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Potential of high-intensity focused ultrasound in resin-dentine bonding

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ABSTRACT

Objective. This study introduced the potential and proof-of-concept of high intensity focused ultrasound (HIFU) technology for dentin-surface treatment for resin-dentin bonding without acid-aided demineralization. This new strategy could provide a way to enhance interface-integrity and bond-durability by changing the nature of dentin-substrate; bonded-interface structure and properties; and minimizing denuded-collagen exposure.
Methods. The interaction between HIFU waves and dentin-surface in terms of structural, mechanical and chemical variations were investigated by SEM, TEM, AFM, nano-indentation and Raman-analysis. The bonding between HIFU-treated dentin and two-step, etch-and-rinse, adhesive was preliminary explored by characterizing dentin-bound proteases activities, resin-dentin interfacial morphology and bond-durability with HIFU exposure at different time-points of 60, 90 and 120 s compared to conventional acid-etching technique.
Results. With the increase in HIFU exposure-time from 60-to-120 s, HIFU waves were able to remove the smear-layer, expose dentinal-tubules and creating textured/rough dentin surface. In addition, dentin surfaces showed a pattern of interlocking ribbon-like minerals-coated collagen-fibrils protruding from the underlaying amorphous dentin-background with HIFU exposure for 90 s and 120 s. This characteristic pattern of dentin-surface showing inorganic-minerals associated/aligned with collagen-fibrils, with 90-to-120 s HIFU-treatment, was confirmed by the Raman-analysis. HIFU-treated specimens showed higher nano-indentation properties and lower concentrations of active MMP-2 and Cathepsin-K compared to the acid-etched specimens. The resin-dentin bonded interface revealed the partial/complete absence of the characteristic hybrid-layer formed with conventional etch-and-rinse bonding strategy. Additionally, resin-infiltration and resin-tags formation were enhanced with the increase in HIFU exposure-time to 120 s. Although, all groups showed significant decrease in bond-strength after 12 months compared to 24 h storage in artificial saliva, groups exposed to HIFU for 90 s and 120 s showed significantly higher μTBS compared to the control acid-etched group.

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Significance. The implementation of HIFU-technology for dental hard-tissues treatment could be of potential significance in adhesive/restorative dentistry owing to its ability of controlled, selective and localised combined tissue alteration/ablation effects.

1. Introduction

The two processes that are involved in dentin-bonding are the complete/partial removal of inorganic-phase, with minimal disruption within the collagen-matrix, and substituting the voids formed by missing inorganic-content with adhesive-resins that have gone through *in-situ* polymerization [1]. This results in the formation of a hybrid-layer which is a 3-dmensional collagen/polymer-network providing a stable-link between the dentin-substrate and bulk-adhesive [2]. Despite intensive research and development in the field of adhesive-dentistry, clinical failure of resin-dentin junctions remains a challenge [3]. Incomplete resin infiltration within the demineralized dentin-matrix results in the exposure of the collagen fibrils-network which is susceptible to enzymatic degradation/hydrolysis leading to failure of the bonding-interface [4]. Accordingly, within the current acid-aided demineralization (etching) resin-dentin bonding strategies, there is a continuous chronic deterioration of the hybrid-layer involving collagen degradation, hydrolysis and leaching of resin-monomers [5]. This said, dentin organic-matrix, based on type-I collagen, is reinforced by the associated apatite-minerals. Nevertheless, the mechanical, chemical and structural integrity/durability of dentin depends on the degree of intrafibrillar/interfibrillar mineralization [6]. Given this, it could be hypothesized that the preservation of the inorganic-content of the dentin-matrix would affect the success and durability of resin-dentin bonding. Moreover, development of alternative clinically relevant strategies for resin-dentine bonding, rather than acid-aided demineralization, is prudent.

One of the new approaches is therapeutic ultrasound that has promising medical potentials and has been acknowledged to produce various bioeffects on biological tissues. Much of the work has been focused on using ultrasonic-energy to induce changes in tissues ultimately leading to therapeutic/modification effects [7]. Very curiously, high-intensity focused ultrasound (HIFU) is high amplitude ultrasound-energy that can be focused from a transducer over the overlying tissue leading to changes created by high tensile-waves inducing a cloud of bubbles [8]. The nonlinear acoustic-effects result in the formation of a shock front creating a significant mechanical stress within the tissue and coalescence of micro-cracks [9]. Further research has shown that presence of these bubbles can mechanically erode/ablate tissues by liquefying-effect [10]. Hydrodynamic pressure-gradients are generated by a stream of variable speed in which nuclei of bubbles migrate producing shock-waves, micro-streaming or micro-jets [11].

The authors are envisioning HIFU-technology for dentin-surface treatment as a potential strategy for resin dentin bonding, and thereby without acid-etching with phosphoric-acid. With the use of an *in-vitro* model, bonded-interfaces created by a two-step, etch-and-rinse, adhesive and HIFU-treated dentin were investigated. It is thought that this new strategy might provide an innovative way to enhance the interface integrity and bond-durability by minimizing denuded-collagen exposure. With the above in mind, the aim of this study was to investigate the interaction between HIFU-waves and the dentin-surface. In addition, the aim was to preliminary explore the potential of HIFU technology on resin-dentin bonding by characterizing the dentin-bound proteases activity, resin-dentin interfacial morphology and bond-durability with HIFU-exposure as a function of time. The null-hypotheses tested were: dentin-surface treatment with HIFU has no effect on MMP-2 and Cathepsin-K activities, and bond strength.

2. Materials and methods

Non-carious/non-restored human molars (n = 70) were collected from patients of age-range of 21-35y. Ethical approval was provided by the Institutional Review Board of the National University of Singapore. The extracted teeth were stored in 0.5% chloramine-T solution for 2wk then in by distilled water at 4 °C to be used within 2mo from the extraction-time. Next, they were sectioned at midcoronal-dentin into disc-shaped specimens with a low-speed diamond-saw (Buehler, Lake-Bluff, USA) under water-cooling followed by wet-grinding with 600-grit silicon-carbide paper.

2.1. *HIFU experimental-setup*

The HIFU experimental setup are presented in Fig. 1. A water tank having dimensions of 15 cm (length) × 15 cm (width) × 25 (depth) cm was used. The distilled water used was degassed by boiling for 30 min and stored in tightly sealed bottles placed at room temperature (20 °C) so that the speed of sound was constant over the experimental operation. Water was used as a medium since ultrasound propagation in water closely approximates the propagation of an ultrasound beam in tissues except for the effect of attenuation. The experimental setup consisted of a bowl-shaped piezo ceramic transducer with driving circuit consisted of a linear voltage-amplifier and a signal generator as we previously described [12]. An arbitrary waveform generator (20 MHz Function/Arbitary Waveform Generator, Agilent Technologies, Santa Clara, CA, USA) from the manufacturer was designed to provide continuous sinusoidal standard waveforms /signal to a power amplifier which in turn is connected to the transducer. Signals were run through a linear voltage amplifier (AG 1021, AG Series Amplifier, T & C Power Conversion, Rochester, NY, USA) to boost the

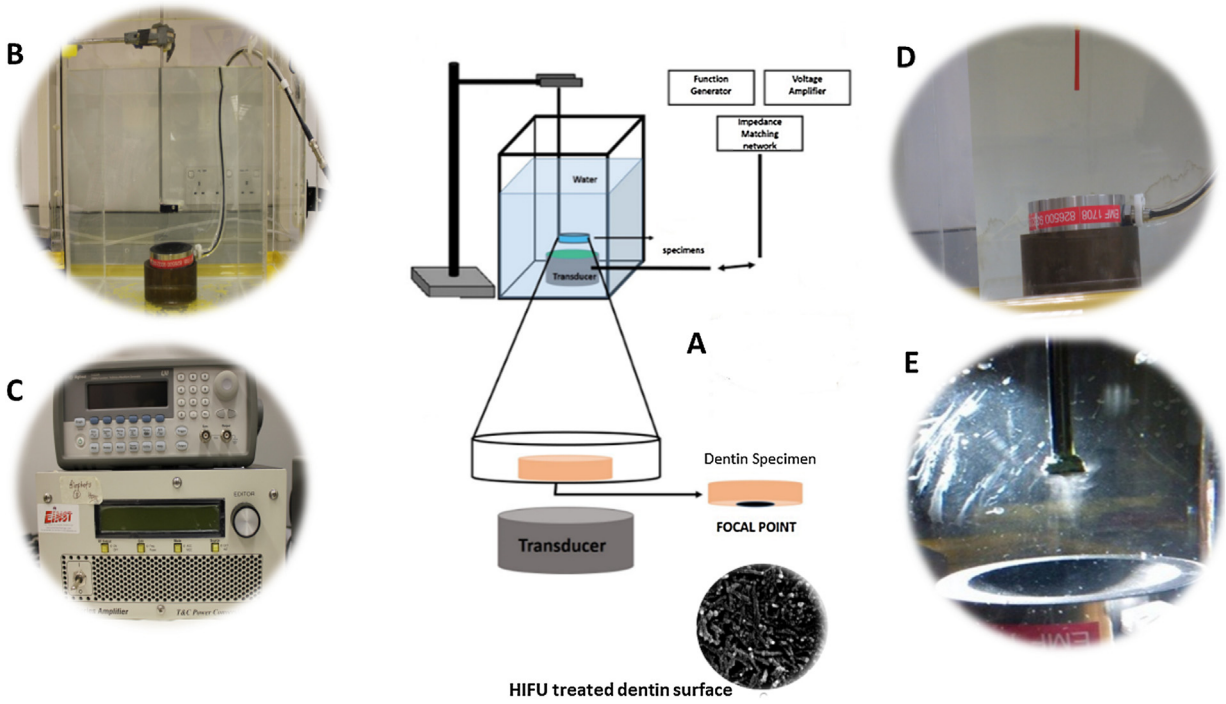

Fig. 1 – The HIFU setup presented: (A) schematic diagram of the full setup showing the transducer and the specimen holder positioned inside the water tank, (B); the driving circuit (C); the needle-hydrophone in position (D); and the exposure of dentin specimen to HIFU (E). The setup consists of a bowl-shaped transducer with the dentin specimen placed at the geometric focus and focal depth of 59.97 and 50.65 mm, respectively. The transducer is supported with a brass backing that is placed in the water tank filled until three-fourths full and driven by a circuit composed of an impedance-matching network, a voltage amplifier and a functional generator. HIFU was generated with a continuous sinusoidal wave of the 120 V amplitude and a frequency of 250 kHz.

power of the signal from the function generator. The amplifier output impedance was matched to the transducer via a matching network provided by the manufacturer of the HIFU source transducer. A piezo-ceramic, bowl-shaped, transducer having 64 mm diameter (H- 115, Sonic Concepts, Bothell, WA, USA) with a resonance frequency of 250 kHz was submerged in the water tank and placed on a brass backing. The transducer has a geometrical focus and focal depths of 59.97 mm and 50.65 mm, respectively. The excitation signal was initiated by the signal generator which gets amplified through the amplifier and then sent to the transducer. The maximum negative pressure at the focal point is measured by a PVDF needle hydrophone (RP-Acoustic, Germany). This needle type hydrophone has a nominal sensitivity of 184 mV/MPa over the 1 kHz –3 MHz frequency range. The peak negative acoustic pressures were determined using the hydrophone system connected to a TDS 420 A digitizing oscilloscope (Tektronix, Beaverton, OR, USA). The digitized signals were made to calibrate pressure values from the needle hydrophone measurements and to get correct values of acoustic pressure (in kPa). The ultrasound field in the tank is a standing wave. Along the axis of the transducer there is a conical shaped stream of cavitation bubbles. This is due to the focusing effect of the transducer. The strongest ultrasonic pressure (about 10 bar) is measured at the focus where the stream is the narrowest. The maximum negative pressure at the focal is measured by a PVDF hydrophone to be about 10 bar.

The HIFU-exposure was conducted on each dentin disc such that each specimen was attached to a metal supporting-plate using cyanoacrylate-adhesive located within the focal-point of the HIFU-transducer. Specimens were divided into three experimental-groups according to the HIFU exposure-time: 60 s, 90 s, and 120 s. Each dentin specimen was positioned to be centred at the focal point of the generated HIFU. The HIFU waves were generated by the transducer with a driving voltage of about 120 V. The source used was a sinusoidal wave of peak-to-peak amplitude of 2 V and 250 kHz. In addition, to the three HIFU experimental groups, dentin specimens etched with 35% phosphoric acid gel (ScotchbondTM Etchant, 3 M ESPE, Minneapolis, MN, USA) for 15 s or left without any surface treatment after grinding were used as controls throughout the study.

2.2. *HIFU dentin-interaction*

The HIFU-dentin interactions were investigated in terms of morphological, structural, mechanical, and chemical changes.

2.2.1. *AFM/Nanoindentation study*

The surface micro-topography and mechanical properties were investigated using an AFM/nano-indentation system. Dentin discs (n = 7; d = 1 mm) were scanned with Multimode-AFM with a J-Scanner (NanoScope-IV, Bruker) using a silicon-probe (RTESP, Bruker) with a nominal tip-radius of

8 nm, 300 kHz resonance frequency, and 40 N/m spring-constant. Nano-indentation testing was performed using a diamond-corner of a cube-shape pyramidal tip with a 40 nm radius (MDNISP-HS, Bruker) in the hydrated-condition of dentin. Up to 14 indentations were made in the intertubular-dentin region for each specimen, and with a lateral spacing not less than 400 nm. The reduced elastic-modulus (*Er*) and surface-hardness (*H*) were calculated [13].

2.2.2. SEM/TEM investigation

Dentin specimens (n = 6) were dehydrated in ascending grades of ethanol, processed, dried, sputter-coated with gold-palladium and viewed by SEM (Hitachi S-3400 N, USA) at different magnifications. For TEM analysis, the specimens were fixed, buffered with 0.1 M sodium-cacodylate for 1 h, treated with 1% osmium-tetroxide in PBS for 1 h. Next, specimens were rinsed with distilled water, dehydrated in ascending concentrations of ethanol, and finally followed by infiltration with araldite-resin. Ultra-microtomy with a diamond-knife was used to cut to ultrathin sections (~90 nm) which were stained with uranyl-acetate and imaged at 100 kV (JEOL1010, Japan).

2.2.3. Proteases detection

The concentrations of MMP-2 and Cathepsin-K in the supernatant, from dentin specimens (n = 9) exposed to HIFU (60 s, 90 s, and 120 s) or demineralised (control), were analysed by ELISA-kits using a spectrophotometer after baseline (24 h and 14 days). The pulverising of dentin powder and demineralisation of the control groups were followed from a previous protocol [14]. Human molars were ultrasonically cleaned and occlusal enamel sectioned perpendicular to the longitudinal axis using Isomet saw under water coolant. After rinsing, dentin beams with the dimensions of 6 mm × 2 mm × 1 mm were sectioned from the centre of each disc and then pulverized in liquid nitrogen into a fine powder using a steel mortar/pestle (Reimiller, Reggio Emilia, Italy). After taking five one-gram aliquot of dentin powder, the specimens were demineralised using 35% phosphoric acid for 10 s and washed for ten minutes using deionised water. Specimens which were to receive the HIFU treatment were devoid of the demineralisation procedure. The HIFU treatment was provided to the rest of the experimental groups by generating HIFU waves by the transducer with a driving voltage of about 120 V. The source used was a sinusoidal wave of peak-to-peak amplitude of 2 V and 250 kHz. Similar dentine powder aliquots were taken for specimens without demineralization.

After specific treatments, dentin powder was re-suspended in extraction buffer [50 mM Tris−HCl at pH 7.5 containing 0.2% Triton X-100, 5 mM $CaCl_2$, and 100 mM NaCl] to extract the proteases inside the vials. Later, the vials were centrifuged for 30 min at 20,000 rpm (4 °C). The supernatants were collaected, dialyzed in bags with 30 kDa molecular cut-off overnight, lyophilized and frozen at 20 °C until they were analysed. MMP-2 and Cathepsin K inside the supernatant were analysed using ELISA (Human MMP2 ELISA Kit – Lot #5619 for MMP-2; Human CTSK/Cathepsin K ELISA Kit – Lot #5614 for cathepsin K, both from Lifespan Biosciences, Seattle, WA, USA). The kits were used according to the manufacturer's instructions with the absorbance recorded with a spectrophotometer at 405 nm (Bio-Rad Laboratories, Hercules, CA, USA). All the tests were conducted in a triplicate and the protease concentrations were expressed as ng/mL.

2.2.4. Raman analysis

Chemical changes of dentin-surfaces were investigated with Raman-spectrometer (Horbia-iHR550, Edison, USA) set to a full scan-range of 200-3200 cm^{-1} having a 785 nm diode laser as an excitation-source. Four accumulations were averaged for each analysis using a 600 lines/mm grafting. A 50X-objective (N-Plan, NA¼0.75, Leica, Germany) was used to focus the light into a ~1 μm spot at 35 mW on the sample. The room-temperature and humidity were maintained at 24.5 °C and 53.5% respectively. For each specimen, two 12 μm × 12 μm areas at different sites were mapped through a 0.5 μm spacing with 10 complete overlapping Gaussian-lines for further calculations. For the centroid cluster measurements, the mineral and organic bands of the dentin-surface were specifically examined within each region (3 measurements). Baseline correction was performed by using polynomial fit to reduce the signal without losing information of small peaks and changing the peak ratio (Matlab 200 iterations, the 5th order polynomial). The mineral and organic bands of the dentin-surface were specifically examined within each specimen.

2.2.5. Resin-Dentin bonding

HIFU-treated and acid-etched dentin discs (4 mm thickness) were used for resin-dentin bonding. An etch-and-rinse adhesive system (Adper™ Singlebond, 3 M ESPE, USA) was applied according to manufacturer's instructions. Resin-composite (Filtek Z350, 3 M ESPE, USA) crowns were built-up, light-cured (600 mW/cm^2; Elipar-S10, 3 M ESPE, USA), and placed in distilled water at 37 °C for 24 h before sectioning.

2.2.6. Microscopic investigation of resin-dentin interface

The bonded specimens were sectioned, at mid-coronal dentin, occluso-gingivally into 1 mm thin dentin-slabs (n = 6) using a low-speed diamond-saw under water cooling. Dentin-slabs were wet-polished, dried, mounted on aluminium-stubs, and viewed by SEM (JEOL JSM-6701F, Japan). For confocal microscopy (CLSM), the adhesive was labelled with 0.05 wt% Rhodamine-B (Sigma-Aldrich, USA) before application. Resin-dentin slabs (n = 5) were prepared and viewed by CLSM (TCS-SL, Leica, Germany) at dual-fluorescence mode using a x40 objective and x2 electronic-zoom.

2.2.7. Bond-strength testing

The bonded specimens were sectioned across the adhesive-interface to obtain resin-dentin beams (1 mm × 0.9 mm). Beams were randomly divided (n = 21) to be stored either for 24 h or 12 months in artificial-saliva at 37 °C [15]. At each time-point, the beams were fixed to a universal testing-machine (Model-4440, Instron, USA) and exposed to a 50 N load-cell at 1 mm/min speed. The μTBS was calculated by dividing the maximum-load to the surface-area which was reconfirmed (using a digital-caliper). After debonding, the fractured surfaces were analysed microscopically to determine the mode of failure.

2.3. Statistical analysis

Data were presented as mean ± standard-deviation and analysed by the analysis of variance (ANOVA), followed by the Tukey-Kramer *post-hoc* test at a significance level of $p \leq 0.05$. Data normality was explored through test of normality (Shapiro-Wilk and Kolmogorov-Smirnov tests) and normal Q–Q plots.

3. Results

The effect of HIFU on dentin-surface by the SEM and AFM is demonstrated in Figs. 2 & 3 . With an increase in exposure-time from 60 s to 120 s, the HIFU-waves were able to remove the smear-layer, expose dentinal-tubules and create a textured dentin-surface. In addition, the dentin-surfaces showed a pattern of interlocking ribbon-like minerals coated collagen-fibrils protruding from the underlaying amorphous dentin background when the HIFU exposure was for 90 s and 120 s. This characteristic pattern of dentin-surface showing inorganic-minerals associated and aligned with collagen-fibrils (with 90 s-to-120 s HIFU treatment) is also revealed by the high magnification SEM (Fig. 2G & H), and TEM (Fig. 3D & E). Moreover, this was confirmed by the Raman-analysis (Fig. 4C) through the accentuation of the phosphate stretching-bands identified at 960 cm^{-1} compared to the acid-etched specimens. Despite of the gradual reduction in mechanical surface-properties reflected by nanoindentation testing (compared to the untreated control specimens), the HIFU-treated specimens showed higher reduced elastic-modulus and hardness compared to the acid-etched specimens (Fig. 4A). However, there was a decrease in nanoindentation properties with the increase in HIFU exposure-time reflecting the gradual minerals removal-effect with the HIFU treatment. Despite of the interaction between the HIFU-waves and dentin-surface, collagen-fibrils preserved their intact structure. Several peaks were observed at 1246 cm^{-1}, 1665–1667 cm^{-1} and 1450 cm^{-1} and they were assigned to the amide III, amide I [16] and C—H alkyl-groups [17] by the Raman-analysis respectively (Fig. 4C).

SEM investigation of the resin-dentin bonded interface (Fig. 5A–E) revealed the partial-to-complete absence of the characteristic hybrid-layer formed with conventional etch-and-rinse bonding strategy. Moreover, resin-infiltration and resin-tags formation were enhanced with the increase in HIFU exposure-time to 120 s as also supported by CLSM investigation (Fig. 5F). The results of μTBS (Table 1) showed no difference between dentin specimens bonded using the etch-and-rinse strategy and those bonded after 120 s HIFU-exposure, after 24 h storage. However, there was a gradual reduction in bond-strength with the decrease of HIFU exposure-time to 60 s. Although, all groups showed significant decrease in bond-strength after 12 months compared to 24 h storage, the groups exposed to HIFU for 90 s and 120 s showed significantly higher μTBS compared to the acid-etch control. All specimens showed combination of different resin-dentin failure mode at both time-points. In addition, all HIFU-treated groups showed lower concentrations (ng/mL) of MMP-2 and Cathepsin-K compared to the acid-etched and mineralised groups (Fig. 4B). However, there was no significant difference between the three experimental HIFU-treated groups at 14 days. Increasing the HIFU effect had no significant effect on cathepsin K activity in specimens. All experimental HIFU groups showed a slight increase in MMP-2 levels which were significantly different than the mineralised dentin groups ($p < 0.05$).

4. Discussion

We have preliminary investigated the interactions of HIFU with the dentin surface and its potential as an alternative surface-treatment strategy for resin-dentin bonding. The gradual ablation-effect of HIFU on dentin surface was dependent on the HIFU exposure-time (Fig. 2 & 3). Within the applied HIFU parameters which all were kept constant, except for exposure-time, HIFU exposure for 120 s showed the maximum ablation-effect on dentin-surface. Phosphoric acid-etching showed a typical pattern of demineralized dentine-surface with exposure of network of denuded collagen-fibrils which was morphologically different than HIFU-treated dentin-surfaces. The formation of interlocking ribbon-like collagen-fibrils protruding from the underlaying amorphous dentin background with the HIFU-exposure was a characteristic morphological pattern especially with the HIFU-exposure for 90–120 s. This might be attributed to the gradual mechanical-removal effect with the HIFU-exposure. However, although of the interaction between the HIFU-waves and dentin-surface, collagen-fibrils preserved their intact structure with clear evidence of inorganic-minerals aligned and associated with the collagen-fibrils and inter-fibril spaces. This was confirmed microscopically (Fig. 2G & H) and (Fig. 3D & E); and spectroscopically (Fig. 4C).

Mineralisation of dentin collagen-fibrils account for their mechanical properties [18]. The mineralized collagen-matrix with a low mineral content is less effective for load-transfer [19] due to the alteration of stress-strain behaviour of the collagen microfibril structure. The formation of salt-bridges between the mineral crystals and collagen provides an eff ;ective load-transfer mechanism between the mineral/organic phases that also enhances energy dissipation [20,21]. Given this, in the current study it was necessary to determine the mechanical properties at the nanoscale with the HIFU-treatment. The previous might explain the higher nanoindentation properties reported with the HIFU treated specimens compared to acid-etched dentin specimens (Fig. 4A) which additionally indirectly reflect the higher mineral content of dentin-surfaces after the HIFU-exposure. The gradual decrease in the mechanical properties of dentin-surface from the sound untreated-dentin (control) and with the increase in the HIFU-exposure time from 60 s-to-120 s, support the gradual mineral ablation potential of acoustic HIFU-waves. Any damage on the focussed area is not expected during treatment with HIFU, as the ultrasound energy passing through the intervening structure is of lower acoustic energy and does not hamper the substrate mechanically. Therefore, adverse effect with HIFU treatment are rare [22]. In contrary, lasers have been previously reported to cause damage in the form of dentin charring or cementum melting [23].

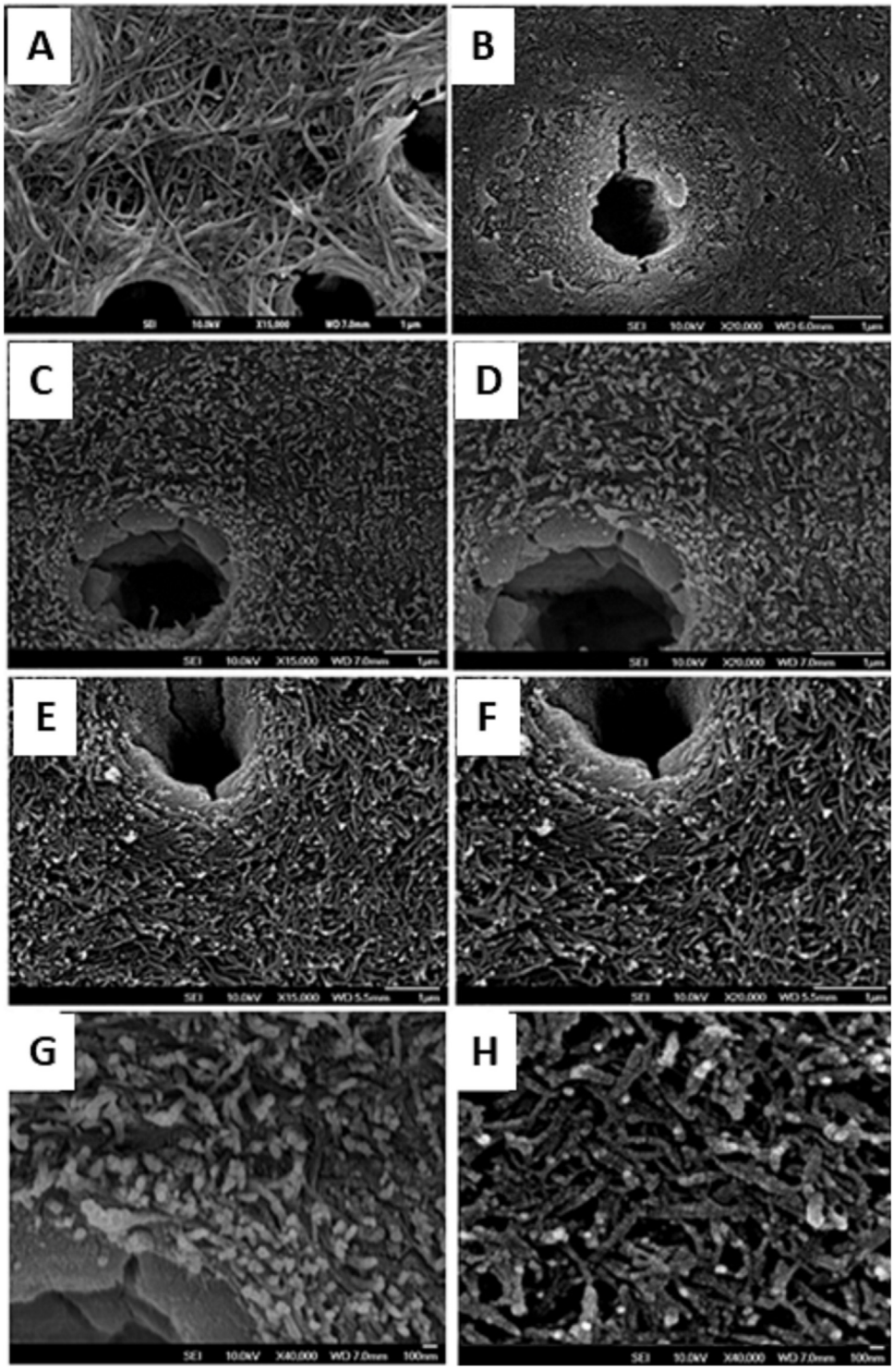

Fig. 2 – (A) SEM micrograph of coronal dentine specimens treated with conventional 35% phosphoric acid gel showing typical demineralized dentin collagen network structure with open interfibrillar spaces at the intertubular dentin and widely opened dentinal tubules with the absence of peritubular dentin. SEM micrograph (B) of specimens exposed to HIFU for 60 s showing more homogenous mineralized dentin surface with partial removal of the smear layer and exposure of dentinal tubules, however, the peritubular dentin structure is clearly preserved. With HIFU exposure to 90 s, SEM revealed (C & D) the removal of the smear layer with the exposure of what is look like mineralized collagen fibrils protruding from the underlaying amorphous dentin background creating coarser textured/rough dentin surface. With the increase of the HIFU exposure to 120 s (E & F), the exposure of the minerals-coated collagen fibrils structure become more visible leading to higher and rougher dentin surface texture and more widely opened dentinal tubules. High magnification SEM images of intertubular dentin surface treated with HIFU for 90 s (G) and 120 s (H) showing the interlocking ribbon-like structure of dentin collagen fibrils coated with the inorganic-minerals.

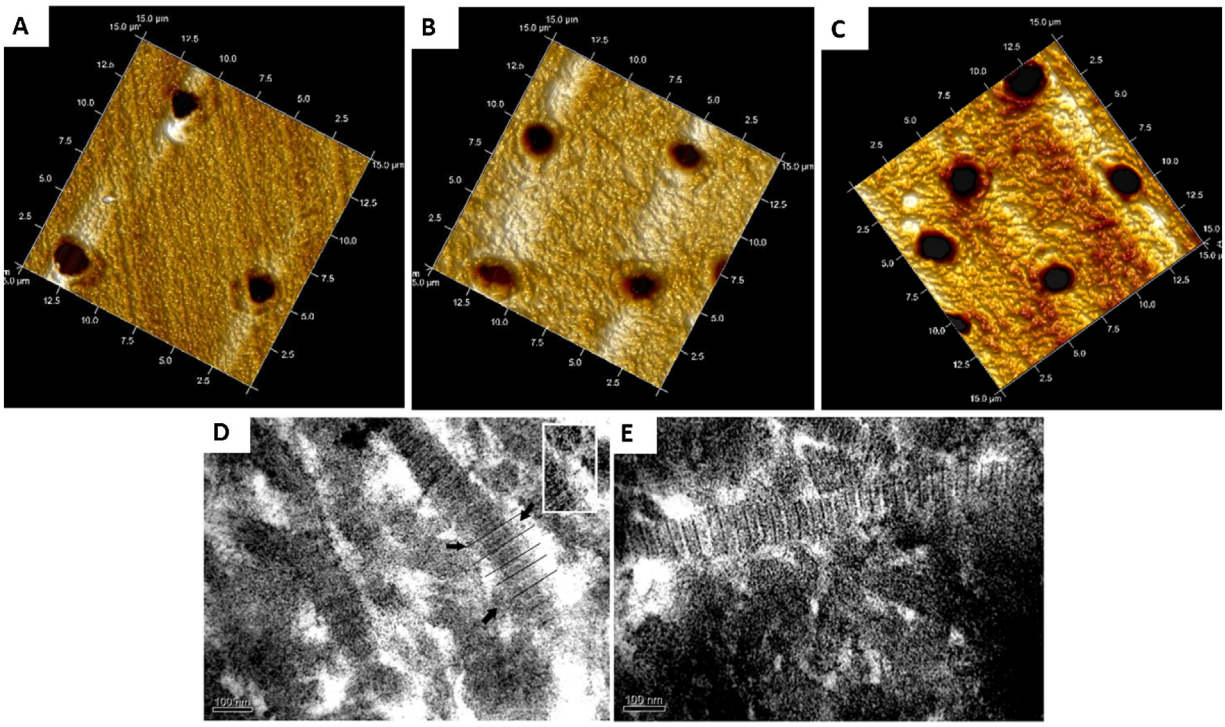

Fig. 3 – AFM 3D images of the HIFU treated dentin specimens for 60 s (A), 90 s (B) and 120 s (C). AFM imaging showed the formation of fine-textured dentin surface with 60 s HIFU exposure with the exposure of open dentinal tubules (A). With the increase in HIFU exposure time, coarser textured dentin surfaces were resulted with widely open dentinal tubules (B & C). TEM investigation (D & E; bar: 100 nm) revealed minerals association and alignment with collagen fibril's axis and D-spacing (black arrows) and at the interfibrillar spaces for dentin specimens treated with HIFU for 120 s.

Table 1 – Estimated micro-tensile bond strength (μTBS) in MPa and percentage distribution of failure mode after 24 h and 12 months storage in artificial saliva. Within each column for μTBS, groups identified by different upper-case letters are statistically different ($p \leq 0.05$). Within each row groups identified by different symbols († and ‡) are statistically different ($p \leq 0.05$).

Group	HIFU Treatment		Failure Mode μTBS (MPa)			
			24 h	12 months	24 h	12 months
I	Control (acid-etched)	A	15	40	37.21 ± 6.3^{AC} †	21.33 ± 7.9^{A} ‡
		M	35	31		
		CD	25	11		
		CC	25	18		
II	HIFU 60 s	A	32	39	26.77 ± 5.1^{B} †	19.61 ± 6.1^{A} ‡
		M	31	44		
		CD	15	9		
		CC	22	8		
III	HIFU 90 s	A	28	32	34.82 ± 5.3^{A} †	27.21 ± 5.2^{B} ‡
		M	45	31		
		CD	13	15		
		CC	14	22		
IV	HIFU 120 s	A	17	27	39.39 ± 4.6^{C} †	34.91 ± 4.8^{C} ‡
		M	41	39		
		CD	19	7		
		CC	12	27		

Failure mode: A = adhesive; CD = cohesive failure in dentine; CC = cohesive failure in resin composite; M = mixed failure.

Moreover, the preservation of the collagen proteins and their structural integrity and the lower expression of active dentin-bound proteases are critical for the potential application of HIFU technology as an alternative to acid-etching. There was a gradual increase in the C—H and amide-band signal regions (Fig. 4C) of the HIFU-treated groups which could support the stability of collagen protein structure after the HIFU interaction with the dentin-surface. The shifts within the amide-regions reflected the stability of collagen structures because of hydrogen-bonding between the prolines

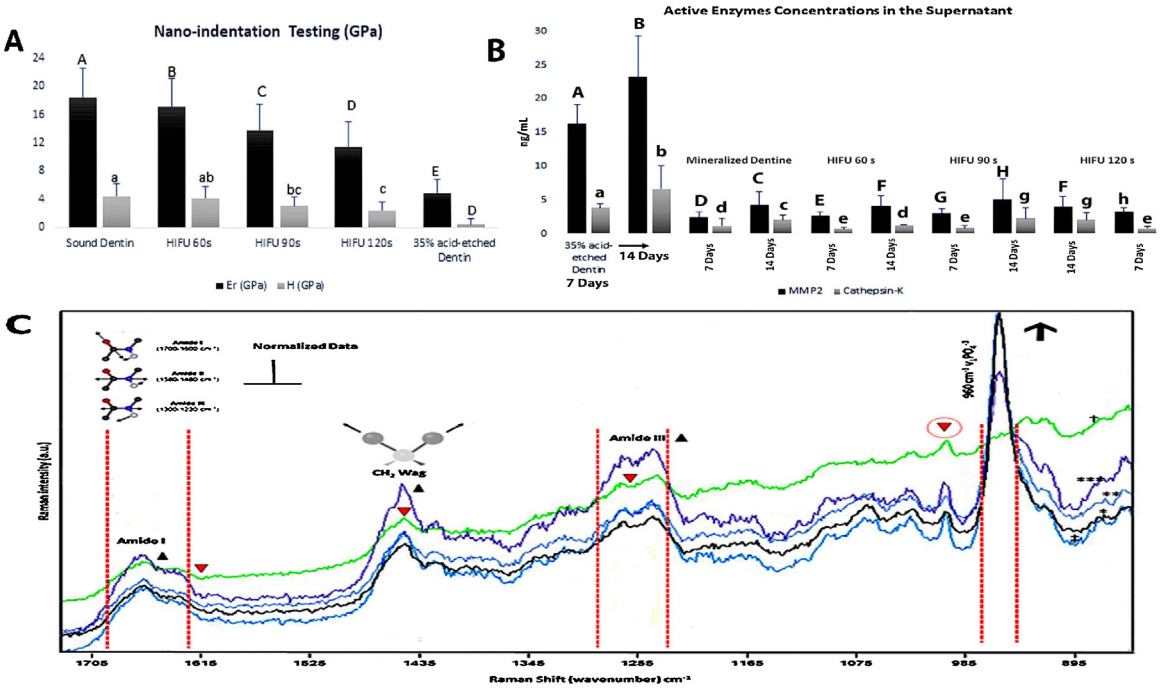

Fig. 4 – (A) Bar-chart showing mean ± standard deviation of the variations in the surface mechanical properties tested by nano-indentation in terms of reduced elastic modulus (E_r) and hardness (H) of the control groups (sound untreated and 35% phosphoric acid etched dentin) and the three experimental HIFU treated groups. Upper-case and lower-case alphabets represent statistical significance at a significance level of $p \leq 0.05$ for reduced elastic modulus (E_r) and hardness (H) respectively. (B) Bar-chart showing mean ± standard deviation of the concentration of active MMP-2 and Cathepsin-K activities (ng/mL) of the phosphoric acid-etched dentin, mineralized dentine and three HIFU treated experimental groups. Upper-case and lower-case alphabets represent statistical significance at a significance level of $p \leq 0.05$ for MMP-2 and Cathepsin-K respectively. (C) Raman spectroscopic analysis obtained from different dentine specimens acquired in the region of 800 cm^{-1} and 1800 cm^{-1} for the Control (sound dentin [‡] and phosphoric acid-etched dentin [†]) and the three HIFU experimental (at 60 [*], 90 [**] and 120 s [***] exposure) groups. The P—O stretching within the dentin minerals is identified at 960 cm^{-1}. The shown spectroscopic region representing the characteristic bands of minerals is most intense to hydroxyapatite showing symmetric phosphate stretching labelled as PO_4^{3}; v_1. Additionally, several peaks were observed at; 1246 cm^{-1}, 1665-1667 cm^{-1} and 1450 cm^{-1} were assigned to the amide III, amide I (Xu et al. 2012) and C—H alkyl group (Ozaki et al. 1992) respectively (red arrows head). The Raman bands scanned across the dentine surface at 960 cm^{-1} (P—O peak) and 1450 cm^{-1} (C—H peak) are assigned to phosphate vibrations of hydroxyapatite and the C—H alkyl group. The phosphate Raman peaks were more accentuated for the sound dentin and the HIFU treated dentin compared to the phosphoric acid demineralized dentin confirming higher inorganic minerals content of dentin surface layer. The amide I region at 1665-1667 cm^{-1} is related to the secondary structure of protein dominated by the α-helix and centred around 1667 cm^{-1} with a shoulder at ~1636 cm^{-1} in our experiment within the control groups and the experimental HIFU groups. Along with the amide bands, the C–H stretching band of alkyl moieties is one of the most widely observed bands. It is also considered another finger print region to investigate the proteins. There was a gradual increase in the C—H and amide band regions of all HIFU treated groups which might indicate that the collagen proteins structure was stable after the use of ultrasound waves on the dentine surface. The pyridinium ring vibration at 1032 cm^{-1} showed no remarkable changes in all groups (circled red arrow head) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

and glycines [24,25]. Additionally, the HIFU-treated specimens indicated a sharp well-defined band at 1450 cm^{-1} (CH_2 wagging) that appeared at a lower intensity among the acid-etched specimens. It was reported previously that the denuded dentin collagen-fibrils after acid-etching are subjected to challenge by active endogenous enzymes, namely MMPs and cysteine cathepsins [26,27]. The lower active enzyme concentrations (MMP2/Cathepsin-K) reported with the HIFU-treatment (Fig. 4B) might be attributed to the preservation of apatite crystallites associated with collagen-fibrils and maintenance of the pH-level through an acid-free dentin treatment protocol. However, further studies will be needed to fully describe the mechanism of HIFU on dentin-bound proteases.

In the current study, a step wise increase in the HIFU exposure time (from 60 s to 120 s) was made to investigate the gradual ablation-effect on the dentin-surface. It is noteworthy that HIFU is generated using a focusing transducer which gives high intensity at its focal-point with minimal damage to

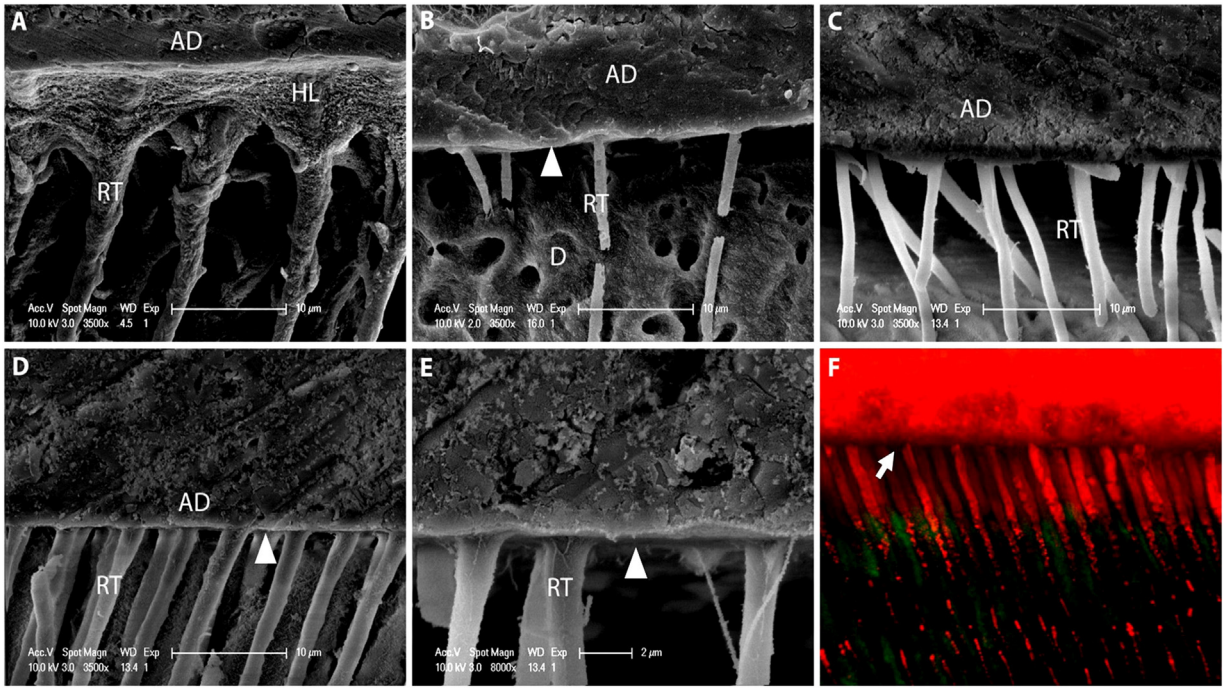

Fig. 5 – Representative SEM micrograph of resin-dentin interface of specimens treated with (A) 35% phosphoric acid gel showing typical hybrid layer and well-formed funnel-shaped resin tags. Representative SEM micrograph of resin-dentin interface of specimens exposed to HIFU for 60 s (B) showing absence of hybrid layer (white arrows) with the formation of few resin tags. In addition, the resin tags exhibited more cylindrical structure due to the preservation of the peritubular dentin. Similar pattern is found when the HIFU exposure is increased to 90 s (C), however, more resin tags could be observed indicated better resin tubular infiltration. Representative SEM micrographs, at two different magnifications, of resin-dentin interface of specimens exposed to HIFU for 120 s (D & E); showing similar interface morphology with complete/partial absence of the hybrid layer typical structure, however, numerous well-formed resin tags are clearly visible reflecting the efficient smear layer removing effect with the exposure of opened tubular structure with higher resin infiltration as supported with the associated confocal microscopy image of the resin-dentin interfacial junction (F).

the surrounding tissues. The fundamental principle behind is the formation of cavitation bubbles [28]. These bubbles are in a non-equilibrium state and will oscillate and collapse releasing high speed jets with kinetic energy towards the adjacent surfaces, such as dentin. They generate shock waves which can be utilized for various localized and controlled tissue alteration and ablation effects [29]. The said effects depend upon both parameters of HIFU operation and the tissue being treated. HIFU parameters, specifically exposure-time and intensity at the focal-point, lead to varying effects on a tissue being targeted. Moreover, different amounts of acoustic-energy will be absorbed by different tissues and/or phases depending on their properties resulting in different biological effects. From the previous, as dentin is a biological-composite of collagen/minerals phases having different physicochemical properties and mechanical-shock absorption capacities, the resulted changes of dentin-surface following HIFU-exposure might be partially explained. However, more in-depth investigations of the interaction between HIFU and dentin-surface taking in consideration the other HIFU parameters, and the variations in dentin-substrate such as age and/or caries related changes, not investigated in this study, are essential to control the selective dentin-surface alteration/ablation for future potential applications in restorative/preventive dentistry. Furthermore, the two main mechanisms for tissue alteration/ablation by HIFU are the thermal and non-thermal pathways through acoustic-cavitation, radiation-pressure and acoustic-streaming [30]. Although it was not reported in the current study, we have previously investigated the variations in temperature following the HIFU exposure having similar parameters [12]. The temperature rise was approximately 1 °C at the time intervals between 15 and 120 s of HIFU-exposure. However, further investigations are vital finding a clinically relevant setup.

The main concern of current dentin-bonding techniques, based on acid demineralization, is the loss of integrity of the bonded-interface and bond-strength. This may be due to the degradation of one (or both) component(s) of the hybrid-layer or adhesive resins and collagen fibrils [31]. Suboptimal resin infiltration of denuded collagen fibrils is very common, especially with etch-and-rinse adhesives [32]. SEM/CLSM investigations (Fig. 5) revealed the morphological pattern of the resin-dentin bonded interface created after the HIFU exposure which was clearly different from the typical hybrid-layer and funnel-shaped resin-tags formed with the traditional acid etching based bonding technique. In addition, the resin infiltration and resin-tags formation were consistent with (and dependent on) changes on the dentin-surface. These observed

changes were associated with the HIFU exposure at different time periods and they were showing better infiltration with the HIFU exposure for 90–120 s. Now, the HIFU exposure for 90–120 s did not show higher μTBS values after 24 h compared to the acid etched control group (Table 1). That said higher bond strength values were found after 12 months storage, especially with 120 s of HIFU-exposure. This might be partially attributed to the higher dentinal organic-matrix degradation resistance due to the preservation of minerals associated with collage fibrils - and to the lower proteases' expression found with the HIFU treatment. In our study, MMP-2 levels began to increase on the 3rd day and declined on the 14th day. As the expression of proteases gets decreased, the arrangement of collagen fibers becomes more orderly [33]. HIFU results in generation of heat and heating beyond a threshold as seen in our mechanical properties of the study can lead to fracturing of the collagen structure [34] resulting in dentin stiffness. Collagen is able to form bridging bonds to other collagen fibers at minimal heat [35] and this is only possible if the free ends of collagen are physically adjacent and there is less effect of proteases. This ordered formation of structure is due to minimal exposure to HIFU, and further leads to an improvement in mechanical properties.

The inferior μTBS values found with the 60 s HIFU exposure (Table 1), could be related to the partial removal of the smear-layer and the relatively fine dentin surface texture (Fig. 2B & 3A) owing to limited dentin-surface ablation as reflected from the observed poor resin infiltration (Fig. 5B). At this point, our investigation of the nature and performance of the bonded-interface between the HIFU treated dentin and adhesive resins considered at the early stage. Nevertheless, further investigations are required. However, within the results, it could be postulated that the mechanism of the interfacial attachment is mainly micromechanical-interlocking between infiltrated resin monomers and the protruded mineral-coated collagen fibrils. AFM scanning (Fig. 3A–C) showed an increase in the dentin-surface roughness with the HIFU exposure. However, quantification of roughness-parameters was not included in this laboratory study. Although the micromechanical-attachment concept does exist in conventional acid-etching technique, the most important aspect is to bond to more stable dentin substrate which would have less-to-no exposed denuded collagen network. This could minimize the well documented degradation process of the bonded interface associated with the acid demineralization strategy. However, the reduction in μTBS after 12 months reported for the 90–120 s HIFU exposure (Table 1) might be partially due to the hydrolysis of resin-phase of the bonded-interface. Given this, it should be noted that the commercial adhesive system used in this study (with its hydrophilic monomer content) is designed to first wet and then infiltrate the acid demineralized dentin substrate which is different from the HIFU-treated dentin substrate. The potential of having chemical bonding contribution with the preserved apatite crystallites associated with collagen fibrils should not be neglected. Future research is expected to investigate the formulation of more stable and well defined hydrophobic and functionalized resin monomers to bond to the highly mineralized HIFU-treated dentin.

Within the results of this study, the null-hypotheses tested should be rejected. The implementation of HIFU technology for dental hard-tissues treatment could be of potential significance in adhesive/restorative dentistry owing to its ability of controlled, selective and localised combined tissue alteration/ablation capabilities and the previously reported biofilm-eradication effect [12]. The successful applications of HIFU in different medical applications led to the engineering of more clinically suitable HIFU devices commercially-available which could be adopted and modified to suite dental-clinical requirements if proved to be significant.

5. Conclusion

This study introduced the proof-of-concept, interaction and potential of high intensity focused ultrasound (HIFU) for the first time, to our knowledge, as a tool for dentin-surface treatment for resin-dentin bonding.

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