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Antibiofilm Activities of Fluoride Releasing Restorative Materials

SUMMARY

Backround/Aim: *The purpose of this in vitro study is to evaluate the antibiofilm and antimicrobial activities of 5 different restorative materials that release fluoride.* ***Material and Methods:*** *Five different fluoride releasing restorative materials [Riva Self Cure (SDI, Australia), Riva Light Cure (SDI, Australia), Riva Silver (SDI, Australia), Dyract® XP (DENTSPLY, Germany) and Beautifil II (SHOFU, Japan)] and one composite resin material (Grandio, VOCO, Germany) were selected for this study. A total of 48 specimens (8 of each) were prepared using Teflon molds (4.0 mm-diameter and 2.0 mm-thickness). The antibacterial and antibiofilm activities of the mentioned restorative materials on Streptococcus mutans were evaluated. The data obtained were evaluated by One-Way analysis and Tukey's Test ($p<0.05$).* ***Results:*** *As a result, no correlation was found in terms of antibacterial and antibiofilm activities of the restorative materials evaluated in the study. While the dental plaque (matrix) accumulation was detected at least on the Grandio resin, the materials with the least cell adhesion were Light Cure and Riva Self Cure since it showed antiadhesive properties for S. mutans.* ***Conclusions:*** *Although the highest antibacterial activity against S. mutans was detected in resin-modified glass ionomers, biofilm matrix (dental plaque) accumulation was mostly detected on these material surface in our study.*

Key words: Fluoride, Biofilm, Streptococcus mutans, Restorative Material, Resin-modified Glass Ionomer Cement, Resin Composite

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Introduction

Tooth decay, one of the most common chronic diseases in the world; It is defined as the pathological condition caused by the continuation of the breaking of the bonds of the organo-inorganic molecules of the enamel and dentin tissues of the tooth with the acid formed as a result of fermenting the carbohydrates of the cariogenic bacteria in the dental plaque[1-5]. As a result of the formation of bacterial plaque in the mouth, the balance between remineralization and demineralization is disrupted, and caries occur as a result of mineral loss in dental hard tissues[3-6,7]. There are four essential factors in the etiology of dental caries. It is known that dental caries occur as a result of the interaction of microflora, diet, time, and host with each other[8,9]. The cause-effect relationship between microorganisms in the oral flora and the onset of caries lesions is not fully understood. Oral bacteria are composed of many colonies and species in a sticky matrix. The metabolic activity of bacteria in the mouth determines the presence of disease in the hard and soft tissue of the tooth. It is known that a small bacterial population is the primary cause of gum disease and caries formation[10,11]. The most important of these bacterial communities is *Streptococcus mutans (S. mutans*). *S. mutans* becomes the most dominant member of mouth flora in individuals with active caries[11].

The definition of the relationship between dental caries and fluorides started with epidemiological research and continued with animal experiments and laboratory studies. Studies showing the relationship between fluorides and dental caries date back to

the 19th century. However, researches on caries prevention in recent years have focused primarily on the effectiveness of fluoride[12-18]. Based on the fact that the enamel is permeable to ions and molecules and the enamel permeability is replaced by physical and chemical factors, fluoride has been added to restorative materials used in dentistry[18-25]. Fluoride raises the pH of the plaque by inhibiting the enzymes by preventing the bacteria from forming acid through the glycolytic route. Due to the high affinity of fluoride to calcium, the adhesion of phosphate and proteins to hydroxyapatite is prevented. This affinity complicates plaque colonization and adhesion. Fluoride, which has a bactericidal effect in high concentrations, prevents the formation of dental caries[26-29]. Developments in restorative materials used in dentistry allow physicians to approach more conservatively in removing caries. As a result of the tissue-protective approach, more infected tissue and microorganisms are left within the borders of the cavity. This reason led to the need to develop materials that increase remineralization and have antibacterial effects with fluoride release. Thus, the materials will prevent the formation of secondary caries and reduce microleakage[30].

Today, fluoride-containing restorative materials release fluoride into the surrounding tooth tissues and mouth environment. These restorative materials include Traditional Glass Ionomer Cement (GIC), Hybrid Glass Ionomer Cements (1- Resin Modified Glass Ionomer Cement (RMGIC), 2- Polyacid Modified Composite Resins (PMCR), High Viscosity Glass Ionomer Cements (HVGICs), Glass Carbomers, Giomers, Nanoionomers, New Generation Glass Hybrid Restorative Material, Amalgomers, fluoride added fissure sealants and fluoride-containing composite resins[31-35].

Our aim in this study is to investigate the effect of fluoride in bacterial adhesion in fluoride-releasing restorative materials in-vitro. Restorative materials releasing fluoride do not have a positive effect in preventing bacterial adhesion, and their acceptance is considered as the H_0 hypothesis.

Material and Methods

Restorative Material Preparation

In our study, HVGIC (Riva Self Cure, SDI, Australia), RMGIC (Riva Light Cure, SDI, Australia), silver reinforced glass ionomer cement (Riva Silver, SDI, Australia), compomer (Dyract® XP, DENTSPLY, Germany), giomer (Beautifil II, SHOFU, Japan) and universal nano-hybrid composite as a control group (Grandio, VOCO, Germany) 6 restorative materials were used (Table 1).

Table 1. Restorative materials used in the study

Material	Type of Material	Content of the material
Riva Self Cure	High Viscosity Glass Ionomer	Strontium Fluoro-Aluminosilicate glass, polyacrylic acid copolymer powders, pigment, Polyacrylic acid copolymer Tartaric acid
Riva Light Cure	Resin Modified Glass Ionomer	1: 2-hydroxyethyl methacrylate (20% –25%) (HEMA), acrylic acid homopolymer (15%-25%), Dimethacrylate crosslinkers (10%-25%), acidic monomer (10% 20%),tartaric acid (1%-5%),glass powder (95%-100%)
Riva Silver	Silver Reinforced Glass Ionomer	Polyacrylic acid, Tartaric acid, Balancing component Alloy powder
Dyract® XP	Polyacid Modified Composite Resin (Compomer)	Urethane dimethacrylate (UDMA), Carboxylic acid modified dimethacrylate (TCB resin), Kamforokino's, Ethyl-4 (dimethylamino) benzoate, Butyl Hydroxy Toluene, UV stabilizer, Strontium-alimino-sodium-fluorine-phosphorus-silicate glass, Silicon dioxide,Strontium fluoride, Iron oxide pigment and Titanium oxide pigment
Beautifil II	Giomer	Bis-GMA (bisphenol A glycidyl ether dimethacrylate), TEGDMA (triethylene glycol dimethacrylate), Aluminofluoroboracilicate glass, Kamforokino
Grandio Resin Composite	Nanohybrid resin composite	Bis-GMA, UDMA, TEGDMA, B-Al-borosilicate glass filler, SiO2 nanodiller

All samples are selected in A2 color to ensure standardization. A total of 48 samples were prepared using cylindrical Teflon molds with a diameter of 4.0 mm and a depth of 2.0 mm. The molds in which the restorative material was placed were placed between two glasses with a thin polyester band placed on them, and light finger pressure was applied. After the excess material was removed and corrections were made, it was polymerized for 20 sec. using an LED light source (Elipar Deepcure-L (3M ESPE, Mapplewood, USA) in line with the manufacturer's recommendation. Each sample was polished dry and unidirectional for 20 seconds using a medium speed tour (Kavo, Warthausen Germany). The discs used in the polishing process are thick (100), medium (29 µm), thin (14 µm), and super thin (8 µm) discs (Sof-Lex, 3M ESPE, Mapplewood, America). After each disc change, the surfaces of the samples were washed, and air dried for 5 seconds. New discs were used each time for all prepared samples[36]. Disc-shaped specimens were sterilized in an autoclave at 121°C for 15 min before being tested with bacteria. Each sample was packaged in dry plastic sterile bags before use in bacterial adhesion and biofilm assays.

Biofilm Producing Optimization for Streptococcus mutans

In bacterial adhesion and biofilm tests, *S. mutans* ATCC 25175 reference strain was preferred. For the activation of the strain stored at -86°C and in 60% glycerol stock, the tubes containing 5 mL of BHI (Brain Heart Infusion, Merck, Germany) broth were inoculated (1% inoculum). The cultures were then incubated at 37°C for 18 h under static conditions. After the first activation in the BHI medium, cultures were activated in the standard TSB medium (Tryptic Soy Broth, Merck, Germany) in subsequent studies.

In the first stage, the TSB medium was modified with different sucrose concentrations in order to determine the ideal biofilm production conditions for *S. mutans* ATCC 25175 strain (Table 2).

Table 2. TSB media containing different sucrose concentrations

Content	Amount
Tryptone enzymatic digest from casein	17.0 g
Soytone (enzymatic hydrolysate of soybean)	3.0 g
D(+)-Glucose (= Dextrose)	2.5 g
Sodium chloride	5.0 g
Potassium phosphate dibasic	2.5 g
Sucrose	0.25%; 0.50%; 0.75%; 1.0%; 1.5%; 2.0%; 3.0%; 4.0%; 5.0%; 6.0%; 8.0%; 10.0%
dH2O	1000 mL

After preparation, it was autoclaved at 121°C for 15 min (pH 7.3± 0.2). The sucrose content was filter sterilized (0.22 µm pore sized membrane filter, Sartorius, France)

The effect of sucrose on *S. mutans* biofilm formation was screened with the modified crystal violet binding methodology of Stepanović *et al*.[37] Inoculation preparation was performed as described above before biofilm sampling. After the inoculum preparation, 10 µL of the culture was transferred into each 96-well polystyrene microtiter plate wells (LP Italiana, Italy) containing 190 µL of TSB medium adjusted with different sucrose concentrations. The plates were then incubated at 37°C for 24 h. At the end of the incubation, the planktonic counterparts were removed by rinsing the wells three times with physiological serum (0.85% NaCl) under aseptic conditions. The plates were fixed with 200 µL of 95% methanol for 15 min. Then, the plates were rinsed again, and 200 µL of 0.1% crystal violet solution was added to the wells. The plates were re-washed with distilled water to remove the unbound dye and dried after 30 min incubation. Finally, 200 µL of the ethanol: acetone (70:30 v/v) solution was added to each well for dissolving bound dye within the biofilm matrix. The optical density of the dissolved crystal violet dye was measured by Elisa reader at 595 nm (BioTek, USA). The amount of biofilm production was calculated by subtracting the averages of the negative control (containing the only medium) wells from the mean of the absorbance values obtained from the test wells (medium and inoculum). Biofilm production capacities of *S. mutans* strain under the effect of different sucrose concentration were categorized as non-producer ($OD \leq OD_{cut\ off}$), weak ($OD_{cut\ off} < OD \leq 2 \times OD_{cut\ off}$), moderate ($2 \times OD_{cut\ off} < OD \leq 4 \times OD_{cut\ off}$), and strong ($4 \times OD_{cut\ off} < OD$) based on the cut-off values obtained from negative control well absorbance values (Stepanović *et al*.[37]; Vestby *et al*.[38]). The 5% sucrose concentration was found to be best for *S. mutans* biofilm formation ($p< 0,05$; One-WAY ANOVA; Tukey's Test, SPSS version 22,0; USA). After the findings obtained from this experiment, future studies were continued by taking into consideration the ideal sucrose concentration.

Antibacterial and Antibiofilm Effects of Restorative Materials

The 6-well polystyrene cell culture plates (Thermo Fisher Scientific, USA) were used for biofilm formation and bacterial adhesion on restorative materials. Two pieces of different restorative materials were transferred to each well. Each well containing restorative materials was then inoculated with 5 mL of TSB medium containing the ideal sucrose (5%) concentration and active *S. mutans* ATCC 25175 culture (5% inoculation rate). Only wells containing medium and each restorative material are designed as negative controls. After these processes, the plates were tightly packed to prevent evaporation and incubated at 37°C for 24 h under static conditions. Before the incubation, the optical density of the culture suspension at time *t0* (OD600 nm) was measured spectrophotometrically (BioTek, USA) and after

serial dilution of the suspension (10-fold), the number of colony-forming units was determined by drop plate method. Petri dishes containing TSA (Tryptic Soy Agar; Merck Germany) were incubated at 37°C for 24 h, and at the end of incubation, Petri dishes containing the ideal number of colonies were included in the count.

After the plates were incubated, the restorative materials were removed from the wells under aseptic conditions and washed twice with sterile saline to remove loosely attached bacterial cells and planktonic counterparts. At this stage, the optical densities of the culture suspensions in wells containing different restorative materials were measured at a wavelength of 600 nm (t_{24}). Thus, it was aimed to determine the bacterial growth difference between t_0 and t_{24} time points. The percentage of optical density changes for bacterial growth was calculated with the given formula: [(C-B)-(T-B)/(C-B)x100)] (C; control group, containing only inoculum and medium-B; blank, only medium-T; test groups, containing different restorative materials, medium, and inoculum).

After the restorative materials were washed, one of each material was transferred to new 6-well plates, and the previously described crystal violet binding method was modified to determine the biofilm accumulation on the materials (1 mL of 95% methanol; 1 mL of 0.1% crystal violet solution; 1 mL of ethanol: acetone solution for each well). All stages were also applied to negative control groups (only groups containing restorative material and sterile media). 200 µL of dissolved crystal violet suspensions were taken and transferred to 96 well microtiter plate wells, and optical density (OD) values at 595 nm were determined. Absorbance values obtained from negative control groups were subtracted from the absorbance value obtained from each test group. Thus, only the amount of dye bound by the biofilm (plaque) accumulated on the material was calculated. The study was carried out in duplicate.

After the crystal violet binding assay, cell counts were made in the biofilms deposited on the remaining restorative materials. At this stage, the method proposed by Giaorouis *et al.* was modified[39]. The restorative materials were placed in a sterile plastic tube containing 5 mL of physiological saline and 3 g of glass beads (R: 3 mm) to remove the biofilm cells. The tubes were then vortexed for 2 min at maximum intensity. For total bacterial counts, ten-fold dilutions in physiological saline were prepared, and each dilution was dropped in 10 µL onto TSA (Tryptic Soy Broth; Merck, Germany) agar plates. The plates were incubated at 37°C for 24 h before colony counting. The results were calculated as colony-forming units per unit area (CFU/cm^2) and then converted to the logarithmic base ($log_{10}CFU/cm^2$). All the experiments were done in duplicate.

Statistical Analysis

As a result of power analysis, the number of samples to be prepared from each group was determined as 8. In this study, the minimum number of samples required was determined by using the effect size of Cohen criteria and taking alpha= 0.05 and power= 0.80. All statistical analyzes were carried out by SPSS (Version 22.0, USA) software. Whether data sets obtained from experimental studies show normal distribution and homogeneity were investigated with the Shapiro-Wilk test. One-Way ANOVA and Tukey's tests were used to compare the averages between groups. Paired T-Test was used to compare the averages between the two groups ($p< 0.05$). While conducting this study, it was acted following the principles of the Helsinki declaration.

Results

Antibacterial activity was detected in all of the restorative materials tested. At the end of the 24 h-incubation period (t_{24}), there was a significant decrease in the optical densities of bacterial growth in culture media containing restorative material compared to the control group. The optical density decrease was highest for Riva Light Cure and Beautifil II (One-Way ANOVA; Tukey's Test $p< 0.05$) (Table 3).

Table 3. Percentage (%) reduction of bacterial growth in culture media containing different restorative materials

Restorative material	Optical density decrease of bacterial growth (%)
Grandio Resin Composite	62.50
Beautifil II	75.10*
Dyract® XP	63.50
Riva Light Cure	75.90*
Riva Self Cure	70.70
Riva Silver	61.80

The amount of biofilm matrix (dental plaque) accumulated on the restorative materials is given in Figure 1. When the amount of plaque accumulated on the restorative materials is examined, it was observed that the plaque accumulation on the Grandio composite, which is considered as the control group, is the least compared to the five different restorative materials tested. The highest plaque accumulation was determined on Riva Light Cure material (One-Way-ANOVA; Tukey's Test, $p< 0.05$). The samples with the least accumulation following the Grandio composite are Riva Self Cure and Riva Silver, and there was no significant difference between these samples (Paired T-Test, $p< 0.05$).

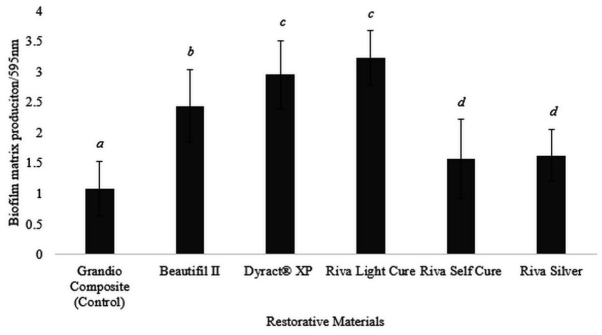

Figure 1. The amount of biofilm matrix (plaque) accumulated on restorative materials (crystal violet binding assay). Letters indicate statistical differences between groups. Bars were given as standard deviation

Counts of attached *S. mutans* biofilm cells on different restorative materials are given in Figure 2. *S. mutans* biofilm cells appear to be less attached to the surfaces of Beautifil II and Riva Silver materials but mostly attached to the surfaces of Riva Light Cure and Riva Self Cure materials.

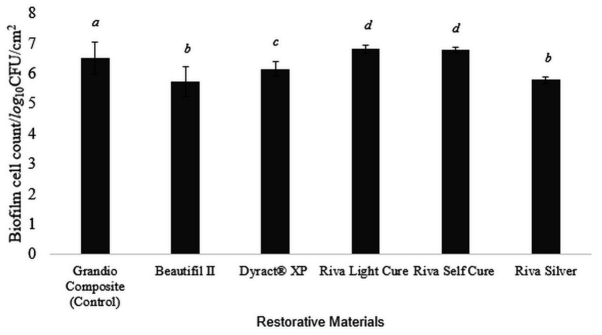

Figure 2. Biofilm cell count results of S. mutans on restorative materials ($log_{10}CFU/cm^2$) Letters indicate statistical differences between groups. Bars were given as standard deviation

Discussions

It has been proven in this study that restorative materials with fluoride release significantly inhibit *S. mutans* adhesion. Thus, the H_0 hypothesis put forward at the beginning of the study: "Fluoride-releasing restorative materials have no effect on *S. mutans* adhesion", has been rejected.

S. mutans has high adhesion capacity on all surfaces in the mouth, dental tissue, restorative materials[40,41]. In addition, *S. mutans* are among the virulent bacteria due to their acidic and aciduric properties[40]. Since *S. mutans* is the most cariogenic, and acidoid bacteria in the oral flora, and it is also found in the dental plaque, *S. mutans* was used as a microorganism in the current study[42].

According to Onom *et al.* study, the dental plaque shows better adhesion and accumulates faster on rough surfaces *in vivo* conditions. These findings reveal that polishing the composite surface is a crucial factor in terms of preventing or slowing biofilm adhesion and growth[43]. Filler and monomer compounds of composites also affect biofilm accumulation. In our study, the composite resin (Grandio) filling the material we use as a control group was found to have the least amount of biofilm accumulation in the biofilm, since the material does not contain fluoride, the number of *S. mutans* in biofilm was found to be high[43].

Biofilms on GICs; It negatively affects the properties of the material such as hardness and roughness, the deterioration of this material surface promotes the formation of more biofilms[44,45]. Also, according to the results of other studies investigating bacterial adhesion and biofilm formation in GICs, glass ionomer-containing materials may affect bacterial adhesion, acidity, and biofilm formation[46,47]. In the current study, biofilm accumulation was mostly observed in Riva Light Cure, which is similar to the traditional glass ionomer cement. The findings of the study are compatible with the current literature[46,47]. Suzana *et al*., in their study, the GICs (Ketac Nano, Vitremer, Ketac Molar Easymix, and Fuji IX) evaluated showed antibacterial activity that prevented the growth of the cariogenic bacteria, possibly selected in connection with the solubility of organic and inorganic components[48]. Factors affecting solubility are filler concentration and average particle size, binders, filler particles solvent type, nature, and degree of monomer conversion[49]. Restorative materials with GIC content used in the current study showed antibacterial activity.

The effects of glass ionomer based restorative materials on cariogenic bacteria are probably due to fluoride release, but this information has not been entirely proven[50]. Vermeersch *et al*. suggested that the low pH of GIC can contribute more to antibacterial properties than fluoride release properties[51]. Yap *et al*.[52] reported that there was no antibacterial activity despite the presence of fluoride in the agar around the test materials. However, different results can be obtained as the antibacterial effect depends on factors such as the size of the diffusion of the material, the shape of the filler forms, and the fluorine concentration in the material. Also, the release of ions (F, GIC from Ca ++, Al +++, OH-) depends on the pH of the medium. In the the present study, the highest antibacterial effect was observed in Riva Light Cure (RMGIC), keeping the pH of the environment and the size of the materials constant. Moreau and Xu and Czarnecka *et al*. have shown in their studies that lower pH conditions increase the release of fluoride from both GICs and RMGICs and can buffer acid. It is thought that if restorative materials that release higher fluoride ions are developed, they can have a more potent antibacterial effect and better prevent secondary caries[53,54]. Eick

et al. reported that RMGIC, HVGIC, compomer, and composite materials all inhibit *S. mutans* growth and the RMGIC they use exhibits the most remarkable antibacterial effect[55]. Among the fluoride-containing restorative materials selected in the present study, the most antibacterial restorative material is Riva Light Cure (RMGIC). Ngoc et al. although GIC reports that the fluoride release shows anti-biofilm activity against *S mutans*, it is not clear that these materials can maintain their fluoride release feature for a long time. Because fluoride release from these materials has been reported to be faster in the biphasic period and gradually decreasing in the later stages[56,57]. Therefore, more research is needed to investigate the relationship between the cariogenic composition of in vivo dental biofilms and fluoride-releasing materials to maintain their anti-cariogenic biofilm activity. In their study, Ngoc *et al.* revealed that the effectiveness of fluoride against acidity, bacterial biovolume, and extracellular polysaccharide formation in *S. mutans* biofilms is related to the rate of fluoride release[56]. They argued that GICs might play an essential role in reducing cariogenic biofilms and subsequent secondary caries formation if certain amounts of fluoride are released from restorative materials during biofilm formation. Although the most antibacterial material in this study was Riva Light Cure (RMGIC), the most biofilm was observed in Riva light cure (RMGIC). Although the high antibacterial efficacy observed due to fluoride release significantly reduces *S. mutans* viability within the biofilm structure, this is not a sufficient effect as biofilm matrix (plaque) accumulation should be reduced simultaneously. In summary, the development of both antibacterial properties and materials that will reduce plaque accumulation becomes important at this point. The data of the current study coincide with Auschill *et al*.[58] that "antibacterial activity does not always show the ability to prevent bacterial adhesion".

The glass ionomer has a more rough and more inorganic, positively charged surface that allows for more protein binding and bacterial adhesion than composite resin[59]. The reduced hardness of ionomeric materials after interacting with biofilm will accelerate the biodegradation process in the oral cavity. The presence of low pH and saliva hydrolases can also contribute to the dissolution of materials that lead to high fluoride levels and reduce bacterial growth[60]. According to our study results, biofilm accumulation in the glass ionomer content hybrid restorative materials is higher than the control group composite. GICs are considered to have more antibacterial properties because they release fluoride, but studies with fluoride have shown that although fluoride prevents softening of the enamel surface and increases plaque pH, it does not reduce the adhesion of *S. mutans*[61,62]. Fluoride is known to inhibit biosynthetic metabolism of bacteria, but these antibacterial effects in the prevention of caries are generally considered too little or insignificant when compared to the direct interaction of fluoride with hard tissue during caries development and progression. Studies on whether antibacterial effects clinically contribute to the anti-caries effect of fluoride and whether fluoride from restorative materials to plaque or saliva is related to these antibacterial effects are still uncertain[60].

More *in vivo* and *in vitro* studies are needed to demonstrate the effects of different factors on bacterial adhesion to fully reveal the causes of bacterial adhesion to fluoride-releasing restorative materials.

Conclusions

Although the highest antibacterial activity against *S. mutans* was detected in resin-modified glass ionomers, biofilm matrix (dental plaque) accumulation was mostly detected on these material surface in our study. It is recommended to use restorative materials containing glass ionomer in individuals with active caries. While the least amount of biofilm matrix was observed on the Grandio composite, it has been one of the surfaces on which *S. mutans* biofilm cells are easily attached. Since the amount of biofilm matrix on the composite surface is minimal, and it is the most preferred permanent restorative material today.

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