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Deproteinization Effects of NaOCl on Acid-etched Dentin in Clinically-relevant vs Prolonged Periods of Application. A Confocal and Environmental Scanning Electron Microscopy Study

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Clinical Relevance

Complete removal of the collagen matrix prior to dentin bonding procedures has been proposed as a strategy to prevent later degradation, which may jeopardize the longevity of resin-dentin bonds. This study demonstrates that a complete removal of the exposed collagen matrix from the etched dentin surface can be achieved by applying a 12 w/v% NaOCl solution, but at this concentration, it requires a far longer reaction time than is clinically acceptable.

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SUMMARY

Complete removal of the collagen matrix with sodium hypochlorite (NaOCl) as an adjunctive step of restorative and adhesive dentistry is still a subject for debate. This study evaluated the efficacy of a 12 w/v% NaOCl solution for complete

removal of exposed collagen matrices from acid-etched dentin surfaces within a maximum clinically possible period of 120 seconds and a longer period of application (10 minutes) using confocal reflection/immuno-fluorescence microscopy and ESEM. An extended period (45 minutes) of NaOCl application was also performed as a negative control. Unstained and immunohistochemically-stained collagen fibrils were imaged using a confocal laser-scanning microscope for the reflection/fluorescence experiment. Fully-hydrated specimens were also examined with an ESEM. Unetched dentin was devoid of exposed collagen fibrils. Conversely, confocal microscopy showed demineralized collagen after acid-etching, which appeared as a hydrogel-like layer during ESEM examination. The application of NaOCl for two minutes left remnants of dentin collagen on intertubular and intratubular surfaces. The ESEM examination confirmed the presence of remnants of a hydrogel-like layer. After 10 minutes of NaOCl application, residual collagen reflection and immuno-fluorescence signals were detected around dentinal tubules, appearing as spike-like projections during the ESEM investigation.

Complete dissolution of the collagen presence was achieved after 45 minutes of NaOCl treatment. Complete deproteinization of acid-etched dentin is unachievable in a maximum clinically possible period of 120 seconds.

INTRODUCTION

Acid etching is used in adhesive dentistry to expose a shallow (*ca* 5-8 µm) layer of completely demineralized dentin collagen matrix for the infiltration of resin monomers.[1-3] This demineralized collagen matrix remains attached to the dentin surface in a denatured state,[4] with residual apatite crystallites at the base of the demineralized collagen network[5-6] and occasional intra-fibril mineral remnants, which may protect unaltered collagen fibrils.[7] The presence of water around the collagen fibrils and at the base of the demineralized dentin layer may prohibit complete resin infiltration.[8-9] This may result in degradation of the exposed collagen matrix, thereby jeopardizing the longevity of the resin-dentin bonds.[10]

Complete removal of the demineralized collagen matrix with sodium hypochlorite (NaOCl), a non-specific deproteinizing agent, has been proposed as an adjunctive procedure following the etch-and-rinse technique to improve the wettability of the bonding substrate,[11-12] thus facilitating intertubular and intratubular resin infiltration in order to minimize the degradation of the denuded collagen matrix.[13-14] The collagen dissolution capability of NaOCl is proportional to the concentration of active chlorine[15] and superoxide radicals.[16] The deproteinizing effects of NaOCl on acid-etched dentin are also time-dependent.[17] Studies covering the deproteinizing effect of NaOCl on collagen fibrils have mostly been conducted using scanning electron microscopy (SEM). However, soft demineralized collagen fibril networks and structures are susceptible to collapse during conventional SEM specimen preparation and examination.[7] Confocal microscopy offers the advantage of minimal specimen preparation[18] and the examination of fibrin polymers and collagen structures under more normal, wet conditions.[19-21] Immunohistochemical labeling enhances confocal microscopy by targeting specific antigens within intact fibrils, positive binding to the matrix or undenatured collagen being confirmed by retention of the fluorescent marker.[22-23] Environmental SEM (ESEM) offers a valuable technique for the non-destructive observation of unfixed and non-dehydrated biologic specimen structures, complimenting confocal imaging in this context by demonstrating structures at higher resolution but without the immunofluorescence functionality.[24-25]

This study evaluated the efficacy of a 12 w/v% NaOCl solution (one the highest concentrations that was commercially available) for the complete removal of exposed collagen matrix from the surface of acid-etched dentin in a relatively short (maximum clinically tolerable) period of 120 seconds (comparable to previous studies[26]) using complementary confocal reflection/immunofluorescence microscopy and ESEM techniques. The null hypothesis tested was that there are no differences in efficacy of collagen removal between the clinically tolerable period (two minutes) and the long period of NaOCl application (10 minutes) on acid-etched dentin.

METHODS AND MATERIALS

Human third molars were collected and stored in phosphate buffered saline (pH 7.4) at 4°C for the experiments, which were conducted within one month of extraction. Local protocols, reviewed and approved by the Ethics Committee of the Academic Health Science Centre at King's College London, were followed, including informed consent for tissue use in research.

Specimen Preparation for Confocal Microscopy

Twenty-five teeth were used for the confocal microscopy part of the study. Two 0.5-0.7-mm thick dentin slices were obtained from the mid-coronal dentin of each tooth using a slow-speed water-cooled diamond saw (Labcut, Agar Scientific, Stansted, Essex, UK). The slices were randomly divided into two groups of 25 slices each for examination with either reflection or immune-fluorescence confocal microscopy. Each group was further divided in five subgroups (N=5) according to the substrate treatment (acid etching with phosphoric acid, no etching/ultrasonicated and/or NaOCl application). The

dentin slices were polished to 1200 grit to make a very thin smear layer that could be removed by ultrasonication for 10 minutes to expose intact mineralized dentin. These latest specimens were considered to be the negative control as well as those that were acid-etched and subsequently treated with a prolonged application of NaOCl (45 minutes). The positive controls were acid-etched with phosphoric acid for 15 seconds and rinsed with PBS to expose a homogeneous layer of collagen fibrillar matrix. NaOCl was also applied on the dentin surfaces for two minutes to simulate a maximum tolerable clinical intervention or for a longer period of 10 minutes.

Each dentin slice was polished with wet 320-grit silicon carbide (SiC) papers for 30 seconds and subsequently conditioned with 35% phosphoric acid solution for 15 seconds. After acid-etching and rinsing with water for 15 seconds, the specimens were deproteinized using 15 mL of 12% NaOCl solution (Sciencelab.com, Inc, Houston, TX, USA) under continuous agitation at 60 cycles/minute for 2, 10 or 45 minutes, then copiously rinsed with phosphate-buffered saline (PBS) for one minute. This NaOCl concentration was selected to increase the chances for complete collagen removal, with a minimum increase in reagent concentration in order to minimize any potential risks to vital underlying pulp tissue. The specimens from the ultrasonication subgroups were polished with 1200-grit SiC papers to create very thin smear layers and immersed in a bath of deionized water and ultrasonicated for 10 minutes to remove the thin smear layer and expose the underlying intact mineralized dentin.

Confocal Microscopy Imaging

The specimens were imaged using a confocal laser-scanning microscope (Leica SP2 CLSM, Heidelberg, Germany) equipped with a 100x, 1.4 NA oil immersion lens using 514 nm argon/helium ion laser illumination. Reflected light was detected with a photomultiplier tube using reflection filters. Dentin slices used for immunohistochemical staining were fixed in a solution of 4% paraformaldehyde for one hour after the acid/NaOCl treatment and washed for five minutes in phosphate buffer solution (PBS, pH 7.6). The dentin slices were then immersed in 0.1% albumin in PBS buffer containing 0.1% Tween 20 (Sigma-Aldrich Corp, St Louis, MO, USA) for 30 minutes to block non-specific binding sites. The specimens were washed for five minutes in PBS and incubated overnight using mouse monoclonal anti-type I human collagen antibodies (Clone Col-1, Sigma Chemical Co) at a concentration of 1:50. The specimens were then washed for 15 minutes in PBS containing 0.1% Tween 20 and immediately incubated with a 1:100 dilution of secondary antibodies, a goat anti-mouse IgG conjugated with Alexa Fluor 568 (Molecular Probes, Inc, Eugene, OR, USA) at room temperature for one hour. Immunohistochemical negative controls were obtained by omitting the primary antibody from the staining protocol. In brief, confocal immuno-fluorescence microscopy was performed using a 561 nm Diode laser and the emission was detected with a 603 nm long-pass filter. A z-step of 1 µm was used to optically section the specimens to depths of up to 20 µm below surface. The three-dimensional scans of the fluorescent antibody-stained exposed collagen were converted into pseudo-color for better visualization and compiled into either a single view projection or a topographic projection using the Leica SP2 CLSM image-processing software (Leica, Heidelberg, Germany). Configuration of the system was standardized and used at the same level for the entire investigation.

Environmental Scanning Electron Microscopy

Fifteen molars were used for the ESEM investigation, yielding 30 dentin slices divided into subgroups as previously described. Wet specimens were examined with an FEI Quanta 200F field emission ESEM (FEI, Eindhoven, The Netherlands) using the gaseous secondary electron detector (GSE) mode at a water vapor pressure of 6.5 Torr.

Three representative images of each dentin slice were obtained by reflection confocal microscopy, immuno-fluorescence microscopy and ESEM. The images were assessed by two blinded examiners using the following criteria for determining the efficacy of collagen removal:

0: Complete absence of collagen fibrils.

1: Presence of remnant collagen within dentinal tubules.

2: Presence of remnant collagen on the intertubular dentin surface and within dentinal tubules.

3: Remnants of demineralized intact collagen on the dentin surface and within dentinal tubules.

4: Demineralized intact collagen network on the dentin surface and within dentinal tubules.

Each image of a dentin slice was considered as a statistical unit. For each microscopy technique, a single value was assigned to each image, resulting in 15 values per subgroup.

As the images of the three microscopy techniques showed similar values in the same subgroup, these values were combined together (n=45) (Table 1). Differences between the relatively short (two minute) and the relatively long (10 minute) NaOCl application groups were determined using Pearson's $\chi 2$ with the significance threshold set at $\alpha=0.05$.

Table 1: *Distribution of the evaluation scores for the collagen removal in the positive control, negative controls, short period of NaOCl application and long period of NaOCl application groups based on the three microscopy techniques involved in the study.*

Evaluation Criteria (scores: 0–4)	Microscopy Techniques	Dentin Treatment Groups				
		Acid-etching Dentin (positive control) [N=15]	No-etch-ultrasonicated Dentin (negative control) [N=15]	NaOCl Application (2 minutes) [N=15]	NaOCl Application (10 minutes) [N=15]	Prolonged NaOCl Application (45 minutes) (negative control) [N=15]
0	RC	--	15	--	--	15
	IFC	--	15	--	--	15
	ESEM	--	15	--	--	15
1	RC	--	--	--	14	--
	IFC	--	--	--	13	--
	ESEM	--	--	3	15	--
2	RC	--	--	8	1	--
	IFC	--	--	10	2	--
	ESEM	--	--	5	--	--
3	RC	15	--	7	--	--
	IFC	15	--	5	--	--
	ESEM	15	--	7	--	--
4	RC	--	--	--	--	--
	IFC	--	--	--	--	--
	ESEM	--	--	--	--	--

Abbreviations: RC–Reflection confocal microscopy; IFC–Immuno-fluorescence confocal microscopy; ESEM–Environmental scanning electron microscopy

Evaluation Scores: 0: Complete absence of collagen fibrils; 1: Presence of remnant collagen within dentinal tubules; 2: Presence of remnant collagen on the intertubular dentin surface and within dentinal tubules; 3: Remnant of demineralized intact collagen on the dentin surface and within dentinal tubules; 4: Demineralized intact collagen network on the dentin surface and within dentinal tubules.

RESULTS

Confocal Microscopy

The immuno-fluorescence and reflection confocal microscopy of unetched/ultrasonicated dentin treated with 12% NaOCl for 45 minutes (negative controls) revealed a dark background of intertubular dentin that was devoid of collagen (Figure 1A), although traces of collagen could be seen inside the tubules. Conversely, strong reflection and fluorescence signals, indicative of the presence of a demineralized collagen matrix, were observed after acid-etching (positive control) (Figure 1B). By contrast, weaker reflection signals identified from the acid-etched dentin surfaces after two minutes of 12% NaOCl application indicated the presence of much residual collagen in the intertubular and intratubular dentin (Figure 1C and 2A).

In the prolonged NaOCl application groups, collagen fluorescence signals were predominantly found along the intratubular dentin surface of the walls of the dentinal tubules (Figures 1D and 2B). Similary, immuno-fluorescence topographic projections revealed incomplete removal of the demineralized collagen matrix from both the intertubular and intratubular dentin surface after a period of two minutes of NaOCl treatment (Figure 3A), but demineralized surface collagen was largely absent after 10 minutes of prolonged NaOCl treatment (Figure 3B). However, it was still possible to detect residual collagen immuno-fluorescence signals along the dentinal tubule walls (Figure 3C). Complete dissolution of the acid-etched dentin collagen matrix was only achieved after 45 minutes of NaOCl treatment, with no reflection or immuno-fluorescence collagen signals being detectable (image not shown).

Environmental Scanning Electron Microscopy

Specimens from the ultrasonicated negative control (no acid or NaOCl treatment) exhibited flat surfaces with no exposed collagen fibrils. The diameter of the dentinal tubules was 1.5-2 µm and the peritubular dentin matrix could be seen lining most tubules (Figure 4A). After acid-etching, the water-filled demineralized dentin matrix appeared smooth and gel-like, with widened tubular orifices (*ca* 3-4 µm) due to acid dissolution of the peritubular dentin (not shown). Treatment of the acid-etched dentin for two minutes with 12% NaOCl treatment (Figure 4B) failed to completely remove the surface hydrogel-like layer. Moreover, after 10 minutes of NaOCl treatment, the dentin surface appeared rough and highly irregular, with a complete absence of the surface hydrogel-like layer. The tubules were widened (4-5 µm) with fine, spike-like[25] projections along the tubular walls (Figure 4C). The dentin substrate appeared very porous after 45 minutes of NaOCl treatment, with a complete absence of the surface gel-like layer and intertubular spike-like projections (Figure 4D).1

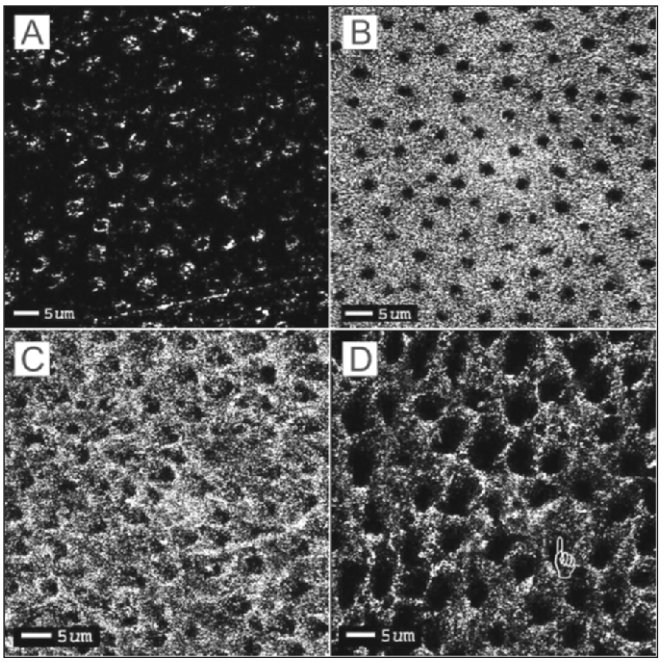

Figure 1: *Confocal reflection microscopy. A: unetched and ultrasonicated dentin (negative control) shows a dark background of intertubular dentin devoid of collagen matrix. B: Acid-etched dentin (positive control). The strong reflection signals indicate the presence of an intact collagen network on the demineralized dentin surface. C: Acid-etched dentin surface after two minutes of NaOCl application, showing a weaker reflection, indicating some loss collagen remnants on the intertubular and tubule surfaces. D: Acid-etched dentin surface tubules after 10 minutes of NaOCl exposure. A weak reflection signal is predominantly observed only along the walls of the dentinal tubules, indicating the remnant presence of collagen (Figure 1D) (100x/1.4 NA oil immersion lens).*

Evaluation scores assigned to the positive control, negative controls, two-minute and 10-minute exposures to 12% NaOCl for each microscopy technique are presented in Table 1.

Analysis of the combined ordinal data obtained from the three microscopy techniques revealed a significant difference in the efficacy of collagen removal between the two and 10 minutes of the NaOCl application ($p<0.001$) (Table 1)

DISCUSSION

Ninety percent of the weight of the organic phase in dentin is type I collagen[27] that is composed of collagen fibrils approximately 100 nm in diameter that form an intricate three-dimensional network around and between the dentinal tubules.[7] The non-destructive nature of confocal microscopy and ESEM minimizes artifacts induced by preparation and dehydration for conventional SEM and TEM microscopy. In particular, thin layers of partially-denatured collagen are difficult to preserve, even when using critical point or hexamethyldisilasane drying for conventional high vacuum SEM techniques. This may explain why incomplete removal of the collagen matrix was reported more often

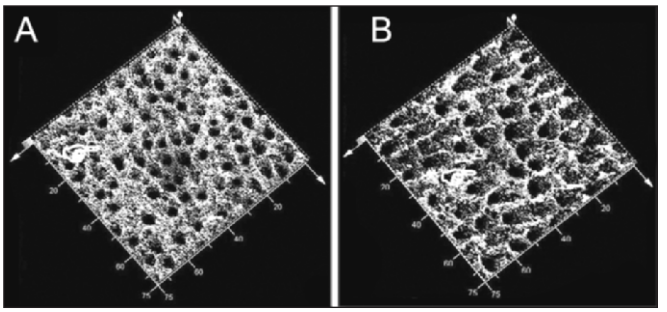

Figure 2: *Confocal reflection microscopy projections. A: Acid-etched dentin surface after two minutes of NaOCl application showing reflection signals indicating the presence of remnant demineralized collagen on both the intertubular and intratubular dentin surfaces (pointer). B: Acid-etched dentin surface after 10 minutes of NaOCl exposure showing a reduced intertubular reflection signal (pointer), suggesting a more thorough but incomplete collagen removal, especially on the tubule walls (100x/1.4 NA oil immersion lens).*

in papers that utilized TEM examination techniques, instead of SEM reports.[11,27-28]

A potential limitation of confocal microscopy and ESEM, as evidenced from the results of the current work, is the difficulty in visualizing the morphology of the collagen fibrils. However, the use of specific fluorescent dye-labeled monoclonal antibodies allowed for the discrimination of intact and denatured collagen fibrils. The reflection and immuno-fluorescence confocal microscopy results confirmed that the efficacy of NaOCl at removal of collagen is time-dependent. After two minutes of 12% NaOCl application, strong immuno-fluorescence and reflection signals, indicative of the presence of residual, non-denatured type I collagen, could be detected on intertubular and intratubular dentin surfaces.

These results are in accordance with those of Oyarzún and others,[22] who reported that two minutes of 5% NaOCl treatment affected the organization of collagen and glycosaminoglycans (GAG) in mineralized and demineralized dentin-extracellular matrix. The authors showed that most of the collagen was removed from the external demineralized dentin, leaving a remnant immuno-fluorescence signal on the intratubular dentin and from the transitional and internal zones of dentin.

The ESEM results obtained in this study after two minutes of NaOCl treatment were comparable to those obtained with confocal microscopy but with some morphological differences. Highly water-absorbent polyanionic non-collagenous proteins and GAGs are contained within the collagen network[29] and probably interfered with visualization of the surface of collagen fibrils by ESEM, resulting in the appearance of a gel-like layer[30] both in acid-etched and NaOCl-treated dentin. The 10-minute treatment with NaOCl removed

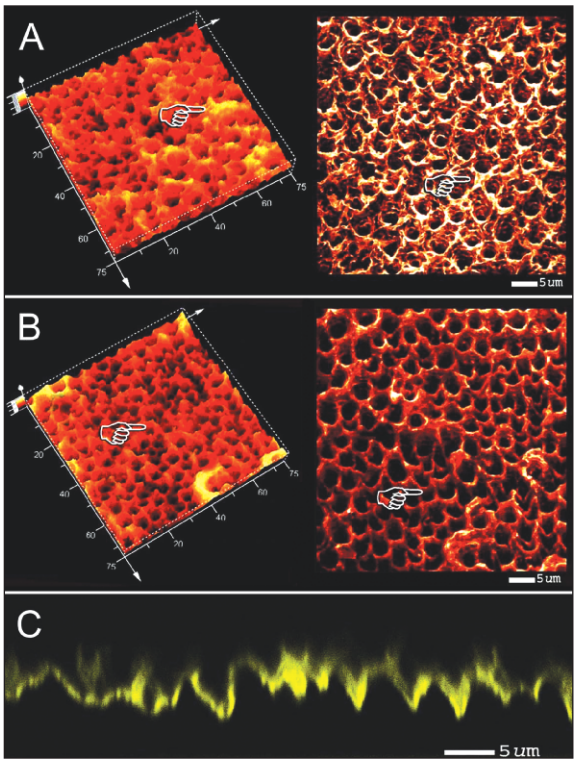

Figure 3: *Confocal immuno-fluorescence microscopy projections. A: Incomplete removal of demineralized collagen from both the acid-etched dentin and from the walls of the dentinal tubules after two minutes of NaOCl treatment (pointer) in 3-D (left) vs 2-D (right). B: Acid-etched dentin treated with NaOCl for 10 minutes. Collagen is completely absent from the dentin surface; however, it was possible to detect the presence of residual collagen immuno-fluorescence signals along the walls of the dentinal tubules (pointer) seen more clearly as bright features in the single projection on the right. C: Single projection reconstruction image parallel (x-z plane) to the treated surface, showing the presence of residual immuno-fluorescence signals of collagen principally along the walls of the dentinal tubules after 10 minutes of NaOCl treatment (100x/1.4 NA oil immersion lens).*

most of the demineralized collagen matrix, but the presence of weak immuno-fluorescent and reflection signals from the intratubular dentin and the presence of spike-like fibrillar structures suggest that the collagen was incompletely removed. Kodaka and others[25] used ESEM to evaluate the effect of 30% hydrogen peroxide in the bleaching of dentin. They reported the presence of collagen fibrillar structures with spike-like morphology even after treatment with 30% H_2O_2.

Dentin demineralization achieved by using acid conditioners (35-37% phosphoric acid) may leave residual apatite crystallites within the collagen matrix[4,31] and residual intrafibrillar mineral in unaltered collagen fibrils.[7] It is believed that these residual minerals may play a protective role for collagen fibrils during NaOCl deproteinization.[22,32]

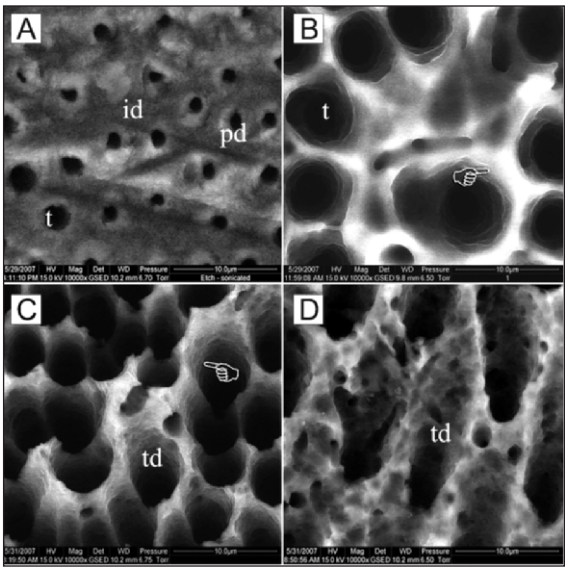

Figure 4: *ESEM images. A: Surface of ultrasonication negative control. There were no exposed collagen fibrils on the intertubular (id) dentin; the diameter of the dentinal tubules (t) was 1.5-2 µm in diameter. B: The micrograph represents acid-etched dentin surfaces treated with NaOCl for two minutes. Dentinal tubules (t) appeared widened to 4-5 µm in diameter due to dissolution of peritubular dentin. It is possible to observe an incomplete removal of the surface gel-like layer (pointer). C: After 10 minutes of NaOCl treatment, the acid-etched dentin surface appears rough and highly irregular, with a complete absence of the surface gel-like layer. Dentinal tubules were enlarged in diameter, with fine spike-like projections along the tubular walls (pointer). D: The micrograph represents the specimens from the acid-etched dentin substrate treated with NaOCl for 45 minutes. It is possible to see a porous dentin substrate with a complete absence of the surface gel-like layer and intratubular spike-like projections.*

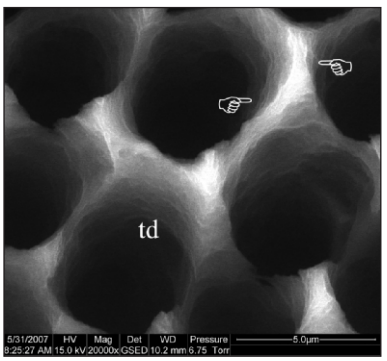

Figure 5: *Higher ESEM magnification of the acid etched dentin after 10 minutes of NaOCl application; the surface appears free from the gel-like layer but, with several fine, spike-like projections (pointers) along the intratubular dentin (td) of the enlarged tubular walls.*

This conjecture supports the results of the current study that, even 10 minutes of NaOCl application was not able to completely remove the collagen from the porous surface of the intratubular and intertubular dentin. These morphological results probably account for the presence of nanoleakage, which, theoretically should not exist in NaOCl-treated acid-etched, bonded dentin.[33] Yiu and others showed vertical, shag-pile carpet-like nanoleakage patterns along junctions between the porous demineralization front and the adhesive.[33] This porous substrate may also be the location of NaOCl retention, which can potentially disrupt adhesive polymerization.

Based on these results, the authors of the current study must accept the experimental null hypothesis for the two- and 10-minute exposures; the data only supporting the hypothesized assertion that there is a significant difference in the efficacy of collagen removal between the two- and 45-minute NaOCl exposure groups on acid-etched dentin.

The results of the current study are in agreement with Perdigão and others,[27] who demonstrated by transmission electron microscopy (TEM) that incompletely removed collagen fibrils were present along the resin-dentin interface and the walls of dentinal tubules after treatment with 10% NaOCl for 60 seconds. Using TEM, Osorio and others[28] also reported that the use of 5% NaOCl for 120 seconds did not completely remove the collagen fibrils from phosphoric acid-etched dentin. These authors observed collagen fibrils when the surface-deproteinized specimens were bonded with dentin adhesives.

The efficacy of collagen removal may be related to the vehicle employed for delivering the NaOCl (gel or solution),[34] the active chloride present in the solution (age of solution) and the type of substrate (depth and organic content of the dentin). In the current study, complete removal of collagen from acid-etched dentin was only consistently achieved sometime between 10- and 45-minutes of NaOCl treatment, when neither reflection signal, immuno-fluorescence signals or spike-like ESEM projections could further be detected from the dentin substrate. Di Renzo and others[17] reported similar results using photoacoustic Fourier transform infrared spectroscopy. The spectroscopic features of the deproteinized acid-etched dentin surface were similar to the original spectrum of untreated dentin only after 15 minutes of NaOCl treatment. Sakae and others[35] demonstrated that the nearly complete removal of organic materials from powdered dentin is only achievable using a 10%-NaOCl treatment for 30 minutes. However, such an extended time of high concentrations of NaOCl treatment may compromise the mechanical and physical properties of the dentin,[36] rendering it unsuitable as a clinical procedure in restorative dentistry.

The complete deproteinization of acid-etched dentin might theoretically offer the possibility of an increase in bond strength of the resin-bonded dentin created by adhesives containing phosphate, phosphonate or carboxylic groups that are potentially capable of interacting with Ca-ions of the apatite of the dentin surface.[37-38] However, in a recent bond strength test conducted after one year of water storage, none of the eight bonding systems that were used showed any significant increase in bond strength after deproteinization of the dentin achieved by immersion of the samples in 0.5w/w%NaOCl for 60 minutes.[39]

CONCLUSIONS

Considering that complete removal of the demineralized collagen matrix with 12% NaOCl from acid-etched dentin was only reliably achieved using application times that exceed 10 minutes, it can be concluded that NaOCl deproteinization of acid-etched dentin cannot be achieved if clinically realistic times of application are used.

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