



<b>Title</b>	<b>A nanoleakage perspective on bonding to oxidized dentin</b>
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## ABSTRACT

The mechanism responsible for sodium-hypochlorite-induced reduction in dentin bond strength and its reversal with reducing agents is unknown. This study examined the relationship between nanoleakage and reversal of compromised bonding to oxidized dentin. Acid-etched dentin was completely depleted of demineralized collagen matrix when sodium hypochlorite was used. Specimens were bonded with two single-bottle dentin adhesives. They were immersed in ammoniacal silver nitrate for 24 hrs before being processed for transmission electron microscopy. For both adhesives, tensile bond strengths of acid-etched dentin were significantly reduced after sodium hypochlorite treatment, but were reversed when sodium ascorbate was used. After sodium hypochlorite application, reticular nanoleakage patterns in hybrid layers were replaced by vertical, shag-carpet-like patterns along the demineralization front. This type of nanoleakage was completely eliminated after sodium ascorbate treatment with the materials tested. Residual sodium hypochlorite within the porosities of mineralized dentin may result in incomplete resin polymerization, and hence compromised bond strength.

**KEY WORDS:** sodium hypochlorite, sodium ascorbate, nanoleakage, microtensile bond strength, ultrastructure.

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# A Nanoleakage Perspective on Bonding to Oxidized Dentin

## INTRODUCTION

Effective dentin bonding depends upon the formation of a hybrid layer that is optimally infiltrated with adhesive resins. Incomplete resin penetration in the hybrid layer permits nanoleakage to occur (Sano *et al.*, 1995a). “Nanoleakage” was originally used to describe microporosities within hybrid layers that allow silver nitrate penetration to occur in the absence of gap formation between resin composite and the hybrid layer (Sano *et al.*, 1995a). The presence of nanoleakage pathways may hasten bond failure by promoting hydrolysis of collagen fibrils and/or degradation of polymerized resins (Carvalho *et al.*, 2000; Hashimoto *et al.*, 2000). Although nanoleakage can be avoided by removal of the acid-demineralized collagen layer with sodium hypochlorite before dentin bonding (Pioch *et al.*, 2001a), no correlation has been found between nanoleakage and the strength of resin-dentin bonds (Paul *et al.*, 1999; Okuda *et al.*, 2001; Pereira *et al.*, 2001).

Compromised bond strengths were observed for some single-bottle adhesives when dentin was treated with sodium hypochlorite either before or after acid-etching (Perdigão *et al.*, 2000; Lai *et al.*, 2001). This drop in bond strength was attributed to the oxidizing instead of the deproteinizing effect of sodium hypochlorite, since the compromised bond strength may be reversed by the application of a reducing agent such as sodium ascorbate to the oxidized dentin (Lai *et al.*, 2001). However, a correlation between the drop in bond strength and ultrastructural changes along the oxidized resin-dentin interface has not been established. Removal of demineralized collagen layer by sodium hypochlorite eliminated nanoleakage formation (Pioch *et al.*, 2001a). Nanoleakage that occurred along the bonded interfaces of oxidized acid-etched dentin and those that were subsequently neutralized with a reducing agent were assessed by means of a silver-staining technique and compared by transmission electron microscopy. The null hypothesis of this study was that there is no difference in the distribution of nanoleakage patterns and tensile bond strengths of single-bottle adhesives bonded to sodium-hypochlorite-treated, acid-etched dentin and those that were further neutralized with sodium ascorbate.

## MATERIALS & METHODS

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 lubrication. The exposed dentin samples were polished with wet 180-grit silicon carbide papers for 60 sec to create thick smear layers in the deep coronal dentin.

## Experimental Design

Two single-bottle adhesives—One-Step (OS, Bisco, Inc., Schaumburg, IL, USA), an acetone-based adhesive, and Gluma Comfort Bond + Desensitizer (GCB, Heraeus Kulzer, Inc., South Bend, IN, USA), an ethanol-based adhesive—were used in this study. Each adhesive consisted of 3 experimental groups with 10 teeth each. Eight restored teeth were used for bond strength evaluation by the microtensile bond test. The other 2 were prepared for nanoleakage evaluation by transmission electron microscopy (TEM). Bonding was performed on the occlusal surfaces of the deep coronal dentin. The 3 experimental groups were shown in the Appendix ([www.dentalresearch.org](http://www.dentalresearch.org)).

The treated teeth were bonded visibly moist with either 2 coats of OS or 3 coats of GCB according to the manufacturers' instructions. Bonded surfaces were air-dried, then light-cured for 10 sec. Composite build-ups were performed with the use of a light-cured composite (Renamel Sculpt, Cosmedent, Inc., Chicago, IL, USA) in 5 1-mm increments. The teeth were stored in distilled water at 37°C for 24 hrs.

## Tensile Bond Strength Evaluation

Bonded teeth were sectioned occluso-gingivally into serial slabs, and further sectioned into 0.9 x 0.9 mm composite-dentin beams, according to the "non-trimming" technique of the micro-tensile test (Shono *et al.*, 1999). Specimens were stressed to failure under tension in a Bencor Multi-T device (Danville Engineering, San Ramon, CA, USA) in a universal testing machine Model 4440 (Instron, Inc., Canton, MA, USA) at a crosshead speed of 1 mm *per* min. For each adhesive, bond strength data from the 3 experimental groups were statistically analyzed with the software package Instat V3.0 for Windows 95 (GraphPad Software, Inc., San Diego, CA, USA). Differences between adhesives were determined by one-way ANOVA and Tukey's multiple-comparisons tests, with statistical significance set at  $\alpha = 0.05$ .

## Nanoleakage Evaluation

A modified silver-staining technique (Tay *et al.*, 2002) was used with basic 50 wt% ammoniacal silver nitrate (pH = 9.5) to avoid the possibility of artifactual dissolution of mineralized dentin along the bases of the hybrid layers (Pashley *et al.*, 2002). We prepared the solution by dissolving 25 g of silver nitrate crystals (Sigma Chemical Co., St. Louis, MO, USA) in 25 mL of distilled water. Concentrated (28%) ammonium hydroxide (Sigma Chemical Co.) was used to titrate the black solution until it became clear as ammonium ions complexed the silver into diamine silver ( $[\text{Ag}(\text{NH}_3)_2]^+$ ) ions (Tay *et al.*, 2002). This solution was diluted to 50 mL with distilled water to achieve a 50 wt% solution.

From the remaining 2 teeth in each group, 0.9-mm slabs were prepared and coated with 2 layers of fast-setting nail varnish applied 1 mm from the bonded interfaces. Before these slabs could become dehydrated, they were immersed immediately in the ammoniacal silver nitrate solution for 24 hrs. The silver-stained slabs were

rinsed thoroughly in distilled water and placed in photodeveloping solution for 8 hrs under a fluorescent light (Wu *et al.*, 1983) to reduce the diamine silver ions into metallic silver grains within potential voids along the bonded interfaces. Undemineralized, epoxy-resin-embedded, 90-nm-thick ultrathin sections were prepared according to the TEM protocol of Tay *et al.* (1999). The unstained sections were examined by means of a TEM (Philips EM208S, Philips, Eindhoven, The Netherlands) operating at 80 kV.

## RESULTS

Both OS and GCB showed significant reduction in microtensile bond strength following application of sodium hypochlorite to dentin ( $p < 0.001$ ) (Table). When sodium ascorbate, the reducing agent, was applied to the oxidized dentin, the compromised bond strength was effectively reversed to pre-treatment levels.

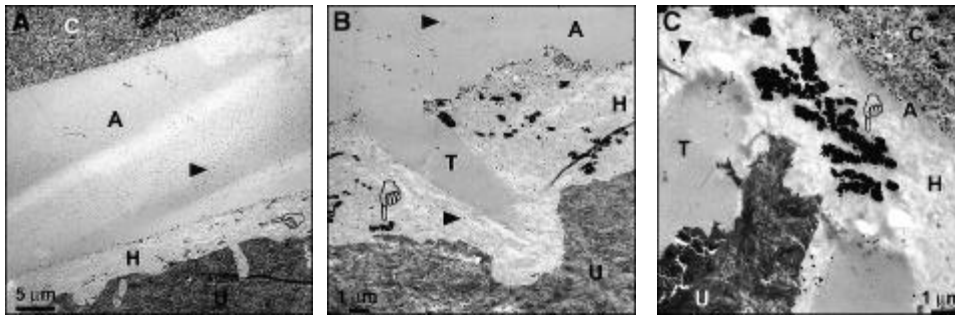
Two types of nanoleakage patterns were suggested by silver staining along the resin-dentin interface, as seen by TEM (Fig. 1). The reticular type consisted of fine, discontinuous islands of silver deposits, which were seen exclusively in the hybrid layer of OS (Fig. 1A). The spotted type, consisting of isolated silver grains, was evident throughout the hybrid and adhesive layers in OS (Fig. 1B). The adhesive layer in GCB was very thin and almost indiscernible from the hybrid layer. A denser reticular type of nanoleakage pattern was seen in the hybrid layer created by this adhesive. A sparsely distributed, spotted type of nanoleakage was evident along the base of the hybrid layer and around the dentinal tubules (Fig. 1C).

After sodium hypochlorite treatment, the demineralized collagen matrix was completely removed, and the associated reticular type of nanoleakage within the hybrid layers could not be observed (Fig. 2A). No hybrid layer was present. For both OS and GCB, a third vertical, shag-carpet-like, nanoleakage pattern was observed along the junction between the demineralization front and the adhesive (Fig. 2A). This pattern originated from the porosities within the underlying mineralized dentin (Fig. 2B). The vertical projections in GCB were less prominent along the dentin surface, but were clearly identifiable along the periphery of the dentinal tubules (Fig. 2C). It is speculated that phase separation of the methacrylic polycarboxylic acid component

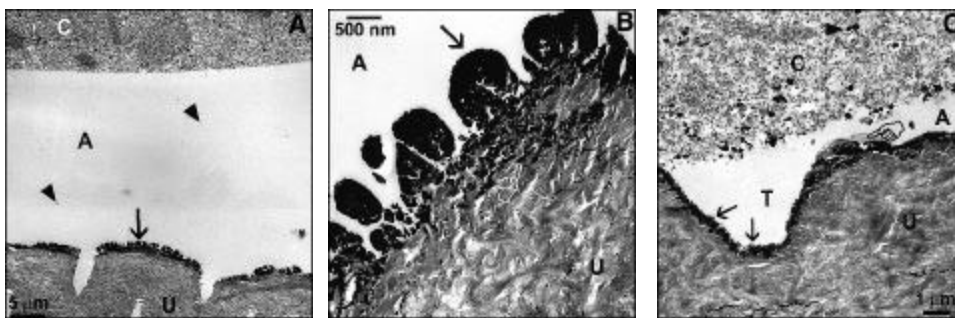
**Table.** Alterations in the Microtensile Bond Strengths of Single-bottle Adhesives Bonded to Acid-etched Dentin Samples Treated with 5% Sodium Hypochlorite and/or 10% Sodium Ascorbate

Treatment Protocol	One-Step Bond Strength (MPa) <sup>a</sup>	Gluma Comfort Bond + Desensitizer Bond Strength (MPa) <sup>a</sup>
Acid-etch only	49.6 ± 7.0 (61) <sup>1</sup>	50.1 ± 8.2 (62) <sup>1</sup>
Acid-etch followed by 5% sodium hypochlorite for 10 min	36.5 ± 7.7 (70) <sup>2</sup>	35.0 ± 6.3 (62) <sup>2</sup>
Acid-etch, 5% sodium hypochlorite for 10 min, rinse, 10% sodium ascorbate for 10 min	48.4 ± 8.8 (63) <sup>1</sup>	49.4 ± 9.2 (56) <sup>1</sup>

<sup>a</sup> Values are means ± standard deviation. Number of specimens tested is included in parentheses. For each adhesive, bond strength results were analyzed by one-way analysis of variance and Tukey's multiple-comparisons tests. Groups identified by different superscripts were significantly different for each adhesive system used ( $p < 0.001$ ).



**Figure 1.** Unstained, undemineralized TEM micrographs of nanoleakage patterns in acid-etched dentin samples that were bonded with single-bottle adhesives. Bonded specimens were previously immersed in ammoniacal silver nitrate before laboratory dehydration and epoxy resin embedding. **(A)** A low-magnification view of the resin-dentin interface in OS. **(B)** A high-magnification view of the hybrid layer in OS. The hybrid layer (H) was only vaguely discerned because the collagen fibrils were unstained. Two different types of silver-staining patterns were discernible. The reticular type, consisting of discontinuous strands of silver deposits (pointer), was observed exclusively within the hybrid layer. The spotted type consisted of isolated spots of silver grains (arrowheads) that were found in both the hybrid layer and the adhesive layer (A). **(C)** A high-magnification view of the resin-dentin interface in GCB. The adhesive layer (A) was very thin. The reticular type (pointer) and the spotted type (arrowhead) of nanoleakage patterns were present within the hybrid layer (H). C, resin composite; U, undemineralized intertubular dentin; T, dentinal tubule.



**Figure 2.** Unstained, undemineralized TEM micrographs of nanoleakage patterns in acid-etched dentin treated with 5% sodium hypochlorite for 10 min, rinsed, and then bonded with single-bottle adhesives. Bonded specimens were previously immersed in ammoniacal silver nitrate. **(A)** A low-magnification view of the resin-dentin interface in OS. **(B)** A high-magnification view of the junction between the adhesive and the demineralization front in OS. The hybrid layer was completely removed, and the reticular type of nanoleakage pattern was absent. The spotted type of nanoleakage (arrowheads) could be only sparsely observed within the adhesive layer (A). A third type of vertical, shag-carpet-like nanoleakage pattern (arrow) was evident along the junction between the adhesive and the undemineralized dentin. This pattern was continuous with silver deposits (pointer) present within the porosities of the undemineralized dentin. **(C)** A high-magnification view of the resin-dentin interface in GCB. The adhesive layer (A) was thin, and the hybrid layer was completely absent. Along the junction between the adhesive and the demineralization front, the shag-carpet-type nanoleakage pattern could be seen (arrows) together with silver deposits within the porosities of the undemineralized dentin. Pointer = phase separation of a relatively electron-dense resin component in the polymerized adhesive; arrowhead = electron-dense ytterbium trifluoride crystals within the resin composite. C, resin composite; U, undemineralized intertubular dentin; T, dentinal tubule.

of this adhesive was observed within the adhesive layer.

When sodium ascorbate was applied to the oxidized and completely deproteinized acid-etched dentin, the vertical shag-carpet type of nanoleakage was entirely eliminated from the demineralization front (Figs. 3A, 3C), although the spotted type of nanoleakage was still sparsely observed within the adhesive layer at high magnification (Fig. 3B). Silhouettes of rhombohedral-shaped crystals (Lai *et al.*, 2001) that were trapped by the adhesive layer could sometimes be seen along the dentin surface and within the dentinal tubules (Fig. 3C). The spotted type of nanoleakage was occasionally observed along the junction of these crystals and the adhesive resin (not shown).

## DISCUSSION

For each single-bottle adhesive, the distributions of nanoleakage patterns and the tensile bond strengths in NaOCl-treated, acid-etched dentin were different from those of either acid-etched dentin or sodium-ascorbate-neutralized, NaOCl-treated acid-etched dentin. Hence the null hypothesis can be rejected.

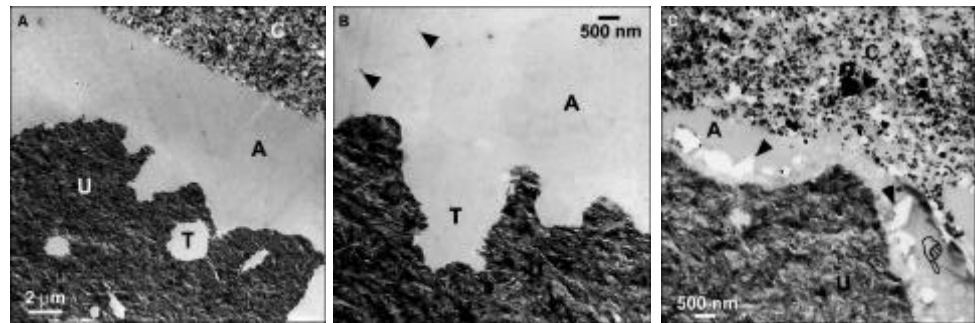
Different types of nanoleakage patterns occurred in the resin-dentin interfaces of all experimental groups with both adhesives, depending on the treatment applied to the dentin surface. The reticular and the spotted types were identified along the resin-dentin interface in acid-etched dentin. The reticular type represents the classic nanoleakage pattern that corresponds to regions of incomplete resin infiltration within hybrid layers (Sano *et al.*, 1995b). This type of nanoleakage was eliminated in the absence of a hybrid layer, but was replaced with the vertical shag-carpet-like pattern after sodium hypochlorite treatment. Although subsequent treatment with sodium ascorbate further eliminated the shag-carpet-like pattern, both sodium hypochlorite and sodium ascorbate treatments did not eliminate the spotted type of nanoleakage pattern. The spotted type probably represents areas of increased permeability within the resin layers (Pashley *et al.*, 2002) that result from the interaction of the hydrophilic resin components with the basic diamine silver ions. The relationship between the hydrophilicity of resin monomers and the permeability

of the adhesive layer requires further exploration.

All previous nanoleakage studies have revealed areas of incomplete resin infiltration within hybrid layers (Pioch *et al.*, 2001b). Incomplete resin penetration of demineralized dentin may leave spaces for penetration of bacterial products and dentinal or oral fluids. This may result in the hydrolytic breakdown of either the adhesive resin or collagen fibrils within the hybrid layer, compromising the long-term durability of resin-dentin bonds (Hashimoto *et al.*, 2000). Although incompletely infiltrated hybrid layers have been shown recently, at least for primates, to be capable of remineralization (Akimoto *et al.*, 2001), complete removal of

the demineralized collagen matrix in acid-etched dentin has been proposed as an alternative way of improving the quality of resin-dentin bonds. A recent study indicated that a one-minute application of 10% sodium hypochlorite to acid-etched dentin completely eliminated nanoleakage at the resin-dentin interface (Pioch *et al.*, 2001a). Other studies indicated the presence of a remnant hybrid layer when a 5% sodium hypochlorite solution (Lai *et al.*, 2001) or a 10% sodium hypochlorite gel (Perdigão *et al.*, 2000) was used for 1 min. A combined sequential two-minute treatment of dentin with maleic acid and sodium hypochlorite was shown to remove the collagen matrix effectively and restore the etched dentin surface to its natural composition (Di Renzo *et al.*, 2001). Pre-treatment with a 10% sodium hypochlorite solution for 2 min was shown to remove organic material from artificial dentin lesions and to increase lesion remineralization (Inaba *et al.*, 1996).

The vertical shag-carpet-like nanoleakage pattern along the demineralization front following application of sodium hypochlorite was completely eliminated after treatment with sodium ascorbate, a reducing agent. Thus, this nanoleakage pattern may be attributed to the elution of either oxygen or residual sodium hypochlorite from the subsurface porous, oxidized mineralized dentin during bonding. A recent micro-Raman spectroscopic study demonstrated spectral shifts in human dentin that was treated with sodium hypochlorite (Tsuda *et al.*, 1996). A previous study (Lai *et al.*, 2001) failed to demonstrate a cause-and-effect relationship between the reduction in bond strength in sodium-hypochlorite-treated dentin and the ultrastructure of resin-dentin bonds. Since the demineralized collagen network had been completely removed from the dentin surface following application of sodium hypochlorite, denatured, remnant collagen matrix could not be responsible for the compromised bonding to sodium-hypochlorite-treated dentin. Influence of organic solvents in the adhesives was not detectable. The use of a nanoleakage approach in the present study thus provides a new perspective on distinguishing among the resin-dentin interfaces in acid-etched dentin, sodium-hypochlorite-treated acid-etched dentin, and acid-etched dentin which was sequentially treated by the oxidizing and then the reducing agent. Since mineralized dentin is porous (Uchtmann and Wilkie, 1997), it is also possible that some of the sodium hypochlorite was retained within the subsurface mineralized tissues even after immersion in water for 10 min before bonding. Elution of the residual sodium hypochlorite could result in incomplete polymerization of resin monomers at the junction between the adhesive and the demineralization front that contributed to the drop in bond strength and the vertical shag-carpet-like nanoleakage pattern at the dentin-adhesive interface. This residual



**Figure 3.** Unstained, undemineralized TEM micrographs of nanoleakage patterns in acid-etched dentin treated with 5% sodium hypochlorite for 10 min, rinsed, immersed in 10% sodium ascorbate for 10 min, and then bonded with single-bottle adhesives. Bonded specimens were previously immersed in ammoniacal silver nitrate. **(A)** A low-magnification view of the resin-dentin interface in OS. **(B)** A high-magnification view of the junction between the adhesive and the demineralization front in OS. Although the spotted type of nanoleakage pattern was occasionally seen within the adhesive layer (A), the vertical shag-carpet-like pattern was absent along the junction of the adhesive and the undemineralized dentin (U). Dentinal tubules (T) were also free of silver deposits. **(C)** A high-magnification view of the resin-dentin interface in GCB. The interface was generally devoid of nanoleakage. Rhombohedral-shaped crystals (arrowheads) were speculated to be sodium ascorbate that were dislodged during sectioning and were trapped by the adhesive (A) along the demineralization front. Silver deposits were occasionally observed along the periphery of these silhouettes (not shown). Pointer: phase separation of a relatively electron-dense resin component in the polymerized adhesive. C, resin composite; U, undemineralized intertubular dentin; T, dentinal tubule.

sodium hypochlorite could have been neutralized by the sodium ascorbate, resulting in a return of the tensile bond strengths to those that were observed in acid-etched dentin.

Both decreases (Nikaido *et al.*, 1999; Frankenberger *et al.*, 2000; Perdigão *et al.*, 2000) and increases (Wakabayashi *et al.*, 1994; Phrukkanon *et al.*, 2000) in bond strengths were reported following application of sodium hypochlorite to acid-etched dentin. Others showed no improvement in bond strength following pre-treatment with NaOCl (Uno and Finger, 1995; Armstrong *et al.*, 1998). This may be due to the specificity of different adhesive systems to the oxidizing effect of sodium hypochlorite. Reduction in bond strength may also be related to changes in the physical and chemical properties of dentin after application of sodium hypochlorite. Reductions in the elastic modulus and flexural strength of dentin were reported after irrigation of the root canals with 5% sodium hypochlorite (Grigoratos *et al.*, 2001; Sim *et al.*, 2001). Similar reductions in the microhardness of root canal dentin were reported when 5% sodium hypochlorite was used for irrigation during endodontic therapy (Saleh and Ettman, 1999). Results from these studies showed that alterations in dentin structure and properties may affect the bonding of adhesives to the treated dentin. It would be interesting to see if physical properties that are reduced after sodium hypochlorite treatment can also be reversed with the use of sodium ascorbate.

In conclusion, the demonstration of different nanoleakage patterns provides an insight into the reduction in bond strength after sodium hypochlorite treatment and its reversal with sodium ascorbate. Although no correlation appears to exist between nanoleakage and bond strength (Okuda *et al.*, 2001; Pereira *et al.*, 2001), nanoleakage may have significant consequences for the long-term stability of adhesive bonds between dentin and restorative materials (Pioch *et al.*, 2001b). Further investigations are required to clarify the relationship between the different types of nanoleakage and the long-term durability of resin-dentin bonds.

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## RESEARCH REPORTS

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### APPENDIX

**Table.** Summary of the 3 Treatment Protocols Investigated in OS and GCB

One-Step (OS)	Gluma Comfort Bond and Desensitizer (GCB)
The bonding surface was etched with a 32% phosphoric acid gel (Uni-Etch, Bisco, Inc., Schaumburg, IL, USA) for 15 sec and rinsed with water for 20 sec before bonding.	The bonding surface was etched with a 20% phosphoric acid gel (Gluma Etch 20 Gel, Heraeus Kulzer, Inc., South Bend, IN, USA) for 20 sec and rinsed with water for 20 sec before bonding.
After being acid-etched, the teeth were immersed in 5.25% sodium hypochlorite (Chlorox, The Chlorox Co., Oakland, CA, USA) for 10 min, for complete removal of the demineralized collagen network. They were placed in distilled water for 10 min before bonding.	
After being acid-etched and receiving a sodium hypochlorite application, the teeth were additionally immersed in 10% sodium ascorbate (Sigma Chemical Co., St. Louis, MO, USA), a reducing agent, for another 10 min to neutralize the oxidizing effect of sodium hypochlorite. <sup>a</sup> They were placed in distilled water for 10 min before bonding.	

<sup>a</sup> Lai *et al.*, 2001.

# A Nanoleakage Perspective on Bonding to Oxidized Dentin