—原著—

Nanoleakage of various adhesive systems and dentin conditioning techniques

—Comparison of Wet and Dry Techniques after Long-term Stored in Water—

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Abstract : The purpose of the study was to evaluate the marginal leakage with wet or dry bonding techniques samples by EPMA after long-term storage in distilled water of the restoration tooth. The adhesive systems, Clearfil Photobond and Single Bond were used. The specimens were prepared as class V cavities having a depth of 2 mm on the CEJ. The cavity wall and floors were treated with the etchant of two bonding systems either by wet or dry bonding techniques. The specimens were viewed with an EPMA of the WAX type for the elemental distribution of calcium, nitrogen and silver on the resin-dentin interface after storage in distilled water for 90-day. Different microleakage and nanoleakage patterns were observed with different adhesive systems and different application techniques. The infiltration of silver particles was observed to be less in specimens where wet bonding technique was used compared with samples where dry bonding technique was utilized. The infiltration of silver particles (in 90-day samples) was notably prominently than day-1 samples[1]. This study showed that the bonding technique and bonding materials are important in establishing a seal for the control of marginal leakage.

抄録：接着修復の技法，材料の発展に伴い，健全歯質の不必要な切削が減少し，複雑な症例の処置法も簡易化されてきた。しかし，接着システムは，エナメル質に対しては安定な接着関係が得られるものの，象牙質に対しては，依然，辺縁封鎖性の問題が残されている。一方，露出根面のような象牙質窩縁を有する齲蝕歯或いは知覚過敏に対しても，接着性レジン修復が多用されていることから，レジン・象牙質間の接着関係を強固で且つ長期安定なものにするために，レジン・象牙質接着界面の構造解明が必要とされる。

レジン・象牙質接着界面において，脱灰コラーゲン層にレジンモノマーが浸透して形成される樹脂含浸層は，レジン・象牙質間の接着強度を高めていると考えられている。しかし、実際の臨床修復例では，レジン充填物の歯頸側に色素の浸入がよく見られ，Van Meerbeekらは，アルゴンエッチング法による樹脂含浸層の象牙質側にモノマーの浸透不充分な部分が残っていると指摘した。また，本研究の第一報では[1]，銀染色とEPMA元素分析により接着界面には窒素に富んだ層が検出され，同部位では脱灰層全層への接着性レジンの浸透は，不充分であるためだと思われる。レジン象牙質接着界面にこのような submicron あるいは nanometer サイズの微少欠陥を介する漏洩経路が存在し，nanoleakage を起こすことが確認された。

そこで，本研究では，２種の接着システムを用いて５級窩洞にコンポジットレジン充填を行った試片を長期水中保管後に硝酸銀染色を行い，波長分散型X線マイクロアナライザー（EPMA）を用いてレジン象牙質接着界面の微細構造学的観察を行い，nanoleakageの発生と接着界面の耐久性との関係について検討を行った。

INTRODUCTION

Many factors challenge the marginal integrity of composite resin bonding restorations. At the time of the initial set, polymerization contraction produces forces, which tend to pull the restoration away from the tooth structure. Thereafter, chemical, thermal and mechanical stresses compromise the integrity of the bond.[2-7] In the clinical situation, these forces may not always result in the loss of the restoration, yet the significance of bond strengths is often overemphasized.[8] The seal of restorative materials against the tooth structure, and the quality and durability of the seal are major considerations for the longevity of restorations. This may influence the selection of restorative materials and can be a significant factor in preventing pulp damage and secondary caries. Bacteria will rapidly colonize any gaps present between the composite resin and tooth structure[9] as bacterial infection has been shown to be mainly responsible for inflammatory pulp reactions. Therefore, the marginal integrity of restorations is of primary importance in maintaining the long-term function of composite restorations. The clinically important issue is to achieve a biocompatible restoration that will not compromise the pulp, with materials where technique sensitivity in not an issue.[10] Thus, the study of bacterial and fluid penetration at the interface between the restoration and tooth structure, that is, microleakage, has been of great concern in restorative dentistry.

The acid etching technique proposed by Buonocore (1955) has proved successful in enamel bonding, has effectively eliminated microleakage at the enamel-restoration interface[11] and has been found to effectively inhibit microleakage at the dentin and cementum margins of cavities.[12] Sano et al.[13-15] have described another pattern of leakage by observing the penetration of silver nitrate along gap-free margins with several dentin adhesive systems using SEM and TEM. They described leakage patterns occurring within the nanometer-sized spaces around the collagen fibrils of the hybrid layer, and termed them nanoleakages. Realizing the presence of such gaps, an appropriate adhesive system applied using an efficient bonding technique is important in the prevention of such microleakages and nanoleakages.

The choice of an appropriate adhesive system together with the bonding technique has always been an important concern for clinical dentistry. A number of adhesive systems are currently available. Many of these current generation dentin adhesive systems involve the removal of the smear layer[16-17] to facilitate the infiltration of hydrophilic resin into acid-conditioned dentin. For instance, the use of the total-etch technique to enamel and dentin surfaces[18] removes the inorganic mineralized material surrounding the collagen fibrils in dentin to a depth of approximately 8-10 μm.[19] However, recently studies have shown that 32% phosphoric acid (All-Bond 2 Bisco corp) and 10% maleic acid (Scotchbond Multi-Purpose plus, 3M) in the presence of excess water will lead to lower resin bonding strength values.[20]

Resin adhesives, along with glass-ionomer cement or amalgam, are widely used as restorative materials in dentistry. Comparatively high bond strengths of resin to enamel and to dentin have been achieved in short-term in vitro experiments; however, unfavorable results such as marginal discoloration, secondary caries, and loss of material have been reported in long-term clinical use.[21] Therefore, the problem regarding the evaluation of the longevity of resin restorations under in vivo conditions has become an important research topic.[22]

The use of EPMA to observe the relationship between the adhesive material and the resin-dentin interface has become popular. A common observation in such studies is the presence of a thick nitrogen layer in regions where the adhesive resin monomer is not able to infiltrate the decalcified layer on the resin-dentin interface. In other studies it was clarified that this was easily generated in regions of leakage on the dentin side of the hybrid layer.[23]

The aim of this study was to use an WDX-type (Wavelength Dispersive X-Ray Spectrometer) electron probe X-ray microanalyzer (EPMA) to characterize ultra-morphologically the resin-dentin interface produced by the two adhesive systems, when either a dry or a wet bonding technique was applied after long-term storage in distilled water.

MATERIALS AND METHODS

For this experiment, two resin bonding systems (Table 1) and four test groups (Table 2) were used.

That are, materials used were the Photo Bond system (CPB) and the Single Bond system (SB); furthermore, two application techniques were used: the dry-bonding technique (with air-drying) and the wet-bonding technique (with blot-drying) for the each material.

Twenty freshly extracted human premolars surgically removed for orthodontic reasons were stored at 4ºC in isotonic saline no longer than 2 months prior to their use. The teeth were collected after patients' informed consent had been obtained under a reviewed protocol. Class V cavities were prepared on the cervical area of enamel and dentin with an air-turbine hand piece and round diamond burs to a depth of approximately 2 mm. The specimens were divided randomly into the four resin-bonding application groups and bonded with one of the bonding techniques (Table 2). The conditioned cavities were each restored with composite resins Clearfil AP-X (Kuraray, Osaka, Japan) and Z250 (3M, Minnesota, U.S.A.), and light cured for 30 seconds by XL 3000 (3M, Minnesota, U.S.A.).

The specimens were stored in distilled water at 37℃ for 90 days after the margins were finished and polished with finishing diamond point and silicone point (Shofu, Tokyo, Japan). The apical root portion of each specimen was sealed with composite resin (AP-X), and the entire tooth, except for the area of the bonded margin and 1 mm of the composite resin restoration adjacent to it, was coated with two layers of nail varnish.

Table 1 Materials, Compositions, Manufacturers and Batch numbers.

Adhesive system	Etchant	Adhesive Resin	Manufacturer
Photo Bond system (CPB) (Bacth No: 302)	37% Phosphoric acid (apply 30 sec.)	MDP, HEMA Photoinitialor (Light cure 10 sec.)	Kuraray, Osaka Japan
Single Bond system (SB) (Bacth No: 19970131)	35% Phosphoric acid(apply15 sec.)	Bis-GMA,HEMA Dimethacrylates Photoinitialor (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 30 sec, water rinse 10 sec. blot-dry by cotton pellet. Bonding: with pellet brush two consecutive gently air thin 2-3 sec. Light cure 10 sec.
Group 2 (Photo Bond, by air-dry)	Etching: apply 30 sec, water rinse 10 sec. air-dry 10 sec by three way syringe. Bonding: with pellet brush, gently air thin 2-3 sec. Light cure 10 sec.
Group 3 (Single Bond, by blot-dry)	Etching: apply 15 sec, water rinse 10 sec. blot-dry by cotton pellet. Bonding: with pellet brush two consecutive gently air thin 2-3 sec. Light cure 10 sec.
Group 4 (Single Bond, by air-dry)	Etching: apply 15 sec, water rinse 10 sec. air-dry 10 sec by three way syringe. Bonding: with pellet brush, gently air thin 2-3 sec. Light cure 10 sec.

A modified silver staining technique[24] was used with basic 50 wt % ammonia silver nitrate (pH=9.5) to avoid the possibility of artifact dissolution of the enamel apatite. The solution was prepared by the dissolution of 25g of silver nitrate crystals in 25 ml of distilled water. Concentrated (28%) ammonium hydroxide was used to titrate the black solution until it became clear as the ammonium ions complexed the silver into diamine silver ($[Ag(NH_3)_2]^+$) ions. The solution was diluted to 50 ml with distilled water to achieve a 50-wt% solution.

The teeth were placed in the 50 wt% silver nitrate solution in total darkness for 24 hours and rinsed in running water (tap water) for 5 min thereafter, immersed in photo-developing solution and exposed to a fluorescent light for 8 hours in order to reduce the silver ions to metallic silver[25] for the 90-day samples. The samples were placed in running water for 5 min after removal from the developing solution. The specimens were longitudinally sectioned 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) under water coolant. A final polish was performed with a soft cloth using an alpha-alumina powder (1 μm Marumoto, Tokyo, Japan) with distilled water. The specimens were analyzed for the elemental distribution of calcium (Ca), nitrogen (N) and silver (Ag) on the resin-dentin interface with the EPMA of WDX type after critical point drying and coating with carbon and aluminum. The EPMA of WDX-type [Wavelength Dispersive X-ray Spectrometer] electron probe X-ray microanalyzer was used to observe the micromorphological changes of the resin-dentin interface.[19, 23]

RESULTS

The relationship between the dentin surface conditioning techniques and the elemental distribution

of Ca, N and Ag on the resin-dentin interfaces are summarized in Figs. 1-4. The evaluation of specimens for resin-dentin interfaces consisted of five teeth in each group.

Fig. 1 (Group 1) shows the resin-dentin adhesive interface of CPB using the blot-dry application technique. The silver deposits of the resin-dentin interface could be clearly noted in the SEI. The decalcified layer (Ca=Calcium) of the specimen was observed as having a width of 8-10 μm (Ca, arrow), while the N layer (=Nitrogen) which is also shown here (was about 8-10 μm; N, arrow). Additionally, the mapping picture of Ag (=Silver) showed a silver stained band (Ag, arrow) of 2-3 μm in the dentin surface, notably around dentinal tubules. However, the electron density of the N and Ag are shown more thickly on the dentin side of the specimen of the 90-day comparing to the 1-day specimen[1].

Fig. 2 (Group 2) shows the resin-dentin adhesive interface of CPB using the air-dry application technique (with a dental unit three-way air syringe). The silver deposits of the resin-dentin adhesive interface was about 6-7 μm thick (SEI), the N layer thickness was about 8 μm (N, arrow) and the silver stained band, 6-8 μm (Ag, arrow). Silver deposits were clearly shown (SEI) in the resin-dentin interface.

Fig. 3 (Group 3) shows the resin-dentin interface of SB by using the blot-drying technique. The silver deposits in the resin-dentin interface showed a thickness of 2-3 μm(±) on the SEI; the N layer has a thickness of about 3-5 μm (N, arrow) and the Ag (Silver) showed a silver stained band of 3-4 μm thickness (Ag, arrow). The thickness of the silver deposits layers was comparable to Group 1 samples.

Fig. 4 (Group 4) showed the resin-dentin adhesive interface of SB using the air-drying technique through a dental unit three-way air syringe. Ag deposits of the resin-dentin interface showed a thickness of 7-10 μm in the SEI while the N layer exhibited a thickness of about 10-12 μm (N, arrow). The Ag (Silver) showed silver deposits (Ag, arrow) that infiltrated deeply into the dentinal tubules. The layer (Ag) forming the same N layer corresponds to the decalcified layer. Most of Group 4 samples show silver deposits resembling CPB sample used with air-dry application technique.

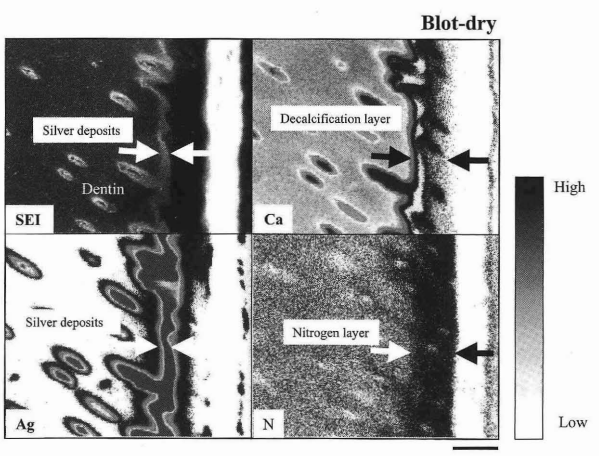

Fig.1 EPMA element mapping of resin-dentin interface with *Photo Bond adhesive system (by wet-bonding technique)* (3000X). Silver deposits were within the resin-dentin interface and were noted along the hybrid layer. The adhesive layer took up numerous silver deposits on the sample that stored in water 90-day (SEI: secondary electron image, Ca: Calcium, N: Nitrogen, Ag: silver)

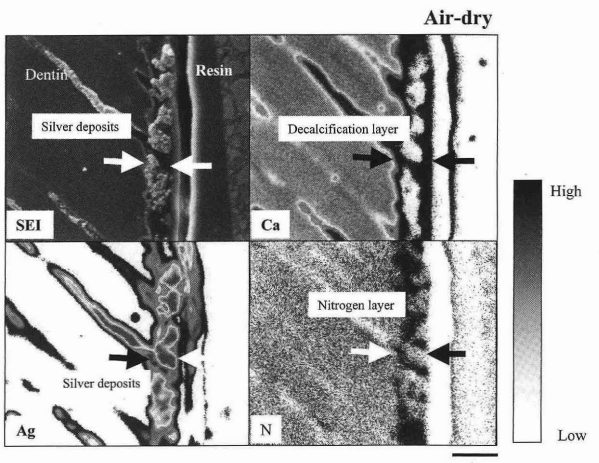

Fig.2 EPMA element mapping of resin-dentin interface with *Photo Bond adhesive system (by dry-bonding technique)* (3000X). Silver deposits were within the resin-dentin interface and were noted along the hybrid layer. The adhesive layer took up numerous silver deposits on the sample that stored in water 90-day (SEI: secondary electron image, Ca: Calcium, N: Nitrogen, Ag: silver)

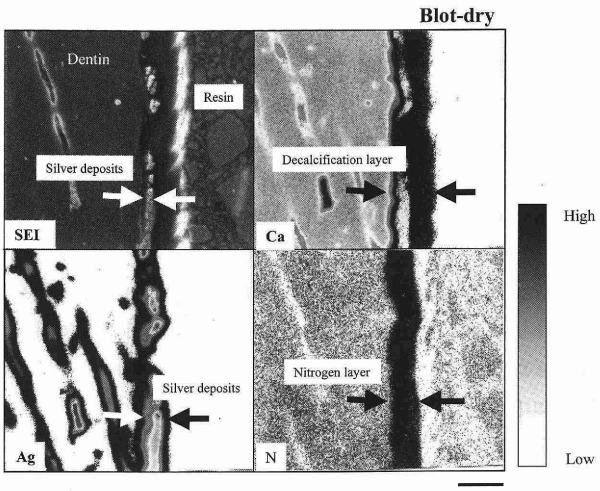

Fig.3 EPMA element mapping of resin-dentin interface with *Single Bond adhesive system (by wet-bonding technique)* (3000X). Silver deposits were within the resin-dentin interface were noted along the hybrid layer. The adhesive layer took up numerous silver deposits on the sample that stored in water 90-day (SEI: secondary electron image, Ca: Calcium, N: Nitrogen, Ag: silver)

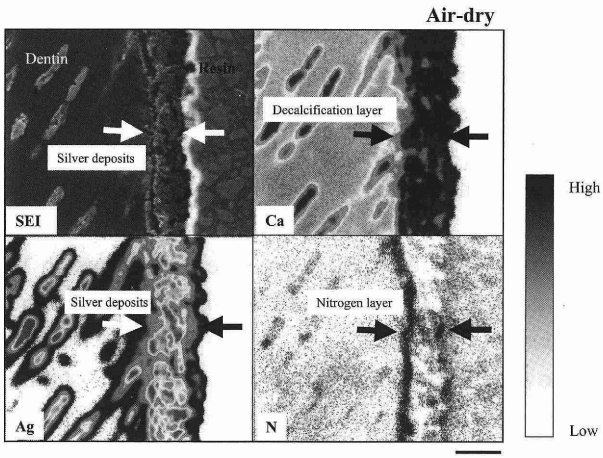

Fig.4 EPMA element mapping of resin-dentin interface with *Single Bond adhesive system (by dry-bonding technique)* (3000X). Silver deposits were within the resin-dentin interface and were noted along the hybrid layer. The adhesive layer, took up numerous silver deposits on the sample that stored in water 90-day (SEI: secondary electron image, Ca: Calcium, N: Nitrogen, Ag: silver)

DISCUSSION

In the present study, there were large differences between specimens of short-term[1] and long-term storage in distilled water. There were also notable differences between specimens of the air-drying and blot-drying application techniques as observed with EPMA. Using higher magnifications of SEI (3000×) and the EPMA of the elemental analyzer, the silver element in this study was observed to have penetrated further into the hybrid layer than could be detected at low magnifications. (EPMA was not only useful for elemental analysis; it also allowed a second electron photograph to be taken).

Marginal leakage may have been not caused, if the adhesive resin monomer infiltrated to deeper layers of the decalcified dentin surface. However, the relative thickness of the decalcified dentin that measured approximately 10-15 μm, and the thickly decalcified layer disturbed the efficient diffusion for the adhesive resin monomer. Additionally, this led to the formation of minute gaps on the dentin side of the decalcified layer resulting in subsequent leakage in the resin-dentin interface, the so-called nanoleakage[14-15]. Afterwards, with the decomposition of the exposed collagen fiber layer, larger gaps are formed and subsequent leakage on the resin-dentin interface of the dentin side, the so-called microleakage. This nanometer-sized space causes nanoleakage immediately following the placement of restorations, while the micrometer-sized space causes microleakage following long-term observation of restored teeth. Both were observed on the dentin side of the resin-dentin interface. The nanoleakage seems to result in microleakage.

Some differences were also seen in the resin-dentin interface bonded under dry bonding versus wet-bonding techniques used EPMA analysis. The resin-dentin interface showed an 8-10 μm thick collagen-rich layer (nitrogen layer) in the wet bonding sample (Fig. 1, N), and silver deposits width ranged from 2-3 μm (Fig. 1, Ag). Moreover, the silver deposits were fragments. This indicates that adhesive resin monomer diffused to deeper parts of the decalcified layer. And, in the dry-bonding technique samples, the resin-dentin interface showed a collagen-rich layer measuring 6-7 μm (Fig. 2, N); silver deposits measured

approximately 6 μm (Fig. 2, Ag). It was also observed that the silver deposits showed a bigger lump (Fig. 2, SEI). This indicates that 1) the top part of collagen fiber layer on the decalcified dentin may have collapsed after being air-dried, because, the diffusion of the adhesive resin monomer were obstructed by the collapsed collagen layer, and 2) the exposed collagen layer underwent decomposition after long term placement of a restoration, and the gap grows, in the resin-dentin interface of the dry-bonding technique samples.

The same phenomenon was observed in SB samples for both dry and wet-bonding techniques. Silver deposits ranged from 3-4 μm (Fig. 3, Ag) and it was found scattered on the resin-dentin interface (Fig. 3, SEI) in SB samples that used the wet-bonding technique. In contrast, the silver deposits in those samples that utilized the dry-bonding technique measured about 10 μm (Fig. 4, Ag and SEI); they also exhibited a higher density (Ag). This indicates that the adhesive resin monomer nearly failed to infiltrate into the decalcified layer. This may be attributed to the exposure and subsequent collapsed of the superficial part of the decalcified layer after the tooth was conditioned with etchant and air-drying. This made it difficult for the adhesive resin monomer to diffuse into the thick decalcified layer. Thus, the collagen layer that supports the adhesive resin layer undergoes dessication over time, resulting into gap formation and eventual microleakage were caused.

Pashley & others (1993)[26] examined the effect of phosphoric acid on dentin and reported that a collagen-rich layer developed as a result of demineralization, which could interfere with adhesive agent penetration. Dehydration of the acid-conditioned dentin surface through air-drying is thought to induce surface tension stress, causing the exposed collagen network to collapse, shrink and form a compact layer that is impenetrable to resin monomer.[19, 23]

The wet-bonding technique can promote efficient diffusion of resin monomers into the decalcified matrix only if excess water on the dentin surface is eliminated with dry cotton after etching, water-rinsing, and then replaced by monomers during subsequent priming steps. In the currently available adhesive systems, hydrophilic primer monomers are dissolved in volatile solvents, such as acetone and ethanol; these aid in the displacement of the remaining water as well as permitting the polymerizable hydrophillic monomers into open dentinal tubules and through the nano-spaces of the collagen web.[27]

The importance of a hybrid layer for resin-dentin adhesion is well recognized among researchers. The hybrid layer is formed by monomer impregnation into exposed collagen fibrils of decalcified dentin surfaces following acid treatment of the dentin.[28-29] However, a resin-free decalcified dentin zone exists at the base of the hybrid layer, due to incomplete resin infiltration.[30-31] Thus collagen fibril networks of resin-free zones have been the cause of nanoleakage or microleakage on resin-dentin interfaces.

Previously, a complete air-drying bonding technique for applied cavity walls was generally used after acid etching and tap water rinsing (by a three-way syringe) on composite resin restorations. At that time, it was thought that the adhesive monomers could not diffuse into wet dentin layer. However, the adhesive monomers diffusion into the air-dried, collapsed collagen layer, maybe need a longer time; however, given the application time of only 2-3 seconds, it was not possible for adhesive monomers to effectively diffuse into the decalcified layer. In contrast, when using blot-drying after etching, the presence of some water in the decalcified layer renders the dentin surface moistened/wet thus keeping the collagen fibrils separated, a suitable condition of hydrophiles for adhesive monomer diffusion[19].

In the present study, silver deposits were found to be intense within the hybrid layer; silver particles were noted along the tubule walls. The silver particles were concentrated mainly in the hybrid layer of the dentin side compared to the adhesive resin side, because the dentin side of the hybrid layer is located farther away from the top of the decalcified layer as well as the adhesive resin layer. The silver layer was thicker on the long-term specimens (compared to the 1-day specimen)[1]. The nitrate of silver staining method is often used because it permits the microleakage pathway to be studied by secondary electron and backscattered scanning electron microscopy.[32-33] Silver staining method provides a much sharper photograph of the penetration along tooth-restoration margins compared with others staining techniques.[34] However, microleakage assessments are usually performed using a low power optical microscope providing only gross information,

such as the depth of penetration.[35]

With regard to the result of the comparison of the wet vs. dry bonding technique, it was noted that the uptake of the silver element increased in the resin-dentin interface of the dry-bonding technique specimens (Fig.2 and 4) compared to the specimens of the wet-bonding technique (Fig.1 and 3). In specimens treated with the wet-bonding technique, water that was required for optimal adhesive resin inter-diffusion and higher shear bond strength generation was available to maintain open the inter-fibrillar spaces.[36] In the specimens of the dry-bonding technique, the presence of a collapsed thick decalcified collagen fibril layer, i.e. a collagen smear layer, interfered with resin infiltration. This limits the adhesive resin infiltration to the surface of the decalcified layer (Fig. 2 and 4).

With the wet-bonding technique, the expanded collagen fibril network[37] allowed for deeper adhesive resin infiltration into the decalcified layer. However, even when using the wet-bonding technique, a nitrogen rich layer was detected by EPMA in the resin-dentin interface (Fig. 1, 3).

The wet or dry condition of the dentin surface was significant for adhesion of restorations such as composite resin. This has an extremely important influence on the prognosis of the restoration of teeth. The results of this study indicate that a variety of factors affect the performance of dentin adhesive systems and application techniques for adhesive. Naturally, the passage of time after restoration also produces an important influence on the resin-dentin interface.

Presumably, if this in vitro demonstration occurred in vivo, it would cause marginal nanoleakage that could progress to microleakage within the adhesive resin monomer uninfiltrated collagen layer. The adhesive resin monomer infiltrate into the expanded decalcified layer would prevent occurrence of nanoleakage or microleakage.[38-39] The wet-bonding technique seems to be more useful for the prevention of the nanoleakage and microleakage.

CONCLUSION

The present findings indicate that moist dentin surfaces are required for the optimum infiltration of adhesive resin into the decalcified layer when the dentin conditioner used is an acidic type, such as phosphoric acid. Adhesive resin monomers should have good fluidity, good permeability and good hydrophilic properties for this to be achieved. In addition, marginal leakage of the composite resin restoration was increase after long-term storage as observed in all test specimens.

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