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Adhesion to chondroitinase ABC treated dentin

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Abstract

Dentin bonding relies on complete resin impregnation throughout the demineralised hydrophilic collagen mesh. Chondroitin sulphate-glycosaminoglycans are claimed to regulate the three-dimensional arrangement of the dentin organic matrix and its hydrophilicity. The aim of this study was to investigate bond strength of two etch-and-rinse adhesives to chondroitinase ABC treated dentin. Human extracted molars were treated with chondroitinase ABC and a double labelling immunohistochemical technique was applied to reveal type I collagen and chondroitin 4/6 sulphate distribution under field emission in-lens scanning electron microscope. The immunohistochemical technique confirmed the effective removal of chondroitin 4/6 sulphate after the enzymatic treatment. Dentin surfaces exposed to chondroitinase ABC and untreated specimens prepared on untreated acid-etched dentin were bonded with Adper Scotchbond Multi-Purpose or Prime & Bond NT. Bonded specimens were submitted to microtensile testing and nanoleakage interfacial analysis under transmission electron microscope. Increased mean values of microtensile bond strength and reduced nanoleakage expression were found for both adhesives after chondroitinase ABC treatment of the dentin surface. Adper Scotchbond Multi-Purpose increased its bond strength about 28%, while bonding made with Prime & Bond NT almost doubled (92% increase) compared to untreated specimens. This study supports the hypothesis that adhesion can be enhanced by removal of chondroitin 4/6 sulphate and dermatan sulphate, probably due to a reduced amount of water content and enlarged interfibrillar spaces. Further studies should validate this hypothesis investigating the stability of chondroitin 4/6 and dermatan sulphate-depleted dentin bonded interface over time.

Keywords

Dental bonding systems; dentin; proteoglycans; microtensile bond strength

INTRODUCTION

Dentin bonding relies on the proper impregnation of the substrate with an adhesive system forming the so-called hybrid layer.¹ The organic portion of the dentin matrix that is infiltrated is a meshwork of type I collagen fibrils. Resin infiltration occurs by diffusion of solvated liquid monomers through spaces between type I collagen fibrils² embedded in a matrix of proteoglycans³, to the dentin demineralization depth. Dentin collagen fibrils are

mineralized to provide a stiff, tough biologic composite structure, similar to other calcified tissues such as bone or cementum. Within the dentin collagen network, proteoglycans are known to link major collagen fibrils⁴.

Dentin proteoglycans are large protein-carbohydrate complexes with a high molecular weight^{8,9} characterized by a polypeptide core attached to one or more glycosaminoglycan (GAG) lateral chains¹⁰ which are claimed to regulate the biophysical properties and three-dimensional structure of proteoglycans.^{11–13} GAGs are usually identified as hyaluronan, heparan sulphate, keratan sulphate, dermatan sulphate and chondroitin sulphate, previously detected in predentin, dentin and cementum.^{6,7,13–17}

Biochemical and immunohistochemical studies on predentin and dentin organic matrices indicated that predominant proteoglycans belongs to the small leucine-rich family (SLRP), characterized by the presence of 7 to 24 leucine-rich tandem repeats in the core protein.^{6,13} Dentin SLRPs are mainly chondroitin 4–6 sulphate-rich, with decorin and biglycan being the most prominent members, whereas the keratan-sulphate rich fibromodulin and lumican showed a more limited distribution.^{6,13,18} In addition, a large aggregating proteoglycan, called versican, has also been described in the form of degradation products in predentin¹³ and more recently in dentin.¹⁹

The role of dentin proteoglycans has been well-investigated. They are known to play functional and structural roles in soft and calcified tissues as key factors of the mineralization process.^{18,20,21} In addition, in mature dentin, they contribute to the final stability and morphology of the demineralized dentin organic matrix.^{5–7}

Due to their polar status GAGs have the ability to bind and organize water molecules and to fill spaces.^{22,23} Among the collagen fibrils, polyanionic GAGs act as sponges soaked with water and this may be one of reason why water cannot be completely eliminated from demineralized dentin matrix, and why areas of the etched dentin occupied by this hydrogel cannot be fully impregnated by hydrophobic monomers.^{19,20} Since collagen is a somewhat hydrophobic polypeptide,²⁶ while demineralized dentin is highly hydrophilic²⁷, non-collagenous hydrophilic molecules, (such as hydrated chondroitin 4/6 sulphate) may be responsible, in part, for the hydrophilicity of demineralized dentin.

Demineralized dentin represents the substrate for etch-and-rinse adhesive systems which are characterized by a preliminary etching step (usually performed with 35–37% phosphoric acid for 15 s) followed by a primer and a bonding agent that are either applied separately (three-step system) or combined (two-step system).¹ Since resin impregnation of demineralised substrates occur via 20–30 nm wide interfibrillar spaces, the determinants of the width of these critical diffusion channels needs further research.^{28–30}

Various factors may affect the three-dimensional arrangement of demineralised collagen fibrils such as the amount of residual water, the degree of cross-linking between collagen molecules and the presence of non-collagenous proteins that also contributes to binding mineral to collagen.³¹ Since a tight relation exists between the three dimensional arrangement of the dentin matrix and bonding mechanisms, especially for etch-and-rinse systems,^{30,32} selective removal of GAGs from the collagen network may affect resin bonding. Even though previous studies investigated the effect of chondroitinase ABC on the dentin organic matrix,^{33,34} little information is available on the adhesion of resins to pure collagen or pure proteoglycans, and very few studies have evaluated their selective removal in relation to resin bonding,^{35,36} and those studies were performed only on bovine dentin. Chondroitinase ABC contains three catalytic domains: a N-terminal β -domain, a α -helix domain and a C-terminal antiparallel β -sheet domain.³⁷ The enzyme, similar to other GAGlyases, cleaves the substrate at the glycosidic linkage between the hexosamine and

uronic acid via a β -elimination mechanism.³⁷ The fundamental steps involved in the enzymatic mechanism are the removal of a proton on the uronic acid, the stabilization of the carbanion intermediate, and the protonation of the anomeric oxygen, breaking the glycosidic bond.³⁸ As dentin contains a large pool of proteoglycans, the enzymatic cleavage does not remove all proteoglycans, but only its constituent GAG chains, while the core protein of the proteoglycans still remains within the matrix. The chondroitin 4–6 sulphate chains of decorin and biglycan are postulated to have a role in the alignment of the collagen fibrils but do not bind the fibrils themselves. The use of chondroitinase ABC will digest both chondroitin 4–6 sulphate and dermatan sulphate, all of which are present in the dentin matrix albeit to different degrees.

Accordingly, the aim of the study was to evaluate the effects on resin adhesion of selective digestion of acid-etched dentin matrix by chondroitinase ABC. The null hypothesis tested was that GAG removal by chondroitinase ABC has no effect on microtensile bond strength and nanoleakage expression of a three-step or a two-step etch-and-rinse adhesive system.

MATERIALS & METHODS

Immunohistochemical analysis

Since proteoglycans can be visualized only under electron microscope following special staining or immunolabelling, in order to verify the effective removal of proteoglycans following chondroitinase ABC treatment, a double immunolabeling technique of additional dentin surfaces was performed in accordance with the method of Breschi *et al.* (2003).³⁹

In brief, caries-free, human third molars were selected for the study after obtaining patient informed consent under a protocol approved by the University of Bologna, Italy. Five mid-coronal dentin disks were created with a low speed diamond saw under water irrigation (Micromet, Remet, Bologna, Italy) and a standardized smear layer was created on the exposed coronal dentin with 180-grit wet silicon carbide paper. Dentin surfaces were sectioned into two equal halves, A and B, and etched with 35% phosphoric acid for 15 s. Half A was additionally treated with chondroitinase ABC (Sigma, Chemical Co., St. Louis, MO, 3U/ml) in Tris phosphate buffer pH 7.2 for 24 h at 37°C, while half B (untreated surfaces) were stored in water. Immunolabeling of the untreated (half B) and chondroitinase-treated (half A) dentin surfaces was performed using two primary monoclonal antibodies, an IgG anti-type I collagen (mouse monoclonal, Sigma Chemical Co.) and an IgM anti-chondroitin 4/6 sulphate (mouse monoclonal, Sigma Chemical Co.). Gold labeling was performed using two secondary antibodies conjugated with gold nano-particles of different sizes: an IgG goat anti-mouse-IgG conjugated with 30 nm gold particles (British BioCell International, Cardiff, United Kingdom) for type I collagen visualization and an IgG goat anti-mouse-IgM conjugated with 15 nm colloidal gold particles for chondroitin 4/6 sulphate identification (British BioCell International).³⁹ Specimens were then fixed, dehydrated in ascending ethanol series, and finally HMDS-dried.⁴⁰

Specimens were mounted on microscope stubs and observed under field emission in-lens scanning electron microscope (FEISEM) JEOL JSM 890 (JEOL, Tokyo, Japan) at 7 kV accelerating voltage and 1×10^{-12} amp probe current. Final images were obtained with both back-scattered and secondary electron signals³⁹ and were performed utilizing the proprietary image-analysis software of the microscope (JSMSCSI, JEOL Italia Spa, Milan, Italy).

Microtensile bond strength analysis

Thirty-two, caries-free, human third molars were selected for the study after obtaining patient informed consent under a protocol approved by the University of Bologna, Italy. The

teeth were stored in a 0.5% chloramine at 4°C and used within one month after extraction. Mid-coronal dentin was exposed with a low speed diamond saw under water irrigation (Micromet, Remet, Bologna, Italy). A standardized smear layer was created on the exposed coronal dentin with 180-grit wet silicon carbide paper.

The teeth were randomly assigned (N=16) to a three-step adhesive (Group 1, Adper Scotchbond Multi-Purpose, 3M ESPE, St. Paul, MN, USA) or (N=16) to a two-step etch-and-rinse adhesive (Group 2, Prime&Bond NT, Dentsply DeTrey, Konstanz, Germany, Table 1). Irrespective of the tested bonding system, all teeth were longitudinally sectioned into two equal halves (randomly named half A and half B) to create two similar bonding substrates in term of tubular density and orientation. All dentin surfaces (A and B halves) were etched with 35% phosphoric acid for 15 s (Etching Agent, 3M ESPE): Half A specimens were additionally immersed in chondroitinase ABC (Sigma, Chemical Co., St. Louis, MO) in Tris phosphate buffer pH 7.2 for 24 h at 37°C (3U/ml), while half B (untreated surfaces) were stored in water under the same conditions. In Group 1, both A and B halves (N=16+16) were bonded with Adper Scotchbond Multi-Purpose, similarly for Group 2 (N=16+16) Prime & Bond NT was used. Both adhesive systems were used in accordance with manufacturers' instructions (Table 1). A 4-mm thick layer of microhybrid resin composite (Filtek Z250, 3M ESPE) was incrementally placed over the bonded dentin surface and polymerized for 20 s using a dental light-curing unit that delivered 600 mW/cm² of blue light.

The pulp chamber was bonded with Prime & Bond NT in accordance with the manufacturer's instructions and filled with Filtek Z250 to provide sufficient bulk for microtensile testing. Sticks with surface areas of approximately 0.9×0.9 mm were created from each half-specimen using the low speed saw under water irrigation. The dimension of each stick was individually measured with a digital caliper to the nearest 0.01 mm and the bonding area was calculated for subsequent bond strength evaluation. The specimens were observed under a stereomicroscope (Stemi 2000-C, Carl Zeiss Jena GmbH, Germany) to avoid the inclusion of sticks containing residual enamel. The sticks were stored in deionized water for 24 h, attached to a modified jig for microtensile testing (Bisco, Schaumburg, IL, USA) and stressed to failure under tension at a crosshead speed of 1 mm/min. Failure modes were evaluated using the stereomicroscope at 50× magnification and classified as cohesive, adhesive, or mixed failure. The number of prematurely debonded sticks per group during specimen preparation was also recorded but not included in the statistical evaluation (Table 2). Means ± standard deviations of micro-tensile bond strength were statistically evaluated with a two-way ANOVA (bonding agent vs. enzyme treatment) at $\alpha = 0.05$ and Scheffè post-hoc test.

Nanoleakage analysis

One slab from the central area of each half-specimen was not stressed and the bonded interface was submitted to nanoleakage analysis. The slabs were covered with nail varnish, leaving only 1 mm free at the bonded interfaces and immediately immersed in a 50 wt% ammoniacal AgNO₃ solution (pH=9.5).⁴¹ After 24 hr, the specimens were rinsed with deionized water for 5 min and placed in a photodeveloping solution for 8 hr under fluorescent light to reduce the diamine silver ions [Ag(NH₃)₂⁺] into metallic silver grains. Slabs were then prepared for TEM examination, 90–100 thick undemineralized section containing the resin-dentin adhesive interfaces, were collected on 100-mesh, carbon- and formvar-coated copper grids and examined without further staining using a transmission electron microscope (TEM, Philips CM-10, Eindhoven, The Netherlands) operating at 70 kV.⁴²

RESULTS

Untreated specimens showed that after the 24 h of chondroitinase ABC treatment (A halves), only 30 nm gold particles identifying collagen type I fibrils were detectable, while no 15 nm labeling (i.e. selective for chondroitin 4/6 sulphate) was evident under high resolution FEI-SEM (Fig. 1a;1c). Conversely, positive labeling both for chondroitin 4/6 sulphate and collagen type I was found on B halves (untreated surfaces; Fig. 1b;1d).

Means of the micro-tensile bond strength of specimens prepared with Adper Scotchbond Multi-Purpose were 41.2 ± 5.4 MPa for dentin surfaces submitted to the preliminary enzymatic treatment and 32.2 ± 4.9 MPa for untreated specimens. Mean values for Prime & Bond NT treated with or without chondroitinase ABC were 57.7 ± 10.4 MPa and 27.2 ± 10.4 MPa, respectively. The statistical analysis showed that microtensile bond strength was significantly influenced by the type of adhesive ($p < 0.001$; $F = 105.852$), by the chondroitinase ABC treatment of the dentin surface ($p < 0.001$; $F = 822.663$), and their interaction ($p < 0.001$; $F = 235.730$). No statistically significant difference was observed in fracture modes between the groups (Table 2).

The TEM analysis (Fig. 2) of the nanoleakage expression showed minimal nanoleakage expression characterized by small scattered silver nitrate particles for Adper Scotchbond Multi-Purpose, irrespective from the enzymatic treatment of the dentin surface (Fig. 2a,2b), i.e. no influence of chondroitinase ABC treatment was found for the three-step etch-and-rinse adhesive. Conversely, nanoleakage expression of Prime & Bond NT applied on chondroitin 4/6 sulphate and dermatan sulphate-depleted dentin was significantly reduced (Fig. 2c) compared to control surfaces (Fig. 2d) in which nanoleakage formation was clearly visible in some areas along the adhesive interface.

DISCUSSION

Previous high magnification immunolabelling studies on dentin GAGs by means of primary monoclonal anti-chondroitin 4/6 sulphate antibodies followed by a secondary antibody conjugated with gold nano-particles.^{3,39} Those studies revealed that dentin GAGs interact with collagen fibrils at specific binding sites, supporting the hypothesis that these molecules play an important role in regulating the spacing between the dentin collagen fibrils. Since different labelling patterns of GAGs were identified on the etched dentin surface in relation to different etching agents, a direct relation between the acid solution aggressiveness (i.e. pKa, pH and concentration) and the three-dimensional morphology of the collagen fibrils and associated chondroitin 4/6 sulphate was hypothesized.³⁹

In the current study, observations under high resolution FEI-SEM were conducted on both A and B halves (i.e. experimental and untreated), to verify the effective removal of chondroitin 4/6 sulphate from the dentin surface after the enzymatic treatment. Since no labelling for chondroitin 4/6 sulphate was found on acid etched, chondroitinase ABC treated dentin (A halves; Fig. 2a,2c), while positive labelling was found in untreated B halves (Fig. 2b,2d), we concluded that the enzyme treatment of human dentin for 24 h at 37°C effectively removes chondroitin 4/6 sulphate from human dentin matrix.

The results of the present study revealed that microtensile bond strength is increased by exposing acid-etched dentin surfaces to chondroitinase ABC enzymatic treatment prior to bonding with either Adper Scotchbond Multi-Purpose or Prime & Bond NT. In addition, while nanoleakage expression of Adper Scotchbond Multi-Purpose was minimal, irrespective of the chondroitinase ABC treatment, Prime & Bond NT showed reduced nanoleakage when applied to chondroitin 4/6 sulphate and dermatan sulphate-depleted acid-

etched dentin surfaces. These data suggest that chondroitinase ABC may either influence dentin matrix or modify the hydration status of the etched dentin surface, or both.

Observations of dentin surfaces exposed to the enzymatic pre-treatment under high resolution-SEM revealed wider empty spaces between the collagen fibrils (Fig. 1a;1c) than untreated specimens (Fig. 1b;1d). This finding was only observational (FEI-SEM images). Thus different interpretations of the chondroitinase ABC effects can be made. In mature dentin, histochemical (using cationic dyes and cationic detergents)^{14,43–45} and immunohistochemical investigations^{3,16,39,46,47} evidence has shown a close association of chondroitin 4/6 sulfate with the so-called hole regions in collagen fibrils. In addition, as shown by Bonucci⁴⁸, in bone and cartilage, proteoglycans are actually components of the crystal ghosts together with some phospholipids, and proteins. In mature dentin, proteoglycans probably protect collagen fibrils from many chemical attacks, including enzymes such as endogenous matrix metalloproteinases^{49,50} and acidic solutions or gels. It is likely that there is a cascade of degradation, acids allowing the total or partial removal of the mineral phase together with some extracellular matrix components. Chondroitinase ABC degradation occurs subsequently, and acidic remnants or intrinsic matrix metalloproteinases^{49,50} degrade the remaining non-collagenous proteins, providing an access to interfibrillar spaces and infiltration of low-viscosity resin, leading to the formation of a better hybrid layer. It can be hypothesised that the enlarged spaces visible under high resolution FEI-SEM are due to the removal of what appears to be an amorphous coating. Together with the acidic attack and the removal of the mineral phase, empty intercollagen spaces are revealed and available for infiltration. This would confirm previous investigations that hypothesized that the empty interfibrillar spaces influence dentin impregnation, since it is well known that they serve as diffusion channels for monomer infiltration leading to formation of a compact and homogenous hybrid layer irrespective from the bonding strategy (i.e. etch-and-rinse or a self-etch approach).^{1,24}

Even if the modified dentin matrix may be responsible for increases in microtensile bond strength, it does not explain the differential effects on the tested adhesives. In fact, while Adper Scotchbond Multi-Purpose only increased its bond strength about 28%, Prime & Bond NT almost doubled its mean value (92% increase). Since the tested adhesives reacted differently to the enzymatic pre-treatment, further work should be done to clarify the mechanisms of these different findings. As we tested a three-step (Scotchbond Multi-Purpose) versus a two-step etch-and-rinse (Prime & Bond NT) adhesive system, the differences may be related to the priming (Scotchbond Multi-Purpose) step. Since Adper Scotchbond Multi-Purpose is characterized by use of a separate primer which contains water, its application may replace the water that is normally bound to the chondroitin 4/6 sulphate and dermatan sulphate in untreated dentin, in the chondroitinase ABC treated dentin. Prime & Bond NT does not use a primer, and its bonding agent is dissolved in acetone. Its application to moist dentin may remove residual water from the dentin matrix surface in both untreated and experimental tooth halves. Such water removal by acetone-solvated systems may be further enhanced by chondroitinase ABC pretreatment that depletes the matrix of GAGs.

Moreover, as Adper Scotchbond Multi-Purpose is a water/ethanol based adhesive, while Prime & Bond NT is an acetone based adhesive, they may differently impregnate the chondroitin 4/6 sulphate and dermatan sulphate-depleted dentin. In fact, the water-chasing ability of acetone and the effect that acetone has on the water/acetone vapour pressure,⁵¹ may explain why the acetone-based Prime & Bond NT performed better on chondroitin 4/6 sulphate and dermatan sulphate-depleted dentin. We speculate that when water/ethanol mixtures are used as a solvent, the comonomers may be less able to displace water from the GAG hydrogel that may occupy the interfibrillar spaces. Residual water may not evaporate

as easily and completely, allowing more water molecules to remain bound to GAGs within the collagen network. Acetone-based adhesive systems may be more effective in dehydrating interfibrillar spaces, especially after removal of the space-occupying GAG hydrogel. This may explain Prime & Bond NT 92% increase in microtensile bond strength values. Scotchbond Multi-Purpose utilizes a primer that contains 35% HEMA and 65% water. It is likely that there is far more residual water⁵² in such resin-dentin bonds than remain in Prime & Bond NT dentin bonds. The interfibrillar GAG hydrogel may prevent full infiltration of Scotchbond MP comonomers compared to Prime & Bond NT.⁵²

Recent studies also indicated that reducing the amount of water within the HL may stabilize the bonded interface over time by reducing water sorption^{53–59} that plasticize copolymers constituting the HL. Similarly, a reduced water content of the adhesive interface may contribute to inhibit the activity of endogenous collagenolytic enzymes, which are hydrolases, that have been recently claimed to degrade the collagen fibrils partially encapsulated by adhesive within the bonded interface.^{60,61} Further investigations are currently ongoing to provide additional evidence of the influence of GAGs on dentin bonding and to validate the hypothesis that the removal of chondroitin 4/6 sulphate and dermatan sulphate from the dentin surface can enhance resin-dentin bond strength.

Although the enzymatic removal of GAGs required a long incubation period that is not clinically relevant, it did demonstrate an important principle. That is, that the interfibrillar space created in dentin matrices by acid extraction of the mineral phase of dentin may not leave empty spaces. Rather, these spaces may be filled by a GAG hydrogel that may serve as a molecular sieve that can restrict the inward diffusion of relatively large comonomers such as BisGMA and UDMA. Acetone-based adhesive may dehydrate the GAG hydrogel so much that it is no longer a diffusion barrier. This may also explain the higher bond strengths reported when five experimental adhesives were bonded to ethanol-saturated dentin compared to water-saturated dentin.⁶² Those authors pretreated water-saturated acid-etched dentin with excess 100% ethanol. This treatment would likely cause severe dehydration of any GAG hydrogel in interfibrillar spaces, thereby enhancing infiltration of all monomers, including BisGMA.

In conclusion, our results showed that the morphological alterations induced by Chondroitinase ABC digestion of dentin GAGs led to higher microtensile bond strength values and reduced nanoleakage due to the enzymatic-modified collagen mesh which probably led to better infiltration by etch-and-rinse adhesives. Further studies should clarify if the enzymatic treatment can be performed in a clinically relevant time, and without any potential adverse effects on odontoblasts when bonding to deep dentin.

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Figure 1.

FEI-SEM images of dentin resulting from mixing a back scattered image, that reveals the gold nanoparticles conjugated with secondary antibodies as 15 nm diameter white spots for chondroitin 4/6 sulphate and 30 nm for type I collagen fibrils, with a secondary electron image, that shows the dentin morphology. Dentin specimens exposed to the enzymatic treatment are represented in Fig. 1a and 1c, while untreated dentin surfaces are shown on Fig. 1b and 1d. Fig. 1a: after 24 h chondroitinase ABC pretreatment, 30 nm gold particles identifying type I collagen fibrils are clearly visible while no 15 nm gold labeling selective for chondroitin 4/6n sulphate can be detected. Fig. 1b: Dentin surface etched with phosphoric acid and exposed to the double immunolabelling technique. Chondroitin 4/6 sulphate appears as globular structures clearly labeled by 15 nm colloidal gold particles, localized along branching minor fibrils (arrows). Collagen fibrils are positively labeled by 30 nm gold particles. Fig. 1c. High magnification view of chondroitinase ABC treated dentin showing enlarged matrix mesh (asterisks), negative labeling for chondroitin 4/6 sulphate but positive for type I collagen fibrils. Fig. 1d: high magnification FEI-SEM micrograph revealing the complex network of major collagen fibrils and globular chondroitin 4/6 sulphate structures (arrows) in untreated dentin specimens.

Figure 2.

TEM micrographs of un-demineralized and unstained specimens showing the interfacial nanoleakage expression of Adper Scotchbond Multi-Purpose (Fig. 2a,2b) and Prime&Bond NT (Fig. 2c,2d). Fig. 2a: Low magnification image of Adper Scotchbond Multi-Purpose applied on chondroitinase 4/6 sulphate-depleted dentin due to Chondroitinase ABC treatment showing minimal nanoleakage expression characterized by small scattered silver nitrate particles; dentin tubule (T); hybrid layer (HL). Fig. 2b: Adper Scotchbond MP applied on untreated control dentin: nanoleakage expression of the bonded interface is similar to chondroitinase ABC pretreated sample with small silver aggregates (arrows), i.e. no influence on nanoleakage expression was found due to the enzymatic treatment for the three-step etch-and-rinse adhesive; Dentin tubule (T); hybrid layer (HL). Fig. 2c: The use of chondroitinase ABC pretreatment before the application of Prime & Bond NT resulted in a strong reduction of silver nitrate expression within the hybrid layer created on intertubular dentin; Dentin tubule (T); hybrid layer (HL). Fig. 2d: application of Prime & Bond NT on untreated dentin showed spotted nanoleakage expression close to the tubular lumen (T) exposed by acid etching. Silver grains (arrows) are also located at the top of the hybrid layer (HL) forming an uniformly distributed electron-dense layer. Dentin tubule (T); hybrid layer (HL). All images were taken at the same magnification (Bar = 1µm).

Composition and modes of application of the dental adhesive systems tested in the study.

TABLE 1

Adhesive	Composition	Mode of application
Adper Scotchbond Multi Purpose	<p><i>Etching:</i></p> <ul style="list-style-type: none"> • 37% phosphoric acid <p><i>Primer</i></p> <ul style="list-style-type: none"> • HEMA • polyalkenoic acid copolymer • water <p><i>Bonding:</i></p> <ul style="list-style-type: none"> • Bis-GMA • HEMA • dimethacrylates • initiators 	<p>1 Acid etching (15 s), rinsing (15 s) and air-drying (10 s) leaving dentin moist</p> <p>2 Application of 2 coats of the primer (10 s with slight agitation)</p> <p>3 Air-dry (10 s at 20 cm)</p> <p>4 Application of 1 coat of the adhesive (10 s with slight agitation)</p> <p>5 Air-dry (10 s at 20 cm)</p> <p>6 Light-activation (10 s–600 mW/cm²)</p>
Prime & Bond NT	<p><i>Etching:</i></p> <ul style="list-style-type: none"> • 35% phosphoric acid <p><i>Primer/Bonding:</i></p> <ul style="list-style-type: none"> • PENTA, • UDMA • T-resin (cross-linking agent) • D-resin (small hydrophilic molecule), • butylated hydroxytoluene, • 4-ethyl dimethyl aminobenzoate, • cetilamine hydrofluoride, • acetone, -silica nanofiller 	<p>1 Acid etching (15 s), rinsing (15 s) and air-drying (10 s) leaving dentin moist</p> <p>2 Application to the dentin surface in copious amounts, leaving the surface wet and undisturbed for 20 s</p> <p>3 Air-dry (10 s at 20 cm)</p> <p>4 Light-activation (10 s–600 mW/cm²)</p>

Microtensile bond strength data obtained applying Adper Scotchbond MP or Prime & Bond NT after enzymatic treatment with chondroitinase ABC versus untreated.

TABLE 2

Adhesive	Chondroitinase ABC-treated etched dentin				Untreated etched dentin			
	A	CC	CD	M	A	CC	CD	M
Adper Scotchbond MP	41.2 ^a ±5.4 MPa [92] (4)				32.2 ^b ±4.9 MPa [87] (7)			
	48.9% N=45	10.9% N=10	0% N=0	40.2% N=37	47.1% N=41	5.8% N=5	2.3% N=2	44.8% N=39
Prime & Bond NT	57.4 ^c ±7.9 MPa [101] (2)				29.9 ^b ±6.6 MPa [84] (8)			
	62.4% N=63	8.9% N=9	0% N=0	28.7% N=29	57.1% N=48	2.4% N=2	0% N=0	40.5% N=34

[†] Values are mean±standard deviation bond strengths in MPa [number of intact sticks tested] (number of prematurely debonded specimens). Groups with the same superscripts are not statistically significant (p>0.05). Premature failures due to preparation procedures were not included in the statistical analysis. Percentages of the fracture modes after microtensile test were analyzed under stereomicroscope. Fracture were classified as: A: adhesive; CC: cohesive in composite; CD: cohesive in dentin, M: mixed.