EPMA Observation Between Dentin and Resin Interfaces.

Part 1. Comparison of Wet and Dry Technique after Short-term Stored in Water

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The study was to evaluate the marginal leakage of wet or dry dentin condition after restoration on short-term. In the study we used Photobond adhesive system and Single Bond adhesive system. The specimens were prepared from premolars. A class V cavity was prepared at the CEJ with a high-speed hand piece and #010 round diamond point. The cavity's walls and floors were etched with phosphoric acids of the test materials by wet and dry bonding techniques. The specimens were cross-sectioned longitudinally through the center of the cavities with a low speed diamond micro-cutter and polished with carbide paper (#600-1200) after storage in distilled water for 1 day, and silver nitrate staining. Specimens were viewed with EPMA for elemental distribution of calcium, nitrogen and silver on the resin-dentin interface.

The uptake of silver particles was less in samples treated with the wet-bonding technique when compared with dry-bonding technique. This *in vitro* study showed that bonding technique is important in establishing a seal along the restoration margins to control marginal leakage.

Key words : Nanoleakage, Dry-bonding technique, Wet-bonding technique

INTRODUCTION

The acid etch technique proposed by Buonocore (1955)¹⁾ has proved successful in enamel bonding and has effectively eliminated microleakage at the enamel-restoration interface²⁾. It has also been found to effectively inhibit microleakage at dentin and cementum margins of cavities³⁾. Sano and others^{4–6)} have described another pattern of leakage. By observing the penetration of silver nitrate along gap-free margins with several dentin adhesive systems by SEM (Scanning Electron Microscopy) and TEM (Transmission Electron Microscopy), they described leakage patterns occurring within the nanometer-size spaces around the collagen fibrils of the hybrid layer. This has been termed nanoleakage. Therefore, a more effective adhesive system and an bonding technique are important to prevent such microleakage-nanoleakage.

The absence of resin in the interfibrillar spaces of the bottom half of these areas suggests that these regions collapse when using the air-dry technique^{7,8)}. Phosphoric acid etching of a dentin surface sometimes modifies the collagen in demineralized

dentin, which collapses easily during the drying process. The collapsed collagen layer does not permit monomer permeation^{9,10}. Therefore, the use of the dry-bonding technique is perhaps not the best choice for composite resin restorations⁷. In addition, the dentin surface is maintained in a hydrated state prior to the application of the hydrophilic primer, leading to efficient infiltration of adhesive resin into the decalcified dentin layer.

In the wet-bonding technique, the use of phosphoric acid (35%) (Single Bond Dental Adhesive System-3M) as an etching agent is recommended. The wet-bonding technique¹¹ is considered as the more effective bonding technique for dentin cavity walls compared to the dry bonding technique. The dentin surface is maintained in a hydrated state prior to the application of the hydrophilic primer, leading to efficient infiltration of adhesive resin into the decalcified dentin layer.

Air-drying of acid etched decalcified dentin has been shown to cause the decalcified unsupported collagen framework to shrink and collapse, thereby preventing the adhesive resin from efficiently infiltrating the conditioned dentin surface^{12,13}. HEMA-containing primers maintained in moist condition may prevent the collapse of the decalcified dentin layer resulting from dehydration, and keep the collagenous network open for infiltration of hydrophilic monomers to form a hybrid layer zone¹⁴. The presence of water is beneficial for optimizing bond strength and in providing a polymeric seal with adhesive resin, but too much water may also be detrimental¹⁵.

Formerly, studies have shown that 32% phosphoric acid (All-Bond 2) and 10% maleic acid (Scotchbond Multi-Purpose plus) in the presence of excess water will lead to lower resin bonding strength values¹⁶.

Recently, we conducted observations using the Electron Probe X-ray Micro Analyzer (EPMA) to look into the adhesive relationship between the resin-dentin interface. A large nitrogen layer (indicating a collagen-rich layer)¹⁷ which changes in quality where the adhesive resin monomer was not able to infiltrate the decalcified layer on the resin-dentin interface, can be observed. It was observed that this was easily generated in regions of the leakage on the dentin side of the hybrid layer¹⁸.

The aim of this study was to determine whether differences occurred in the leakage patterns of two types of adhesive systems: Photo Bond (Kuraray, Osaka, Japan) and Single Bond (3M Dental Products, St Paul, MN), and by two dentin surface applications (dentin bonding techniques): the dry-bonding technique (by air-drying) and the wet-bonding technique (by blot-drying) (as described by Van Meerbeek *et al.*)¹⁹.

MATERIALS AND METHODS

Two adhesive systems were studied (Table 1) for four test groups (Table 2).

EPMA analysis of resin dentin interface after silver staining

Twenty human premolars freshly extracted for orthodontic reasons were stored at 4°C in isotonic saline no longer than 2 months prior to their use. Class V cavities were prepared at the CEJ, of enamel and dentin with a high-speed hand piece and

Table 1 Materials, Manufacturers, batch numbers and composition

Adhesive system	Etchant	Adhesive Resin	Manufacturer
Photo Bond system (CPB) (Batch No: 302)	37% Phosphoric acid (apply 30 sec.)	MDP, HEMA Photoinitiator (Light cure 10 sec.)	Kuraray, Osaka Japan
Single Bond system (SB) (Batch No: 19970131)	35% Phosphoric acid (apply 15 sec.)	Bis-GMA, HEMA dimethacrylates Photoinitiator (Light cure 10 sec.)	3M dental Products, Division St Paul, MN, USA

Table 2 Adhesive procedures all of the test groups

Group 1 (Photo Bond, by blot-dry)	Etching: apply 30s, water rinse 10s blot-dry by cotton pellet, Bonding: with pellet brush two consecutive gently air thin 2-3 sec. Light cure 10s
Group 2 (Photo Bond, by air-dry)	Etching: apply 30s, water rinse 10s air-dry 10s by three way syringe, Bonding: with pellet brush, gently air thin 2-3 sec. Light cure 10s
Group 3 (Single Bond, by blot-dry)	Etching: apply 15s, water rinse 10s blot-dry by cotton pellet, Bonding: with pellet brush two consecutive gently air thin 2-3 sec. Light cure 10s
Group 4 (Single Bond, by air-dry)	Etching: apply 15s, water rinse 10s air-dry 10s by three way syringe, Bonding: with pellet brush, gently air thin 2-3 sec. Light cure 10s

#010 round diamond point 2 mm in depth. The twenty teeth were divided randomly into the four treatment groups and bonded with one of the bonding techniques (Table 2). The conditioned cavities were restored with composite resin of Clearfil AP-X for Photo Bond adhesive system (Kuraray, Osaka, Japan) and Z250 for Single Bond adhesive system (3M, MN, USA) and lightly cured for 30 seconds by a visible light unit (XL3000, 3M, MN, USA).

The margins were finished and polished with a diamond finishing point and a silicone point (SHOFU, Tokyo, Japan) after storage in distilled water at 37°C for 24 hours. Root apices of each specimen were sealed with inlay wax, and the entire tooth, except for the margin and 1 mm of the tooth surface adjacent to the interface, was coated with two layers of nail varnish. The teeth were placed in a 50% (W/V) silver nitrate solution in total darkness for 24 hours, rinsed in running water for 5 min, immersed in photo developing solution and exposed to a fluorescent light for 8 hours in order to reduce the silver ions to metallic silver²⁰. After removal from the developing solution, the teeth were placed under running water for 5 min. The specimens were cross-sectioned longitudinally through the center of the cavities with a low speed diamond micro-cutter (Micro Cutter 201, Marumoto, Tokyo, Japan), making a

total of 10 specimens for each adhesive conditioning group. All surfaces of the specimens were polished with silicone carbide papers of decreasing abrasiveness (# 600, 800 and 1200-grit; Maruto, Tokyo, Japan) with a water coolant. A final polish was performed with a soft cloth using alpha-alumina powder ($1\mu\text{m}$; Marumoto, Tokyo, Japan) with distilled water. The specimens were cleaned ultrasonically, and examined with Electron Probe X-ray Micro Analyzer (EPMA 8705, Shimadzu, Kurashiki, Japan).

SEM observation of dentin surface with CPB and SB treatment

Dentin specimens were treated with CPB and SB conditioner (the conditions for testing and dentin surface treatment were the same as in the first study). These were subsequently fixed simultaneously in 2% paraformaldehyde and 1.25% glutaraldehyde (PA-GA) for 12 hours, and dehydrated in ascending grades of ethanol (50%, 70%, 80%, 90%, 95% and 100%) for 10 min. with double application for each. All fragments were then transversally fractured before being critically point dried (Critical Point Dryer; HITACHI, Tokyo, Japan) and gold coated. These sections were observed using a scanning electron microscope (S-430, HITACHI, Tokyo, Japan).

RESULTS

Element analysis of EPMA

The relationship between the procedure of dentin surface conditioning techniques and the elemental distribution of the Calcium, Nitrogen and Silver on the resin-dentin interfaces are summarized in Fig. 1-4. (The specimens for resin-dentin interface evaluation consisted of five teeth in each group).

Fig. 1 (Group 1) shows the resin-dentin adhesive interface by blot-dry technique (with cotton) using the Photo Bond adhesive system. The silver deposits of resin-dentin adhesive interface could be clearly noted in the SEI. The decalcified layer is shown slightly with a width of $6\text{--}8\mu\text{m}$ (Ca, arrow). Nitrogen layer is shown having a width of about $4\text{--}5\mu\text{m}$ (N, arrow) and Ag showed a $4\text{--}5\mu\text{m}$ silver stained band (Ag, arrow). The Nitrogen layer exhibited the same thickness of high electronic density. Silver deposits could be seen slightly on dentin side of the decalcified layer.

Fig. 2 (Group 2) shows the resin-dentin adhesive interface by air-dry technique with a dental unit three way air syringe using the Photo Bond adhesive system. The silver deposits of the resin-dentin adhesive interface showed $5\text{--}6\mu\text{m}$ thickness in the SEI. The nitrogen layer thinness is about $8\text{--}10\mu\text{m}$ (N, arrow) and Ag showed a silver stained band measuring $6\text{--}8\mu\text{m}$ (Ag, arrow). The Nitrogen layer exhibited the same thickness of high electronic density.

Fig. 3 (Group 3) shows the resin-dentin adhesive interface after acid conditioning for 15 seconds and by blot-dry technique with cotton using the Single Bond adhesive system. The silver deposits of resin-dentin adhesive interface showed a thickness of $1\mu\text{m}(\pm)$ in the SEI. Nitrogen layer has a in thickness of about $3\text{--}4\mu\text{m}$ (N, arrow) and Ag is showed a silver stained band of $2\text{--}3\mu\text{m}$ thickness (Ag, arrow). The

Fig. 1 Photograph of EPMA secondary electron image on the resin-dentin interface, and the sample bonded with *wet-bonding technique* of *Photo Bond adhesive system* (SEI).

The photograph of EPMA elemental analysis mapping show the decalcified layer (Ca, Calcium), exposed collagen layer=adhesive resin monomer uninfiltreated layer (Nitrogen, N) and Silver staining (Ag) of elementals. The color bar shows the element concentration (measurement line=10 μm).

Fig. 2 Photograph of EPMA secondary electron image on the resin-dentin interface, and the sample bonded with *dry-bonding technique* of *Photo Bond adhesive system* (SEI).

The photograph of EPMA elemental analysis mapping show the decalcified layer (Ca, Calcium), exposed collagen layer=adhesive resin monomer uninfiltreated layer (Nitrogen, N) and Silver staining (Ag) of elementals. The color bar shows the element concentration (measurement line=10 μm).

Fig.3 Photograph of EPMA secondary electron image on the resin-dentin interface, and the sample bonded with *wet-bonding technique* of *Single Bond adhesive system* (SEI).

The photograph of EPMA elemental analysis mapping show the decalcified layer (Ca, Calcium), exposed collagen layer=adhesive resin monomer uninfiltreated layer (Nitrogen, N) and Silver staining (Ag) of elementals. The color bar shows the element concentration (measurement line=10 μm).

Fig.4 Photograph of EPMA secondary electron image on the resin-dentin interface, and the sample bonded with *wet-bonding technique* of *Single Bond adhesive system* (SEI).

The photograph of EPMA elemental analysis mapping show the decalcified layer (Ca, Calcium), exposed collagen layer=adhesive resin monomer uninfiltreated layer (Nitrogen, N) and Silver staining (Ag) of elementals. The color bar shows the element concentration (measurement line=10 μm).

Fig. 5 Photograph of the dentin surface, the samples was treated with Photo Bond dry-bonding technique (a) and wet-bonding technique (b); and with the Single Bond dry-bonding technique (c) and wet-bonding technique (d).

The samples showed that collagen fibers was collapsed or were compacted on the decalcified dentin surface with the air-dry bonding technique (black arrow). In the samples with wet-bonding technique collagen fibers of uncollapse or uncompact on the decalcified dentin surface were observed (white arrow).

Nitrogen layer exhibited the same thickness of high electron density.

Fig. 4 (Group 4) shows the resin-dentin adhesive interface by the air-dry technique with a dental unit three way air syringe using the Single Bond adhesive system. The silver deposits of resin-dentin adhesive interface showed a thickness of $6-7\mu\text{m}$ in the SEI. Nitrogen layer exhibited a thickness of about $8-9\mu\text{m}$ (N, arrow) and Ag is shown having a silver stained band measuring $4-6\mu\text{m}$ (Ag, arrow) that infiltrated deeply into the dentinal tubule, forming the same Nitrogen elemental distribution area; this corresponds to the decalcified layer.

SEM observation

Representative SEM micrographs (Figs. 5a, c) show the respective etched dentin surfaces produced by the CPB (Fig. 5a) and SB (Fig. 5b) adhesive systems following the two experimental post-conditioning procedures. Figs. 5a and c, represent the acid etched dentin surface of the CPB and SB using the dry-bonding technique. This shows a collapse of the decalcified collagen matrix associated with enlarged orifices of the tubules up to $4-5\mu\text{m}$ in diameter due to the loss of peritubular dentin. Figs. 5b and d shows the result of the wet-bonding technique (blot-drying) using the CPB and SB adhesive system; they showed an intact matrix with open dentinal tubules. The tubule orifices measured $2-3\mu\text{m}$ in diameter owing to an intact collagen matrix.

DISCUSSION

The results of this study indicate that factors of the bonding techniques affect the

performance of dentin adhesive systems and adhesive application techniques. The type of etchants used to condition the dentin surface, the presence of dry or moist dentin surfaces, and the type of adhesive system all have significant effects on the diffusion of silver particles into the resin-dentin adhesive interface, as shown by EPMA and SEM photographs.

Silver nitrate staining is one of the most commonly used methods for microleakage evaluation. Compared with other staining techniques the silver staining method provides a much sharper photograph of penetration along tooth-restoration margins²⁰⁾. However, microleakage assessments are usually performed using a low power optical microscope, providing only gross information, such as the depth of penetration²¹⁾.

Traditional microleakage evaluation methods cannot identify the exact location where the microleakage occurs at the tooth-resin interface, for example, between the hybrid layer and the bonding agent, or between the bonding agent and the restorative material²²⁾. It was noted in this study that using higher magnifications (3000 $\times$) and EPMA of an elemental analyzer, the silver element was observed to have penetrated further into the hybrid layer than could be detected at low magnifications. The silver nitrate penetration method combined with high magnification SEM by means of secondary electron or backscattered electron mode can provide much better information concerning the sealing ability of the restorations and the quality of the hybrid layer⁴⁻⁶⁾. In this study, observations of the nanospace of the resin-dentin interfaces were made using a WDX type of electron probe X-ray microanalyzer (EPMA). The EPMA was not only used for element analysis but also allowed for a second electron micrograph to be taken.

In the present study, there were large differences between specimens exposed to air-drying and wet-bonding techniques when viewed under an SEI. The dentin surface conditioners used by the CPB or SB systems are very similar in that they are both phosphoric acid gels with silica. The moist or dry condition changes the morphology of the dentin surfaces (Fig. 5a and b). With the dry-bonding technique the dentin surface consists of a collapsed, shrunken collagen network (Fig. 5a)

Some differences in the resin-dentin interface bonded under dry vs. wet bonding techniques were seen using WDX analysis. The resin-dentin interface showed an 8-10 μm thick collagen-rich layer in the dry bonding (Group 2), while the same layer was only 3-4 μm thick in the wet bonding (Group 1). The collagen-rich layer suggests the presence of a decalcified collagen rich layer that was not infiltrated by adhesive resin (Fig. 2). The thickness of the collagen-rich layer may cause the collagen network to collapse and form a compact coagulate after air-drying (Fig. 5a) which forms an impenetrable decalcified layer for the adhesive resin monomer. The collagen fiber network that was exposed following acid etching was affected by the adhesive resin, which diffused into the collagen fiber layer, and penetrated into the nano-spaces (collagen web space) previously occupied by the apatite mineral; together, these formed a resin-collagen complex which was fully polymerized by the hybrid layer.

In the present study, SEI (EPMA SE images) of the interface bonded with CPB

and SB to the decalcified dentin, silver deposition was found to be intense within the hybrid layer and silver particles were noted along the tubule walls. The silver particles were concentrated mainly in the hybrid layer of dentin side than resin side because the dentin side of hybrid layer is located farther away from the top decalcified layer. Van Meerbeek & others²³⁾ found a gradual transition of resin concentration in the decalcified superficial dentin; the concentration of the resin was greatest at the top of the hybrid layer and lowest near the base. If the resin does not penetrate to the full depth of the decalcified zone, the uninfiltreated weak collagen layer at the base of the zone may be susceptible to long-term hydrolytic degradation^{23,24)}.

In this study, nitrogen elements were detected on the resin-dentin interface of all samples, and the thickness of this layer was related to silver uptake. Uptake of the silver element increased in resin-dentin interface when the Nitrogen layer was thick. The Nitrogen layer was thinner in samples that were treated using the wet-bonding technique when compared with dry-bonding technique. In samples treated with wet bonding technique, water that was required for optimal adhesive resin inter-diffusion and higher shear bond strength generation was available to maintain open interfibrillar spaces²⁵⁾. In the dry-bonding technique, the presence of a collapsed thick decalcified collagen fibril layer, *i.e.* collagen smear layer, interfered with resin infiltration. This limits the adhesive resin infiltration to the surface of the decalcified layer. With the blot-drying technique, the expanded collagen fibril network²⁶⁾ allowed for deeper adhesive resin infiltration into the demineralized layer (Fig. 1, 3). However, even when using the wet-bonding technique, a Nitrogen rich layer was detected by WDX in the lower half of the resin inter-diffusion zone. This supports the recent findings of Spencer and Swafford²⁷⁾, who demonstrated an unprotected dentin matrix in the lower half of hybrid layers.

The moist or dry condition of the dentin surface was important for the adhesion technique. Presumably, if this occurred *in vivo*, it would cause marginal microleakage and increased nanoleakage in the nanometer sized spaces within the uninfiltreated collagen layer. The infiltration of adhesive resin into the expanded decalcified layer would prevent microleakage and nanoleakage^{28,29)}. In the present study, there were large differences between specimens exposed to the air-drying and wet-bonding techniques when viewed under SEM (Fig. 5).

CONCLUSION

The present findings indicate that a moist dentin surface is required for optimum infiltration of adhesive resin into the decalcified layer when the dentin conditioner used is of the phosphoric acid type. Additionally, adhesive resin monomer should have a optimum fluidity, permeability and hydrophilic property for this to be achieved. In addition, it seems that the element analysis on the resin-dentin interface where EPMA was used is valuable in evaluating microleakage and nanoleakage research.

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