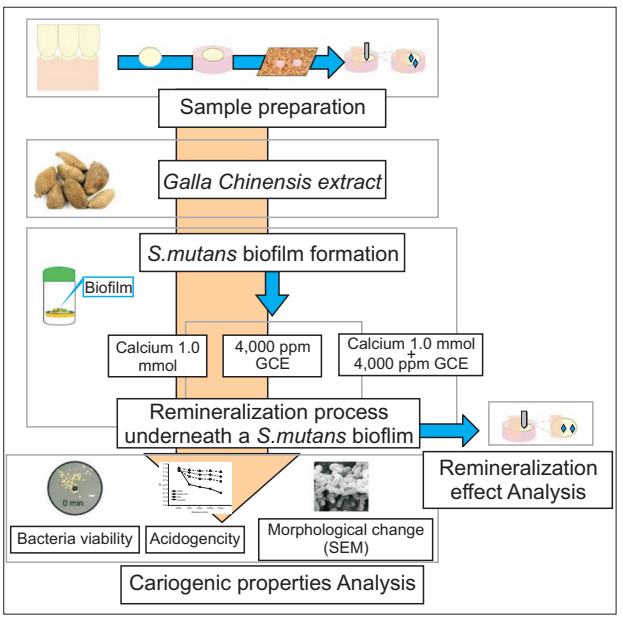

Fig. 1. Flowchart of the in vitro experimental study design.

Table 1. Comparison of the surface microhardness of different groups after remineralization

Condition	N	VHN	VHN	ΔVHN	*P*
		Baseline	After artificial caries formation	After remineralization	
GCE	24	308.8±5.1	48.70±5.00	41.8±3.9[a]	0.009*
GCE+ CA	24	308.9±6.3	46.85±4.95	44.2±3.9[a]	
CA	24	303.2±6.3	42.72±5.19	27.1±5.4[b]	
Control	12	307.3±7.3	44.85±4.85	0.5±3.9[c]	

Values are mean±SD.
ΔVHN = After remineralization VHN - Before treatment VHN (baseline).
CA, immersed in 1.0 mol $CaCl_2$ for 10 min; GCE, immersed in 4,000 ppm GCE for 10 min; CA+GCE, immersed in 1.0 mol $CaCl_2$ and 4,000 ppm GCE for 10 min; Control, no treatment.
*Statistically significant by repeated measured ANOVA at the α=0.05 level.
[a-c]The different lower case letters indicate statistically significant differences between same groups by Tukey's HSD post-hoc test at $P<0.05$.

Table 2. Antibacterial effects of the GCE against *S. mutans* biofilms

Condition	Exposure time		
	CFU (×10^8)		
	0 min	5 min	10 min
GCE	48.8±10.6	5.2±1.4[a]	3.1±0.9[a]
GCE+CA	49.5±6.7	7.8±0.2[a]	6.5±2.2[a]
CA	51.2±8.8	34.1±7.8[b]	30.1±8.4[b]
Control	50.5±9.7	56.5±9.7[c]	60.5±10.8[c]

The data shown are the Mean±SD.
The different superscripts in the same column indicate statistically significant difference from each group ($P<0.05$).

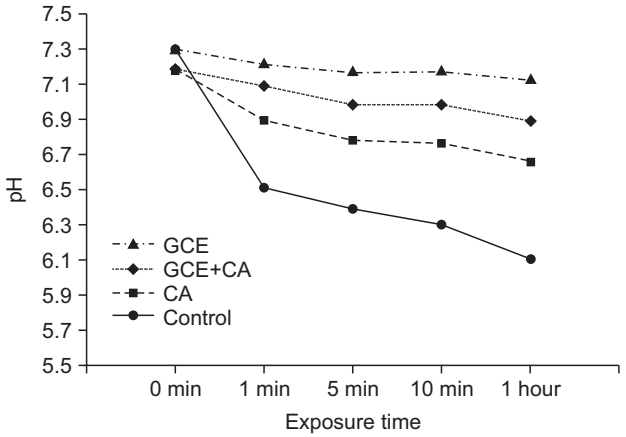

Fig. 2. Acidogenicity of *S. mutans* biofilms was determined by measuring the pH of media.

fect was found in the CA group; 27.1 ΔVHN. As a result of one-way ANOVA analysis, the CA group were significantly different from GCE group and GCE+CA group ($P<0.05$).

2. Antibacterial activity of GCE for *S. mutans* biofilm

After the formation of *S. mutans* biofilm on bovine enamel species, the biofilm was treated with three different solutions. The GCE and GCE+CA groups showed significantly lower numbers of surviving *S. mutans* colony forming units (CFU) than those of the control groups ($P<0.05$). The GCE showed the highest level of antibacterial activity for *S. mutans* biofilm, and the GCE+CA also significant antibacterial activity but less than GCE. The GCE+CA exhibited similar bactericidal activity to GCE. The GCE and GCE+CA groups for 5 min showed 91.0% and 87.5% fewer CFU, respectively than the control group. The GCE group for 10 min showed greater bactericidal activity (94.6%) than that of the GCE+CA and CA group exposed at the same time. However, the CA group (39.3%) showed antibacterial activity but not as much as GCE or GCE+CA (Table 2).

3. Inhibition of acid production

The pH of culture medium was recorded during 1 hour after each solution treatment to determine the effect of GCE on acid production. The pH patterns of the GCE were significantly different from control group after 1 min ($P<0.05$). Both GCE and GCE+CA groups maintained a pH of approximately 7.0 for 1 hour whereas the pH of the control group decreased rapidly from pH 7.3 to pH 6.1 after 1 hour (Fig. 2).

4. Morphological changes in *S. mutans* biofilms

The mechanism responsible for the antimicrobial activity of GCE was investigated by observing the morphological changes of *S. mutans* by SEM after treating the biofilm with the treatment solution for 10 min. SEM showed less morphological and intracellular content in the GCE and GCE+CA groups for 10 min compared to the control group. Also, GCE and GCE+CA groups showed irregular cell wall structure and showed fewer cells in the chain than the typical long chains observed in the control group (Fig. 3).

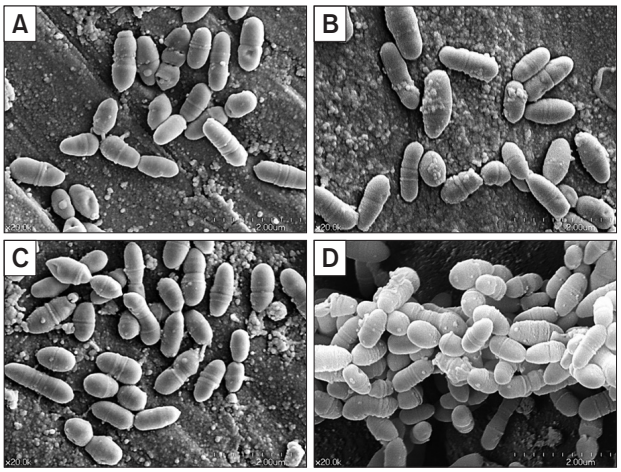

Fig. 3. SEM images of the *S. mutans* biofilm after 10 min treatment. (A) GCE, (B) GCE+CA, (C) CA, and (D) Control.

Discussion

Dental plaque is a representative example of a biofilm, which plays an essential role in the pathogenesis of dental caries. A biofilm is a multicellular aggregate of microorganism attached and accumulates on the surface. One approach to controlling oral biofilm is to remove or reduce the biofilm mass or its acidogenicity by using an antimicrobial agent. Synthetic compounds such as chlorhexidine have been used as an antimicrobial agent to inhibit the growth of bacteria and reduce the adhesion of biofilm to prevent dental caries. However, excessive use leads to side effects of synthetic compounds including alteration of the oral cavity, the bacterial tolerance, taste disorders, dry mouth, and tooth discoloration[15,16]. The search for natural antiplaque agents with safe efficacy and potent activity has focused on reducing the use of synthetic antimicrobials in daily oral care products[17]. *G. Chinensis* has been widely studied as a Chinese herbal medicine to reconstruct tooth enamel following enamel mineral loss and discussed as an effective preventative caries agent due to its unique potential remineralization effects[18,19] and antibacterial effects[6,7]. Also, previous studies on the safety of *G. Chinensis* showed that *G. Chinensis* did not cause toxicity to the cells[20]. However, some studies on antibacterial activity on biofilm model have been conducted, none of the studies investigate the effect of chemical compounds of *G. Chinensis* on the remineralization and antibacterial effects underneath a biofilm model. Thus, the present study investigated the effect of *G. Chinensis* and the combined effect of *G. Chinensis* with calcium on the remineralization and antibacterial effects of enamel underneath a *S. mutans* biofilm *in vitro*.

Our data showed that the GCE exhibits an apparent remineralization effect on bovine enamel. In the present study, surface hardness change was assessed with microhardness measurement. Since the enamel surface is not uniform to remineralization, we tried to measure it at the same spot, and it was repeatedly measured three times when measurement after experiment process, for reduce errors during the measurement process. The measurement of surface enamel hardness using microhardness determination is judged as a suitable tool to investigate the surface softening of enamel[21]. The main finding in this study was that enamel remineralized with GCE and GCE+CA showed more deposited than those of CA group. The remineralization effect of GCE was previously observed by Chu et al.[22], Huang et al.[23]. It showed a thick layer was formed on the surface of the enamel in the GCE group. The reason for this is not apparent, but maybe these results indicate that GCE has more ion channels to the lesion body, so makes minerals to deposit more. In addition, when GCE was combined with calcium, a higher remineralization effect was seen compared to the calcium group (Table 2). It indicated that the combined use of calcium and GCE has a synergistic effect in improving remineralization on enamel underneath biofilm model. The results of this study corresponded well with those of an earlier study that reported that a chemical compound in GCE might act as a calcium ion carrier, supplying the caries lesion with calcium ions from the remineralization solution[24]. Cheng et al.[8] also proposed that some component of GCE might combine with the enamel crystals of a surface layer and inhibit the demineralization of enamel. This may be mainly due to their different mechanisms of action for remineralization. Based on all these results, it can be shown that GCE can directly affect remineralization of the enamel surface and can affect the calcium deposition on the demineralized enamel surface in the combined group during the remineralization process.

There were significantly fewer CFUs of *S. mutans* in the group exposed to GCE and GCE+CA than in the group exposed to CA and control group ($P<0.05$). In addition, the biofilm exposed to GCE and GCE+CA maintained a constant pH around 7. This result shows that GCE can stop the additional acid production of *S. mutans*. This is similar to the results of previous studies on the antibacterial effect of CHX, the most effective antibacterial agent[25]. From the above results, GCE has potential as an antimicrobial agent against *S. mutans* instead of CHX, which has many side effects when used for a long time. According to the SEM images in this study, GCE might have destroyed *S. mutans* chain. *S. mutans* exposed

to GCE also showed morphology change compared with the control group. The antimicrobial activity of *G. Chinensis* on common oral bacteria has been confirmed, as its main component, gallotannins, was found to be bactericidal for *S. mutans* strains[26]. GCE may additionally function by adjusting biofilm structure, composition, and glucosyltransferase activity besides directly inhibiting both bacteria growth and lactic acid formation. Also, GCE has been proven to limit acidic accumulation from carbohydrate metabolism and reduce the proportion of cariogenic bacteria in the biofilm and inhibit demineralization. These results are similar to those of previous studies of natural materials. A grapefruit seed extract is a naturally antibacterial material. It can weaken the function of the physiologically active enzyme in microorganism cells and also destroy the cell wall function[27]. And also, Kim et al.[25] studied about *Curcuma Xanthorrhiza* extract, it has strong bactericidal acidity, inhibitory effects on acidogenesis, and alters the microstructure of *S. mutans* biofilm.

There were several limitations to this study. This study performed using bovine enamel instead of human enamel specimens. However, bovine enamel specimens instead of human enamel specimens were primarily chosen due to several reasons. Human enamel is often difficult to obtain in sufficient quantity and with adequate quality, due to extensive caries lesions or other defects. And also, it can cause large variations in the outcome measures due to the source and age of the collected human teeth[28]. Bovine teeth have been used instead of human teeth, as in other investigations[8,29]. Camargo et al.[27] revealed no significant difference between bovine and human teeth in the pH measurement. That is why we used bovine teeth in this study. Since this study only analyzed the antimicrobial effect of *S. mutans*, further studies will be needed to investigate the efficacy of *G. Chinensis* on other cariogenic bacteria other than *S. mutans*. Further, since this study is an *in vitro* study, future studies using clinical trials will be needed to determine clinical relevance.

Conclusion

This study found that natural *G. Chinensis* has a significant effect on enhancing the remineralization of enamel lesion, and it had combined synergic effects with calcium in improving remineralization. And *G. Chinensis* also has potent bactericidal activity, inhibits acid production, and changes the microstructure of *S. mutans* biofilm. These results suggest that *G. Chinensis* may act as a preventive dental caries agent.

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