Deproteinization Effectiveness on Occlusal Enamel Surfaces and Resultant Acid Etching Patterns: An *in vitro* Study

Valencia R * / Espinosa R** / Borovoy N ***/ Pérez S **** / Ceja I***** / Saadia M******

Purpose: *The goal of this in vitro study was to identify whether occlusal enamel deproteinization is effective in the removal of organic material in order to obtain quality etching patterns using phosphoric acid (H_3PO_4) and sodium hypochlorite (NaOCl) compared to phosphoric acid alone.* ***Study design:*** *Nine extracted third permanent molars were polished with pumice and water. Every pit and fissure was evaluated as a unit, resulting in 40 individual units and then these were divided into five treatment groups. The occlusal enamel surface of each group was subjected to the following treatments: Group 1 (C) Control: No treatment; Group 2 (P): Polish and rinse; Group 3 (PD): Polish, rinse, and sodium hypochlorite (NaOCl) 5.25% for 60 seconds; Group 4 (PA): Polish, rinse, and acid etching with H_3PO_4 37% for 15 seconds; and Group 5 (PDA): Polish, rinse, sodium hypochlorite (NaOCl) 5.25% for 60 seconds, and acid etching with H_3PO_4 37% for 15 seconds.* ***Results*** *showed no significant statistical difference in the organic material present between groups 1 (C) (30.18%) and 2 (P) (36.61%), but there was a statistical difference ($p < 0.002$) between Groups 1 and 2, and Group 3 (PD) (16.50%). In the acid etching group, the undesirable Type-III pattern (discussed later) was found in Group 4 (PA) (33.54%), while this was only 7.70% in Group 5, nearly five times more than Group 4, with a significant statistical difference (0.05). When differences were sought for Types I and II etch patterns (discussed later) for Groups 4 and 5, Group 4 (PA) obtained 26.29% (Type I) and 1.75% (Type II) etch patterns, compared to Group 5 (PDA) with 33.4% (Type I) and 38.97% (Type II) etch patterns.* ***Conclusions:*** *The enamel deproteinization technique is an effective way to remove organic material on the occlusal surfaces of teeth, obtaining after phosphoric acid application, up to 72.38% of Types I and II etch patterns. Etching Types I or II can also be determined by the removal of organic material in between enamel crystals.*

Key words: *Enamel, sodium hypochlorite, phosphoric acid, etch pattern, occlusal surfaces.*

INTRODUCTION

Dental caries remains one of the most common diseases found in humans. Different tooth surfaces show variable susceptibility to dental caries, [1,2] with the tooth's occlusal surfaces being the most prone to develop them. Dental caries is related to the depth and morphology of tooth pits and fissures, considered to be caused by an imperfect cuspal coalition during odontogenesis. Pit and fissure sealants are frequently used on these teeth to prevent occlusal caries. A key factor for successful caries prevention with these materials is for the sealant to remain intact over time. [3-5]

Since the late 1800s, efforts to prevent the disease in these occlusal surfaces have been performed by the dentist, blocking these defects either with cements such as zinc phosphate, or the chemical treatment of pits and fissures with silver nitrate for proposed inclusive mechanical-fissure eradication. [6, 7]

Finally Buonocore, in 1955, introduced a truly promising method to prevent pit and fissure carious lesions. His classical study, "A simple method of increasing the adhesion of acrylic filling materials to the enamel surfaces" was published, presenting an innovative method for mechanical bonding of acrylic resin to enamel previously etched using phosphoric acid. [8]

* Roberto Valencia, DDS, Associate Professor, Pediatric Dentistry, Universidad Tecnológica de México.
** Roberto Espinosa, DDS, Professor, epartment of Oral Rehabilitation, Health Science and Enviromental Center, Universidad de Guadalajara, Mexico.
*** Nilly Borovoy, DDS, Associate Professor, Department of Pediatric Dentustry, University of Baylor Texas.
**** Sandra Pérez, DDS, Private Practice, Mexico City.
***** Israel Ceja, PhD, Researcher Exact Science and Engieneering Center, Universidad de Guadalajara, Mexico.
****** Marc Saadia, DDS.MS, Private Practice, Mexico City.

Send all correspondence to:
Roberto Valencia,
Rodríguez Saro 100-201
Col Del Valle, México City CP. 03100
Phone. (525)555349789
E-mail: rmval@hotmail.com

Ten years after Cueto and Buonocore wrote the first specific paper on pit and fissure sealants, utilizing 50% H_3PO_4 buffered with 7% ZnO as an etchant and along with this a mixture of methylmethacrylate monomer and grain from silicate cement as a sealant. [9]

Silverstone *et al* in 1975 described for the first time, different enamel etch patterns. Type-I and Type-II are considered ideal for adequate bonding. [10,11]

Acid etch techniques work by removing contaminants, increasing enamel surface energy, in turn creating dissolution of prism cores, which will result in microporosities where resin can flow and create a mechanical bond with enamel during polymerization.[12]

Studies conducted with scanning electron microscopy by Hobson (2002; 2005) in enamel adhesion have showed that the topographic quality of enamel etching with H_3PO_4 is not achieved over the entire treated adhesion surface: more than 69% of the treated surface was not etched; 7% presented tenuous etching, and only 2% was ideally etched. To counteract these limitations, various invasive and non-invasive techniques were utilized without satisfactory results. [13,14]

Resin composite is the most commonly used sealant material. The caries-preventive effect of resin-based sealants depends on the sealing of pits and fissures through the micro-retention of tags created by the acid etching of the enamel. Sealants are indicated for primary and permanent teeth with pits and fissures predisposed to plaque retention and are set in place to reduce the risk of caries in these susceptible areas of teeth. Because the risk of caries may increase at any time during a patient's life due to changes in habits, oral microflora, or physical conditions, unsealed teeth thus might benefit from sealant application. [15,16]

The required properties of an ideal fissure sealant include biocompatibility, adequate bond strength, anticariogenecity, resistance to wear and abrasion, good marginal integrity, and cost effectiveness. [17]

Retention and good marginal integrity also depend on the conditioning of enamel, techniques of application, and the characteristics of the sealant material such as viscosity, surface tension, and proper adhesion. [18,19]

Various studies suggested that the organic material and salivary proteins present in saliva are normally found on the superficial zone, and may create hindrance both in the conventional etching technique and in deepest penetration of resin. [20-22] It is noteworthy that Gurney and Rapp in 1946 published an original paper entitled "A Technique for Observing Minute Changes on Tooth Surfaces". These authors etched enamel and dentin previously treated with a sodium hypochlorite (NaOCl) derivate, a sodium hydroxide (NHCl) solution, at different concentrations to obtain better surfaces observed under Scanning Electronic Microscopy (SEM). The specimens were then immersed in etching solutions and the effects were studied by making additional micro-impressions at suitable times. After application of the NHCl solution, the surfaces appeared smoother, presumably because some of the organic coats had been dissolved off these surfaces. [23]

The concept of enamel deproteinization for clinical use was first introduced by Espinosa *et al* in 2008. These authors demonstrated that removing organic content from an enamel surface with 5.25% sodium hypochlorite (NaOCl) as a deproteinizing agent prior to phosphoric acid etching doubled the enamel's retentive surface significantly, from 48.8% to 94.47% and increased Type-I and Type-II etch patterns. [5]

This technique could significantly improve adhesion by removing the organic elements of both the enamel structure and the acquired pellicle. In support of their preceding study, Espinosa *et al* in 2010 evaluated qualitative and quantitative resin-tag penetration with a resin replica model, and concluded that enamel deproteinization with 5.25% NaOCl for 60 seconds prior to H_3PO_4 etching nearly doubled the enamel's retentive surface, from 46% to 73%, In addition, the topographical features of the resin-replica penetration surface technique increased significantly with Type-I and Type-II etch patterns.[6]

Since the deproteinization concept has not, to our knowledge, been utilized on occlusal surfaces previously and because there is no agreement, to our knowledge, regarding the role of intermediate bonding in the retention of pit and fissure sealants, this study was undertaken to evaluate the effect of enamel deproteinization and the use of pit and fissure sealants.

Contrariwise, Ahuja *et al* in 2010 concluded that 5.25% NaOCl enamel deproteinization did not significantly alter the topographical surface features of the enamel surface before acid etching in relation to Type-I and Type-II etch patterns. [24] During the same time period, Justus *et al* in 2010 concluded, once again from their *in-vitro* study, that enamel deproteinization with 5.25% NaOCl prior to acid etching significantly increased bracket bond strength with regard to Fuji Ortho LC and Transbond XT as well. [25,26]

The need for sealant placement should be reassessed at a periodic preventive-care appointment, and then repaired or replaced as needed. Unfortunately, after all these years, we continue to face adhesive failures and perform repetitive dentistry. We sometimes struggle with insurance companies, who often restrict re-application prior to 5 years of the restoration's initial placement. [27]

We predicted that pretreating occlusal enamel with sodium hypochlorite would render the enamel crystals more accessible to the etching solution, resulting in a clinically more favorable etched surface. Thus, the present study was carried out to evaluate the effect of NaOCl occlusal-surface deproteinization and enamel acid etching employing Scanning Electron Microscopy (SEM) analysis.

MATERIALS AND METHOD

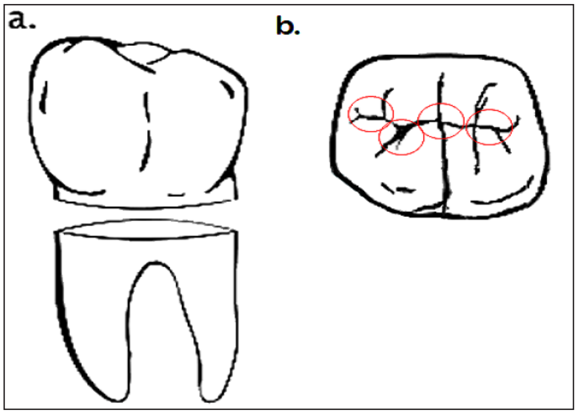
Nine extracted human mandibular third permanent molars were selected with the following exclusion criteria: teeth with enamel cracks or fractures along their occlusal aspect; dental pathology; malformations; carious lesions; restorations; and erosions.

This study was conducted in accordance with the guidelines established by the Mexican Ministry of Health Code of Bioethics for Dentists, in the Official Mexican Standard Regulations, and in the bioethics regulations enforced by the Universidad Tecnológica de México (Technological University of Mexico).

After extraction, all samples were stored in saline solution at 37ºC. Each tooth was polished with pumice and rinsed with distilled water for 10 seconds. Roots were amputated with a low-speed double-sided diamond disk (Shofu #S23-1164, Japan) under copious irrigation with water .

Each pit and fissure was then evaluated as a unit, resulting in 40 individual units, and then divided into five treatment groups as follows: [(2)]

Figure 1



Group	# Samples
Control group 1 (C)	8
Polish and rinse Group 2 (P)	6
Polish, rinse, and NaOCl Group 3 (PD)	7
Polish, rinse, and acid etching Group 4 (PA)	10
Polish, rinse, NaOCl, and acid etching Group 5 (PDA)	9

All occlusal pits and fissures were encoded for identification purposes and prepared to receive one of the following five treatments: **Group 1 (C)** Control: No treatment; **Group 2 (P): P**olished and rinsed; **Group 3 (PD): P**olished + **D**eproteinization with Sodium Hypochlorite (NaOCl) 5.25% applied with a sterile cotton pellet for 60 seconds, rinsed with sterile water, air sprayed for 10 seconds, and then dried; **Group 4 (PA): P**olished + **A**cid Etching with H_3PO_4 37% gel (3M ESPE Scotch bond etching gel, St. Paul, MN, USA) applied with a microbrush for 15 seconds, rinsed with sterile water, air sprayed for 20 seconds, and then dried with oil-free compressed air; and **Group 5 (PDA)**: **P**olished + **D**eproteinization + **A**cid.

All samples were coated with gold electrodepositing, using a Sputtering Effacoater (Ernest Fullam 18930, NY, USA) and prepared for surface SEM analysis (JEOL JSM 5400LV, Japan). The observation zone for all samples was standardized at the middle pit and fissure section (2 mm) of the tooth. (Fig. 1)

Microphotographs at 500X magnification were obtained from each enamel specimen, covering the entire pit and fissure sample surface.

To maintain a standard among the samples (considering that there was a total of 40 pits and fissures), each was divided into five groups and each pit or fissure was exposed to one of the different treatments, ensuring that this handling technique was applied to teeth with the same enamel quality. The images were submitted to double-blind evaluation by two investigators, with an (r = 0.78 correlation). In order to obtain quantitative results, the samples were calculated using Auto-CAD 2005 Software (Microsoft Corporation, Macrovision Corp.) to evaluate each of the images.

RESULTS

The total surface of each pit and fissure image (μm^2) was calculated, finding an average area as follows: 6047.8132 μm for Group (C); 2150.8332 μm for Group (P); 4851.6526 μm for Group (PD); 3057.4964 μm for Group (PA); and 3603.2892 μm for Group (PDA).

The data obtained was submitted to statistical analysis, which was performed using the SPSS (Statistical Package for Social Sciences) version 12 statistical software for Windows to find the extent of the relation between the groups with the Pearson co-relation test and the paired Student t test to compare the two groups; the level of significance was set at 0.05 (5%). (Tables 1 and 2)

Table 1. Descriptive statistics according fissure area in μm for Groups 1, 2, and 3

Group	# Samples	Fissure average area	SD
1 (C)	8	6047.8132	2150.43467
2 (P)	6	2150.8332	719.03708
3 (PD)	7	4851.6526	1657.53989

Fig 1. A. Clinical photograph of the occlusal sample. B. Scanning Electronic Microscope (SEM) photographic composition of the same molar.

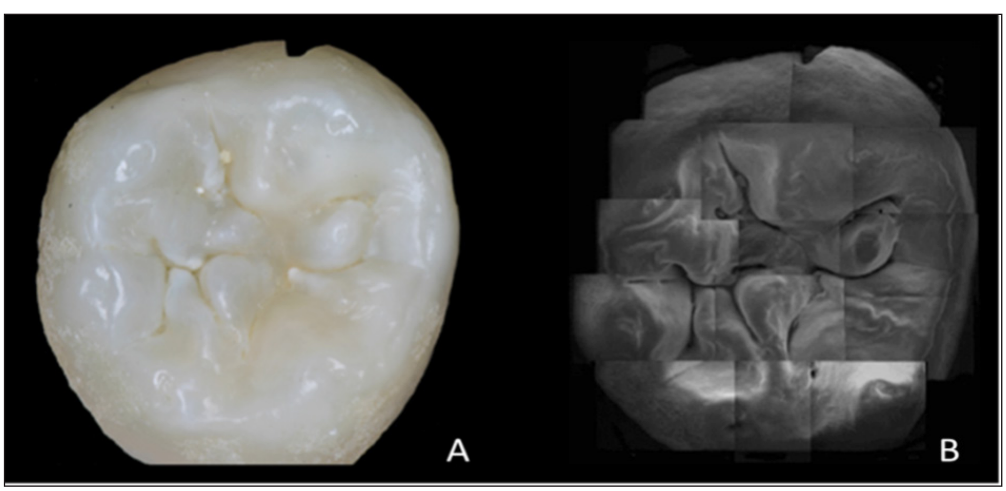


Table 2. Descriptive statistics according fissure area in µm for Groups 4 and 5

Group	# Samples	Fissure average area	SD
4 (PA)	10	3057.4969	1766.36161
5 (PDA)	9	3603.2892	1113.88452

Each sample (pit and fissure area surface unit) in Groups 1, 2, and 3 was evaluated for organic material, obtaining mean values and measured percentages. The results obtained for Group 1 (C) with no treatment were 30.18%, for Group 2 (P) 36.61%, and for Group 3 (PD), 16.50%.

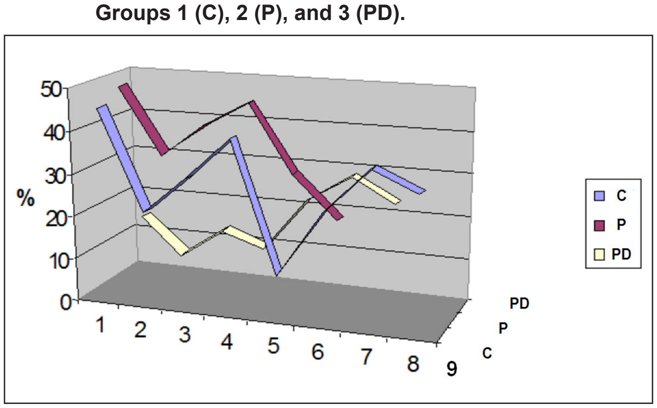
Table 3. Descriptive statistics according to the percentage of organic material found in the pits and fissures areas for Groups 1, 2, and 3

Group	# Sam-plus	(%) Organic material	S.D.	Minimum	Maximum	Mean
1 (C)	8	30.1	11.0	20.9	39.3	30.1
2 (P)	6	36.6	10.3	25.6	47.4	36.5
3 (PD)	7	16.5	8.3	8.7	24.1	16.4

P <0.002 between groups.

The Pearson correlation test revealed no statistically significant differences according the organic material present, with percentages between Control group 1 (C) and Polish group 2 (P), but there was a statistically significant difference within Group 3 (PD) (p >0.002) (Table 3, Graph 1). (Figs. 1 and 2)

Graph 1. The tendency of the percentage of organic material for Groups 1 (C), 2 (P), and 3 (PD).



When the percentage of etch Types I and II was compared between Group 4 (PA) and Group 5 (PDA), a statistically significant correlation was found (Table 4 and Graph 2) (Figs. 3 and 4).

Table 4. Descriptive statistics in percentages found for etch Types I and II at the pit and fissure areas for Groups 4 (PA) and 5 (PDA)

	# Samples	% Etch surface Types I and II	Standard deviation	Standard error
4 (PA)	10	66.4	17.0	5.4
5 (PDA)	9	92.2	4.3	1.4

P <0.05 between groups.

Regarding the acid etching groups, each sample (pit and fissure surface-area unit) in Groups 4 and 5 was evaluated. The undesirable Type-III pattern found in Group 4 (PA) was 33.54%, while this was only 7.70% for Group 5 (PDA), that is, nearly five times more for Group 4 (PA), with a statistically significant difference 0.05. (Table 4 and Graph 2) (Figs. 3 and 4).

Graph 2. Depicts etch Type-I and Type-II areas in percentages found in pits and fissures for Groups 4 (PA) and 5 (PDA).

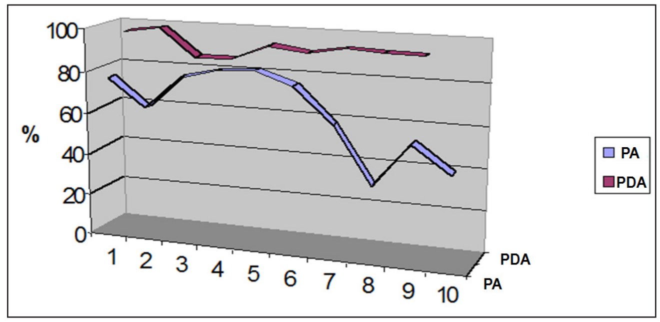


Table 5. Descriptive statistics in percentages found for etch Types I, II, and III in pit and fissure areas for Groups 4 (PA) and 5 (PDA).

Group 4 (PA)	etch type I etch type II	(26.29%) (1.75%) ------------- 28.04 %	etch type III (71.96 %) or not etch
Group 5 (PDA)	etch type I etch type II	(33.41%) (38.97%) -------------- 72.38 %	etch type III (27.62 %) or not etch

When the percentage of acid etching on the enamel's occlusal surface is distributed in the acid etching groups, the results included Group 4 (PA) etch Type I (26.29%), etch Type II (1.75%), and etch Type III (71.96%), while for Group 5 (PDA), we observed etch Type I (33.41%), etch Type II (38.97%), and etch Type III (27.62%) (Table 5).

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Figure 1. A. SEM 50X microphotograph of the occlusal enamel surface polished with the pumice and distilled water technique (Group 2 P). It can be observed that the organic pellicle remained around the occlusal fissure on the enamel surface; this could not be removed with polishing and pumice. B. SEM 100X microphotograph from the same area of the sample. Image shows an organic pellicle around and in the interior of the occlusal enamel-surface fissure. C. SEM 150X microphotograph of the same area of the same sample but a different position. D. SEM 350X microphotograph of the same sample demonstrating organic material in the interior of the fissure.

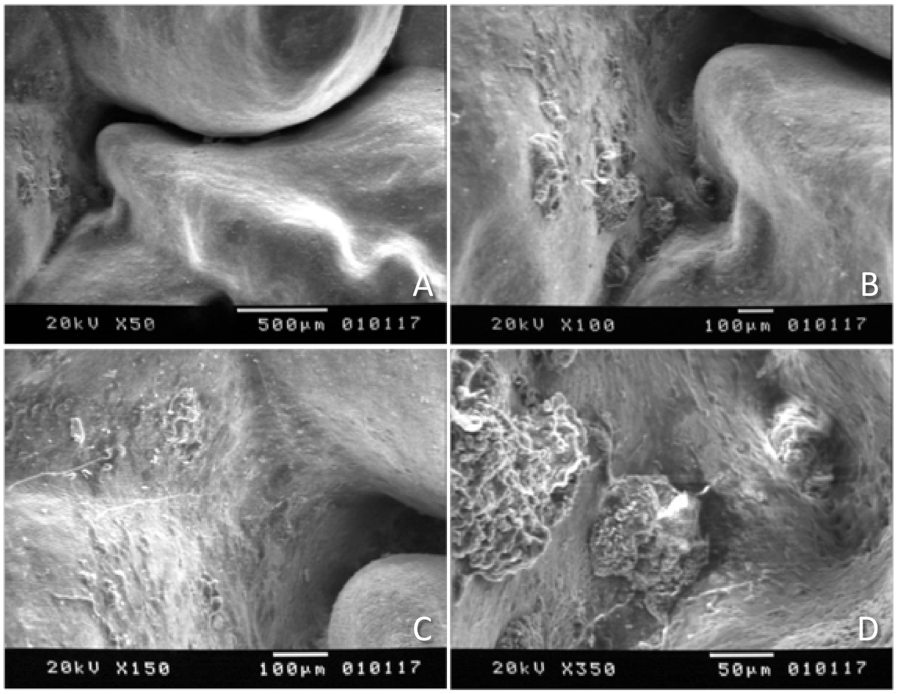


Figure 2. A. SEM 50X microphotograph of the occlusal enamel surface polished with pumice, distilled water, and the deproteinization technique. (Group 3 PD). A significantly cleaner occlusal fissure on the enamel surface is observed where the sodium hypochlorite was utilized after polishing and pumice. B. SEM 350X microphotograph from the same area of the sample. Image shows an absence of organic material around and in the interior of the occlusal enamel-surface fissure. C. SEM 500X microphotograph of the same area of the same sample in a different position. D. SEM 750X microphotograph of the same sample revealing very clearly the interior of the fissure. Areas that may appear as acid-etched surface are observed, and it is indeed an etched enamel, but with the etching carried out by the bacterial organic acids. This surface is not observed in Group P because the organic material is covering its irregularities.

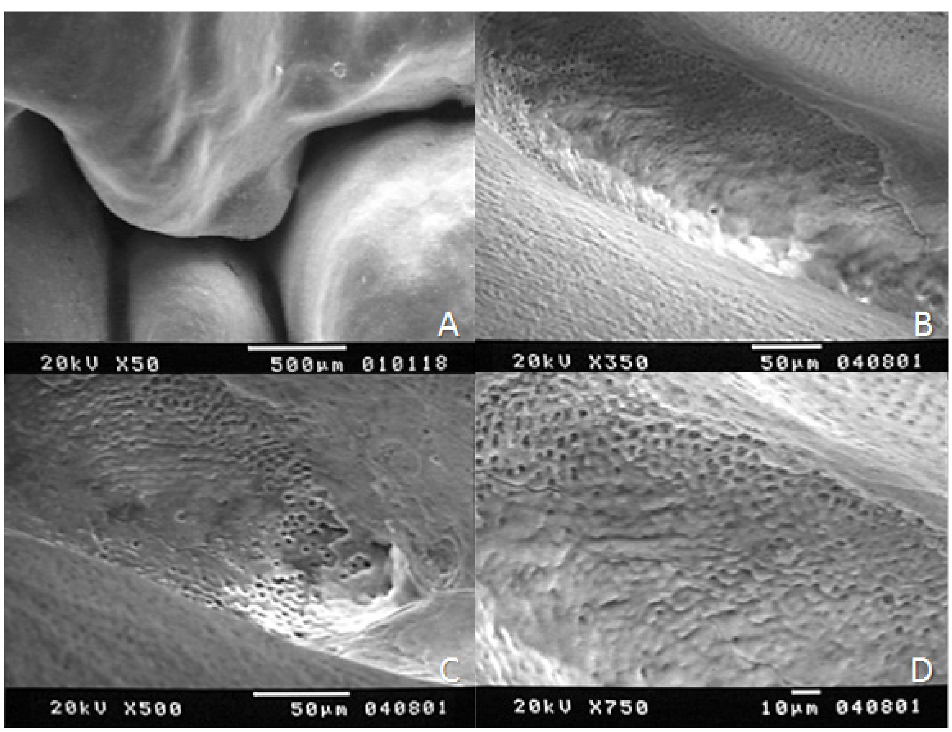


Figure 3. A. SEM 35X microphotograph of the occlusal enamel surface polished with pumice, distilled water, and the acid etching technique (Group 4 PA). A. great amount of organic material in the occlusal enamel fissure that could not be removed with polishing, pumice, and acid is observed. B. SEM 150X microphotograph from the same area of the same sample. Image depicts an organic pellicle around and in the interior of the occlusal enamel-surface fissure. C. SEM 350X microphotograph of the same area exhibiting a great amount of organic material in the interior of the fissure. D. SEM 5 00X microphotograph of the same sample showing organic material in the interior of the fissure.

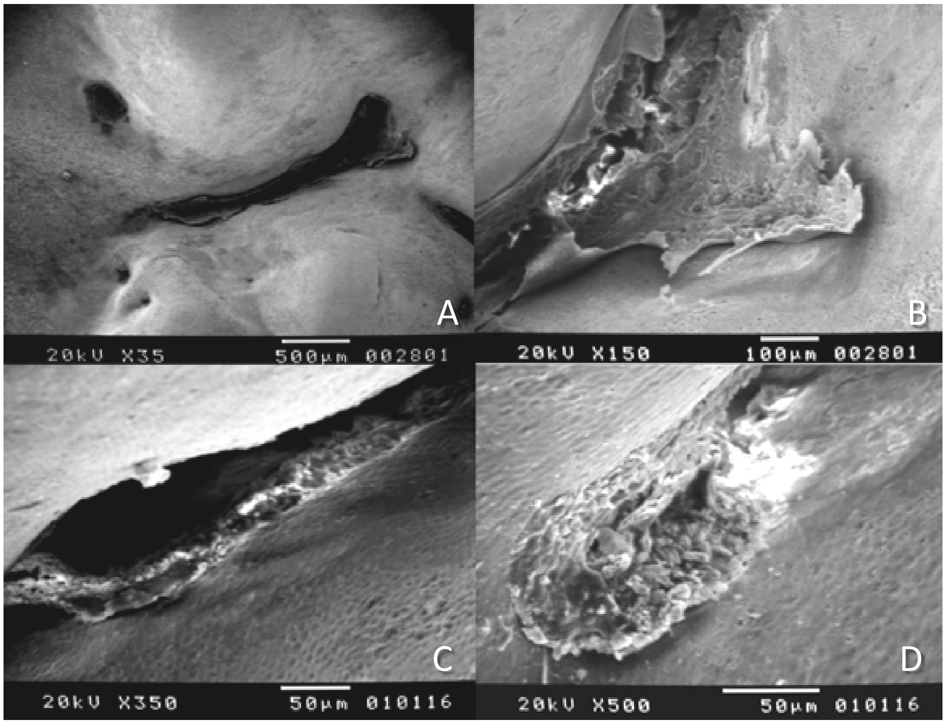
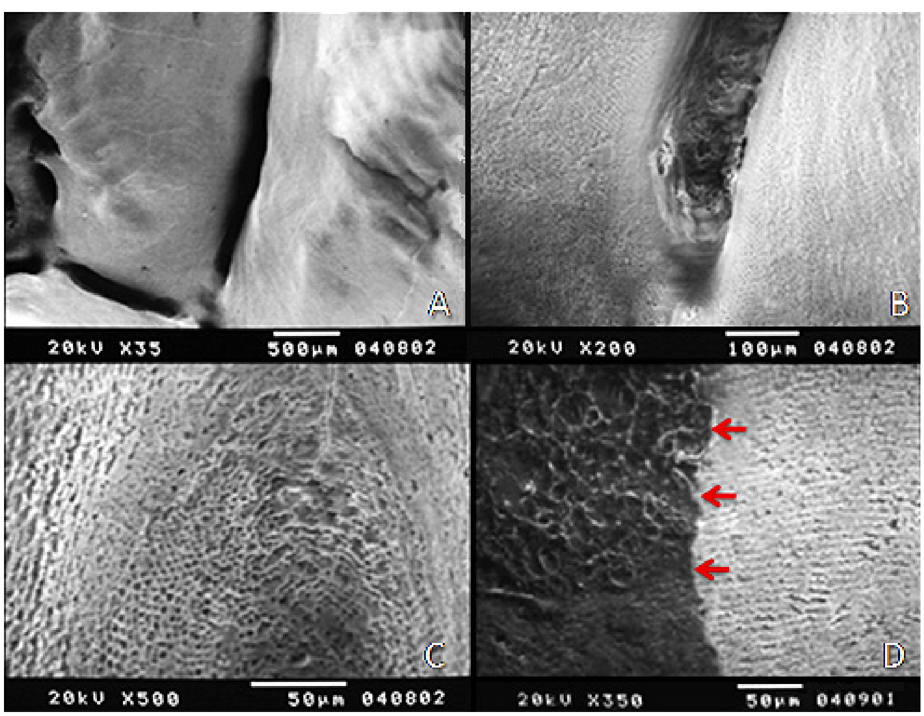


Figure 4. A. SEM 35X microphotograph of the occlusal enamel surface polished with pumice, distilled water, deproteinization, and the acid-etching technique (Group 5 PDA) and on comparison, with the last group (in Fig. 3), a very clean occlusal fissure surface can be observed. B. Same area sample can be observed in the SEM 200X microphotograph. This image demonstrates a clean and etched enamel in the interior surface of the occlusal fissure. C. Same area sample can be observed in the SEM 500X microphotograph, where etched enamel can be observed all along the fissure. D. SEM 350X microphotograph of the same sample showing a much cleaner interior of the fissure. We can appreciate two different surface appearances. On the right side, this figure shows an enamel surface polished with pumice, distilled water, and the acid-etching technique. This is possible to observe on the left side, with the drop of sodium hypochlorite reaching this point.



DISCUSSION

Appropriate enamel etching depends on the type and concentration of the acid, time of exposure, and the composition of the enamel surface including the organic material. After many years, we continue to debate how to etch deciduous and permanent teeth.[(28)] Even after all these years, and with all of the knowledge and technology available, we still face the burden of adhesive failures,[29-31] which requires re-doing part of our previous work.

It has been known since the beginning of 1900s that occlusal surfaces account for up to 43-45% of the carious lesions of all surfaces,[32] and that there are carious lesions on occlusal surfaces in up to 75-92%.[33] It is not a new concept that caries occur in direct proportion to the irregular form and depth of pits and fissures.[34, 35]

Occlusal pits and fissures are considered an incomplete coalescence of the occlusal cusp lobes when ameloblastic activity develops independently and finally becomes welded. After one or more lobes begin to merge, a depression or valley is formed between them. The ameloblasts are accumulated at the bottom of the fissure and, at the same time, the activity of the ameloblasts ceases, accumulating great amounts of organic material. On the other hand, the ameloblasts of the valley sides continue their activity, approaching the walls of the future fissure into three Types: V; I, and Y.[36]

Previous studies have been carried out on smooth surfaces, while occlusal surfaces have large amounts of organic material accumulated on top of the crown, mainly in pits and fissures. These surfaces become a challenge to clean with simple prophylaxis, the latter a possible reason for the failure of sealants.

This study showed that occlusal enamel deproteinization with 5.25% NaOCl for 60 seconds prior to enamel etching with H_3PO_4 exhibited best results for the pit and fissure organic-material removal. Table 3 shows a percentage of 30.1% of organic material present in pits and fissures for the Control group, while the Polish group shows 36.5% and the Deproteinization group presents only 16.4% (Table 3) (Graph 1).

When the occlusal surface was polished and etched, the percentage of organic material present pit and fissure areas was 33.54%, while the polished, etched, and deproteinization groups only presented 7.70%. (Table 4) (Graph 2).

Enamel deproteinization with sodium hypochlorite was able to remove the organic material effectively on occlusal enamel surfaces; hence, there is a significant increase in the retention of all adhesive restorations.

The pit and fissures of immature permanent teeth are highly susceptible to tooth decay. Therefore, pit-and-fissure sealants are frequently applied to these teeth to prevent occlusal caries. A key factor for successful caries prevention with these materials comprises that the sealant should remain intact over time.[37]

Resin composite is the most commonly used sealant material.[38] The caries-preventive effect of resin-based sealants depends on the sealing of pits and fissures through microretention in tags created by the acid etching of enamel. Frequently, these sealants are utilized on teeth with early caries, the latter appearing as a white spot.

Sealants are indicated for primary and permanent teeth with pits and fissures that are predisposed to plaque retention, and they are applied to reduce the risk of pit and fissure caries in susceptible teeth. Because the risk of caries may increase at any time during a patient's lifetime due to hormonal factors, changes in habits, oral microflora, or other physical conditions, unsealed teeth might benefit from sealant application. The need for sealant placement should be reassessed during periodic preventive care, being repaired and replaced as needed. Unfortunately, after all these years, we continue to face adhesive failures and engage in repetitive dentistry. We sometimes need to struggle with insurance companies, who restrict the re-application prior to 5 years of the restoration's initial placement.[27]

It is reasonable to think that pretreating occlusal enamel with sodium hypochlorite would render enamel crystals more accessible to the etching solution, resulting in a clinically more favorable etched surface.

Silverstone and Luria launched the hypothesis that the Type-III acid etching pattern is obtained due to the quality and area of the enamel, the pH of th ace, the quantities of fluoride present, or to the prismatic substance, among others.[10, 39]

However, we have found that when deproteinization is not conducted, the acid etching more easily attacks the center of the prism crystals because it possesses a smaller amount of organic material, while when it is deproteinized, acid etching affects the interprismatics spaces, which contain more organic material between the crystals. This makes us think etch Types I, II, or III can also be determined by removal of the enamel's organic material (biofilm, enamel pellicles, and intercrystal proteins).

CONCLUSIONS

- Deproteinization is an appropriate procedure to eliminate the organic material of the enamel structure that prevents optimal acid-etch action.
- Enamel deproteinization with 5.25% sodium hypochlorite for 60 seconds is an effective way to remove organic material on the occlusal surfaces of teeth, obtaining up to 72.38% of all surfaces with Types I and II etch patterns.
- When surfaces where compared according to ideal etch patterns (Types I and II), the more frequent of these was etch-pattern Type II.
- Groups (P) polish and rinse or (PA) polish, rinse and acid etching do not comprise the best option for effective removal of the organic material on occlusal enamel surfaces.
- Etch Types I, II, and III may be determined by removal of the inter-organic enamel crystals.

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