Effects of low concentration fluoride released from fluoride-sustained-releasing composite resin on the bioactivity of Streptococcus mutans

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This study aimed to determine the effects of the low concentration of fluoride sustained-released from the composite resin on properties of Streptococcus mutans (S. mutans). The proliferation, adhesion, biofilm formation, and lactic acid production of the bacterium were investigated after the treatment with fluoride from the composite resin. The activity and expression of ATPase, glucosyltransferase (GTF), and enolase were also determined by colorimetric assay and western blot analysis. Fluoride from the sustained-releasing composite resin significantly inhibited biofilm formation, adhesion, and acid production of S. mutans but did not influence the growth and ATPase activity of the bacterial strain. Fluoride-sustained-releasing composite resin inhibited adhesion, biofilm formation, and acid production of S. mutans through the suppression of GTF expression as well as the expression and activity of enolase. These results suggest the clinical significance of fluoride-sustained-releasing composite resin.

Keywords: Biofilm, Phosphopyruvate hydratase, Composite resins, Glucosyltransferases, Streptococcus mutans

INTRODUCTION

Streptococcus mutans (S. mutans) is considered an important contributor to the pathogenesis of dental caries. It is generally known that S. mutans causes dental caries due to its adhesion to tooth surfaces, acid-producing ability, and acid tolerance5. S. mutans synthesizes extracellular polysaccharides such as glucan from sucrose by cell-associated extracellular enzymes such as glucosyltransferase (GTF), contribute to adhesion and biofilm formation2,6. The bacterium produces acids during the metabolism of dietary carbohydrates, which lowers the pH of the biofilm. The bacteria are acid-tolerant; therefore, they can grow in low-pH environments. The ATPase activity of oral bacterial membranes is strongly associated with bacterial acid tolerance8. Fluoride inhibits demineralization and promotes remineralization of tooth surfaces by binding to hydroxyapatite and forming fluorohydroxyapatite6. Fluoride also inhibits bacterial growth and acid production in vitro. In particular, fluoride reportedly inhibits enolase, an enzyme that converts 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic system9. Therefore, fluoride is included in a variety of dental materials with the expectation of preventing dental caries. The amount of fluoride used in dental materials ranges from a few ppm to several thousand ppm, and its action has been verified mainly at high concentrations6,7.

Many fluoride-releasing dental materials, including toothpaste, composite resins, and resin cements, have been developed. Fillers mediate fluoride release. Surface reaction-type pre-reacted glass-ionomer (S-PRG) fillers reportedly inhibit S. mutans growth, adhesion, and acid production. The antimicrobial activity of the S-PRG fillers is attributable to the fluoride and borate released from the fillers, and the mechanism of inhibition has been investigated using filler extracts9. However, the effect of the filler-containing composite resins and the low concentration of sustained fluoride release from composite resins on the properties of S. mutans have not fully been clarified.

A composite resin that releases a low concentration of fluoride in a sustained manner have developed. This study investigated the effects of fluoride released from the composite resin on the biofilm formation and cariogenic abilities of S. mutans.

MATERIALS AND METHODS

S. mutans strain and proliferation assay
S. mutans JCM 5175 obtained from the Riken BioResource Research Center (Ibaraki, Japan) were used in this study. The strain was routinely anaerobically grown at 37°C (90% N2, 5% CO2, and 5% H2) in a brain–heart infusion broth (BHI; Difco, Sparks, MD, USA).

S. mutans (1.0×107 CFU/mL) was cultured in BHI containing fluoride solution (1, 10, 50, 100, 1,000 ppm) or composite resin eluates at 37°C for 24 h. The suspension was measured for absorbance at 570 nm.

Composite resins and assay of released fluoride ion
A fluoride sustained-release composite resin, TMR-Z Fill 10. (YAMAKIN, Osaka, Japan), was used in this study. TMR-Z Fill 10. has the sustained fluoride release property because it is mixed with sustained fluoride release fillers. A composite resin with no fluoride sustained release, Luna-Wing (YAMAKIN), was used...
as a control material. These composite resins were molded to a diameter of 12 mm and a thickness of 1 mm, and they were polished with a P2000 water-resistant abrasive paper.

Composite resin pellets were immersed in 15 mL of distilled water for 24 h, and the fluoride ion concentration of the eluates was measured using an ion meter (F-55, HORIBA Advanced Techno, Kyoto, Japan).

Preparation of S. mutans lysates
S. mutans was permeabilized according to the method described by van Loveren et al.\(^9\). Permeabilized cells were obtained from cultures in the early stationary phase of growth. Twenty-five-milliliter culture samples were centrifuged for 10 min at 3,000×g at 4ºC and the pellets collected. Each pellet was resuspended in 2.5 mL of phosphate-buffered saline (PBS), at a pH of 7.0, and toluene was added to a final concentration of 10% (v/v). The suspension was vigorously vortexed and incubated at 37ºC for 15 min. The cells were frozen twice in liquid nitrogen with thawing at 37ºC. The permeabilized cells were harvested by centrifugation for 5 min at 3,000×g at 4ºC and resuspended in 1 mL of PBS at a pH of 7.0 and stored at −80ºC.

Assays for ATPase, GTF, and enolase activities
The ATPase activity of the permeabilized cells was measured using an ATPase Activity Assay Kit (Colorimetric) (BioVision, Milpitas, CA, USA). Assays were performed according to the manufacturer’s protocol. Briefly, 50 µL of the permeabilized cells and 50 µL of each concentration (1, 10, 50, and 100 ppm), eluate of TMR-Fill 10, (TMR-eluate), or eluate of Luna-Wing (Luna-eluate) was added to each well. Then, 100 µL of the Reaction Mix was added to each well, and the mixtures were incubated for 30 min at 25ºC. After adding 30 µL of ATPase Assay Developer to each well, the plate was incubated for 30 min at 25ºC and measured for absorbance at 650 nm.

S. mutans was cultured in BHI for 24 h at 37ºC. The supernatant was collected by centrifugation at 12,000×g for 30 min at 4ºC, concentrated by 50% (v/v) saturated ammonium sulfate precipitation, and dialyzed overnight at 4ºC to give crude GTF\(^10\). Three hundred microliters of crude enzyme and 900 µL of 0.1 M potassium phosphate buffer containing 0.01% (w/v) sodium azide and 0.1 M sucrose were mixed and 0.1 M potassium phosphate buffer containing 0.01% (w/v) sodium azide and 0.1 M sucrose were mixed and 0.1 M potassium phosphate buffer containing 0.01% (w/v) sodium azide and 0.1 M sucrose were mixed and incubated at 37ºC for 18 h. The absorbance at a wavelength of 550 nm was measured.

Enolase activity in the permeabilized S. mutans was determined by Enolase Activity Colorimetric/Fluorometric Assay Kit (BioVision). Assays were performed according to the manufacturer’s protocol. After the mixture of 25 µL of the permeabilized cells and 25 µL of each concentration of fluoride solution (0.5, 1, 5, 10, and 50 ppm), TMR-eluate, or Luna-eluate, 50 µL of the Reaction Mix inside wells was added to each well. Then the absorbance at a wavelength of 570 nm was measured in kinetic mode for 20–60 min at 25ºC.

Adhesion assay
The adhesion of S. mutans to composite resin pellets was measured using the Microbial Viability Assay Kit-WST (Dojindo, Kumamoto, Japan), which provides colorimetric detection of microbial metabolism. The amount of water-soluble formazan dye generated by WST-8 that was employed as a colorimetric indicator is directly proportional to the number of living microorganisms. Each composite resin pellet was placed inside wells of a 24-well microtiter plate. S. mutans (1.0×10^7 CFU/mL) suspended in BHI containing 1% (w/v) sucrose were incubated on composite resin pellets with fluoride solution (0.5, 1, 5, 10, and 50 ppm) or TMR-eluate at 37ºC for 24 h. Then the pellets were washed with PBS (−) and placed in clean wells. After the addition of 950 µL of 0.1% (v/v) PBS (−) and 50 µL of coloring reagent, the plate was incubated at 37ºC for 2 h and the absorbance at a wavelength of 450 nm was measured.

Biofilm formation assay
Each composite resin pellet was placed inside wells in a 24-well microtiter plate. S. mutans (1.0×10^7 CFU/mL) was cultured in BHI containing 1% (w/v) sucrose on the composite resin pellets with fluoride solution (1 and 5 ppm) or TMR-eluate at 37ºC for 24 h. Then, the pellets were washed with PBS (−) and placed in a clean well. After the staining of the pellets with 300 µL of 0.1% (w/v) crystal violet solution for 5 min, the pellets were washed twice with PBS (−) to remove excess stain. The dye incorporated by the cells forming a biofilm was dissolved with 200 µL of 33% (v/v) glacial acetic acid, and the absorbance at a wavelength of 540 nm was measured.

Microscopic analysis
Each composite resin pellet was placed inside a well in a 24-well microtiter plate. S. mutans (1.0×10^7 CFU/mL) was cultured in BHI containing 1% (w/v) sucrose on composite resin pellets with fluoride solution (1 and 5 ppm) or TMR-eluate at 37ºC for 24 h. The cells were fixed with 2% (v/v) glutaraldehyde and observed with a VHX-6000 (Keyence, Selangor, Malaysia) digital microscope.

Western blot analysis for GTF and enolase
GTF in S. mutans was measured using the supernatant, and enolase in S. mutans was measured using the permeabilized cells through western blot analysis. S. mutans were cultured in BHI containing each fluoride concentration or eluates of composite resins for 24 h at 37ºC. Then, the culture supernatants were collected by centrifugation at 12,000×g for 30 min at 4ºC followed by ultrafiltration. After adjusting the protein concentration in the culture supernatants to a constant level, proteins were separated on their molecular weight on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the proteins were transferred onto a polyvinyl difluoride membrane (Millipore, Billerica, MA, USA). The membranes were incubated with anti-gtfC antibody (CUSABIO TECHNOLOGY, Houston, TX, USA) or anti-ENO1 rabbit polyclonal antibody
Fig. 1 Effects of composite resin eluates on the growth of S. mutans. S. mutans (1.0×10^7 CFU/mL) was cultured in BHI containing resin eluates or fluoride (1, 10, 50, 100, and 1,000 ppm) at 37ºC for the indicated periods. The growth of S. mutans was measured by absorbance at 570 nm of the suspensions. Continuous are presented as the mean±standard error of triplicate determinations from a representative experiment which was undertaken three times. The standard deviations were less than 0.05. *: p<0.05 against control.

Fig. 2 Effects of composite resin eluates on the ATPase activity of S. mutans. S. mutans (1.0×10^7 CFU/mL) was cultured in BHI containing resin eluates or fluoride (1, 10, 50, and 100 ppm) at 37ºC for 24 h. Then, ATPase activity of the permeabilized cells was measured using the ATPase Activity Assay Kit. Data are presented as the mean±standard error of triplicate determinations from a representative experiment which was undertaken three times.

RALTIC ACID PRODUCTION TEST

Lactic acid produced by S. mutans was measured using a Lactate Assay Kit-WST (Dojindo). S. mutans (1.0×10^7 CFU/mL) was cultured in BHI containing 1% (w/v) sucrose on composite resins with fluoride solution (0.5, 1, 5, 10, and 50 ppm) or TMR-eluate at 37ºC for 24 h. The supernatants were collected by centrifugation for 10 min at 5,000×g at 4ºC. Twenty microliters of the supernatant and 80 µL of the coloring reagent were added into 96-well culture plates and incubated at 37ºC for 30 min, and the absorbance at a wavelength of 450 nm was measured.

STATISTICAL ANALYSIS

The analysis was undertaken three times with triplicate determination. The sample size for this study was determined based on the mean and standard deviation calculated from the preliminary study (n=3).

Continuous data are presented as mean±standard deviation, while categorical data are presented as frequencies and percentages. The data were subjected to the Kolmogorov-Smirnov tests and Levene tests (α=0.05) to confirm normality and homoscedasticity, respectively. The intergroup differences were estimated using the one-way analysis of variance, followed by a post hoc multiple comparison (Tukey test) to compare multiple means. A p-value of <0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 12 (SPSS, Chicago, IL, USA).

RESULTS

INFLUENCES OF COMPOSITE RESIN ELUATES ON THE GROWTH AND ATPASE ACTIVITY OF S. MUTANS

The fluoride ion concentrations in eluates of the fluoride-sustained-releasing composite resin, TMR-eluate, and the fluoride non-releasing composite resin, Luna-eluate, were 3.19 ppm and 0.43 ppm, respectively. Fluoride concentrations of less than 50 ppm did not inhibit the proliferation of S. mutans (Fig. 1). Also, the proliferation of S. mutans was not suppressed in the presence of TMR-eluate and Luna-eluate. On the other hand, 50 ppm or higher concentrations of fluoride significantly inhibited the growth of S. mutans. The ATPase activity of S. mutans was not affected by any concentrations of fluoride as well as TMR-eluate and Luna-eluate (Fig. 2). The results of normality and homoscedasticity analysis of variance both showed the p-value of >0.05. The normality of the data in groups control, fluoride (1, 10, 50, and 100 ppm), Luna-eluate and TMR-eluate showed the p-values of 0.110, 0.797, 0.829, 0.167, 0.114, 0.815 and 0.605. The results of homoscedasticity analysis of variance showed the p-value of 0.306.

INFLUENCES OF THE FLUORIDE-SUSTAINED-RELEASE COMPOSITE RESIN ON ADHESION AND BIOFILM FORMATION OF S. MUTANS

The adhesion of S. mutans to the fluoride-sustained-releasing composite resin, TMR-Z Fill 10, was significantly suppressed as compared to its adhesion to the fluoride non-releasing composite resin, Luna-Wing (Fig. 3). Fluoride at concentrations of 0.5, 1, 5, 10, and
Fig. 3 Influence of composite resin with or without fluoride on the adhesion of *S. mutans*. *S. mutans* (1.0×10^7 CFU/mL) were incubated on each composite resin pellet in the presence or absence of fluoride at 37°C for 24 h. The adhesion of the bacterium to the pellets was measured using the Microbial Viability Assay Kit-WST. Continuous data are presented as the mean±standard error of triplicate determinations from a representative experiment which was undertaken three times. *: p<0.05 against Luna-Wing without fluoride.

Fig. 4 Influence of composite resins with or without fluoride on the biofilm formation of *S. mutans*. *S. mutans* (1.0×10^7 CFU/mL) were cultured on each composite resin pellet in the presence or absence of fluoride at 37°C for 24 h. After the pellets were stained with crystal violet solution and microscopic observation was performed (upper panels). Then, the incorporated dye into the bacterium was measured at a wavelength of 570 nm. Data are presented as the mean±standard error of triplicate determinations from a representative experiment which was undertaken three times. *: p<0.05 against Luna-Wing without fluoride.

50 ppm also significantly suppressed the adhesion of *S. mutans* to the fluoride non-releasing composite resin. Biofilm formation of *S. mutans* on TMR-Z Fill 10 was significantly suppressed compared with the formation on Luna-Wing (Fig. 4). Biofilm formation on TMR-Z Fill 10 was more strongly suppressed compared to the formation on Luna-Wing in the presence of 1 ppm fluoride. Figure 5 presents microscope images of biofilm formation on each composite resin in the presence or absence of fluoride. 

Biofilm formation on TMR-Z Fill 10 was significantly inhibited compared to the formation on Luna-Wing without fluoride, similar to the formation on Luna-Wing in the presence of 5 ppm of fluoride. The normality of the data in groups control, fluoride (0.5, 1, 5, 10, and 50 ppm) and TMR-Z Fill 10 showed the *p*-values of 0.094, 0.802, 0.106, 0.067, 0.199, 0.143 and 0.301 in adhesion assay.

Fig. 5 Biofilm formation of *S. mutans* on fluoride-sustained-releasing composite resin. *S. mutans* (1.0×10^7 CFU/mL) was cultured on Luna-Wing pellets (A, B, and C) or TMR-Z Fill 10 pellets (D) with (B: 1 ppm and C: 5 ppm) or without (A and D) fluoride 37°C for 24 h. Then, microscopic observation was performed.

Fig. 6 Effects of composite resin eluates on the GTF activity of *S. mutans*. *S. mutans* (1.0×10^7 CFU/mL) was cultured in BHI containing resin eluates or fluoride (1, 5, 10, and 100 ppm) at 37°C for 24 h. Then, the GTF activity in the culture supernatants was measured as described in the Materials and Methods section. Continuous data are presented as the mean±standard error of triplicate determinations from a representative experiment which was undertaken three times.
Influences of composite resin eluates on the activity and expression of GTF from S. mutans

The GTF activity of S. mutans was not influenced by any concentration of fluoride and eluates of both composite resins (Fig. 6). However, the expression of GTF in S. mutans incubated with fluoride at a concentration of 100 ppm or eluate of fluoride-sustained-releasing composite resin, TMR-Z Fill 10, was decreased (Fig. 7). On the other hand, fluoride at low concentrations such as 1, 5, and 10 ppm and eluate of the fluoride non-releasing composite resin, Luna-Wing did not affect the GTF expression of S. mutans. All of these assays showed p-values >0.05 for both normality and homoscedasticity analysis of variance. The normality of the data in groups control, fluoride (1 and 5 ppm) and TMR-Z Fill 10. showed the p-values of 0.963, 0.291, 0.554 and 0.084 in biofilm formation assay. The data of the adhesion assay and biofilm formation assay were homoscedasticity (p=0.052 and 0.077).

Lactic acid production by S. mutans on fluoride sustained releasing or non-releasing composite resins

The production of lactic acid by S. mutans on the fluoride-sustained-releasing composite resin, TMR-Z Fill 10, was significantly lower than the fluoride non-
Fig. 10 Effects of composite resin eluates on the enolase expression of S. mutans. 
*S. mutans* (1.0×10⁷ CFU/mL) was cultured in BHI containing resin eluates or fluoride (1, 5, 10, and 100 ppm) at 37°C for 24 h. Then, the GTF expression in the culture supernatants was analyzed using western blot (top panel). CBB staining was used as a loading control for western blot analysis (middle panel). Each bar represents arbitrary units of GTF activity by densitometric analysis (bottom panel). Our results are representative of three separate experiments with similar results.

Influences of composite resin eluates on the activity and expression of enolase from *S. mutans*

The enolase activity of *S. mutans* was significantly inhibited by the fluoride-sustained-releasing composite resin, TMR-Z Fill 10. compared with the fluoride non-releasing composite resin, Luna-Wing (Fig. 9). However, enolase activity is significantly inhibited in the presence of fluoride at concentrations of more than 1 ppm. The expression of enolase in *S. mutans* incubated with fluoride at low concentrations such as 0.5, 1, and 5 ppm or eluate of the fluoride-sustained-releasing composite resin, TMR-Z Fill 10, was decreased (Fig. 10). On the other hand, the eluate of the fluoride non-releasing composite resin, Luna-Wing did not affect the enolase expression of *S. mutans*. There was a positive correlation between enolase activity and lactate production in *S. mutans* (Fig. 11). The normality of the data in groups control, fluoride (0.5, 1, 5, 10, and 50 ppm), Luna-eluate and TMR-eluate showed the *p*-values of 0.172, 0.318, 0.892, 0.715, 0.367, 0.513, 0.456 and 0.918. The results of homoscedasticity analysis of variance showed the *p*-value of 0.146.

DISCUSSION

Fluoride suppresses dental caries formation. The mechanisms involved are reducing demineralization, inhibiting *S. mutans* growth, and suppressing biofilm formation. To prevent secondary caries, composite resins that continuously release fluoride have been developed. However, the effect of such materials depends on the amount of fluoride. Therefore, the present study was designed to examine the antibacterial activity of a fluoride-sustained-releasing composite resin compared with a fluoride non-releasing composite resin.

The concentration of fluoride released from the fluoride continuously releasing resin used in this study was approximately 3 ppm, which was lower than the concentration used in previous studies on fluorine. The fluoride continuously releasing resin did not influence the growth and ATPase activity of *S. mutans*. The bacterium can grow under acidic conditions, and the ATPase activity of the bacterial membranes is strongly associated with bacterial acid tolerance. Fluoride reportedly inhibits the growth of *S. mutans* at 300 ppm[12], and ATPase activity is reportedly inhibited at 50 ppm[13]. In the present study, high concentrations (more than 50 ppm) of fluoride inhibited the growth of *S. mutans*, but not low concentrations (10 ppm or less) of fluoride. The reason why the fluoride continuously releasing composite resin, TMR-Z Fill 10, did not inhibit growth and ATPase activity of *S. mutans* seems to be the low concentration of fluoride released from the material.
The first step of dental caries is the adhesion of \textit{S. mutans} to the tooth surface and the formation of biofilm on it. Therefore, we investigated the influence of the fluoride sustained-release composite resin on \textit{S. mutans} adhesion and biofilm formation. The results showed that the adhesion and biofilm formation on the fluoride sustained-release composite resin was significantly inhibited compared with them on the fluoride non-releasing composite resin. On fluoride non-releasing material, low concentrations of fluoride inhibited bacterial adhesion and biofilm formation. There are many reports on the inhibition of \textit{S. mutans} adhesion by fluoride; however, there is no consensus presently. Previously, it has been reported that adhesion was inhibited at a fluoride concentration of 500 ppm; however, it has also been reported that the adhesion was inhibited only at higher concentrations\textsuperscript{14-17}. This discrepancy seems to be due to the difference in experimental methods. In the present study, we used the metabolic activity of the bacteria to estimate the adhesion of the bacteria to the material surface, which is considered to be more sensitive than the conventional method. Cai \textit{et al}. reported that the biofilm formation of \textit{S. mutans} was inhibited at fluoride concentrations of 4.75 ppm\textsuperscript{19}. Our results indicated that the biofilm formation of \textit{S. mutans} on the fluoride sustained-release composite resin, releasing approximately 3 ppm fluoride, was almost completely inhibited compared with that on fluoride non-releasing materials.

\textit{S. mutans} produces insoluble glucan by GTF, which is involved in its adhesion and biofilm maturation on the tooth surface\textsuperscript{5}. Then, we examined the influence of the fluoride sustained-release composite resin on the activity and expression of GTF in \textit{S. mutans}. However, no significant suppression of GTF activity per the same amount of protein was observed in the presence of the eluate from the fluoride sustained-release composite resin as well as any concentrations of fluoride. These results are consistent with previous reports that fluoride did not suppress GTF activity\textsuperscript{13,16,19}. However, our result showed that GTF expression in \textit{S. mutans} was suppressed by the eluate from the fluoride sustained-release composite resin as well as more than 1 ppm of fluoride. Further studies are needed to clarify the reason why the GTF expression of \textit{S. mutans} was inhibited by fluoride, including the eluate from the material, but GTF activity was not.

The second step in the formation of dental caries is that the \textit{S. mutans} adhering to the tooth surface produce lactic acid, which lowers the pH and promotes demineralization. Then, we investigated the influence of the eluate from the fluoride sustained-release composite resin on lactate production in \textit{S. mutans}. Lactic acid production by \textit{S. mutans} was inhibited by the eluate from fluoride-sustained-releasing composite resin, but not the eluate from the fluoride-non-releasing composite resin. More than 1 ppm of fluoride also inhibited lactic acid production by \textit{S. mutans}. The previous study reported that the lactate production in \textit{S. mutans} was inhibited by fluoride concentrations of 50 ppm\textsuperscript{20}. The difference in obtained results might be due to the difference in fluoride origins.

Fluoride inhibits enolase activity. Enolase is an enzyme that converts 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic system in \textit{S. mutans}. In the present study, the activity and expression of enolase in \textit{S. mutans} was inhibited by the eluate from the fluoride-sustained-releasing composite resin and fluoride concentrations of more than 1 ppm, not the eluate from the fluoride-non-releasing composite resin. A previous study reported that fluoride inhibits enolase activity at 0.5 ppm\textsuperscript{21}, which is consistent with the results of this study. Furthermore, there was a positive correlation between the amount of lactic acid produced and enolase activity in \textit{S. mutans}, suggesting that fluoride inhibits lactic acid production by inhibiting the activity of enolase.

\section*{CONCLUSION}

These results indicate that fluoride released from fluoride-sustained-releasing composite resin inhibits adhesion and biofilm formation in \textit{S. mutans} through the suppression of GTF expression, as well as the expression and activity of enolase. This suggests the clinical significance of the fluoride-sustained-releasing composite resin.

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\section*{CONFLICT OF INTEREST}

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\section*{REFERENCES}

1) Islam B, Khan SN, Khan AU. Dental caries: From infection to prevention. Med Sci Monit 2007; 13: RA196-RA203.
2) Tamesada M, Kawabata S, Fujiwara T, Hamada S. Synergistic effects of streptococcal glucosyltransferases on adhesive biofilm formation. J Dent Res 2004; 83: 874-879.
3) Bender GR, Sutton SV, Marquis RE. Acid tolerance, proton permeabilities, and membrane ATPases of oral streptococci. Infect Immun 1986; 53: 331-338.
4) Buzalaf MAR, Pessan JP, Honório HM, ten Cate JM. Mechanisms of action of fluoride for caries control. Monogr Oral Sci 2011; 22: 97-114.
5) Hüther FJ, Psarros N, Duschner H. Isolation, characterization, and inhibition kinetics of enolase from \textit{Streptococcus rattus} FA-1. Infect Immun 1990; 58: 1043-1047.
6) Han J, Cv E, Li M, Niwano K, Ab N, Okamoto A, \textit{et al}. Effect
of fluoride mouth rinse on fluoride releasing and recharging from aesthetic dental materials. Dent Mater J 2002; 21: 285–295.

7) Nassar HM, Gregory RL. Biofilm sensitivity of seven Streptococcus mutans strains to different fluoride levels. J Oral Microbiol 2017; 9: 1328265.

8) Nomura R, Morita Y, Matayoshi S, Nakano K. Inhibitory effect of surface prereacted glass-ionomer (S-PRG) eluate against adhesion and colonization by Streptococcus mutans. Sci Rep 2018; 8: 5056.

9) van Loveren C, Hoogenkamp MA, Deng DM, ten Cate JM. Effects of different kinds of fluorides on enolase and ATPase activity of a fluoride-sensitive and fluoride-resistant Streptococcus mutans strain. Caries Res 2008; 42: 429-434.

10) Wiater A, Choma A, Szczodrak J. Insoluble glucans synthesized by cariogenic streptococci: A structural study. J Basic Microbiol 1999; 39: 265-273.

11) Song JH, Kim SK, Chang KW, Han SK, Yi HK, Jeon JG. In vitro inhibitory effects of Polygonum cuspidatum on bacterial viability and virulence factors of Streptococcus mutans and Streptococcus sobrinus. Arch Oral Biol 2006; 51: 1131-1140.

12) Mayhew RR, Brown LR. Comparative effect of SnF2, NaF, and SnCl2 on the growth of Streptococcus mutans. J Dent Res 1981; 60: 1809-1814.

13) Pandit S, Kim JE, Jung KH, Chang KW, Jeon JG. Effect of sodium fluoride on the virulence factors and composition of Streptococcus mutans biofilms. Arch Oral Biol 2011; 56: 643-649.

14) Meurman JH. Ultrastructure, growth, and adherence of Streptococcus mutans after treatment with chlorhexidine and fluoride. Caries Res 1988; 22: 283-287.

15) Rölla G, Melsen B. Desorption of protein and bacteria from hydroxyapatite by fluoride and monofluorophosphate. Caries Res 1975; 9: 66-73.

16) Shani S, Friedman M, Steinberg D. The anticariogenic effect of amine fluorides on Streptococcus sobrinus and glucosyltransferase in biofilms. Caries Res 2000; 34: 260-267.

17) Streckfuss JL, Perkins D, Horton IM, Brown LR, Dreizen S, Graves L. Fluoride resistance and adherence of selected strains of Streptococcus mutans to smooth surfaces after exposure to fluoride. J Dent Res 1980; 59: 151-158.

18) Cai Y, Liao Y, Brandt BW, Wei X, Liu H, Crielarda W, et al. The fitness cost of fluoride resistance for different Streptococcus mutans strains in biofilms. Front Microbiol 2017; 8: 1630.

19) Koo H, Sheng J, Nguyen PTM, Marquis RE. Co-operative inhibition by fluoride and zinc of glucosyl transferase production and polysaccharide synthesis by mutans streptococci in suspension cultures and biofilms. FEMS Microbiol Lett 2006; 254: 134-140.

20) Exterkate RAM, Crielarda W, Ten Cate JM. Different response to amine fluoride by Streptococcus mutans and polymicrobial biofilms in a novel high-throughput active attachment model. Caries Res 2010; 44: 372-379.

21) Cimasoni G. The inhibition of enolase by fluoride in vitro. Caries Res 1972; 6: 93-102.