Original Article

pISSN, eISSN 0125-5614
M Dent J 2021; 41 (2) : 179-186

Benefits of pit and fissure sealants on fluoride release, buffering capacity, and biofilm formation

Arthit Klaophimai[1], Anisha Komalsingsakul[2], Ratchapin Laovanitch Srisatjaluk[1], Pisol Senawongse[2]

[1] *Department of Oral Microbiology, Faculty of Dentistry, Mahidol University, Bangkok, Thailand*
[2] *Department of Operative Dentistry and Endodontics, Faculty of Dentistry, Mahidol University, Bangkok, Thailand*

Objective: Antibacterial property of pit and fissure sealant enhance the positive effect for caries prevention. The aims of this study were to investigate the fluoride release, buffering capacity, and biofilm formation of sealants.
Materials and Methods: Specimens from BeautiSealant (giomer-based sealant), Clinpro™ (fluoride releasing resin-based sealant), Fuji VII (glass-ionomer-based sealant), and Concise (nonfluoride releasing resin-based sealant) were prepared. Studies of the specimen were conducted to examine fluoride released, buffering capacity and formation of *S. mutans* biofilm, which involved examining the biovolume and live/dead cell ratios.
Results: Fuji VII released the highest amount of fluoride (11.71 ppm), followed by BeautiSealant (6.20 ppm), Clinpro (1.27 ppm), and Concise (0.02 ppm). The smallest change in pH was found in BeautiSealant (-1.35), followed by Fuji (-1.85), Clinpro (-2.11), and Concise (-2.67). There was a higher biovolume of biofilms on Concise (0.0177 mm^3mm^{-2}) than the other materials. Thus, the live/dead cell ratio on the BeautiSealant biofilms (0.5554) was less than on the other materials.
Conclusions: BeautiSealant demonstrated benefit in reducing the biovolume of biofilm and the live/dead cell ratio with a high buffering capacity.

Keywords: biofilm formation, fluoride release, pH, *S. mutans,* sealant

How to cite: Klaophimai A, Komalsingsakul A, Srisatjaluk R L, Senawongse P. Benefits of pit and fissure sealants on fluoride release, buffering capacity, and biofilm formation. M Dent J 2021; 41: 179-186.

Introduction

Dental caries develops when bacterial plaque cannot be removed from the tooth surface. Approximately 90% of carious lesions are found in the pits and fissures of permanent molars [1]. Sealants have been used for decades as a preventive measure against caries developing in susceptible pits and fissures as they form a physical barrier between the oral environment and deep fissures [2-4]. Once the pit and fissure are covered with a sealant, the bacteria are isolated, and the number of cariogenic bacteria, including *Streptococcus mutans*, decreases to 50% [1].

This positive effect of caries prevention can be enhanced by adding antibacterial agents to the sealant material [5]. In recent years, chlorhexidine, bioactive glass, silver and zinc oxide nanoparticles, fluoride compounds, and surface reaction type pre-reacted glass-ionomer (S-PRG) filler have been added to sealants as antibacterial agents [6-10]. These materials may be classified as materials with bioactivity.

Thus, the prevention of pit and fissure caries by using the sealant should be considered not only physical barrier, but also bioactivity of antibacterial effect.

The purpose of this study was to evaluate the antibacterial properties of commercial bioactive

Correspondence author: Pisol Senawongse
Department of Operative Dentistry and Endodontics Faculty of Dentistry, Mahidol University
6 Yothi Road, Ratchathewi, Bangkok 10400, Thailand
Tel. +66 2200 7997 E mail: pisol.sen@mahidol.ac.th
Received: 14 June 2021 Revised: 17 July 2021 Accepted: 18 July 2021

sealants containing different additive agents to determine their capacity of buffering and inhibiting bacterial growth in biofilm formation. The null hypothesis of this study was: there was no effect of different bioactive sealants on fluoride released, pH change, biovolume of *S. mutans* biofilms, and live/dead cell ratios of *S. mutans* biofilms.

Materials and methods

This study was performed using protocols approved by the Faculty of Dentistry/Faculty of Pharmacy, Mahidol University Institutional Review Board (COE 2019/041.2607).

A BeautiSealant containing S-PRG filler (giomer-based sealant), a fluoride releasing Clinpro™ sealant (fluoride-releasing resin-based sealant), a glass-ionomer sealant Fuji VII (glass-ionomer-based sealant), and a nonfluoride releasing Concise sealant (resin-based sealant) were used in this study. The compositions and manufacturers of these materials are shown in Table 1.

Twenty cylindrical specimens from each material were prepared. The specimens were 6 mm in diameter and 2 mm thick and were prepared with a plastic mold. Each sealant was loaded into the plastic mold, the mold was placed on a mylar strip above a glass slide, and an LED light curing unit (Bluephase New, Ivoclar Vivadent AG, Schaan, Liechtenstein) was used to polymerize it from the top for 20 sec with the curing tip close to its surfaces. The curing unit was set to a high-power program with an intensity of approximately 1,200 mW/cm^2. Glass-ionomer sealant was allowed to self-cure for 2 minutes and 20 seconds. All specimens were sterilized in a UV chamber for 1.5 hours per side.

The cariogenic bacteria *S. mutans* (ATCC 25175, Thai Can Biotech, Bangkok, Thailand) were cultured in brain-heart infusion agar (BHI) in a 5% CO_2 chamber at 37 °C for 48 hours. Bacteria were then cultivated in brain-heart infusion broth supplemented with 5% sucrose to achieve the desired turbidity at a cell density of 1x10^8 CFU/mL or 0.5 McFarland in a 5% CO_2 chamber at 37 °C.

Table 1 List of the materials selected for this investigation and their manufacturers.

Groups	Materials	Composition	Manufacturer
1	Concise™sealant	TEGDMA, Bis-GMA (Nonfluoride releasing resin-based sealant)	3 M ESPE, USA
2	Clinpro™sealant	TEGDMA, Bis-GMA, Tetrabutyl-ammoniumtetrafluoroborate, Silane-treated silica (Fluoride releasing resin-based sealant)	3 M ESPE, USA
3	BeautiSealant	TEGDMA, UDMA, Fluoroboroaluminosilicate glass, Micro fumed silica (S-PRG filler containing giomer-based sealant)	Shofu Inc., Japan
4	Fuji VII	Fluoroaluminosilicate glass, CPP-ACP, pigment, distilled water, polyacrylic acid, polybase carboxylic acid (Glass-ionomer-based sealant)	GC, JAPAN

TEGDMA, triethylene glycol dimethacrylate; UDMA, urethane dimethacrylate; Bis-GMA, bisphenol A glycidylmethacrylate; S-PRG, Surface pre-reacted glass-ionomer; CPP-ACP, Casein Phosphopeptide-Amorphous Calcium Phosphate

Amount of fluoride releasing

Five specimens from each material underwent fluoride release determination tests. The specimens were placed in 24-well culture plates, immersed in 1 mL of deionized water and incubated in a 5% CO_2 chamber at 37 °C for 24 hours. The specimen was removed. The sample solution was transferred into a clean plastic tube and mixed with 100 mL TISAB III (total ionic strength adjustment buffer, 940911, Thermo Scientific Orion, Beverly, MA, USA). The solution was used to measure the amount of fluoride released three times from each specimen of each material using a fluoride-specific ion electrode (Orion EA940 expandable, Orion Research, Beverly, MA, USA) connected to an ion analyzer (Orion ion analyzer EA940, Beverly, MA, USA). The fluoride electrodes can detect fluoride concentrations down to 0.02 ppm. The mean fluoride concentration in part per million (ppm) of each specimen was calculated and used as representative data of each specimen.

Evaluation of buffering capacity

Five specimens from each group were randomly selected for biofilm formation assays. The specimen was placed into a customized polymethyl methacrylate model and incubated with 1 mL filtrated saliva for 16 hours at 37 °C to form an acquired pellicle.

InLab® pH electrodes (H+ ion-sensitive area, length 6 mm, diameter 3 mm) (Mettler-ToLedo, Greifensee, Switzerland) and pH meters (ORION Star A111 mV/pH METER, BAS, Tokyo, Japan) were used to determine the pH on the material surfaces. Before use, the pH electrode was calibrated with buffer solution at pH 4, pH 7, and pH 10.

After 16 hours of pellicle formation, filtrated saliva was drawn from the model. Ten saliva-coated specimens from each subgroup were immersed and incubated with 1 mL of bacterial suspension in a 5% CO_2 chamber at 37 °C for 24 hours. Then, the planktonic bacteria were removed, and the tip of the pH electrode was inserted into the customized polymethyl methacrylate model and placed onto the surfaces of the biofilm-coated specimen. A 140 µL sample of 5% sucrose was added into the model. The pH measurement was started after adding 140 µL of 5% sucrose into the model, and the measurements were taken after every hour with incubation under 5% CO_2 at 37 °C for 24 hours. The maximum pH changes calculated from the 0 to 24 hours after the start of incubation were used for statistical analysis.

Biofilm formation of S. mutans

The remaining ten specimens from each material were used for biofilm formation assays. The specimen was placed in a 24-well culture plate and incubated with 1 mL filtrated saliva for 16 hours at 37 °C to form an acquired pellicle. Then, filtrated saliva was drawn from the culture plate. Ten saliva-coated specimens from each subgroup were immersed and incubated with 1 mL of bacterial suspension in a 5% CO_2 chamber at 37 °C for 24 hours. After incubation, the specimens were washed with 1 mL distilled water 3 times and transferred to a clean 24-well culture plate.

Five specimens from each subgroup were used to rule out whether a biofilm formed using a crystal violet (CV) assay, which will be described later. When biofilm formation was found in the CV assay, further study on biofilm formation on the other five specimens from the same group was carried out using a confocal laser scanning microscope (CLSM, Fluoview 10i; Olympus Corp, Shinjuku-Ku, Tokyo, Japan) to determine the biovolume and the live and dead cell ratio.

The specimen was placed in a petri dish and stained using the Live/Dead Bac Light™ Bacterial Viability kit (Molecular Probes, Eugene, USA). The staining kit is composed of two fluorescent nucleic acid-binding dyes: SYTO 9,

which can penetrate all bacterial membranes, stain the cells and produce green fluorescing cells, and isopropidium iodide, which penetrates only cells with damaged membranes. Cells with damaged membranes were subjected to a combination of the two stains, producing red fluorescing cells. Three microliters of each dye were mixed into a tube containing 1 mL of filtrated distilled water. Mixed dyes were placed onto a specimen in which a biofilm was formed, and the cells were stained for 20 minutes under light protection.

Three neighboring points at the center of each specimen were analyzed with CLSM for each specimen. An excitation wavelength of 488 nm was used, and the emitted light between 500 and 560 nm was collected by different filters. The specimens were observed using optical lenses with magnifications of 60x. The observational sections were then reconstructed to a 3-dimensional model using FV 10-ASW (V 1.7a, Olympus, Tokyo, Japan) software. The biovolume or the bacterial densities over a studied area (45000 μm^2) were calculated using the color segmentation method. In addition, the live and dead numbers of *S. mutans* cells in biofilms were calculated from the total number of green and red pixels from FV10-ASW software.

Statistical analysis

Statistics were calculated using PASW statistics 18 (SPSS Inc., Chicago, IL, USA). The means and the standard deviations of all groups were calculated. Testing for a normal distribution of data with Komolgorov-Smirnov test and for homogeneity of variance of samples with Levene's test were performed. One-way analysis of variance was used to examine the effect of material types on the amount of fluoride released, pH change, biovolume and bacterial live/dead ratio. Post hoc tests were performed using Tukey's multiple comparison test for fluoride release, pH change, biovolume and Dunnett T3 test for live/dead bacteria with a significance level of *p*-value 0.05.

Results

According to the analysis of fluoride release, one-way ANOVA revealed the effect of materials on fluoride release at $p<0.01$. Means and standard deviations of fluoride release (ppm) are demonstrated in Table 2. Fuji VII, a glass-ionomer sealant, showed the highest amount of fluoride release (11.71 ± 1.29 ppm), followed by BeautiSealant (6.20 ± 0.25 ppm), Clinpro (1.27 ± 0.16 ppm), and Concise (0.02 ± 0.02 ppm). There were statistically significant differences among the tested materials ($p<0.05$).

Table 2 Means ± standard deviations of fluoride released in part per million (ppm), pH change, biovolume of *S. mutans* biofilms (mm^3mm^{-2}) and live/dead cell ratios of *S. mutans* biofilms

		Materials			
		Concise	Clinpro	BeautiSealant	Fuji VII
Fluoride releasing		0.02 ± 0.02 ^{d}	1.27 ± 0.16 ^{c}	6.20 ± 0.25 ^{b}	11.71 ± 1.29 ^{a}
pH change		-2.67 ± 0.20 ^{d}	-2.11 ± 0.09 ^{c}	-1.35 ± 0.07 ^{a}	-1.85 ± 0.10 ^{b}
Biofilms	Biovolume	0.0177 ± 0.0006 ^{a}	0.0158 ± 0.0009 ^{b}	0.0159 ± 0.0008 ^{b}	0.0152 ± 0.0010 ^{b}
	Live/dead cells	0.6757 ± 0.0069 ^{a}	0.6445 ± 0.0287 ^{a}	0.5554 ± 0.0230 ^{b}	0.6980 ± 0.0488 ^{a}

The data with the same superscript letter of the same row demonstrate no statistically significant difference.

The biofilm pH measurements that were taken every 1 hour for 24 hours are shown in Figure 1. A gradual decrease in pH was found for all materials. A relatively low rate of change and the least overall pH change were observed in the BeautifilSealant group. The highest pH change was found for the Concise sealant.

According to the analysis of pH change, one-way ANOVA revealed the effect of materials on fluoride release at $p<0.01$. The means and standard deviations of pH changes are demonstrated in Table 2. The least change in pH was found for the BeautifilSealant (-1.35 ± 0.07), followed by the Fuji VII (-1.85 ± 0.10), Clinpro (-2.11 ± 0.09), and Concise (-2.67 ± 0.20).

According to the biovolume analysis of the *S. mutans* biofilms, one-way ANOVA revealed the effect of the materials on the biovolume of *S. mutans* at $p<0.01$. The biovolume means and standard deviations of *S. mutans* (mm^3mm^{-2}) are demonstrated in Table 2. The biovolume of *S. mutans* biofilm on Concise (0.0177 ± 0.0006) was higher than that of the other materials ($p<0.05$). There were no significant differences in the biovolume of *S. mutans* biofilms among Clinpro (0.0158 ± 0.0009), BeautiSealant (0.0159 ± 0.0008) and Fuji VII (0.0152 ± 0.0010), which were less than that of Concise.

According to the analysis of live/dead cells of *S. mutans* biofilms, one-way ANOVA revealed the effect of materials on the biovolume of *S. mutans* at $p<0.01$. Means and standard deviations of live/dead cell ratios of *S. mutans* are demonstrated in Table 2. The live/dead cell ratio of *S. mutans* biofilms on BeautiSealant (0.5554 ± 0.0230) was significantly less than that on other materials. There were no significant differences in the live/dead cell ratios of *S. mutans* among Concise (0.6757 ± 0.0069), Clinpro (0.6445 ± 0.0287) and Fuji VII (0.6980 ± 0.0488), which were higher than those of BeautiSealant.

The representative images of biovolume and live/dead cells staining are shown in Figure 2. The intensity of green (live cells) and red (dead cells) were somehow in consistent with the biovolume, which were lower in the group of BeautiSealant.

The null hypothesis was rejected at a significance level of 0.05.

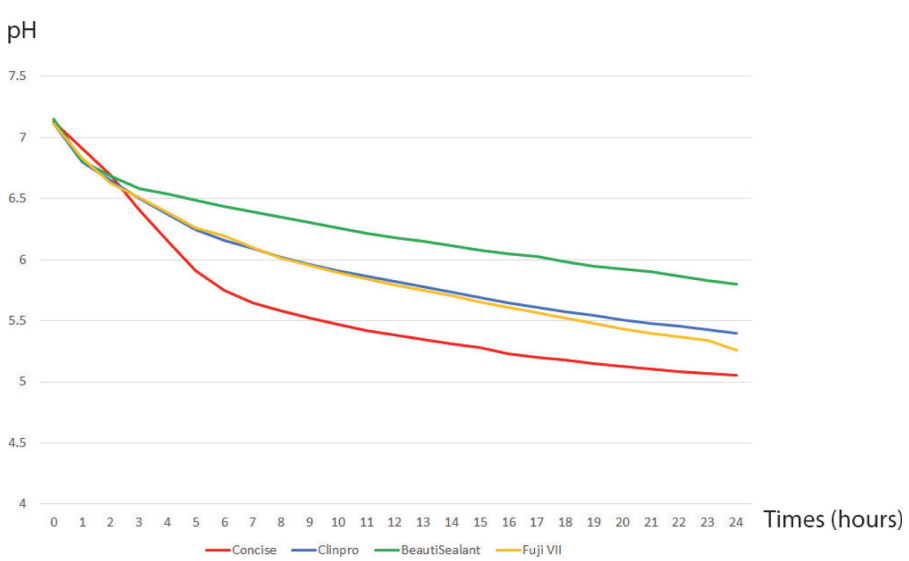

Figure 1 Continuous pH measurement every 1 hour for 24 hours

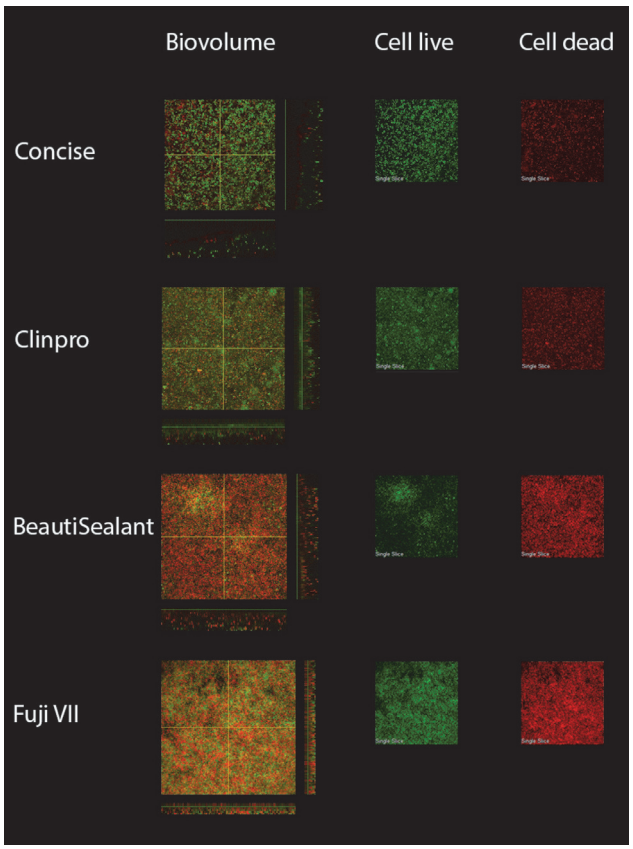

Figure 2 The representative images of biovolume and live/dead cells under a confocal laser scanning microscope. Live and dead cells are shown in green and red, respectively.

Discussions

Traditionally, pit and fissure sealants have been used to seat pits and fissures, leading to the prevention of cariogenic bacterial invasion and plaque accumulation [11]. For pit and fissure sealants with fluoride-releasing properties, the capability for caries prevention, reduction of enamel demineralization and promotion of remineralization has been proposed [12]. Therefore, the limitation of fluoride as a protective agent for caries control has been reported [13].

The fluoride released was 11.71 ± 1.29 ppm from Fuji VII, a glass-ionomer sealant; 6.20 ± 0.25 ppm from BeautiSealant, a giomer sealant; 1.27 ± 0.16 ppm from Clinpro, a fluoride releasing resin-based sealant; and 0.02 ± 0.02 ppm from Concise, a nonfluoride releasing resin-based sealant. The amounts of fluoride released agreed with previous studies in which the highest amount of fluoride was from glass-ionomer sealants followed by giomer-based sealants and fluoride-releasing resin-based sealants [14-15]. In addition, various ions, such as aluminum (Al^{3+}), sodium (Na^{+}), strontium (Sr^{2+}) and borate (BO^{3-}), have been proven to be released from BeautiSealant [16-17]. In addition to the cariostatic effect of fluoride being released, which is based on the antibacterial effects [18-19] and the inhibition of bacterial acid production [20], there is a bactericidal effect from the borate ions released from PRG filler in BeautiSealant [17,21]. Both of these properties contribute to the pH increase of the plaque around materials. The lowest pH change was found with BeutifilSealant, followed by Fuji VII, Clinpro and Concise. The amount of fluoride and borate ions released might be an important explanation. The result of this study agreed with the results of Kaga and co-worker [22]. Thus, lower live/dead cell ratios were found in the giomer-based sealant group (BeautiSealant) than in other groups.

By using any sealants that had the ability to release not only fluoride (Fuji VII, Clinpro) but also borate ions (BeautiSealant), the amount of *S. mutans* biovolume could be reduced significantly in contrast to nonreleasing resin-based materials (Concise). Thus, lower live/dead cell ratios were found in the giomer-based sealant group than in other groups (BeautiSealant). The high effectiveness of BeautiSealand for antibacterial effects was expected, which was in agreement with a previous study [23]. The effect of various ions released from this sealant might play an important role in this phenomenon [14-21].

Generally, interference of the caries process, reduction of demineralization and enhancement of remineralization of enamel and dentin have been proposed with the presentation of fluoride [24]. When the pH in biofilm fluid dropped due to sugar consumption, mineral dissolution was reduced in the presence of fluoride because the fluoride returned part of the demineralized hydroxyapatite to the fluoroapatite [25]. The metal ions released from these materials provide a combination of antibacterial effects [23], and materials that can release various ions, including fluoride ions, calcium ions, aluminum ions, and borate ions [14-17] may enhance the effective reduction of dental caries.

Pit and fissure sealants are used to prevent the accumulation of food debris and plaque and to minimize the exchange of metabolic products between fissure micro- organisms and the oral environment. Therefore, perfect penetration of sealant materials deep into pits and fissures is difficult to clinically obtain because of the complicated structures of pits and fissures on the occlusal surfaces of posterior teeth. The application of a sealant that can release substantial amounts of ions may be beneficial for protecting sealed teeth because of its effectiveness in neutralization and anti-demineralization.

Conclusions

Amount of fluoride-releasing from sealants was an important property that allow them to be effective in reducing *S. mutans* biofilm formation. Giomer-based sealants were more beneficial than other materials in reducing the biovolume of *S. mutans* biofilms and reducing the live/dead cell ratio with a high buffering capacity.

References

1. Griffin SO, Gray SK, Malvitz DM, Gooch BF. Caries risk in formerly sealed teeth. ***J Am Dent Assoc*** 2009; 140: 415-23.
2. Ahovuo-Saloranta A, Forss H, Walsh T, Nordblad A, Mäkelä M, Worthington HV. Sealants for preventing dental decay in the permanent teeth. ***Cochrane Database Syst Rev*** 2013; 3: Cd001830.
3. Mejàre I, Lingström P, Petersson LG, Holm AK, Twetman S, Källestål C, *et al*. Caries-preventive effect of fissure sealants: a systematic review. ***Acta Odontol Scand*** 2003; 61: 321-30.
4. Matalon S, Peretz B, Sidon R, Weiss EI, Slutzky H. Antibacterial properties of pit and fissure sealants combined with daily fluoride mouth rinse. ***Pediatr Dent*** 2010; 32: 9-13.
5. Loyola-Rodriguez JP, Garcia-Godoy F. Antibacterial activity of fluoride release sealants on mutans streptococci. ***J Clin Pediatr Dent*** 1996; 20: 109-11.
6. Petrovski KR, Caicedo-Caldas A, Williamson NB, Lopez-Villalobos N, Grinberg A, Parkinson TJ, *et al*. Efficacy of a novel internal dry period teat sealant containing 0.5% chlorhexidine against experimental challenge with Streptococcus uberis in dairy cattle. ***J Dairy Sci*** 2011; 94: 3366-75.
7. Yang SY, Piao YZ, Kim SM, Lee YK, Kim KN, Kim KM. Acid neutralizing, mechanical and physical properties of pit and fissure sealants containing melt-derived 45S5 bioactive glass. ***Dent Mater*** 2013; 29: 1228-35.
8. Al-Naimi RJ, Al-Alousi WS, Al-Nema M. Effect of silver and zinc oxide nanoparticle addition on microhardness and depth of cure of resin-based pit and fissure sealants. ***IJERSTE*** 2015; 4: 157-63.
9. Naorungroj S, Wei HH, Arnold RR, Swift EJ Jr, Walter R. Antibacterial surface properties of fluoride-containing resin-based sealants. ***J Dent*** 2010; 38: 387-91.
10. Shin SW, Kim JS. Microleakage and anticariogenic effect of S-PRG filler-containing pit and fissure sealant. ***J Korean Acad Pediatr Dent*** 2013; 40: 247-52.
11. Mickenautsch S, Yengopal V. Caries-preventive effect of high-viscosity glass ionomer and resin-based fissure sealants on permanent teeth: a systematic review of clinical trials. ***PLoS One*** 2016; 11: e0146512.

12. Kühnisch J, Mansmann U, Heinrich-Weltzien R, Hickel R. Longevity of materials for pit and fissure sealing - results from a meta-analysis. ***Dent Mater*** 2012; 28: 298-303.
13. Featherstone JD, Doméjean S. The role of remineralizing and anticaries agents in caries management. ***Adv Dent Res*** 2012; 24: 28-31.
14. Patil SS, Kontham UR, Kontham RK, Patil SS, Kamble SP. Fluoride release and fluoride-recharging ability of three different sealants. ***J Indian Soc Pedod Prev Dent*** 2020; 38: 247-52.
15. Salmerón-Valdés EN, Scougall-Vilchis RJ, Alanis-Tavira J, Morales-Luckie RA. Comparative study of fluoride released and recharged from conventional pit and fissure sealants versus surface pre-reacted glass ionomer technology. ***J Conserv Dent*** 2016; 19: 41-5.
16. Chen L, Zeng SJ, Li YH, Du MQ. Evaluation of ion release from four dental sealants. ***Curr Med Sci*** 2018; 38: 524-29.
17. Fujimoto Y, Iwasa M, Murayama R, Miyazaki M, Nagafuji A, Nakatsuka T. Detection of ions released from S-PRG fillers and their modulation effect. ***Dent Mater J*** 2010; 29: 392-97.
18. Duque C, Negrini Tde C, Hebling J, Spolidorio DM. Inhibitory activity of glass-ionomer cements on cariogenic bacteria. ***Oper Dent*** 2005; 30: 636–40.
19. Tegginmani VS, Goel B, Uppin V, Horatti P, Kumar LS, Nainani A. Comparison of antibacterial activity of glass-ionomer cement and amalgam in class two restorations by Streptococcus mutans count analysis at fixed intervals: an in vivo study. ***J Contemp Dent Pract*** 2013; 14: 381-86.
20. Nakajo K, Imazato S, Takahashi Y, Kiba W, Ebisu S, Takahashi N. Fluoride released from glass-ionomer cement is responsible to inhibit the acid production of caries-related oral streptococci. ***Dent Mater*** 2009; 25:703-708.
21. Najma Hajira NSW, Meena N. GIOMER- The Intelligent Particle (New Generation Glass Ionomer Cement). ***Int J Dent Oral Health*** 2015; 2: doi http://dx.doi.org/10.16966/2378-7090.166
22. Kaga M, Kakuda S, Ida Y, Toshima H, Hashimoto M, Endo K, *et al.* Inhibition of enamel demineralization by buffering effect of S-PRG filler- containing dental sealant. ***Eur J Oral Sci*** 2014; 122: 78–83.
23. Kondo Y, Ito S, Uehara O, Kurashige Y, Fujita Y, Saito T, *et al.* Chemical and biological properties of new sealant-use cement materials. ***Dent Mater*** 2019; 35: 673-85.
24. Cury JA, Tenuta LM. Enamel remineralization: controlling the caries disease or treating early caries lesions? ***Braz Oral Res*** 2009; 23(Suppl 1): 23-30.
25. Fejerskov O, Cury JA, Tenuta LMA, Marinho V. Fluorides in caries control. In: Fejerskov O, Kidd E, editors. Dental caries: the disease and its clinical management. third ed. Oxford: Wiley Blackwell; 2015. p. 245–76.