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Comparative evaluation of structural changes in enamel induced by 37% phosphoric acid before and after enamel deproteinization with 5.25% sodium hypochlorite – An sem study

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Abstract:

Context: The use of 5.25% sodium hypochlorite (NaOCl) as a deproteinizing agent might remove organic elements of both enamel structure and acquired enamel pellicle, thereby enhancing the retentive and sealing abilities of restorative materials on acid etched enamel surface**.** Aims: To assess the effect of deproteinization before and after acid etching on micromorphological features of enamel of permanent teeth compared to acid etching alone using SEM analysis.

Settings and Design: Forty enamel blocks were randomly distributed into 4 groups (10 each).According to the surface treatment in the form of deproteinized with 5.25% sodium hypochlorite (NaOCl) before and after acid etching with 37% phosphoric acid (H3PO4)

compared to application of H3PO4 alone. Group1 (10 blocks) - No treatment was carried out. Group2 (10 blocks): Acid etched with 37 % phosphoric acid (H_3PO_4) for 15 seconds. Group 3 (10 blocks): treated as in Group2 followed by application of 5.25% sodium hypochlorite (NaOCl) for 60 seconds. Group 4 (10 blocks): 5.25% sodium hypochlorite (NaOCl) was applied for 60 seconds and then treated as in Group2 .The samples were subjected to SEM analysis Statistical analysis used: Analysis of variance, and Tukey's honest significance difference test were used. All statistical analyses were established with a significance level of $P < 0.05$.

Results: The highest percentage of type1-type2 acid etch pattern was recorded in Group 4(NaOCl + H3PO4) and the lowest percentage was recorded for Group 3(H3PO4+NaOCl). SEM evaluation showed different topographical features of deproteinized enamel surface

Conclusions: Conventional acid etching might be adequate to produce ample retentive surface area on enamel, as the differences in total etched area after the three different surface treatment regimens were not statistically significant.

Keywords: Enamel deproteinization, Acid etching, Sodium hypochlorite, Phosphoric acid.

Introduction

Adhesion of resin based restorative materials on enamel demands a uniform retentive area over the entire acid etched enamel surface. Enamel, the hardest tissue in the human body is composed of 96% inorganic component and the rest 4% is organic component and water[1] .In 70% of permanent teeth and all primary teeth structure less layer of enamel has been described, which is prismless enamel. The prismless layer is more resistant to acid attack[1].

Buonocore in 1955, proposed that acid etching changes the enamel topography from a low reactive to a surface that is more susceptible to adhesion[2]. Acid etching removes approximately 10µ of enamel surface and creates a morphologically porous layer (5µ to 50µ deep) which increases the surface energy and thus fluid resin contact surface[3].

The etching pattern on teeth was first described by Pool and Johnson (1967)[4] which was further classified by Silverstone et al. (1975). Three patterns of enamel surface etching were noticed. Gallil et al**.** (1979) classified enamel etching patterns into 5 types[5]:

1) Preferential dissolution of prism cores; resulting in a honeycomb like appearance
2) Preferential dissolution of prism peripheries giving rise to cobblestone appearance
3) A mixture of type 1, type 2 pattern
4) Pitted enamel surface as well as structures that look like unfinished maps or networks
5) Flat, smooth surfaces.

Of these types, type 1 and type 2 etching patterns shows greatest retention with adhesive materials[5,6].

However, the studies have shown that topographic quality of enamel etching with phosphoric acid is not achieved over the entire adhesion surfaces. More than 69% of treated surface had no etching. 7% presented tenuous etching; only 2% was ideally etched[7]. This was the main reason for increased failure rate of sealants and adhesive restorations .

Etching quality depends mainly on etching agent, acid concentration, etching time and composition of enamel surface. Organic deposits such as surface cuticle and stained pellicle cover the enamel surface[8]. These remnants might interfere with etching process, resulting in lower resin adhesion[9].

Deproteinization of enamel is a noninvasive way by which organic content of enamel can be removed[13].

Since 1920, sodium hypochlorite solution has been used as an endodontic irrigant due to its excellent protein denaturing and anti-bacterial action. Hand et al. observed that dilution more than 5.25% greatly reduced the tissue dissolving property of sodium hypochlorite[10]. Venezia et al. first proposed enamel deproteinization with 5% sodium hypochlorite after acid etching, which resulted in improved bonding of orthodontic bracket to hypomineralized enamel. Removal of excess proteins may provide an advantage on bonding of restorations[11]. Gordon et al. observed that most of the tissue dissolving activity of sodium hypochlorite was lost after two minutes of contact with organic tissue[12].

Espinosa et al. showed that enamel deproteinization with 5.25% sodium hypochlorite for 1 minute prior to phosphoric acid etching doubles enamels retentive surface to 94.47% and there was an increase in type 1 & type 2 etching pattern[13].

Other deproteinizing agents like papain gel, bromelain enzyme and chlorine dioxide are included in the literature. Pithon et al. evaluated the effect of bromelain in association with 10% papain gel as deproteinizing agent on orthodontic bracket bonding and found an increase in the shear bond strength of bracket bonded with RMGIC[14]. Hasija et al. compared the effect of different deproteinizing agents on shear bond strength of composite to primary teeth enamel and observed that deproteinization with bromelain gel showed effective bond strength[15].

However, studies dealing with effect of sodium hypochlorite pre or post treatment on the topographic feature of acid etched permanent tooth enamel have not been reported yet.

Therefore, the purpose of this in-vitro study was to identify the topographic feature of enamel deproteinized with 5.25% sodium hypochlorite before and after acid etching with 37% phosphoric acid compared to application of 37% phosphoric acid alone using scanning electron microscope.

Materials and Methods:

This study was carried out in the Department of Pediatric and Preventive Dentistry , Mar Baselios Dental College, Kothamangalam, Kerala ,India. Ten permanent mandibular first or second molars extracted for periodontal reasons were selected as sample. The teeth with enamel cracks or fractures along their buccal aspect, malformations, carious lesions, restorations or erosions were excluded. Samples were stored in saline solution at 37^0C, after extraction and were polished with pumice and rinsed with distilled water for 10 seconds. Roots were amputated and separated with a low speed double sided diamond disk (Shofu, Japan) under continuous water spray irrigation. To obtain enamel samples comparable among themselves and with uniform physical and chemical characteristics, the buccal surface of each crown was marked with 2 horizontal lines dividing the crown portion into 3 parts and the middle section was taken as the study specimen[13]. In that middle section, 3 vertical lines were marked equidistant to each other and was cut with the same disc and trimmed to $1mm^2$ giving 4blocks per sample. Thus, 40 enamel blocks of $1mm^2$ was obtained from 10 teeth. These blocks were the divided into their respective groups depending on the treatment given. To maintain uniform standard between samples, each tooth was divided into four sections, which formed three treatment groups and one control group. Each tooth was subjected to 3 different treatments ensuring that the surface

treatments were applied to teeth with the same enamel quality[12].

Preparation of the sample for study

Group 1 (No treatment group): 10 enamel blocks of 1 mm^2 obtained from middle portion of all teeth were utilized for SEM analysis. All the blocks were observed under a Scanning Electron Microscope for any defects or cracks produced during sectioning process.

Group 2 (Control-acid etch group): The specimens were etched with 37% H_3PO_4 gel, applied with a micro brush for 15 seconds, washed with sterile water and air sprayed for 10 seconds, then dried with oil free compressed air.

Group 3 (H_3PO_4 followed by NaOCl): The enamel surface was treated with 37% H3PO4 gel applied with microbrush for 15 seconds, washed with sterile water and air sprayed for 10 seconds, then dried with oil free compressed air. The etched enamel surface was then treated with 5.25% NaOCl applied with a sterile cotton pellet for 60 seconds, washed with distilled water and dried with oil free compressed air for 10 seconds.

Group 4 (NaOCl followed by H_3PO_4): The enamel surfaces were treated with 5.25% NaOCl , applied with sterile cotton pellet for 60 seconds, washed with sterile water for 10 seconds, then dried and etched with 37% H3PO4 gel applied with a microbrush for 15 seconds, washed with sterile water and then dried with oil free compressed air for 10 seconds.

After the assigned treatment, all the specimens were prepared for SEM analysis.

Preparation of specimen for sem analysis

All the samples were prepared for Scanning Electron Microscope (SEM) analysis. The samples were coated with gold electrodepositing, using a Sputtering Effacoater (JEOL JFC-1600 AUTO FINE COATER) and prepared for surface SEM analysis using Scanning Electron Microscope (JEOL JSM 5610LV, Japan).

The observation zone for all samples was standardized at middle upper section of tooth. 5 microphotographs at 500x magnification were obtained from each enamel specimen covering the entire treated sample surface. A total of 20 microphotographs for each molar were obtained in a consecutive order. Thus, a total of 200 images or 50 images per group were generated for its analysis. The acquired images were subjected to descriptive analysis. The SEM photographs were interpreted by two separate examiners who were blinded to the treatment rendered and well trained to analyse the SEM views. To obtain quantitative results, the sample were evaluated using Auto CAD 2014 software to grade each of the image.

Results and Discussion

Results: The surface area of Type 1 and Type 2 etching patterns were determined for each image. The highest percentage of Type 1- Type 2 acid etch pattern was noticed in group 4 as shown in figure 4. Descriptive statistics are presented in Table 2. Specific Post Hoc test is in line with ANOVA test and there is no significant difference among each group.

Discussion: The enamel surface is highly resistant to acid dissolution due to the presence of prismless enamel[1]. The thickness of this prismless layer is about 15 to 20µm[21]. The enamel surface is usually in a low energy, weakly reactive, and hydrophobic state. When exposed to acid, it becomes a high energy, low reactive and hydrophilic surface. This high energy provides a favorable environment for bonding of resin to tooth structure[17,18]. Buonocore in 1955, first demonstrated that acid conditioning with 85% phosphoric acid for 60 seconds, increased resin adhesion to enamel[3]. The acid etching produces increased retentive surface area due to

selective demineralization of hexagonal prisms. Since 1960, different types of acid with various concentrations or etching time have been investigated. Various acids such as citric, maleic, hydrofluoric and hydrochloric acids were compared to phosphoric acid, among which phosphoric acid was found to be the most effective in promoting enamel adhesion to dental materials in vitro[21]. When phosphoric acid was used in vitro, at concentration less than 30% .It was considered to be inefficient in providing adequate enamel dissolution for bonding, while acid concentration more than 50% presented fewer surface morphological changes. Chow and Brown observed that when 50% phosphoric acid was applied for 60 seconds on enamel, a precipitate of mono calcium phosphate monohydrate was formed, which could be rinsed off easily and at a concentration less than 27%, a precipitate of dicalcium phosphate dihydrate was produced, which could not be easily rinsed off[25].Thus a concentration of phosphoric acid between 30% and 40% was most preferred for achieving favorable retentive area.

Acid etching time as long as 60 seconds was recommended in early 1970s. Silverstone in 1974 observed that phosphoric acid solution in concentration between 20 % and 50% when applied for 60 seconds, produced the most retentive conditions and 30 % unbuffered solutions produced the most consistent and evenly distributed etch pattern[6]. During the subsequent decades, studies ascertained that shorter etching time did not influence the clinical performance of enamel adhesion. In-vitro assessment by Nordenvall et al. in 1980, Brannstrom et al. in 1982**,** Fuks et al. in 1984 suggested that 15 to 20 seconds of acid etching might be acceptable to provide increased retentive surface area. Clinical studies by Stephen et al**.** in 1982, Eidelman et al. in1984, Tandon et al.in 1989 verified that 20 seconds etching time produces retention rate similar to 60 seconds etching time. However, Zhu et al. 2014 reviewed that even though etching time varied broadly in the past, 15 to 30 seconds produced preferable etching pattern for enamel adhesion.

Clinically, the acid etched surface appears as chalky white and opaque. Platia et al**.** evaluated the acid etch patterns on permanent enamel after different pretreatment modalities under SEM and concluded that type 1, type 2 and type 3 etching patterns can appear randomly at any point on the enamel and be found together in the same enamel zone. The morphological changes generated vary from tooth to tooth with a prevalence of type 3 etching pattern, which significantly decreases the ability of material to bond effectively to enamel. Similar result was obtained in the present study, with different patterns of etching occurring together in a specimen[15].

Acid etching has 2 distinct actions on human enamel.

- Removes superficial plaque, debris and chemically inert enamel crystallites.
- Rendering the enamel surface more porous[19].

Phosphoric acid acts mainly on mineralized tissue. Unfortunately, the acid does not eliminate the organic matter such as enamel pellicle which was proven by Nakabayashi in 1998[15]. The effect of acid etching on enamel depends on the way in which etching is activated and if enamel is instrumented before acid etching procedure. The effect of acid etching might also vary in fluoridated, hypo mineralized or stained enamel surface in primary or permanent teeth [20].

Hobson et al. observed that only 2% of the surface was ideally etched when enamel surface was conditioned with 37% phosphoric acid for 30 seconds. However, Espinosa et al, observed 49% of enamel surface as ideally etched when treated with the same concentration

of phosphoric acid for 15 seconds. Bhoomika et al. and Lopez et al. reported, with similar concentrations and time of acid etching, less than 50% of enamel surface was ideally etched. The result of the present study is in agreement with aforementioned studies.

Organic materials and salivary pellicle are found on the superficial zone, which may interfere with the conventional etching technique[21]. Polishing the enamel surface is intended to eliminate the organic components that hinder effective enamel etching. However, Abreu et al**.** observed that organic pellicle could not be removed entirely with pumice prophylaxis[22].Thus to counteract these limitations, various invasive and noninvasive techniques such as air abrasion and CO_2 laser, were used but no good results were obtained[23,24].

Dakin suggested that 0.5% of sodium hypochlorite solution can be used as an antiseptic for infected wounds. Crane AB in 1920 advocated sodium hypochlorite as an endodontic irrigant due to its bactericidal and proteolytic properties. Gurney and Rapp observed in their study that pretreatment of sodium hydroxide, (which is a derivative of sodium hypochlorite solution) at different concentrations before acid etching, provided a smoother surface enamel because, the organic coats over the enamel surface had been thoroughly dissolved off[25].Ultra-structurally, hypocalcified enamel was found to have rougher crystallite structure than normal enamel. Hypocalcified enamel also has elevated protein content due to protein retention during development. All these factors render bonding composite resin to hypo calcified enamel more difficult. Espinosa et al. suggested that prior enamel deproteinization with 5.25% NaOCl for 60 seconds in permanent enamel doubled the retentive surface of acid etched enamel to 94.47%[15].

Estrela et al. discussed the antimicrobial and physicochemical properties of sodium hypochlorite and suggested that sodium hypochlorite exhibited 3 types of chemical reactions:

Saponification reaction: Sodium hypochlorite degrades fatty acids, transform them into fatty acid salts(soap) and glycerol (alcohol) that reduces the surface tension of remaining solution.

Chloramination reaction: Hypochlorite ions leads to amino acid degradation and hydrolysis.

Amino acid neutralization

It neutralizes amino acids forming salt and water.

These reactions occur simultaneously and synergistically leading to liquefaction of organic tissue.

Thus, based on these observations, 5.25% of sodium hypochlorite was used in the present study to evaluate the effect of enamel deproteinization on acid etch patterns in permanent enamel. Topographic features of enamel were observed under Scanning Electron Microscope. In the conventional acid etch group irregular etching pattern was predominantly noticed. In Group 4 mean percentage of 37.5% of total area was ideally etched, while in Group 2, 29.16% of total area exhibited the ideal etching pattern.

Bhoomika et al. stated that enamel deproteinization prior to acid etching, did not grossly alter the surface topographic features. Similar results were obtained in the present study. Even though, there was an increase in the retentive surface area, the result was statistically insignificant.

On the contrary, Espinosa et al**.**[15] and Christopher A et al. observed that enamel surface pretreated with 5.25% sodium hypochlorite prior to acid etching dramatically increased the retentive surface area. The increase in retentive surface area might be due to removal of organic

smear layer from surface of enamel by deproteinization which cannot be achieved by acid etching alone.

Ramakrishna et al. observed in their study that there was no enhancive effect of enamel deproteinization , after acid etching on the topographic quality of enamel, rather indiscriminate pattern of etching was predominantly seen in the total etched surface area. The present study was also in accordance with the above mentioned study, where a mean percentage of 22.34% of total area was ideally etched in Group 3 (H3PO4 followed by NaOCl). In almost all specimens of group3 as evident in figure3, indiscriminate etching patterns or clogging of etched surface was noticed. This might be due to accumulation of organic debris in the etched prism surface.

Mechanical tests performed on teeth to evaluate and compare the effect of deproteinization on acid etching confirms that deproteinization significantly improves the values of shear bond strength both in permanent and primary teeth, as it increases the surface area of adhesion of resin to dental surface. However, studies conducted by Harleen et al., Ramakrishna et al**.** and Mowiena et al. found that there was no significant effects of deproteinization with NaOCl on shear bond strength of resin to enamel. One of the limitations of the present study is that in vitro setting may not simulate the effect of deproteinization on acid etching in-vivo. In addition, possible concerns of sodium hypochlorite are the taste, chlorinated odor, tolerance by young children and possible soft tissue reactions. Finally, the clinical implications of these findings are important. Based on the results of the present study, it can be inferred that, acid etching is adequate to produce retentive area as the differences in total etched area after the three different surface treatment regimen were not statistically significant.

Table 1: Association of type of surface treatment and total acid etched surface area in Group 2, Group 3 and Group 4. (in μm^2)

Descriptive statistics for type 1- type 2 total etched surface patterns (μm^2)								
					95% Confidence Interval for Mean			
Group	N	Mean	Std Deviation	Std. Error	Lower Bound	Upper Bound	Min	Max
Group 2	50	63762	59511.46	16656.9	20481.78	91318.51	10933	148000
Group 3	50	48937	46577.41	14729.1	15617.12	82256.07	7872	112000
Group 4	50	81955	76650.51	24239	27122	136787.3	8747	206000

*__Group 2__ (Acid etch only); __Group 3__ (H_3PO_4 followed by NaOCl); __Group 4__ (NaOCl followed by H_3PO_4).

Statistical analysis showed that:

1. The mean of total etched surface displaying type 1-type 2 etch pattern in Group 2 is 63762 μm^2
2. The mean of total etched surface displaying type 1-type 2 etch pattern in Group 3 is 48937 μm^2
3. The mean of total etched surface displaying type 1-type 2 etch pattern in Group 4 is 81955 μm^2

Inference

The total etched surface area was maximum in Group 4 with a mean area of 63762 μm^2 and minimum in group3 with an area of 81955 μm^2.

Multiple Comparisons						
Type 1 Type 2 acid etched surface patterns in micrometer Square						
Tukey HSD						
					95% Confidence Interval	
(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Acid Etch Group	H3PO4 followed by NaOCl	6963.55	26452.6	0.96	-58623.61	72550.71
	NaOCl followed by H3PO4	-26054.65	26452.6	0.59	-91641.81	39532.51
H3PO4 followed by NaOCl	Acid Etch Group	-6963.55	26452.6	0.96	-72550.71	58623.61
	NaOCl followed by H3PO4	-33018.2	26452.6	0.43	-98605.36	32568.96
NaOCl followed by H3PO4	Acid Etch Group	26054.65	26452.6	0.59	-39532.51	91641.81
	H3PO4 followed by NaOCl	33018.2	26452.6	0.43	-32568.96	98605.36

Table 2: Post hoc test for comparing acid etched surface patterns in 3 different groups: - Group 2 (Acid etch only), Group 3 (H_3PO_4 followed by NaOCl), Group 4 (NaOCl followed by H_3PO_4)

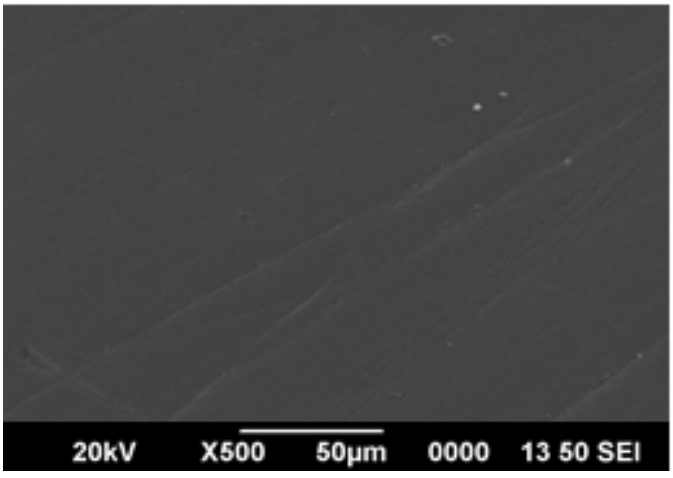

Figure 1: SEM microphotograph of Group 1(No treatment group) with magnification 500x

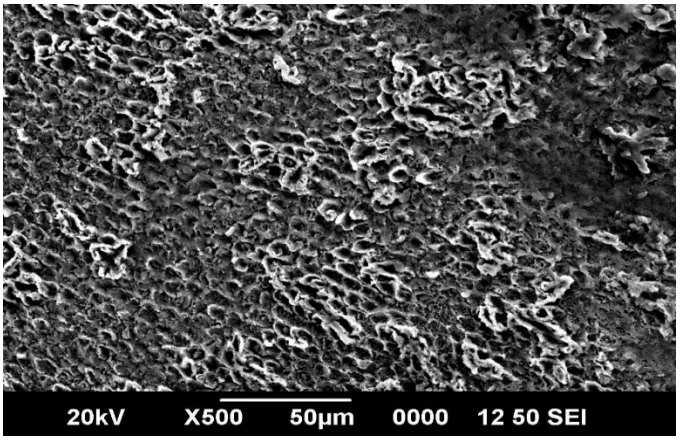

Figure 2: SEM microphotograph of Group 2 (Acid etch group) with magnification 500x. Observations: Indiscriminate pattern of etching.

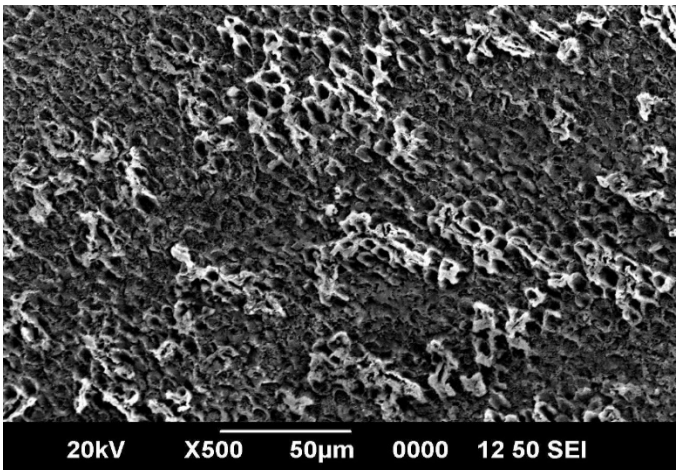

Figure 3: SEM microphotograph of Group3 (H_3PO_4 followed by NaOCl) with magnification 500x.

Observations: Indiscriminate pattern of etching.

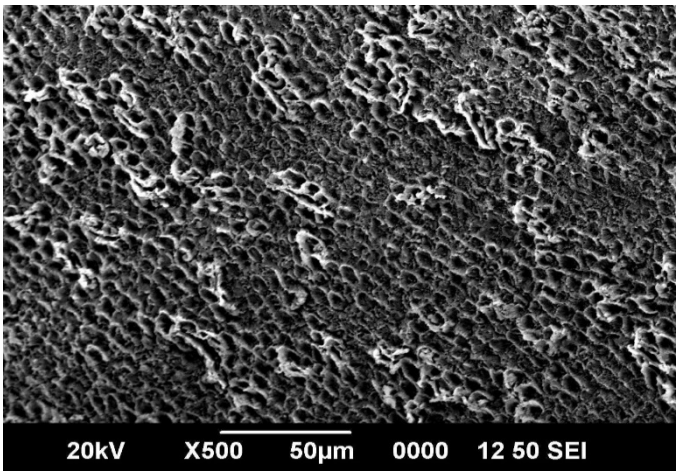

Figure 4: SEM microphotograph of Group 4 (NaOCl followed by H_3PO_4) with magnification 500x.

Observations: Type 1 etching pattern predominantly seen.

Conclusion

Within the limitations of the present in vitro study, the following conclusions can be drawn:

- Surface treatment with 5.25% NaOCl followed by acid etching, produced the highest marginal improvement in retentive surface area comparatively.
- The retentive area produced by conventional acid etching, was comparatively lesser than that produced by surface treatment with 5.25 % NaOCl followed by acid etching.
- Enamel deproteinization after acid etching produced the least retentive area.

Enamel pre-treatment with conventional acid etching is adequate to produce retentive area, as the differences in total etched area after the three different surface treatment regimens were not statistically significant.

Appendix

Abbreviations	Descriptions
NaOCl	Sodium hypochlorite
H_3PO_4	Phosphoric acid
SEM	Scanning Electron microscope
%	Percentage
μm^2	Micrometer square
SBS	Shear Bond Strength
Sd	Standard deviation

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