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# In vitro assessment of retention and microleakage in pit and fissure sealants following enamel pre-etching with sodium hypochlorite deproteinisation

## ABSTRACT

**Aim** The purpose of this study was to assess and compare the rate of sealant retention and microleakage after placement on etched enamel with and without prior deproteinisation.

**Materials and methods** Study design: 75-five freshly extracted third molars were randomly assigned to either of two pit and fissure treatment methods. Samples from both groups were etched with 37% phosphoric acid gel for 15 seconds, followed by placement of a sealant, and then subjected to thermocycling for evaluation of sealant retention. After that, specimens were immersed in rhodamine B, sectioned longitudinally, and examined under a confocal laser scanning microscope for assessment of microleakage. Collected data were statistically analysed using chi-square and Fisher exact tests with an  $\alpha$  level of 0.05.

**Results** The rate of sealant retention was similar between the two study groups ( $P = 0.073$ ), but the rate of sealant microleakage was significantly lower in the enamel deproteinisation group ( $P < 0.001$ ) than in the control group.

**Conclusion** Based on these findings, we recommend the deproteinisation method prior to enamel acid etching to obtain better clinical results with sealants.

**Keywords** Deproteinisation; Pit and fissure sealants; Prevention.

## Introduction

Prevention of childhood dental caries is still one of the most important challenges for paediatric dentists all around the world. This challenge remains despite research, clinical advances and improvements that have arisen in the last 60 years, most importantly the advent of Buonocore's enamel acid etching method in 1955 and the introduction of adhesive pit and fissure sealants that act as a physical barrier and are placed over the occlusal surface of vulnerable primary and permanent molars. The term "vulnerable" means higher susceptibility to dental caries due to the complex morphology of occlusal pits and fissures, which are ideal sites to trap food remnants, cariogenic bacteria and harmful carbonate byproducts [Agrawal and Shigli, 2012]. It has been shown that placement of sealants can decrease the number of viable cariogenic bacteria up to 100% [Kramer et al., 1993].

Sealant placement is very technique sensitive. There is a risk of leaving residual material, moisture, or air bubbles inside of the fissures, preventing proper sealant penetration and endangering optimal retention and structural integrity. Complete sealant retention is associated with increased longevity of the micro-mechanically retained protective barrier between the enamel surface and the oral environment. Recent studies have reported that sealant retention rates in permanent molars decrease to 85% and 50% one and five years after placement, respectively (in primary molars, the retention rate ranges from 85% at 1 year to 73% at almost 3 years) [Shapira and Eidelman, 1986; Castro and Galvão, 2004; Beauchamp et al., 2008]. The lack of integrity of the peripheral enamel-sealant interface may cause microleakage of bacteria, nutrients, and the resulting acidic metabolic products, which may allow caries initiation or recurrence or postoperative pulp inflammation [Bagherian et al., 2013]. Regardless of the type of sealant applied, any sign of the loss of retention or integrity in the sealant eventually leads to failure, seriously compromising the long-lasting, preventive effects of such materials. The testing of invasive and non-invasive treatment methods for cleaning and preparing the enamel fissures prior to the etching and sealant application have delivered contrasting and even contradictory results. Dry brushing; pumice prophylaxis

with rubber cups; fissure burs; adhesive agents; lasers; abrasion with air, sodium bicarbonate, or aluminum oxide particles; and prolonging the etching time are some examples of techniques currently suggested in paediatric dentistry [Agrawal and Shigli, 2012; Muller-Bolla et al., 2006].

The non-invasive method of pretreating the enamel surface by deproteinising with 5.25% sodium hypochlorite (NaOCl) for 60 seconds prior to etching has proven to be a promising method for improving the quality of conditioning by significantly removing organic elements and denaturing proteins present in both the enamel and the acquired pellicle. Espinoza et al. [2008] demonstrated *in vitro* that the deproteinisation process increases the conditioned area and improves the quality of enamel acid etching, optimizing the retentive surface and sealant bond strength. In contrast, Ramakrishna et al. [2014] do not support those findings and concluded that deproteinisation prior to or after etching did not significantly alter the superficial enamel topographic characteristics; thus, they concluded that "...use of 37% phosphoric acid alone still remains the best method for pretreatment of the enamel".

Given these contradictory opinions, we conducted an *in vitro* trial with the aim of assessing and comparing the rate of sealant retention and microleakage with and without 5.25% NaOCl deproteinisation prior to enamel acid etching. Our hypothesis was that enamel deproteinisation prior to phosphoric acid etching produces better retention and lower rates of microleakage than traditional acid etching and sealant placement.

## Materials and methods

### Design and selection criteria

An *in vitro* trial was conducted; patients who donated their extracted third molars for this study signed a written informed consent. During a period of 4 months, 75 sound human upper or lower third molars, freshly extracted for surgical reasons, were selected for this study. Exclusion criteria were molars with fractures or cracks, occlusal caries, malformations, enamel hypoplasia, or erosions. Immediately after the extraction, all molars were completely washed with tap water and cleaned with hand scalers to remove blood and attached organic debris. They were then preserved in a glass container with distilled water at room temperature until ready for use; the aqueous preservation medium was changed weekly.

### Enamel preparation and sealant placement

All molars were mounted on cubic acrylic blocks and randomly assigned to two study groups of 36 molars each using permuted-block randomisation with six blocks of four letters (A, B, A, B): Group A (experimental) was

subjected to enamel deproteinisation and etching acid. Pits and fissures were cleaned with a brush and pumice/water using a low-speed hand piece for approximately 10 seconds. The occlusal enamel surface was rubbed with sterile cotton impregnated with 5.25% NaOCl for 60 seconds, rinsed with a sterile distilled water slurry for 10 seconds, and thoroughly dried with oil-free compressed air spray. Then, the enamel was etched with 37% phosphoric acid gel (Scotchbond™ 3M ESPE, Mexico) for 15 seconds, washed and thoroughly dried. Group B (control) underwent only enamel acid etching (no deproteinisation). Pits and fissures were cleaned with a brush and pumice/water using a low-speed hand piece for approximately 10 seconds. Then, the enamel was etched with 37% phosphoric acid gel (Scotchbond™ 3M ESPE, Mexico) for 15 seconds, washed and thoroughly dried.

After acid etching, the pits and fissures appeared frosted. Regardless of the experimental group, all molars were sealed (Clinpro™ 3M ESPE, Mexico) according to the manufacturer's instructions by employing a syringe-type tip. By using the tip of the probe, the sealant was gently placed into the etched fissures, and obvious air voids were simultaneously removed. Then, the sealant was light-cured for 20 seconds (Optilux-500 Demetron, output of 800 mW/cm<sup>2</sup>; Kerr Corp, USA) with the tip of the curing light held perpendicularly to the sealant surface and approximately 1 mm away.

### Laboratory procedures

Samples were then incubated in sterile distilled water for 24 hours at room temperature. Afterwards, they underwent thermocycling 500 times in artificial saliva-filled baths (Viarden S. A., Mexico) at 4°C, 37°C, and 57°C with an exposure time of 30 seconds each to simulate the oral environment. Pieces of each molar were then carefully sealed with sticky wax, followed by the application of two layers of acid-resistant nail varnish over the axial surfaces of each molar, leaving 1 mm free around the sealant borders. Specimens were immersed for 24 hours in a rhodamine B (Sigma-Aldrich, Mexico) dye solution (0.02 mg in 200 ml of sterile distilled water) followed by thorough washing under tap water for 1 min to eliminate excess dye from the enamel surface. The sticky wax and varnish were removed by scraping. Each specimen was then sectioned longitudinally through the sealant in a buccolingual direction with a water-cooled diamond disk. The sections were kept dry until microscopic examination.

### Outcome evaluation

Two dependent variables were considered. The first was sealant retention. After thermocycling, a trained, independent, and pre-calibrated examiner passed a 0.5 mm diameter probe along the margins of the sealant to verify integrity, failure, or loss of continuity. The results of sealant retention were classified based on

Simonsen’s criteria as follows:

1. complete retention;
2. partial loss;
3. complete loss [Simonsen, 1989].

The second variable considered was microleakage. The prepared sections from each specimen were carefully examined under a confocal laser scanning microscope (Leica® DMI 4000B, Germany). Microleakage was determined by observing the degree of penetration of rhodamine B on photographs (10x magnification;  $\lambda_{\text{exc}}$  553 nm,  $\lambda_{\text{em}}$  627 nm) and scored by the same pre-calibrated examiner according to a dichotomous criterion: 1. absent (no marginal penetration of dye) or 2. present (marginal or deep penetration of dye).

### Statistical analysis

First, a descriptive data analysis was performed. Then, to evaluate and compare the effect of NaOCl deproteinisation on sealant retention prior to enamel acid etching in the two study groups, a non-parametric chi-square test for three independent sample groups was carried out.

A Fisher exact test was conducted to compare groups according to the presence or absence of microleakage. To confirm intra-observer reliability, a Cohen’s Kappa test and Landis-Koch criteria were used. To assess both dependent variables, the observer twice evaluated 20 randomly-selected molars before and after sectioning (10 day interval between examinations). Kappa statistics demonstrated excellent intra-observer reliability: 100% for retention and 97.3% for microleakage based on the Landis-Koch criteria [Landis and Koch, 1977]. All experimental data were subjected to statistical analysis using SPSS (Statistical Package for the Social Sciences) version 15.0 for Windows. The level of significance was set to 0.05.

## Results

In total, 75 third molars (36 upper and 39 lower) were included in the present study. The randomisation process yielded two homogeneous study groups with respect to

Study groups	Retention			Microleakage	
	Total	Partial loss	Total loss	Yes	No
Deproteinisation group	100%	0%	0%	79%	21%
Control group	91.9%	8.1%	0%	83.7%	16.3%
p value	0.073			< 0.001	

TABLE 1 Summarised results from retention and microleakage outcomes.

intraoral molar location (chi square test,  $P < 0.05$ ).

Regarding to sealant retention and microleakage outcomes, results are summarised in Table 1. When retention was compared, there was not a significant difference between the study groups ( $P = 0.073$ ). On the other hand, the microleakage in the desproteinisation group was significantly lower than the control group ( $P < 0.001$ ). Figure 1 shows a representative image of the evaluation.

## Discussion

In addition to oral hygiene techniques, topical fluoride application, and dietary modifications, light-cured and flowable resin pit and fissure sealants have played an important role in the prevention of enamel demineralisation in children and adolescents. This has been demonstrated and confirmed by high-quality research designs including meta-analyses and well-conducted randomised clinical trials [Beauchamp et al., 2008; Muller-Bolla et al., 2006]. However, low long-term retention rates of sealants have been reported, so a variety of pit and fissure treatment methods, such as enamel surface deproteinisation, have been used prior to acid etching in an attempt to successfully maximise sealant retention. The objectives of etching the enamel with 30%-40% phosphoric acid are:

1. to clean the enamel surface by eliminating contaminating material and the smear layer;

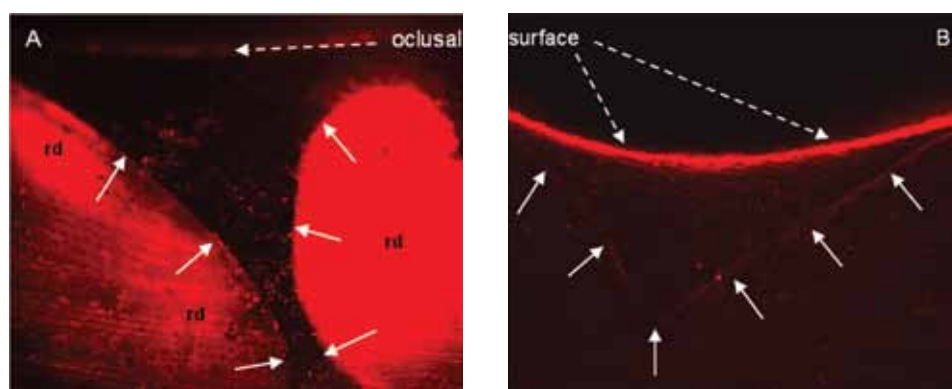


FIG. 1 Confocal microscopic view (10X) of two sealants placed using A. the Conventional technique, B. the NaOCl deproteinisation technique. The white solid arrows show the bottom of the sealant and the rhodamine (rd) penetration underneath. There is no penetration in B.

2. to selectively dissolve the prismatic and interprismatic mineral crystals, producing an irregular and porous surface;
3. to increase the surface free energy of enamel up to 72 dynes/cm, which enables a deeper resin flow into the porosities through capillary action [Espinoza et al., 2008].

Acid etching removes approximately 10 µm of the enamel surface and produces a 5-50-µm-deep rough and porous layer, which improves enamel retentive properties, resulting in better penetration and mechanical adhesion of the sealant [Ahuja et al., 2010; Harleen et al., 2011]. Sealant retention strongly depends on the quantity and quality of the etched enamel surface. In addition, the type of acid, concentration, etching time, form of the etchant (gel, semi-gel or aqueous solution), rinsing time, and enamel surface structural features are factors determining the quantity and quality of the acid etching pattern [Ramakrishna et al., 2014].

The major finding of the present *in vitro* study is that enamel surface treatment with NaOCl deproteinisation for 60 seconds prior to acid etching resulted in high pit and fissure sealant retention rates and significantly less microleakage compared to conventional phosphoric acid etching alone. Traditionally, NaOCl solution has been used in endodontics for its antibacterial effects and capacity to dissolve and remove the organic smear layer from the root canal space without damaging healthy hard tissue or the tooth structure. When NaOCl comes in contact with organic material, specific chemical reactions occur, including saponification and neutralization; these reactions—acting simultaneously and synergistically—lead to liquefaction of organic matter in dental hard tissue, including protein, a process known as deproteinisation [Ramakrishna et al., 2014]. Acid etching does not eliminate the organic content on the enamel surface, which is primarily composed of protein. This was shown by Nakabayashi and Pashley [1998], who observed that the collagen fibril network remained undamaged after dentin demineralisation with phosphoric acid. The organic content comprises only 1% of the enamel layer, but its presence can affect the quality of the etching pattern; the organic layer cannot be entirely removed without considering the proteins immersed in the enamel crystals. Espinoza et al. [2008] demonstrated that acid etching alone achieved a homogeneous retentive morphology only in less than 50% of the enamel surface.

Several factors can explain the results from this study. Enamel surface NaOCl deproteinisation for 60 seconds (rather than 30 seconds) before phosphoric acid conditioning doubles the retentive etched surface area (48.8% to 94.5% according to Espinoza et al. [2008]) and the quality of the resulting topographic pattern because of the elimination of the organic components when compared to conventional etching. Outer

enamel proteins prevent effective etching, resulting in uneven and unreliable patterns for resin adhesion [Espinoza et al., 2008]. Furthermore, it has been observed that protein debris and pellicle remnants that have accumulated at the base of fissures can clog the porosities created after etching, decreasing the enamel retentive strength [Ramakrishna et al., 2014]. Another likely effect of surface protein removal is the production of longer adhesive tags into the enamel, increasing the mechanical retention to the enamel surface [Barbieri et al., 2004; Justus et al., 2010].

Silverstone et al. [1975] classified enamel etching into three different types based on the resulting surface micromorphological pattern: type 1, enamel prism cores are dissolved; type 2, enamel prism peripheries are dissolved; and type 3, some superficial dissolution without affecting prisms. The most retentive etching patterns are types 1 and 2 because the porous areas are of a larger size and depth. Increased area with these two retentive patterns can be obtained through NaOCl 60-second deproteinisation prior to etching when compared to conventional etching [Espinoza et al., 2008]. Therefore, 5.25% NaOCl deproteinisation may increase the retention of a resin sealant [Ramakrishna et al., 2014].

The main limitations present in the current study were the preservation of test specimens in distilled water and the employment of thermocycling with artificial saliva to simulate the oral environment. Justus et al. [2010] mentioned that when tooth specimens are stored in distilled water, the organic content of the enamel surface may be partially lost. Therefore, our results regarding enamel deproteinisation might be slightly inflated compared to those previously reported. On the other hand, Harleen et al. [2011] reported that although *in vitro* tests do not completely predict how dental materials will behave in the oral cavity, these tests are still valuable; most research into dental composite bond strength is carried out *in vitro* because it is difficult to expose the materials and subsequently retrieve them from the oral environment. According to the International Organization for Standardization (2003), thermocycling is the best process for mimicking thermal changes in the oral environment during *in vitro* studies. Since 1994, they have provided specific guidelines for conducting and standardising adhesiveness tests for dental materials to enable investigators to interpret and compare reproducible results that would support *in vivo* testing. These guides indicate that a thermocycling regimen composed of 500 cycles in water between 5 and 55 °C is considered an appropriate simulated aging test [Mohammed-Salih, 2013]. Given this, we decided to use artificial saliva instead of water in an attempt to more reliably reproduce the oral environment.

Our results confirm some previous studies, but are different from those of others. The diversity of the materials and methods (such as the use of different



dye agents, including methylene blue, basic fuchsin, or rhodamine B as in our study) employed by different authors to assess microleakage may explain the contradictions between results. The wide variation in the results from bond strength tests has also been attributed to many influencing factors, both in specimen preparation and testing [Harleen et al., 2011]. Enamel deproteinisation has been previously employed in the management of enamel hypomineralisation and amelogenesis imperfecta, where the enamel protein content is excessive compared to normal enamel. However, before using this technique, paediatric dentists should consider some drawbacks of NaOCl, including its possible soft tissue reactions, taste, odor, and tolerance by pediatric patients [Espinoza et al., 2008].

In summary, although additional studies should be performed, we conclude that retention and adhesion to enamel pit and fissures are improved by NaOCl deproteinisation prior to acid etching by increasing the surface area of etched enamel, among other factors. Additionally, microleakage is significantly reduced when a resin sealant is placed over the pretreated occlusal enamel after 60 seconds of NaOCl deproteinisation prior to etching when compared to conventional acid etching without deproteinisation. Therefore, we suggest this enamel treatment method prior to acid etching, based on valid evidence, to increase the useful clinical life of resin sealants placed on primary or permanent molars.

### Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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