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<th>Aging affects two modes of nanoleakage expression in bonded dentin</th>
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ABSTRACT

Water sorption into resin-dentin interfaces precedes hydrolytic degradation. We hypothesized that these processes are morphologically manifested by the uptake of ammoniacal silver nitrate, which is thought to trace hydrophilic domains and water-filled channels within matrices. Water sorption is thought to be nonuniform and can be traced by the use of silver nitrate. Human teeth bonded with an experimental filled-adhesive were aged in artificial saliva (control) and in ammoniacal silver nitrate, which is thought to trace ammoniacal silver nitrate. The term “restricted” was used since it is impossible to eliminate water artifacts that are prone to artifactual silver uptake (Agee et al., 2002). However, changes in the extent of nanoleakage that occurred within hybrid layers (Sano et al., 1995; Pioch et al., 2001) did not correlate well with the time-dependent deterioration of these bonds (Okuda et al., 2001).

The objective of this study was to examine, with the use of transmission electron microscopy (TEM), the changes in silver uptake that occur with aging of resin-dentin bonds. The specimens were subjected to a 12-month period of aging, in the presence of unrestricted and restricted water sorption. The term “restricted” was used since it is impossible to eliminate water entirely from bonded hydrated dentin without causing dehydration artifacts that are prone to artifactual silver uptake (Agee et al., 2002). The null hypothesis tested was that water sorption has no effect on the objective of this study was to examine, with the use of transmission electron microscopy (TEM), the changes in silver uptake that occur with aging of resin-dentin bonds. The specimens were subjected to a 12-month period of aging, in the presence of unrestricted and restricted water sorption. The term “restricted” was used since it is impossible to eliminate water entirely from bonded hydrated dentin without causing dehydration artifacts that are prone to artifactual silver uptake (Agee et al., 2002). The null hypothesis tested was that water sorption has no effect on the manifestation of potential water-binding domains within resin-bonded dentin.

MATERIALS & METHODS

Ten extracted human third molars were collected after the patients’ informed consent had been obtained under a protocol reviewed and approved by the institutional review board of the Medical College of Georgia, USA. Within one month of extraction, the occlusal enamel of the teeth was removed by means of a slow-speed saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water-
organic fillers (Metafil CX, Sun Medical Co. Ltd., Shiga, Japan). A microfilled resin composite that contains pre-polymerized methacrylate, and an ionic monomer with carboxylic functional groups (biphenyl dimethacrylate). Camphorquinone is used as the photosensitizer, and the tertiary amine dimethylaminobenzoic acid that are commonly used in dentin adhesives: a relatively hydrophobic monomer (bisphenol A diglycidyl ether dimethacrylate), a hydrophilic monomer (2-hydroxyethyl methacrylate), and an ionic monomer with carboxylic functional groups (biphenyl dimethacrylate). Camphorquinone is used as the photosensitizer, and the tertiary amine dimethylaminobenzoic acid is used as the accelerator. The rationale for using an experimental water-free adhesive was to develop a baseline of the changes in nanoleakage expression that may occur during aging of resin-dentin interfaces, to facilitate future evaluation of commercially available three-step total-etch, two-step total-etch and self-etch, and one-step self-etch adhesives that contain various amounts of water.

Each tooth surface was etched with a 32% phosphoric acid (Uni-Etch, Bisco, Inc., Schaumburg, IL, USA) for 15 sec and rinsed with water for 20 sec. Two coats of the experimental adhesive were applied to the visibly moist acid-etched dentin, air-dried, and light-activated for 10 sec. A microfilled resin composite that contains pre-polymerized organic fillers (Metafil CX, Sun Medical Co. Ltd., Shiga, Japan) was used to facilitate ultramicrotomy. It was applied to the bonded interfaces and light-activated in three 1-mm increments.

After the bonds were allowed to mature for 24 hrs, each tooth was sectioned longitudinally across the resin-dentin interface into 0.9-mm-thick serial slabs. Two slabs from the center part of each tooth were used for the experiments. Ten slabs, one from each tooth, were stored in 5 mL of artificial saliva (Wataha et al., 1999) and incubated at 55°C to accelerate aging. The other 10 slabs were wiped with lint-free gauze, briefly air-dried to remove excess water, and incubated at 55°C in 5 mL of oil (Dow-Corning 200 Fluid, Dow-Corning Corp., Midland, MI, USA). This served as the control group and provided a non-aqueous medium in which no additional water sorption could occur apart from the small amount that was initially present in the slabs during the period of aging and the sectioning process. The experimental specimens were examined at 24 hrs, 1, 3, 6, and 12 mos, while the control specimens were examined at 6 and 12 mos.

**Evaluation of Silver Uptake**

A 50 wt% ammoniacal silver nitrate solution was used as the tracer, following the silver-staining protocol of Tay et al. (2002). The rationale for using ammoniacal silver nitrate was based on the potential of the diamine silver ([Ag(NH₂)₂]⁺) ion complex to trace water-filled regions and/or hydrophilic, water-binding polymer domains within both hybrid layers and the overlying adhesive layers (Tay and Pashley, 2003). Each slab was coated with two layers of nail varnish applied 1 mm from the bonded interfaces. Before the slabs could become dehydrated, they were immediately immersed in the tracer solution for 24 hrs. The silver-stained slabs were rinsed and placed in photodeveloping solution for 8 hrs under a fluorescent light to reduce the diamine silver ions into metallic silver grains. Three blocks were prepared from each experimental slab and 2 blocks for each control slab that were retrieved at a specific time interval. Undemineralized, epoxy-resin-embedded, 90- to 100-nm-thick ultrathin sections were prepared according to the TEM protocol of Tay et al. (1999). The 35 unstained sections were examined by means of a TEM (Philips EM208S, Philips, Eindhoven, The Netherlands) operating at 80 kV.

Five images from each of the 7 groups were analyzed with image analysis software (NIH Image 1.60, Scion Corp., Frederick, MD, USA) for evaluation of the percentage distribution of silver.
deposits within the hybrid layers. Due to the interference from glass fillers, the distribution of silver deposits in the adhesive layers was not determined. Data were statistically analyzed by Kruskal-Wallis ANOVA on ranks and Dunn's multiple comparison tests with statistical significance set at \( \alpha = 0.05 \).

**RESULTS**

Two modes of nanoleakage expression could be recognized within resin-dentin interfaces: reticular and spotted types. Both of these patterns exhibited substantial changes when specimens were aged in artificial saliva, but to a much slower extent when specimens were aged in oil. In specimens that were aged in artificial saliva, the reticular type consisted originally of discontinuous islands of silver deposits that were randomly distributed within the bulk of the unstained hybrid layers (Fig. 1A). These incompletely resin-infiltrated zones (Fig. 1B) gradually reduced in their dimensions at 1 mo (Fig. 1C) until they became truly reticular in appearance by the end of 3 mos (Fig. 1D). While the reticular silver deposits within the hybrid layer continued to diminish with time, there was a gradual accumulation of similar silver deposits along the hybrid layer-adhesive interface (Fig. 1E), until they occupied almost the entire top half of the hybrid layer at 12 mos (open arrowheads, Fig. 1F). Water trees (i.e., water-filled channels; Tay and Pashley, 2003) that began to form along this interface at 6 mo (Fig. 1E) became clearly evident at 12 mos (Fig. 1F). There was also an increase in the size and density of the spotted type of nanoleakage (i.e., isolated silver grains) within the hybrid layers during the entire storage period.

High-magnification views of these specimens also revealed the existence of isolated silver grains within the adhesive layers of the resin-dentin interfaces. At 24 hrs, only very fine grains could be identified within the filled adhesive and hybrid layers (Fig. 2A). They increased in size and density at 1 mo (Fig. 2B). At 3 mos, additional rosettes of silver grains were formed around some of the original silver grains (Fig. 2C). Precursors of water trees that were created by the aggregation of the silver rosettes could be seen at 6 mos (Fig. 2D). Frank water trees were evident in the adhesive layers at 12 mos (Fig. 2E).

For specimens that were aged in oil for 6 mos (Fig. 3A), the original discontinuous islands of silver deposits within the hybrid layers were similar in dimension to those in the 24-hour artificial saliva specimens (Fig. 1B). However, a more extensive distribution of isolated silver grains could be identified. The bulk silver deposits within the hybrid layers were considerably diminished at 12 mos and appeared as truly reticular patterns (Fig. 3B). The dimensions of these reticular patterns (Fig. 3C) were comparable with those of specimens that were stored in artificial saliva after 1 mo (Fig. 1C). Fine, sparsely distributed isolated silver grains could also be observed in the adhesive layers of the 12-month mineral oil specimens (Fig. 3D). Features such as rosette formation and water trees that were identified from the experimental specimens were absent from the control specimens stored in oil.

Since we could not differentiate between the two modes of nanoleakage expression during image analyses, only the total percentage distributions of silver tracer within the hybrid layers are shown in Fig. 4. The 12-month artificial saliva group has a significantly higher silver tracer distribution than do the one-month artificial saliva group and the 12-month oil group (P < 0.05).

**DISCUSSION**

Since the two modes of nanoleakage expression were affected by aging of resin-dentin bonds, this led to a rejection of the null hypothesis. The changes observed provided a working model for future comparisons of commercially available three-step, two-step, and one-step dentin adhesives. Some of these changes were observed in both the experimental and control specimens, such as the initial reduction of the reticular silver tracer patterns within the bulk of the hybrid layer, and the increase in size and density of the isolated silver grains within the adhesive. However, these features were manifested much more slowly when the control specimens were aged in a non-aqueous medium. This may be attributed to restricted water uptake from the minimal amount of water that was initially trapped within
the specimens by the oil.

The transition from the original isolated silver grains to frank water trees in the adhesive resin matrices may be viewed as a series of events that is initiated by water sorption. Water that is retained within hydrophilic polymers via hydrogen bonding exits as a spectrum of intermediate states, ranging from strongly bound primary hydration spheres to less-ordered bonding exits as a spectrum of intermediate states, ranging isolated water-filled channels (Zaikov, 1988). Depending on the state of water that exists, water movement in polymer membranes may begin as a slow Fickian diffusion-type mechanism through the entire matrix, and then become more rapid as convective transport routes develop through the isolated water-filled channels (Zaikov et al., 1988; Steward et al., 1995). Thus, the original isolated silver grains may represent sites where water molecules are structurally bound as hydration spheres to the polar and ionic functional groups of the hydrophilic adhesive. The rosettes of silver grains that were subsequently formed around the original silver grains (Fig. 2C) may represent the secondary hydration shells that, with time, may coalesce and form the water-filled channels. This spectrum of intermediate states, ranging from strongly bound primary hydration spheres to less-ordered bonding exits as a spectrum of intermediate states, ranging

Figure 3. TEM micrographs showing the changes in silver tracer penetration along the resin-dentin interfaces after aging in oil (control). (A) 6 mos, (B-D) 12 mos. Pointer, reticular patterns of silver deposits; arrow, isolated silver grains; C, resin composite; FA, filled adhesive; H, hybrid layer; T, dentinal tubule; U, undemineralized dentin; G, fluoroaluminosilicate glass fillers. (A) Up to 6 mos, islands of heavy silver deposits could still be seen in the hybrid layers. (B) An overall view of a specimen examined after aging in oil for 12 mos revealed a considerable reduction of these bulk deposits to reticular silver patterns. (C) A high-magnification view of the hybrid layer in the 12-month specimen, showing that the extent of the reticular silver deposits within the hybrid layer was similar to that in the one-month specimen that was stored in artificial saliva (Fig. 1C). (D) A high-magnification view of the adhesive layer in the 12-month specimen revealed fine, sparsely distributed isolated silver grains that were devoid of rosettes or water tree formation. This study further demonstrated that water trees may emerge with time in an aged water-free adhesive that is initially devoid of these water-conducting channels. This water must be slowly absorbed and accounts for the increased porosities observed in adhesive layers after in vivo aging (Sano et al., 1999; Takahashi et al., 2002). Unlike the original silver grains that were segregated from one another, unrestricted water movement within the water trees along the hybrid layer-adhesive interface permits a more rapid rate of resin leaching to occur and expedites hydrolytic degradation along these channels.

The significant increase in silver tracer that extended from the hybrid layer-adhesive interface into the hybrid layer in the 12-month experimental specimens supported the findings of Li et al. (2001) and may be interpreted as the morphological manifestation of hydrolytic degradation of resin-infiltrated hybrid layers. Unlike the reticular silver patterns seen within the hybrid layers of the 24-hour experimental specimens that were the original sites of incomplete resin infiltration, the highly concentrated reticular pattern of silver did not appear until the isolated silver grains had increased in size and density, sometime between 6 and 12 mos. This is a reasonable expectation, since water sorption should precede hydrolytic degradation. Expansion of the resin within the hybrid layer, caused by water sorption, seems to have started in the lower half of the hybrid layer and slowly closed those porosities but opened up others closer to the top of the hybrid layer after 1 mo of aging in artificial saliva and at the top by 6 mos. A similar trend was also observed in specimens that were aged in oil, albeit to a much smaller extent. Such a phenomenon supports the absence of correlation between incompletely infiltrated hybrid layers and the decline in resin-dentin bond strengths over time (Okuda et al., 2001) seen in total-etch adhesives. Since the original sites of incomplete resin infiltration may not correspond to the sites of hydrolytic degradation, this finding complements those of Hashimoto et al. (2002), who reported that resins were slowly released from the hybrid layer-adhesive interface when exposed dentin specimens were aged in water for 12 mos. It is possible that, as resins continue to leach components via the water trees along this interface, endogenous matrix metalloproteinases such as MMP-2,-8,-9, and -20 (Birkedal-Hansen et al., 1993) are slowly released from the denuded
deminerlalized dentin matrix, further contributing to the degradation of the hybrid layer in a way that is similar to caries progression (Tjäderhane et al., 1998; Sulkala et al., 2002). This hypothesis merits further investigation by aging exposed dentin in an aqueous medium that contains protease inhibitors.

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REFERENCES


