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ABSTRACT

In ethanol wet bonding, water is replaced by ethanol to maintain dehydrated collagen matrices in an extended state to facilitate resin infiltration. Since short ethanol dehydration protocols may be ineffective, this study tested the null hypothesis that there are no differences in ethanol dehydration protocols for maintaining the surface roughness, fibril diameter, and interfibrillar spaces of acid-etched dentin. Polished human dentin surfaces were etched with phosphoric acid and water-rinsed. Tested protocols were: (1) water-rinse (control); (2) 100% ethanol-rinse (1-min); (3) 100% ethanol-rinse (5-min); and (4) progressive ethanol replacement (50-100%). Surface roughness, fibril diameter, and interfibrillar spaces were determined with atomic force microscopy and analyzed by one-way analysis of variance and the Student-Newman-Keuls test ($\alpha = 0.05$). Dentin roughness and fibril diameter significantly decreased when 100% ethanol (1-5 min) was used for rinsing ($p < 0.001$). Absolute ethanol produced collapse and shrinkage of collagen fibrils. Ascending ethanol concentrations did not collapse the matrix and shrank the fibrils less than absolute ethanol-rinses.

KEY WORDS: atomic force microscopy, acid-etched dentin, ethanol-saturated, surface topography, water replacement.

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Ethanol Wet-bonding Technique Sensitivity Assessed by AFM

INTRODUCTION

The presence of water in acid-etched dentin is critical to prevent collapse of the mineral-depleted collagen matrix (Toledano *et al.*, 1999; Osorio *et al.*, 2009). Water molecules form hydration clusters around the functional groups of collagen peptides (Pashley *et al.*, 2002) and proteoglycans (Breschi *et al.*, 2002). This results in full expansion of the interfibrillar spaces available for infiltration of resin monomers.

Resin infiltration of acid-etched dentin is incomplete and leaves collagen fibrils exposed. Nanoleakage studies have identified water-filled channels within hybrid layers, indicating that not all residual water is removed (Tay and Pashley, 2003). Recent studies that demonstrated intrafibrillar remineralization of collagen fibrils within hybrid layers further imply that contemporary dentin adhesives cannot replace free and loosely bound water from the intrafibrillar spaces, even when resin monomers are able to encapsulate the collagen fibrils (Tay and Pashley, 2009; Kim *et al.*, 2010a). Water sorption also plasticizes hydrophilic adhesives and lowers their mechanical properties (Ito *et al.*, 2005).

The bonding philosophy “ethanol wet bonding” (Pashley *et al.*, 2007) embraces the important concept of water replacement from interfibrillar and intrafibrillar spaces (Tay *et al.*, 2007; Kim *et al.*, 2010b). Although it represents a concept rather than a technique, this bonding philosophy has improved our understanding of the deficiencies associated with contemporary etch-and-rinse adhesives. In ethanol wet bonding, water is replaced by ethanol, which suspends the demineralized collagen matrix in its dehydrated but fully extended state. The ethanol-suspended collagen matrix is rendered less hydrophilic and prevents phase separation of ethanol-soluble hydrophobic resin monomers (Wang and Spencer, 2003). In one aspect of the bonding philosophy, water within a 5- μ m-thick layer of demineralized collagen matrix was slowly replaced with ascending concentrations of ethanol over a period of 5 to 10 min (Sadek *et al.*, 2008). In other aspects, simplified ethanol dehydration protocols were used (Hosaka *et al.*, 2009; Sauro *et al.*, 2009), based on the rationale that the prolonged dehydration with ascending ethanol concentrations is clinically unrealistic. Bond strengths of hydrophilic and hydrophobic resins increased significantly when etched dentin was saturated with ethanol instead of water (Nishitani *et al.*, 2006; Hosaka *et al.*, 2009). However, the results with direct application of absolute ethanol to water-saturated dentin were more variable (Sadek *et al.*, 2008; Sauro *et al.*, 2010).

It is speculated that rinsing with absolute ethanol only is very technique-sensitive. Collapse of the collagen matrix caused by water evaporation during the transition from the water to the ethanol phase would have resulted in stiffening and stabilization of the matrix in its collapsed state. Conversely, progressive water replacement would have provided an opportunity for

re-expansion of the collagen fibrils when the latter is initially suspended with water-containing ethanol. Since there are no studies that examine the influence of ethanol dehydration protocols on acid-etched dentin topography, the aforementioned hypothesis requires validation. Thus, the objective of this atomic force microscopy study was to examine the effects of three ethanol dehydration protocols on etched dentin surface topography. The null hypothesis tested was that there are no differences in the different ethanol dehydration protocols in maintaining the surface roughness, fibril diameter, and interfibrillar spacings of acid-etched dentin created by phosphoric acid-etching.

MATERIALS & METHODS

Extracted human third molars were obtained following a protocol that was reviewed and approved by the institutional review board and with the informed consent of the donors. The molars were stored in 0.5% chloramine T at 4°C and used within 1 mo after extraction. Each tooth was sectioned perpendicular to its longitudinal axis and at 1 mm below the dentino-enamel junction to create a flat bonding surface in mid-coronal dentin. Twelve 2-mm-thick dentin disks were prepared. Each disk was polished with 0.25 μm diamond spray (Struers A/S, Ballerup, Denmark) to provide a flat, highly polished surface for atomic force microscopy (AFM). Each dentin surface was acid-etched with 32% phosphoric acid gel (Bisco Inc., Schaumburg, IL, USA) for 15 sec and rinsed with water for 30 sec. Four experimental groups were formed:

(1) Water-saturated etched dentin (control): acid-etched dentin specimens were immersed in water until they were examined by AFM under water coverage.

(2) 100% ethanol for 1 min: After the etchant was rinsed with water, each specimen was rinsed with absolute ethanol for 1 min and then examined immediately with AFM while immersed in absolute ethanol.

(3) 100% ethanol for 5 min: After being rinsed with water, each specimen was rinsed with absolute ethanol for 5 min and examined immediately with AFM while immersed in absolute ethanol.

(4) Progressive water replacement: After specimens were rinsed with water, water within the demineralized collagen matrix was progressively replaced with increasing ethanol concentrations (50%, 70%, 80%, 95%, and 100% 3 times for 30 sec each). The specimen was examined immediately with AFM while immersed in absolute ethanol.

Figure 1. (continued)

Three dentin surfaces (4 x 4 mm) *per* group were evaluated by AFM (Multimode Nanoscope V, Veeco Metrology Group, Santa Barbara, CA, USA) in the tapping mode, with a calibrated vertical-engaged "E" piezo-scanner (Digital Instrument, Santa Barbara, CA, USA). Digital images were taken in a liquid environment (water-covered or ethanol-covered). A 10-nm-radius silicon nitride tip (Veeco) was attached to the end of an oscillating cantilever that came into intermittent contact with the surface at the lowest point of the oscillation. Changes in vertical position of the AFM tip at resonance frequencies near 330 kHz provided the height of the images registered as bright and dark regions. Three 15 x 15 μm and three 500 x 500 nm digital images were recorded from each surface, with a slow scan rate (0.1 Hz). The 15 x 15 μm images were analyzed quantitatively. For each image, 5 randomized boxes (3 x 3 μm) were created for examination of the surface roughness of the demineralized intertubular dentin (N = 45). Nanoroughness (Ra, in nanometers) was measured with proprietary software (Nanoscope Software, version V7).

Collagen fibril diameter and interfibrillar space dimensions (in nanometers) were determined from the 500 x 500 nm images by section analysis with data that had been modified only by plane-fitting. The collagen fibril diameter was preferentially determined from fibrils that were exposed along their complete widths. Five fibrils and their interfibrillar spacings were analyzed from each image. Measurements were corrected for

Figure 1. AFM images of (a) water-saturated acid-etched dentin surface, and (b) after 100% ethanol application for 1 min. Left, 15 x 15 μm top-view and surface plot images of intertubular dentin topography. Right, AFM phase image of the dentin collagen fibrils of the acid-etched dentin surfaces and surface plot of the intertubular dentin (500 x 500 nm).

tip-broadening (Habelitz *et al.*, 2002) by the equation $e = 2r$, where e is the error in the horizontal dimension and r is the tip's radius (Takeyasu *et al.*, 1996).

Since the normality and homoscedasticity assumptions of the data were valid, the data were analyzed with one-way analyses of variance and Student-Newman-Keuls multiple-comparison tests, with statistical significance pre-set at $\alpha = 0.05$.

RESULTS

Four AFM images are shown for each group (Figs. 1, 2): top-view and 3-D images of the intertubular dentin taken at 15 x 15 μm and 500 x 500 nm resolutions. Surface roughness of the acid-etched dentin was affected by the ethanol-water replacement protocols ($F = 24.07$; $p = 0.0000$) (Table). Intertubular dentin roughness (R_a) significantly decreased after the use of 100% ethanol for 1 min (69.38 ± 9.06 nm) and 5 min (59.38 ± 10.94 nm) when compared with water-saturated etched dentin (98.66 ± 5.97 nm). The difference between the water-saturated etched dentin and the progressive water replacement group (108.46 ± 14.65 nm) was not significant.

Differences in the surface topography of etched dentin specimens among the 4 groups are illustrated with the 3-D AFM

images (15 x 15 μm). Water-saturated etched dentin and the use of a progressive water replacement protocol produced similar, more undulating surface topography (3-D, Figs. 1a, 2b). Conversely, the use of 100% ethanol for 1 min (3-D, Fig. 1b) and 5 min (3-D, Fig. 2a) produced relatively smooth, collapsed surfaces with marked decrease in nanoroughness. These topographical differences are also shown at higher resolution with images captured with a 500-nm scan size.

Fibril diameters were influenced by the ethanol-water replacement protocols ($F = 58.70$; $p = 0.0000$; Table). Significant differences exist for the fibril diameter in all groups, in decreasing order: water-saturated etched dentin > progressively increasing ethanol concentrations > ethanol-rinse for 1 min > ethanol-rinse for 5 min. Interfibrillar spacings were influenced by the ethanol-water replacement protocols ($F = 49.83$; $p = 0.0000$; Table). Some differences exist between groups, in decreasing order: water-saturated etched dentin = progressively increasing ethanol concentrations > ethanol-rinse for 1 min = ethanol-rinse for 5 min. From the 500 x 500 nm top-view images, it is possible to

observe distinct collagen fibrils in water-saturated etched dentin (top-view Fig. 1a) with the widest fibril diameter. Narrower fibril diameters were encountered when 100% ethanol was used for 1 min or 5 min (Figs. 1b, 2a).

DISCUSSION

Increases in surface roughness of the etched-dentin surfaces improve dentin wettability for optimizing resin-dentin bond strength (Osorio *et al.*, 2009). However, the mechanisms involved in this observation are difficult to predict in a biological tissue. Adhesion is mainly guided by thermodynamic properties (*i.e.*, roughness and wettability) of the etched dentin surface, which depends on the concavity/convexity of the attained topographical features (Pegueroles *et al.*, 2010).

Regarding the relation between surface roughness and porosity, it appears that the heights/depths of the peaks/valleys at the extreme of a surface profile affect the so-called "barrier layer continuity", even if they occupy a small fraction of the surface area. Surface morphology can affect the ability of this barrier to spread conformally on the surface area. Increasing pore size produces greater surface roughness, which by itself can have a dramatic influence on the barrier continuity (Sun *et al.*, 2003).

There is a concern about the diffusion of liquid into the porous structures, especially with surfaces possessing big open pores. The barrier layer continuity appears to be thinner locally and becomes a “leaky” surface for the liquid to escape. Surface roughness and average pore size indeed affect the barrier layer thickness of the surface with interconnected porosity, because the surface roughness is also inherently correlated with pore size (Sun *et al.*, 2003). Roughness could improve spreading by the capillary force mechanism. Newitt and Conway-Jones (1958) explained the prevailing influence of the liquid-to-solid ratio in terms of the stages of liquid distribution that may occur inside a granule. These stages may vary from liquid bridges at points of contact to the capillary state, when all pores are completely filled with liquid.

After apatite dissolution by acids in etched dentin, water fills the intrafibrillar and interfibrillar spaces. Ideally, resin monomers should replace water in these spaces (Vaidyanathan and Vaidyanathan, 2009). Nevertheless, incomplete replacement of water usually occurs during adhesive application and leaves an exposed collagen matrix that is partially encapsulated by polymerized adhesive resins. In ethanol wet bonding (Pashley *et al.*, 2007), a polar solvent with less hydrogen bonding capacity than water (Becker *et al.*, 2006) is used for chemical dehydration of the demineralized collagen network (Nishitani *et al.*, 2006). It produces shrinkage of the collagen fibrils, enlarging the interfibrillar spaces for hydrophobic monomers to infiltrate the matrix more optimally (Tay *et al.*, 2007). This could extend the longevity of resin-dentin bonds (Hosaka *et al.*, 2009).

Since there were topographical changes in etched dentin surfaces when the different ethanol-water replacement protocols were applied, the null hypothesis—that there are no differences in the different ethanol dehydration protocols in maintaining the surface roughness, fibril diameter, and interfibrillar spacings of acid-etched dentin created by phosphoric-acid-etching—has to be rejected. In the AFM images (15 × 15 μm), the use of 100% ethanol for 1 min and for 5 min produced smoother topographies than water-saturated etched-dentin and when increasing ethanol concentrations were applied. For the former two protocols, evaporation of water from the water-saturated collagen matrix prior to the rinsing with absolute ethanol could have resulted in the collapse of the matrix. Since no adhesive resins were used after ethanol rinsing in the present study, rapid evaporation of absolute alcohol during transfer of the ethanol-saturated specimen to the AFM stage (*ca.* 10 min) could also have accounted for the collapse of

Figure 2. (continued)

the collagen matrix. Both of these technical flaws in the application of ethanol wet bonding could be responsible for the extreme technique sensitivity inherent in this bonding philosophy. However, since collapse of the collagen matrix was not seen in the progressive ethanol-water replacement group, it is more likely that initial water evaporation from the etched dentin prior to replacement by absolute ethanol is accountable for the observed results. This technique-sensitivity issue was not seen in the progressive ethanol-water replacement group, since the collapsed collagen matrix could have been rehydrated and re-expanded, to a certain extent, with the 50% water present in the 50% ethanol. Thus, the progressive ethanol-water replacement protocol may be considered a less operator-demanding technique, although it is considerably more time-consuming. Shrinkage of collagen fibrils may be proportional to the alcohol concentration in the final mixture (Becker *et al.*, 2006), with more complete chemical dehydration achieved with higher volume fractions of water-free solvent in the replacement medium. This probably explains why collagen fibril diameters in the ‘100% ethanol for 1 min’ group were between those in the ‘water-saturated etched dentin’ group and the ‘100% ethanol for 5 min’ group, and those in the ‘100% ethanol for 5 min’ group were narrower than those in the ‘progressive ethanol-water replacement’ group. Incomplete extraction of water from the dentinal tubules by the absolute ethanol and the presence

Figure 2. AFM images of acid-etched dentin (a) after 100% ethanol for 5 min, and (b) after being progressively rinsed with increasing concentrations of ethanol solutions. Left, 15 x 15 μm top-view and surface plot images of intertubular dentin topography. Right, AFM phase image of the dentin collagen fibrils of the acid-etched dentin surfaces and surface plot of the intertubular dentin (500 x 500 nm).

of residual water in the demineralized intertubular dentin could have accounted for the observation of at least some collagen fibrils with widths similar to those of the water-saturated etched dentin group. A similar feature could be observed when ethanol wet-bonded hybrid layers were subjected to biomimetic remineralization (Kim *et al.*, 2010b); incomplete removal of free or loosely bound water from the intrafibrillar compartments of the collagen fibrils resulted in their eventual intrafibrillar remineralization in the presence of biomimetic analogs. Conversely, when intrafibrillar and interfibrillar water was meticulously removed, interfibrillar and interfibrillar collagen remineralization did not occur.

Water plays an important role in protein structure, interaction, and function. Strong “hydration” forces are present among many macromolecules resulting from the energetic cost of re-organizing the hydrogen-bonding network of water near macromolecular surfaces (Kuznetsova *et al.*, 1997). Collagen molecules are surrounded by a highly ordered inner layer of tightly bound structural water and water bridges created by hydrogen bonds (Ramachandran and Chandrasekharan, 1968). This water compartment may be responsible for maintaining finite spacings among the peptide

molecules (Bella *et al.*, 1994) and prevents formation of interpeptide hydrogen bonds among the collagen fibrils (Becker *et al.*, 2006). Thus, the collagen network remained fully expanded in water-saturated acid-etched dentin (Fig. 1a). Intact collagen fibrils could also be observed in the water-saturated acid-etched dentin surface, by AFM (Yang *et al.*, 2005).

When absolute ethanol was applied in the ethanol wet-bonding philosophy, it removed free and loosely bound water, but probably preserved the innermost layer of structural water from collagen fibrils which otherwise could have disrupted the triple helical structure. Intermolecular hydrogen bonds cause shrinkage of the fibrils by reduction in the lateral spacing of the collagen molecules (Miles and Ghelashvili, 1999). This could enlarge the interfibrillar spaces in the case of progressive water replacement with increasing ethanol solutions. The interfibrillar space sizes were similar to those of water-saturated etched dentin. This also explains why 100% ethanol was unable to rehydrate the collapsed collagen matrix caused by water evaporation.

Within the limits of the present study, it may be concluded that the ethanol wet-bonding philosophy is highly technique-sensitive in its execution. Reducing application time by the use of a simplified absolute ethanol

replacement protocol may result in the collapse of the demineralized collagen matrix, either by evaporation of water or absolute ethanol, when the procedure of ethanol-water replacement is not meticulously followed. Nevertheless, the principles of removal of free and loosely bound water from the intrafibrillar compartments of the collagen matrix behind this philosophical bonding regime have brought to fruition a resin-dentin interfacial remineralization scheme (Tay and Pashley, 2009; Kim *et al.*, 2010a) that is based on a similar principle of progressive water replacement (Magne *et al.*, 2001) by intrafibrillar remineralization of collagen fibrils with apatite nanocrystals generated *via* the use of biomimetic analogs.

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Table. Means and Standard Deviations of Dentin Surface Roughness (nm), Interfibrillar Space Sizes (nm), and Collagen Fibril Diameter (nm) and after Different Ethanol-Water Replacement Protocols

Ethanol-Water Replacement Protocols	Surface Roughness, Mean (SD)	Interfibrillar Space Dimension, Mean (SD)	Fibril Diameter, Mean (SD)
Distilled water immersion (control)	98.66 (5.97) a	12.15 (1.28) B	108.66 (15.78) ^a
100% ethanol immersion for 1 min	69.38 (9.06) b	8.78 (1.27) A	72.75 (21.18) ^c
100% ethanol immersion for 5 min	59.38 (10.94) b	7.63 (1.40) A	53.68 (10.68) ^d
Increasing ethanol concentration immersion	108.46 (14.65) a	19.79 (3.37) B	91.53 (12.03) ^b

For each column, different letters represent significant differences among groups ($p < 0.05$).

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