Effects of brief sodium fluoride treatment on the growth of early and mature cariogenic biofilms

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Research Article

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Abstract

Although fluoride has been widely used in the prevention of dental caries, the effect of fluoride on the activity of biofilm in different stages of cariogenic biofilm formation is less studied. This study aimed to investigate the antibiofilm activity of sodium fluoride during early and mature *Streptococcus mutans* (*S. mutans*) biofilms formation. *S. mutans* biofilms were formed on saliva-coated hydroxyapatite disks. In the early (0 ~ 46 h) and mature (46 ~ 94 h) biofilm stages, the biofilm was treated with different concentrations of fluoride (250, 500, 1000, 2000 ppm; 5 times in total, 1 min/treatment). Acidogenicity, dry weight, colony-forming units, water-soluble/insoluble extracellular polysaccharides (EPS), and intracellular polysaccharides were analyzed and confocal laser scanning microscopy images were obtained of the two stages of biofilms (early and mature biofilms). To determine the antibiofilm activity of sodium fluoride during the formation of early and mature biofilms, and to evaluate the relationship between different concentrations of sodium fluoride and antibiofilm activity. In the early cariogenic biofilm formation stage, all fluoride concentration test groups (250, 500, 1000, 2000 ppm) significantly inhibited the growth of *S. mutans* biofilm. The antibiofilm and anti-EPS formation activities of the brief fluoride treatment increased in a concentration-dependent pattern. At the mature biofilm stage, only the 2000 ppm fluoride treatment group significantly inhibited biofilm accumulation, activity, and intracellular/extracellular polysaccharide content compared with the control and other fluoride treatment groups. The antimicrobial activity of fluoride is related to the formation stage of cariogenic biofilm. The early formation stage of cariogenic biofilm is more susceptible to the inhibition of fluorine than the mature stage. The fluoride treatment in the early formation stage of cariogenic biofilm may be an effective means to control the development of cariogenic biofilm and prevent caries.

Introduction

Dental caries is an oral disease associated with dental biofilm[1]. The increase of acidogenic and aciduric bacteria in the microorganism plays an important role in the pathogenesis of dental caries[1]. When biofilms exist on the surface of teeth and expose to dietary sugar, acidogenic bacteria reduce pH in the dental biofilm utilizing glycolysis[1]. The low pH environment further increased the growth of acidity and acidogenic bacteria including mutans streptococci, other acidogenic streptococci, lactobacilli, and bifidobacteria[2]. The composition of cariogenic biofilm microflora is complex[3]. Although there may be other acidogenic and aciduric bacteria[4], *S. mutans* is considered one of the important etiological agents for the development of dental caries[5, 6]. *S. mutans* can produce glucosyltransferases (GTFs) and can synthesize extracellular polysaccharides (EPSs) and intracellular polysaccharides (IPSs) with sucrose as substrate. EPSs promote bacterial adhesion on the tooth surface, colonization, and formation of plaque biofilm, which is an important cariogenic factor of *S. mutans*[7]. The other major cariogenicity of *S. mutans* is determined by acid production (using carbohydrates to produce acid) and acid tolerance (living at low pH), resulting in the loss of local hard tissue and the start of the cariogenic process[7]. Reducing the adhesion of *S. mutans* to tooth surfaces, acid production, and EPSs formation of *S. mutans* biofilms will reduce the incidence of dental caries.

Fluoride is a widely used dental caries preventive agent. Fluoride products, such as fluoride water, mouthwash, ordinary toothpaste, and prescription toothpaste, play an important role in preventing dental caries[8]. The anti-caries mechanism of fluoride is to reduce the enamel solubility and promote enamel remineralization[9, 10]. Some studies have shown that fluoride can inhibit the adhesion of *S. mutans* to the surface of hydroxyapatite[11], acid production, acid tolerance, and glucosyltransferase (GTF) production of *S. mutans* biofilms[12, 13, 14]. Previous studies have reported the effect of fluoride on cariogenic biofilm virulence. When fluoride concentration ≥ 300 ppm, a single brief fluoride treatment (1 minute) can affect cariogenic characteristics of *S. mutans* biofilm[15]. However, a single brief fluoride treatment (1 minute) did not provide sustained antiviral activity because *S. mutans* can recover from fluoride shock over time[16]. Periodical brief fluoride treatment (1 minute) can reduce the dry weight and acid production of *S. mutans* biofilms in a concentration-dependent manner[17].

Although there are many studies on the mechanism of fluoride inhibiting cariogenic biofilm growth of *S. mutans*, there are few studies on the relationship between anti-cariogenic biofilms activity of fluoride and biofilms growth stage, and the effect of continuous brief fluoride treatment on the virulence characteristics of early and mature cariogenic biofilm. It is necessary to test the hypothesis that brief fluoride treatment can show anti-cariogenic biofilm activity, which may depend on the early stage of biofilm formation. Therefore, to demonstrate the effect of fluoride on the development of *S. mutants* biofilms depend on the biofilm formation stage, two stages were used in this study: early biofilm formation stage (0 ~ 46h) and mature biofilm stage (46h ~ 94h)[18, 19]. *S. mutan*s biofilm was treated with different concentrations of fluoride, and the effects of different concentrations of fluoride on the development of *S. mutans* biofilm in two stages were compared.

This study aimed to evaluate the anti-cariogenic biofilm activity treated with brief fluoride (1 min/treatment, five treatments were performed) during early and mature cariogenic biofilm formation using the *S. mutans* biofilm model. Also investigated changes in anti-cariogenic biofilm activity associated with treatment concentrations (0, 250, 500, 1000, 2000 ppm).

Result

Effects of brief fluoride treatments during early *S. mutans* biofilm formation.

The microbiological and biochemical analyses in early S. mutans **biofilms formation (0 ~ 46 h).** The effects of brief fluoride treatment on biofilm dry weight, bacterial activity, and polysaccharides were related to the fluoride concentration. As shown in Fig. 1, the dry weight (all biofilm components except water) (Fig. 1A; $p < 0.05$), CFUs (Fig. 1B; $p < 0.05$), water-insoluble extracellular polysaccharides (ASP) (Fig. 1C; $p < 0.05$), water-soluble extracellular polysaccharides (WSP) (Fig. 1D; $p < 0.05$), and intracellular polysaccharides (IPS) (Fig. 1E; $p < 0.05$) values of 46-h-old *S. mutans* biofilms decreased with the increase of brief fluoride concentration and showed the lowest value in the 2000 ppm fluoride treatment group.

Relationship between fluoride concentration and acid production of early *S. mutans* biofilms. Figure 2A showed the pH changes in the culture medium during the formation of early *S. mutans* biofilms (0 ~ 46 h) after brief fluoride treatment. During the formation of early *S. mutans* biofilms, brief fluoride treatment affected its acidogenicity ($P < 0.05$). The acidogenicity of the biofilm was inhibited in all fluoride concentration test groups during fluorine treatment (2 ~ 31 h; $P < 0.05$; 1 min/treatment, a total of five times) and without fluorine treatment (31 ~ 46 h; $P < 0.05$). The inhibition of acidogenicity of biofilms was enhanced with the increase of brief fluoride concentration, and the highest inhibition ability was shown in the 2000 ppm fluoride treatment group. The glycolytic pH drop assay was used to determine the effect of brief fluoride treatment on the acid production and acid tolerance of 46-h-old *S. mutans* biofilm. As shown in Fig. 3A and 3B, the initial acid production rate and total acid production concentration of the 46-h-old *S. mutans* biofilm were affected by the brief fluoride treatment, which was closely related to the fluoride treatment concentration. Compared with the control group, acid production was inhibited in all fluoride concentration treatment groups. With the increase of fluoride concentration, the anti-acidogenic activity was gradually enhanced. The 2000 ppm fluoride treatment group showed the lowest initial rate of H^+ production and total produced concentrations of H^+ (Fig. 3B). Also, the final pH value of the 2000 ppm fluoride treatment group was the highest, reflecting the decrease of acid tolerance of cells (Fig. 3A).

Confocal laser scanning microscope study of early *S. mutans* biofilms. To further evaluate the effect of brief fluoride treatment on early *S. mutans* biofilm formation components and structure, the CLSM analysis was performed. As shown in Fig. 4, the live/dead cells bacterial biovolume, bacterial thickness, and total biovolume of 46-h-old *S. mutans* biofilms decreased with the increase of brief fluoride treatment concentration, showing the lowest values in the 2000 ppm fluoride treatment group (Fig. 4A, 4B, 4C; $P < 0.05$). The three-dimensional image of bacterial microcolonies of 46-h-old *S. mutans* biofilms showed that with the increase of brief fluoride concentration, the morphology of the live/dead cells microcolonies changed, the volume and number decreased, and the arrangement was loose (Fig. 4D). Brief fluoride treatment also affected EPS formation of *S. mutans* biofilms, as shown in Figs. 5A and 5B. Brief fluoride treatment reduced EPS biovolume ($P < 0.05$) and thickness ($P > 0.05$) (Fig. 5A, 5B). With the increase of fluoride concentration, the anti-EPS formation increased in a concentration-dependent manner after transient fluoride treatment. EPSs and bacterial microcolony images of 46-h-old S. mutans biofilms showed minimal concentrations of homogeneous structures of EPSs covering and surrounding bacterial microcolonies in the 2000 ppm fluoride treatment group (Fig. 5C).

Effects of brief fluoride treatments during mature *S. mutans* biofilm formation.

The microbiological and biochemical analyses in mature *S. mutans* biofilms formation. As shown in Fig. 1, mature *S. mutans* biofilm formation (46 ~ 94 h) was also affected by the 2000 ppm fluoride treatment group (1 min/treatment, a total of five times). There was no significant difference in the dry weight of 94-h-old biofilm in the 250 ppm, 500 ppm, and 1000 ppm fluoride treatment groups compared with the control group. In the 2000 ppm fluorine treatment group, the dry weight of 94-h-old biofilm was

significantly lower than that in the control group and other fluoride treatment groups. Compared with the control group, biofilm dry weight was 12% less in the 2000 ppm fluoride treatment group (Fig. 1A; $p > 0.05$). Also, the values of extracellular polysaccharides (ASP) (Fig. 1C; $p > 0.05$), water-soluble extracellular polysaccharides (WSP) (Fig. 1D; $p < 0.05$), and intracellular polysaccharides (IPS) (Fig. 1E; $p < 0.05$) in 94-h-old biofilm showed the lowest values in the 2000 ppm fluorine treatment group. The CFUs count in 94-h-old *S. mutans* biofilm decreased with the increase of brief fluoride concentration (Fig. 1B; $p > 0.05$). Compared with the control group, the CFUs count of biofilm was reduced by about 80% in the 2000 ppm fluoride treatment group (Fig. 1B).

Relationship between fluoride concentration and acid production of mature *S. mutans* biofilms. Figure 2B showed the pH changes in the culture medium during the formation of mature *S. mutans* biofilms after brief fluoride treatment. In the mature *S. mutans* biofilm stage, the acidogenicity of the biofilm was inhibited after brief fluoride treatment (46 ~ 94 h; $P < 0.05$). The acidogenicity of the biofilm was inhibited in all fluoride concentration test groups during fluoride treatment (46 ~ 55 h; 70 ~ 79 h; 1 min/treatment, a total of five times; $P < 0.05$) and without fluoride treatment (55 ~ 70 h; 79 ~ 94 h; $P < 0.05$). The inhibition of acidogenicity of biofilms was enhanced with the increase of brief fluoride concentration, and the highest inhibition ability was shown in the 2000 ppm fluoride treatment group. As shown in Fig. 3C and 3D, the initial acid production rate and total acid production concentration of the 94-h-old *S. mutans* biofilms were affected by the brief fluoride treatment, which was closely related to the fluoride treatment concentration. Compared with the control group, acid production was inhibited in all fluoride concentration treatment groups. With the increase of fluoride concentration, the anti-acidogenic activity was gradually enhanced. The 2000 ppm fluoride treatment group showed the lowest initial rate of H^+ production and total produced concentrations of H^+ (Fig. 3D; $P < 0.05$). In addition, the final pH value of the 2000 ppm fluoride treatment group was the highest, reflecting the decrease of acid tolerance of cells (Fig. 3C).

Confocal laser scanning microscope study of mature *S. mutans* biofilms. As shown in Fig. 4, the live/dead cells bacterial biovolume, bacterial thickness, and total biovolume of 94-h-old *S.mutans* biofilms decreased with the increase of brief fluoride treatment concentration, showing the lowest values in the 2000 ppm fluoride treatment group (Fig. 4A, 4B, 4C; $P < 0.05$). Three-dimensional images of 94-h-old *S. mutans* biofilms showed that the live/dead cells of the 94-h-old biofilms showed larger microcolonies than the 46-h-old biofilms, with uniform morphology and compact arrangement. With the increase of brief fluoride concentration, the number of live/dead cells microcolonies decreased and the arrangement of microcolonies was loose, but the morphology of microcolonies did not change significantly, and there were still some microcolonies in each treatment group similar to the control group (Fig. 4E). Brief fluoride treatment also affected EPS formation of *S. mutans* biofilms, as shown in Figs. 5A and 5B. Brief fluoride treatment reduced EPS biovolume and thickness (Fig. 5A, 5B; $P < 0.05$). With the increase of fluoride concentration, the anti-EPS formation increased in a concentration-dependent manner after brief fluoride treatment. EPSs and bacterial microcolony images of 94-h-old *S. mutans* biofilms showed minimal

concentrations of homogeneous structures of EPSs covering and surrounding bacterial microcolonies in the 2000 ppm fluoride treatment group (Fig. 5D).

Discussion

Several studies have reported that fluoride can affect acidogenicity, acid tolerance, and EPS formation of cariogenic biofilms, such as *S. mutans* biofilms[20, 21, 22]. Although widespread studies of the effects of fluoride on the biological activity of oral microorganisms, the effects of fluoride on the activity of biofilm at different stages of cariogenic biofilm formation are rarely studied. The *S. mutans* biofilm model was used in this study. The effects of different fluoride concentrations on the growth, virulence (EPSs and acidogenicity), and activity of early and mature *S. mutans* biofilms were compared. Although the *S. mutans* biofilms model used in the present study does not mimic the complex microbial community found in dental biofilms precisely, the mono-species biofilm is advantageous in examining the mechanisms of actions of *S. mutans* in biofilms, also the model could provide significant benefit in establishing the reproducibility of data and reducing variance[10, 23, 24].

Biofilms are typically characterized by dense, highly hydrated clusters of bacterial cells. Bacterial adhesion and early biofilm formation are the first steps of cariogenic biofilm formation[25, 26]. In the early formation stage of biofilm (0 ~ 46h), the bio-volume and average thickness of bacterial microcolonies and extracellular polysaccharides of biofilm continued to increase[19]. Since biomass density of dental biofilms increases from the saliva-biofilm interface inwards[27]. At the mature biofilm stage (≥ 46 h), the CFU count and acid production capacity of the biofilm remained stable, as did the bacterial microflora of the biofilm and the bio-volume and average thickness of the extracellular polysaccharides. the standard deviation of bio-volume (bacteria or EPS) and mean biofilm thickness (bacteria or EPS) did not change over time[19].

According to some reports, the effect of antibacterial agents applied in the mature biofilm stage is lower than that in the early biofilm stage, and the antibacterial activity of mature biofilm is limited to the surface layer of the biofilm substrate[28, 29]. Mature biofilms have thicker and more complex biofilm structures, which may affect the diffusion and penetration of antibacterial agents[27, 30]. Therefore, the antimicrobial membrane activity of *S. mutans* treated with brief fluoride during biofilm formation was studied in this study. As shown in Fig. 1, in the 46-h-old *S. mutans* biofilms, brief fluoride treatment reduced biofilm dry weight accumulation and bacterial activity at all fluoride test concentrations. The dry weight accumulation of biofilm decreased in a concentration-dependent manner, and there were significant differences between each treatment group and the control group (Fig. 1A; $P < 0.05$). When the fluoride concentration ≥ 500 ppm, the activity of biofilm bacteria was significantly affected compared with the control group (Fig. 1B; $P < 0.05$). In the 94-h-old *S. mutans* biofilms, only the 2000 ppm fluoride treatment group significantly inhibited biofilm dry weight accumulation and bacterial activity compared to the control and other fluoride treatment groups (Fig. 1A, 1B; $P > 0.05$).

In the studies of confocal laser scanning scope, the 46-h-old and 94-h-old biofilms live/dead cells bacterial biovolume, total biovolume, and bacterial thickness showed a concentration-dependent decrease in brief fluoride treatment. The lowest values were found in the 2000 ppm fluoride treatment group. There were significant differences between each treatment group and the control group at 46-h-old biofilms, while there were significant differences between the treatment group and the control group at 94-h-old biofilms when fluoride concentration ≥ 500 ppm (Fig. 4A, 4B, 4C; $P < 0.05$). The morphology of 46-h-old biofilms live/dead cell microcolonies was uniform and tightly arranged in the control group. With the increase of brief fluoride concentration, the morphology of live/dead cell microcolonies changed, and the size and number of microcolonies decreased, and the arrangement of microcolonies was loose. These results indicate that high fluoride treatment at the early stage of biofilm can affect the structural integrity of *S. mutans* biofilm cells (Fig. 4D). The live/dead cells of the 94-h-old biofilms showed larger microcolonies than those of the 46-h-old biofilms with uniform morphology and compact arrangement. With the increase of brief fluoride concentration, the number of live/dead cell microcolonies decreased and the arrangement of microcolonies was loose, but the morphology of microcolonies did not change significantly, and there were still some microcolonies in each treatment group similar to the control group (Fig. 4E). These results indicate that brief fluoride treatment is closely related to antibacterial biofilm activity. The antimicrobial biofilm activity of brief fluoride treatment depends on the biofilm formation stage and fluoride treatment concentration. In the early formation stage of biofilm, it is more susceptible to the influence of fluoride than in the mature stage. The antibacterial biofilm activity of the high-concentration fluoride treatment group is higher than that of the low-concentration fluoride treatment group. Also, according to the results of live/dead cell microcolonies, the brief fluoride concentration used in this study had no bactericidal or killing effect on the growth of *S. mutans* biofilms. Studies have reported that fluoride concentration of 3040–5700 ppm or fluoride concentration of > 5000 ug/ml has a bactericidal effect on *S. mutans*[31, 32]. In this study, the highest fluoride concentration was 2000 ppm, which was lower than the fluoride concentration with bactericidal activity.

Up to 40% of the dry weight of dental biofilms is composed of polysaccharides, which are mostly synthesized by microbial GTFs[33]. Therefore, the reduction of biofilm biomass is directly related to the reduction of polysaccharides in the whole biofilm matrix. The complex structural integrity of biofilm is mainly determined by the density of extracellular polysaccharides in the biological matrix, and extracellular polysaccharides contribute to the formation, volume, structural integrity, and stability of the biofilm matrix[7, 34]. In this study, the effects of brief fluoride treatment on the synthesis of water-soluble/insoluble extracellular and intracellular polysaccharides during the formation of *S. mutans* biofilms were investigated. In the 46-h-old *S. mutans* biofilm, the synthesis of water-soluble/insoluble extracellular and intracellular polysaccharides was significantly inhibited in all fluoride treatment groups compared with the control group. The anti-EPS formation activity increased in a concentration-dependent manner (Fig. 1C, 1D, 1E; $P < 0.05$). In the 94-h-old *S. mutans* biofilm, only the 2000 ppm fluoride treatment group significantly reduced the synthesis of water-insoluble extracellular and intracellular polysaccharides compared with the control group (Fig. 1C, $P > 0.05$; Fig. 1E, $P < 0.05$). In terms of water-soluble extracellular polysaccharides, 1000 ppm and 2000 ppm fluoride treatment groups significantly

inhibited the synthesis of water-soluble extracellular polysaccharides compared with the control group (Fig. 1D; $P < 0.05$). Since the biofilms are mainly composed of bacterial cells and polysaccharides[7], the decrease in dry weight in all fluoride concentrations tested in the 46-h-old *S. mutans* biofilms and the 2000 ppm fluoride treatment group in the 94-h-old *S. mutans* biofilms may be due to the reduction in the number of bacterial cells and EPS formation. This result was also supported by the bio-volume and thickness of EPS of biofilm studied by the confocal laser scanning microscope. Brief fluoride treatment has an obvious damage effect on EPS formation of biofilm, and the decrease of bio-volume and thickness of EPS may affect the growth height of bacterial cells (Fig. 5). These results indicate that brief fluoride treatment can inhibit the synthesis of biofilm polysaccharides and is dependent on the biofilm formation stage and fluoride treatment concentration. Compared with the early stage of biofilm, the cariogenic biofilm maturation stage requires a higher concentration of fluoride (≥ 1000 ppm) to significantly inhibit the biofilm polysaccharide synthesis. The inhibition ability of the high concentration fluoride treatment group was higher than that of the low concentration fluoride treatment group. The mechanism of brief fluoride treatment inhibiting biofilm polysaccharide synthesis may be to inhibit GTFs activity or reduce GTFs production in *S. mutans* biofilm cells. However, further studies are needed to determine the mode of action of transient fluoride treatment on GTF activity and production in *S. mutans* biofilm cells.

Acid production (using carbohydrates to produce acid) and acid tolerance (living at low pH) are one of the main toxicity characteristics of *S. mutans*[7], which are closely associated with enamel demineralization and the formation of dental caries[35, 36, 37]. It is well known that fluoride has inhibitory effects on the acidogenicity of cariogenic biofilms[14]. In this study, the acid production and acid tolerance of *S. mutans* biofilms formation by brief fluoride treatment were studied. The results showed that fluoride inhibited the acid production and acid tolerance of *S. mutans* biofilm cells (Fig. 2, 3). In the pH changes of the old culture medium, it was found that after brief fluoride treatment, all fluoride concentration test groups inhibited the acid-producing capacity of the biofilm in the early formation and mature stages of the biofilm during fluoride treatment and without fluoride treatment. The concentration of acid in the old medium decreased in a concentration-dependent manner, and the highest inhibition ability was shown in the 2000 ppm fluoride treatment group (Fig. 2A, 2B). The lower the acid concentration may be related to the decrease of biofilm cell viability or physiological capacity. In the glycolytic pH drop experiment, the initial pH value reflects the acid-producing capacity of the cell, and the final pH value reflects the acid-tolerant capacity of the cell[37]. As shown in Fig. 4, brief fluoride treatment inhibited acid production in the early and mature stages of cariogenic biofilms. The acidification activity of the biofilms was inhibited in all fluoride treatment groups, and the 2000 ppm fluoride treatment group showed the lowest initial rate and total acid production (Fig. 3B, 3D). Also, brief fluoride treatment reduced the acid resistance of cariogenic biofilm cells, and the 2000 ppm fluoride treatment group showed the highest final H^+ concentration, which showed the highest effect on the acid resistance of biofilm cells (Fig. 3A, 3C). The change of H^+ concentration may be due to the inhibition of glycolytic acid production by fluoride. These results indicate that the acid production and acid tolerance of the biofilm can be reduced by brief fluoride treatment during the early biofilm formation and mature biofilm. The anti-acidification activity was

related to the concentration of fluoride, and the highest anti-acidification activity was shown in the 2000 ppm fluoride treatment group.

In general, brief fluoride treatment significantly inhibited early *S. mutans* biofilm formation (0 ~ 46 h). At the early stage of biofilm formation, biofilm dry weight, bacterial activity, water-soluble/insoluble polysaccharides, intracellular polysaccharides, and acid-producing capacity were significantly inhibited in all fluoride treatment groups compared with the control group (Fig. 1, 2, 3; $P < 0.05$). The inhibition degree of brief fluoride treatment on early biofilm growth of cariogenic bacteria was proportional to fluoride concentration. At the mature biofilm stage, only the 2000 ppm fluoride treatment group significantly inhibited the growth of biofilm compared with the control group. These results indicate that brief fluoride treatment can exhibit anti-*S. mutans* biofilm activity, which depends on the stage of biofilm formation. In the early stage of biofilm formation, low fluoride concentration treatment can significantly inhibit the growth of biofilm, while in the mature stage, higher fluoride concentration treatment can significantly inhibit the growth of biofilm. At the same time, the experimental results showed that the inhibition ability of fluoride on the growth of early and mature biofilm of *S. mutans* was related to the concentration of fluoride treatment. Compared with other fluoride treatment groups and the control group, the inhibition ability of the 2000 ppm treatment group was the strongest. This study provides a theoretical basis for the timing of fluoride in preventing caries. However, further research is needed to reveal the exact mechanism by which brief fluoride therapy affects early cariogenic biofilm formation.

Conclusion

The antimicrobial activity of fluoride is related to the stage of biofilm formation. Brief fluoride treatment significantly inhibited the growth of early *S. mutans* biofilms. The growth of *S. mutans* biofilm is more easily inhibited by fluoride in the early formation stage than in the mature stage. The fluoride treatment in the early stage of cariogenic biofilm formation may be an effective means to control cariogenic biofilm development and prevent dental caries.

Materials And Methods

***Streptococcus mutans* biofilms formation, fluoride, and experimental scheme.** Figure 6 shows *S. mutans* biofilm preparation and experimental scheme for the present study. *Streptococcus mutans* UA159 (ATCC 700610; serotype c) biofilms were formed on saliva-coated hydroxyapatite (sHA) discs (2.93 cm^2; Clarkson Chromatography Products, Inc., South Williamsport, PA, USA) placed in a vertical position in 24-well plates. Briefly, an adult male was selected for oral saliva collection. HA discs were incubated in filter-sterilized (0.22-μm low protein-binding filter) saliva (3 ml/disc) for 1 h at 37°C. For biofilms formation, the sHA discs were transferred to a 24-well plate containing brain heart infusion (BHI; D-ifco, Detroit, MI, USA) broth with 1% (w/v) sucrose and *S. mutans* UA159 ($5–7×10^6$ colony-forming unit (CFU)/ml) (3 ml/disc). The biofilms were grown at 37°C with 5% CO^2 for biofilms growth. After 22 h of biofilm growth, the culture medium was changed twice daily (9 a.m. and 6 p.m. Oral sugar levels rise after 9 a.m. for breakfast and 6 p.m. for dinner) until it was 46 h (0 ~ 46 h early biofilm formation) or 94 h (46 h ~ 94 h mature biofilm

formation) of age. In the present study, the biofilms of ≥ 46 hours were defined as mature biofilms[18]. This study is approved by the ethics committee/institutional review board of the Department of Preventive Dentistry, School of Dentistry, Institute of Oral Bioscience, Jeonbuk National University. All experimental protocols were approved by the Department of Preventive Dentistry, School of Dentistry, Institute of Oral Bioscience, Jeonbuk National University. The author confirms that all methods were carried out in accordance with relevant guidelines and regulations. The author confirms that informed consent had been obtained from all subjects.

The fluoride source in this study was NaF. NaF was purchased from Sigma-Aldrich (St Louis, MO, USA). The solutions were made with NaF and purified water. Fluoride products at concentrations between 1 to 2000 ppm are recommended[8]. In this study, four concentrations of fluoride were used for the experiment, respectively: 250 ppm, 500 ppm, 1000 ppm, 2000 ppm.

Considering that in practical applications, the secretion, swallowing, and spitting of saliva after fluoride products enter the oral cavity may reduce the initial fluoride concentration[38], the 1:3 dilution method is used to simulate the concentration of fluoride products in the oral cavity[39, 40]. The four fluoride concentrations selected in this study can simulate the oral concentrations of four commonly used fluoride products after 1:3 dilution, respectively: 250 ppm (fluoride content in mouth rinse after a 1:3 dilution), 500 ppm (fluoride content in regular toothpaste after a 1:3 dilution), 1000 ppm (fluoride content in prescription toothpaste after a 1:3 dilution), 2000 ppm (fluoride content in the topical dental gel after a 1:3 dilution)[41].

To determine the anti-cariogenic biofilm activity of brief fluoride treatments during early *S. mutans* biofilm formation, the saliva-coated HA disks were treated with 0 ppm (control group), 250 ppm, 500 ppm, 1000 ppm, 2000 ppm for 1 min, a total of 5 times treated during the early *S. mutans* biofilm formation (at 2, 7, 22, 26, 31 hours; The corresponding time is 9 a.m., 1, 6 p.m.). Then, the treated saliva-coated HA disks were transferred into the original 24-well plates containing a 1% sucrose culture medium. The incubated time of the *S. mutans* biofilms was 46 hours.

To determine the anti-cariogenic biofilm activity of brief fluoride treatments during mature *S. mutans* biofilm formation, *S. mutans* biofilms were not treated at the early biofilm growth stage (0 ~ 46h). After mature biofilm formation, the saliva-coated HA disks were treated with 0, 250, 500, 1000, 2000 ppm for 1 min, a total of 5 times treated during the mature *S. mutans* biofilm formation (at 50, 55, 70, 74, 79 hours; The corresponding time is 9 a.m., 1, 6 p.m.). Then, the treated saliva-coated HA disks were transferred into the original 24-well plates containing a 1% sucrose culture medium. The incubated time of the *S. mutans* biofilms was 94 hours.

The effects of different concentrations of fluoride on the dry weight, colony-forming unit, water-soluble/insoluble extracellular polysaccharide, intracellular polysaccharide, and acidogenicity of early (46-h-old biofilms) and mature biofilms (94-h-old biofilms) were compared. The images of biofilms at 46-h-old and 94-h-old were obtained by confocal laser scanning microscopy.

Microbiological and biochemical biofilm analyses. The dry weight and colony-forming units (CFUs) in the homogenized suspension were analyzed. Briefly, the 46/94-h-old biofilms on the sHA disc were transferred into 2 ml of 0.89% NaCl and sonicated in an ultrasonic bath for 10 min to disperse the biofilms. The dispersed solution was re-sonicated at 7W for 30 s after adding 3 ml of 0.89% NaCl (VCX 130PB; Sonics and Materials, Inc., Newtown, CT, USA). For the determination of CFUs count, an aliquot (0.1 ml) of the homogenized solution (5 ml) was serially diluted, plated onto brain heart infusion (BHI; Difco, Detroit, MI, USA) agar plates, and then incubated under aerobic conditions at 37°C to determine the CFUs count[15, 42].

For the determination of the dry weight and amount of water-insoluble extracellular polysaccharides (water-insoluble EPSs) (ASP), water-soluble extracellular polysaccharides (water-soluble EPSs) (WSP), intracellular polysaccharides (IPS), the remaining solution (4.9 ml) was centrifuged (3000 ×g) for 20 min at 4°C. The biofilm pellet was resuspended and washed twice in the same volume of water. Mix the water washed the biofilms pellet with 95% alcohol and put it in a refrigerator at -20°C for at least 18 hours to precipitate the water-soluble EPS. Then calculate the content of water-soluble EPS in the biofilms. The washed biofilms pellet was evenly divided into two portions, lyophilized, and weighed to determine the dry weight. One part used 1 N sodium hydroxide to extract water-insoluble EPS from the dried precipitate. The other part was used to calculate the content of intracellular polysaccharides, as detailed elsewhere[23].

Acid production analysis. The final pH values of the old culture media were also determined during the experimental period using a glass electrode (Beckman Coulter Inc., Brea, CA, USA) to investigate the change in acidogenicity of *S. mutans* biofilms by the treatments. The effect of brief fluoride treatment on the acidogenic and aciduric activity of early and mature *S. mutans* biofilms was determined by the glycolytic pH drop assay. Briefly, *S. mutans* biofilms were not treated with fluoride during the formation stage. the 46/94-h-old *S. mutans* biofilm was incubated in 20 mM potassium phosphate buffer (pH 7.2) for 1 h to deplete endogenous catabolites. They were then washed with salt solution (50 mM KCl + 1 mM MgCl2, pH 7.0) and treated with fluoride (0, 250, 500, 1,000, 2,000 ppm F-). After the fluoride treatment, the biofilms were dip-washed with salt solution and transferred into a 6-well plate containing salt solution. The pH was adjusted to 7.2 with a 0.2 M KOH solution. Glucose was then added to the mixture to give a final concentration of 1% (w/v). The decrease in pH was assessed using a glass electrode over 120 min (Futura Micro Combination pH electrode, 5 mm diameter; Beckman Coulter Inc., CA, USA). The effect of fluoride on the acid production of the biofilm was determined according to the acid production rate, calculated by the change in pH values over the linear portion (0 ~ 20, 30, 120 min) of the pH drop curves[43].

The initial rate (0 ~ 20 min) of H^+ production (y1) and initial rate (0 ~ 30 min) of H^+ production (y2) was derived from the equation:

y1 = (H^+ concentration at 20 min – H^+ concentration at 0 min)/20.

y2 = (H^+ concentration at 30 min – H^+ concentration at 0 min)/30.

The total produced concentration of H^+ (y3) was derived from the equation:

y3 = H^+ concentration at 120 min – H^+ concentration at 0 min.

Confocal Laser Scanning Microscopy Analysis

Live and dead bacterial cells staining. Confocal laser scanning microscopy (CLSM) analysis was performed to confirm the results of microbiological and biochemical studies. To investigate the difference in bacterial cells, the 46/94-h-old biofilms were stained at room temperature in the dark for 30 min using the Film Tracer LIVE/DEAD Biofilm viability kit L10316 (Invitrogen, Molecular Probes Inc., Eugene, OR, USA). The final concentrations of SYTO®9 and propidium iodide (PI) were 6.0 and 30 μM, respectively. This viability kit was based on plasma membrane integrity to determine live and dead cells. In this study, we regarded the cells with intact membranes (green) as live cells, whereas cells with damaged membranes (red) were regarded as dead cells. The excitation/emission wavelengths were 480/500nm for SYTO®9 and 490/635nm for PI for collecting the fluorescence. The stained live and dead bacterial cells were observed with an LSM 510 META microscope (Carl Zeiss, Jena, Germany) equipped with argon-ion and helium-neon lasers. All confocal fluorescence images were taken with an EC Plan-Neofuar 10x/0.30 M27 objective lens. A stack of slices in 6.4 μm step sizes was captured from the top to the bottom of the biofilms. The biovolume and thickness of live and dead cells were quantified from

the entire stack using COMSTAT image-processing software. The biovolume is defined as the volume of the biomass (μm^3) divided by the substratum (hydroxyapatite surface) area (μm^2). The three-dimensional architecture of the biofilms was visualized using ZEN 2.3 (blue edition) (Carl Zeiss Microscopy GmbH, Jena, Germany). The original confocal data was uploaded to ZEN 2.3 software and the intensity of green and red fluorescence in the full thickness of biofilms layers were captured automatically. The software reconstructed the 2-dimensional intensity of fluorescence in all the layers to a 3-dimensional volume stack[44].

EPS staining. The EPSs of 46/94-h-old biofilms were also investigated by simultaneous in situ labeling as described elsewhere[45]. Briefly, Alexa Fluor® 647-labeled dextran conjugate (1 μM, 10,000 MW; absorbance/fluorescence emission maxima 647/668 nm; Molecular Probes Inc., Eugene, OR, USA) was added to the culture medium during the formation of *S. mutans* biofilms (at 0, 22, 31 h of 46-h-old biofilms; at 0, 22, 31, 46, 55, 70, 79 h of 94-h-old biofilms) to label the newly formed EPSs. As described above, the stained EPSs were observed with an LSM 510 META microscope (Carl Zeiss, Jena, Germany) (objective: EC Plan Neofuar 10x/0.30 M27) equipped with argon-ion and helium-neon lasers and visualized using ZEN 2.3. A stack of slices in 7.8 μm step sizes was captured from the top to the bottom of the biofilms. Four independent experiments were performed, and five image stacks per experiment were collected. The EPSs biovolume and thickness were quantified from the confocal stacks using COMSTAT.

Statistical Analysis. All experiments (except CLSM and SEM) were performed in duplicate, and at least six different experiments were conducted. The data are presented as mean ± standard deviation. Inter-group differences were estimated using a one-way analysis of variance, followed by a post hoc multiple comparison (Tukey) test to compare multiple means (SPSS® software, IBM). Values were considered statistically significant when the p-value was < 0.05.

References

1. Marsh, P. D. Are dental diseases examples of ecological catastrophes? *Microbiology*. **149**, 279–294(2003).
2. Marsh, P. D. Controlling the oral biofilm with antimicrobials. *J Dent.* **38**, 11-15(2010).
3. Takahashi, N. & Nyvad, B. Caries ecology revisited: microbial dynamics and the caries process. *Caries Res*. **42**, 409-418(2008).
4. Beighton, D. The complex oral microflora of high-risk individuals and groups and its role in the caries process. *Community Dent Oral Epidemiol*. **33**, 248–255(2005).
5. Hojo, K., Nagaoka, S., Ohshima, T. & Maeda, N. Bacterial interactions in dental biofilm development. *J Dent Res*. **88**, 982–990(2009).
6. Lemos, J. A., Quivey, R. G., Koo, H. & Abranches, J. *Streptococcus mutans*: a new Gram-positive paradigm? *Microbiology*. **159**, 436–445(2013).
7. Bowen, W. H. & Koo, H. Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. *Caries Res.* **45**, 69–86(2011).
8. Centers for Disease Control and Prevention. Recommendations for using fluoride to prevent and control dental caries in the United States. *MMWR Morb Mortal Wkly Rep*. **50**, 1–42(2001).
9. ten Cate, J. M. Current concepts on the theories of the mechanism of action of fluoride. *Acta Odontol Scand.* **57**, 325–329(1999).
10. Pandit, S., Kim, J. E., Jung, K. H., Chang, K.W. & Jeon, J. G. Effect of sodium fluoride on the virulence factors and composition of *Streptococcus mutans Archives of oral biology*. **56**, 643-649(2011).
11. Loskill, P. *et al*. Reduced adhesion of oral bacteria on hydroxyapatite by fluoride treatment. *Langmuir*. **29**, 5528–5533(2013).
12. Bowen, W. H. & Hewitt, M. J. Effect of fluoride on extracellular polysaccharide production by *Streptococcus mutans*. *J Dent Res*. **53**, 627–9(1974).
13. Marquis, R. E., Clock, S. A. & Mota-Meira, M. Fluoride and organic weak acids as modulators of microbial physiology. *FEMS Microbiol Rev*. **760**, 1–18(2003).
14. Pandit, S., Kim, H. J., Song, K. Y. & Jeon, J. G. Relationship between fluoride concentration and activity against virulence factors and viability of a cariogenic biofilm: in vitro study. *Caries Res*. **47**, 539-547(2013).
15. Pandit, S., Cai, J. N., Jung, J. E. & Jeon, J. G. Effect of 1-Minute Fluoride Treatment on Potential Virulence and Viability of a Cariogenic Biofilm. *Caries Res*. **49**, 449-457(2015).

16. Dang, M. H., Jung, J. E., Lee, D. W., Song, K. Y. & Jeon, J. G. Recovery of Acid Production in *Streptococcus mutans* Biofilms after Short-Term Fluoride Treatment. *Caries Res*. **50**, 363–371(2016).
17. Pandit, S., Jung, J. E., Choi, H. M. & Jeon, J. G. Effect of brief periodic fluoride treatments on the virulence and composition of a cariogenic biofilm. *Biofouling*. **34**, 53-61(2018).
18. Lee, D. W., Jung, J. E., Yang, Y. M., Kim, J. G., Yi, H. K. & Jeon, J. G. The antibacterial activity of chlorhexidine digluconate against *Streptococcus mutans* bioflms follows sigmoidal patterns. *Eur J Oral Sci*. **124**, 440–446(2016).
19. Chul, O., Pandit, S. & Jeon, J. G. Changes in the composition of artificial cariogenic biofilms over time. *J Korean Acad Oral Health*. **43**,118-123(2019).
20. Bradshaw, D. J., Marsh, P. D., Hodgson, R. J. & Visser, J. M. Effects of glucose and fluoride on competition and metabolism within in vitro dental bacterial communities and biofilms. *Caries Res*. **36**, 81–86(2002).
21. Pandit, S., Kim, H. J., Park, S.H. & Jeon, J. G. Enhancement of fluoride activity against *Streptococcus mutans* biofilms by a substance separated from Polygonum cuspidatum. *Biofouling*. **28**, 279-287(2012).
22. Neilands, J., Troedsson, U., Sjödin, T. & Davies, J. R. The effect of delmopinol and fluoride on acid adaptation and acid production in dental plaque biofilms. *Arch Oral Biol*. **59**, 318–323(2014).
23. Lemos, J. A., Abranches, J., Koo, H., Marquis, R. E. & Burne, R. A. Protocols to study the physiology of oral biofilms. *Methods Mol Biol.* **666**, 87–102(2010).
24. Klein, M. I. *et al*. Dynamics of *Streptococcus mutans* transcriptome in response to starch and sucrose during biofilm development. *PLoS One.* **5**, e13478(2010).
25. Song, J., Choi, B., Jin, E. J., Yoon, Y. & Choi, K. H. Curcumin suppresses *Streptococcus mutans* adherence to human tooth surfaces and extracellular matrix proteins. *Eur J Clin Microbio.* **31**, 1347-1352(2012).
26. Ronn, S. *et al*. Bacterial flagella explore microscale hummocks and hollows to increase adhesin. *PNAS*. **110**, 5624-5629(2013).
27. Robinson, C. *et al*. Plaque biofilms: the effect of chemical environment on natural human plaque biofilm architecture. *Arch Oral Biol*. **51**, 1006-1014(2006).
28. Zaura-Arite, E., Marle, J. V. & ten Cate, J. M. Conofocal microscopy study of undisturbed and chlorhexidine-treated dental biofilm. *J Dent Res*. **50**, 1436-1440(2001).
29. Donlan, R. M. & Costerton, J. W. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Mrcrobiol Rev*. **P**, 167–193(2002).
30. Filoche, S., Wong, L. & Sissons, C. H. Oral biofilms: emerging concepts in microbial ecology. *J Dent Res.* **89**, 8–18(2010).
31. Bowden, H. W. Effects of fluoride on the microbial ecology of dental plaque. *J Dent Res.* **69**, 653–659(1990).

32. Cai, J. N., Kim, M. A., Jung, J. E., Pandit, S., Song, K. Y. & Jeon, J. G. Effects of combined oleic acid and fluoride at sub-MIC levels on EPS formation and viability of *Streptococcus mutans* UA159 biofilms. *Biofouling*. **31**, 555-563(2015).
33. Koo, H., Xiao, J., Klein, M. I. & Jeon, J. G. Exopolysaccharides produced by *Streptococcus mutans* glucosyltransferases modulate the establishment of microcolonies within multispecies biofilms. *J Bacteriol*. **192**, 3024–3032(2010).
34. Koo, H. & Jeon, J. G. Naturally occurring molecules as alternative therapeutic agents against cariogenic biofilms. *Adv Dent Res*. **21**, 63–68(2009).
35. Kwon, Y. R., Son, K. J., Pandit, S., Kim, J. E., Chang, K. W. & Jeon, J. G. Bioactivity-guided separation of anti-acidogenic substances against *Streptococcus mutans* UA 159 from Polygonum cuspidatum. *Oral Dis*. **16**, 204–209(2010).
36. Zhou, L., Ding, Y., Chen, W., Zhang, P., Chen, Y. & Lv, X. The in vitro study of ursolic acid and oleanolic acid inhibiting cariogenic microorganisms as well as biofilm. *Oral Dis*. **19**, 494–500(2013).
37. Gregoire, S., Singh, A. P., Vorsa, N. & Koo, H. Influence of cranberry phenolics on glucan synthesis by glucosyltransferases and *Streptococcus mutans J Appl Microbiol*. **103**, 1960–8(2007).
38. Marsh, P. D. Microbiological aspects of the chemical control of plaque and gingivitis. *J Dent Re* **71**, 1431–1438(1992).
39. Hara, A. T., Gonzalez-Cabezas, C., Creeth, J., Parmar, M., Eckert, G. J. & Zero, D. T. Interplay between fluoride and abrasivity of dentifrices on dental erosion-abrasion. *J Dent*. **37**, 781–785(2009).
40. Nassar, H. M., Lippert, F., Eckert, G. J. & Hara, A. T. Dentifrice fluoride and abrasivity interplay on artificial caries lesions. *Caries Res.* **48**, 557-565(2014).
41. Hani, M., Nassar, H. M. & Gregory, R. L. Biofilm sensitivity of seven *Streptococcus mutans* strains to different fluoride levels. *J Oral Microbiol*. **9**, 1328265(2017).
42. Koo, H. *et al*. Apigenin and tt-farnesol with fluoride effects on *mutans* biofilms and dental caries. *J Dent Res.* **84**, 1016–1020(2005).
43. Belli, W., Buckley, D. & Marquis, R. E. Weak acid effects and fluoride inhibition of glycolysis by *Streptococcus mutans* GS-5. *Can J Microbiol*. **41**, 785–791(1995).
44. Zhang, T., Wang, Z., Hancock, R. E. W., Fuente-Núñez, C. D. L. & Haapasalo, M. Treatment of Oral Bioflms by a D-Enantiomeric Peptide. *PLoS One*. **23**, e0166997(2016).
45. Hwang, G. *et al*. Simultaneous spatiotemporal mapping of in situ pH and bacterial activity within an intact 3D microcolony structure. *Sci Rep*. **6**, 32841(2016).

Figures

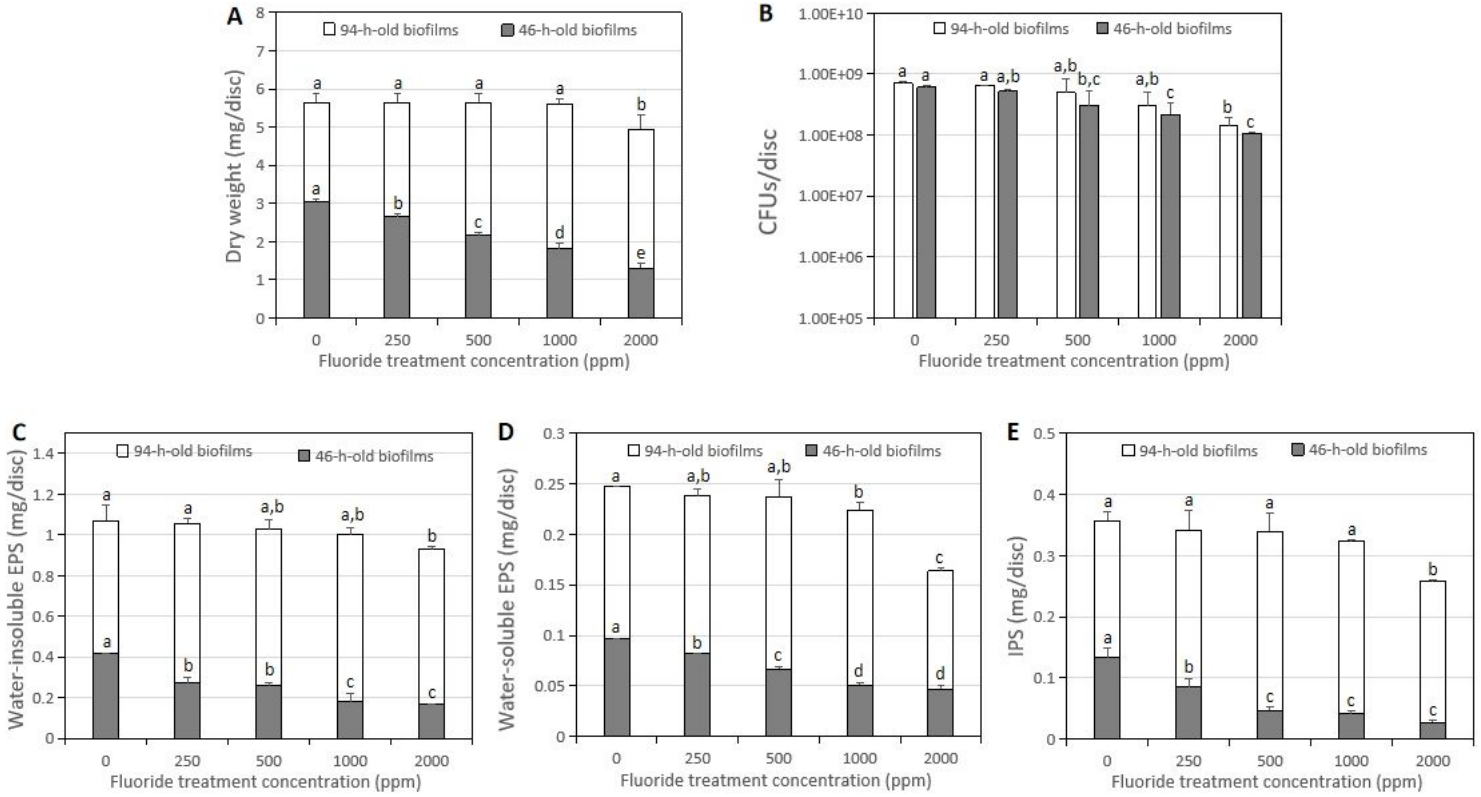

Figure 1

Change in the microbiological and biochemical composition of the 46/94-h-old S. mutans UA159 biofilms treated with different concentrations of fluoride. (A) Dry weight. (B) CFUs. (C) Water-insoluble EPS. (D) Water-soluble EPS. (E) Intracellular polysaccharides (IPS). Data represent mean ± standard deviation. In the 46-h-old S. mutans UA159 biofilms, figure 1A, 1B, 1C, 1D, 1E, $p < 0.05$. In the 94-h-old S. mutans UA159 biofilms, figure 1A, 1B,1C, $p > 0.05$; 1D, 1E, $p < 0.05$. *P < 0.05: significantly different from each other. P > 0.05: values followed by the same superscript are not significantly different from each other.

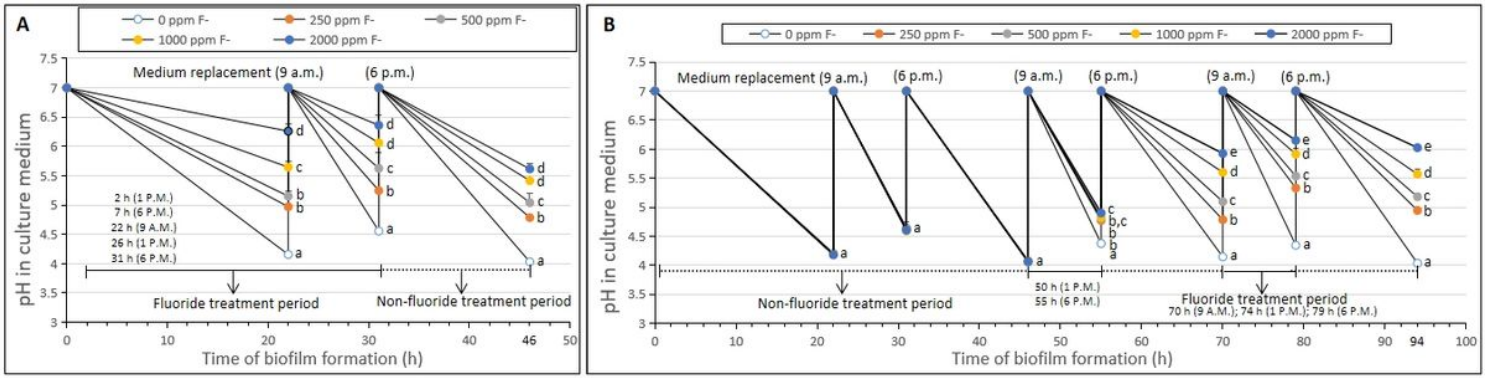

Figure 2

Change in the pH values of old culture medium treated with different concentrations of fluoride during 46/94-h-old S. mutans UA159 biofilms formation. (A) 46-h-old S. mutans biofilms ($p < 0.05$). (B) 94-hour-old S. mutans biofilms (22h, 31h, 46h, $p > 0.05$; 55h, 70h, 79h, 94h, $p < 0.05$). Data represent mean ±

standard deviation. *P < 0.05: significantly different from each other. P > 0.05: values followed by the same superscript are not significantly different from each other.

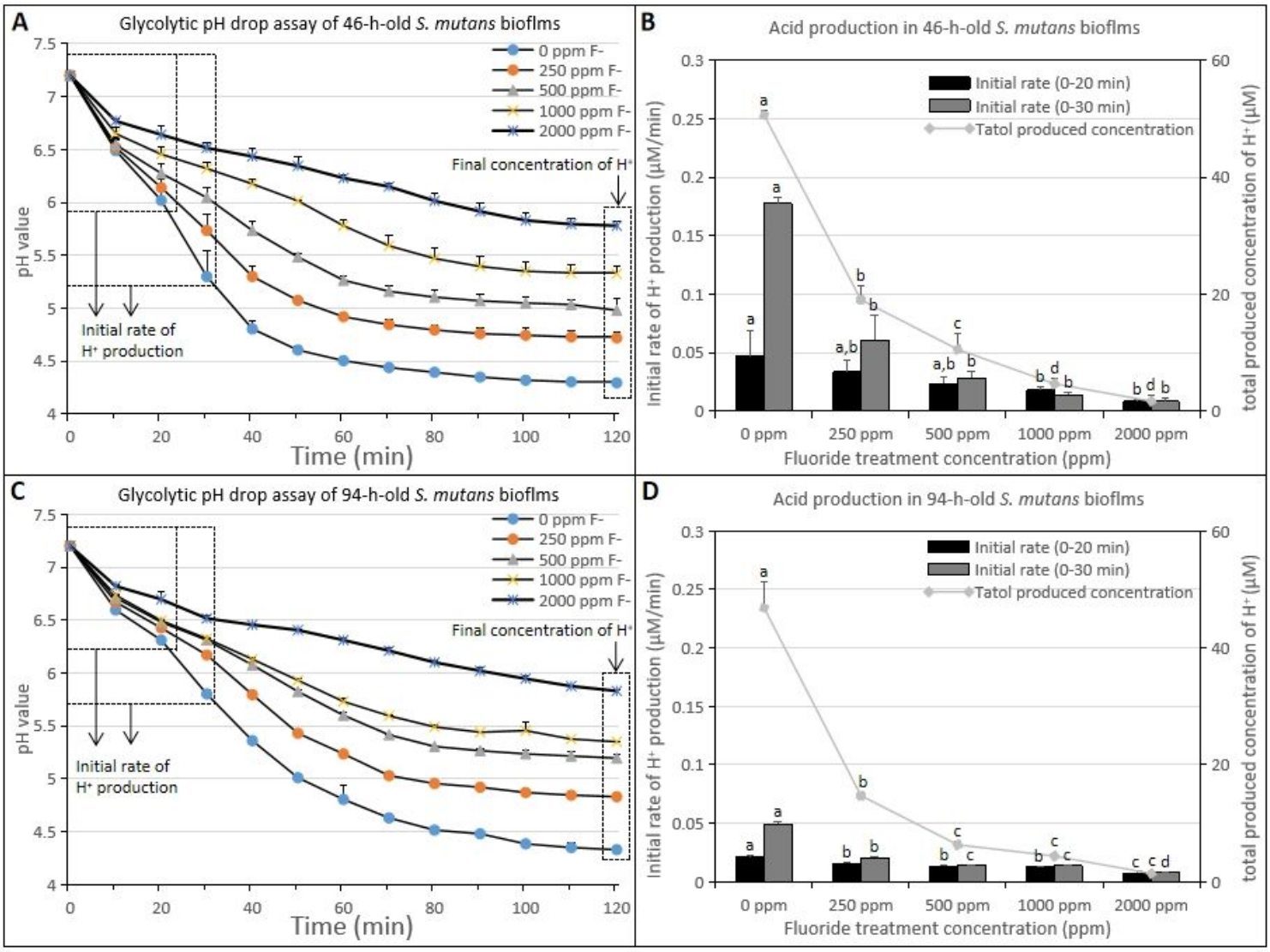

Figure 3

The effect of different concentrations of fluoride treatment on the acidogenicity of 46/94-h-old S. mutans UA159 biofilms cells. Changes in the initial rate of H+ production (0 ~ 20min; 0 ~ 30min) and total produced concentration of H+ (120min) in 46/94-h-old S. mutans biofilms, calculated from biofilm glycolytic pH drop assay data. (A) Change in acid production of 46-h-old S. mutans biofilms ($p < 0.05$). (B) Initial rate of H+ production (0 ~ 20min; 0 ~ 30min) and total produced concentration of H+ (120min) in 46-h-old S. mutans bioflms ($p < 0.05$). (C) Change in acid production of 94-h-old S. mutans biofilms. (D) Initial rate of H+ production (0 ~ 20min; 0 ~ 30min) and total produced concentration of H+ (120min) in 94-h-old S. mutans bioflms ($p < 0.05$). Data represent mean ± standard deviation. *P < 0.05: significantly different from each other.

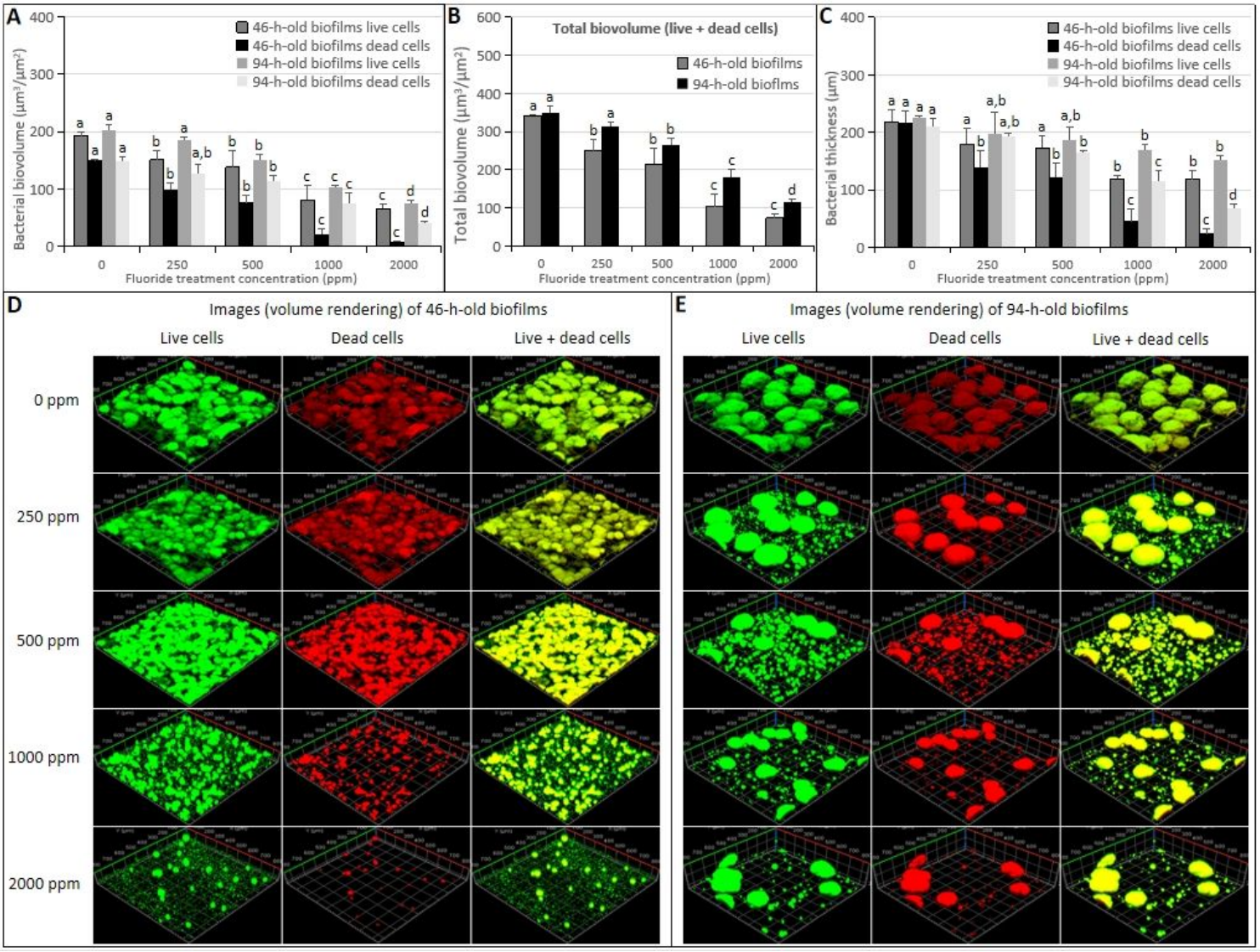

Figure 4

Change in CLSM of bacterial cells in the 46/94-h-old S. mutans UA159 biofilms treated with different concentrations of fluoride. (A) Bacterial biovolume. (B) Total biovolume (live + dead cells). (C) Bacterial thickness. (D) Representative confocal images of 46-h-old S. mutans biofilms. (E) Representative confocal images of 94-h-old S. mutans biofilms. Data represent mean ± standard deviation. Significantly different from each other ($p < 0.05$).

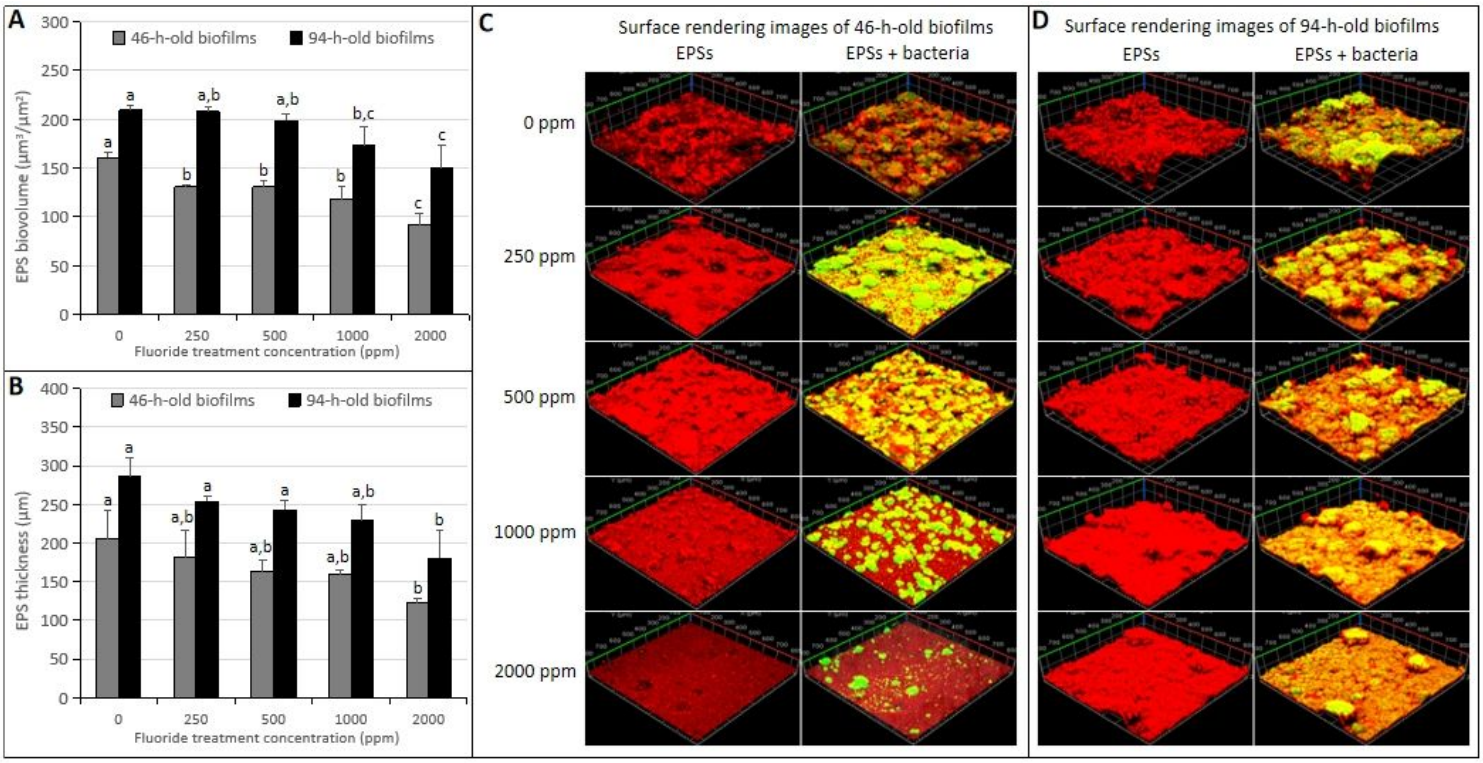

Figure 5

Change in CLSM of EPSs in the 46/94-h-old S. mutans UA159 biofilms treated with different concentrations of fluoride. (A) EPS biovolume. (B) EPS thickness. (C) Representative confocal images of 46-h-old S. mutans biofilms. (D) Representative confocal images of 94-h-old S. mutans biofilms. Data represent mean ± standard deviation. Significantly different from each other ($p < 0.05$).

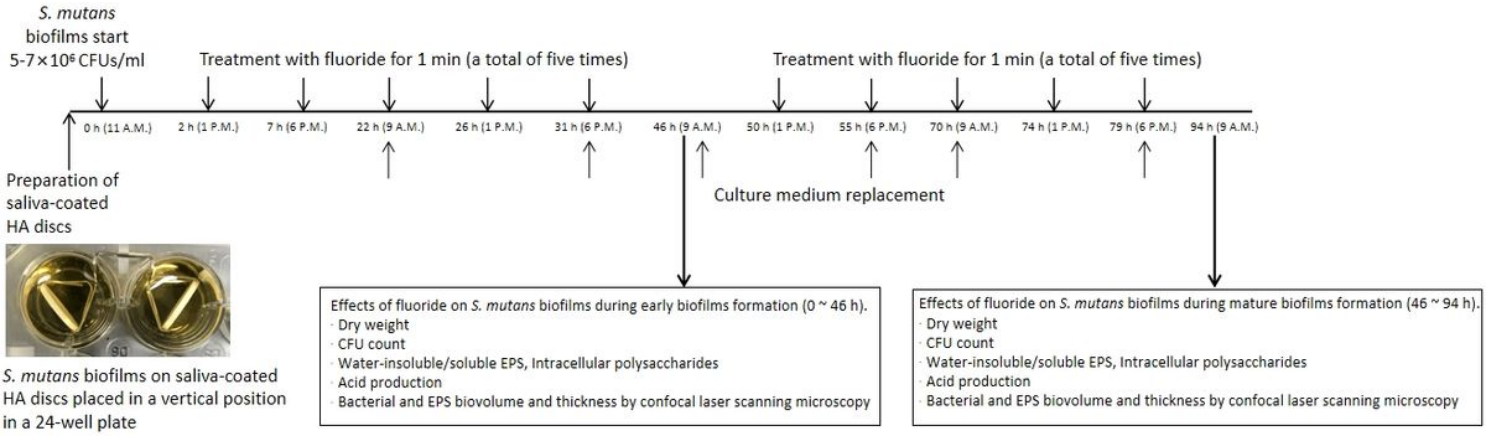

Figure 6

S. mutans biofilms formation and experimental scheme.