# Deproteinization of primary enamel with sodium hypochlorite before phosphoric acid etching

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***ABSTRACT***

*The aim of this study was to evaluate the deproteinization of primary enamel by analyzing etching pattern types, with and without the application of 5% NaOCl before acid etching with 37% $H_3PO_4$. Fifteen extracted human primary molars were randomly selected for the present in vitro study; 1mm x 1mm blocks were prepared and divided into two groups (n = 21). These groups were treated as follows: Group A- Acid Etching with 37% $H_3PO_4$ gel for 15 s; Group B- 5% NaOCl for 60 s + Acid Etching with 37% $H_3PO_4$for 15 s. The specimens were prepared for scanning electron microscopy analysis. The images were evaluated for quality types I and II etching of the enamel surface using ImageJ software. Datasets were checked for normality by Kolgomorv-Smirnov test and the non-parametric unpaired Mann-Whitney test was applied. The mean surface area of type I and II etching pattern values was 1922.314 $\mu m^2$for Group A and 3840.473 $\mu m^2$Group B. We conclude that deproteinization with 5% NaOCl prior to acid etching can be used to increase the area of adhesion and the quality of the etching pattern.*

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***Keywords:*** *Dental enamel, tooth, deciduous, dental etching, sodium hypochlorite.*

## Desproteinización del esmalte primario con hipoclorito de sodio antes del grabado con ácido fosfórico

***RESUMEN***

*El objetivo del estudio fue evaluar la desproteinización del esmalte primario a través de los tipos de patrones de grabado, con y sin NaOCl 5% utilizado antes del grabado ácido con H3PO4 37%. Quince dientes primarios humanos extraídos se seleccionaron al azar para el presente estudio in vitro, se prepararon bloques de 1mm x 1 mm y se dividieron en dos grupos (n = 21). Estos grupos se trataron de la siguiente manera: Grupo A: Grabado ácido con H3PO4 37% en gel durante 15 segundos; Grupo B: NaOCl 5% durante 60 segundos + Grabado ácido con H3PO4 37% durante 15 segundos. Las muestras se prepararon para el análisis de microscopía electrónica de barrido. Las imágenes obtenidas se evaluaron principalmente por la calidad de los grabados tipo I y II de la superficie del esmalte primario, utilizando el software Image J. Los datos se analizaron en cuanto a su normalidad mediante la prueba de Kolgomorv-Smirnov, se utilizó pruebas no paramétricas: Prueba de Mann-Whitney no pareada. Como resultado, se encontró que el área de superficie media de los valores de patrón de grabado de tipo I y II para el Grupo A era 1922,314 $\mu m^2$ y el Grupo B era 3840,473 $\mu m^2$. Finalmente, llegamos a la conclusión de que se puede usar la desproteinización con NaOCl 5% antes del grabado ácido para aumentar el área de adhesión y la calidad del patrón de grabado.*

***Palabras clave:*** *Esmalte dental, diente deciduo, grabado ácido, hipoclorito de sodio.*

## INTRODUCTION

For decades, the dental community has endeavored to obtain and use materials and techniques that increase the bond strength of restorative materials and orthodontic devices to the tooth enamel surface. A good bonding technique requires appropriate preparation of the enamel surface, which includes removing the pellicle and roughening the surface,

in a process called conditioning[1]. Enamel is conditioned using one of two techniques: (a) acid etching, which uses an acid gel to produce micro-etching and (b) the abrasive technique, using abrasive air and resulting in macro-etching[2].

The introduction of the acid etching technique in 1955 by Buonocore marked a milestone in dentistry[2]. This concept is based on the acid dissolution of the dental enamel surface to create micro-porosities on the surface, thereby improving micro-mechanical adhesion. Subsequent modifications and improvements have been made, including the dilution of phosphoric acid concentration from 80% to 37% and a timereduction from 60 s to 15 streatment[3]. Phosphoric acid etching removes a 10 μm to 50 μm layer from the enamel surface, while the rough surface increases from 10 μm to 200 μm[4, 5]. However, phosphoric acid alone does not produce a total adhesion surface. Espinosa et al.[6] found that phosphoric acid etched less than 50% of the enamel area, while Hobson et al.[7] found up to 69% of the surface intact, with only 2% of the ideal surface etched.

The adhesion of restorative materials and orthodontic devices to the enamel surface of the tooth depends on the type of agent used, the duration of the use of acid and the enamel surface[8]. Comparing the enamel of primary and permanent teeth, it is logical to expect differences in the quality of the etching process and in bond strength. Several studies have shown that this process is negatively affected by a high amount of organic structures and by the presence of a prism less layer on the enamel surface of deciduous teeth[9-11].There is therefore a need to modify the organic content of the enamel of primary teeth. This can be done by using a recently developed method: deproteinization.

Enamel deproteinization involves the removal of the organic content (proteins) from the enamel surface. NaOCl has been shown to degrade protein efficiently and to be capable of eliminating excess protein[12]. Proteins interfere in the establishment of a clinically successful acid etching pattern and their elimination improves the bond[13]. LA A study with electron microscopy by Espinosa et al.[12] showed that the use of 5% NaOCl improves both the quantity and the quality of the etching surface, suggesting that this method has the potential to optimize adhesion and improve bond strengths. This process duplicates the retentive surface of the enamel up to 94.47% and produces an increase in patterns I and II, which have greater retentive capabilities than the type III etching pattern, thus improving retention[12].

Several deproteinizing agents are currently used, such as papain gel from the papaya plant, bromelain enzyme from the leaves of the pineapple plant and calcium hypochlorite. Pithon et al.[14] used 10% papain gel as an enamel-protecting agent before the cementing process and achieved good results with respect to the bond strength of the cemented brackets with modified glass ionomer. Dayem and Tameesh[15] evaluated the deproteinizing effects of the enzyme bromelain, which decreased the filtration score of the adhesive system.

Deproteinization has the potential to become a convenient, non-invasive, low-cost method to increase the binding forces of restorative materials and orthodontic devices, which can be quickly and easily used in daily clinical practice[16].

However, few studies evaluating deproteinization of the enamel surface have found significant improvements in adhesion properties[17,18], so further studies are needed to elucidate this issue. The aim of this study was therefore to evaluate the deproteinization of primary enamel by analyzing etching pattern types, with and without the application of 5% NaOCl before etching with 37%$H_3PO_4$.

## MATERIALS AND METHODS

### Study Design

This study reports the findings of an experimental *in vitro* study. The samples consisted of fifteen asymptomatic primary molars without caries and with different degrees of root resorption, collected from children who visisted the Postgraduate Unit of the Faculty of Dentistry of the *Universidad Nacional Mayor de San Marcos*, Lima-Peru. Prior to tooth donation, signed parental consent and child assent agreement was requested. Teeth were extracted under clinical indication and immediately stored in saline solution. This study was approved by the Ethics Committee of the Faculty of Dentistry of the *Universidad Nacional Mayor de San Marcos*, Lima -Peru.

The fifteen teeth were washed with distilled water for 10 s, and then cut with a Giflex-TR diamond disc and a low-speed handpiece(NSK Ex 203C), discarding the roots. The buccal surface of the crown was divided into four 1 mm x 1 mm thickness

fragments[12]. All samples were stored in saline solution at 37 °C and then randomly assigned to two groups:

Group A: The enamel surface was etched with 37% $H_3PO_4$ (Scotchbond™ universal etchant gel; 3M ESPE, St Paul, MN, USA), applied for 15 s with a microbrush, washed with sterile water for 20 s, and then dried with compressed air[12].

Group B: The enamel surface was treated with5% NaOCl (Clorox®) applied with a sterile cotton swab for 60 s, washed with sterile water, then dried for 10 s, and etched with 37% $H_3PO_4$ (Scotchbond™ universal etchant gel; 3M ESPE, St Paul, MN, USA), applied for 15 swith a microbrush, washed with sterile water for 20 s then dried with compressed air[12]. This procedure was performed in the Postgraduate Unit of the Faculty of Dentistry of the *Universidad Nacional Mayor de San Marcos*, Lima-Peru.

Samples were analyzed with a scanning electron microscope (SEM,Faculty of Biology of *Universidad Nacional Mayor de San Marcos*, Lima-Peru, FEI model Inspect S50). All samples were sputter-coated with gold and taken to the SEM.A total 180 micrographs were taken at different magnifications: 150x, 500x, 4000x.

Two researchers received theoretical, practical and clinical specialized training and were calibrated for the evaluation of the SEM images. Intra-and inter-examiner reliability was assessed by kappa statistics (Kappa>0.80).

**Etched area measurement protocol**

The etched area, as defined by Silverstone, was measured using ImageJ[19] software, according to the following protocol:

1) The type of etching observed in each image was determined and marked according to the following color code: type I (green), type II (yellow), type III (red), type IV (blue), type V (orange).
2) With the aid of ImageJ software, the area corresponding to each color was measured.

**Statistical analysis**

The results of area measurements and type of etching were recorded in an electronic database with controlled entry fields in Microsoft Office Excel 2016. A total 42 images were analyzed, 21 from Group A and 21 from Group B. The data were analyzed with statistics and graphical tests using the Stata Statistical Software Release 14. College Station, TX: StataCorp LP.

Before determining significance, the variables were subjected to normality tests, using Kolgomorv-Smirnov graphical methods. It was found that the variables did not comply with this assumption of normality because the analysis with Stata Statistical Software (Release 14, College Station, TX: Stata Corp LP)gave a p value <0.05. Therefore, the nonparametric Mann-Whitney U test was used to determine the significance.

## RESULTS

To evaluate the deproteinization of primary enamel through etching pattern types, the etching pattern was analyzed on a total 42 photographs, of which 21 corresponded to Group A ($H_3PO_4$ 37%) and 21 to group B (NaOCl 5% +$H_3PO_4$ 37%), at magnification 4000x. All samples were analyzed to determine whether their pattern was type I, II, III, IV or V. Table 1 shows average etched area in groups A and B, according to etching types. Only one sample from Group A and one sample from Group B had type V etching pattern. Only one sample from Group B had type IV etching pattern. No sample from Group A had type I etching pattern. Group A (1922.314 μm$^2$) hada lower etched area consistentwithtypes I and II compared to Group B (3840.47μm$^2$).The etched area of Group A exceeded the area of Group B, when the areas were of types III, IV and V. The etching type with the largest area in Group A was type IV, followed by type III. The etching types with the largest area in Group B were types I and II, almost in the same proportion. The area of type III in group A was three times greater

**Table 1: Average etched surface in Groups A and B according to etching type (μm$^2$).**

| Group | Type I | Type II | Type III | Type IV | Type V |
|---|---|---|---|---|---|
| Group A | - | 1922.314 | 3411.056 | 1989.716 | 4797.826 |
| Group B | 3870.061 | 3831.226 | 1066.414 | 458.037 | 450.939 |
| Total | 3870.061 | 3310.614 | 2169.775 | 1893.986 | 2624.383 |

than in Group B. Table 2 shows the type I and II etching surfaces per group.

Only 6 out of the 21 samples from Group A (28.57%) presented a type II etching pattern, with an average of 1922.314 $\mu m^2$, while all samples from Group B presented a type I or II etching pattern, with an average of 3840.473 $\mu m^2$. Figures 1 and 2 show that Group B has a larger area of type I and II compared to the mean (Fig. 1) and compared to the median (Fig. 2).

The Mann-Whitney U test showed significant differences between groups ($p = 0, 0004$). Table 3 shows the etching pattern of the surface (types III, IV and V) per group.

All samples from Group A had type III, IV or V surface etching patterns, while in Group B, this percentage was 85.71%. However, the type III, IV or V etched area was four times smaller in group B than in Group A. Figs. 3 and 4 show that Group A has a larger area of type III, IV or V, compared to the mean (Fig. 3) and to the median (Fig. 4).

Mann-Whitney U test showed significant differences in the etched area between the two groups, ($p< 0.001$). Figs. 5 and 6 show some examples of the etching patterns found in this study.

## DISCUSSION

Studies have shown that enamel etching patterns depend on the type of substance used and its concentration, as well as the time of etching and the composition and morphology of the surface of the enamel to be etched[6]. Good adhesion of composite materials requires a good amount of etching to ensure maximum retentive capacity, as well as a good etching pattern (types I and II). Phosphoric acid has long been used for this purpose. However, $H_3PO_4$alone does not produce a total adhesion surface[7]. Espinosa et al.[6] found that $H_3PO_4$etched

**Table 2: Descriptive statistics of etched surface type I and II per group ($\mu m^2$).**

| Group | N | Mean | Standard Deviation | Minimum | Maximum | Median | Interquartile range |
|---|---|---|---|---|---|---|---|
| Group A | 6 | 1922.31 | 574.490 | 1278.72 | 2599.196 | 1868.21 | 1149.605 |
| Group B | 21 | 3840.47 | 754.799 | 2502.12 | 4797.826 | 3939.90 | 1021.807 |

**Table 3: Descriptive statistics of etched surface type III, IV and V per group ($\mu m^2$).**

| Group | N | Mean | Standard Deviation | Minimum | Maximum | Median | Interquartile range |
|---|---|---|---|---|---|---|---|
| Group A | 21 | 4248.59 | 935.07 | 2198.63 | 4797.83 | 4797.83 | 1278.72 |
| Group B | 18 | 1116.91 | 693.95 | 107.88 | 2295.70 | 1069.35 | 1049.80 |

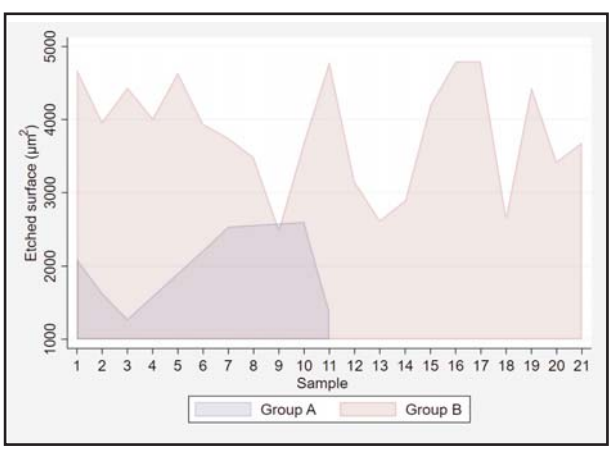


*Fig. 1: Type I and II etched surfaces per group ($\mu m^2$).*


*Fig. 2: Box plots for type I and II etched areas per group.*

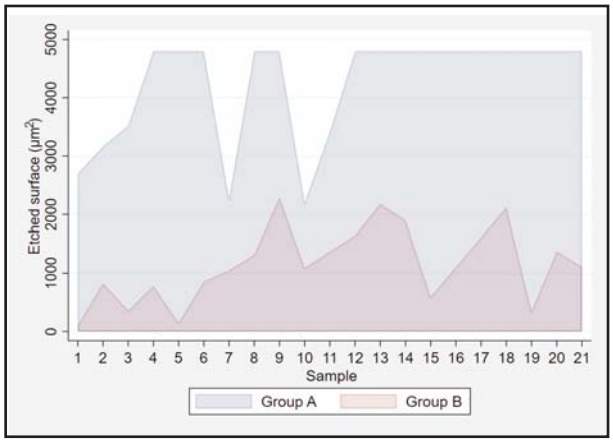


*Fig. 3: Type III, IV and V etched areas per group (µm²).*

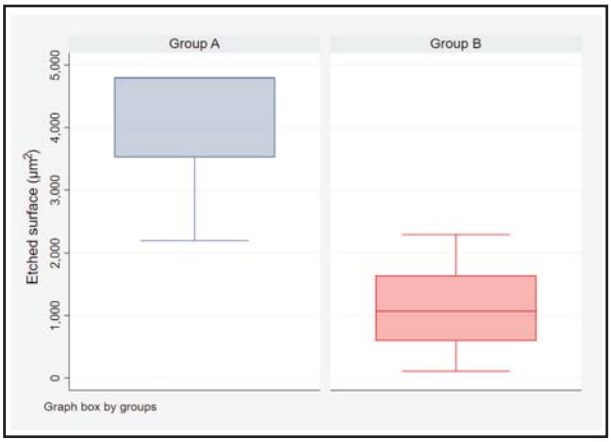


*Fig. 4: Box plots for types III, IV and V etched areas per group.*

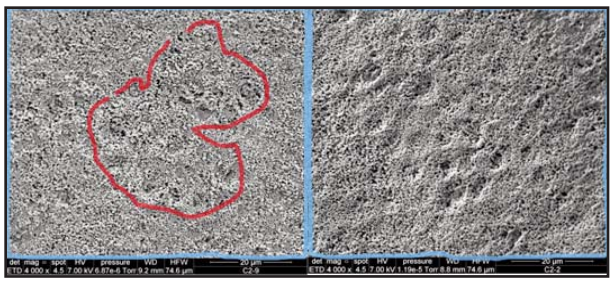

*Fig. 5: Example of etching pattern types III (red) and IV (blue) found in Group A.*

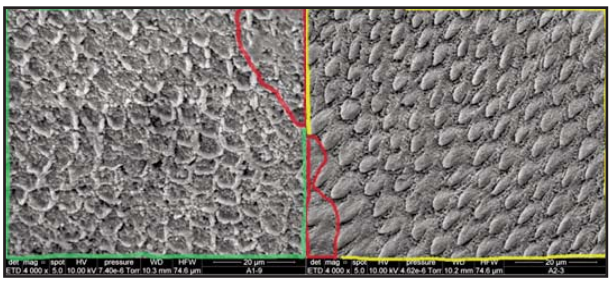

*Fig. 6: Example of etching pattern types I (green) and II (yellow) found in Group B.*

less than 50% of the enamel area, while Hobson et al.[7] found up to 69% of the surface intact, with only 2% of the surface ideally etched. In our study, phosphoric acid used alone produced 11.44% of etching surface type I and II patterns (considered the ideal etching types) with respect to the total area of all samples analyzed. This highlights the fact that the $H_3PO_4$alone did not produce a single sample with a type I etching pattern.

This poor etching action is a consequence of phosphoric acid acting only on the mineralized surface (inorganic material) and not on the organic material that usually coats the enamel. So, a previous process to remove this organic material is needed before starting the etching protocol. Etching is often hampered by the presence of proteins seated between the enamel crystals. In order to overcome this difficulty and obtain a greater retentive etching, Espinosa et al.[12] and Ananthi et al.[20] developed a pre-treatment for all the enamel to be etched based on enamel deproteinization with 5.25% NaOCl for one minute. This step has been successful in etching enamel surface, increasing the etched retentive surface by more than 50%[12,20].

We found that in group B (5% NaOCl +37% $H_3PO_4$), 80.04% of the total area was etched in all of the samples with type I and II patterns. This was higher than the etched area in group A (only 37% $H_3PO_4$). Moreover, only some of the Group A samples had type I or II etching patterns, with an average of 1922.314 µm², while all the Group B samples had etching types I or II, with an average of 3840.473 µm². In other words, the type I and II area etched by 37% $H_3PO_4$ combined with sodium hypochlorite was much larger than the type I and II area etched by $H_3PO_4$ alone. These differences were statistically significant.

The opposite occurred for etched surface types III, IV and V by group. Types III, IV and V are considered weaker etching patterns. Group A had a larger etched area for each of these types (III, IV and V). Etching patterns Type III, IV or V were four times higher in group A than Group B. In addition, in Group B, only one sample was found with

type IV etching and only one sample with type V etching, clearly showing that 5% sodium hypochlorite and 37% phosphoric acid almost exclusively generated the best etching patterns (I and II) in the samples.

These results agree with Ananthi et al.[20] who also found a larger type I and II etching surface when $H_3PO_4$was used in combination with NaOCl (45051.34 $\mu m^2$) compared to $H_3PO_4$alone (39608.18 $\mu m^2$), and with the study by Sharma et. al.[16] who found a greater amount of type I and II patterns in the group deproteinized with 5.25% NaOCl in a study to determine the effect of deproteinization using sodium hypochlorite on the bond strength of orthodontic brackets.

Ekambaram et al.[21] have even observed type I and II etching patterns in hypomineralized teeth after pre-treatment with 5% NaOCl. These data also agree with an earlier study conducted by Espinosa et al.[12],who found the largest etched area with type I and II patterns when the combination was used for 30 s(559.681 $\mu m^2$) and for 60 s (718.244 $\mu m^2$), compared to the area etched when phosphoric acid alone was used (368,689 $\mu m^2$). The difference in the numerical values can be explained by the differences in the magnification and the calibration used. For example, Espinosa et al.[12] used samples with 500x magnification, while we used 4000x magnification.

Our results do not agree with Ramakrishna et al.[17], who concluded that there was no significant improvement in the deproteinization of enamel after acid etching with respect to the appearance of Type I-II etching patterns. Neither do our results agree with Bhoomika et al.[18], who found no significance in etching pattern values in a group treated only with phosphoric acid, while another group treated with sodium hypochlorite and phosphoric acid had53.58%. Bhoomika et al.[18]even claim that the use of 37% $H_3PO_4$for15 s is still the best method for the pre-treatment of enamel.

The best retention area is obtained with Type I and I etching patterns, which have larger, deeper retentive areas compared to other types of patterns which do not haveneat, deep morphology, and lack the micro-mechanical retention that guarantees proper adhesion of restorative resins.

Another way to evaluate and compare the effect of deproteinization is through tensile tests performed on teeth that have been subjected to any of the etching procedures. The mechanical tests confirm that deproteinization before etching significantly improves the values of shear bond strength both in permanent teeth and in immature primary teeth, as it increases the surface area of the adhesion of the composite material tothe dental surface and presents better etching patterns, thereby increasing the adhesion strength of orthodontic appliances[5,8,16,21-24]. However, other researchers such as Poggioet al.[25] and Monjarás-Avila[26]did not find significant effects of the deproteinization of the enamel with NaOCl on shear bond strength.

Taken together, these data indicate that the deproteinization of the tooth structure before acid etching increases the etched area of type I and II patterns, which are considered the best etching patterns. This, in turn, enables better adaptation of the material to be bonded and longer survival of restorations. The latter was demonstrated in the study by Espinosa et al.[6], which found a lower loss in percentage of sealing material in molars with deproteinization compared to teeth that had not received this treatment, when the teeth were checked six months after treatment.

Shear tests have also found the same phenomenon, showing more cohesive failures when enamel is deproteinized before acid etching [11]. Additionally, Ekambaram et al.[22] have found that deproteinization of the enamel with NaOCl results in some cohesive failures within the enamel structure.

It should be noted that other studies have used software outside the field of medicine to measure etching, which implies certain limitations. For instance, AutoCAD is a software used to develop 3D structures, which is widely used in engineering and requires quite a long learning time. Photoshop software, even though it provides tools for manipulation, is also not designed for the field of medicine. In this study, we used the ImageJ program to determine the recorded area. ImageJ is a free software which is widely used in the field of biomedicine for densitometry morphometric analyses. It can be used to calculate area, perimeter or length of particles contained in a digital image[19].

Finally, the clinical implications of these findings are important. Based on the results (a greater amount of type I and II etching after the use of NaOCl and $H_3PO_4$),deproteinization of the surface of enamel with 5% NaOCl for 60 s before acid