

Original Article

In vitro evaluation of the effect of deproteinization on the marginal leakage of resin restorations using three bonding agents

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ABSTRACT

Background: The perfect sealing of the tooth/restoration interface is important to prevent bacteria penetration that may lead to secondary caries and also, when dentin is involved, prevent excessive fluid movement in the dentinal tubules that may cause hypersensitivity. The aim of this study was to evaluate the effect of deproteinization and application of reducing agent on the marginal integrity of composite restorations using three different bonding agents (Prime & Bond NT, AdheSE and G-Bond).

Materials and Methods: Class V cavities were prepared on the buccal surface of 90 recently extracted human premolars and were divided into three groups (I, II, and III) based on the adhesives. Each group was subdivided into three subgroups of 10 each according to the surface treatment: application according to clinical protocol; etching with 37% phosphoric acid for 15 seconds/5% NaOCl; 10% sodium ascorbate after etching/NaOCl. The cavities were restored with Filtek Z 350 nanocomposite. The specimens were sectioned and evaluated under stereomicroscope. The morphology of the resin-dentin interface was visualized using SEM. Statistical analysis was done using Kruskal-Wallis one-way ANOVA followed by a Mann-Whitney U- test ($P < 0.05$).

Results: Group I showed significantly least microleakage among the groups. No significant difference in microleakage was found between groups I and II. Within the subgroups for each group, no significant difference in microleakage scores was observed. SEM micrographs presented gap free areas in group I and varying degrees of gaps in the other two groups.

Conclusion: Etch and rinse adhesives were tenable for deproteinization than self etch adhesives.

Key Words: Bonding, deproteinization, etch and rinse adhesives, microleakage

Received: April 2012

Accepted: June 2012

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INTRODUCTION

The basic foundation for adhesive dentistry was laid by Buonocore who proposed that acids could be used to alter the surface of enamel to render it more receptive to adhesion.^[1] Various concentrations of phosphoric acid have been evaluated as enamel etchants. Adhesion of restorative materials to enamel

has become a routine and reliable technique in modern restorative dentistry, but dentinal adhesion has proved to be more difficult and less predictable. The difficulty in bonding to dentin is the result of the complex histologic structure and variable composition of dentin itself since enamel contains 92% and dentin 45% by volume, of hydroxyapatite respectively. Further, hydroxyapatite crystals in enamel are arranged in regular pattern, whereas dentinal hydroxyapatite is randomly arranged in an organic matrix that consists primarily of collagen.^[2]

Recent SEM and transmission electron microscopy (TEM) studies have provided significant information regarding the current generation of adhesive systems. It has been reported that acid etching removes the smear

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layer, opens the dentinal tubules, increases dentinal permeability, and decalcifies the intertubular and peritubular dentin. Further the depth of decalcification may be affected by various factors, including the pH, concentration, viscosity, and application time of the etchant. Removal of hydroxyapatite crystals leaves a collagen *network* that can collapse and shrink because of the loss of inorganic support.^[3] The concept of acidic primers is attractive and subsequent to smear layer incorporation; these systems simultaneously infiltrate the collagen fibrils while decalcifying the inorganic component to the same depth, minimizing the existence of a region of demineralized dentin not encapsulated by resin.^[4]

Many raised concern regarding the efficacy of these systems, since smear layers reinforced by impregnated resin may be too weak to provide strong, durable mechanical properties. The presence of thick smear layer may interfere with the diffusion of self etching primers into the underlying intact dentin.^[5,6] Also, it has been claimed that the exposed collagen web not impregnated with adhesive monomers is highly susceptible to hydrolytic degradation over a long period, leading to reduction in bond strength and increased microleakage.

Hence a separate conditioning step should be considered to remove the smear layer and also produce a wettable surface, helping resin monomers to infiltrate through the demineralized interfibrillar spaces. It has been suggested that removal of the collagen matrix with a proteolytic agent such as sodium hypochlorite could have beneficial effect on etch and rinse as well as self-etch adhesives, facilitating the infiltration, and possibly promoting a chemical interaction.^[7]

Sodium hypochlorite has been tried to promote the exposure of lateral network and amplifies the dentinal tubules rendering dentin similar to etched enamel, which is favorable for adhesion.^[8] But, residual sodium hypochlorite may interfere with the polymerization of resin. This residual sodium hypochlorite could be neutralized by the application of sodium ascorbate to the oxidized dentin, which acts a reducing agent, restoring the redox potential of dentin and converting the microenvironment of the dentin from an oxidized substrate to reduced substrate, thus facilitating complete resin polymerization.^[9]

The present *in vitro* study has been undertaken to evaluate the effect of deproteinization and application

of reducing agent on the marginal integrity of composite restorations using three different bonding agents, based on etch and rinse, two step self etch and one step self etch approach.

MATERIALS AND METHODS

Recently extracted 90 human noncarious premolars without enamel fractures were selected for the study. The teeth were cleaned and stored in a saline solution (0.9%) at room temperature until use. A class V cavity was prepared on the buccal surface of each tooth with occlusal margin in enamel and cervical margin in dentin, with the dimensions of 3 mm width, 1.5 mm depth, and 2 mm height, with well-defined line angles and walls.

The teeth were divided into three groups of 30 each based on the bonding agent used. Group I comprised fifth-generation one-bottle-acetone-based etch and rinse adhesive (Prime & Bond NT, Dentsply, USA), group II comprised sixth-generation, a two-bottle two-step self-etching adhesive (AdheSE, Ivoclar Vivadent), and group III comprised seventh-generation all-in-one self-etching adhesive (G-Bond, GC, Asia). Each group was subdivided into three subgroups of 10 teeth each. In subgroup I, the adhesive was applied according to the manufacturer's direction; in subgroup II, the adhesive applied according to the manufacturer's direction after collagen removal. This procedure was carried out by application of 37% phosphoric acid for 15 seconds, followed by rinsing for 15 seconds, blot-drying for 2 seconds. This was followed by application of 5% sodium hypochlorite (NaOCl, Dentpro, INDIA) solution with cotton using tweezers for two minutes, and rinsed with water for additional 2 minutes prior to the application of adhesive system. In subgroup III 10% sodium ascorbate was applied for 1 minute after collagen removal as in subgroup II then rinsed for 30 seconds, followed by the application of adhesive system. Following application of the adhesive, the cavities were restored with Filtek Z 350(3M, ESPE) nanocomposite. Light curing of the composite was done using QHL75 halogen curing unit (Dentsply, USA) with a minimum output of 450 mw/cm².

Finishing and polishing of the restoration was accomplished with the assistance of abrasive disks from coarse to super fine (Shofu super-snap, mini-kit). The restored specimens were stored for 24 hours in a saline solution (0.9%) to allow the resin composite to expand hygroscopically. The specimens were

subjected to a thermocycling regimen of 500 cycles between $5(\pm 5)^{\circ}\text{C}$ and $55(\pm 5)^{\circ}\text{C}$ water bath.

For dye penetration, the specimens were air dried and coated with two layers of nail varnish, leaving a 1 mm window around the cavity margins. The specimens were immersed in a chemical marker (basic fuchsin solution 0.5%) for 24 hours at room temperature. Then specimens were washed, cleaned, dried, and sectioned into two halves using hard tissue microtome in a buccolingual direction by a cut through the center of the restoration. The section with clearest chemical marker was evaluated with a stereomicroscope at a magnification of $20\times$ starting from the gingival margin of restoration and moving toward the axial wall.

The following scoring system was used.

Degree of leakage depth of dye penetration

0= indicates no evidence of microleakage

1= indicates dye penetration up to half the cavity depth

2= indicates dye penetration of more than half the cavity depth

3= indicates dye penetration along the axial wall.

Specimen preparation for scanning electron microscopy

For scanning electron microscopic evaluation, two representative specimens from each subgroup were taken. The sections were fixed in 10% formalin for 24 hours and decalcified in 6N hydrochloric acid (HCl) for 30 seconds, rinsed in distilled water, and deproteinized by 10-minute immersion in 1% NaOCl, then rinsed in distilled water. After acid base treatment, the specimens were subjected to dehydration in ascending grades of ethanol up to 100% (25% for 20 minutes, 50% for 20 minutes, 75% for 20 minutes, 95% for 30 minutes, and 100% for 60 minutes). The specimens were mounted on aluminum stubs and further dried in vacuum before sputter coating with gold. Gold sputter coating was carried out under reduced pressure. The gold-coated samples were examined under scanning electron microscope (J.E.O.L, USA). Micrographs of the axial resin dentin interface were taken at $1000\times$ to observe the quality of bonding between the restorations and dental hard tissue.

Statistical analysis

The microleakage scores were tabulated and statistically analyzed using Kruskal-Wallis one-way

ANOVA followed by a Mann-Whitney U-test with $P<0.05$ as the level of significance.

RESULTS

The microleakage score of all the three groups is shown in Table 1. Table 2 shows the mean and standard deviation of the subgroups in each study group using Kruskal-Wallis one-way ANOVA followed by a Mann-Whitney U-test. In the present study, $P<0.05$ was considered as the level of significance. For group I, the mean score in subgroup I (0.8 ± 1.0) is the highest followed by subgroup II (0.7 ± 1.1) and the lowest in subgroup III (0.6 ± 1.1). The test of significance by Kruskal-Wallis one-Way ANOVA showed that there was no significant difference in mean scores among three subgroups ($P = 0.79$).

For group II, the mean score in subgroup I (1.8 ± 1.0), subgroup II (1.9 ± 1.0), and in subgroup III (1.8 ± 1.2). The test of significance by Kruskal-Wallis one-way ANOVA showed that there was no significant difference in mean scores among three subgroups ($P = 0.98$). For group III, the mean score in subgroup I was 2.1 ± 1.1 , in subgroup II 2.0 ± 1.2 , and in subgroup III 2.4 ± 1.0 . The test of significance by Kruskal-Wallis one-way ANOVA showed that there was no significant difference in mean scores among three subgroups ($P = 0.75$).

Two representative samples from each subgroup were analyzed using scanning electron microscope at the

Table 1: Number and percentages of microleakage score at dentin margin in each study group and subgroup

Group	Score	Subgroup I		Subgroup II		Subgroup III	
		Number	%	Number	%	Number	%
Group I	0	5	50	6	60	7	70
	1	3	30	2	20	1	10
	2	1	10	1	10	1	10
	3	1	10	1	10	1	10
Group II	0	1	10	1	10	2	20
	1	3	30	2	20	2	20
	2	3	30	4	40	2	20
	3	3	30	3	30	4	40
Group III	0	1	10	2	20	1	10
	1	2	20	1	10	0	-
	2	2	20	2	20	3	30
	3	5	50	5	50	6	60

Group I – Prime & Bond NT, group II – AdheSE, group III – G-Bond. Subgroup I - Adhesive applied according to the manufacturer's directions. Subgroup II - Adhesive applied after acid etching and NaOCl. Subgroup III - 10% sodium ascorbate applied after collagen removal as in subgroup II

Table 2: Mean, standard deviation (SD) and test of significance of mean values among different subgroups in each study group

Group	Subgroup	Mean± S.D	P value	Significance at 5%
Group I	I	0.8±1.0	0.79	NIL
	II	0.7±1.1		
	III	0.6±1.1		
Group II	I	1.8±1.0	0.98	NIL
	II	1.9±1.0		
	III	1.8±1.2		
Group III	I	2.1±1.1	0.75	NIL
	II	2.0±1.2		
	III	2.4±1.0		

Group I – Prime & Bond NT, group II – AdheSE, group III – G-Bond. Subgroup I - Adhesive applied according to the manufacturer's directions. Subgroup II - Adhesive applied after acid etching and NaOCl. Subgroup III - 10% sodium ascorbate applied after collagen removal as in subgroup II

axial wall along the resin dentine interface. Gap-free margins were noted in the specimens of all subgroups for group I (Prime & Bond NT) [Figures 1-3]. Elongated resin tags with multiple lateral branchings were visualized in the specimens that were acid etched and deproteinized [Figures 2 and 3]. Gaps were visualized in the specimens of all subgroups for group II (AdheSE) [Figures 4-6] and group III (G-Bond) [Figures 7-9], but were pronounced in group III when compared with group II.

DISCUSSION

Dentinal bonding is complicated by the formation of a smear layer as debris is burnished onto the dentinal surface while the dentin is cut or ground. The smear layer occludes the orifices of the dentinal tubules and act as “diffusion barriers” that decrease the permeability of dentin.^[10] Some investigators report that treatment of dentin with acids can cause collapse of exposed collagen fibers due to removal of the supporting hydroxyapatite and/or denaturation of collagen.^[11] The ensuing matted collagen surface becomes more difficult to impregnate with adhesive monomers. To overcome this problem, investigators have used priming agents to restore the permeability of acid treated dentin.^[12,13]

Microleakage may influence marginal permeability to bacterial, chemical and molecular invasion at the tooth/material interface and is the result of a breakdown of the tooth-restoration interface, causing discoloration, recurrent caries, pulpal inflammation, and possible restoration replacement.^[14] The possible reasons for microleakage at the dentin restoration margin include cavity configuration (C-factor),

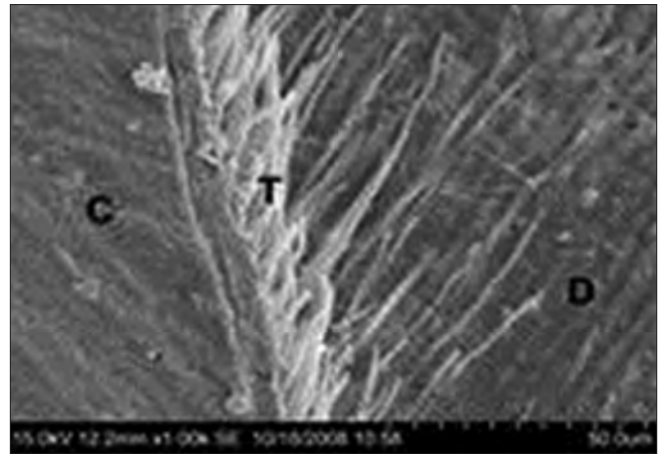


Figure 1: Representative specimen of group I (Prime & Bond NT), subgroup I (clinical protocol) seen under scanning electron microscope. C: composite resin, T: resin tags, D: dentin

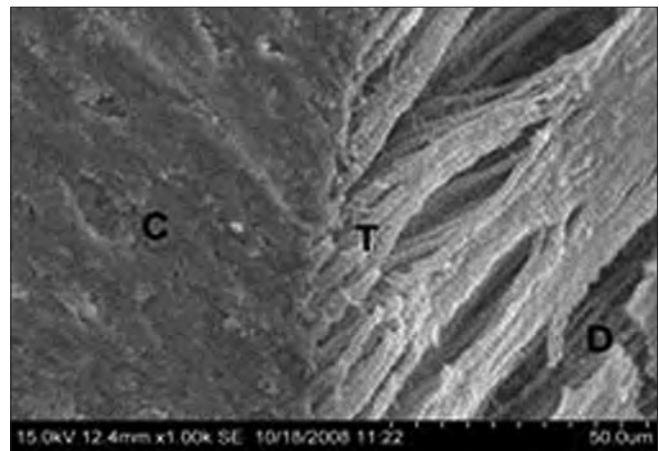


Figure 2: Representative specimen of group I (Prime & Bond NT), subgroup II (acid etchant, NaOCl) seen under scanning electron microscope

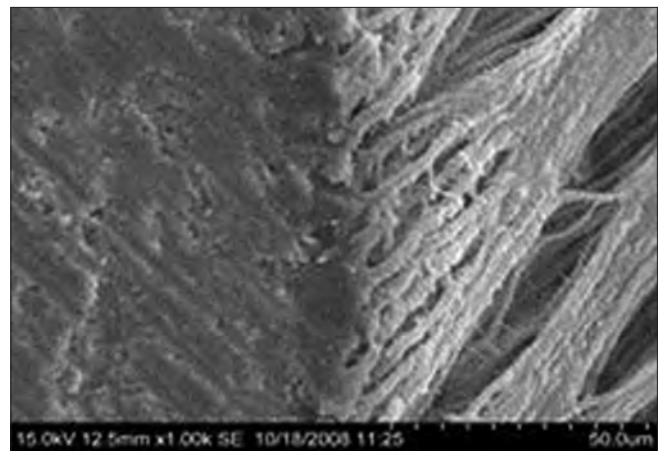


Figure 3: Representative specimen of group I (Prime & Bond NT), subgroup III (acid etchant, NaOCl, sodium ascorbate) seen under a scanning electron microscope

dental tubule orientation to the cervical wall (CEJ), organic content of dentin substrate and movement of

dental tubular fluids, incomplete alteration/removal of smear layer by acidic primers (selfetch systems)

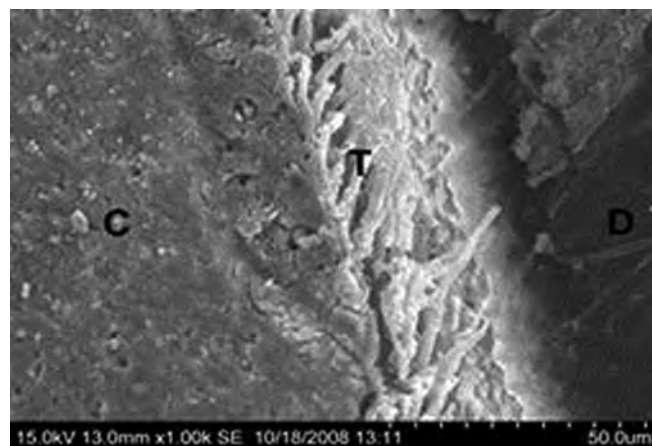


Figure 4: Representative specimen of group II (AdheSE), subgroup I (clinical protocol) seen under a scanning electron microscope

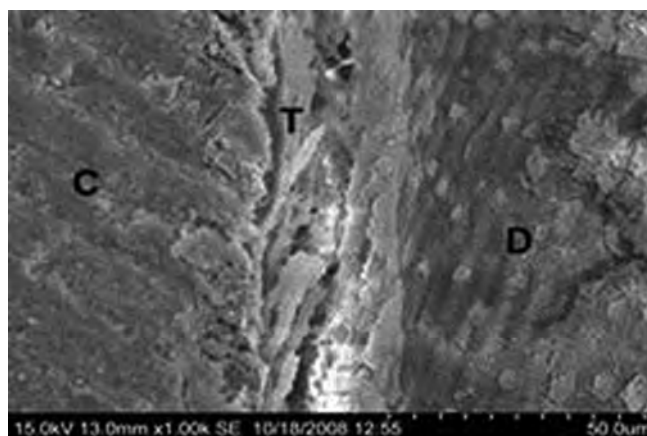


Figure 5: Representative specimen of group II (AdheSE), subgroup II (acid etchant, NaOCl) seen under a scanning electron microscope

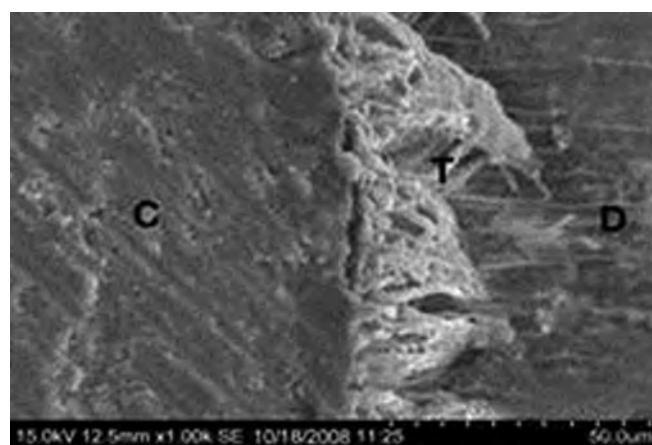


Figure 6: Representative specimen of group II (AdheSE), subgroup III (acid etchant, NaOCl, sodium ascorbate) seen under a scanning electron microscope

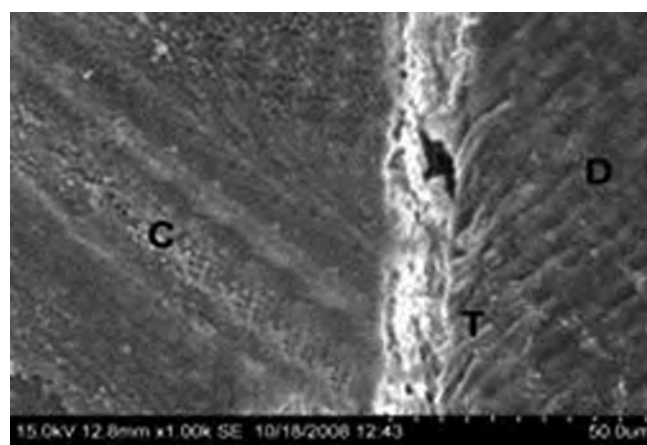


Figure 7: Representative specimen of group III (G-Bond), subgroup I (clinical protocol) seen under a scanning electron microscope

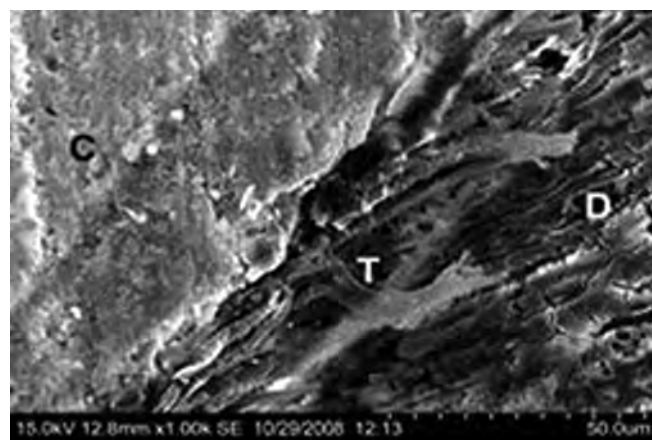


Figure 8: Representative specimen of group III (G-Bond), subgroup II (acid etchant, NaOCl) seen under a scanning electron microscope



Figure 9: Representative specimen of group III (G-Bond), subgroup III (acid etchant, NaOCl, sodium ascorbate) seen under a scanning electron microscope

for adequate demineralization and hybrid layer formation; inefficient infiltration/penetration of primer components into the demineralized collagen fibrillar network, dentin substrate hydration level (solvent carriers [water, alcohol, acetone] in the adhesive agent reacting differently with varying degrees of surface “moisture”).

Other reasons include incomplete evaporation of the solvent from the dentin surface prior to attachment of adhesive monomers, acid component composition (pH, osmolality, thickening agent), polymerization contraction of the resin composite, physical characteristics of restoration material (filler loading, volumetric expansion, modulus of elasticity), inadequate margin adaptation of the restorative material, and instrumentation and finishing/polishing effects.^[15]

In the present study the effect of retaining or removing collagen fibrils on the marginal integrity of composite restorations following deproteinization and antioxidant application using three different bonding agents based on etch and rinse (two-step), self-etch (two-step), and self-etch (one-step) approaches were evaluated.

Class V cavities were prepared on the buccal surface of premolars because they have high C-factor and consequent higher polymerization shrinkage and hence have a higher risk for marginal gap formation and microleakage. The teeth were divided into three groups, which were further divided into three subgroups each and the first subgroup (I) was bonded using the respective bonding systems following the manufacturer's directions. In the second subgroup (II), deproteinization was done following acid etching using 5% sodium hypochlorite solution for two minutes prior to bonding.^[16] Sodium hypochlorite as a solution was found to be more effective in collagen fibril removal than gel, whereas in the third subgroup (III) 10% sodium ascorbate was applied, for 1 minute, following the application of 5% sodium hypochlorite, prior to bonding.^[17]

In the present study 5% sodium hypochlorite was applied for 2 minutes. NaOCl is a nonspecific proteolytic agent that effectively removes organic components at room temperature. It is reported that deproteinization transforms dentin as a porous structure with multiple irregularities, which allow good mechanical retention.^[18]

After NaOCl treatment, an increase in wettability

is expected because deproteinization leads to a mineralized, naturally hydrophilic surface, exposes a network of lateral canals on superficial dentin, and widens the aperture of the dentinal tubules on superficial and deep dentin, which should produce stronger resin tags. The “large” tubules visualized in deproteinized dentin would permit more resin to engage the dentin.^[19]

This deproteinization results in enlarged resin tag formation and numerous sidelong resin projections.^[20] This is evident in the scanning electron micrographs of the specimens that were acid etched and deproteinized in the present study [Figures 2 and 3].

In the present study Prime & Bond NT (group I) showed least microleakage among the three groups irrespective of different dentin treatment protocols. No significant difference in microleakage was observed among composite restorations bonded with Prime & Bond NT according to manufacture protocol, collagen removal by deproteinization and deproteinization followed by sodium ascorbate, which is in agreement with a previous study by Toledano *et al.*^[21] Though there was no significant difference in microleakage score, a decrease in microleakage was found following deproteinization and also following sodium ascorbate probably due to the composition of the bonding system.

Acetone which is present as a solvent in Prime & Bond NT shows higher rate of diffusion and greater ability to displace water on deproteinized dentin surfaces, causing a slight decrease in microleakage. Also, there might have been better monomer interaction with the intertubular dentin structure exposed through sodium hypochlorite treatment, enabling penetration of the monomer into substratum porosities. Another consequence of the higher acetone level in Prime & Bond NT would be in affecting the solvent's ability to promote the volatilization of free radicals of oxygen released by NaOCl, which could interfere with the bonding agent's polymerization process. In addition, the presence of phosphate terminals from a phosphoric acid ester (PENTA) in the composition of Prime & Bond NT was verified. Phosphate terminals may establish some kind of interaction with calcium ions left over after collagen removal from the dentin surface.

In group II (AdheSE) there was no difference in microleakage scores among the subgroups which may be due to the fact that water present as solvent in the bonding agent could not vaporize the free radicals

formed after deproteinization. This is in agreement with a previous study done by Abo *et al.*^[22] Though group II showed less microleakage than group III this was not statistically significant.

In group III (G-Bond) no significant difference in microleakage was found between different dentin protocols. Although there was no statistical difference, G-Bond showed more microleakage than AdheSE and Prime & Bond NT, even when the manufacturer's instructions were followed, (subgroup I) probably due to the phenomenon of phase separation at the interface. This is due to the presence of hydrophilic monomers that separated from the hydrophobic resins following evaporation of the organic solvent acetone. Since this might have been the overriding factor in G-Bond, the beneficial effects of acetone in displacing water, increasing monomer penetration, and volatilization of the free radicals are offset. Also G-Bond has water as a solvent in addition to acetone.

Also degradation of the dentin bonding interface is known to occur in G-Bond. This may be due to the hydrolytic degradation of resin components in the hybrid layer.^[23] Water can also plasticize the resin matrix, which decreases the mechanical properties of the polymer. There may be other factors responsible for the inadequate penetration of the acetone-based G-Bond into dentin. Porosities (or blisters) occur at the bonding interface, because most simplified all-in-one adhesives behave as semipermeable membranes. These interfacial defects are usually observed when interfaces are stained with heavy metals. The porosities may be a result of water accumulation either caused by an osmotic gradient or by monomer-solvent phase separation upon evaporation of the acetone. The number and size of these blisters may also depend on the intensity of the air-drying step.^[24]

The leakage score was prominent for the deproteinization/sodium ascorbate subgroup (III). As the samples were not rinsed with deionized water, the trapped sodium ascorbate crystals could have interfered in the polymerization of the resin monomers which might have contributed to voids in the resin dentin interface.^[25]

The bond strength and microleakage relationship is complex and poorly understood. Although there is no statistically significant correlation between these phenomena, they are strongly associated with each other. But in the present study there was no significant reduction in microleakage though previous studies

have shown improvement in bond strength. Hence bond strength analysis would not be a true indicator of microleakage.

Specimens bonded with Prime & Bond NT showed least microleakage scores regardless of whether deproteinization and antioxidant application were done, which is confirmed by predominantly gap free resin dentin interface seen in the SEM. This is probably because etch and rinse systems are more tenable to deproteinization treatment of dentin than self-etch systems.^[26]

CONCLUSION

There is no significant difference in the microleakage scores whether collagen fibrils are retained or removed by deproteinization treatment. Etch and rinse systems showed significantly less microleakage than self-etch adhesives. Etch and rinse systems show a slight decrease in microleakage after deproteinization, though not statistically significant, probably due to the presence of acetone and PENTA that promote better penetration of monomers, and interaction with dentinal tubules. In the self-etch adhesives the two-step approach shows no difference in microleakage before and after collagen fibril removal using deproteinization treatment. Self-etch (one step) appears to have more microleakage upon deproteinization probably due to the effect of phase separation and increased interfacial gap formation, though this is not statistically significant.

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How to cite this article: Ravishanker P, Chaitanya K. *In vitro* evaluation of the effect of deproteinization on the marginal leakage of resin restorations using three bonding agents. *Dent Res J* 2012;9:452-9.

Source of Support: Nil. **Conflict of Interest:** None declared.