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Selective demineralisation of dentine extrafibrillar minerals—A potential method to eliminate water-wet bonding in the etch-and-rinse technique

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ABSTRACT

Objective: The present study tested the central hypothesis that selective demineralisation of dentine extrafibrillar minerals by lowering the phosphoric acid concentration improves the quality of the resin-dentine interface.

Methods: Dentine surfaces were etched with different concentrations of phosphoric acid (1, 5, 10, 20, 30 or 40 wt%). Scanning electron microscopy was used to observe the micromorphology of the etched dentine surfaces. Energy dispersive X-ray analysis was performed to determine the residual Ca-content of the demineralised dentine matrix. Atomic force microscopy-based nanoindentation was used to analyse the nanomechanical properties of the treated dentine surfaces. The influence of H_3PO_4 concentration on resin-dentine bond strength was evaluated by microtensile bond strength testing. One-way ANOVA was used to compare the residual Ca-content ratio, reduced elastic modulus (Er) of the treated dentine surfaces and microtensile bond strength among groups.

Results: Collagen fibrils appeared to be wider in diameter after etching with 5% and 10% H_3PO_4. The partially-demineralized collagen scaffold retained part of its rigidity to maintain an uncollapsed three-dimensional structure. Etching with 1% H_3PO_4 resulted in the highest residual Ca-content ratio and Er of demineralised dentine matrix, followed by 5% H_3PO_4. Those values were all significantly higher than values derived from the other groups. Etching with 30% H_3PO_4 resulted in the lowest Ca-content ratio and Er. Using 5% H_3PO_4 as etchant resulted in the highest resin-dentine bond strength.

Conclusions: Selective demineralisation of the dentine matrix may be achieved by lowering the H_3PO_4 concentration to 5 wt%, to achieve better bonding performance.

Clinical relevance: By retaining intrafibrillar minerals, more through air-drying of the partially demineralised collagen matrix may be accomplished without the need to worry about collapsing a mineral-free collagen matrix during air-drying. This may result in the elimination of water-wet bonding during the application of etch-and-rinse adhesives.

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1. Introduction

Dentine adhesives are functionally classified as etch-and-rinse adhesives or self-etch adhesives. Although both classes of adhesives can achieve high immediate bond strength, many studies showed that the high immediate bond strength achieved with the etch-and-rinse technique are not durable [1]. Improvement of resin-dentine bond durability remains a timely issue even after more than 60 years of intensive research on dentine bonding.

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Lack of resin-dentine bond durability in etch-and-rinse adhesives has been attributed to the involvement of water during the formation of hybrid layer [2]. With the advent of the water-wet bonding concept in 1992, resin-dentine bond strengths achieved with the use of etch-and-rinse adhesives dramatically increased from 10 MPa to more than 40 MPa [3]. Nevertheless, this bonding concept also introduced undesirable consequences. After acid etching, the apatite crystallites in the mineralised dentine matrix are almost completely dissolved by the 30–40% phosphoric acid to a depth of 5–8 μm. Prior to the application of resin monomers, it is critical to keep the microporosities patent within the demineralised collagen matrix. When water is evaporated from the demineralised collagen matrix, the relatively flexible collagen fibrils rapidly form interpeptide hydrogen bonds with their nearest neighbours, causing the matrix to collapse and form an impermeable membrane-like structure that prevents optimal resin monomer infiltration [4]. Water has the highest Hansen's solubility parameter for hydrogen bonding (δ_h; 40.4 $(J/cm^2)^{1}/_{2}$), which can easily break the hydrogen bonds between collagen fibrils to provide room for the infiltration of resin monomers [5]. Because dimethacrylates such as triethylene glycol dimethacrylate are almost insoluble in water, hydrophilic resin monomers such as 2-hydroyethyl methacrylate (HEMA) are introduced into dentine adhesives as a co-solvent, and to enhance the dispersion of hydrophobic resin monomers within the wet demineralised dentine substrate. This noble objective, however, is accomplished at the expense of creating potentially permeable, unstable resin matrices that are prone to water sorption, leaching of resinous components and hydrolysis over time [6,7].

Bonding of contemporary self-etch adhesives is also based on the water-wet bonding concept since these adhesives contain 20–35% water [8]. Self-etch adhesive formulations usually include hydrophilic monomers with acidic functional groups, water, HEMA, and bifunctional dimethacrylates. Water is indispensable for ionisation of the acidic resin monomers in self-etch adhesives. The process releases hydronium ions that etch through the smear layer to partially demineralise the underlying dentine. After comonomer infiltration, the volatile solvents and water are partially removed by air-drying. As a water-miscible co-solvent, HEMA decreases the vapour pressure of water and interferes with water evaporation [9]. Prior to polymerisation, HEMA may form a homogenous hydrogel containing unbranched polymer [10] and create water channels (i.e. water trees) within the polymerised resin matrix [11]. After polymerisation, the residual water, incompletely polymerised HEMA, and acidic monomers that are retained within the polymer network can absorb water, resulting in the plasticisation of the polymers and decreases in the physical properties of the polymerised adhesive [12,13].

Contemporary etch-and-rinse adhesives, self-etch adhesives as well as universal adhesives cannot eliminate the adverse effect of water and do not produce perfect hybrid layers. As long as the dentine is completely demineralised, it is difficult for resin monomers to completely infiltrate the interfibrillar and intrafibrillar spaces within the demineralised collagen matrix. Whereas the interfibrillar spaces (i.e. spaces around the collagen fibrils) are comparatively easier to be infiltrated by resin monomers, infiltration of the intrafibrillar spaces (i.e. spaces around the microfibrils and between collagen triple helices with a collagen fibril) is a formidable task even for the most hydrophilic monomers with the smallest molecular dimensions. It is surmised that if mineralised collagen fibrils can be selectively demineralised to remove only the interfibrillar minerals while keeping the intrafibrillar minerals intact, there will be no need for resin monomers to infiltrate the nanoscopical intermolecular spaces within the collagen fibrils. An additional advantage is that the interfibrillarly-demineralised collagen fibrils retain their strength because of the presence of intrafibrillar minerals [14] and will not collapse during the process of air-drying.

The simplest way to selectively demineralise dentine with the etch-and-rinse technique is to lower the acidity of the etchant. Hence, the objective of the present study was to examine the central hypothesis that selective demineralisation of dentine extrafibrillar minerals by lowering the phosphoric acid concentration improves the quality of the resin-dentine interface. This objective was accomplished by testing three null hypotheses: 1) there is no difference in dentine surface morphology after the application of different concentrations of phosphoric acid; 2) there are no differences in the Ca^{2+} content and modulus of elasticity of dentine surfaces after they are etched with different concentrations of phosphoric acid; and 3) there are no differences in the tensile bond strengths of resin-dentine bonds prepared from etching dentine with low (below 10%) or high concentrations (10–40%) of phosphoric acid.

2. Materials and methods

2.1. Teeth collection

Unerupted, non-carious third molars were collected from adult patients with their informed consent, under a protocol approved by the Peking University School of Stomatology Institutional Review Board (PKUSSIRB-201522043). The teeth were stored at 4 °C in saline containing 0.02% sodium azide to prevent bacterial growth and used within 1 month after collection.

2.2. Preparation of etchants

The etchants employed for etching dentine surfaces consisted of phosphoric acid solutions with different concentrations (1, 5, 10, 20, 30 or 40 wt%). They were prepared from 85 wt% phosphoric acid (Millipore Sigma, St. Louis, MO, USA) by dilution with deionised water. The pH values of these etchants are shown in Table 1.

2.3. Scanning electron microscopy

The occlusal enamel of 18 teeth was removed with a water-cooled low-speed Isomet saw (Buehler Ltd., Lake Bluff, IL, USA) under copious water cooling. Sectioning was performed perpendicular to the tooth axis. A second parallel cut was conducted at 1 mm below the dentinoenamel junction to expose the mid-coronal dentine. Eighteen dentine discs (700 ± 100 μm thick) were obtained and randomly divided into 6 groups. A stereomicroscope (SMZ 1500, Nikon, Japan) was used to ensure that the dentine discs were free of enamel. The occlusal side of the dentine discs were polished with 320-, 800-, 1200-, 2400- and 4000-grit wet silicon carbide papers under running water for 20 s each. Each occlusal

Table 1
Residual Ca-content and reduced elastic modulus of the dentine surface after etching with different concentrations of phosphoric acid.

Groups	pH value	Ca-content ratio (%)* N = 5 teeth	Reduced elastic modulus (GPa)* N = 10 points
Control	–	–	19.65 ± 2.43^{a}
1%	1.48	54.71 ± 2.58^{A}	2.04 ± 0.29^{b}
5%	1.01	42.49 ± 2.49^{B}	1.00 ± 0.22^{c}
10%	0.82	32.93 ± 0.71^{C}	0.40 ± 0.10^{d}
20%	0.55	28.82 ± 3.35^{C}	0.32 ± 0.08^{d}
30%	0.35	17.38 ± 3.68^{D}	0.19 ± 0.05^{e}
40%	0.46	31.99 ± 1.47^{C}	0.36 ± 0.10^{d}

* Values are means ± standard deviations. For each parameter, values identified by different superscripts letters are significantly different ($P < 0.05$).

dentine surface was then etched with a different concentration of phosphoric acid for 15 s. The treated dentine surfaces were rinsed with running water for 30 s. Immediately after rinsing, the dentine discs were dehydrated in an ascending series of ethanol (25%, 50%, 75%, 95% and 100%). Changing of ethanol was performed without allowing the demineralised dentine surface to be exposed to air, that would have resulted in the collapse of the demineralised collagen matrix by surface tension. After drying with a critical point dryer (EM CPD300, Leica, Germany) for 4 h, the dentine discs were mounted on aluminum stubs and sputter-coated with gold. The specimens were observed using a field emission scanning electron microscope (Helios Nanolab 600i, FEI, Hillsboro, OR, USA) at 5 kV.

2.4. Energy dispersive X-ray analysis

The residual calcium content on the surface of the phosphoric acid-treated dentine discs was analysed using an energy dispersive X-ray micro-analyser (EDX). The dentine discs were polished using the same method as described previously. Prior to acid etching, the dentine discs were sectioned into two halves. One-half was etched with a respective concentration of phosphoric acid. The other half served as the control and was not etched. The dentine surfaces were analysed with scanning electron microscopy (SU8020, HITACHI, Japan) EDX (X-Max M, Oxford Instrument, Wiesbaden, Germany). Ten points were randomly selected on each dentine disc and analysed using an accelerating voltage of 10 kV and a counting time of at least 3 min for each point. The residual Ca-content on each etched dentine surface was expressed as a percentage of the content derived from the control half of the same tooth. Measurements were conducted on five teeth for each group to obtain the mean measurement for that group [15].

2.5. Nanoidentation with atomic force microscopy

Seven 5 mm-long, 1.5 mm-wide and 1.0 mm-thick dentine beams were obtained from one mid-coronal dentine disc that had been polished using the previously described method. Six dentine beams were randomly selected and etched with different concentrations of phosphoric acid. The remaining dentine beam served as the control group and was not etched. After etching, the nanomechanical properties of the treated dentine surfaces were evaluated with a pyramidal Berkovich diamond indenter (Hysitron Inc., Minneapolis, MN, USA; tip radius of curvature 150 nm) attached to an atomic force microscope (Nanoscope III Digital Instruments, Santa Barbara, CA, USA). Ten points from the intertubular dentine of each treated dentine surface were randomly selected and tested.

Because dentine possesses viscoelastic and viscoplastic time-dependent behaviour, the demineralised collagen matrix may be subjected to adhesive interaction with the Berkovich tip. Accordingly, a multi-cycling method was used in the present study to eliminate thermal drift/creep in order to obtain stable and reliable readings. Each specimen was partially unloaded to 10% of the maximum load at every cycle and then reloaded to the next peak using the same constant loading/unloading rate (400 μN/s). The load ranged from 100 μN to 1000 μN with an incremental load of 100 μN per cycle. Loads and displacements were recorded during the loading and unloading cycles. The reduced elastic modulus (Er) of the exposed dentine matrix, being an elastic-plastic material, was calculated using the following equation:

$$Er = \frac{\sqrt{\Pi}}{2\beta}\frac{s}{\sqrt{A}}$$

where β is the correction factor for the indenter shape (for a Berkovich indenter, $\beta \approx 1.05$), A is the contact area at the point of unloading, and S is the slope of the unloading curve [16,17].

2.6. Microtensile bond strength testing

Twenty-four molars were allocated randomly to six groups according to the phosphoric acid concentration. After exposing the mid-coronal dentine surface, a standardised smear layer was created by grinding with 600-grit silicon carbide paper under running water for 60 s. Each dentine surfaces was etched with a different concentration of phosphoric acid for 15 s, and then rinsed with water spray for 30 s. The dentine surface was blot-dried with moist tissue paper (Kimberly-Clark, Roswell, GA, USA) to keep the demineralised collagen matrix visibly moist, in accordance with the water-wet bonding technique. Two separate layers of an ethanol-based two-step etch-and-rinse adhesive (Single Bond Plus, 3 M ESPE St. Paul, MN, USA) was applied on the moist dentine surfaces. This was followed by evaporation of the adhesive solvent for 15 s and light-curing of the adhesive for 20 s at 800 mW/cm^2 using a visible light-curing unit (Elipar FreeLight 2, 3 M ESPE). Resin composite build-up was subsequently performed using three 1.5 mm increments of a hybrid resin composite (Clearfil AP-X, Kuraray Noritake Dental Inc., Osaka, Japan). Each increment was individually light-cured for 20 s.

The bonded teeth were stored in water at 37 °C for 24 h. Each bonded tooth was sectioned in the x and y directions across the adhesive interface to obtain 16 resin-dentine beams, each with cross-sectional dimensions of approximately 0.7 × 0.7 mm. The exact dimensions were measured with a pair of digital callipers. Each beam was individually fixed to a custom-made testing jig with cyanoacrylate adhesive (Zapit, Dental Ventures of America Inc., Corona, CA, USA) and subjected to tensile loading using a universal testing machine (EZ-L-1kN, Shimadzu, Japan) at a crosshead speed of 1.0 mm/min until failure.

2.7. Statistical analysis

The residual Ca-content ratios, reduced elastic modulus of the treated dentine surfaces and microtensile bond strengths were separately analyzed using one-way analysis of variance, after ascertaining the normality and homogeneity of variance assumptions of the respective data sets. Microtensile bond strengths obtained from beams derived from each of the 4 teeth of each group (6 groups) were pooled together to obtain the mean bond strength value, with each tooth treated as a statistical unit. For each parameter, post-hoc pairwise comparisons were performed using the Student-Newman-Keuls statistic. When either of the aforementioned statistical assumptions was violated, the data set was non-linearly transformed to satisfy those assumptions prior to the use of parametric statistical methods. For each analysis, statistical significance was pre-set at $\alpha = 0.05$.

3. Results

3.1. Scanning electron microscopy

Representative images of the treated dentine surfaces are shown in Fig. 1. The effect of acid-etching, dissolution of the peritubular cuff, patency of dentinal tubules and exposure of the collagen fibrillar network were visible in all groups except for the 1% H_3PO_4 group (Fig. 1A/a). In the 1% group, remnant smear plugs could be identified within the dentinal tubules. Minerals were precipitated around the surface of the exposed collagen matrix.

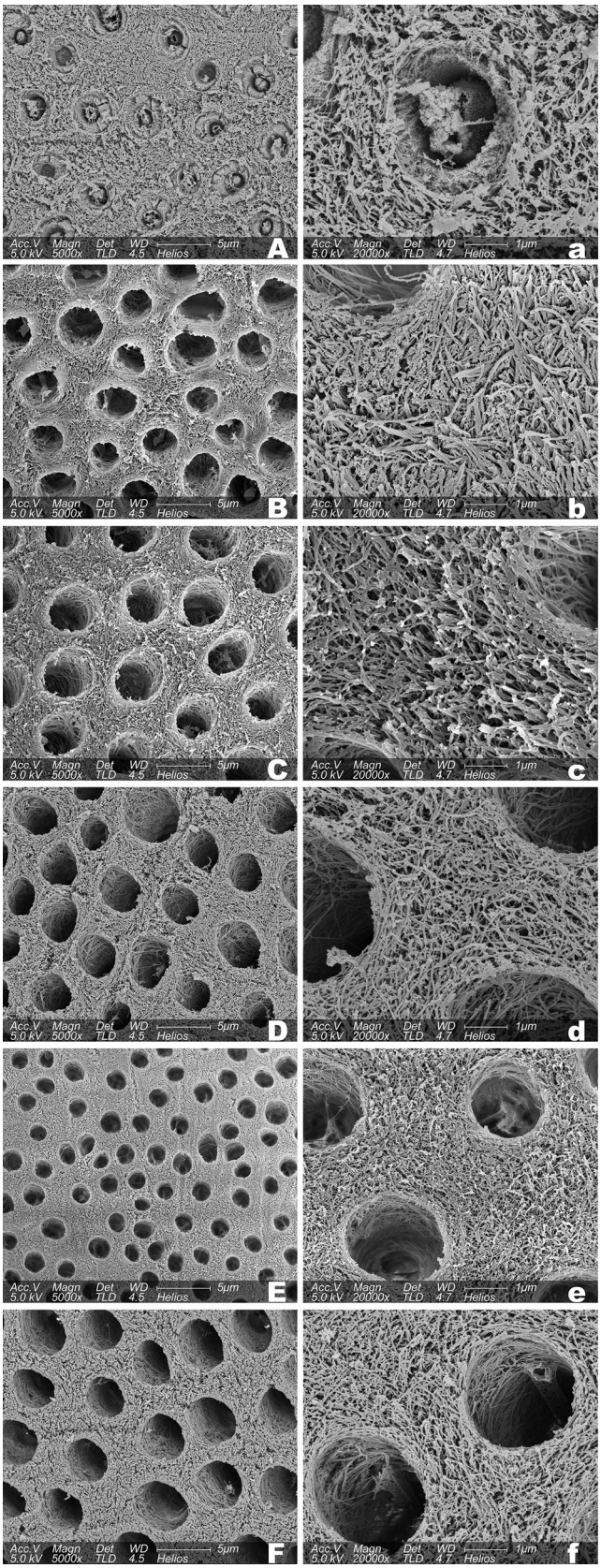

Fig. 1. Representative scanning electron microscopy images taken from dentine surfaces that were etched by different concentrations of aqueous phosphoric acid. 1% group (A, a): the collagen fibril network is only partially visible, with precipitations within the interfibrillar spaces. Peritubular cuffs are partially dissolved and smear plugs are not completely removed from the dentinal tubular orifices. 5% group (B, b): the diameter of the exposed collagen fibrils are apparently larger than those present in the other groups etched with higher concentrations of phosphoric acid. 10% group (C, c): surface morphology is similar to that of 5% H_3PO_4 group, except that the apparent diameter of the collagen fibrils is smaller qualitatively. 20% group (D, d), 30% group (E, e), 40% group (F, f): similar features are seen in these groups. Smear plugs and peritubular cuffs are completely removed and extensively shrunken collagen fibrils can be identified in the intertubular dentine with minimal precipitates present in the interfibrillar spaces.

The exposed collagen matrix exhibited a cleaner, precipitate-free appearance in the 5% H_3PO_4 group (Fig. 1B). In this group, the collagen fibrils were able to support their own weight without collapsing even when examined using dehydrated and high vacuum conditions (Fig. 1b). The dentine surface morphology in the 10% H_3PO_4 group was similar to that observed in the 5% group except that the diameter of collagen fibrils decreased due to shrinkage of the mineral-sparse fibrils. Characteristic morphologies of the 5% and 10% group were not observed in the three higher H_3PO_4 concentration groups. The apparent diameter of the collagen fibrils was considerably lower in the 20%, 30% and 40% H_3PO_4 groups, with splitting of the ends of individual fibrils (Fig. 1D-F/d-f).

3.2. Energy dispersive X-ray analysis

The Ca-content ratios of dentine surfaces etched with different concentrations of phosphoric acid are listed in Table 1. The Ca-content ratios decreased from 54.71% to 17.38% when the concentrations of phosphoric acid increased from 1% to 30%. The values in these groups were significantly different from one another ($P < 0.05$). The Ca-content ratios increased to 31.99% when the concentrations of phosphoric acid increased to 40%; these values were not significantly different from the values obtained for the 10% and 20% H_3PO_4 groups ($P > 0.05$).

3.3. Atomic force microscopy-based nanoidentation

Sequential polishing with up to 4000-grit silicon carbide paper resulted in removal of the smear layer and smear plugs, exposure of the tubule orifices, peritubular dentine and intertubular dentine. These features were confirmed with atomic force microscopy using the tapping mode on a $10 \times 10\ \mu m^2$ field area (Fig. 2A and B, images of the unetched group). These images helped to ensure that indentations were always positioned on intertubular dentine that was devoid of the smear layer.

Fig. 2C represents the typical load-displacement curve of multi-cycling nanoidentation on etched dentine surfaces. Hysteresis loops, attributed to viscoelastic behaviour [16,18], are observed between the loading and unloading curves. Multi-cycling delivers a set of data that includes the entire material response, from the first indenter-specimen contact to the maximum penetration [19]. Because of the viscoelastic properties of the demineralised dentine matrix and the strong surface roughness effect, the Er values were not stable within 30–60 nm of indenter displacement. Those surface effects disappeared with increase contact pressure and the increase in depth-displacement resulted in larger contact areas and decreased modulus values [20]. As shown in Fig. 2D, representative Er values increased abruptly at the first indenter-specimen contact and then reduced to a relatively constant level. To obtain reliable and unbiased results, the more consistent values obtained from deeper displacements were averaged to generate mean values representative of a particular indentation episode.

Because the deformed three-dimensional domains of protein molecules cannot completely recover during cyclic indentation, the results obtained from multi-cycling indentation are usually lower than those derived from using the single-cycle mode [16]. In the present work, sound, unetched intertubular dentine had a Er value of 19.65 ± 2.43 GPa, which is slightly less than the 20.1 GPa

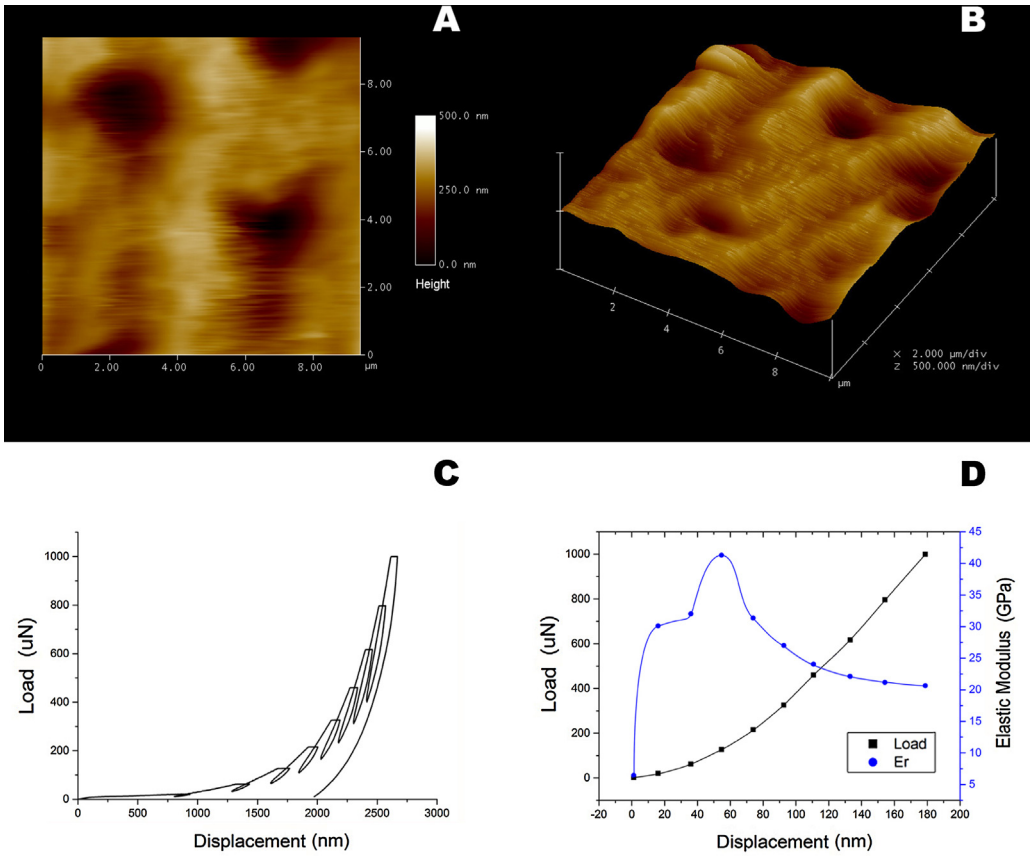

Fig. 2. (A and B) Atomic force microscopy mappings performed using the tapping mode, confirming that the smear layer was removed through sequential polishing with silicon carbide papers of increasing fineness. (C) Representative load-displacement curve generated from multi-cyclic nanoindentation of the demineralised dentine surface. Hysteresis loops can be identified between loading and unloading. (D) Increasing the indentation load of the Berkovich indenter results in an abrupt increase in the reduced elastic modulus (Er) the first 30–60 nm of indenter displacement that subsequently reduces to a relatively constant value with further increases in the displacement load.

reported by Kinney et al. using single-cycle indentation. Among the experimental groups, specimens etched with 1% H_3PO_4 exhibited the highest Er within the partially demineralised dentine matrix (2.04 ± 0.29 GPa). This was followed by specimens etched with 5% H_3PO_4 (1.00 ± 0.22 GPa). Those values were all significantly higher than values obtained from the other groups with higher H_3PO_4 concentrations ($P < 0.05$). Of those values, the lowest Er value was obtained from the 30% H_3PO_4 group (0.19 ± 0.05 GPa).

3.4. Microtensile bond strength

Fig. 3 shows the results of microtensile bond strength evaluation. The 5% H_3PO_4 group had the highest tensile bond strength (66.2 ± 1.14 MPa), which was significantly higher than the values obtained from all the other groups ($P < 0.05$). The tensile bond strengths obtained for the 10% H_3PO_4 group (61.5 ± 1.2 MPa) and the 20% H_3PO_4 group (59.5 ± 1.2 MPa) were not significantly different from one another ($P > 0.05$). Values obtained for the 1% H_3PO_4 group (57.2 ± 2.3 MPa), the 30% H_3PO_4 group (58.0 ± 1.9 MPa) and the 40% H_3PO_4 group (56.6 ± 1.2 MPa) were the lowest, and were not significantly different from one another ($P > 0.05$).

4. Discussion

Scanning electron microscopy imaging indicated that dentine surfaces exhibited different morphological appearance after etching with different concentrations of phosphoric acid for the same time period. Under the same dehydration and high vacuum examination conditions, the collagen fibrils are qualitatively wider in diameter when dentine was etched with 5 or 10% phosphoric acid, compared to those observed with the use of higher phosphoric acid concentrations. In the 5 and 10% H_3PO_4 groups, the collagen fibrils were erect and supported their own weight, with the absence of split ends that were commonly identified in the higher concentration groups. These results suggest that collagen fibrils are stronger in the 5 or 10% H_3PO_4 groups. Hence, the first null hypothesis that "there is no difference in dentine

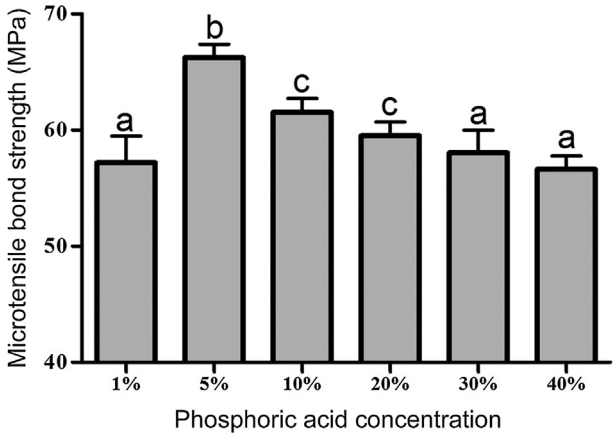

Fig. 3. The results of microtensile bond strength test. Values are means and standard deviations (N = 16). Columns identified by different letters are significantly different ($P < 0.05$).

surface morphology after the application of different concentrations of phosphoric acid" has to be rejected.

The difference in surface morphologies in the 5% or 10% H_3PO_4 groups is likely to be attributed to incomplete removal of intrafibrillar apatite crystallites. Kinney and colleagues reported that dentine etching proceeded in two stages; about 70–75% of the mineral component was demineralised at a rapid rate, while the remaining minerals were removed slowly [21]. Since intrafibrillar minerals account for 25–30% of the total mineral content in mineralised collagen [14], the two-stage etching process may be attributed to the loss of intrafibrillar minerals at a substantially slower rate compared with extrafibrillar minerals. This hypothesis was subsequently confirmed by Balooch et al. using atomic force microscopy-based nanoindentation, Raman spectroscopy and small angle X-ray scattering [22]. Whilst additional confirmation with the use of transmission electron microscopy is strongly desirable, it is reasonable to speculate at this stage that, the more dissolution-susceptible extrafibrillar minerals will be dissolved initially during acid-etching with phosphoric acid. As long as the acidity of the etchant is lowered by reducing the concentration of phosphoric acid to a certain point, it is theoretically possible to selectively demineralise the extrafibrillar minerals and keep the intrafibrillar minerals at least partially, if not completely intact. The results of the energy dispersive X-ray analysis in the present study also supported this hypothesis, which showed that the Ca-content on the etched dentine surfaces was much higher in the low H_3PO_4 concentration groups than in high H_3PO_4 concentration groups.

Dentine is composed of 30 vol% protein, predominantly in the form of type-I collagen fibrils, 50 vol% mineral and 20 vol% water-based fluid. The mineral phase which reinforces the organic phase is conceptually divided into: 1) interfibrillar minerals that are bound to fibril surface and separate the mineralised collagen fibrils from one another, and 2) intrafibrillar minerals which are initially formed within the gap zones between the collagen triple helices and eventually extend into the spaces between the microfibrils [22,23]. Kinney and colleagues reported that interfibrillar minerals account for 67%, while intrafibrillar minerals account for only 25–30% of the total mineral content in the mineralised dentine matrix [14]. The mechanical properties of collagen fibrils are positively correlated with the degree of mineralisation. Although intrafibrillar minerals cannot be identified using scanning electron microscopy, they contribute substantially to the mechanical properties of dentine. In type-II dentinogenesis imperfecta that lacks intrafibrillar minerals within the dentine matrix, the indentation Young's modulus is only a quarter of the magnitude of normal dentine even though the mineral concentration is 70–90% of normal dentine [24].

When collagen fibrils are supported by intrafibrillar apatite crystallites, the dentine collagen matrix can be strengthened tremendously. Balooch and colleagues reported that the modulus of elasticity of mineralised dentine with variable intrafibrillar mineral contents ranges from 0.2 to 2 GPa. In contrast, the modulus of elasticity of completely demineralised dentine is 0.008–0.02 GPa [22]. In the present study, the reduced elastic modulus (Er) of the dentine surface in the 5% H_3PO_4 group is 1.00 ± 0.22 GPa, which is much higher than the Er of completely demineralised dentine matrix. This is indirect, albeit convincing proof that there are remnant intrafibrillar minerals within the collagen fibrils after etching with low concentrations of phosphoric acid. The Er value of the 5% H_3PO_4 group is also significantly higher than that of cross-linked demineralised collagen (0.4 GPa) [25]. This implies that the proposed selective etching technique may be a better alternative than the application of cross-linking agents to improve dentine bonding. Admittedly, this paradigm is speculative at best and requires further documentation and validation with future studies. Based on the results of energy dispersive X-ray analysis and atomic force microscopy-based nanoindentation, the second null that "there are no differences in the Ca^{2+} content and modulus of elasticity of dentine surfaces after they are etched with different concentrations of phosphoric acid" has to be rejected.

Intrafibrillar apatite crystallites are completely removed when dentine is etched with relatively strong acids such as 30–40% phosphoric acid. The porosities created by the loss of both interfibrillar and intrafibrillar minerals are replaced by water molecules, resulting in the presence of 70 vol% water surrounding the mineral-deleted collagen matrix. It is much easier for resin monomers to infiltrate the interfibrillar space, compared with the nanoscopical intermolecular gaps within the intrafibrillar water compartments. Luiz and colleagues opined that after etching with 37% phosphoric acid, the intermolecular space between the microfibrils of a collagen fibril is 1.26–1.33 nm [24]. The smallest resin monomer used in dental polymers such as TEGDMA has an approximate length of 2 nm. Hence, the authors argued that complete infiltration of the adhesive resin monomers into the intrafibrillar milieu is unrealistic at the molecular level [26]. Although this notion was subsequently challenged by the size exclusion experiments performed in Pashley's laboratory [27], severe restriction remains in the ability of solvated resin monomers to completely replace all the unbound intrafibrillar water, particular in the overlap region of the collagen molecules, during the formation of hybrid layer [28,29]. Demineralised dentine collagen matrices also contain negatively-charged proteoglycans, which can bind a large amount water to produce hydrogels [30]. Water-bound proteoglycan hydrogels may be responsible for "molecular sieving" of larger dimethacrylate resin monomers such as BisGMA, preventing their infiltration into the intrafibrillar compartments of collagen fibrils [22]. Thus, as long as dentine is completely demineralised, it would be very difficult for resin monomers to infiltrate and encapsulate the mineral-deleted water-rich collagen scaffold completely. At best, one may expect to have optimal infiltration of the interfibrillar spaces, while the intrafibrillar spaces is still filled with water. This scenario was reported in hybrid layers created by some universal adhesives when they were applied using the etch-and-rinse technique; the collagen fibrils were surrounded by a resin sheath that did not completely infiltrate the intrafibrillar spaces [31]. Entrapment of intrafibrillar water within a resin sheath provides a functional environment for endogenous proteolytic proenzymes, already activated by acids, to break down the mineral-depleted collagen molecules within the resin-ensheathed fibril. If one can selectively demineralise extrafibrillar minerals while leaving the intrafibrillar minerals intact, there would be no need for resin monomers to infiltrate the nanoscopical intrafibrillar spaces.

Within the short-term bonding conditions in the present study, significantly higher bond strength was observed in the 5% H_3PO_4 group. This is likely attributed to the reinforcement of the partially demineralised collagen matrix by remnant intrafibrillar minerals and better intrafibrillar infiltration of resin monomers. In this group, the predominant mode of failure was cohesive failure within resin or dentine (data not shown), indicating the actual interfacial bond strength should be higher. Thus, the third null hypothesis that "there are no differences in the tensile bond strengths of resin-dentine bonds prepared from etching dentine with low (below 10%) or high concentrations (10–40%) of phosphoric acid" has to be rejected. The relationship among the residual Ca content, Er and microtensile bond strength is summarised in Fig. 4. In general, increases in phosphoric acid concentration result in reductions of the residual Ca content, Er and microtensile bond strength. The only exception is the 1% H_3PO_4 group, which has the highest residual Ca content and Er, while the bond strength in this group was not significantly different from the 30% and 40% H_3PO_4 group. The low bond strength obtained from

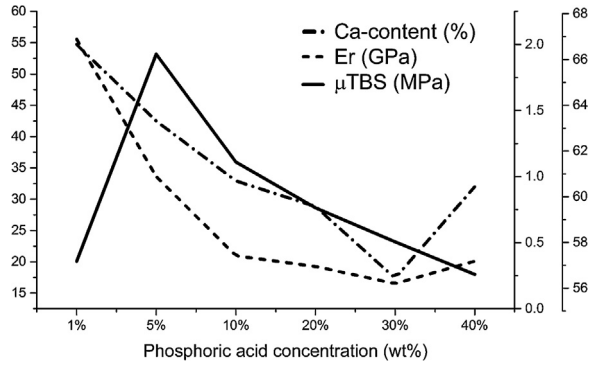

Fig. 4. The relationship among residual Ca-content, reduced elastic modulus (Er) of the demineralised dentine surface and microtensile bond strength (μTBS).

the 1% H_3PO_4 group may be attributed to the weak demineralising ability of 1% phosphoric acid and the formation of dicalcium phosphate dehydrate which were retained within collagen matrix, obstructing the microporosities and preventing optimal resin infiltration.

The use of dentine etchants dated back to the time when the presence and implication of the smear layer on dentine bonding were recognised [32–35]. Different etchants have been tested, including hydrochloric acid, maleic acid, ferric oxalate, pyruvic acid, nitric acid, low-pH EDTA and citric acid. There is no direct evidence indicating that phosphoric acid is more advantageous than the other etchants. The popularity of using phosphoric acid is probably related to its initial adoption for enamel etching. Etching enamel with 30–40% phosphoric acid was found to produce the most consistent etching patterns in prismatic enamel. About 35 years ago, Pashley had already identified the minimum time and acid concentration required to remove smear layers from cut dentine [35]. To date, the only other phosphoric acid concentrations tested are 10% and 85%. Few studies have examined the concentrations of phosphoric acid below 10%.

There are two clinically relevant questions that have to be addressed concerning the use of 5% phosphoric acid as an etchant in dentine bonding. The first question is whether low concentrations of phosphoric acid would adversely affect enamel bonding. This question had already been addressed as early as 1986 by Zidan and Hill [36]. The authors reported that etching enamel with 2%, 5% and 35% H_3PO_4 resulted in similar resin-enamel bond strengths, irrespective of the etching patterns produced. This question should be revisited using contemporary dentine adhesive systems that are considerably more hydrophilic than the enamel bonding resins available in the pre-dentine bonding era.

The second, equally salient question is whether the technique of using 5% phosphoric acid etchant followed by the application of an etch-and-rinse adhesive is redundant, in light of the clinically favourable results achieved with mild self-etch adhesives that etch and prime/bond simultaneously [23]. As the concentration of phosphoric acid is reduced to 5%, its pH value approximates those of the moderately aggressive contemporary self-etch adhesive containing acidic functional resin monomers ($pH \approx 1$; Table 1). Admittedly, the low concentration phosphoric acid etching technique involves the use of an extra step, which may not be readily appreciated by time-conscious clinicians. However, dentine bonded with self-etch adhesives are not immune to bond degradation, as seen in the use of universal adhesives in the self-etching mode [31], due to incorporation of water, hydrophilic and acidic functional resin monomers in these adhesives. Shortening the etching time for etch-and-rinse adhesives has been reported to increase bond stability in sound and caries-affected dentine of deciduous teeth [37]. In the present context of using a similar technique on sound permanent dentine, retention of intrafibrillar minerals prevents collapse of collagen fibrils via the formation of interfibrillar hydrogen bonds that prevent resin monomers from infiltrating the interfibrillar spaces. Delinquencies associated with vigorous air-drying during the application of etch-and-rinse adhesives have led to the rise of the water-wet bonding technique; the latter has been acquainted with controversial, indeterminate issues such as “how wet is wet dentine”. By retaining intrafibrillar minerals, more through air-drying and fluid displacement of the partially demineralised collagen matrix may be accomplished without the need to worry about collapsing a mineral-free collagen matrix during air-drying. More thorough fluid displacement may be achieved using the recently-reported augmented pressure adhesive displacement technique, if desired [32]. This may ultimately result in the elimination of water-wet bonding that has been the predominant technique employed for bonding of etch-and-rinse adhesives for decades. When fossilised by intrafibrillar minerals [23] within a milieu that contains only tightly-bound water to maintain the integrity of collagen triple helices [29], endogenous proenzymes that reside along the telopeptide region of the collagen molecules [38] cannot be reactivated to degrade the collagen matrix. These factors, when working in concert, are conducive to the maintenance of long-term resin-dentine bond integrity.

The limitations of the present study are apparent. Despite the use of low phosphoric acid concentrations to etch dentine, bonding in all groups was performed by blot-drying the water-rinsed acid-etched dentine with moist tissue paper prior to the application of the etch-and-rinse adhesive. The feasibility of using the selective demineralisation technique with air-drying should be evaluated in future studies using water-free adhesives, to ascertain that the water-wet bonding technique can be really eliminated during the bonding process. For scanning electron microscopy imaging, critical point drying was performed on all groups in order to identify the condition of the collagen fibrils after they were etched with different concentrations of phosphoric acid. While such a protocol was necessary in the present preliminary investigation, it is important to re-examine the acid-etched specimens in future studies without the use of critical point drying or drying accompanied by stiffening of the collagen fibrils with hexamethyldisilasane. Under such a condition, selectively-demineralised collagen fibrils that can support their own weight without collapsing after air- or vacuum-drying should be visible as discrete fibrillar structures when examined with a high-vacuum scanning electron microscope. In contrast, completely-demineralised collagen fibrils will adhere together to form an indistinguishable amorphous surface sheet when they were examined using the same conditions. This should provide more convincing evidence to justify that selective demineralisation of dentine is an alternative to replace the water-wet bonding technique. Energy dispersive X-ray analysis and atomic force microscopy-based nanoindentation only provide indirect evidence that intrafibrillar minerals are retained with the use of low concentrations of phosphoric acid. The notion that dentine can be selectively demineralised by lowering the acidity of the etchant requires confirmation using transmission electron microscopy, and preferably, supplemented with electron tomography and 3-D reconstruction to visualise mineral distribution within the interfibrillar and intrafibrillar volumes. Because the hydronium cation is relatively small, the etching process may not be predictably controlled. Thus, it is prudent to identify larger molecules with etching capability, such as polymeric chelating agents, to selectively demineralise dentine with precision. Selection of optimal polymeric chelating agents should be based on investigation of their size exclusion characteristics; those that are large enough to be prevented from entering the intrafibrillar

compartments of demineralised collagen fibrils should be chosen to confine the process of demineralisation exclusively to the interfibrillar spaces. Because only immediate dentine bong strength was evaluated in the present work, ageing studies should be performed in the future to validate that the "selective etching technique" improves resin-dentine bond durability.

5. Conclusion

The intrafibrillar mineral phase is responsible for the strength and stability of mineralised dentine collagen. Within the limitations of the present study, it may be tentatively concluded that lowering the concentration of phosphoric acid to 5% selectively demineralises dentine extrafibrillar minerals, reduces the need for using cross-linking agents or the incorporation of cross-linking agents in the etchant to strengthen a completely demineralised collagen matrix, and results in better short-term bond performance.

Conflict of interest

The authors denied conflict of interest associated with the materials employed in the study.

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