

Original

Effects of different antibacterial agents on enamel in a biofilm caries model

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Abstract: Using a mature biofilm model, the aim of this study was to evaluate the effectiveness of different antibacterial agents in comparison with silver diamine fluoride (SDF). Forty-eight saliva-coated enamel slabs were inoculated with *Streptococcus mutans* monospecies biofilm. The biofilms were then exposed to 10% sucrose in tryptone yeast-extract culture medium, 8 times per day for 7 days. After the biofilm growth period, the enamel slabs were treated with one of the following substances: 1) distilled water; 2) SDF; 3) acidulated phosphate fluoride (APF); 4) ammonium hexafluorosilicate (AHF); 5) ammonium hexafluorosilicate + cetylpyridinium chloride (AHF+CPC); or 6) 0.2% chlorhexidine (CHX). After these treatment procedures, the samples were incubated at 37°C for 2 days, and the numbers of viable microorganisms in the biofilms were counted. The number of viable bacteria was significantly reduced by all of the antibacterial agents ($P < 0.05$). However, SDF showed the highest antibacterial activity ($P < 0.05$), and the effectiveness of the other agents was lower ($P < 0.05$). SDF has a highly effective antibacterial action against cariogenic *Streptococcus mutans* biofilm; none of the other fluoride agents used in this study, or 0.2 CHX agent, showed an antibacterial effect comparable to that of SDF. (J Oral Sci 57, 367-372, 2015)

Keywords: dental caries; fluoride; oral biofilm;
Streptococcus mutans.

Introduction

The incidence of dental caries has declined in the industrialized world and developing countries; however, it is still the most prevalent chronic infectious disease, especially in disadvantaged and poor populations (1-3).

Caries is a biofilm-related disease that can be identified by localized destruction of dental hard tissues by the acidic products of oral bacteria (4,5). Because biofilm bacteria are the driving force of demineralization and caries development, control of dental plaque has become an important strategy for caries prevention (6-8).

One of the most effective methods of preventing caries progression is application of fluoride (6,9,10). The anti-caries activity of fluoride-containing products can be attributed to inhibition of the demineralization processes, enhancement of enamel remineralization, and effects on the biological activities of cariogenic microorganisms (6,9,10). Several different fluoride agents, such as acidulated phosphate fluoride (APF), sodium fluoride, stannous fluoride, and amine fluoride have been used clinically (9-13). However, the antibacterial activity of these materials is limited, and attempts to develop more effective antibacterial materials are ongoing (11,14,15). One of these materials, silver diamine fluoride (SDF), is a safe, cost-effective, efficient, and noninvasive caries-preventive agent that has been used for treatment of both primary and permanent teeth (16,17). It has been hypothesized that SDF exerts an antibacterial action and is able to reduce enamel surface mineral loss and increase

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the enamel surface microhardness (16,17). Although a number of studies have indicated that SDF is effective, its clinical use is restricted due to tooth staining caused by silver deposition (18). For this esthetic reason, the use of ammonium hexafluorosilicate (AHF) solution, which replaces the silver component with silica, has been suggested for prevention of caries progression (19). Several studies have indicated that AHF treatment does not produce tooth staining; upon application, a large amount of precipitate forms on the tooth surface, and the acidity of the solution causes etching of the hard tissues of the tooth and re-precipitation of calcium and phosphate ions as silica-calcium-phosphate crystals (18-21). It has been emphasized that the antibacterial activity of AHF solution may be increased when it is used along with several antibacterial agents, such as cetylpyridinium chloride. However, only a limited number of studies have investigated the antibacterial activity of AHF, and its effects on dental biofilms are unclear (18).

Biofilm models are important tools for evaluating the biochemical and microbiological composition of biofilms formed under different conditions (6,14). Biofilm models make it possible to create standardize procedures for comparing the effectiveness of different materials. To our knowledge, many of the previous studies have investigated the antibacterial activity of SDF solution, but none have evaluated the effectiveness of different antibacterial agents on mature dental biofilm in comparison with SDF. Therefore, the aim of this *in vitro* study was to evaluate the effects of various antibacterial agents in a validated and tested *S. mutans* biofilm caries model simulating highly cariogenic conditions on enamel. The null hypothesis was that there would be no statistically significant differences among the agents tested.

Materials and Methods

Sample preparation

Forty-eight enamel blocks were prepared from sound, caries-free bovine incisors. The roots of the teeth were removed by sectioning approximately 2 mm below the cement-enamel junction and perpendicular to the long axis, using a water-cooled diamond disk. The buccal surface of the crown was polished and flattened using 600, 800, and 1,200 sanding paper under water. Forty-eight standardized enamel specimens ($7 \times 4 \times 1$ mm) were prepared from the buccal surfaces of the teeth. After preparation, the specimens were divided into six groups of eight enamel slabs each, according to the test agents employed. The tooth specimens were suspended in the wells of two 24-well culture plates and sterilized with ethylene oxide. Then, the slabs were immersed in

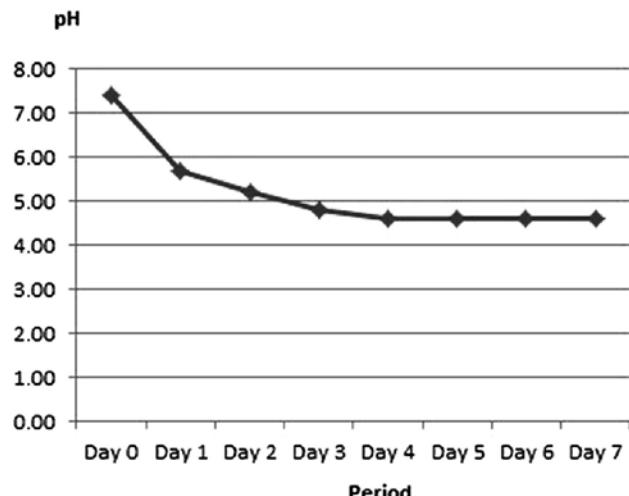


Fig. 1 Culture medium pH during the experimental biofilm growth period.

filtered, pooled human saliva and agitated (60 rpm for 30 min at 37°C) to simulate pellicle formation.

The present study was approved by the Medical Ethics Committee of Izmir Katip Celebi University, under report no. 2013/204.

Biofilm growth

Ultrafiltered tryptone–yeast extract broth (UTYEB) was used as the culture medium. *S. mutans* UA159 (American Type Culture Collection 700610, Rockville, MD, USA) colonies were transferred to the UTYEB, containing 1% glucose, and incubated at 37°C under 10% CO₂ to reactivate the microorganisms. For biofilm formation, *S. mutans* UA159 was inoculated into 1% glucose containing UTYEB and incubated (37°C and 5% CO₂) until an optical density of 1.5 at 600 nm/mL was obtained. The enamel samples with human salivary pellicles were positioned individually in wells containing 2.0 mL of the inoculum and incubated at 37°C under 10% CO₂ to allow bacterial adhesion. The next day, the enamel slabs with biofilms were transferred to fresh UTYEB containing 0.1 mM glucose and exposed for 1 min, 8 times per day (at 8:00, 9:30, 11:00, 12:30, 14:00, 15:30, 17:00, and 18:30), to 10% sucrose for 7 days. This 7-day period allowed the bacteria to grow and mature in the biofilm. After each sucrose exposure, the enamel slabs were washed 3 times in 0.01% NaCl. To assess the acidogenicity of the biofilm, the pH of the culture medium was recorded before each change of medium using a microelectrode coupled to a pH meter (inoLab pH/ION 7320; WTW GmbH, Weilheim, Germany) directly inside the wells (Fig. 1). The culture medium was changed daily, after the first and last sucrose exposures. On the 7th day of the experimental

Table 1 Chemical compositions and application procedures of the tested materials

	Chemical composition	Application methods
Distilled water	Chlorine, ammonium, sulfate, magnesium, calcium, sodium (<2 ppm)	Apply with a brush to all surface for 4 min
38% Silver diamine fluoride (Saforide; Toyo Seiyaku Kasei Co. Ltd., Osaka, Japan)	AgFH_6N_2	Apply with a brush to all surface for 3 min
1.23% Acidulated phosphate fluoride (Sultan Topex APF gel, Sultan Dental Products, Englewood, NJ, USA)	Sodium fluoride, orthophosphoric acid	Apply with a brush to all surface for 4 min
Ammonium hexafluorosilicate (0.476 mol/L) (Sigma Aldrich, St. Louis, MO, USA)	$\text{H}_8\text{F}_6\text{N}_2\text{Si}$	Apply with a brush to all surface for 3 min
Ammonium hexafluorosilicate (0.9 wt%) + Cetylpyridinium chloride (0.1 wt%) (Sigma Aldrich, St. Louis, MO, USA)	$\text{H}_8\text{F}_6\text{N}_2\text{Si} + \text{C}_{21}\text{H}_{38}\text{CIN-H}_2\text{O}$	Apply with a brush to all surface for 3 min
0.2% Chlorhexidine (Klorhex, Drogasan, Ankara, Turkey)	Water, alcohol, glycerin, sodium saccharin, chlorhexidine gluconate	Apply with a brush to all surface for 4 min

Table 2 The total viable microorganism in the biofilm and the medium pH values after treatment procedures

	Total viable microorganism in the biofilm Mean (SD)	Medium pH Mean (SD)
Distilled water (control)	$3.94 \times 10^8 (3.29 \times 10^7)^a$	5.14 (0.29) ^a
Acidulated phosphate fluoride	$2.62 \times 10^8 (1.72 \times 10^7)^b$	5.70 (0.16) ^b
Silver diamine fluoride	0 ^c	7.46 (0.19) ^c
Ammonium hexafluorosilicate	$2.65 \times 10^8 (2.04 \times 10^7)^b$	5.56 (0.10) ^d
Ammonium hexafluorosilicate + Cetylpyridinium chloride	$2.48 \times 10^8 (2.42 \times 10^7)^b$	5.40 (0.15) ^e
0.2% Chlorhexidine	$3.21 \times 10^8 (3.01 \times 10^7)^d$	5.27 (0.12) ^e

*SD: Standard deviation

*Lowercase letters indicate statistically significance differences within columns ($P < 0.05$).

growth period, it was decided that a sufficient cariogenic and mature biofilm layer had been obtained (22,23).

Treatment

The 48 enamel blocks were removed for treatment procedures, and the biofilms were treated with one of the following: 1) distilled water (control); 2) SDF (Saforide; Toyo Seiyaku Kasei Co. Ltd., Osaka, Japan); 3) acidulated phosphate fluoride (APF) (Sultan Topex APF gel, Sultan Dental Products, Englewood, NJ, USA); 4) ammonium hexafluorosilicate (AHF) (Sigma Aldrich, St. Louis, MO, USA); 5) ammonium hexafluorosilicate [0.9 wt%] + cetylpyridinium chloride [0.1 wt%] (AHF + CPC) (Sigma Aldrich); or 6) 0.2% chlorhexidine (CHX) (Klorhex, Drogasan, Ankara, Turkey). The chemical compositions, manufacturers, and application procedures for the treatment agents are presented in Table 1.

Biofilm assessment

After the treatment procedures, all of the enamel blocks were returned to the 24-well plates and incubated for 48 h, after which they were washed three times in 0.9% NaCl and transferred individually to sterile glass tubes containing 9 mL of 0.9 NaCl. The tubes were sonicated to detach the biofilms that had formed on the slabs. To determine the total bacterial colony forming units

(CFUs), an aliquot (1 mL) of the homogenized suspension was serially diluted in 0.9 NaCl (10^0 – 10^8) and a 1-mL suspension from each dilution was inoculated onto the plates, which contained brain-heart infusion agar. To determine the number of viable microorganisms, the plates were incubated in 10% CO₂ at 37°C for 48 h, and then the number of CFU was counted.

Statistical analysis

All of the data were assessed for a normal distribution using the Shapiro-Wilk test for normality. A normal distribution of all data was verified, and values obtained for the different treatment groups were compared by analysis of variance followed by Tukey's test, using SPSS 22.0 statistical software (SPSS, Chicago, IL, USA) ($\alpha = 0.05$).

Results

The total bacteria counts and the medium pH values for all groups are shown in Table 2. All of the antibacterial agents significantly reduced the number of viable bacteria ($P < 0.05$). Among the tested agents, SDF showed the highest antibacterial activity ($P < 0.05$); after the evaluation period, the bacterial count in the *S. mutans* biofilm decreased to zero. The smallest reduction in the number of live bacteria occurred in the 0.2% CHX group ($P <$

0.05), and there were no significant differences among the AHF, APF, and AHF+CPC groups ($P > 0.05$).

Discussion

The process of biofilm development involves initial bacterial adhesion and accumulation, causing enamel demineralization, which indicates that the incidence of dental caries can be reduced by control of biofilm formation and accumulation (4,6,7). *Streptococcus mutans* is the most cariogenic microorganism in dental biofilm due to its acidogenic and aciduric properties, as well as its capacity to utilize dietary carbohydrates and synthesize extracellular polysaccharides (24,25). Although the *S. mutans* biofilm model used in the present study does not exactly simulate the complex microbial community in the oral cavity, previous studies have indicated that the improved model of *S. mutans* biofilm growth is sufficiently sensitive to show biofilm changes in the presence of antimicrobial substances (6,14). Therefore, in this study, the antibacterial effectiveness of different agents was tested on the validated cariogenic *S. mutans* biofilm caries model. Based on the results obtained, the null hypothesis was rejected, since differences were observed in the antibacterial activities of the various agents tested.

The present results revealed significant differences between the negative control and the five treatment groups (SDF, APF, AHF, APF+CPC, and CHX), and the number of viable microorganisms was reduced in the samples treated with the antibacterial agents. The differences in the observed antibacterial activities between the control and tested material groups suggested that the number of viable microorganisms was affected by the use of different antibacterial agents. The highest antibacterial activity was observed in the SDF group, and no viable microorganisms were detected in the biofilms treated with this agent. This result is consistent with those of many previous studies (16,23,25). Chu et al. reported the bacterial count decreased to zero after treatment of mature *S. mutans* biofilm with SDF (22). In contrast, confluent growth of live *S. mutans* and high CFU counts were observed in the negative control group. At the end of the experiment, pH values were found to be higher in the SDF treatment group than in the control group. Similarly, Mei et al. mentioned that 38% SDF inhibited multispecies cariogenic biofilm formation on dentin carious lesions and reduced the process of demineralization (23). In the same study, the pH value in the negative control group was 3.5–4.0 and that in the SDF group was 6.0–6.5. The antibacterial activity of SDF is attributable to the silver and fluoride ions in the structure of the material (16,17,23,25). SDF contains high concentrations of

silver (253,870 ppm) and fluoride (44,800 ppm) ions (16,17). Silver ions are bactericidal metal cations that inhibit biofilm formation by inactivating and interfering with the bacterial synthesis of cellular polysaccharides through inactivation of the glucosyltransferase enzymes responsible for the synthesis of soluble and insoluble glucan (16,17). Fluoride ions can also bind to bacterial cell constituents and influence enzymes, such as enolase and proton-extruding adenosine triphosphatase. High concentrations of fluoride can inhibit biofilm formation (16,17).

When we evaluated the antibacterial efficacy of the other tested fluoride-containing agents, all of them were found to have higher activity than that in the control group. However, in comparison with SDF, their antibacterial effect against the *S. mutans* biofilm was lower. Although no reported study has compared the antibacterial effect of these agents with that of SDF on a mature biofilm caries model, the findings are consistent to those of previous differently designed studies (14,18,25). Shah et al. reported that SDF application reduced the saliva *S. mutans* count to a greater degree than APF gel or 6% NaF fluoride varnish (15). They found that APF and fluoride varnish had a similar effect on microorganisms. Another study that tested the antibacterial activities of SDF, AHF, and AHF+CPC solutions on bacterial adhesion found that SDF was the most effective agent, while AHF had limited efficacy (18). However, it was emphasized that addition of CPC to the AHF solution significantly increased the antibacterial activity of the agent, and that the amount of bacterial adhesion was significantly decreased to the same level as that with SDF. CPC is a quaternary ammonium salt of the pyridinium group and bears a positive charge. CPC exerts its antibacterial action by combining with negatively charged proteins on bacterial cells and destroying their cell membrane by disturbing the electrical balance (18). In that study, the antibacterial agents were applied just before seeding of bacteria on hydroxyapatite pellets, allowing only 12 h for plaque accumulation. It is thought that, unlike this previous study, the lack of difference between the AHF and AHF+CPC groups in the present study was because the agents were unable to sufficiently penetrate the mature biofilm within the seven-day period.

In addition to fluoride-containing materials, CHX solution, with proven antibacterial activity on oral bacteria, was also used in the present study. Of all available antimicrobials used in dentistry, CHX is still the agent used most frequently to reduce plaque for the purpose of caries control, due to its broad antimicrobial spectrum (14,26–28). In the present study, application of CHX

solution to the *S. mutans* biofilm significantly reduced the count of viable microorganisms in the dental biofilm. However, its bactericidal activity was found to be significantly lower than that of the other agents tested. This low antibacterial effect of a single application of CHX on *S. mutans* biofilm is consistent with the findings of many other published studies (26-28). Pratten et al. reported that a five-minute application of 0.2% CHX had an insufficient ability to reduce the viability of microorganisms in a mature biofilm (26). However, they emphasized that in the group treated for 60 minute, a marked reduction of bacterial viability was evident. Zaura-Arite et al. reported that 0.2% CHX was able to affect 6-h biofilm and was effective for inhibiting *S. mutans*; however, its effect was limited to the outer layer of 48-h dental plaque (27). Similarly, Vitkov et al. reported that application of 0.1% CHX for 1 or 5 min had an insufficient effect on 24-h oral biofilm (28). These consistent findings demonstrate that at low concentrations and with short single-application times, CHX has only a limited effect on mature dental biofilm.

Acid production is an important virulence factor of *S. mutans* biofilms that warrants attention in studies of dental caries prevention (7). The present results showed that all of the tested materials reduced acid production by *S. mutans* biofilms during the experimental period; the highest pH was detected in the SDF group. This reduction of acid production was closely related to reduction of the CFU count. In addition, the differences in plaque pH reduction among the other test agents may be explained by their various anti-physiological effects on the biofilm cells, as there were no differences in CFU counts among the groups.

The present study is the first to have investigated the antibacterial efficacy of four different fluoride-containing topical agents and 0.2% CHX on a mature *S. mutans* biofilm caries model in comparison with SDF. SDF is known to be a highly effective topical fluoride agent against bacterial dental biofilm, and the present findings clearly demonstrated that none of the other fluoride agents or 0.2% CHX showed an antibacterial effect comparable to that of SDF. Although the present study could not completely simulate the complex oral environment, the results provide useful information on the antibacterial effects of different agents.

Within the limitations of this study:

1. The present study showed that SDF is an effective antibacterial material that can penetrate mature biofilm, and it is suggested that it would be useful as an anti-caries agent.
2. The lower antibacterial activity of the other fluoride-

containing materials in comparison with SDF indicates that fluoride alone is not as effective as the synergistic effect of fluoride and silver ions.

3. The plaque pH in the treatment groups was found to correlate with the number of viable microorganisms in the biofilm. This finding, along with the known relationship between dental plaque pH and caries formation, suggests that remineralization agents would also have antibacterial effects.

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Conflict of interest

The authors have no conflicts of interest to declare.

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