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MICROLEAKAGE ASSESSMENT AND EFFICIENCY OF TWO ANTIMICROBIAL PIT AND FISSURE SEALANTS: AN IN-VITRO STUDY

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ABSTRACT

The purpose of this study was to evaluate microleakage and antimicrobial efficiency of two pit and fissure sealants, SeLECT Defense® and Ultra Seal XT Plus.

Methods: Intact human premolars were selected for the microleakage test and assigned randomly to two groups (n=20) according to the sealant used. After sealants application, the specimens were thermocycled then subjected to silver nitrate staining. Thin sections were obtained from each specimen, photo-developed then observed with polarizing light microscope. Microleakage was assessed according to a (0-4) scoring method where 0 indicated no leakage and 4 indicating most leakage to the base of the fissures. For evaluation of antimicrobial efficiency, tooth specimens were obtained from intact human molars and treated with Select Defense sealant (Group A), Ultra Seal XT Plus (Group B), while tooth specimens in Group C were left as control (n=20). Bacterial viability was evaluated after 24h, 48h, 72h and 96h, and was conducted by staining the adherent plaque with live/dead bacterial stain then observed with confocal laser scanning microscopy. Data obtained from microleakage and bacterial viability testes were analyzed using one-way ANOVA, Chi-square tests and Yuen-Welch tests (α=0.05).

Results: Microleakage scores were significantly lower with SeLECT Defense compared to Ultra Seal XT Plus ($p<0.01$). Analysis of bacterial viability data indicated both sealants tested showed significantly lower live/dead ratios means compared to the control group at 24h. The difference was not as significant after 96h of incubation.

Conclusion: The results of the current study indicate that both sealants tested show an antimicrobial efficiency that is more marked at 24h, nevertheless SeLECT Defense sealant could have a lower chance of failing due to microleakage compared to Ultra Seal XT Plus sealant.

KEYWORDS: Microleakage, Pits and fissures, Sealants, Thermocycling, Antimicrobial, selenium

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INTRODUCTION

One of the major goals of an oral health care program is the prevention of buildup of dental plaque, the biofilm on the tooth surface that may cause the formation of dental caries and gum disease[1]. Carious disease is caused by the demineralization of tooth tissue by organic acids formed on tooth surface through the metabolism of fermentable carbohydrate by cariogenic bacteria, particularly *Streptococcus mutans*[2]. The acids, mainly lactic acid, are formed within the bacterial plaque, and diffuse onto the tooth surface. If the carious process is not interrupted in its early stages, it may lead to cavitation of the affected tooth.

Many proactive steps could be taken to decrease the chances of forming dental caries, of which flossing, the use fluoride toothpaste and fluoride rinses are the most commonly advised. Nevertheless, in cases of deep pits and fissures, thorough brushing is not sufficiently effective, as the bristles of the toothbrush may not reach the depth of the fissures[3]. Thus, the application of dental sealants to the occlusal surfaces of molars and premolars may be a more effective and proactive step to protect the areas susceptible to stagnation of plaque and bacteria by converting them into shallow self-cleansing areas[4].

Over the last few decades, dental sealants have come to play an essential role in preventive dentistry[5,6]. They have proven to be effective in caries prevention where cleaning of the tooth may be difficult[7]. Introduced in 1967, their effectiveness was later recognized by the American Dental Association in 1971[8]. Dental sealants now come in many variants from resins to glass ionomers, with or without fillers and with or without a fluoride release potential. Yet, for the sealant to be effective, it should show high retention rates to the tooth surface, be able to resist shrinkage stresses and result in minimal marginal leakage, all of which are common causes of sealant failure[9].

The application of pit and fissure sealants is technique-sensitive and salivary contamination may have a major detrimental effect on the seal and retention of sealant. Trapped fermentable carbohydrates underneath a partially retained sealant may result in a fissure system more difficult to clean and facilitate the progression of caries[10]. In this regard, sealants that possess an antimicrobial property could be of great benefit in reduction of microleakage and caries[11]. Ultraseal XT Plus sealant (Ultradent Products, Inc) a fluoride-releasing filled-resin sealant has proven to inhibit the bacterial growth of *Streptococcus mutans* and *Lactobacillus acidophilus*. However, several studies have questioned the addition of soluble fluoride salts in sealants because of their possible dissolution which may weaken the sealant and reduce its usefulness as a preventive agent[12]. Furthermore, others[13,14] have even shown that the retention rate of fluoride releasing sealants is lower than that of non-fluoride releasing.

More recently an antimicrobial, non-fluoride releasing sealant is "SeLect Defense" (Element 34 Technologies) has been introduced. The sealant utilizes a "selenium" based organic compound that creates a barrier with an antimicrobial effect, thus the name "Se"lect Defense. Tran et al[15] reported that the organic selenium polymerized into the dental sealant SeLect Defense is effective in inhibiting bacterial attachment and biofilm formation by two main oral pathogens, *Streptococcus mutans* and *Streptococcus salivarius*, thus rendering the sealant more potent in preventing dental caries and plaque formation. While the antimicrobial effect SeLect Defense has been recognized[15], few studies have evaluated the possible associated microleakage when used in sealing pits and fissures.

The aim of the present study was to evaluate and compare microleakage at pits and fissures of human permanent premolars when sealed with either SeLECT Defense or Ultra Seal XT Plus sealant and to assess their antimicrobial efficiency against cariogenic plaque. The null hypotheses tested were that: 1/ SeLect Defense will not show lower microleakage scores compared to UltraSeal XT Plus sealant, 2/ SeLECT defense adhesive will not show a greater antimicrobial effect compared to UltraSeal XT Plus sealant and control.

MATERIALS AND METHODS

Teeth Selection and Preparation

Non-carious extracted human permanent premolars were used in this study. The teeth were selected from pooled and unidentified teeth, extracted for orthodontic purposes and collected for research purposes at the department of Oral Surgery of the Faculty of Dentistry, Cairo University. In accordance to the World Medical Association Declaration of Helsinki, it was impractical to obtain consent for the use of the unidentified teeth for research work and therefore, ethics committee approval was not required. Collected teeth were cleaned with hand scaler and pumice prophylaxis to remove calculus and soft tissue deposits, then examined using Fiber-optic transillumination. The inclusion criteria were the absence of carious lesions, fractures or restorations, complete root formation and presence of deep occlusal pits and fissures. Selected teeth were sterilized by immersion in 2% chloramine T solution for one week then stored in non-ionized water until use. All teeth were used within 3 months of extraction.

Microleakage Test

Forty teeth were used for microleakage testing and randomly assigned to two experimental groups, according the sealant being used "SeLECT Defense" (Element-34 Technologies Inc., Lubbock, TX, USA) and "Ultra Seal XT Plus" (Ultradent Products, USA), (n=20). Each group of teeth was set in a stone cast inside a Petri dish in order to facilitate the handling.

Sealant Placement

All laboratory procedures were performed by the same operator. Prophylaxis was performed on the occlusal surface of the teeth with pumice and water. Once cleaned, the teeth were thoroughly rinsed with water then dried with air syringe for 15 seconds. The occlusal surfaces of all teeth were etched using 35 % phosphoric acid gel (UltraEtch, Ultradent Products, USA) for 20 s, rinsed with water for 15 s and air dried to reveal a frosty and chalky appearance. The assigned sealant was then applied to the etched occlusal surfaces according to the manufacturers' recommendations. Care was taken to cover all pits and fissures and to avoid air bubbles inclusion. The occlusal surfaces were then light cured for 2x20 s using a light emitting diode (LED) curing unit (Demi Ultra LED Ultracapacitor Curing Light, SDS Kerr Corp., Middleton, WI, USA). The intensity of light delivered was premeasured and regularly checked to be 1000mW/cm^2 or greater. The tip of the light guide was placed in direct contact with the occlusal surface while curing to ensure distance standardization.

Thermocycling

Immediately after sealing, the teeth were placed in distilled water at 37 °C for 24 h and then subjected to a thermocycling regimen of 350 cycles between 5˚ and 55˚C for 60 s each with a dwell time of 15s, simulating a little over three and a half months of exposure in the oral cavity.

Microleakage Testing

Microleakage was assessed via the silver nitrate tracer penetration method. The teeth were coated with two layers of nail varnish interposed by a layer of wax leaving a 2 mm window around the sealant, and the roots were embedded in an acrylic resin cylinder. The teeth were then soaked for 8 hours in 50 % silver nitrate solution in the dark.

Each tooth was then gently removed from the stone casts and embedded in a cold-cure epoxy resin, Epo-Thin® epoxy resin (Buehler Ltd., IL, USA).

Following embedding in resin, the teeth were sectioned longitudinally through the central fissure in a buccolingual direction using a water-cooled diamond saw (Isomet, Buehler, Lake Bluff, IL, USA) to obtain a thin section (around150 μm thick) from each tooth. Each side of the cut slice was polished to attain 100 μm thickness. The thickness of each specimen was confirmed to the nearest 0.01mm using a digital caliper (Mastercraft electronic caliper, Canadian Tire Corporation, Ltd, ON, Canada) at different points of the specimen. The specimens were then immersed in photo development fluid for 8 hours, followed by 16 hours of exposure to fluorescent light. Each specimen was then examined with a polarizing-light microscope (40x) by an examiner who was unaware of the sealant used. The tracer penetration was distinguished by the blackening effect of silver nitrate on tooth tissue and each specimen was given a microleakage score according to the criteria described in table 1.

TABLE (1) Microleakage scoring criteria

Score	Definition
0	No Tracer Penetration
1	Tracer penetration to 1/4 of the fissure depth
2	Tracer penetration to 1/2 of the fissure depth
3	Tracer penetration to ¾ of the fissure depth
4	Tracer penetration reaching the fissure floor

Antimicrobial Test

Sixty tooth specimens (blocks 3 x 4 mm wide) were cut from the selected and prepared teeth by using the water cooled diamond saw. The specimens were sterilized once more using ethylene dioxide, then assigned to 3 experimental groups (n=20) according to the treatment applied to the enamel surface of the tooth specimen in each group. In Group A, the enamel surface of the tooth blocks was treated with SeLECT Defense Pit and Fissure Sealant, in Group B with Ultra seal XT Plus Pit and Fissure Sealant, while in Group C, no enamel surface treatment was performed and the group was left as control. In groups B and C, enamel surfaces of the tooth blocks were etched and treated with the assigned sealant, as recommended by the manufacturers and as described above for the microleakage test.

Experimental procedure for antimicrobial testing

After the surface treatment as per the assigned group, each tooth specimen was individually placed in a separate well of a 24 well microtiter plate. Two milliliters of Bacto brain heart infusion broth (BHI) with Glutathione (150μm/l) was then added to each well which was then inoculated with 20ml of mixed organism consortium of Streptococcus mutans (ATCC 55677) and Lactobacillus acidophilus (ATCC 11975). Every 12 hours of incubation at 37C, the specimens were washed with PBS to remove non-adhering cells and the old BHI medium was replaced by a new BHI medium. At 24 hours, 48 hours, 72 hours, and 96 hours, 5 tooth blocks from each group were collected for confocal laser scanning microscope (CLSM) analysis.

Confocal laser scanning microscope (CLSM) processing and analysis

Once the specimens were collected from the BHI medium, they were transferred to a new wells containing phosphate buffered solution (PBS). The viability of plaque biofilm that adhered to the enamel surface of each specimen was determined by enumeration of microorganisms after applying live/dead stain (L13152 BaclightTM 2 Bacterial Viability Kit, Invitrogen) following the manufactures' protocol, where 6 μl of Component A (Syto 9) and 6 μl of Component B (propidium iodide) were separately diluted in 5ml of distilled water each, then the solutions obtained combined to form the staining solution. Each specimen was subjected to the 500μl of staining solution for 15 minutes at room temperature in the dark prior to CLSM analysis. Following staining, the specimens were then gently removed from the staining solution using sterile forceps, and placed in a sterile petri dish containing approximately 5 mL of PBS for the microscopic evaluation. Then samples were immediately examined using Olympus FV1000 confocal system on an Ix81 microscope with a 40X dipping objective lens (Olympus Life Science, Center Valley, PA) and an excitation wavelength of 488 and 543. For each specimen, microscopic images from two representative locations were captured for the live/dead ratio analysis. The ratio of the layers of live vs. dead (green vs. red) stained bacteria for each microscopic image was determined by volumetric rendering of red and green pixels using image analysis software Optimas 6.2 (Meyer Instruments, Houston, USA).

Statistical Analysis

Statistical analysis was performed using SPSS Version 14.0 (IBM SPSS for Mac OS, IBM corp, Chicago, IL). Microleakage scores obtained with the use of the two sealants were compared by one-way ANOVA. Differences in the frequency of score distribution between groups were assessed using the Chi-square test. For the antibacterial test, statistical analysis was performed on the live/dead cell ratios of all specimens from each group. Each group contained five specimens of which two random locations were selected for confocal image analysis (n=10). All pairwise comparisons were performed among the treatment groups using the Yuen-Welch test and utilizing trimmed means (20% trimming). Bonferroni correction was used for performing multiple comparisons and reported the adjusted p-values. All testing was done at p<0.05 level of significance.

RESULTS

Table 2 shows the mean and standard deviation of microleakage scores for each experimental group while score distribution and percentages of each score are shown in table 3. One-way Anova test indicated that there was a significant difference (p<0.01) in the rating scores between the groups. The mean rating score for Ultra Seal XT Plus was 3.6±0.82 while that of SeLECT Defense was 2.55±1.7. Only 20% of specimens from the SeLect Defense group showed no signs of tracer penetration (score 0), while 0% of that score was recorded with the use of Ultra Seal XT Plus sealant. 60% of the specimens in the SeLect Defense group were recorded showing between 3 and 4 scores compared to 90% with the Ultra Seal XT Plus group. Sample images of the sectioned teeth with various levels of tracer penetration are shown in Fig. 1.

TABLE (2) Mean and standard deviation (SD) of microleakage scores obtained with the different pit and fissure sealants

	SeLect Defense	Ultra Seal XT Plus
Mean (SD)	**2.55 (1.7)^{a}**	**3.60 (0.82)^{b}**

Different superscript letters denote statistically significant differences (α=0.05)

The antibacterial test results are shown in figure 2. At 24 hours, both SeLECT Defense sealant, and Ultra Seal XT Plus showed statistically lower live/dead ratios compared to the control. At 48 hours, there was no significant difference between the control and all other comparisons. At 72 hours, Ultra Seal XT Plus showed a significantly lower live/dead ratio. At 96 hours, there was no significant difference between any groups due to wide variability in data. However, at a slightly higher p-value (p<0.10), SeLECT Defense Sealant and Ultra Seal XT Plus were significantly lower than the control.

TABLE (3) Frequency distribution of microleakage in the three groups

Microleakage Score	SeLect Defense		Ultra Seal XT Plus	
	Frequency	Percentage %	Frequency	Percentage %
0	4	20	0	0
1	3	15	1	5
2	1	5	1	5
3	2	10	3	15
4	10	50	15	75

Fig. (1) Sample images of the sectioned teeth with various levels of tracer penetration. a: score 0, no tracer penetration; b: score 1; c: score 2; d: score 3 and e: score 4 showing tracer penetration to full depth of the fissure. Arrows indicating depth of tracer penetration.

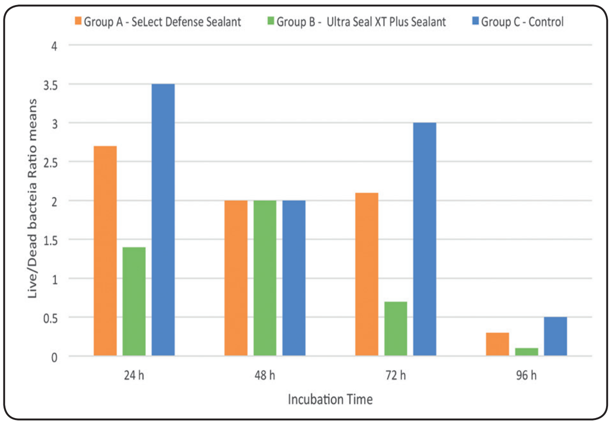

Fig. (2) Means of ratios of live/ dead bacteria adhered on the enamel surface of tooth specimens subjected to different sealant materials.

DISCUSSION

The complexity of the occlusal pits and fissures morphology renders them more susceptible to caries, accounting for approximately 90 % of all caries-affected tooth surfaces[16]. Resin sealants prevent the development and progression of carious lesions by forming a mechanical barrier between the tooth surface and the oral environment, thus interrupting metabolic exchange[17]. The success of pit and fissure sealants in protecting these surfaces is mainly dependent on the quality of adhesion between the sealant and occlusal enamel surface and hence its resistance to microleakage of saliva and microorganisms at the interface[18,19,20].

In the current study, microleakage of two pit and fissure sealants marketed as having antimicrobial properties was compared. In preparing the teeth to receive the sealant application, only pumice prophylaxis was performed, without enameloplasty. While some authors[21] emphasized the importance of enameloplasty prior to sealant application allowing for better resin infiltration, others[22,23] argue its benefit. In fact, the later believe that enameloplasty would increase the sealant surface area resulting in a greater margin more susceptible to microleakage.

Although both sealant materials used in the current study are claimed to have antimicrobial potential, they differ substantially in their composition and mechanism of action. Ultra Seal XT Plus is filled resin, fluoride releasing, opaque sealant, obtaining its antibacterial property through its fluoride release[24]. On the other hand, SeLect Defence is a non-fluoride releasing sealant owing its antimicrobial property to its organic selenium content[15]. It has previously been shown that the organo-selenium molecule serves as a catalytic generator of superoxide radicals ($O2•−$)[25,26] which appears to account for most of selenium's toxicity toward different bacteria[27].

Within the experimental set up of the current study, only 20% of all specimens showed no microleakage, the other 80% showed variable degrees of microleakage being significantly lower with the use of SeLect Defense sealant, which warrants the rejection of the null hypothesis. The higher microleakage scores found with Ultra Seal XT Plus are in in contrast with the findings of Kwon et al[28] and Hatibovic-Kofman et al[29] who reported significantly lower microleakage scores with its use when compared to other sealant materials. Although microleakage testing is widely used, the methods are often not standardized (thermocyling regimen, type of dye, immersion duration), which may explain the inconsistency in the reported results[30]. In the current study, silver nitrate tracer was used rather than 1% methylene blue dye. Silver nitrate is considered a more severe test because of the small diameter of the silver ions (0.059 nm) and thus its higher penetration capacity[31], possibly accounting for the result discrepancy with the finding the other studies[28,29].

The significant difference in microleakage scores observed between SeLect Defense and Ultra Seal XT Plus in the current study, could have resulted from several factors related to the bond strength of the material to the enamel surface after thermal challenge, the polymerization shrinkage of the materials and resulting stresses at the interface, factors that warrant further investigation and that were beyond the scope of the current study. Another factor that deserves attention is the possible leaching out of the fluoride particles from Ultra Seal XT Plus[12,13,14], which is not a matter of concern with the non-fluoride releasing SeLect Defense sealant.

The antimicrobial efficiency of the tested sealants was evaluated by determining bacteria viability. A lower life/dead cell ratio would indicate a greater antimicrobial potential of the sealant used and could be translated clinically in less chances for recurrent caries development[32]. Both sealants tested resulted in significantly lower life/dead cell ratios after 24h (at $p<0.05$) and 96h (at $p<0.10$) incubation periods compared to the control group. No significant difference was found with the use of the different sealants at all evaluation times, expect at 72h, where Ultra Seal XT Plus resulted in significantly lower ratios compared to the SeLect Defense and control groups. This necessitates the partial rejection of the second nul hypothesis tested. It was interesting to note that even though the sealants tested differ in their antimicrobial mechanism, their antimicrobial efficiency was generally not different. These results are in disagreemt with those found by Tran et al[15] who found that SeLect Defense completely inhibited streptococcus mutans growth even after long incubation periods. This contrast in the results may be attributed to the lack of standardization in the antimicrobial testing methods where quantification of the antimicrobial activity may yield to very different results[32].

Nevertheless, it may be speculated that the antimicrobial potential of both SeLect Defense[15] and Ultra Seal XT Plus sealants, may prohibit bacterial growth underneath the sealant despite the conceivable microleakage at the interface. In this regard, and with the lower microleakage scores obtained, the use of SeLECT Defense sealant may offer protection against carious attacks at the occlusal pits and fissures, requiring less frequent replacement compared to Ultra Seal XT Plus.

Within the limitations of the current study, it can be concluded that:

1- The organo-selenium containing sealant (SeLect Defense) shows less microleakage at the tooth surface interface when compared to fluoride releasing sealant (Ultra Seal XT Plus).

2- Variable degrees of microleakage scores were recorded in all teeth sealed with Ultra Seal XT Plus, while only in 80% of teeth sealed with SeLect Defense sealant.

3- Both SeLect Defense and Ultra Seal XT Plus sealants show an antimicrobial potential that is more pronounced after 24h of application.

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