AN *IN VITRO* EVALUATION OF THE EFFECTS OF PRE-TREATMENT PROTOCOLS ON FISSURE SEALANT MICROLEAKAGE

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Abstract

Background: The use of pit and fissure sealants has been shown to be an effective preventive measure in reducing the incidence of occlusal caries. Optimal marginal-sealing ability is highly dependent on pre-treatment protocols, the use of adhesives, and the sealant material used. Various studies on enamel surface preparation and placement methods prior to the application of fissure sealants have produced mixed results. **Objective**: To evaluate and compare *in vitro* the effects of applying different enamel pre-treatments with conventional acid-etch, etch-and-rinse adhesive, and self-etch adhesive on microleakage around fissure-sealant margins in primary and permanent molar teeth. **Materials and methods:** 30 extracted human permanent third molars and 30 extracted primary second molars were randomly assigned to three groups of ten teeth each. Their pits and fissure surfaces were sealed with a resin-based fissure sealant (Clinpro, 3M) after undergoing one of these pre-treatment protocols: 1) Phosphoric acid etching; 2) Acid etching + Prime & Bond ^{NT} (etch-and-rinse adhesive); and 3) Single Bond Universal (self-etch adhesive). The teeth were then stored in water for 30 days following which they underwent thermocycling and then immersed in 0.5% basic fuchsin solution. Each tooth was sectioned into four slices. A total of 180 surfaces were identified and examined under a stereomicroscope by two calibrated examiners. Inter-examiner reliability revealed very good agreement (0.925). A microleakage scoring method was applied based on the following: score 0, no dye penetration; score 1, up to one-half or less of sealant depth penetrated; score 2, more than one-half penetrated; and score 3, penetration to the sealant base. Statistical analysis was done using SPSS version 23.0 and the Kruskal-Wallis test was used for comparison between the three pre-treatment protocols. **Results**: This study showed that there was significant difference ($p < 0.05$) between the permanent and primary teeth groups with all showing some degree of microleakage. In the permanent teeth, the acid etching technique showed the highest score of 0 or no leakage (at 96.7%) followed by the self-etch and etch-and-rinse adhesive at 76.7% and 46.7%, respectively. This was replicated in the primary teeth where the acid etching technique had the highest group score of 0 or no leakage (93.3%) followed by the self-etch (73.3%), and the etch-and-rinse adhesive (63.3%). **Conclusion**: Within the limitations of this study, it can be concluded that fissure sealants applied with conventional acid etching technique showed the least microleakage followed by the self-etch and the etch-and-rinse adhesive technique for both permanent and primary teeth.

Keywords: Fissure sealant, microleakage, acid etch, self-etch adhesive, etch-and-rinse adhesive

ABSTRAK

Latar Belakang Kajian: Kegunaan pengapan liang dan fisur gigi sebagai sebagai kaedah pencegahan telah menunjukkan keberkesanan dalam penurunan kadar karies. Optimum pengapan liang dan fisur bergantung kepada protokol pra-rawatan, penggunaan pelekat, dan bahan pengapan fisur. Banyak kajian yang telah dibuat di seluruh peringkat dunia untuk persiapan permukaan enamel gigi, dan pelbagai kaedah peletakan sebelum aplikasi pengapan liang dan fisur, tetapi, hasil yang diperolehi adalah bercampur aduk. **Objektif**: Kajian ini dijalankan untuk menguji dan membandingkan dalam makmal, kesan daripada aplikasi protokol pra-rawatan yang berlainan, iaitu dengan menggunakan kaedah “asid etch” konvensional, kaedah adhesif “etch-and-rinse”, dan kaedah adhesif “self-etch” kepada ketirisan mikro di sekitar pengapan liang dan fisur pada gigi geraham susu dan gigi geraham kekal. **Kaedah Kajian**: Kajian *in vitro* melibatkan 30 batang gigi geraham bongsu yang telah dicabut dan 30 gigi geraham susu kedua yang telah dicabut telah dibahagikan secara rawak kepada tiga kumpulan yang terdiri dari sepuluh gigi dalam satu kumpulan. Permukaan liang dan fisur gigi telah diletak dengan bahan pengapan fisur yang berasaskan resin iaitu (Clinpro, 3M) setelah menjalani salah satu daripada protokol pra-rawatan seperti di bawah; 1) 37 peratus fosforik asid etch; 2) Prime & Bond ^{NT} (adhesif “etch-and-rinse”) dan 3) Single Bond Universal (adhesif “self-etch”). Kemudian, gigi-gigi tersebut direndam dalam air suling selama 30 hari. Selepas itu, gigi tersebut diletakkan pada kitaran suhu berlainan dan kemudian, direndam dalam 0.5 peratus pewarna “fucshin”. Kemudian, setiap gigi akan dipotong kepada empat kepingan. Secara keseluruhannya, sebanyak 180 permukaan gigi telah dikenalpasti dan diperiksa di bawah stereomikroskop oleh dua pemerhati yang dikalibrasi. Kebolehpercayaan antara dua pemeriksa menunjukkan skor yang sangat baik iaitu (0.925). Kaedah pemarkahan ketirisan mikro dibuat berdasakan kepada empat skor iaitu: (skor 0) – tiada penembusan pewarna; (skor 1)- sehingga satu perdua atau kurang dari kedalaman kedap ditembusi; (skor 2) – lebih daripada satu perdua kedalaman kedap ditembusi; (skor 3)- penembusan pewarna sehingga ke dasar pengedap. Analisis statistik telah dibuat dengan menggunakan SPSS versi 23.0. Ujian Kruskal-Wallis telah digunakan bagi membandingkan di antara tiga protokol pra-rawatan yang berlainan. **Keputusan**: Kajian ini menunjukkan bahawa ada perbezaan yang signifikan ($p < 0.05$) di antara kumpulan gigi susu dan gigi kekal. Semua kumpulan menunjukkan beberapa darjah ketirisan mikro. Dalam gigi kekal, kaedah "asid etch" konvensional menunjukkan peratusan (skor 0) tiada ketirisan yang paling tinggi iaitu (96.7%), diikuti oleh kaedah adhesif "etch-and-rinse", dan kaedah adhesif "self-etch" secara berturutan (76.7%) dan (46.7%). Keputusan bagi gigi susu pula menunjukkan keputusan yang sama iaitu kaedah "asid etch" adalah yang paling tinggi menunjukkan tiada ketirisan iaitu skor 0 sebanyak (93.3%), diikuti oleh kaedah adhesif "self-etch" iaitu (73.3%), dan kaedah adhesif "etch-and-rinse" iaitu (63.3%). **Kesimpulan**: Di dalam pembatasan kajian ini, kesimpulan yang boleh dibuat adalah pengapan liang dan fisur yang diaplikasi secara teknik "asid etch" konvensional menunjukkan ketirisan mikro yang paling rendah, diikuti oleh teknik adhesif "self-etch", dan teknik adhesif "etch-and-rinse" di dalam kedua-dua kumpulan iaitu gigi kekal dan gigi susu.

Kata kunci: Pengapan liang dan fisur, ketirisan mikro, "asid etch", adhesif "self-etch", adhesif "etch-and-rinse"

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LIST OF SYMBOLS AND ABBREVIATIONS

AAPD	:	American Association of Paediatric Dentistry
ADA	:	American Dental Association
ARBS	:	Auto polymerizing resin based sealant
ART	:	Atraumatic restorative technique
BisGMA	:	Bisphenol A-glycidyl methacrylate
EARA	:	Etch-and-rinse adhesive
FRBS	:	Fluoride releasing resin based sealant
FS	:	Fissure sealant
GIC	:	Glass ionomer cement
ISO	:	International Standardization of Organization
LRBS	:	Light activated resin based sealant
MDP	:	Methacryloyloxydecyl dihydrogen phosphate
NOHP	:	National Oral Health Plan
NOHPS	:	National Oral Health Survey of Preschool Children
NOHSS	:	National Oral Health Survey of School Children
OHD	:	Oral Health Division
PAE	:	Phosphoric acid etching
PENTA	:	Dipentacry-thritolpenta-acrylatemonophosphate
RBS	:	Resin based sealant
RMGIC	:	Resin modified glass ionomer cement
SBU	:	Single Bond Universal
SEA	:	Self-etch adhesive
TES	:	Total-etch system
UDMA	:	Urethane dimethacrylate

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CHAPTER 1

INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1 Background of the study

The 2007 Malaysian National Oral Health Survey of School Children (NOHSS) by the Oral Health Department, Ministry of Health, Malaysia, showed a decline in the prevalence of caries among 6-year-old children from 80.9% in 1997 to 74.5% in 2007 (NOHP, 2011). This was mainly due to the implementation of the community water fluoridation and fissure sealant programme in school children in 1972 and 1999, respectively (OHD). The recent National Oral Health Survey of Preschool Children reported a caries prevalence rate of 71.3% in 5-year-old preschool children (NOHPS, 2015). The high caries prevalence emphasizes the need to expand the utilization of a fissure sealant programme in both dentitions as a preventive strategy.

Pit and fissure caries were reported at 90% and 44% in permanent and primary teeth respectively, (AAPD, 2014; Beauchamp et al., 2008), and higher than interproximal caries during childhood and adolescence (Manton & Messer, 1995). The pits and fissures are more prone to caries formation (Batchelor & Sheiham, 2004) even though these sites constitute only 12.5% of all tooth surfaces (Waggoner & Siegal, 1996).

Pit and fissure sealant application have been described to be effective in reducing occlusal caries in the permanent teeth of children and adolescents compared to topical fluoride applications (Ahovuo-Saloranta et al., 2016). The decline in the caries incidence in sealed permanent molars were observed to exceed 50% after 4.5 years of placement (Ahovuo-Saloranta et al., 2008). The sealant forms a coating to prevent access by new bacteria and nutrients into the occlusal surface thus arresting the formation of caries (Dean, 2015; Powell et al., 1976). Good quality dental sealant materials should be easily available, inexpensive, bacteriostatic, offer high bond strength, provide good marginal seal, and have strong resistance to wear and abrasion (Beauchamp et al., 2008).

The efficacy of fissure sealants is determined by their ability to perfectly seal and be retained on the occlusal surfaces for as long as possible (Waggoner & Siegal, 1996). Sealant retention relies on the complexity of the occlusal surfaces of the posterior teeth, tooth isolation protocol, enamel etching technique, use of bonding agent, operator's skill, sealant application techniques, and sealant viscosity (Eliades et al., 2013). Micromechanical bonding from resin-tag formation between resin and enamel surface is the key to resin-based sealant retention and success (Straffon et al., 1985). In order to achieve micromechanical bonding, the etched enamel surfaces should be free from salivary contamination (Tulunoglu et al., 1999).

Sealant failure rates were reported at 5-10 % annually due to leakages and partial or total loss of sealant (Feigal, 1998). Sealant microleakage is a significant concern because it leads to further bacterial invasion and caries progression. The breakdown of the bonding between the sealant and tooth surfaces leads to the formation of micro gaps at the sealant enamel interface and contributes to microleakage. As such, proper methods should be employed to improve sealing ability and sealant retention (Ahovuo-Saloranta et al., 2008; Beauchamp et al., 2008).

The application of phosphoric acid etching is the gold standard for enamel surface roughening prior to sealant placement (Ciucchi et al., 2015; Garcia-Godoy & Gwinnett, 1987). Adhesive systems include the etch-and-rinse adhesive technique and the self-etch adhesive technique. The etch and rinse or total-etch adhesive includes the placement of phosphoric acid for the demineralisation of the enamel followed by rinsing, drying, and adhesive application (De Munck et al., 2003; Garcia-Godoy et al., 1997). Several studies recommend the use of an etch-and-rinse adhesive system prior to sealant application

(Borsatto et al., 2004; Cehreli & Gungor, 2008; Feigal, 1998; Feigal et al., 2000; Feigal et al., 1993; Tulunoglu et al., 1999; Waggoner & Siegal, 1996).

An *in vitro* study by Cehreli and Gungor in 2008 examined the effects of water storage of 48-hour and 48-month on the microleakage of a fluoride fissure sealant (Helioseal F) applied with different bonding agents and without bonding agents. A total of 192 extracted humans third molars were divided into eight groups with 24 teeth each and pre-treated as follows: phosphoric acid etching (PAE) only, (PAE) and Single Bond, (PAE) and Prime & Bond ^{NT}, Clearfil SE Bond, FL Bond, One Up Bond F, Prompt L-Pop, and Mac Bond II. The etch-and-rinse adhesive (Prime & Bond ^{NT}) was found to has reduced microleakage compared to the self-etch adhesive or acid etching alone.

However, the etch-and-rinse adhesive system required longer chair-side time and is technique sensitive, and it is challenging to maintain a dry-etched enamel after etching in a child (De Munck et al., 2003; Perdigao et al., 2003). Others note that the use of the etch-and-rinse adhesive has no benefit compared to traditional etching without adhesives (Marks et al., 2009; Tunc et al., 2012). Marks (2009) investigated, *in vitro*, the effect of bonding agent and morphology of the fissure on microleakage of three different pit and fissure sealants (Conseal F, Admira Seal, and Aegis). 90 human permanent molars were divided into 9 groups of 10 teeth each. Three groups each were prepared with phosphoric acid and fissure sealant, Optibond Solo Plus (total-etch system) and fissure sealant, and Clearfil S Bond (self-etch) and fissure sealant. The Aegis and phosphoric acid applications reported 100% no leakage thus indicating that adhesives are not needed in the fissure sealant application.

Self-etch adhesive, simplified etching, rinsing, and adhesive application were done in a single step. This technique is mostly preferred because it is time-effective, less technique-sensitive and practical in paediatric dentistry (Pashley & Tay, 2001). Al-

Sarheed (2006) compared the shear bond strength of different fissure sealants (Dyract Seal, Concise, Visio-Seal, Helioseal) on 56 extracted first permanent molars (seven teeth for each of eight groups) using acid etch and self-etch adhesive (Prompt-L-Pop). They reported that the self-etch adhesive (Prompt-L-Pop) used with Dyract Seal and Concise fissure sealant showed stronger bonding to enamel compared to the conventional acid-etch technique.

The use of pit and fissure sealant in preventing caries in permanent teeth is well established (Ahovuo-Saloranta et al., 2017). However, the existing evidence from trials and *in vitro* study regarding effectiveness of pit and fissure in preventing occlusal caries in primary teeth is lacking (AAPD, 2013). There is uncertainty regarding the use of sealants in primary molars owing to flatter fissures of primary molars which do not support long term sealant retention (Horowitz & Frazier, 1982). However, recently, the use of sealants in primary teeth are increasingly being recommended as part of preventive measures for young children. Prevention of caries in primary molars is important as the progression of caries is faster in primary teeth compared to permanent molars, due to thinner enamel and greater porosity. Thus, further clinical and *in vitro* study should be conducted to investigate the microleakage of pit and fissure sealant specifically in primary molars (Ramamurthy et al., 2018).

1.2 Rationale of the Study

Studies have been done globally for enamel surface preparation and placement methods prior to fissure sealant application (Baygin et al., 2012; Ciucchi et al., 2015; Hitt & Feigal, 1992; Khogli et al., 2013; Marimuthoo et al., 2017). A few compared the microleakage of sealants after adhesive placement (Botsali et al., 2015; Cehreli & Gungor, 2008; Hebling & Feigal, 2000; Memarpour & Shafiei, 2014; Sakkas et al., 2013; Tulunoglu et al., 1999) but the results were mixed.

The resin-based fissure sealant was chosen as it is widely used and accepted worldwide (Ahovuo-Saloranta et al., 2017) and has better retention over other sealant materials (Forss et al., 2013). Clinpro sealant is a resin based unfilled sealant with fluoride properties and show higher fracture resistance compared to other resin based sealants (Fernandes et al., 2012). A study reported that the unfilled sealant Clinpro retained more than the filled sealant (Helioseal F) (Reddy et al., 2015).

For etch-and-rinse adhesives, Prime & Bond ^{NT}, a 5th generation bonding agent, was chosen due to it being an acetone-based solvent, less moisture sensitive, and having the greatest tensile bond strength (24.42 Mpa) to enamel and dentine compared to Adper Single Bond 3 (3M ESPE) (23.26 Mpa) (Kamble et al., 2015). It was also shown to have less microleakage in sealant applications (Cehreli & Gungor, 2008).

The Single Bond Universal adhesive can be used as self-etching without significant differences in bond strength (Jayasheel et al., 2017). The unique property of Single Bond Universal (SBU) is that it contains 10 methacryloyloxydecyl dihydrogen phosphate (MDP) which can interact with hydroxyapatite to form a chemical bond and maintain a stable and durable seal (Yoshida et al., 2004). The higher shear bond strength of SBU is attributable to the presence of MDP (Jayasheel et al., 2017). However, little information is found in the literature since SBU is a new material and further investigations are needed to evaluate its ability to prevent fissure sealant microleakage, as suggested by Jayasheel (2017).

Currently, comparative information is lacking on the effectiveness of conventional acid etching, etch-and-rinse adhesives, and self-etch adhesives in their sealant microleakage ability for permanent and primary teeth. Further, to the best of our knowledge, no fissure sealant microleakage study has been conducted using a universal adhesive applied as a self-etch in both permanent and primary teeth.

Accordingly, this study conducts an *in vitro* evaluation and comparison of the effects of applying different enamel pre-treatments with conventional acid-etch, etch-and-rinse, and self-etch adhesives on microleakage around fissure sealant margins in primary and permanent molar teeth. The findings will enable us to improve pit and fissure sealant application techniques in order to prevent microleakage as well as enhance the clinical effectiveness of sealants in terms of long-term retention and in reducing dental caries lesions in both permanent and primary teeth.

1.3 PURPOSE OF THE STUDY

1.3.1 Aim of the Study

To conduct an *in vitro* investigation on microleakage around fissure sealant margins in primary and permanent molar teeth following the application of different enamel pre-treatments prior to the placement of the fissure sealants.

1.3.2 Objectives of the Study

The objectives of the *in vitro* study were to:

1. investigate microleakage from resin-based fissure sealants in acid-etching groups of permanent teeth;
2. investigate microleakage from resin-based fissure sealants in etch-and-rinse adhesive groups of permanent teeth;
3. investigate microleakage from resin-based fissure sealants in self-etched groups of permanent teeth;
4. compare microleakage from resin-based fissure sealants between groups of permanent teeth;
5. investigate microleakage from resin-based fissure sealants in acid etched groups of primary teeth;
6. investigate microleakage from resin-based fissure sealants in etch-and-rinse groups of primary teeth;
7. investigate microleakage from resin-based fissure sealants in self-etched groups of primary teeth; and

8. compare microleakage from resin-based fissure sealants between groups of primary teeth.

1.3.3 Research Questions

1. Is there any difference in the level of microleakage from resin-based fissure sealants on permanent teeth after acid etching?

2. Is there any difference in the level of microleakage from resin-based fissure sealants on permanent teeth after application of the etch-and-rinse adhesive technique?

3. Is there any difference in the level of microleakage from resin-based fissure sealants on permanent teeth after application of a self-etch technique?

4. Is there any difference in the level of microleakage from resin-based fissure sealants between groups of permanent teeth *in vitro*?

5. Is there any difference in the level of microleakage from resin-based fissure sealants on primary teeth after acid etching?

6. Is there any difference in the level of microleakage from resin-based fissure sealants on primary teeth after application of the etch-and-rinse adhesive technique?

7. Is there any difference in the level of microleakage from resin-based fissure sealants on primary teeth after applying a self-etch technique?

8. Is there any difference in the microleakage from resin-based fissure sealants between groups of primary teeth *in vitro*?

1.3.4 Null Hypothesis

There is no difference in the degree of microleakage between the groups after acid etching, etch-and-rinse adhesive application, and self-etch adhesive systems in permanent and primary teeth *in vitro*.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2: LITERATURE REVIEW

2.1 Definition of Fissure Sealant Microleakage

A fissure sealant microleakage or marginal leakage is defined as "the entry of oral bacteria and fluids into the space between the enamel surface and the sealant material" (Hatibovic-Kofman et al., 1998). The microleakage of dental restorations is also referred to as "the clinically undetectable passage of bacteria, fluids, molecules, or ions between the surface of the tooth and restorative materials" (Kidd, 1976).

2.1.1 Factors Contributing to Microleakage

Factors contributing to the microleakage of dental restorations include polymerization shrinkage, mechanical and thermal changes, and water absorption. Microleakage can lead to caries progression, staining around the margin of the restorations, and partial or total loss of the restoration (Cehreli & Gungor, 2008). Different thermal expansion coefficients between the dental sealants and enamel as well as differences in the expansion and contraction of teeth and sealants cause microleakage and marginal fissure formations. The fracture resistance of dental sealants is related to salivary flow while oral acidic pH affects the properties of sealants. Any fracture in the sealant contributes to microleakage (Theodoridou-Pahini et al., 1996).

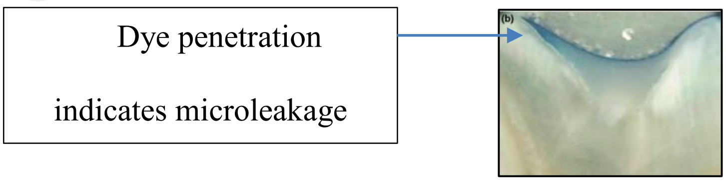


Figure 2.1. Resin-based sealant microleakage as seen with an optical microscope Adapted from Khogli et al., 2013

2.2 Introduction on Pit and Fissure Sealants

2.2.1 Definition of Pit and Fissure and Pit and Fissure Sealants

"Pits and fissures are deep grooves on the tooth structure or anatomical landmarks where the enamel folds inwards" (Nelson, 2014).

Figure 2.2. Pits and fissures on a molar tooth (Adapted from OHD, 2003)

A pit and fissure sealant is a dental material that is applied on the occlusal surfaces of the posterior teeth, followed by polymerization which is achieved through chemical, auto polymerization, or through a visible light curing technique. Pit and fissure sealants act as thin layers of coating on the occlusal surfaces to prevent the ingress of cariogenic bacteria and discontinue nutrient supply in order to stop the progression of caries (Simonsen, 1978).

2.2.2 History of Pit and Fissure Sealants

A yet well-known approach to "extension for prevention" was introduced in past centuries using amalgam restoration in the additional preparation of the pit and fissure areas as a protective procedure against occlusal caries (Black, 1914). This was followed by the acid-etching technique. In 1955, a pioneer study advocated the use of 85% phosphoric acid for 30 seconds to enhance bonding of self-curing methyl methacrylate resin materials to enamel surfaces (Buonocore, 1955). Later, in the 1960s, methyl cyanoacrylate, was introduced as a first sealant material although it was found to be susceptible to bacterial disintegration in the oral environment with the passage of time

(Cueto, 1965). After that, a viscous resin material with improved property was developed namely bisphenol-a-glycidyldimethacrylate (BIS-GMA). It provides good adhesion to the etched enamel and is able to withstand degradation (Bowen, 1965). Later on, the use of BIS-GMA with ultraviolet light has been proven to be successful as a dental sealant material (Buonocore, 1970).

2.2.3 Pit and Fissure Sealant Materials

Numerous sealant materials are readily available in the market nowadays, such as resin-based fissure sealants (RBS), glass ionomer cement (GIC), and resin-modified glass ionomer cement (RMGIC). Currently resin-based sealants are the most commonly used followed by glass ionomer-based sealants (Anusavice et al., 2014).

Resin-based sealants are categorized based on the polymerisation of their monomers and contain monomers namely urethane dimethacrylate (UDMA) or bisphenol A-glycidyl methacrylate (Bis-GMA). The polymerization of the monomers is achieved through chemical or light activation.

Glass ionomer cement-based sealants are another type of pit and fissure sealant material and are the second most-used sealant clinically. Improvements in dental materials have resulted in a new resin-modified glass ionomer cement which is of a light curable type followed by compomers which are also known as polyacid-modified resins (Nicholson, 2007).

2.2.3.1 Resin Based Sealants (RBS)

RBS have been endorsed by the American Dental Association (ADA) as the first choice of dental sealant material (Beauchamp et al., 2008). This is the most often selected sealant by dentists worldwide for fissure sealant applications (Ahovuo-Saloranta et al., 2017). The polymerization of resin-based sealant materials is attained through auto or chemical polymerization, and visible light polymerization (Ripa, 1993). Resin based sealants are further classified into:

2.2.3.1 (a) First Generation Resin Based Sealants

The first generation RBS used an ultraviolet (UV) light initiating system. Ultraviolet rays activate the initiators in the material for initiation of the polymerization. The first dental material developed by Buonocore was Nuva-Seal® (USA) though it is no longer available (Dean, 2015).

2.2.3.1 (b) Second Generation Resin Based Sealants

Auto or chemically cured resin-based sealants (ARBS) are of the second generation type after ultraviolet resin based sealant. The presence of the activator (tertiary amine) in their content produces free radicals upon mixing which later activate the polymerization. The setting time is within one to two minutes (Dean, 2015).

2.2.3.1 (c) Third Generation Resin Based Sealants

The third generation is the light activated resin-based sealants (LRBS). The exposure of the sealant material to the blue light within 470 nanometres wavelength will activate the photo initiators for polymerization induction, and the setting time is within ten to twenty seconds (Santini et al., 2013).

2.2.3.1 (d) Fourth Generation Resin Based Sealants

The fourth generation resin-based sealants contain fluoride and are also known as fluoride-releasing resin based sealants (FRBS). The added fluoride in the FRBS prevents caries progression (Naaman et al., 2017). However, a review reported that this type of sealant does not provide long term fluoride release (Muller-Bolla et al., 2006).

2.2.3.1 (e) Viscosity of Resin Based Sealants

Viscosity is the opposition of a liquid to move along or flow. Based on resin viscosity, RBS can also be divided into filled and unfilled groups. Filler particles greatly influence sealant flow or penetration with higher resin viscosity (filled sealant) producing lower sealant adaptation and penetration. Thus, low resin viscosity (unfilled resin) will flow rapidly, spread deeper into the pit and fissure, and retain more than their counterparts (Rock et al., 1990).

2.2.3.1 (f) Translucency of Resin Based Sealants

Another classification of sealant materials is colour-dependent and being either opaque or transparent (Simonsen, 2002). Opaque sealants are white in colour and can be seen in the mouth clearly during follow up while transparent sealants are difficult to detect (Dean, 2015). Examples of coloured sealants are 3M Concise White Sealant, Helioseal Clear Chroma, USA, and ClinPro, 3M ESPE, USA.

2.2.3.1 (g) Disadvantages of Resin Based Sealants

Despite their good retention characteristics resin based sealants are extremely technique sensitive and expensive compared to glass ionomer cements (Beauchamp et al., 2008).

2.2.3.1 (h) Clinpro Sealant

Clinpro sealant by 3M ESPE, USA is a resin-based, coloured sealant with fluoride release and has been shown to possess higher fracture resistance compared to Helioseal and Conseal F fissure sealants (Fernandes et al., 2012). Bond strength between the ClinPro resin sealant and enamel is greater (24.37 Mpa) than the Helioseal F resin sealant (13.07 Mpa).

Clinpro sealants are made of Bis-GMA (Bisphenol A Diglycidyl methacrylate) monomers and contain a six percent filler load. Filler loading contributes to the viscosity of the sealant. Low sealant viscosity having better penetration into deep pits and fissures, while high viscosity produces sealant stability and homogeneity (Fernandes et al., 2012).

Another study reported higher retention rates and better performance of unfilled (ClinPro) rather than filled sealants (Helioseal F) (Reddy et al., 2015). Owing to these factors, this study further investigated the microleakage properties of the ClinPro sealant.

2.2.3.2 Glass Ionomer Cement (GIC)

2.2.3.2 (a) Basic Chemistry

Glass ionomer based sealant are the second most normally used sealant materials (McLean, 1974). Conventional glass ionomer cements comprise of polyacrylic acid and fluoro alumina silicate glass particles, and acid-based reactions occur once the powder and liquid are mixed (Mount, 1991).

GIC can also be categorized based on whether the cement is of low or high viscosity. Former GIC based sealant studies used low viscosity types (Fuji III) which have poor physical properties. Examples of high viscosity types include Fuji Triage (VII) and Fuji IX from Japan, and Ketac Molar from Germany. These improved materials have greater fluoride release and better physical properties and used extensively in the atraumatic restorative (ATR) technique and as fissure sealants using the finger press method.

2.2.3.2 (b) Adhesion of Glass Ionomer Adhesives

Glass ionomer cements directly attached to the enamel surface through chemical bonding obtained from an ionic exchange formed between dental hard tissue and cement interface. Polyalkenoate chains of the glass ionomer cement exchange with the phosphate ions in the hydroxyapatite and at the same time calcium ion is removed in order to achieve a balanced equilibrium. This condition results in an improved cement that tightly attached to the dental hard tissue (Wilson et al., 1983). The micromechanical bonding of the glass ionomer is attained through a hybridization of the hydroxyapatite and collagen fibril network (Van Meerbeek et al., 2003).

2.2.3.2 (c) Advantages of Glass Ionomer Cements

The fluoride properties of glass ionomer materials make them superior to resin-based sealants and halt the progression of caries through fluoride release and uptake. It has been shown that fluoride discharge from the glass ionomer sealant help to prevent the formation of caries even though the glass ionomer sealant is detached. Glass-ionomer sealed teeth are better protected from demineralization than those without sealants due to remineralisation effects of fluoride produced by the glass ionomer and the remaining cement left in the pits and fissures (Seppä & Forss, 1991).

Fluoride is released from the GICs without damaging the physical features starting from the mixing stage and then remain within the cement (Mount, 1993). Fluoride can be re-absorbed into the cement during topical fluoride application and discharged again; thus, glass ionomer cements act as a fluoride reservoir in the mouth for a relatively long period (Forstein, 1990). The cariostatic effect is provided from fluoride reservoir properties (Mount, 1994). A previous *in vitro* study showed that glass ionomer cements used as restoration have less demineralization (Swift, 1988).

Glass ionomer cements can be placed on the tooth structure without acid etching. Good marginal adaptation can be achieved from the glass ionomer restorations as the thermal expansion of the cements is comparable to hard dental tissues.

According to (Beauchamp et al., 2008), a glass ionomer sealant is recommended whenever the placement of a resin-based sealant is not possible. The sealants retained less compared to the resin-based sealants, but GIC has proven successful in arresting the development of caries (Simonsen, 1996).

Glass ionomer sealants are indicated for high-caries-risk patients where tooth isolation is difficult especially in erupting teeth when the distal part of the occlusal surface is still covered by the operculum (Gilpin, 1997; Raadal et al., 2001).

A study on resin-based and glass ionomer fissure sealants using Fuji Triage White, GC America showed similar retention rates over two-years, and suggest that the former can be applied when salivary contamination is hard to control (Antonson et al., 2012).

2.2.3.2 (d) Disadvantages of Glass Ionomer Cements

Drawbacks reported on the glass ionomer sealants were inadequate retention and microleakage occurring at the margins of the tooth surfaces and the cement. The glass ionomer sealant showed loss of retention that was five times greater than resin-based sealants. A comparison of GIC and RMGIC sealants reveals the former's loss retention at three times than the RMGI sealant (Wright et al., 2016). Conventional glass ionomer cements show low shear bond strength to the tooth structure at within 3 to 7 MPa (Erickson & Glasspoole, 1994). Fractures usually occurred within the glass ionomer cement with the remainder of it still being left on the tooth structure (Mount, 1991).

2.2.3.3 Resin Modified Glass Ionomer Cement (RMGIC)

Its high level of compressive and tensile strength, surface hardness, and fluoride release from RMGIC make this material the preferred option for restorations and as a pit and fissure sealant. However, microleakage studies using Resin-modified glass ionomer sealants are limited. RMGI sealants offered different results in microleakage tests, and this may be due to higher thermal expansion compared to conventional GICs though both types show lower expansion compared to composite-resins (Pereira et al., 2002).

2.3 Effectiveness of Fissure Sealant As A Preventive Measure

2.3.1 Use of Pit and Fissure in Non-Cavitated Lesions

A previous review of the efficacy of pit and fissure sealants in preventing caries development in the occlusal surfaces of the permanent posterior teeth showed that the median annual percentage of non-cavitated lesions progressing was 2.6% for sealed and 12.6% for unsealed carious teeth. Griffin et al., 2008 reported that preventive fraction for randomized controlled trial (RCT) was 71.3% (95%CI: 52.8%–82.5, no heterogeneity) up to 5 years after placement. Thus, they summarized that sealing non-cavitated caries in permanent teeth is effective in reducing caries progression (Griffin et al., 2008). Another review in 2006 which studied the effects of sealants on non-cavitated enamel surfaces showed reduced caries progression within one to five years (Bader & Shugars, 2006).

A systematic review reported the ability of the fissure sealant to stabilise and reduce the number of bacteria in the cavitated occlusal lesions. The pit and fissure sealants administration on the occlusal surfaces reduced number of viable bacteria about hundred to thousand-fold. The percentage of sealed occlusal surfaces with viable bacteria was 47% in comparison to the teeth without sealants and 89% within two weeks to five years of sealant application (Oong et al., 2008).

2.4 Clinical Effectiveness of Fissure Sealants Application

2.4.1 Caries Preventive Effect of Resin Based Sealant (RBS)

A review of the clinical trials of resin based sealant placement on the first permanent molar teeth noted a reduction in caries by 78% and 60% after 2 and 4 to 4.5 years respectively, compared to teeth without sealants (Ahovuo-Saloranta et al., 2008). Historically, the policy for sealant application began during the 1950s to 1970s after a review reported that 70% of pits and fissures of permanent molars would become cavitated within ten years of eruption (Eklund & Ismail, 1986) with the first three years being the most vulnerable period for caries formation. These findings led to the institution of policies for sealants application on newly erupted permanent molar teeth.

The literature reports various ranges of caries reduction at approximately 86% over one year of fissure sealant placement, 78.6% and 58.6% after two years, and four years respectively, following pit and fissure sealant application in children and adolescents. In children with sealed first permanent molars, occlusal caries reduction was reported at 76.3% four years after application, and after 9 years of fissure sealant placement about 65% caries reduction was reported without reapplication in the last five years (Beauchamp et al., 2008).

Extensive reviews and meta analyses have been attempted on the effects of resin based sealants on dental caries (Ahovuo-Saloranta et al., 2008; Kühnisch et al., 2012; Llodra et al., 1993; Mejàre et al., 2003). A review in 2017 investigated clinical trials on the effects of caries after resin based sealant was applied on the first permanent molars compared with control teeth (no sealant). Caries reduction was reported within a range of 11% and 51% on them compared to those without sealant placement within 48 months follow-up (Ahovuo-Saloranta et al., 2017). The placement of pit and fissure sealant reduced caries formation by about 6.25% if 40% of the caries formed in the occlusal surfaces within a two-year period (Ahovuo-Saloranta et al., 2017).

2.4.2 Caries Preventive Effect from Glass Ionomer Cement (GIC)

Occlusal caries was reported to be declined after 2 years of application of the glass ionomer based sealants on the first permanent molars of children aged 7 to 8 years compared with no sealant (Songpaisan et al., 1995).

A comparison study of high viscosity GIC and resin based sealants within a 5-year duration showed that the finger press technique of the former was four times more effective in protecting teeth from occlusal caries than the latter (Beiruti et al., 2006). This protection is attributable to the fluoride discharge from the glass ionomer cement even though if it is partially detached, with the residual cement protecting the occlusal surface from caries.

However, a systematic review in 2017 documented inconclusive findings for comparison of caries protection from glass ionomer sealants with control teeth after a two-year follow up (Ahovuo-Saloranta et al., 2017). Latest meta-analysis which compares resin based sealants and glass ionomer sealant found no significant differences in caries reduction after subsequent observations at two, three, and four years. Nonetheless, a high-risk bias of clinical trials was detected in the analysis (Ahovuo-Saloranta et al., 2017).

Previous clinical trials evaluating resin- and glass ionomer-based sealant retention and marginal staining capabilities and caries formation in partially erupted teeth show that of 39 molar pairs sealed with the resin based sealant group, two had caries and the rest showed decalcification while the glass ionomer based sealants showed no caries. This supports glass ionomer sealants as the best choice instead of resin based one, for use in partially erupted molars (Antonson et al., 2012).

Table 2.1. Evidence-based findings on different sealant materials

Comparison of Sealant Materials	Caries Reduction/ Sealant Retention	Significance Level	Quality of Evidence
Glass Ionomer (GI) Based Sealant with Resin Based Sealant	GI sealant decreased caries incidence by 29%	Non-significant	Very low
	GI sealant has a 5x higher risk of lack of retention	Significant	Very low
Glass Ionomer (GI) Based Sealant with Resin Modified Glass Ionomer Based Sealant	GI sealant enhanced caries incidence by 41%	Non-significant	Very low
	GI sealant has 3x higher risk of lack retention	Significant	Very low

Adapted from (Wright et al., 2016)

2.5 Retention of Different Sealant Materials

2.5.1 Retention Rate of Resin-Based Sealants

The sealant retention rate showed a declining trend through time of 79% to 92% at 12 months, 71% to 85% at 24 months, 61% to 80% at 36 months, 52% at 48 months, and 39% at 9 years (Ahovuo-Saloranta et al., 2008).

Resin-based sealants were reported to have the highest retention rates (Kühnisch et al., 2012) with a mean of 76% compared to glass ionomer sealants (8%) over a 3- to 4-year observation period. The retention rate of the resin based sealant was observed to be around 70% after 48 to 54 months (Forss et al., 2013).

2.5.1.1 Retention Rate of RBS (Auto Polymerization versus Light Initiated Sealants)

Light polymerizing sealants retained more (83.8%) than auto polymerizing ones (64.7%) over 5 years (Kühnisch et al., 2012) although an earlier study did not find any significant difference after 2.5 years (Houpt & Eidelman, 1983). A comparative study on the retention rates and effectiveness of the sealants showed that light-cured sealants (Helioseal) were comparable to self-cured sealants and both were superior to UV light-cured sealants (De Craene et al., 1989).

2.5.1.2 Retention Rate of RBS (Filled versus Unfilled Sealants)

A study comparing unfilled (Clinpro) and filled (Helioseal) sealants after one year found that unfilled light-cured resin sealants have higher retention (64.39%) than filled light-cured resin (53.57%) sealants. Unfilled sealants refer to those without filler particles. Clinpro sealants rapidly flow into the pits and fissure surfaces due to their low viscosity compared to filled sealants (Helioseal) (Reddy et al., 2015). A sealant microleakage study using different enamel surface preparations reported unfilled resin sealants having less microleakage than filled sealants (Hatibovic-Kofman et al., 1998). A reported drawback of filled resin was the high abrasion wear over one-to-two days (Handelman et al., 1987).

2.5.1.3 Retention Rate of FRBS (Fluoride Resin-based Sealant)

The clinical effectiveness of fluoride-releasing sealants in reducing caries progression is less clear (Carlsson et al., 1997). A meta-analysis on the retention rate of fluoride-containing sealants showed a 5-year retention rate of 69.9% (Kühnisch et al., 2012). A four-year evaluation study on the retention rates and caries progression between a fluoride containing sealant (Fluroshield) and non-fluoride containing sealant (Delton) noted that the former had a low retention rate compared to its counterpart although both showed no differences in total sealant loss and caries formation (Garcia-Godoy et al., 1997). In fact, fluoride release declined significantly on the second day of sealant placement and then gradually reduced the day after. Thus, fluoride reservoir is not applicable to fluoride based sealants (Hicks et al., 2002). Moreover, fluoride-based sealants retained less after 2 years (Simonsen, 2002).

2.5.2 Retention Rate of Glass Ionomer (GI) Sealants

A meta-analysis found that GI sealants had a 5.2% retention rate after 5-year observation times. Within 3 years of follow up, GIC-based fissure sealants (high viscous) showed a higher retention rate of 72% compared to 50% for the GIC (low viscous) (van t Hof et al., 2006). A review of clinical trials investigating the retention rate between resin based and GI based sealants reported that in three out of eight trials showed the former were better retained on the occlusal surfaces of the posterior teeth (Kervanto-Seppala et al., 2008; Poulsen et al., 2001). In contrast, two out of eight trials reported that the glass ionomer based sealant was retained more than the resin based sealant (Arrow & Riordan, 1995; Beiruti et al., 2006) while other trials showed similar findings on retention rates (Forss & Halme, 1998; Ganesh & Tandon, 2007; Mills & Ball, 1993). Thus the findings are inconclusive.

2.5.3 Retention Rates of Resin Modified Glass Ionomer (RMGI) Sealants

RMGI sealants wear out more than resin-based sealants (Winkler et al., 1996). A few studies were conducted to investigate and compare the retention rate and efficacy of resin-modified glass ionomer sealant and conventional glass ionomers sealant in preventing occlusal caries. Examples of resin modified glass ionomer and conventional glass ionomer cement are Vitremer and Ketac Bond respectively. These both materials have been used as preventive measure against caries on the pit and fissure surfaces. A study reported higher retention rates of the RMGI sealant (Vitremer) at 59% and 36% after six and twelve months respectively compared to conventional GI sealants (Ketac Bond). Both materials successfully prevent caries formation as evidenced by their lack through one year of sealant application (Pereira et al., 2001; Pereira et al., 1999).

2.6 Fissure Sealants on Primary Teeth

If a child is considered to have a very high caries risk or if the consequences of dental treatment or disease carry a significant risk to their general well-being, thus, the use of fissure sealant in that individual should be considered in the primary dentition (Smallridge, 2010). The occurrence of occlusal caries in the primary dentition is an indicator of future caries development in the next mixed and permanent dentition (McGuckin et al., 1994). Thus, primary molar sealants are indicated in high caries-risk patients, in complex occlusal fissures and deep grooves, in the socioeconomically vulnerable, and children with special health care needs (Casamassimo, 2013; Chi, 2013).

2.6.1 Retention of Fissure Sealants on Primary Molars

A clinical trial using the split-mouth technique conducted in 4 to 7-year-old children reported a larger retention rate (95%) of flowable composite used as a sealant in comparison to a resin based sealant (77.5%) after twelve-month of placement (Corona et al., 2005). The glass ionomer-based sealant had fewer retention rates compared to the resin-based equivalent (Chadwick et al., 2005).

Generally, pit-and-fissure sealant retention rate on the primary molars show a declining trend over time dropping from between 74% to 96.3% within one year to 70.6% to 76.5% after 2.8 years (Beauchamp et al., 2008).

In regard to the use of bonding agents in primary teeth before sealant application, some researchers noted that the use of self-etch adhesive systems resulted in a lower sealant retention rate compared to conventional acid etching without an adhesive (Maher et al., 2013).

2.7 Pit and Fissure Sealant Application Techniques

The retention of dental sealants is dependent on the micromechanical resin tags formed between the sealant material and the enamel surface thus requiring careful sealant placement by a skilled operator. The method of application for pit-and-fissure sealants and adhesive systems should strictly follow the manufacturer's instructions to prevent any potential clinical errors.

2.7.1 Tooth Cleaning Methods

Prior to sealant application, the tooth surface should be cleared of gross plaque and debris to ensure good sealant penetration (Waggoner & Siegal, 1996).

Few studies have been undertaken on different tooth cleaning methods before sealant placement. Tooth cleaning can be done either through a toothbrush or a hand piece. Supervised tooth brushing by patients was shown to be comparable to oral prophylaxis using a hand piece (Gray et al., 2009). Tooth brushing is more effective in terms of cost and time compared to hand piece prophylaxis especially in extensive fissure-sealant school programmes.

A review of clinical trials reported similar outcomes in the retention rates of pit and fissure sealants following different cleaning methods using a prophylaxis brush with pumice on a slow speed handpiece, use of explorer, and lastly use of an air water syringe to remove the debris on the occlusal surfaces (Muller-Bolla et al., 2006).

The standard and current practice before sealant placement is cleaning with slurry pumice on a rotary brush with a hand piece. Regardless of the cleaning method employed, the aim is to ensure the tooth surface is clean of plaque and debris prior to sealant application.

2.7.2 Mechanical Preparation of Enamel Surfaces Prior Sealant Application

Various studies have been undertaken on different methods of enamel preparation before sealant placement such as acid etching, enameloplasty, air abrasion, and use of lasers. (Kramer et al., 2008).

2.7.2.1 Enameloplasty

Enameloplasty is an invasive technique involving the widening of the fissures with a rotary instrument using a round diamond bur (size two) to open the fissure and remove the enamel layer overlying the dentin to clean the fissure and check for caries extension at the bottom.

A study showed reduce microleakage in enameloplasty surface preparation (Feigal et al., 2006). However, this invasive method was not recommended as it exposes the prepared tooth surfaces to risk for caries in case of sealant loss (Kramer et al., 2008; Subramaniam, 2009).

2.7.2.2 Air Abrasion

Air abrasion is a minimally invasive concept of the occlusal surface preparation prior to sealant placement (Waggoner & Siegal, 1996; Yazici et al., 2006) using silica-modified aluminium oxide (Al2O3) airborne particles sized from 30–50 µm together with silanization. The air pressure propels the particles to abrade the tooth surface thereby removing the debris so that the incipient caries can be excavated. In this technique, the etching and rinsing step is eliminated (Kramer et al., 2008).

2.7.2.3 Laser Devices

Lasers are an alternative to acid etching in order to open dentinal tubules to achieve good adhesion. The laser acts by modifying the calcium-to-phosphorus ratio of dental hard tissue, producing more stable and acid resistant compounds thus reducing caries formation (Usumez et al., 2013). Sungurtekin et al. (2010) investigated microleakage of resin-based sealants in primary and permanent teeth after the use of Er,Cr:YSGG laser as tooth conditioning. Their study reported that the teeth treated with laser had greater microleakage at the sealant-enamel interface, thus, concluding that a combination of both acid etching and laser offered optimum results compared to acid etching per se in achieving a perfect marginal seal (Sungurtekin & Öztaş, 2010).

2.7.3 Isolation of the Tooth

2.7.3.1 Cotton Roll versus Rubber Dam Isolation Technique

Salivary contamination of etched enamel surfaces contributes to sealant failure especially for resin-based sealants which are hydrophobic and extremely sensitive to saliva. Exposure of the etched enamel surface with the saliva even for 0.5 seconds will prevent resin tags formation and reduce sealant retention (Deery, 2013).

There are two techniques used for tooth isolation namely cotton roll isolation with saliva ejector and rubber dam isolation (Welbury et al., 2004) with the latter being superior. However, a review reported that cotton roll isolation was the ideal isolation method used with auto polymerized RBS or light cure polymerized RBS (Muller-Bolla et al., 2006).

2.7.4 Etching/ Conditioning

The application of orthophosphoric acid was reported as a standardized method to eliminate the smear layer and achieve efficacious bonding (Buonocore, 1955). Acid etching selectively dissolves hydroxyapatite thereby facilitating resin tag formation ranging from 6-12 µm in length. The common concentration of acid etching used is within 35% to 37% and the current practise of the technique is the use of 37% phosphoric acid for 15 seconds (Van Meerbeek et al., 1996).

After etching, rinsing and drying, a dry, dull, white, chalky etched enamel is required to achieve good bonding between the enamel surfaces and sealant. Contamination of the etched enamel surface to saliva leads to occluded micro pores and subsequent sealant failure (Hormati et al., 1980). In 1955, an 85% concentration of phosphoric acid was introduced for etching and later reduced to 50% (Buonocore, 1970).

The timing for acid-etching has been reduced from 60 to 20 seconds (Griffin et al., 2008). Previous researchers recommended a two-fold etching time for primary teeth at 120 seconds and 60 seconds for primary and permanent enamels, respectively. The longer etching time (120 seconds) was justified due to the existence of prismless enamel in the primary teeth thus requiring extra time to achieve an appropriate etching pattern. A study comparing the effects of etching with a 37% phosphoric acid solution after 15 and 60 seconds of application on enamel surfaces from primary and young and old permanent teeth reported no significant difference between two etching times studied in the primary teeth. In contrast, 15 seconds of etching created more retentive conditions on young permanent enamels than 60 seconds, while old permanent teeth showed a reversed condition (Nordenvall et al., 1980).

An *in vivo* study evaluated sealant retention after 60- and 120-seconds etching time in 56 children aged 3 to 8 years. A total of 373 deciduous first and second molars were used in which 178 teeth received 60-second etching while 195 were applied acid-etch for 120 seconds, following which all were sealed and reviewed after six months. The study found comparable retention rates at 100% and 99% for both groups after 60 and 120 seconds etched time, respectively (Simonsen, 2002). The conclusion was that a shorter etching time probably reduces the risk of contaminated etched enamel surfaces particularly in less cooperative child.

An earlier research investigated the effects of etching depth and bonding strength of hundred and thirty exfoliated primary teeth after different etching times of 15, 30, 60, and 120 seconds. The researcher found no significant difference of mean bond strengths obtained for the all four etching times. The results showed no significant different even after addition of etching depth after 120 seconds (Redford et al., 1986).

Duggal et al., in 1997 conducted a study to investigate the effect of different etching times on the retention of fissure sealants in second primary and first permanent molars. The study involved eighty-four children with a total of 144 second primary molars and 264 first molars. Different etching times of 15, 30, 45 and 60 seconds were used and the fissure sealants were evaluated at 6 and 12 months. The results showed that the overall retention rate of fissure sealants in second primary molars was 73.0% at 6 months and 64.7% at 12 months, whereas in first permanent molars the retention rates were 60.7% at 6 months and 44.1% at 12 months respectively. They reported that there was no significant difference in the retention of fissure sealants either on second primary molars or on first permanent molars at a 6-and 12-month follow-up with the different etching times. It was concluded that the different etching times did not appear to affect the retention of fissure sealants on the first permanent molars or second primary molars. Therefore, they suggested to etch the teeth for a much shorter period than conventionally recommended (Duggal et al., 1997). A 30-second rinsing followed by 15-second drying to remove remaining acid etchant and to obtain the required characteristic of chalky white appearance of enamel, is a critical step in sealant applications (Dean, 2015).

2.7.5 Adhesive/Bonding System

The use of dental adhesives has resulted in increased sealant retention over the long term (Cehreli & Gungor, 2008). There are two types of adhesive systems available namely, total-etch adhesive or etch-and-rinse adhesive, and self-etch adhesive (De Munck et al., 2003).

In 1993, Feigal et al., investigated the usage of hydrophilic bonding agents before sealant placement in a moist environment. Later, they suggested using etch-and-rinse adhesive systems before fissure sealant application to promote stronger bonding at the sealant-enamel interface. The formation of micromechanical interlocks in between dental adhesives and enamel surface are obtained from the infiltration of the resin monomers into the tiny porosities produced from the acid-etch dissolution of enamel. Later, the exposed hydroxyapatite crystals were enclosed with polymerized monomers within the enamel micro porosities (Feigal et al., 2000).

Another classification of the bonding or adhesive system is based on the mechanism of action on the smear layer. Three mechanisms of adhesion are currently developed: etch-and-rinse adhesives, which eliminate the smear layer and superficial hydroxyapatite from acid etching technique; self-etch adhesives, which modify the smear layer and make the smear layer permeable without completely removing it; and lastly, glass ionomer adhesives, which are self-adhesive to the tooth structure.

2.7.5.1 Etch-and-Rinse Adhesive System

Etch-and-rinse adhesive systems also known as the total-etch adhesive technique, or multi-bottle adhesive system involves separated etching techniques. The etch and rinse adhesion system, involves two or three steps. It started with the application of acid etchant followed by rinsing, drying, and then, application of a primer, and adhesive resin (De Munck et al., 2003). Etch-and-rinse adhesive technique involve the placement of a 30% to 40% concentration of phosphoric acid etchants prior to the application of the primer or adhesive followed by light curing (Perdigao et al., 2003).

Some studies suggest the use of etch-and-rinse adhesive strategy as a standard procedure before fissure sealant application (Cehreli & Gungor, 2008; Feigal et al., 2000). Earlier literature noted that the placement of dentine bonding agents before the application of sealants helped reduce microleakage and enhanced bond strength (Tulunoglu et al., 1999).

An *in vitro* study evaluating the bond strength of a resin-based pit and fissure sealant to enamel after cariogenic challenge. The authors used three bonding protocols namely; Group 1 (applied with 37 % phosphoric acid gel), Group 2 (applied with total-etch adhesive system), and Group 3 (applied with one-step self-etch adhesive system). The authors found out that, the total-etch group showed greater tensile bond strength with incipient enamel caries formation (Kalra et al., 2015). A former study reported that the use of total-etch adhesives were associated with greater clinical performance in comparison to the self-etch adhesive techniques and conventional acid-etch protocols (Sakkas et al., 2013).

A previous clinical trial conducted to compare the fourth generation of bonding agent involving (three-step etch-and-rinse), and the fifth generation of bonding agent involving (two-step etch-and-rinse) adhesives used before sealant application. They reported that the latter retained more than the former one. Three step etch-and-rinse diminished the sealant loss by half of the subjects. Surprisingly, the three-step adhesives had reduced sealant retention rate. This may be explained by the water-based composition as water has a detrimental effect on the adhesion at sealant-enamel interface. The two-step etch-and-rinse adhesive is made up of acetone-or ethanol-based which functioned to increase bonding to the etched enamel surface (Feigal et al., 2000).

However, some drawbacks reported with etch-and-rinse adhesives include its time-consuming process, technical sensitivity, insufficiently eradication of existing debris and pellicles by conventional prophylaxis technique, as well as acid etching method. In children with behaviour issues, rubber dam isolation is not possible. Often clinicians have difficulty in maintaining dry etched enamel surfaces especially with the cotton roll isolation technique as tongue movements and swallowing can cause salivary contamination and ultimately sealant loss (De Munck et al., 2003; Perdigao et al., 2003).

In etch-and-rinse adhesive methods, over conditioning can cause uncontrolled demineralization of the tooth structure involving enamel, dentine, and a collapse of the collagen network. Dentinal tubules completely changed and became a channel thus, increasing the flow of dentinal fluids which in turn cause post treatment sensitivity (De Munck et al., 2003; Perdigao et al., 2003). Considering these disadvantages, various researches have sought to improve and simplify this method.

Commercially available total-etch bonding materials include Prime & Bond ^{NT} (Dentsply, USA), Opti Bond^{R} Solo (Kerr, USA), and Adper^{TM} Single Bond (3M ESPE, USA).

2.7.5.1 (a) Prime and Bond [NT]

The development of tooth bonding agents has yielded a variety of products for the market. Thus, continued research on tooth bonding materials is needed to assess retention rates and the ability to prevent microleakage. Prime & Bond [NT], a fifth-generation bonding agent, is an acetone-based adhesive and a total-etch bonding agent which requires a moist dentine surface for adequate bonding (Gangurde et al., 2014). The unique combination of micromechanical and chemical bonding for long-term adhesion with nano-filler technology reinforces hybrid and adhesive layers, protects against microleakage, and ensures marginal seal and integrity. Prime & Bond is less technique-sensitive to intraoral moisture and shows greatest tensile bond strength (24.42 Mpa) to enamel and dentine compared to Adper Single Bond 3 (3M ESPE) (23.26 Mpa) (Kamble et al., 2015). PENTA (dipentacry-thritolpenta-acrylatemonophosphate) composition contributes to higher-bond strength and better adhesion (Albaladejo et al., 2010). The history of bonding developments is summarised in Table 2.2.

2.7.5.2 Self-Etch Adhesive

In the self-etch adhesive system, all the primary components (etchant, primer, and adhesive resin) are combined into a single solution or 1-bottle step (De Munck et al., 2005). The self-etch adhesive eliminates acid etch step, as it etches and primes enamel and dentin simultaneously without requirement for washing. It contains organic solvents, water and both hydrophobic and hydrophilic acidic functional monomers, combined into single mixture or solution (Wang & Spencer, 2004).

Self-etch adhesives technique have been described as being simpler to use, faster, and more user-friendly compared to total-etch system (Tunc et al., 2012). Their other advantages prior to sealant placement include being less technique-sensitive, requiring less operation time, and less likely to contaminate the etched surfaces (Asselin et al., 2008; Pashley & Tay, 2001). Since the self-etch adhesive offers less chair-side time, it is the best option for surface preparation prior to sealant placement particularly in children and uncooperative patients.

Self-etch adhesive systems typically act by partially dissolving hydroxyapatite to allow resin infiltration with minerals incorporated. It comprises of a mixture of acidic monomers, mainly phosphoric acid and or carboxylates. Self-etch adhesive systems have higher pH than that of conventional phosphoric acid etchants. Thus, self-etch adhesives technique produce less demineralization. In this type of adhesives, tooth surface demineralisation and dentine hybridization is achieved by the functional acidic monomer (Van Meerbeek et al., 2011).

A laboratory study was conducted earlier with aims to determine and compare the sealant microleakage after the placement of a light-activated sealant to ungrounded permanent enamel which had previously been treated using three pre-treatment protocols: conditioning of the enamel with 38% phosphoric acid; conditioning with phosphoric acid gel followed by one-bottle dentine bonding agent; and conditioning with a self-etch adhesive technique. The study found that the use of a bonding agent before fissure sealant application showed significantly reduced microleakage compared to the conventional acid etch technique with 38% phosphoric acid (Asselin et al., 2008).

A clinical trial over a three-year period, investigated the retention rate and caries preventive effect of resin based sealant using three adhesive methods i.e., fourth bonding generation (three-step-etch-and-rinse), fifth bonding generation (two-step-etch-and-rinse), and sixth bonding generation (one-step, two-component-self-etch), with the acid etching technique without use of adhesive as a control group. Significant differences were reported in between the groups. Observation at 3-years recall revealed that, the greatest retention rates of sealants on the first permanent molars were at the fourth and fifth bonding generation adhesive systems at 80.01% and 74.27%, respectively. The sixth generation adhesive system was found to have the lowest retention rate (42.84%) followed by the use acid-etch technique (62.86%). This study also described that the first permanent molars that had been sealed with the sixth generation of bonding agent showed significantly larger (34.28%) incidence rate of occlusal caries than the other types of adhesive systems used (Sakkas et al., 2013). Burbridge et al., (2007) reported that the etch-and-rinse adhesive system (fifth bonding generation) showed significantly greater retention rate compared to the self-etch adhesive system (sixth bonding generation) over one-year of observation.

Recently, a systematic review was conducted to investigate the retention rates of resin based pit and fissure sealant applied with or without the use of bonding agent. The authors also compared the sealant retention rate after the use of etch-and-rinse adhesive technique (fourth or fifth bonding generation) and self-etching adhesive technique (sixth or seventh bonding generation). The adhesive techniques were found to have a beneficial effect on the fissure sealant retention. The bonding agents enhance sealant penetration into the enamel micro porosities and, in turn, improving sealant-enamel adhesion. Thus, the authors concluded that the etch-and-rinse adhesive techniques are preferable than that of self-etch adhesive ones concerning the sealant retention capacity (Bagherian et al., 2016).

Self-etch adhesive systems can be classified based on the acid dissociation constants (pKa values) and, also depend on the acidity of the etching process. There are four class of them namely “strong self-etch” ($pH < 1$), “intermediately strong self-etch” ($pH \approx 1.5$), “mild self-etch” ($pH \approx 2$) and “ultra-mild self-etch” ($pH \geq 2.5$) (B Van Meerbeek et al., 2010).

The stronger acidity of the self-etch adhesive leads to deeper demineralization of the tooth structure. A "strong" self-etch adhesive offers better bonding properties (Perdigão et al., 2008). "Intermediately strong self-etch" is an intermediate pattern between "strong" and "mild" self-etching with evidence of demineralized top zone and partially demineralized base zone (Van Meerbeek et al., 2003). "Mild self-etch" removes the smear layer slightly, thus forming a delicate hybrid layer, while "ultra-mild" self-etching eliminates only a small amount of the smear layer and superficially discloses the collagen interconnections to produce a nanometer hybrid zone (Koshiro et al., 2006). Various studies have examined the bonding performance of self-etch adhesives but the findings are still debatable (Hiraishi et al., 2005; Lima et al., 2008; Reis et al., 2005).

2.7.5.2 (a) Universal Adhesive

Advancements in adhesive technology have led to the evolution of various bonding generations starting from non-etch technique to etch-and-rinse technique (4^{th} and 5^{th} generation), and then, followed by self-etch technique (6^{th}, 7^{th}, and 8^{th} generation). New adhesive techniques have been developed since 2011 with the latest being the 8^{th} generation bonding agents known as multi-mode or universal adhesives. It could be used on etched enamel or un-etched enamel surfaces as well as on dentine surfaces. The application has been broadened to selective enamel etch, etch-and-rinse adhesives, and self-etch adhesives. Nanofillers incorporation has led to improved mechanical properties of universal adhesive systems. The different composition of the other adhesive systems allows for more chemical and micromechanical bonding. The self-etch adhesive systems display a dual bonding mechanism with combination of chemical bonding and micro-mechanical bonding with the latter providing protection against mechanical stress while the former ensures a perfect marginal seal by preventing hydrolytic degradation (Perdigão et al., 2008; Van Meerbeek et al., 2011).

2.7.5.2 (b) Single Bond Universal Adhesive (SBU)

A new-generation of adhesive system, known as Single Bond Universal adhesive (3M ESPE) or the universal or multi-mode adhesive was introduced in 2011. It was claimed by the constructors to be applied as etch-and-rinse adhesive, self-etch adhesive, and selective etch application. It is considered ultra-mild due to its 2.7 pH (Van Meerbeek et al., 2010). Functional monomers which are 10-methacryloyloxydecyl dihydrogen phosphates (10-MDP) consist of a lengthy linear alkyl chain and phosphoric acid ester group. It is the main component of self-etch adhesives which form chemical bonding by interacting with the hydroxyapatite in the demineralized tooth surfaces. Chemical bonding between 10-MDP and enamel/dentine in a Single Bond adhesive forms a stable and durable interface (Mena-Serrano et al., 2013). 10-MDP interacts chemically with hydroxyapatite to produce stable and durable bonding (Yoshida et al., 2004). Moreover, the greater shear bond strength (SBS) of the Single Bond Universal is attributable to the presence of 10-MDP (Jayasheel et al., 2017).

Apart from chemical bonding, the physical properties and amazing conversion rate of its hydrophobic resin also produce excellent micro-mechanical bonding (Mena-Serrano et al., 2013). *In vitro* studies of caries-free cervical restorations using Single Bond Universal applied with self-etch adhesive and etch-and-rinse adhesive techniques after one-and-a-half-year observation period reported that, both modes resulted in a low incidence of clinical failures (Perdigão et al., 2014). The history of bonding developments is summarised in Table 2.2

Generation	Year	Mechanism/Steps	Description	Examples
1	1960s	Not used anymore	Enamel etch only and adhesive application - poor adhesion.	Cervident (S.S. White, Lakewood, NJ, USA)
2	1970s	Not used anymore	Enamel etch only followed by placement of adhesive, improved adhesion due to alterations in coupling agent.	Clearfil™ 2 Bond System F (Kuraray, Tokyo, Japan) Scotchbond™ (3M ESPE, Saint Paul, MN, USA) Bondlite (Kerr, Orange, CA, USA)
3	1980s/ 1990s	Selective-etch/ Multi-Step (Etch & Rinse)	Removal of smear layer by half. Acid etching, primer, then unfilled adhesive resin placement.	Scotchbond™ 2 (3M ESPE, Saint Paul, MN, USA) Clearfil™ New Bond (Kuraray, Tokyo, Japan)
4	1990s	Multi step (3 Step) (Etch & Rinse)	Completely removed the smear layer and formation of hybrid layer. Total-etch technique (etching enamel and dentin, rinsing, primer and adhesive.	Scotchbond™ Multi-Purpose (3M ESPE, Saint Paul, MN, USA) All-Bond 2^{R} (BISCO, Schaumburg, IL, USA)
5	Mid 1990s	Multi step (2 Step) (Etch & Rinse)	Separate etching step, rinsing enamel and dentin, followed by placement of a mixture of primer-adhesive solution.	OptiBond^{R} Solo (Kerr, Orange, CA, USA) Adper™ Single Bond (3M ESPE, Saint Paul, MN, USA) Prime & Bond^{R}, (Dentsply, York, PA, USA)
6	Late 1990s	2 Step Self-etch adhesive	Modify the smear layer forming a thin hybrid layer. Acidic primer (etchant + primer in single bottle) followed by bonding application (skipped rinsing step).	Clearfil™ SE Bond (Kuraray, Tokyo, Japan) OptiBond^{R} Solo Plus Self-Etch (Kerr, Orange, CA, USA)
	Early 2000s	Single Step 2 component Self-etch adhesive	Combination of etchant, primer and adhesive in one step, but requires pre-mixing before application.	Adper™ , Prompt™, L-Pop™ (3M ESPE, Saint Paul, MN, USA) Xeno^{R} III (Dentsply, York, PA, USA)
7	2000s	Single Step Self-etch Adhesive	Combination of etchant, primer and adhesive in one bottle.	Clearfil™ S^3 Bond (Kuraray, Tokyo, Japan) G-Bond™ (GC America, Alsip, IL, USA) (Kerr, Orange, CA, USA) iBond^{R} (HeraeusKulzer, Hanau, Germany)
8	2011	Total-etch/Self-etch/Selective-Enamel etch	Universal/Multimode Phosphoric acid pre- etching in total or selective etching.	Single Bond Universal (3M, ESPE, Saint Paul, MN, USA) Futurabond U (Voco, Cuxhaven, Germany)

Table 2.2. History of Bonding Agents, adopted from (Naaman et al., 2017)

2.8 Methods

2.8.1 Storage Medium for Extracted Teeth

Extracted teeth are in high demand in dentistry for *in vitro* investigations such as on microleakage, determining the shear and tensile bond strength of various bonding agents, dentine permeability, and marginal adaptation (Sandhu et al., 2012). Extracted teeth used for research and pre-clinical work must be kept in a storage medium prior to use. They must be cleaned and disinfected to prevent the spread of infectious, particularly blood-borne, diseases involving the Hepatitis B and Human Immunodeficiency Virus, and to protect them from dehydration (Tate & White, 1991).

Selection of the best storage medium is important to preserve dental hard tissue for standardization, reliability and reproducibility of laboratory-based results. Ideal storage medium should not influence the organic and inorganic components of the enamel and dentine substrate. Different storage mediums could probably transform the mechanical and physical properties of dental hard tissue by affecting adsorption, dissolution, and diffusion (Western & Dicksit, 2016).

Various methods applied for sterilization and disinfection of the extracted teeth include use of different concentrated of chemical solutions such as distilled water, normal saline, 1:10 household bleach, chloramine, thymol, alcohol, glutaraldehyde, formalin, and sodium hypochlorite. Other methods employed such as autoclaving, dry heat, ethylene oxide sterilization, and gamma radiation (Lee et al., 2007).

Guidelines by the Centres for Disease Control and Prevention (CDC) in the United States recommend that extracted teeth with existing amalgam restorations should be kept for 2 weeks in 10% formalin prior use. For counterparts, teeth with no amalgam restorations could be autoclaved for forty minutes at 121°C/20 psi (CDC, 2004).

However, this method is only applicable to investigations that evaluate the mechanical or physical properties of dental hard tissues which are not affected by heating from autoclaving procedures.

Enamel and dentine have different structural compositions. By weight, enamel has 95% inorganic contents, 4% organic contents, and 1% water. In contrast, the composition of dentine by weight is 70% mineral, 20% organic substances, and 10% water. Thus, dentine is more affected than enamel by storage regimes due to differences in structural composition (Habelitz et al., 2002). It has been proven that the mineral content of teeth such as calcium, potassium, and sodium are significantly affected by storage mediums and storage time (Secilmis et al., 2011).

The use of saline solution results in rapid mineral dissolution from the dentine due to the low concentration of calcium and phosphates and eventually leads to demineralization (Secilmis et al., 2011). A study recommended that the extracted teeth should be placed in distilled water, kept in a freezer soon after extraction to prevent post mortem changes in the dentine. Earlier researchers found comparable results for resin dentine bond strength of extracted teeth kept in different storage mediums such as in chloramine T, distilled water, neutral buffered formalin, and sodium hypochlorite (Titley et al., 1998). Another research reported that formaldehyde was not a good medium for extracted teeth as it caused the formation of formic acid from the oxidation process and subsequently led to a drop in the pH of the solution (Rueggeberg, 1991).

The International Organization of Standardization ISO11405:2015 recommends chloramine T as a storage medium for extracted teeth prior to use laboratory studies. Other studies use chloramine T solution for disinfection ranging from 0.1 to 1% concentration with a wide range of storage times from 1 week to months prior to use (Arslan et al., 2012; Ciucchi et al., 2015; Memarpour & Shafiei, 2014).

Storage duration after sealant placement directly influences sealant microleakage with a study reporting only minor changes in the marginal gap over time with adhesive materials (Gwinnett & Yu, 1995).

2.8.2 Microleakage Detection Methods

Clinically, microleakage are difficult to detect and various methods have been explored to evaluate marginal integrity such as dye penetration, use of bacteria, chemical tracers, radioactive isotopes, fluid permeability. Other methods used are by detections of marginal flow of water, use of air pressure, electrical application as well as neutron activation analysis (NAA) (Schmid-Schwap et al., 2011).

2.8.2.1. Dye Penetration Method

The dye penetration method is a widely accepted, practical, and inexpensive method and offers an accepted degree of reliability (Taylor & Lynch, 1992). Coloured dye agents can penetrate and stain the sealant-enamel interface and be detected after longitudinal tooth sectioning and assessing under a stereo optical microscope or scanning electron microscopy (SEM). The stain appears in contrasting colours to both the tooth and sealant. This method is effective in evaluating the sealing capability of the sealant material at the enamel-sealant interface (Yavuz et al., 2013).

The dye penetration method is inexpensive, readily available, easily reproducible, and highly feasible. It does not require another chemical series of events and not has any disclosure to potentially unsafe radiation. However, their disadvantages include subjectivity and sample destruction (Subramaniam & Pandey, 2016).

2.8.2.2 Types of Dye

Different dyes act differently based on their particle sizes which affect penetration into the tooth and sealant interface. Fuchsin dye has been widely used by researchers in previous decades (Cehreli et al., 2006; Derelioglu et al., 2014; Sungurtekin & Öztaş, 2010). Moreover, the dye infiltration rate of basic fuchsin remain nearly consistent and steady over a duration of 4.5 months (Mueninghoff et al., 1990).

A total of 144 microleakage studies has been reported in the literature with fuchsin being the most frequently used (40.7%) followed by methylene blue (22%) and silver nitrate (17%). The study found no statistically significant difference in tracer penetration between three tested tracers at the couple of enamel and dentinal margins. However, only fuchsin and silver nitrate penetration was found to have acceptable correlation with a scanning electron microscope with quantitative marginal analysis resulting at dentinal margins (Raskin et al., 2001).

The concentration ranges of tracers frequently used were 0.5-5% methylene blue (Dalli et al., 2013; Meller et al., 2015) and 50% silver nitrate solution (Guedes Pontes et al., 2002). The other tracers reported in the literature include 2% fluorescence, 0.5% crystal violet, 50% India ink, erythrosine B, and 1% rhodamine B with propylene glycol (Schmid-Schwap et al., 2011). Methylene blue is unstable at room temperature and when exposed to surrounding light. It is easily turned into leuco methylene blue in which it becomes colourless in existence of hydroxyl ion (Heintze et al., 2008).

As reported in the literature dye immersion times ranged from 1 to 48 hours though the most current practise was 24 hours at room temperature (37º C) (Schmid-Schwap et al., 2011).

2.8.3 Thermocycling

Thermocycling is an artificial aging technique used to pretend the natural changes of the intra oral condition such as temperature and moisture. It is defined as the *in vitro* process of applying an extracted tooth after placement of restoration to intense temperature to mimic those thermal changes in the mouth. This method has been used extensively to study the microleakage of dental materials as well as the marginal adaptability of dental restorative materials. The use of temperature changes in a thermocycling machine is important as the thermal expansion of a dental material contributes to microleakage.

Clinically, intra-oral temperature changes could probably generate crack through bonded interfacing between the dental hard tissue and restorative or sealant materials; hence the formation of micro gaps at margins allows the ingress of pathogenic microorganisms from the oral cavity which, in turn, lead to microleakage and failure of certain restorative materials. In the literature, a wide range of thermocycling cycles has been reported from 100 to 30,000 cycles with min temperature of 5º C to a maximum of 70º C. Travel and dwell times in between baths as reported in previous studies were 3-36 seconds and 10-60 seconds, respectively (Schmid-Schwap et al., 2011).

2.9 *In Vitro* Pit and Fissure Sealant Microleakage Studies

2.9.1 *In Vitro* Fissure Sealant Microleakage Studies in Permanent Teeth

Marks et al., (2009) evaluated the effect of adhesive agents and fissure morphology on the microleakage and penetrability of pit and fissure sealants *in vitro*. Sealants used in their study was Aegis (Bosworth), Conseal F (Southern Dental Industries), and Admira Seal (Voco). Adhesive agents used was Optibond Solo Plus (sds/Kerr) and Clearfil S³Bond (Kuraray). Ninety extracted permanent molars were randomly assigned to 9 groups. A sealant and control group (phosphoric acid etch only) was also included. Dye penetration (microleakage), penetrability, and fissure morphology assessment was performed for the treatment groups through microscopic evaluation. Results showed that there was a significant (P = .003) differences in microleakage, with the Aegis + control and Aegis + Optibond Solo Plus groups displaying less leakage, while significant (P = .03) differences were also noted between groups regarding penetrability. Fissure morphology was not a significant (P = .82) factor affecting microleakage; however, fissure type did significantly (P < .001) impact penetrability. No correlation was found between the extent of microleakage and penetrability. Therefore, they concluded that the application of sealants using phosphoric acid as a conditioning agent revealed superior results, while the use of adhesives was found to be unnecessary.

Memarpour and Shafiei in 2014, investigated *in vitro*, the fissure sealant microleakage after application of antibacterial adhesive and bonding agent prior to fissure sealant placement on intact enamel. The study included hundred twenty sound third mandibular molar teeth, which were randomly divided into 6 groups of 20 teeth each. Occlusal pits and fissures were sealed with unfilled sealant material (Clinpro, 3M) after pretreatment with 1. phosphoric acid etching (control); 2. acid etching + Adper Single Bond 2 (SB, 3M); 3. chlorhexidine digluconate (CHX, Ultradent) + acid etching; 4. CHX

+ acid etching + SB; 5. acid etching + Clearfi l Protect Bond (CPB, Kuraray) 6. CPB alone. After 6-month water storage and thermocycling, the specimens were placed in 0.5% fuschin, sectioned and evaluated under a digital microscope. They found out that there was a significant difference between groups at $p < 0.05$. Acid etching alone and with SB showed the lowest microleakage, followed by acid etching + CPB. Chlorhexidine with and without bonding agent showed the greatest microleakage. Therefore, it was concluded that conventional acid etching alone or with a one-bottle adhesive were the two most effective methods of reducing microleakage from fissures. Acid etching together with a self-etching adhesive showed better results than self-etching alone. Appling CHX increased microleakage in sealed teeth (Memarpour & Shafiei, 2014).

Another *in vitro* study investigated the marginal leakage and the infiltration ability of pit-and-fissure sealants by applying the conventional sealing technique and compared with fissure sealant applied with additional bonding agent. Extracted non-carious permanent molars (n = 60) were included, teeth were stored in sterile saline solution initially, and then assigned to one of two groups: group C (control) was sealed (Helioseal F) by using the conventional technique, while in group BA (bonding agent), a bonding agent (OptiBond FL) was additionally applied prior to sealing. The teeth were thermocycled (1000 cycles, 5°C to 55°C, dwell time 30 seconds), then varnished and immersed in 5% methylene blue solution for 24-hour. After embedding and sectioning each tooth into 6-12 slices, the presence of microleakage, unfilled areas, and air bubbles trapped in the sealant were assessed with a stereomicroscope. The authors reported that a higher proportion of microleakage was found under sealants applied without the additional use of the bonding agent. A statistically significant difference in microleakage was noted between the groups ($p = 0.045$). Thus, they summarized that, fissure sealant applied with an additional use of bonding agent reduced fissure sealant microleakage (Meller et al., 2015).

Baygin et al., (2012) examined the effects of different techniques of surface treatment on the microleakage of a fissure sealant in permanent molar teeth. A total of 50 freshly extracted non-carious human third molars were randomly assigned to one of five groups. Occlusal fissures were treated with one of the following: acid etching with 35% orthophosphoric acid (group 1); fissurotomy with a Fissurotomy Micro NTF metal bur (group 2); laser etching with an Er,Cr:YSGG laser at 2 W and 20 Hz (group 3); laser etching with an Er,Cr:YSGG laser at 2 W and 40 Hz (group 4); and air abrasion for 20 s with 30-µm Al_2O_3 particles via a CoJet Prep device (group 5). After surface pretreatment, a resin-based sealant was applied to the fissures. The sample teeth were subjected to thermocycling and stored in distilled water at 37°C for 1 month. Following immersion in 0.5% basic fuchsin solution for 24-hour, three buccolingual slices of each sample tooth were scored under a stereomicroscope, and the morphological appearance of the area between the enamel surface and fissure sealant was examined under a scanning electron microscope. The Kruskal-Wallis test and one-way ANOVA revealed significant differences in marginal leakage, as follows: group 1 showed significantly lower scores than groups 2 and 5, the scores of groups 1, 3 and 4 were not significantly different, and group 2 showed significantly higher scores than groups 3 and 4. Laser irradiation, the metal bur, and the CoJet Prep device did not eliminate the need for acid etching of the enamel prior to placement of a fissure sealant. However, laser etching at 2 W (20 Hz or 40 Hz) may be an alternative to conventional acid-etching.

Khogli et al., (2013) conducted an *in vitro* study to compare the microleakage and penetration depth of a hydrophilic sealant and a conventional resin-based sealant using one of the following preparation techniques: acid etching (AE) only, a diamond bur + AE, and Er:YAG laser combined with AE, and to evaluate the microleakage and penetration depth of the hydrophilic pit and fissure sealant on different surface conditions. Eighty extracted third molar teeth were randomly assigned to eight groups of ten teeth each according to the material, preparation technique, and surface condition. For saliva contamination, 0.1 mL of fresh whole human saliva was used. All samples were submitted to 1000 thermal cycles and immersed in 2% methylene blue dye for 4-hour. Sections were examined by a light microscope and analysed using image analysis software (Sigmascan®). The researchers found out that the combination of Er:YAG + AE + conventional sealant showed the least microleakage. Er:YAG ablation significantly reduced the microleakage at the enamel–sealant interface compared to the non-invasive technique. The hydrophilic sealant applied on different surface conditions showed comparable result to the conventional resin-based sealant.

Recent *in vitro* study aimed to evaluate and compare the marginal leakage and penetration ability of a moisture-tolerant (Embrace Wet Bond ™) and a conventional (Clinpro) resin-based sealants under three different enamel surface preparations (acid etched, acid etched and saliva contaminated and bur preparation and acid etched). One hundred and twenty extracted caries free human premolars teeth were cleaned and randomly divided into six groups of equal numbers, according to the type of sealants used and surface preparations. All the sealed teeth were subjected to thermocycling and immersed in a methylene blue dye. Each tooth was then embedded into acrylic resin before it was sectioned into four sections per tooth. Marginal leakage and unfilled surface area (indicating penetration depth of resin) were then measured using an optical 3D measurement device (Alicona Infinite Focus®). Both sealants exhibited comparable proportion of marginal leakage on acid etched only surfaces. Moisture-tolerant sealant showed the least proportion of marginal leakage on bur prepared and etched surfaces. Presence of saliva has detrimental effect on adhesion of both sealants. Nevertheless, depth of penetration of sealant into the fissures is comparable with both sealant types irrespective of the surface preparations (Marimuthoo et al., 2017).

2.9.2 *In Vitro* Fissure Sealant Microleakage Studies in Primary Teeth

Tulunoglu et al., in 1999, conducted an *in vitro* study to investigate the effect of use of three dentine bonding agents: Scotchbond Multi-Purpose Plus® (3M Dental Products, St. Paul, Minnesota, U.S.A.), Syntac® (Vivadent, Schaan, Liechtenstein), Optibond Dual Cure® (Kerr, Romulus, MI, U.S.A.) on microleakage and shear bond strength of a fissure sealant (Helioseal F®, Vivadent, Schaan, Liechtenstein) bonded to either dry or wet (saliva contaminated) enamels of primary teeth. Newly extracted 112 non-carious primary teeth were sectioned and embedded in resin blocks. Eight groups were formed for each test. Each group consisted of 14 specimens. Group 1 and 2: fissure sealant was applied directly to etched enamel in dry and wet condition, respectively; Group 3 and 4: fissure sealant was applied onto etched and Scotchbond Multi-Purpose Plus® treated enamel in dry and wet condition, respectively; Group 5 and 6: fissure sealant was applied onto etched and Syntac® treated enamel in dry and wet condition, respectively; Group 7 and 8: fissure sealant was applied onto etched and Optibond Dual Cure® treated enamel in dry and wet condition, respectively. The results revealed that the use of an enamel–dentine bonding agent under fissure sealant increased the bond strength and decreased the microleakage. The use of enamel–dentine bonding agents under sealant in moisture contaminated conditions gave better results than applying sealant alone onto non-contaminated teeth. Scotch Bond Multi-Purpose Plus® yielded the best results for both tests.

Recent *in vitro* study in primary second molar teeth aimed to compare the microleakage and penetration depth of hydrophilic and hydrophobic sealants using acid-etching on dry and moist surfaces. Extracted 28 second primary molars were assigned to two groups (hydrophobic group I; hydrophilic group II) depending on the surface condition (dry group: A1 and B1; moist group: A2 and B2) of 7 teeth in each group.

Samples from group A1 and B1 were cleaned and dried with a 3-way syringe and etched with etching gel, and sealant was applied to the fissures and cured with visible light. Sample from A2 and B2 were immersed in 0.1 mL of fresh whole human saliva for 20 seconds and dried using a pellet cotton, and the same procedure was carried out. All samples were subjected to 1000 thermal cycles and sectioned to compare the depth of penetration and microleakage. Sections were then examined under light microscope and analyzed using an image analysis software (SigmaScan). The results showed that the least microleakage was seen with hydrophilic sealant under moist surface condition, and the depth of penetration of hydrophobic sealant was found to be better than that of hydrophilic sealant in both dry and moist surface conditions. Therefore, hydrophilic pit and fissure sealants showed higher tolerance to saliva contamination with less microleakage, but in terms of penetration ability, hydrophobic sealants were found to be superior (Gawali et al., 2016).

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3: MATERIALS AND METHOD

3.1 Ethical Approval

Ethical approval for the present study was obtained from the Research Ethics Committee, Faculty of Dentistry, University of Malaya with medical ethics committee reference number DF CD 1612/0070 (P).

3.2 Study Design

This is an experimental laboratory study to determine and to compare the effects of different treatment protocols (acid etching, etch-and-rinse adhesive, and self-etch adhesive) on microleakage around fissure sealant margins in primary and permanent molar teeth. All fissure sealant applications were carried out at the Paediatric Dental Clinic while all laboratory works were conducted in the Biomaterial Research Laboratory (BRL) of the Research and Post-graduate Tower, Faculty of Dentistry, University of Malaya.

3.3 Sample Size Calculation

Sample size calculation was done after discussion and consultation with the statistician using a PS software: Power and Sample Size Calculation programme version 3.1.2 Vanderbilt Biostatistics (Dupont WD). The calculation of the sample size required for this study is shown in Table 3.1

Table 3.1. Sample size calculation

Article	α	Power	σ	δ	M	n
Memarpour and Shafie, 2014	0.05	0.80	0.23	0.17	1	30

α = the alpha error level

Power = the probability of correctly rejecting the null hypothesis when the null hypothesis is false

σ = estimated standard deviation of the sample being studied

δ = a minimum difference taken from a previous study with the same methodology

n = estimated required sample size

m = the ratio of control to experimental subjects

Following the sample size calculations, a total of 30 analysable tooth sections is needed per group based on the above shown parameters: alpha error level of 5%, power of 80%, a minimum difference of 0.17 and a standard deviation of 0.23, to enable rejection of the null hypothesis and to find statistically significant results between the groups with a probability of 80% and a significance level of 0.05.

3.4 Sample/Tooth Collection

A total of 60 sound extracted teeth consisting of thirty human permanent mandibular third molars and primary mandibular second molars each were collected for this study. The permanent mandibular third molar teeth were collected from the Oromaxillofacial Clinic and day care operation theatre (OT) after approval from the Head of Department of Oromaxillofacial Surgery. The extracted human mandibular second primary molar teeth were taken from the Paediatric Dental Clinic, Faculty of Dentistry, University of Malaya. All teeth used for microleakage testing were within one-month post extraction, as recommended by (ISO, 2015). Extracted teeth can be used within six-month period after extraction, to avoid degenerative changes in dentinal protein.

3.5 Inclusion and Exclusion Criteria

Tooth selection and collection followed the inclusion and exclusion criteria as shown.

Table 3.2. Inclusion and exclusion criteria

Inclusion Criteria	**Exclusion Criteria**
Sound human permanent mandibular third molar tooth	Tooth with existing restoration
Sound human primary mandibular second molar tooth	Carious tooth
	Tooth with enamel defects
	Fractured tooth

3.6 Preliminary Study

A preliminary study was carried out on ten samples from January to April 2017. This aided in familiarizing the researcher with the laboratory techniques involved in this study. Apart from intra-examiner and inter-examiner calibration, it provided an idea of estimation times needed throughout the procedure starting from tooth cleaning, sealant placement, aging for one month, thermocycling, immersion in dye, tooth embedding in epoxy resin, tooth sectioning to examination under a stereomicroscope.

It was a very time-consuming process involving more than a month to complete all laboratory procedures including the one-month aging. Difficulties encountered included detachment of the nail varnish, prolonged time taken for epoxy resin to set after tooth embedding, detachment of the tooth from the epoxy resin, and fractured samples during tooth sectioning. All these issues were resolved through frequent practice and guidance as well as discussions with the laboratory technician and supervisor.

3.7 Specimen Preparation

3.7.1 Tooth Disinfection and Storage Media

All the extracted teeth were disinfected in 0.5% chloramine T trihydrate solution for 1-week as recommended by (ISO, 2015). They were cleaned of gross debris with ultrasonic scaler (Piezon® Master 400, Switzerland) and stored in distilled water at 4º C until use.

3.7.2 Tooth Cleaning Method

The occlusal surfaces of all teeth were cleaned with a slow-speed hand piece and a brush with slurry pumice. All (n=30) permanent and (n=30) primary molars teeth were then randomly selected into three groups of ten each to receive the different treatment protocols on the occlusal surfaces before fissure sealant application, as shown in Table 3.3. Details of the materials used are shown in Table3.4. The summary of the research flow is shown in Figure 3.9.

Table 3.3. Different enamel surface treatment protocols

Permanent Group	Dental Materials Used	Enamel Surface Treatment Protocol
1		Use of 37% orthophosphoric acid etching (Scotchbond™, 3M, ESPE) for 15s + rinse for 15s, dry for 5s + Clinpro fissure sealant, light cure for 20s
2		Use of 37% orthophosphoric acid etching (Scotchbond™, 3M, ESPE) for 15s + rinse for 15s, dry for 5s + bonding agent (Prime&Bond) for 20s, gentle air dry 5s, light cure 10s + Clinpro fissure sealant, light cure for 20s
3		Use of self-etch adhesive (Single Bond Universal Adhesive) for 20s, gentle air dry for 5s, light cure 10s + Clinprofissure sealant, light cure for 20s
Primary Group		**Enamel Surface Treatment Protocol**
4		Use of 37% orthophosphoric acid etching (Scotchbond™, 3M, ESPE) for 15s + rinse for 15s, dry for 5s + Clinpro fissure sealant, light cure for 20s
5		Use of 37% orthophosphoric acid etching (Scotchbond™, 3M, ESPE) for 15s + rinse for 15s, dry for 5s + bonding agent (Prime&Bond) for 20s, gentle air dry for 5s, light cure 10s + Clinpro fissure sealant, light cure for 20s
6		Use of self-etch adhesive (Single Bond Universal Adhesive) for 20s, gentle air dry 5s, light cure 10s + Clinpro fissure sealant, light cure for 20s

Table 3.4. Composition of materials and manufacturers' instructions

Material	Chemical Compositions	Manufacturer's Instructions	Manufacturer
Clinpro™ Sealant	Bisphenol A Diglycidyl methacrylate (Bis-GMA), Triethyleneglycol dimethacrylate(TEGDMA), Titanium Dioxide (TiO_2), DL-Camphorquinone, Ethyl 4-(dimethylamino)benzoate (EDMAB),	Etch for 15 seconds, but no more than 60 seconds. Thoroughly wash teeth with air/water spray to remove etchant. Then, dry the etched surfaces. Using the syringe needle tip or a brush, apply sealant into the pits and fissures. Light curing for 20s.	3M, St. Paul, MN, USA
Prime & Bond NT Total-etch	PENTA; UDMA resin; resin R5-62-1; T-resin; D-resin; nanofiller; initiators; stabilizer;cetylamine hydrofluoride;acetone; hydroxyethylmethacrylate.	Etch for 15 seconds. Then, rinse with water spray for 15 seconds and remove water with a soft blow of air. Leave a moist surface. Apply the adhesive to saturate the surface, reapply if necessary. Leave the surface undisturbed for 20 seconds. Remove solvent by blowing gently with air for at least 5 seconds. Light cure for 10 seconds	Dentsply / De Trey GmbH, Konstanz, Germany.
Single Bond Universal Self-etch 1 step	MDP Phosphate monomer, Di-methacrylate resins, HEMA,Vitrebond™ Copolymer,Filler, Ethanol, Water, Initiators, Silane	Apply adhesive to the tooth surface for 20 seconds, then gentle air drying for 5 seconds, followed by 10 seconds of light curing	3M, St. Paul, MN, USA
Scotchbond™ Etchant	Etching Liquid contains 37 % by weight phosphoric acid	Clean with a pumice-water slurry. Dispense the etchant onto a dispensing pad or dappen dish and apply to surfaces to be etched with a brush. Leave etchant or etching liquid in place for 15 seconds. Then, thoroughly rinse for 15 seconds. Dry for 5 seconds. Depending on the adhesive system used, air drying may not be recommended	3M ESPE St. Paul, USA

(a) Clinpro resin based sealant

(b) 37% phosphoric acid etch

(c) Etch-and-rinse adhesive

(d) Self-etch adhesive

Figure 3.1. Materials used: (a) Clinpro sealant (3M, ESPE), (b) Scotchbond etchant (3M, ESPE), (c) Prime & Bond etch and rinse (Denstply), (d) Single Bond Universal (3M, ESPE)

3.7.3 Resin Based Fissure Sealant Placement

After applying the different enamel surface treatment protocols as described in Table 3.3, all teeth were sealed with strict adherence to the manufacturers' instructions with an unfilled resin-based fissure sealant (Clinpro, 3M ESPE; St. Paul, MN, USA) directly onto the occlusal surface from sealant dispensing tips. The sealants were stirred with the syringe tip during or after placement to remove any existing bubbles and to improve sealant flow into deep pits and fissures. Subsequently, light curing was done for 20 seconds using a Light Emitting Diode (LED), Kerr, Demi^{TM} Plus light curing model (921638) with an output intensity of 450 mW/cm^{2}with 450 nm wavelength by placing the curing tip in close proximity to the sealant, without affecting the sealant.

As recommended by the manufacturer, the curing light must have a minimum output of 400 mW/cm^2. For standardization, only one light cure unit was used for all groups and it was calibrated and checked with a hand-held LED curing radiometer (Model 100 Curing Radiometer; Demetron Research Corporation, Danbury, USA), with serial number 130628 to ensure standardized and adequate output intensity for each group. The sealant margins were checked with a sharp explorer to ensure complete fissure sealant coverage and marginal adaptation to the enamel surface, and to inspect for voids.

3.7.4 Aging and Thermocycling

After resin-based sealant application, all teeth/specimens were kept in distilled water at 37º Celcius for one-month of aging. The distilled water need to be regularly changed on a weekly basis to prevent infection. After the aging process, the samples underwent thermocycling (ATDM T6PD UM, Malaysia) following the ISO (2015). The teeth were subjected to 1000 thermocycles at 5º C and 55º C and a dwell time of 20 seconds with a 10-second transfer interval.

3.7.5 Coating with Nail Varnish

The apices of the teeth and all tooth surfaces were sealed with double layer of waterproof nail-varnish except for the sealant and a 1-mm rim surrounding the fissure sealant and allowed to dry. The same nail varnish, brand name; Gel it! with bloop was used for all sample groups. It was a professional gel manicure within minutes, and no ultra violet (UV) light needed. The apical area of the teeth was then covered with sticky wax.

3.7.6 Immersion in Dye Solution (0.5% basic fuchsin)

Fuchsin solution was prepared by a laboratory technician using the formula prescribed by the United States Food and Drug Administration, Bacteriological Analytical Manual (BAM) (FDA, 1995). 0.5 g basic fuchsin dye was dissolved in 20 ml of 95% ethanol and the solution was then diluted to 100 ml with distilled water.

The teeth were subsequently placed in the prepared (0.5% basic fuchsin) dye solution (Batch number: HX68102415, Merck; Darmstadt, Germany) for 24 hours at room temperature (37º C) to allow dye diffusion into possible micro gaps at the enamel-sealant interface.

After 24-hour immersion in the dye, the teeth were washed thoroughly under tap water and each specimen were fixed vertically in clear cold curing epoxy resin (Quickmount 2 fast epoxy). Epoxy resin was mixed by the researcher using EHF-3000-32 hardener and ERF-3000-128 resin using 1:10 ratio. The sealed teeth were fixed in the prepared epoxy resin in the embedding-form with two small moulds of 7 mm in length as shown in Figure 3.6. The epoxy resin was allowed to set prior to sectioning.

3.7.7 Tooth Sectioning

Tooth sectioning was done using a low speed precision cutter (Micracut 125) as shown in Figure 3.8 (a) with a water-cooled Pace 5” diamond wafering blade (WB-0055HC). A lubricant was needed in each tooth-cutting to avoid drying and fracturing of the samples. Each tooth was cut longitudinally in a buccolingual orientation or direction through the fissure sealant producing four tooth slices. Based on sample size calculations, 30 samples were needed for each group to be evaluated and scored for microleakage resulting in a total of 180 surfaces for all groups. All slices of each tooth were labelled and placed in individual containers. Figure 3.7 shows the three surface areas to be examined under a stereomicroscope. The samples were then rinsed and allowed to dry.

3.7.8 Observations of Microleakage under a Stereomicroscope

The stereomicroscope (Olympus, Japan) was calibrated prior to use and the images were then captured at 20-fold magnification. The images were scored based on microleakage criteria from the previous study (Baygin et al., 2012) as shown in Table 3.5. Two examiners under blinded conditions evaluated each slice twice.

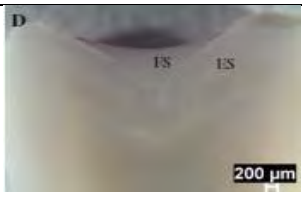
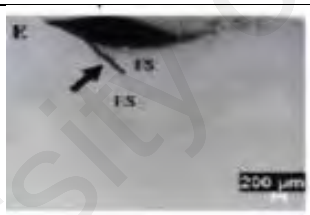
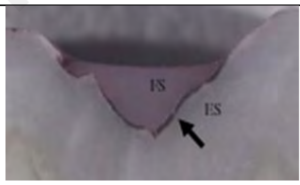
3.7.9 Inter- and Intra-examiner Reliability

Reliability between two observers (SP) and (SM) was assessed in a pilot study conducted earlier using 10 samples. To determine intra-examiner reliability, the microleakage scores were assessed again after a two-week interval.

3.8 Microleakage Scores

The evaluation of the dye penetration is described in Table 3.5.

Table 3.5. Definition and diagram of microleakage score used in the study

Microleakage Score	Image	Microleakage Definition
0		No dye penetration at sealant enamel interface
1		Dye penetration up to one-half or less of the sealant depth penetrated
2		Dye penetration more than one-half penetrated, but not up to the sealant base
3		Dye penetration up to the sealant base

Adapted from (Baygin et al., 2012)

FS- Fissure sealant

ES- Enamel surface

Figure 3.2. The extracted teeth after cleaning with a slow-speed hand piece and a brush with slurry pumice

Figure 3.3. The extracted teeth after sealant placement according to the treatment protocol

Figure 3.4. The apices of the teeth and all teeth surfaces were sealed with double layers of nail-varnish except for the sealant and a 1-mm rim surrounding the fissure sealant

Figure 3.5. 0.5% basic fuchsine dye solution (Merck; Darmstadt, Germany)

Figure 3.6. The samples were fixed vertically in clear cold curing epoxy resin in the embedding-form with 2 small moulds

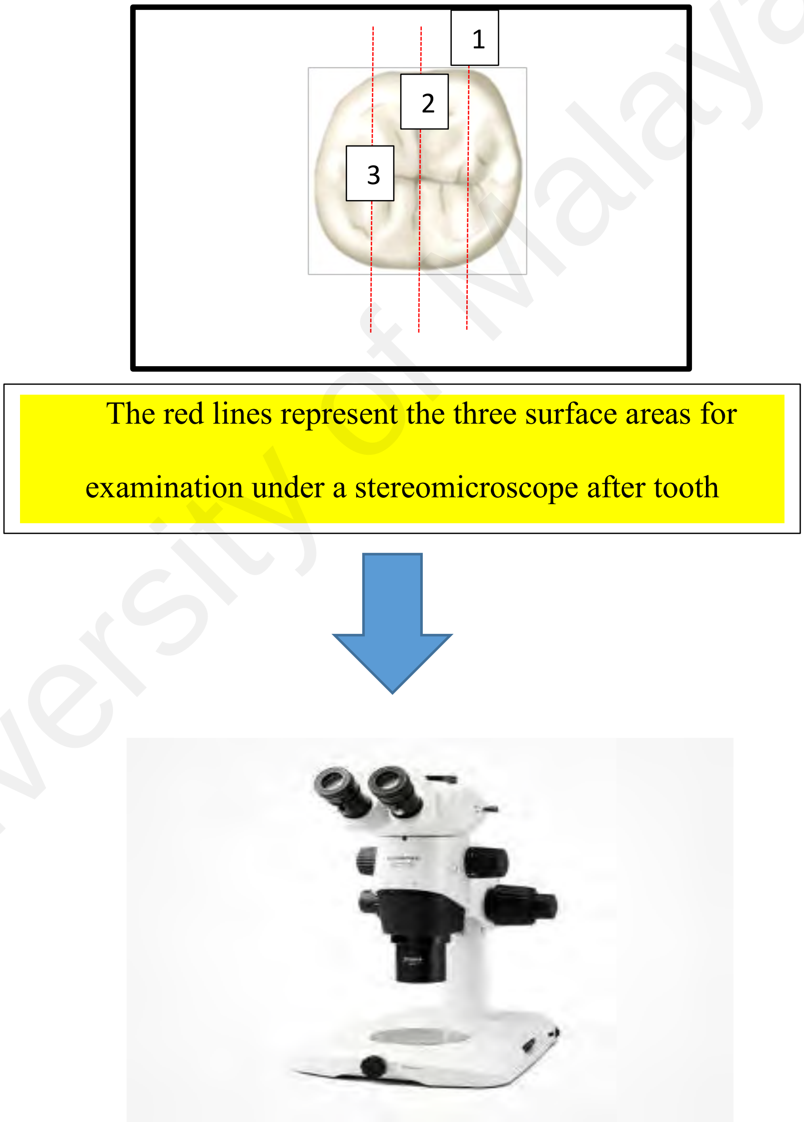


Figure 3.7. The three surfaces used for examination under a stereomicroscope

(a)

(b)

(c)

Figure 3.8. Laboratory equipment used: (a) Low speed precision cutter (Micracut 125), (b) Thermocycling machine, (c) Stereomicroscope (Olympus)

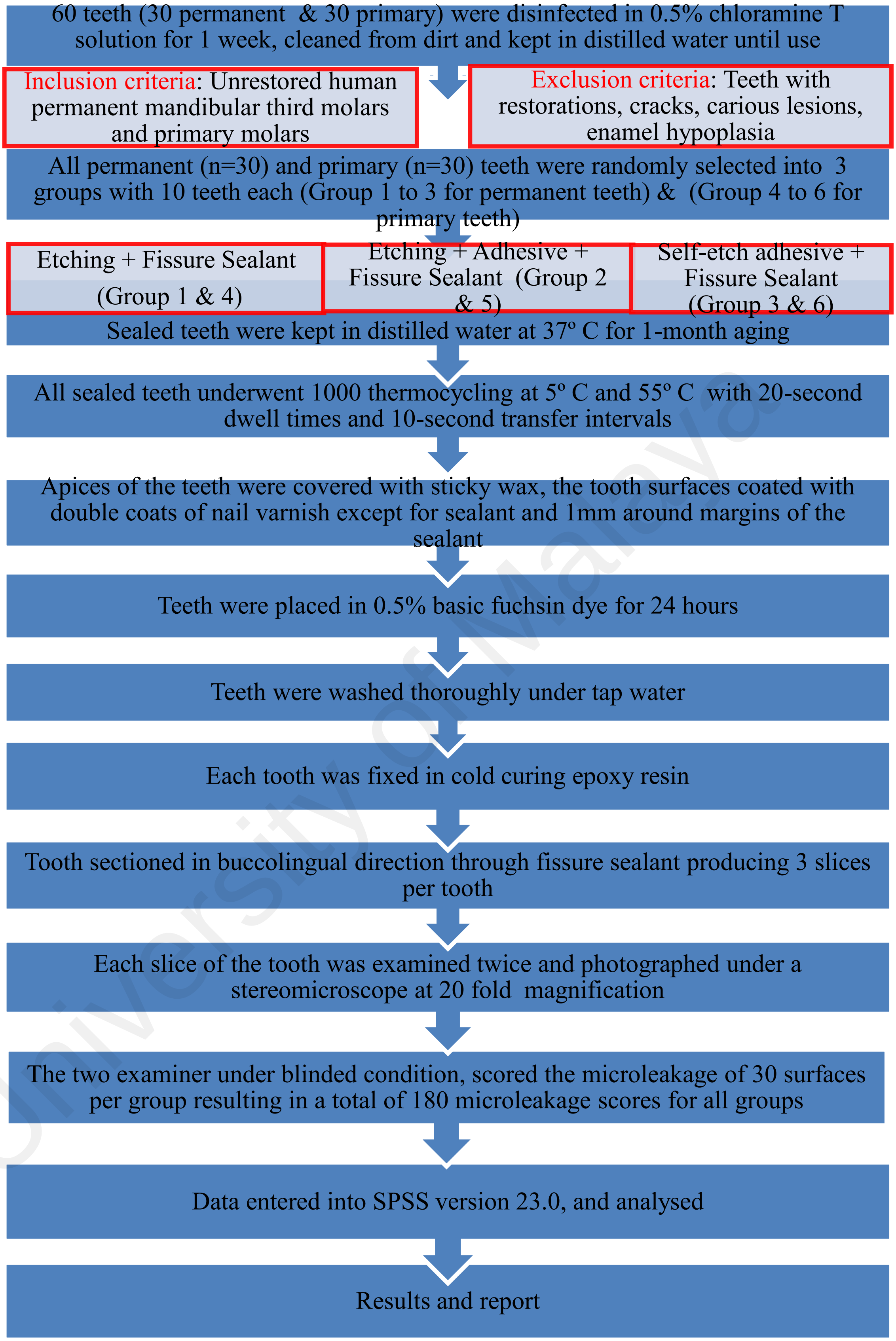


Figure 3.9. Summarized flow chart of the research methodology

3.9 Data Analysis

Inter-examiner and intra-examiner reproducibility was carried out with Cohen kappa statistics. The results were tabulated and analysed using IBM SPSS Data Editor Version 23.0 (SPSS Inc. Chicago, USA). Microleakage distributions were assessed and presented in frequency and percentage and the normality of the data was determined using Shapiro-Wilk test. Since the data were not normally distributed, a non-parametric Kruskal-Wallis test (comparison between groups with three different pre-treatment protocols) was used for statistical evaluation. A p value < 0.05 was set to be significant.

CHAPTER 4

RESULTS

CHAPTER 4: RESULTS

4.1 Inter-examiner and Intra-examiner Reliability

Table 4.1: Cohen's kappa (κ) values for inter- and intra-examiner reliability

	Inter-examiner Reliability	Intra-examiner Reliability
Value of κ	0.925	0.931

Table 4.1 showed that there was very good agreement for both inter- and intra-examiner assessments for microleakage scoring. The kappa value (***K)*** were interpreted as below:

Value of *K*	**Strength of agreement**
< 0.20	Poor
0.21 - 0.40	Fair
0.41 - 0.60	Moderate
0.61 - 0.80	Good
0.81 - 1.00	Very good

(Altman, 1990)

Table 4.2. Normality of the data

Group	Kolmogrov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	Df	Sig.
1	.539	30	.000	.180	30	.000
2	.354	30	.000	.637	30	.000
3	.473	30	.000	.526	30	.000
4	.537	30	.000	.275	30	.000
5	.406	30	.000	.612	30	.000
6	.457	30	.000	.554	30	.000

a. Lilliefors Significance Correction
b. $p < 0.001$
FS - Fissure sealant

Group 1- Permanent phosphoric acid etching and FS

Group 2- Permanent etch-and-rinse adhesive and FS

Group 3- Permanent self-etch adhesive and FS

Group 4- Primary phosphoric acid etching and FS

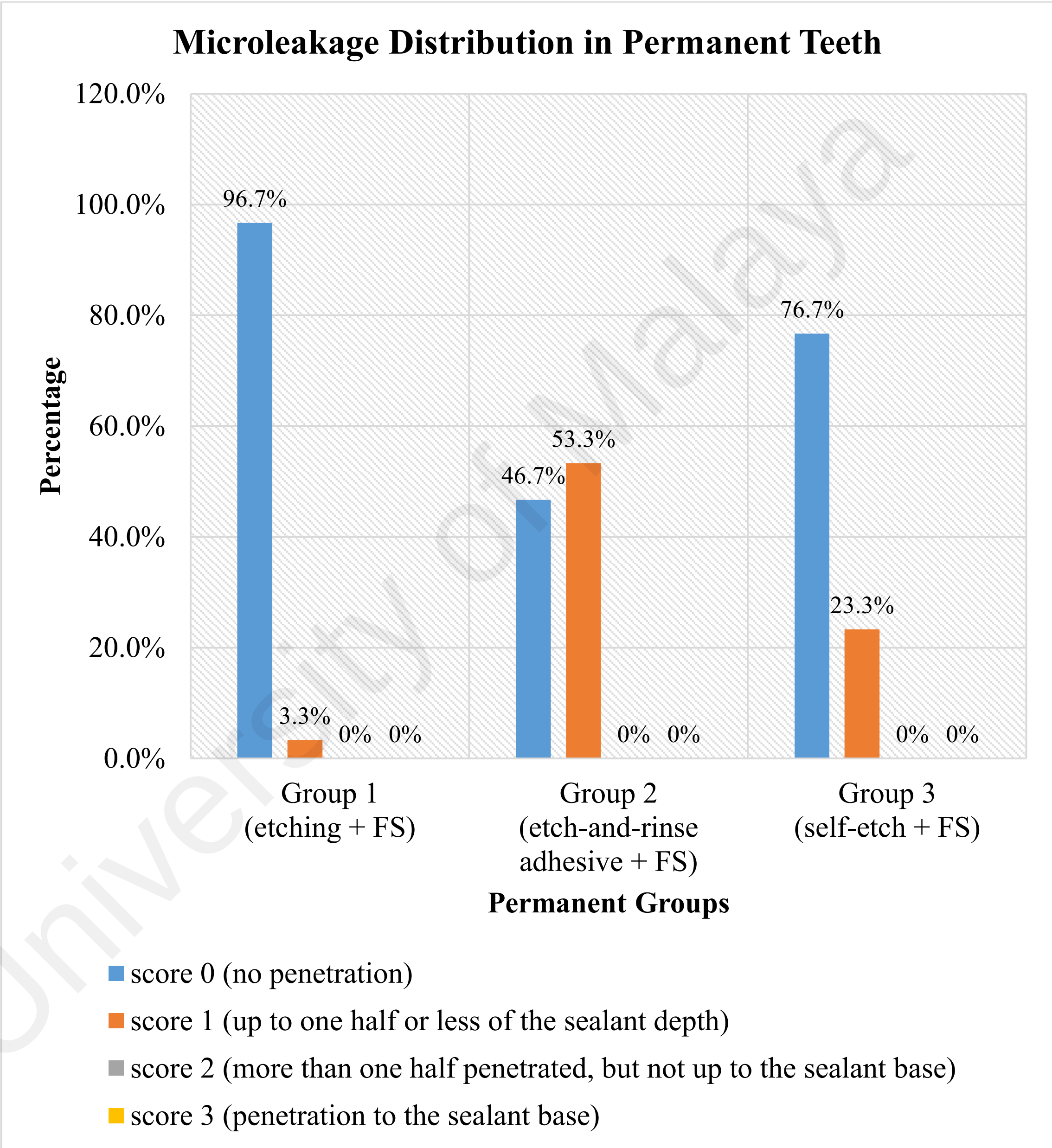
Group 5- Primary etch-and-rinse adhesive and FS

Group 6- Primary self-etch adhesive and FS

Table 4.2 showed normality of the data. In this study, the Shapiro-Wilk test was used due to the sample size being less than 50 per group. Since $p < 0.001$, which is less than 0.05, the assumption of data normality was not met. Therefore, a non-parametric Kruskal-Wallis test (comparison between groups with three different surface preparations) was used for statistical evaluation. A p value < 0.05 was considered to be significant.

4.3 Distribution of Microleakage Scores in Permanent Teeth

There was a total of 6 groups in the study comprising 3 groups each for the permanent and primary teeth with ten molars per group. Following sectioning, 30 samples were included per group yielding a total of 180 tooth surfaces.



FS-Fissure Sealant

Figure 4.1. Percentage of microleakage distribution in the permanent teeth

Table 4.3. Distribution of microleakage scores of the resin-based sealant (Clinpro) after different pre-treatment protocols in the permanent teeth groups

Sample group	Microleakage Score				Total
	Score 0	Score1	Score 2	Score3	
	N (%)	N (%)	N (%)	N (%)	N(%)
Group 1 (Acid Etching and FS)	29 (96.7)	1 (3.3)	0	0	30 (100)
Group 2 (EARA and FS)	14 (46.7)	16 (53.3)	0	0	30 (100)
Group 3 (Self-etch and FS)	23 (76.7)	7 (23.3)	0	0	30 (100)
Total	66 (73.3)	24 (26.7)	0	0	90 (100)

FS- fissure sealant

EARA- Etch-and-rinse adhesive

Table 4.3 exhibited the distribution of microleakage scores of the resin-based sealant after different pre-treatment protocols in the permanent teeth. Dye penetration was noted in all three permanent teeth groups. However, only scores of 0 and 1 were observed for all microleakage scores. No microleakage (score 0) was observed on 66 surfaces (73.33%). The acid etching protocol (Group 1) followed by self-etch group (Group 3) showed the highest score of no leakage (score 0) at 96.7% and 76.7%, respectively. The highest microleakage score (53.3%), which is score 1, was observed in the etch-and-rinse adhesive group (Group 2) followed by group 3 (self-etch adhesive) at 23.3%. 0% was observed for score 2 and 3. The descriptive statistics for the marginal leakages in the permanent teeth are shown in Table 4.4.

4.4 Descriptive statistics of sealant microleakage in permanent teeth groups

Table 4.4. Descriptive statistics for resin-based sealant microleakage in the permanent teeth

Sample Group	Median	IQR	Min	Max
Group 1 (Acid Etching + FS)	0	0	0	1
Group 2 (Etch-and-rinse adhesive + FS)	1	1	0	1
Group 3 (Self-etch + FS)	0	0	0	1

FS- fissure sealant

Table 4.5. Comparison of microleakage of the resin-based sealant (Clinpro) after different pre-treatment protocols in permanent teeth

Proportion	Mean Rank			p-value
Microleakage	Group 1 (Acid Etching and FS)	Group 2 (Etch-and-rinse adhesive and FS)	Group 3 (Self-etch adhesive and FS)	
	35.00	57.50	44.00	p <0.001*

*p-value< 0.05, FS- Fissure sealant

Table 4.5 showed the comparison of microleakage of the resin-based sealant after different pre-treatment protocols in permanent teeth. The Kruskal-Wallis test revealed that there was a significant difference in the microleakage between permanent teeth groups ($p < 0.05$). The highest proportion with a mean rank of 57.50 was observed in the etch-and-rinse adhesive group (Group 2) followed by self-etch group (Group 3) with the mean rank of 44.00.

4.5 Pair-wise comparisons of the sealant microleakage after different pre-treatment protocols in the permanent teeth

Table 4.6. Pair-wise comparisons of the microleakage proportions of the resin-based sealant (Clinpro) between different pre-treatment protocols in the permanent teeth groups

Comparison Groups	p-value
Group 1 (Acid etching and FS) and Group 3 (self-etch adhesive and FS)	0.245
Group 1 (Acid etching and FS) and Group 2 (EARA and FS)	p <0.001*
Group 3 (self-etch adhesive and FS) and Group 2 (EARA and FS)	0.027*

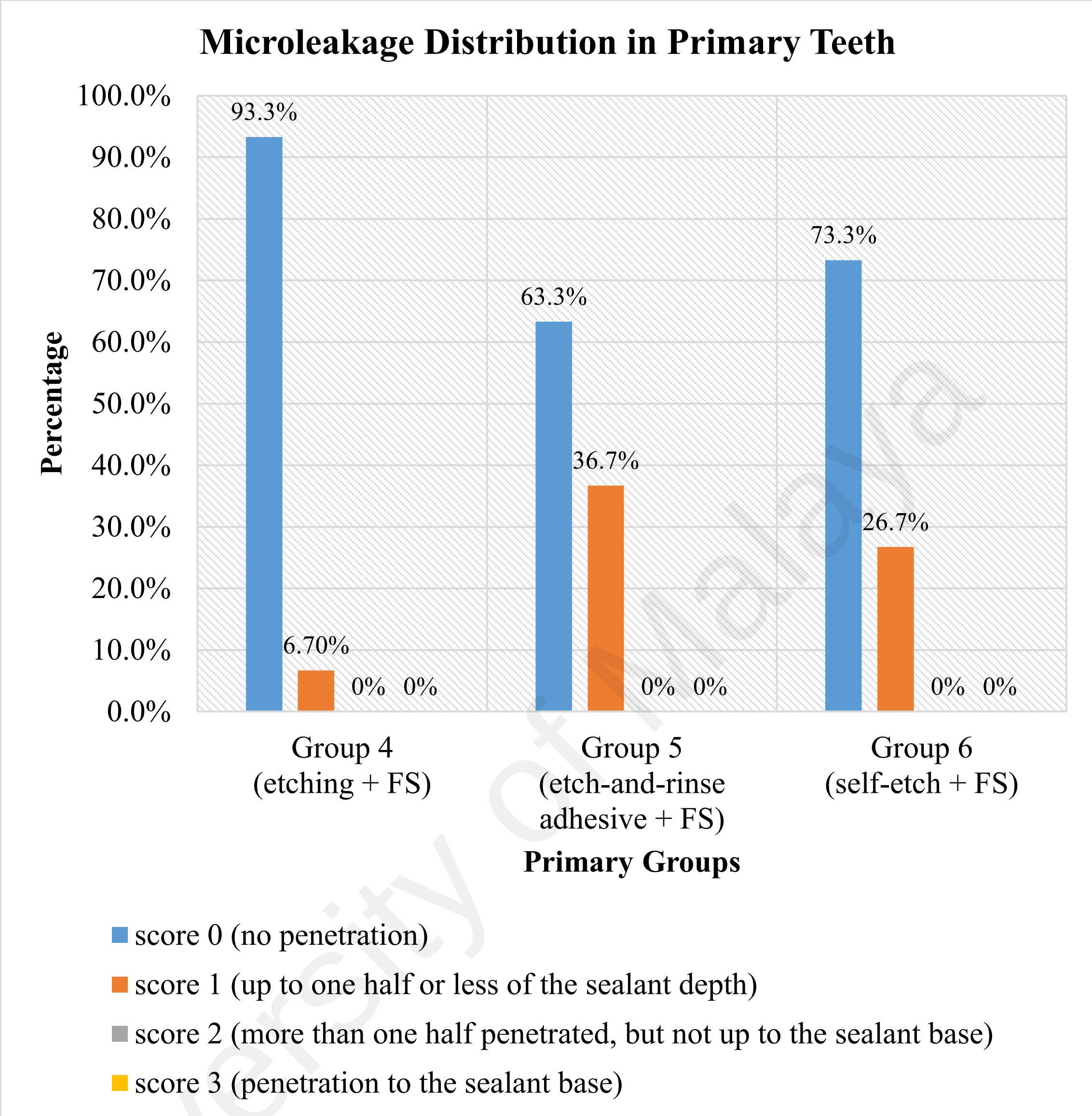
*p-value < 0.05

FS - Fissure sealant

EARA- Etch-and-rinse adhesive

Table 4.6 showed the pair-wise comparisons of the microleakage proportions of the resin-based sealant between different pre-treatment protocols in the permanent teeth. The pair-wise comparisons showed a statistically significant difference with (p value < 0.05) between Group 1(acid etching) and Group 2 (etch-and-rinse adhesive) and also between Group 3 (self-etch adhesive) and Group 2 (etch-and-rinse adhesive).

4.6 Distribution of Microleakage Scores in Primary Teeth



FS-Fissure Sealant

Figure 4.2. Percentage of microleakage distribution in the primary teeth

Table 4.7. Distribution of microleakage scores of the resin-based sealant (Clinpro) after different pre-treatment protocols in the primary teeth groups

Sample Group	Microleakage Score				Total
	Score 0	Score 1	Score 2	Score 3	N (%)
	N (%)	N (%)	N (%)	N (%)	
Group 4 (Acid Etching and FS)	28 (93.3)	2 (6.7)	0	0	30 (100)
Group 5 (EARA and FS)	19 (63.3)	11 (36.7)	0	0	30 (100)
Group 6 (Self-etch and FS)	22 (73.3)	8 (26.7)	0	0	30 (100)
Total	69 (76.7)	21 (23.3)	0	0	90 (100)

FS- Fissure sealant

EARA- Etch-and-rinse adhesive

Table 4.7 showed the distribution of the microleakage scores of the resin-based sealant after different pre-treatment protocols in primary teeth. Dye penetration was observed in all three primary teeth groups. However, only scores of 0 and 1 were exhibited from all microleakage scores. No microleakage (score 0) was found on 69 surfaces (76.67%). The acid etching protocol (Group 4) was found to show the highest score of no leakage (score 0) at 93.3% followed by the self-etch adhesive group (Group 6) at 73.3%. Group 5 (etch-and-rinse adhesive) showed the highest (36.7%) of the microleakage score (score 1) followed by the group 6 (self-etch adhesive) at 26.7%. The descriptive statistics for the marginal leakages in the primary teeth are shown in Table 4.8.

4.7 Descriptive statistics of sealant microleakage in primary teeth groups

Table 4.8. Descriptive statistics for resin-based fissure sealant microleakage score in primary teeth

Sample Group	Median	IQR	Min	Max
Group 4 (Acid Etching and FS)	0	0	0	1
Group 5 (Etch-and-rinse adhesive and FS)	0	1	0	1
Group 6 (Self-etch and FS)	0	1	0	1

FS- Fissure sealant

Table 4.9: Comparison of the microleakage of the resin-based sealant (Clinpro) after different pre-treatment protocols in primary teeth

Proportion	Mean Rank			p-value
Microleakage	Group 4 (Acid Etching and FS)	Group 5 (Etch-and-rinse adhesive and FS)	Group 6 (Self-etch adhesive and FS)	
	38.00	51.50	47.00	0.021*

*p-value < 0.05

FS- Fissure sealant

Table 4.9 exhibited the comparison of the microleakage of the resin-based sealant after different pre-treatment protocols in primary teeth. The Kruskal-Wallis test revealed that there was a significant difference in the microleakage between primary teeth groups ($p < 0.05$). Group 5 (etch-and-rinse adhesive) presented the highest proportion with a mean rank of 51.50 followed by self-etch group (Group 6) with a mean rank of 47.00.

4.8 Pair-wise comparisons of the sealant microleakage after different pre-treatment protocols in the primary teeth

Table 4.10. Pair-wise comparisons of microleakage proportions of the resin-based sealant (Clinpro) between different pre-treatment protocols in the primary teeth

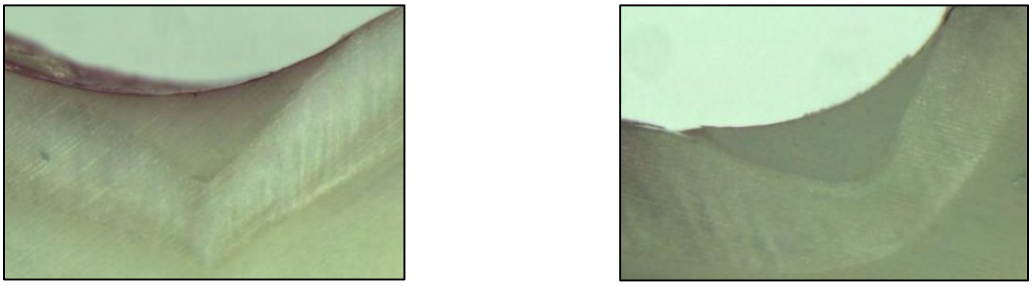
Comparison Groups	p-value
Group 4 (acid etching and FS) and Group 6 (self-etch adhesive and FS)	0.206
Group 4 (acid etching and FS) and Group 5 (EARA and FS)	0.019*
Group 6 (self-etch adhesive and FS) and Group 5 (EARA and FS)	1.000

*p-value < 0.05

FS-Fissure sealant

EARA- Etch-and-rinse adhesive

Table 4.10 showed pair-wise comparisons of the microleakage proportions of the resin-based sealant between different pre-treatment protocols in primary teeth. Pair-wise comparisons showed that there was a statistically significant difference ($p < 0.05$) between Group 4 (acid etching) and Group 5 (etch-and-rinse adhesive).

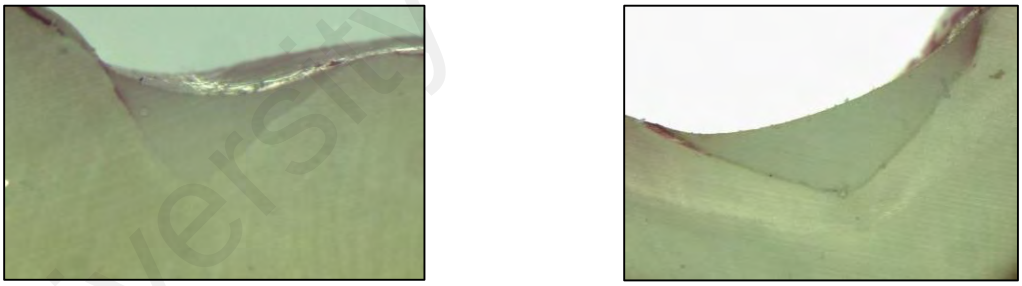


(a) Group 4 (b) Group 6

Figure 4.3. Stereomicroscope image showing no dye penetration (score 0) at enamel-sealant interface (at 20-fold magnification)

(a) Group 4 (primary teeth: use of acid etching and fissure sealant)

(b) Group 6 (primary teeth: use of self-etch and fissure sealant)



(c) Group 3 (d) Group 6

Figure 4.4. Stereomicroscope image showing microleakage score of 1 at enamel-sealant interface (at 20-fold magnification):

(c) Group 3 (permanent teeth: use of self-etch and fissure sealant)

(d) Group 6 (primary teeth: use of self-etch and fissure sealant)

4.9 Testing the Null Hypothesis

In the present study, the null hypothesis is that there is no significant difference in the degree of microleakage between the groups after acid etching, etch-and-rinse adhesive application, and self-etch adhesive systems in both permanent and primary teeth *in vitro*.

The results show that there is a significant difference in the degree of microleakage between groups after conventional acid etching, etch-and-rinse adhesive application, and self-etch adhesive systems in both permanent and primary teeth *in vitro*.

Therefore, the null hypothesis is rejected.

CHAPTER 5

DISCUSSION

CHAPTER 5: DISCUSSION

5.1 DISCUSSION

A resin-based fissure sealant, Clinpro (3M, ESPE) was employed in this study. The only group of resin based sealant material was used because the present study aimed to evaluate the different pre-treatment protocols on resin-based fissure sealant microleakage. Resin based sealants are claimed to be most frequently used despite their moisture sensitivity (Ahovuo-Saloranta et al., 2013) and have been proven to be the best compared to other sealant materials (Kühnisch et al., 2012). All teeth were cleaned with slurry pumice on a slow speed hand piece for standardisation prior to commencement of treatment.

Freshly extracted human teeth are the best samples to test and evaluate adhesive systems *in vitro* (Lee et al., 2007). A study reported that human and bovine teeth are comparable especially in their enamel radio densities (Fonseca et al., 2008). However, another study notes that human and bovine tooth structures are totally different (Jaffer et al., 2009).

In this study, extracted sound human mandibular third molars and extracted mandibular second primary molars were utilized and those with caries, cracks, restorations, and enamel defects were excluded. There are a number of studies using extracted human third molars as their samples (Baygin et al., 2012; Botsali et al., 2015; Dalli et al., 2013; Haller et al., 1993; Khogli et al., 2013; Memarpour & Shafiei, 2014). None of the studies mentioned the justification for third molar teeth selection apart from Bagheri et al. (2017).

The human permanent mandibular third molar teeth in this study were mostly obtained from third molar surgery where the extraction was due to periodontal issues and for orthodontic purposes. The permanent third molar teeth were chosen as our sample because they have more variations in the fissure and groove patterns.

The permanent third molar tooth is the last tooth to erupt into the oral cavity, thus it is less likely to be in functional occlusion for long and anticipated to have developmental prismless enamel that have not worn off (Bagheri et al., 2017) from the occlusal force and repeated cycles of remineralisation and demineralization in the oral environment. In addition, greater mesiodistal width and complicated fissure systems exerted by human third molar teeth in comparison to premolars allow for easier and increased tooth sectioning for microscopic evaluation. According to the ISO technical specification 11450:2015(E), it is preferable to use human permanent third molars from 16 to 40-year-old individuals if possible for microleakage and bond strength tests. For standardisation of fissure pattern variations, only mandibular human permanent third molars were selected in this study.

Fewer studies found in the literature investigated fissure sealant microleakage in primary teeth. A limited number of laboratory studies done on microleakage in primary teeth used mandibular primary molars as specimens (Cehreli et al., 2006; Gawali et al., 2016; Tulunoglu et al., 1999).

In this study, about 10 teeth were allocated per group. A review that investigated the microleakage study found that about two-third of the previous studies used ten teeth per group (Raskin et al., 2001). Recent studies also used the same number of teeth in evaluating fissure sealant microleakage (Guclu et al., 2016; Mehrabkhani et al., 2015). However, after tooth sectioning, this study used 30 specimens per group as suggested by a previous researcher as being sufficient in finding statistically significant differences among groups (de Almeida et al., 2003).

After extraction, all collected teeth were placed in a storage solution in order to maintain humidity and avoid dehydration of dental hard tissue (Tosun et al., 2007). Numerous storage solutions were investigated such as thymol solution, distilled water, and Hank's Balanced Salt Solution (HBSS) (Habelitz et al., 2002) glutaraldehyde, formalin, distilled water, sodium hypochlorite, chloramine-T, physiological saline solution, artificial saliva, phosphate buffered saline, and ethanol (Secilmis et al., 2013). The use of solutions such as formalin influences microleakage (George et al., 2006) and is not recommended for such studies.

A study investigated the microhardness of dental hard tissue using twelve extracted teeth in different storage solutions between two- and twelve-month periods. Storage mediums used were 0.1% sodium hypochlorite, 0.1% thymol solution, distilled water, 0.2% glutaraldehyde solution, and Hanks Balance Salt Solutions (HBBS). They reported a reduction in microhardness of 47–63% in enamel and 35–53% in dentine (Aydın et al., 2015).

In terms of weight, dentine has 70% inorganic content, 20% organic substrates, and 10% water, while 95% of enamel consists of inorganic contents or minerals, mainly (carbonate apatite), (4%) organic substances and water (1%) by weight (Habelitz et al., 2002). The storage medium can affect the mechanical properties of dental hard tissue by leaching of the mineral content from the dentine and enamel (Tesch et al., 2001).

Any alteration of the mineral content will influence the results of mechanical testing *in vitro* because the effectiveness of the sealant material depends on enamel properties. Secilmis et al. (2013) evaluated alterations in the mineral composition of the enamel namely calcium, sodium, potassium, phosphorus, and magnesium in different storage solutions and durations. They reported that calcium was significantly influenced by different storage mediums but not duration while sodium, potassium, magnesium and phosphorus were affected by both.

A study reported that the largest shear bond strength between resin and dentin were observed in extracted teeth stored by freezing in distilled water to maintain their freshness. In fact, water is easily available, widely used, simple, and cost effective (Titley et al., 1998).

Extracted teeth are a possible source of infectious diseases and blood-borne viruses like Hepatitis B and as such, should be disinfected prior to educational use and research. In this study, all collected human molars teeth were disinfected with 0.5% chloramine T solution for one-week duration prior to use as recommended by (ISO, 2015).

Chloramine T and thymol solutions are most commonly used for storage media as well as for disinfection purposes and they have no adverse effects on tooth structures (Boruziniat et al., 2017) nor affect bond strength (Jorgensen et al., 1985). Chloramine T solution has been demonstrated to produce no changes on collagen (Mobarak et al., 2010). Studies show that extracted teeth stored in 1% chloramine T solution were comparable to newly extracted teeth (Haller et al., 1993; Soderholm, 1991). A variety of storage durations were used ranging from one week to seven months (Boruziniat et al., 2017; Haller et al., 1993; Lee et al., 2007; Titley et al., 1998).

All teeth in this study were used no longer than one-month post extraction as recommended by ISO (2015), all extracted teeth should be tested immediately after extraction but this is presumably very hard to achieve. Thus, (ISO, 2015) suggested that the extracted teeth should be used within one month and not more than six-months post extraction. This is because extracted teeth used after six months might have undergone degenerative processes in the dentinal structure that might interfere with the findings of an *in vitro* study.

For aging purposes, following the resin-based fissure sealant placement based on different surface preparations and groups, all teeth were kept in distilled water at 37º Celcius for one-month with the water changed periodically to prevent infection as recommended by ISO (2015). The storage duration of sealed teeth prior to thermocycling is needed to assess bond durability and to accelerate degradation of the resin sealant-enamel interface. Water storage presumably causes water sorption in the tooth substance and the resin itself, which later leads to expansion of the restoration.

Various aging durations have been studied covering 24 hours, 1, 3, and 6 months, and 1 and 4 years (Birlbauer et al., 2017; Carrilho et al., 2005; Cehreli & Gungor, 2008; Gwinnett & Yu, 1995; Memarpour & Shafiei, 2014). However, for this study, a one-month storage period in distilled water at 37º C was applied to accelerate aging. Only a few earlier *in vitro* fissure sealant microleakage studies employed a similar aging period (Bani & Tirali, 2016; Baygin et al., 2012; Sungurtekin & Öztaş, 2010).

Long term water storage is one of the causative component to the failure of interfacial integrity and power of bonding between the resin composite and tooth surfaces. Water absorption has been shown to gradually degrade the resin-based material over time (Gwinnett & Yu, 1995). A study reported that a six-month to twelve-month storage in distilled water significantly reduces resin bonding capacity to dentine despite the use of adhesives and different etching times (Carrilho et al., 2005). Cehreli and Gungor (2008) found that four-year water storage resulted in higher fissure sealant microleakage.

Thermocycling mimics the thermal changes in the oral cavity by producing extreme hot and cold temperatures to the sealed teeth that show a linear thermal expansion coefficient between tooth and sealant materials (Abdalla & Davidson, 1993). Thermal changes may enhance crack propagation through sealant enamel interface and, consequently, microleakage formation leads to the ingress of pathogenic oral fluids and defective sealant performance (Schmid-Schwap et al., 2011). Although not representative of a truly clinical environment such as having occlusal forces, different ranges of salivary pH, salivary buffering and flow rates, by-products of oral pathogens, and sealant abrasion resistance of the sealant, it has been used widely in *in vitro* studies to detect microleakage.

A wide range of temperatures, from 0º C to 68º C, was used in the previous study (Shortall, 1982). 0º C represents ice temperature and the maximum oral temperature depends on a patient's tolerance threshold. However, other studies reported oral thermal tolerance from 4º C to 60º C (Kidd, 1978; Morley & Stockwell, 1977). Consecutive drinking of a hot liquid and iced water caused thermal changes from 15º C to 45º C (Schmid-Schwap et al., 2011).

Other contributory elements that might affect the *in vitro* microleakage study include frequency and period of thermal cycles per unit time. The rationale behind the thermal cycle period is that the maximum exposure period of a tooth in the oral cavity to extreme temperature changes (cold or hot food or drink) should only be within two to five seconds after which the oral temperature returns to normal (Schmid-Schwap et al., 2011).

According to the recommendation by ISO (2015), tested specimens/restored or sealed teeth should be applied with 500 cycles of thermal changes between 5º C and 55º C in water as this range provides the clinical relevance.

Former *in vitro* studies have documented that the number of thermal cycles does not affect dye penetration (Crim et al., 1985; Pazinatto et al., 2003). However, after a couple of years, thousands of thermal cycles would have occurred and this replicates an *in vitro* study in which extracted teeth were subjected to 2000 cycles of thermal cycles that produced *in vivo* enamel crack lengths due to abrasion (Lloyd et al., 1978). A wide range of thermal cycles ranging from 50 to 30,000 have been used in previous studies with the selection of cycle number depending on the researchers' preferences (Schmid-Schwap et al., 2011).

In this study, we applied 1000 cycles of thermocycling at 5º C and 55º C in a water bath with a dwell time of 20 seconds and 10 seconds transfer time intervals. That number was selected as, according to the literature, it is equivalent to 36.5 days of intra-oral use clinically (Gale & Darvell, 1999). Some studies applied similar cycles (1000 cycles) of thermocycling at 5º C and 55º C in water for aging in *in vitro* fissure sealant microleakage investigations (Gawali et al., 2016; Khogli et al., 2013; Memarpour & Shafiei, 2014; Tulunoglu et al., 1999).

The dwell time or thermal cycle duration is the bath or immersion period of the specimen at a particular temperature. A short dwell time of 20 seconds was applied in this study in line with an earlier study which reported that it is more practical and clinically realistic (Causton et al., 1984).

Tooth sectioning is a crucial aspect of laboratory work. As in recent investigations, tooth sectioning in a buccolingual direction for this study involved 3 slices from each tooth to be examined for microleakage scoring. A study which investigated the effect of the tooth sectioning frequency on reliability of *in vitro* microleakage evaluations reported that, various numbers of cuts were made per tooth with 50% of the studies performing one cut per tooth while 20% had two cuts per tooth (Raskin et al., 2001). The same author recommended that three surfaces per tooth are sufficient to represent the fissure sealant variation. A recent study by Guclu et al. (2016) also used three surfaces per tooth to evaluate the marginal leakage of an UltraSeal XT, which is a recent hydrophilic fissure sealant.

However, the more the tooth sectioning the more reliable and precise the dye penetration evaluation, as marginal qualities differ from one to another. Multiple tooth cutting produces more tooth slices for microscopic evaluation thus representing more leakage patterns (AlHabdan, 2017; Heintze et al., 2008). Accordingly, future studies should consider more tooth sectioning so as to obtain more precise and reliable microleakage evaluations.

In vitro microleakage studies are extremely beneficial for evaluating new dental and adhesive materials and to determine the marginal sealing ability of new restorative or sealant materials. Various methods for microleakage detection have been studied such as dye penetration, utilization of radioactive tracers, use of bacteria, and air pressure (AlHabdan, 2017). Of these methods, dye penetration is the most common and widely used by various researchers in microleakage studies (Baygin et al., 2012; Birlbauer et al., 2017; Khogli et al., 2013; Marimuthoo et al., 2017; Memarpour & Shafiei, 2014).

In this study, a conventional qualitative penetration method using dye solution was employed to detect resin-based fissure sealant microleakage as suggested by (ISO, 2015). Microleakage scoring criteria used in the present study in accordance with the study by Baygin et al., (2012) as it is well-accepted worldwide, feasible, non-toxic, and economical (Taylor & Lynch, 1992). In this study, the microscopic evaluation of (0.5% basic fuchsin) dye penetration at the enamel-sealant interface was done using a stereomicroscope (Olympus, Japan) at 20-fold magnification, as in other researches (Baygin et al., 2012; Birlbauer et al., 2017; Derelioglu et al., 2014).

There might be some debate over the merits of using quantitative or qualitative methods; however, to date, qualitative microleakage scoring has been used by various researchers since it is reproducible and reliable in determining the degree of the fissure sealant microleakage in laboratory studies (Guclu et al., 2016).

No specific dye is suggested in the technical specification by ISO (2015) although an immersion time of 24 hours at 37º C is recommended. The specimens in this study were soaked in 0.5% basic fuchsin solution for 24-hours at 37º C. Infiltration of 0.5% basic fuchsin into the sealant-enamel interface indicated a defective marginal seal or microleakage. This solution was selected for this study over other materials because the smaller particle of 0.5% basic fuchsin provides better penetration into the sealant-enamel interface, thus producing a more accurate microleakage score (Sungurtekin & Öztaş, 2010; Theodoridou-Pahini et al., 1996). Further, the dye infiltration or penetration rates of basic fuchsin remained comparatively stable over a duration of 18 weeks (Mueninghoff et al., 1990). 0.5% fuchsin solution was chosen instead of silver nitrate which has tiny size measurement (0.059 nm) than the normal size of bacteria (0.5-1.0 μm) which could have resulted in over penetration and misinterpretation of the microleakage evaluation. In addition, another dye solution, methylene blue, is not persistent at room climate as it transforms into leuco methylene blue and becomes colourless in the presence of hydroxyl ion (Mueninghoff et al., 1990).

Based on the results of the present study, the greatest percentage score 0 (no leakage) of microleakage of the resin-based fissure sealant was observed in the conventional acid etch group followed by the self-etch adhesive group in the couple of permanent and primary teeth groups. The application of acid etching is the initial step in resin based fissure sealant applications. The etching or conditioning step aims to get rid of the smear layer by selectively dissolving the enamel rods, producing macro and micro porosities. Capillary attraction makes the porosities ready for penetration by a hydrophobic bonding agent later. Light polymerisation induces micromechanical interlocking between the etched enamel surfaces and tiny resin tags (Van Meerbeek et al., 2003).

A fissure sealant with etchants and without adhesive is considered to be the gold standard and shows the lowest microleakage compared to the etch-and-rinse and self-etch adhesive techniques. The conventional acid etch technique is identified to be fewer technique sensitive in contrast with the use of adhesive and thus has less clinical error. Moreover, in this study, the lack of salivary contamination led to a greater depth of resin tags formation which eventually produced a good marginal seal compared to other techniques.

A previous research by Derelioglu et al., (2014) reported identical findings when they distinguished the marginal leakage of different sealant materials using a resin-based sealant, a glass ionomer-based sealant, and a self-etch adhesive resin-based sealant with or without application of acid-etching. They found that the phosphoric acid-etching group with the resin-based sealant displayed the least microleakage and was statistically different from other groups.

Similarly, Memarpour and Shafie (2014) also found conventional acid etching alone or together with Single Bond (single bottle) of etch-and–rinse adhesive yielded an effective sealing method with the least microleakage. The findings of this study are also supported by (Ciucchi et al., 2015; Gomez et al., 2008; Memarpour & Shafiei, 2013; Pinar et al., 2005).

A study corroborated that a perfect marginal seal due to optimal resin tag formation could be achieved with phosphoric acid etching compared to etching with a combination of (Xeno III) from self-etch adhesive system (Burbridge et al., 2006). Bagheri et al. (2017), investigated the different pre-treatment effects before fissure sealant application concluding that bioglass air-abrasion enhanced enamel etching and minimized fissure sealant microleakage irrespective of the usage of adhesives.

The findings of this study is supported clinically by Botton et al. (2015) who investigated the retention rate of fissure sealants in primary and permanent molars in self-etch adhesives technique and conventional acid etch technique. They reported that the application of conventional phosphoric acid-etching earlier to the fissure sealant produced maximum retention rate compared to the self-etch adhesive technique.

In the present study, the use of a dental adhesive (Prime Bond NT), which is of the fifth bonding generation, had the highest microleakage (score 1) for both dentition groups (permanent and primary teeth). This is contrary to previous studies which suggested that the use of a dental adhesive before sealant application reduced fissure sealant microleakage (Feigal et al., 2000; Hebling & Feigal, 2000; Tulunoglu et al., 1999). The use of a bonding/adhesive agent helped to enhance the surface wetting of the acid etch enamel and ensured deeper penetration by the sealant material thereby reducing sealant microleakage (Cehreli & Gungor, 2008; Feigal et al., 2000; Hebling & Feigal, 2000; Meller et al., 2015).

In agreement with the findings of the present study, Mehrabkhani et al. (2015) investigated and reported no reduction on the effect of bonding agents and sealant viscosity on sealant microleakage although low-viscosity sealants had less microleakage.

The finding of this study contradict previous ones which claim that the etch-and-rinse technique is the well-recognised and accepted method for bonding of materials to the tooth structure. Some more, it has superior long term clinical performance (Erickson et al., 2009; Rotta et al., 2007; Van Meerbeek, 2007) and thus, suggested them for use in resin-based fissure sealant placements. The etch-and-rinse technique is the earliest multi-generation bonding agent. It was established in the early 1990s. It involves the application of acid etchant, a primer, followed by separate adhesives, known as the three-step etch-and-rinse adhesive method.

The primer comprises a hydrophilic monomer such as 2-Hydroxy ethyl methacrylate (HEMA) melted in variety of solvents either water, acetone, or ethanol. HEMA encourages surface wetting and the re-extension of the collagen fibril network while the solvent prepares the collagen network for resin infiltration (Carvalho et al., 2003).

An earlier study reported that priming is a critical part of the etch-and-rinse technique where the acetone-based solvent was considered a highly sensitive technique requiring "wet bonding" (Tay et al., 1996). In contrast, ethanol or a water based adhesive is considered a less sensitive technique involving "dry bonding" post acid-etch application, thus ensuring strong adhesion between the resin and enamel (Van Meerbeek et al., 1996). In this study, the Prime and Bond^{NT} used in the etch-and-rinse adhesive group. It generally contained acetone as the solvent, and this may explain the highest microleakage compared to their counterparts.

On the other hand, over-dried dentin in the etch-and-rinse technique results in lower hybrid layer formations due to the collapsed demineralized collagen fibres as well as lower diffusion of the resin monomer into the collagen network. Further, small fluid filled bubbles and small spherical voids formed in between resin sealant and enamel surfaces due to separation between the hydrophilic and hydrophobic components of the adhesive under "over-wet" conditions (Tay et al., 1996). In addition, excess humidity in the "over-wet" condition causes water adsorption in the hybrid layer and incomplete polymerization of monomers (Hashimoto et al., 2006). All the above factors, possibly explain the failure of dental adhesion and higher microleakage formation in the etch-and-rinse adhesive groups in comparison with the etching-only and self-etch groups in the present study. Yet, no clinical standardization criteria have been established for what are considered "over-dry" or "over-wet" conditions, and it remain controversial (Van Meerbeek et al., 1998).

Later, with advancements in technology, a simplified two-step etch-and-rinse adhesive technique was introduced in which the primer and the adhesive resin were mixed together into single bottle or solution (Sofan et al., 2017). Some drawbacks have been reported with the two-step etch-and-rinse adhesive technique such as smaller extent of resin penetration into the demineralised tooth substrate owing to the simplification of the primer and adhesive resin into the only solution.

Lesser resin infiltration leads to lesser optimal hybridization compared to the three-step etch-and-rinse technique. The two-step etch-and-rinse adhesive is hydrophilic in nature, allowing for greater water sorption and vulnerable to hydrolytic degradation. Moreover, the difficulty of evaporation of the organic solvents entraps them in the bonding agent after the light-activation process (Van Meerbeek et al., 2005).

Recent ongoing developments of bonding agents have led to the production of the universal adhesive system. The multi-mode system is applied to this universal adhesive because it has a broader mode of application compared to the 7th generation bonding agent. It can be applied as an etch-and-rinse adhesive, self-etch adhesive, and selective enamel etching using a single bottle of adhesive solution (Perdigão et al., 2014).

In this study, the Single Bond Universal (SBU) was applied as a self-etch adhesive before fissure sealant application. The application of the self-etch adhesive reduces the feasibility of operator induced clinical errors throughout the acid etching procedure, started from acid gel placement, rinsing, and drying that might have occurred with the etch-and-rinse adhesive.

Interestingly, our findings showed less microleakage being exhibited by the self-etch adhesive in both the primary and permanent groups compared to the etch-and-rinse adhesive. The results of this study are in agreement with those by (Eminkahyagil et al., 2005; Pashley & Tay, 2001). However, sealant microleakage is higher with the use of self-etch adhesives compared to 37% conventional phosphoric acid etching due to less pH being exerted by the former leading to lower microporosity in the enamel rods and less resin tag formations that produce marginal leakage at the enamel-sealant interface.

The compositions of the universal adhesive are biphenyl dimethacrylate (BPDM), dipenthaerythrolpethacrylate phosphoric acid ester (PENTA), and polyalkenoic acid copolymer (Tay et al., 2001). These components help to upgrade bond energy, durability and marginal sealing ability to the tooth surfaces (De Munck et al., 2003; Hashimoto et al., 2004). These factors clarify the small microleakage observed in the self-etch category in both permanent and primary groups and make the self-etch adhesive superior to the etch-and-rinse in terms of its ability to resist microleakage.

However, in contrast to this, a study comparing microleakage between phosphoric acid with strong self-etch adhesive (Adper Prompt L-Pop) found no significant difference in both groups. Thus, it concluded that self-etch adhesives are comparable with phosphoric acid etching. This might be attributable to the great acidity offered by Adper Prompt L-Pop that produced an acid-etch depth similar to that of the conventional phosphoric acid etch (Asselin et al., 2008).

A study determined and compared the shear bond strength of four different fissure sealant systems to enamel using conventional acid etch protocol and self-etching primer technique (Prompt-L-Pop). It found that the shear bond strength of the four sealants applied with self-etching primer technique were higher and had statistically different bond strengths in comparison with fissure sealants applied with conventional phosphoric acid etching (Al-Sarheed, 2006).

The difference between the universal adhesive and self-etch system is because it contains specific monomers like carboxylate and phosphate monomer that having capacity of inducing chemical bonding with the calcium ion found in the mineral storage form of dental hard tissue (hydroxyapatite crystals) (Van Landuyt et al., 2008).

The presence of methacryloyloxydecyl dihydrogen phosphate (MDP) is another unique characteristic of the universal adhesive which allows for the application of phosphoric acid etching in the etch-and-rinse and the selective enamel-etch mode. The formation of stable MDP-calcium salts improves the durable bonding with enamel and this advantage overcomes the drawback of the previous self-etch adhesive system (Yoshida et al., 2012; Yoshihara et al., 2014).

The self-etch mode application of the universal adhesive enables clinicians to benefit from simplified clinical steps in dealing with very young patients, difficult tooth isolation situations, and limited chair-side time (Sofan et al., 2017).

However, the simplification, as well as complex formulation of the universal adhesive and the high solvents composition may prevent solvent volatilization and subsequently adhesion failure (Yoshihara et al., 2016). This presumably contributed to the high microleakage of the self-etch adhesive system compared to conventional phosphoric acid etching in the present study.

5.2 Clinical implication

The findings of the present *in vitro* study could be used to predict the coronal/marginal sealing capability of resin-based fissure sealant following different enamel pre-treatment protocols. This study found that the use of phosphoric acid etching without adhesive prior to resin-based fissure sealant showed least microleakage compared to the use of adhesive. Therefore, the simplified procedure involving phosphoric acid etching without the use of adhesive prior to resin-based fissure sealing is preferable in ensuring a good marginal seal. The current findings may be useful for clinicians and paediatric dentists in selecting an appropriate technique for fissure sealant administration.

The application of fissure sealant as an occlusal protective method should be simple, economical, and time effective. Thus, a simplified technique which is less technique-sensitive as well as requiring less chair-side time would be most beneficial in managing paediatric patients with short attention spans. A simplified technique without the additional use of an adhesive is more cost effective especially for school-based fissure sealant programmes and in comprehensive dental treatments under general anaesthesia. Moreover, in the operations room time is a critical factor and a simplified clinical workflow involving less general anaesthesia time indirectly offers lower risks to patients.

5.3 Limitations of the study

1. This *in vitro* study was conducted in a condition of no salivary contamination and mechanical stress which is not truly representative of the real oral environment.

2. The time frame between tooth extraction and analysis could also have affected the results of this study.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

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6.1 Conclusion

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

1. The application of fissure sealants using the conventional acid-etching technique has the lowest microleakage (3.3%) in permanent teeth;
2. The application of fissure sealants using the etch-and-rinse adhesive technique has the highest microleakage (53.3%) in permanent teeth;
3. Fissure sealant applications using the self-etch adhesive technique produce the second-highest microleakage (23.3%) in permanent teeth;
4. In permanent teeth, fissure sealants placement using the conventional acid-etching technique have the least microleakage, followed by the self-etch adhesive technique, and the etch-and-rinse adhesive technique;
5. Fissure sealant applications using the conventional acid-etching technique show the lowest microleakage (6.7%) in primary teeth;
6. Fissure sealant applications with the etch-and-rinse adhesive technique show the highest microleakage (36.7%) in primary teeth;
7. Fissure sealant applications with the self-etch adhesive technique show the second highest microleakage (26.7%) in primary teeth;
8. In primary teeth, fissure sealants applied with the conventional acid-etching technique show least microleakage, followed by the self-etch adhesive technique, and the etch-and-rinse adhesive technique.

6.2 Recommendations

Future *in vitro* studies on fissure sealant microleakage should take into consideration methodological issues in terms of having larger sample size, more recordings of tooth sectioning, and the use of a quantitative dye penetration method using computer facilities to enhance the reliability of the results. Long term water storage as an aging process should be considered as water storage accelerates degradation of the resin sealant-enamel interface. The use of artificial saliva is also recommended since it has clinical relevance to salivary contamination.

Long-term randomized clinical trials or *in vivo* studies could also be conducted to clarify real clinical contributions in terms of sealant retention and the long-term effectiveness of fissure sealants using a similar approach to this study. Also, future *in vivo* studies should investigate the clinical efficacy of sealants applied with conventional acid-etching and self-etch adhesive technique as a preventive measure against tooth decay specifically occlusal caries.

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