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ORIGINAL RESEARCH

Evaluation of Hybrid Layer and Bonding Interface after Water Storage with and without the Usage of 2% Chlorhexidine: A Scanning Electron Microscope Study

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ABSTRACT

Aim: Restorative dentists employ different bonding systems between the resin and the dentin and other dentinal tissues to achieve the goal of micromechanical retention. Studies have shown that the bond between composite and dentin degrades over time because of the action of matrix metalloproteinases (MMPs) on collagen fibrils left unprotected by acid etching. The MMPs may be partially responsible for hybrid layer degradation. Since chlorhexidine (CHX) inhibits MMPs, we hypothesized that CHX would decelerate the loss of resin–dentin bonds. Hence, this *in vitro* study is intended to evaluate the effects of 2% CHX on hybrid layer and bonding interface.

Materials and methods: Totally, 40 freshly extracted molars were randomly divided into four experimental groups. In all 40 specimens, class II cavities were prepared to a depth of 1 mm below the dentinoenamel junction with no axial wall, but the elimination of the proximal enamel ridge. The teeth were then randomly divided into four experimental groups, i.e., All Bond 2 without 2% CHX (group I), All Bond 2 with 2% CHX (group II), One Coat 7.0 without 2% CHX (group III), and One Coat 7.0 with 2% CHX (group IV). All the specimens were derooted and sectioned mesiodistally into two halves and placed under water at 37°C for 3 months and observed under scanning electron microscope for the hybrid layer and resin tag formation.

Results: Groups I and II showed statistically significant difference when the presence/absence of resin tags was compared. When groups III and IV were compared for the presence/absence of hybrid layer and resin tags, the results were statistically significant.

Conclusion: Between all the four experimental groups, irrespective of the bonding systems used, we concluded that groups with 2% CHX usage showed promising results with presence/absence of hybrid layer and resin tags formation.

Clinical significance: Studies suggest that the bond between composite and dentin degrades over time because of the action of MMPs on collagen fibrils left unprotected by acid etching. Measures should be taken to prevent this from happening and thus allow bond between composite and dentin last longer.

Keywords: Chlorhexidine, Dentin bonding agents, Hybrid layer, Matrix metalloproteinases.

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INTRODUCTION

For successful bonding to dentin, it is widely accepted that the formation of a hybrid layer must be achieved through the full infusion of resin monomers into water-saturated acid-etched dentin. The integrity and stability of collagen fibrils are the structural bases for hybrid layer matrices, as well as vital for their durability. The premature degradation of hybrid layers has been associated with the inability of current adhesives to durably seal the dentin substrate.[1] This abasement process is quite likely to be the consequence of a myriad of factors, including deficient resin monomer infiltration of demineralized dentin and elution of unpolymerized monomers from polymerized adhesives. This results in zones of exposed

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collagen fibrils within hybrid layers that are prone to be attacked by host-derived proteolytic/hydrolytic enzymes.[2] Evidence of collagenolytic/gelatinolytic activity in dentin demineralized with etch-and-rinse adhesives in the absence of bacteria highlights the potential involvement of host-derived proteases in the disruption of incompletely infiltrated collagen fibrils within hybrid layers.[1]

Long-term dentin bonding is threatened by the disaggregation of the hybrid layer owing mainly to the activation of dentin MMPs.[3] Host-derived MMPs, found both in saliva and etched dentin, have been shown to be involved in the degradation of the unprotected collagen fibrils within the hybrid layer.[3] These proteases are secreted by odontoblasts during dentinogenesis and remain inactive within the dentin extracellular matrix. The acidic environment, resulting from adhesive systems or the biologic carious process, activates different dentinal MMPs.[4] The MMPs are a family of Zn- and Ca-dependent enzymes that regulate the physiological and pathological metabolism of collagen-based tissues. As in other collagen-based tissues, dentin contains different MMPs: Collagenase MMP-8, gelatinases MMP-2 and -9, stromelysin MMP-3, and enamelysin MMP-20. However, when the dentin matrix mineralizes, MMPs become covered with apatite nanocrystals, making them immobile and nonfunctional.[5] As long as dentin is mineralized, its proteases remain structurally stable.[5]

It would be advantageous from a clinical perspective to be able to inhibit the breakdown of deficient resin-impregnation collagen fibrils by host-derived MMPs in the dentinal hybrid layer. Tissue inhibitors of metalloproteinases are the major endogenous inhibitors of MMPs.[6]

Chlorhexidine gluconate has been shown to be effective against various oral bacteria. Due to its broad antimicrobial spectrum (i.e., against Gram-positive/negative bacteria and fungi), CHX has been used to adjunctively treat either endodontic or periodontal diseases and to arrest/prevent caries progression.[7] Besides its antimicrobial properties, CHX is applied to treat dentin before the use of etch-and-rinse adhesives to reduce the breakdown of collagen fibrils.[3] The CHX has been applied in different sequences, including before etching, after etching (with or without rinsing), or CHX-containing phosphoric acid. Even at low concentration (0.2%), CHX functions as MMPs inhibitor that can prevent bond degradation of collagen and disintegration of the bonding interface.[8] The use of 0.2% CHX gluconate for 60 s was found to inhibit collagenolytic activity thus, maintaining the resin–dentin interface.[8-11] Pashley et al[8] recommended the use of CHX on acid-etched dentin before using total-etch adhesives. It did not affect the *in vitro* bond strength of aged specimens tested in microtensile testing, and there were less cohesive failures in dentin or the hybrid layer when dentin was treated with CHX than without such application.[12] Thus, the aim of this *in vitro* study is intended to evaluate the effects of 2% CHX on hybrid layer and bonding interface.

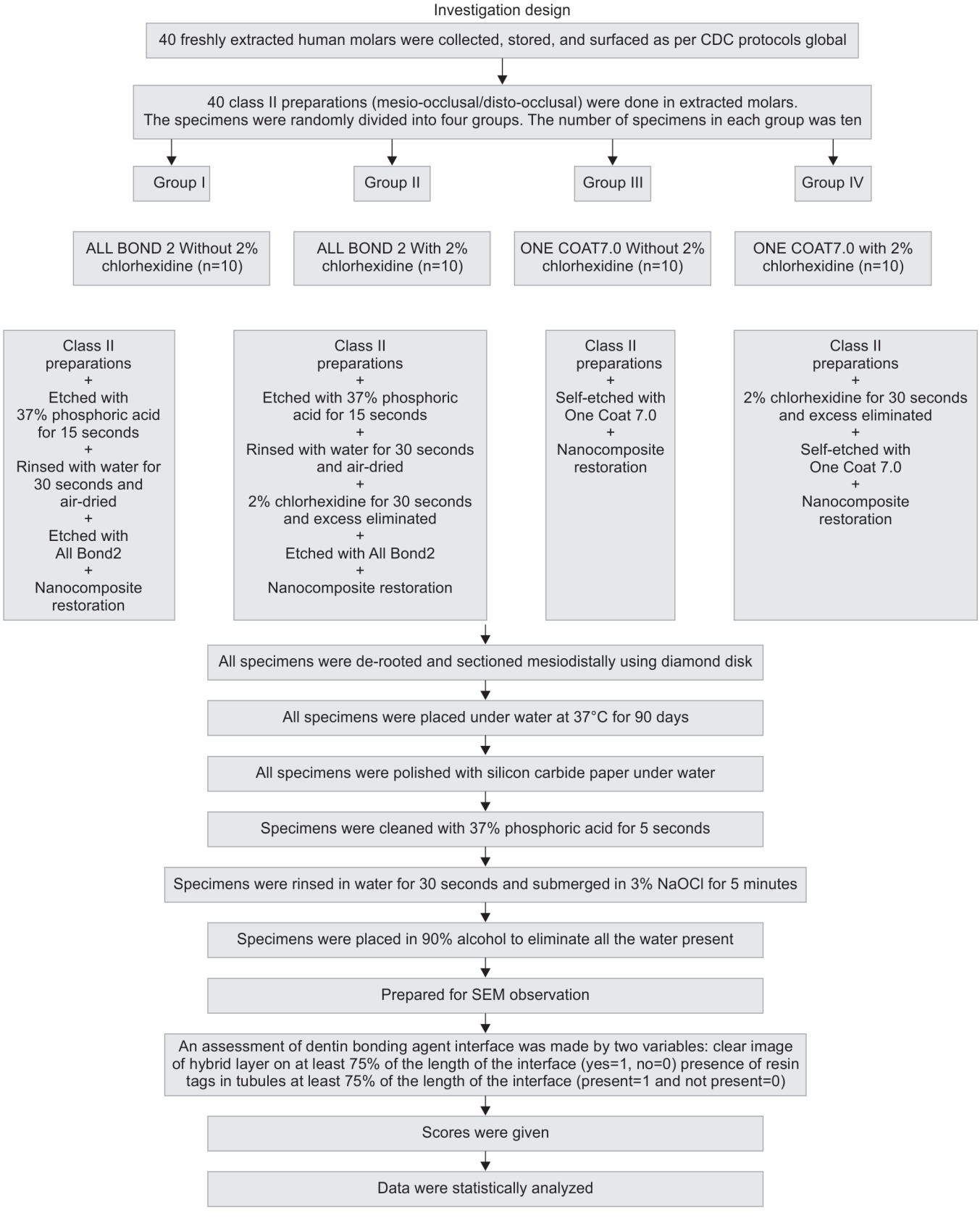
MATERIALS AND METHODS

The details of materials used in the study are given in Table 1. Totally, 40 freshly extracted molars were randomly divided into four experimental groups. In all 40 specimens, class II cavities (mesio-occlusal/disto-occlusal) were prepared using a No. 245 carbide bur with a high-speed aerator handpiece under air–water spray in all teeth. Cavities were prepared to a depth of 1 mm below the dentinoenamel junction with no axial wall, but with elimination of the proximal enamel ridge. The

Table 1: Details of materials used in the study

Product/manufacturer	*Type/function*	*Composition*
All Bond 2 (Bisco)	Dual-cured universal dental adhesive (fourth-generation dentin bonding agent)	Acetone Ethanol Na-N-tolyglycine Glycidylmethacrylate PRIMER A contains N-Tolyglycine glycidyl meth acrylate resin in an acetone solvent PRIMER B contains biphenyl dimethacrylate dentin/enamel RESIN is a light-cured, unfilled bonding resin PRE-BOND is an unfilled resin
One Coat 7.0 (Coltene)	Light-cured, self-etching adhesive (seventh-generation bonding agent)	Methacrylates, photoinitiators, ethanol, and water
SureFil high-density posterior restorative(nanocomposite resin) (Dentsply)		Dimethacrylate resin; Tri ethylene glycol di meth acrylate dimethacrylate multifunctional polymethacrylate; camphorquinone; ethyl-4 (dimethylamino) benzoate; butylated hydroxytoluene; ultraviolet stabilizer; silanated barium-boron-fluoro-aluminosilicate glass, highly dispersed silicon dioxide; fluorescent agent, iron oxide pigments, titanium dioxide
RC-CHLOR (Azure Laboratories Pvt. Ltd)	Root canal disinfectant Antimicrobial agent	2% CHX gluconate

Flow Chart 1: Schematic diagram of methodology



teeth were then randomly divided into four experimental groups (in each group 10, n = 10), i.e., All Bond 2 without 2% CHX (group I), All Bond 2 with 2% CHX (group II), One Coat 7.0 without 2% CHX (group III), and One Coat 7.0 with 2% CHX (group IV). All groups were restored with SureFil high-density posterior nanocomposite resin (Dentsply). Schematic diagram of methodology is shown in Flow Chart 1.

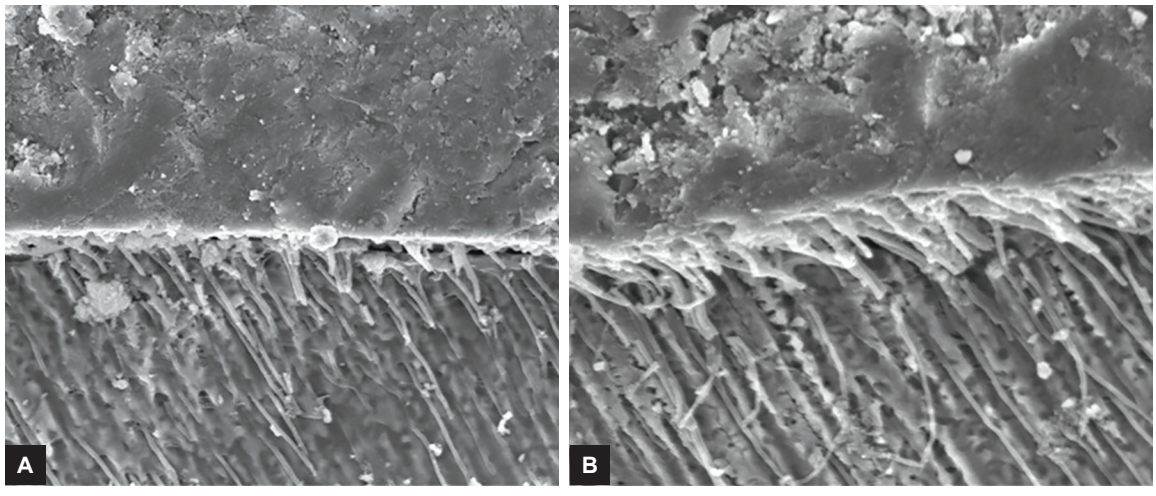
All the specimens were derooted and sectioned mesiodistally into two halves using a low-speed diamond disk (Edenta). Specimens were placed under water at 37°C for 3 months. Then, the specimens were prepared for SEM (JOEL Ltd) evaluation. All specimens were polished with silicon carbide paper under water. The specimens were cleaned with 37% phosphoric acid (Sigma Aldrich) for 5 s, rinsed in water for 30 s, and submerged in 3% NaOCl (Prime Dental, India) for 5 minutes. Then, the specimens were placed in 90% alcohol (Sigma Aldrich) to eliminate all water present before being desiccated and prepared for SEM observation.

All specimens were observed under ×800 magnification, and an assessment of the dentin bonding interface was made by two variables: Clear image of hybrid layer on at least 75% of the length of the interface (yes = 1, no = 0) and the presence of resin tags in tubules at least 75% of the length of the interface (present = 1, not present = 0). Scores were given for each parameter. This methodology was followed by Lafuente.[13]

RESULTS

Experimental Groups

- There was no significant difference in the presence/absence of hybrid layer between groups I and II (Fig. 1), but groups I and II showed statistically significant difference when the presence/absence of resin tags was compared (Tables 2, 3 and Fig. 2).



Figs 1A and B: The SEM shows no significant difference in the presence/absence of hybrid layer between groups I and II

Table 2: Presence/absence of hybrid layer between the experimental groups

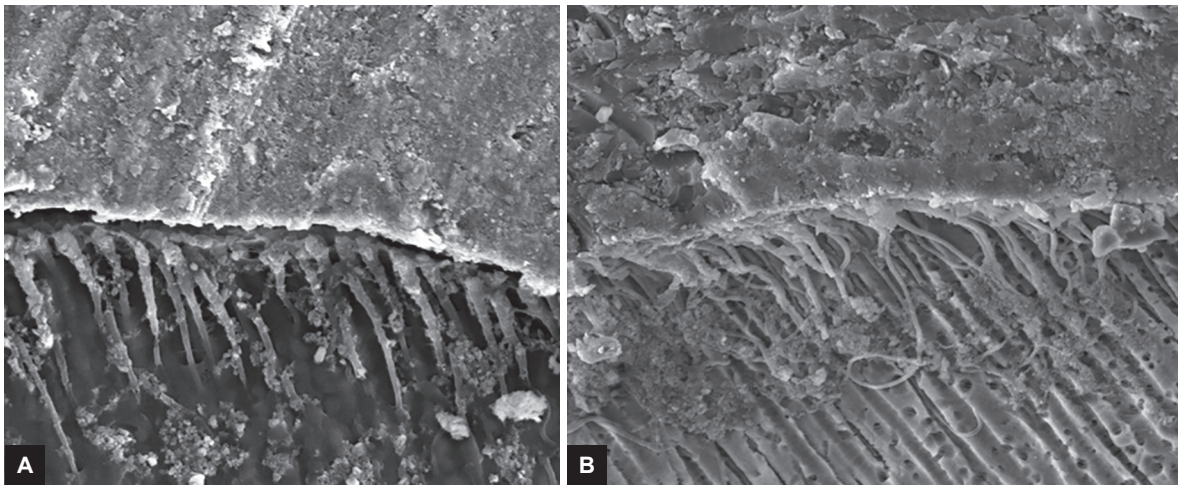
	Group I	*Group II*	*Group III*	*Group IV*	
	All Bond 2 without 2% CHX (n = 10)	*All Bond 2 with 2% CHX (n = 10)*	*One Coat 7.0 without 2% CHX (n = 10)*	*One Coat 7.0 with 2% CHX (n = 10)*	*Total (n = 40)*
Absence					
Count (%)	3 (30.0)	0 (0)	4 (40)	0 (0)	7 (17.5)
Presence					
Count (%)	7 (70.0)	10 (100)	6 (60)	10 (100)	33 (82.5)
Total					
Count (%)	10 (100.0)	10 (100.0)	10 (100.0)	10 (100.0)	40 (100.0)

$\chi^2 = 8.831$; p = 0.032 significant

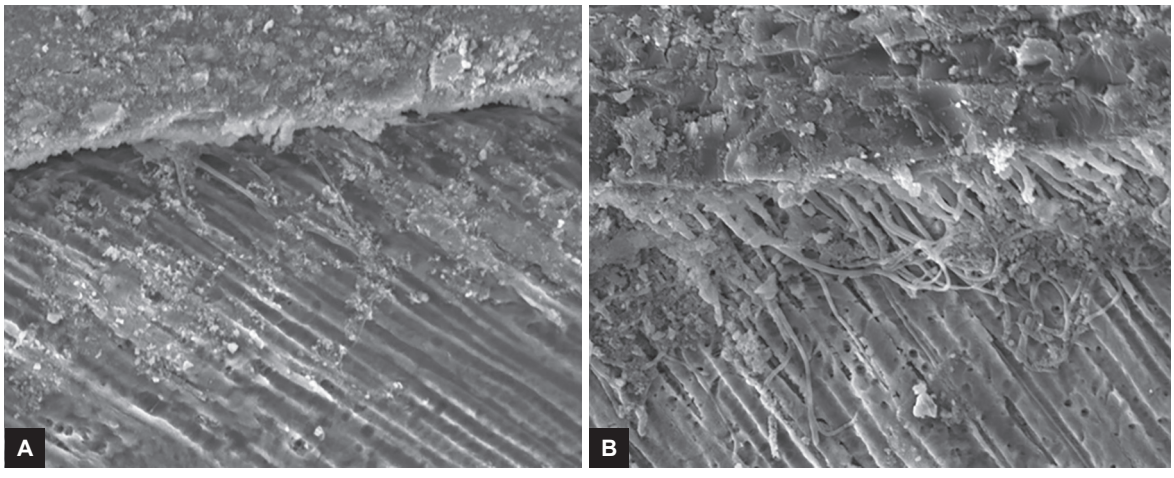
Table 3: Presence/absence of resin tags between the experimental groups

	Group I	*Group II*	*Group III*	*Group IV*	
	All Bond 2 without 2% CHX (n = 10)	*All Bond 2 with 2% CHX (n = 10)*	*One Coat 7.0 without 2% CHX (n = 10)*	*One Coat 7.0 with 2% CHX (n = 10)*	*Total (n = 40)*
Absence					
Count (%)	4 (40.0)	0 (0)	5 (50.0)	0 (0)	9 (22.5)
Presence					
Count (%)	6 (60.0)	10 (100.0)	5 (50.0)	10 (100.0)	31 (77.5)
Total					
Count (%)	10 (100.0)	10 (100.0)	10 (100.0)	10 (100.0)	40 (100.0)

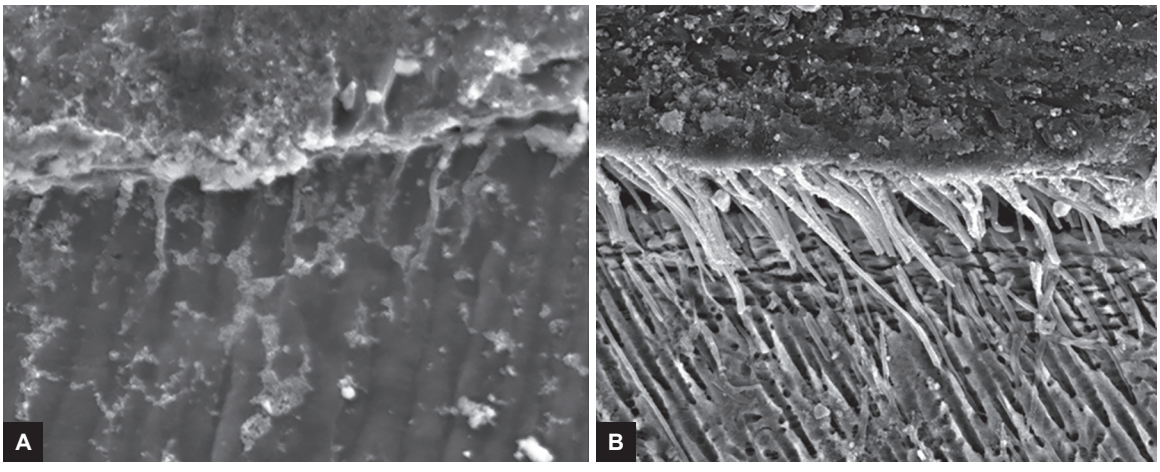
$\chi^2 = 11.9$; p = 0.008



Figs 2A and B: The SEM shows groups I and II have a difference in the presence/absence of resin tags



Figs 3A and B: The SEM shows difference in hybrid layer formation between groups III and IV



Figs 4A and B: The SEM shows difference in resin tag formation between groups III and IV

- When groups III and IV were compared for the presence/absence of hybrid layer (Fig. 3) and resin tags (Figs 4), the results were statistically significant (Tables 2 to 4).

The intercomparison between the four groups for the presence/absence of hybrid layer and resin tags was performed using Mann–Whitney U test (Table 5).

Table 4: Test statistics

	Hybrid layer	*Resin tags*
Kruskal–Wallis test	8.610	11.602
	0.035 significance	0.009 SS

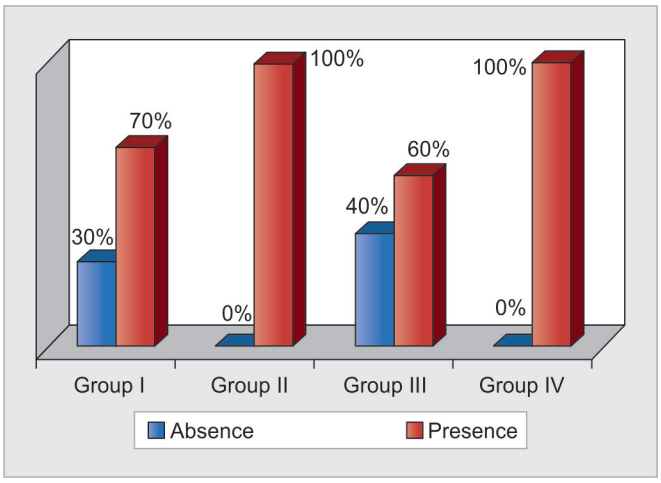
SS: Statistically significant

- There was no significant difference in the presence/absence of hybrid layer between groups I and II, but

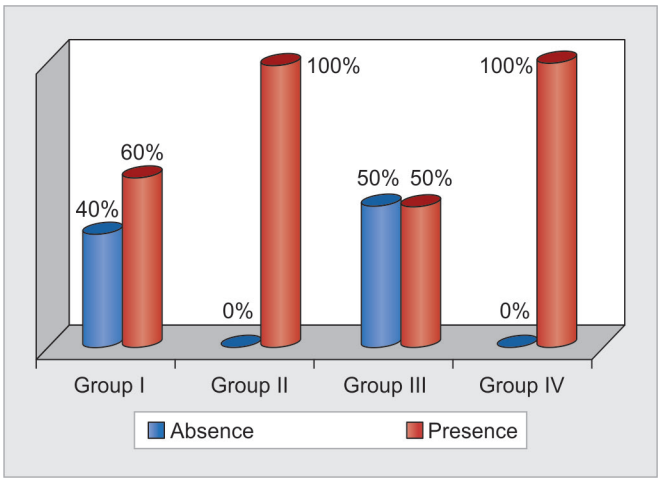
Table 5: Intercomparison between the four groups with and without the use of 2% CHX (Mann–Whitney U test)

	Hybrid layer			*Resin tags*		
Bonding agents with and without 2% CHX	*Z-value*	*p-value*	*Significance*	*Z-value*	*p-value*	*Significance*
All Bond 2 without 2% CHX *vs* All Bond 2 with 2% CHX	1.831	0.067	NS	2.179	0.029	SS
All Bond 2 without 2% CHX *vs* One Coat 7.0 without 2% CHX	0.457	0.648	NS	0.438	0.061	NS
All Bond 2 without 2% CHX *vs* One Coat 7.0 with 2% CHX	1.831	0.067	SS	2.179	0.029	SS
All Bond 2 with 2% CHX *vs* One Coat 7.0 without 2% CHX	2.179	0.029	SS	2.517	0.012	SS
All Bond 2 with 2% CHX *vs* One Coat 7.0 with 2% CHX	0	1	NS	0	1	NS
One Coat 7.0 without 2% CHX *vs* One Coat 7.0 with 2% CHX	2.179	0.029	SS	2.517	0.012	SS

NS: Not significant; SS: Statistically significant



Graph 1: Presence/absence of hybrid layer between the groups (n = 40)



Graph 2: Presence/absence of resin tags between the groups

groups I and II showed a statistically significant difference when the presence/absence of resin tags was compared (Graphs 1 and 2).

- When groups I and III were compared for the presence/absence of hybrid layer and resin tags, the results were not statistically significant (Graphs 1 and 2).
- When groups I and IV were compared for the presence/absence of hybrid layer and resin tags, the results were statistically significant (Graphs 1 and 2).
- When groups II and III were compared for the presence/absence of hybrid layer and resin tags, the results were statistically significant (Graphs 1 and 2).
- When groups II and IV were compared for the presence/absence of hybrid layer and resin tags, the results were not statistically significant.
- When groups III and IV were compared for the presence/absence of hybrid layer and resin tags, the results were statistically significant.

DISCUSSION

Enamel bonding adhesives have a long track record, and the process is very well understood after years of research beginning with Buonocore and probably still continuing with Bowen et al.[14] Dentin has been characterized as a biologic composition of collagen matrix filled with submicron- to nanometer-sized, calcium-deficient, and carbonate-rich appetite crystals dispersed between parallel micron-sized hypermineralized collagen-poor hollow cylinders. Pashley and Carvalho[14] speculated that regional differences in dentinal tubules density, dentin permeability, calcium concentration, presence of abnormal dentin, varying smear layer thickness, result in nonuniform resin infiltration into the collagen fibrils of intertubular dentin thus, leading to nonuniform bond strength values.

Most laboratory researchers have time and again indicated that there is a premature loss of bond strength, which affects adhesive restorations, due to degradation of the hybrid layer at the dentin–adhesive interface and deterioration of dentin collagen fibrils.[11]

The CHX, though a controversial antiseptic today, remains a gold standard as an antiplaque agent, and its efficacy in caries prevention is clinically well documented. Studies done with SEM and other methods have conclusively proved that CHX is found to be an MMPs' inhibitor, preserving the humidity necessary for keeping the collagen network expanded.[15]

Tjäderhane et al[16] mentioned that endogenous dentin collagenolytic enzymes, MMPs, and cysteine cathepsins are responsible for time-dependent hydrolysis of collagen

matrix of hybrid layers. He further stated that as collagen matrix integrity is essential for the preservation of long-term dentin bond strength, inhibition of endogenous dentin proteases is necessary for durable resin-bonded restorations.[16] The vast majority of the experiments aimed to improve the durability of dentin bonds using enzyme inhibition have been performed with CHX, a potent antimicrobial agent. The CHX inhibits effectively MMPs-2, -9, and -8.[16]

Moon et al[11] discussed the effect of hybrid–dentin bond layer stability and use of CHX to prevent bond failure. He states that application of acid resins of dentin-bonding agents, after the activation is depleted by the phosphoric acid etchant in the etch-and-rinse bonding agents, more than restores the MMPs activity in dentin. In addition, activated MMPs may find their way to leakage at bond gaps from MMPs in saliva, crevicular fluid, and through dentinal tubule fluid from the pulp over a time. If collagen fibrils are left exposed in the hybrid layer, unprotected by resin, they can be degraded by the activated MMPs. This degradation *in vitro* may show as loss of retention clinically or a decrease in bond strength.

In the present study, we utilized two main groups, All Bond 2 representing the fourth-generation bonding system and One Coat 7.0 representing the seventh-generation bonding system with and without application of 2% CHX gluconate.

Based on the statistical results analyzed, there was no statistical difference in the presence/absence of hybrid layer between groups I and II, but groups I and II showed statistically significant difference when the presence/absence of resin tags were compared. When groups III and IV were compared for the presence/absence of hybrid layer and resin tags, the results were statistically significant. Pashley and Carvalho.[14] studied the deterioration of hybrid layer due to aging and came to a conclusion that there is deterioration of hybrid layer after water storage. This is in agreement with the study done by Lafuente.[13] In the present study, the effect of water storage on the hybrid layer produced a reduction in thickness of hybrid layer, and it was difficult to isolate the hybrid layer in some of the samples that were not pretreated with 2% CHX. Gendron et al[17] as early as in 1999, stated that there is complete inhibition of MMP-2 and -9 gelatinase activity with CHX concentrations as low as 0.03%. In this study, 2% CHX is used as disinfecting solution to acid-etched dentin before the use of total-etch adhesives. This can have additional potential merits in preventing the degradation of collagen fibrils in the dentin–hybrid layers.

According to Liu et al,[2] there were two major mechanisms involved in the degradation of resin-dentin bonds over time. One mechanism is slow hydrolysis of resin components caused by water sorption or esterases. The other is degradation of water-rich, resin-sparse collagen fibrils within the hybrid layers by the activation of host-derived MMPs and possibly cysteine cathepsins during bonding procedures. They state that complete replacement of free and loosely bound water within the collagen water compartments and inactivation or silencing the collagenolytic enzymes appeared to be the ultimate goals for comparing the durability of resin–dentin bonds.

According to Tjäderhane et al,[16] enzyme inhibition with CHX is to improve the durability of dentin bonds and reduce time-dependent reduction in dentin bond strength.

Some studies have also revealed that the topical application of CHX after acid etching has no effect on the immediate bond strength.[18-22] Dutra-Correa et al[23] reported that the application of CHX before the application of dentin adhesives did not influence their clinical performance up to 18 months of service.

The results of the present study are in agreement with the studies done by several authors including Lafuente,[13] Gendron et al,[17] and Liu et al.[2] In the present study, we have only looked into one parameter to increase the service life of the resin-based bonding procedure. There are many other factors that the authors have recommended, including the use of broad-spectrum inhibitors of collagenolytic enzymes, ethanol-wet bonding with hydrophobic resins, as well as biomimetic remineralization of water-filled collagen matrix using analogs of matrix proteins to progressively replace water with intra- and extrafibrillar appetites to exclude exogenous collagenolytic enzymes.[2] Most authors have recommended a combination of several of these strategies that would result in overcoming the critical barrier to progress, i.e., currently encountered in dentin bonding.

More studies are required to combine all the factors involved in dentin bonding to come to a conclusion as to what helps in resin–dentin bonding.

CONCLUSION

Under the conditions of this *in vitro* study:

- On comparison between the groups where the fourth-generation bonding system (All Bond 2) was used, the group with 2% CHX showed better results when the presence/absence of resin tags were compared, whereas there was no significant difference in presence/absence of hybrid layer between the groups.
- When groups III and IV were tested with seventh-generation bonding system (One Coat 7.0), group with 2% CHX showed statistically significant results in comparison with group without 2% CHX when compared for the presence/absence of hybrid layer and resin tags.

- However, on overall comparison between all the four experimental groups, irrespective of the bonding systems used, we concluded that groups with 2% CHX usage showed promising results with presence/absence of hybrid layer and resin tags formation.

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