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ORIGINAL RESEARCH

Polyphenols Can Improve Resin-Dentin Bond Durability by Promoting Amorphous Calcium Phosphate Nanoparticles to Backfill the Dentin Matrix

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Objective: To investigate the effects of proanthocyanidins (PA), myricetin, resveratrol, and kaempferol on the modification of dentin collagen and the inhibition of matrix metalloproteinase (MMP) activity, and to evaluate their contributions to the biomimetic remineralization and resin-dentin bonding performance.

Methods: Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) and in situ zymography were applied to verify the collagen modification and MMP activity inhibition induced by these four polyphenols. Scanning electron microscopy/energy dispersive spectrometer (SEM/EDS) analysis, X-ray diffraction (XRD), ATR-FTIR, Vickers hardness numbers (VHN), and micro-computed tomography (micro-CT) were performed to characterize the remineralized dentin. Microtensile bond strength (μTBS) and nanoleakage were investigated to evaluate the effects of the four polyphenols on resin-dentin bonding durability.

Results: ATR-FTIR and in situ zymography confirmed that these four polyphenols could modify dentin collagen and inhibit MMP activity, respectively. Chemoanalytic characterization exhibited the efficacies of the four polyphenols in promoting dentin biomimetic remineralization. The surface hardness of PA-pretreated dentin was the greatest. Micro-CT results demonstrated that the PAs group possessed the highest amount of dentin surface minerals and the lowest amount of deep-layer minerals. The surface and deep-layer mineral contents of the Myr group were higher than Res and Kae groups. Treatment with these four polyphenols significantly increased the initial μTBS compared with the control group without primer conditioning. μTBS decreased significantly during aging, and the decrease was more severe in the PAs and Kae groups than in the Myr and Res groups. With or without aging, the polyphenol groups exhibited relatively less fluorescence. However, the Myr and Res groups showed less serious nanoleakage after aging.

Conclusion: PA, myricetin, resveratrol, and kaempferol can modify dentin collagen, inhibit MMP activity, promote biomimetic remineralization, and improve resin-dentin bond durability. Compared with PA and kaempferol, myricetin and resveratrol are more effective in improving resin-dentin bonding.

Keywords: polyphenols, collagen, amorphous calcium phosphate nanoparticles, remineralization, resin-dentin bonding

Introduction

The dynamic process of demineralization-remineralization occurs throughout life in the human body even after bone development is complete. When demineralization is dominant, mineral crystals dissolve and inorganic ions such as Ca^{2+} and PO_4^{3-} are removed from the organic matrix, exposing type I collagen fibers; thus, the mechanical strength of the bone is reduced, potentially leading to the occurrence of fractures and bone deformities.[1] In humans, dentin and bone tissues

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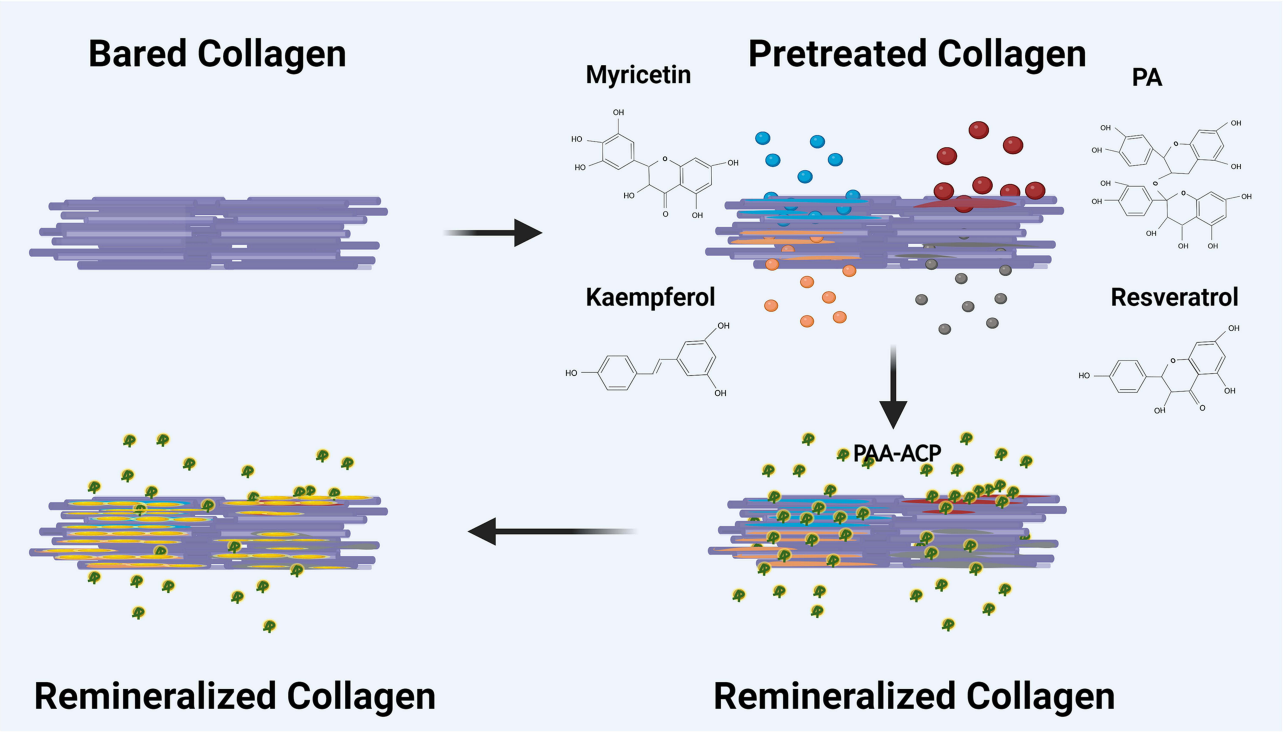
Graphical Abstract



have similar compositions and inorganic contents; hence, the dynamic balance of demineralization-remineralization also exists in dentin.[2,3]

The demineralization of dentin occurs during the bonding procedure, regardless of the self-etch or etch-and-rinse mode. The presence of residual water and acidic ions in the dental adhesive systems as well as the activation of matrix metalloproteinases (MMPs) lead to exposed type I collagen fiber degradation in the deep layers that are not completely encapsulated by the resin matrix. This is the source of the persistent problem of dentin bond durability within the range of available bonding techniques based on the hybrid layer (HL) theory.[4,5] Reducing the degradation of collagen fibers in the HL and inducing biomimetic remineralization of demineralized dentin areas are considered as two viable approaches that can be applied individually or combinedly to improve dentin bond durability.[5,6] Biomimetic remineralization is based on the fact that mineral backfilling of collagen fibril interstices can replace water molecules to encapsulate collagen fibrils, and thus protect collagen from degrading over time.[7] The currently accepted theory of dentin biomimetic remineralization is based on the hypothesis of the polymer-induced liquid precursor (PILP) process, in which liquid-like amorphous calcium phosphate (ACP) nanoparticles are delivered into type I collagen fiber matrices and gradually attract Ca^{2+}, leading to hydroxyapatite (HA) formation.[8–10] However, the biomimetic remineralization process through the PILP alone is slow, and only a few HA crystals are formed within 4 weeks; this provides little resistance to the rapid degradation effect of water and MMPs on bare collagen fibers.[11,12] Therefore, a compound that resists enzymatic degradation while accelerating the formation of minerals around exposed collagen fibers to further protect collagen is extremely attractive.

Natural polyphenols, such as proanthocyanidins (PA),[13] quercetin,[14] catechins,[15] myricetin,[16] resveratrol,[17] and kaempferol[18] contain multiple phenolic -OH groups, which can form hydrogen bonds with collagen amide carbonyl groups to modify the collagen and inhibit MMPs. These -OH groups at the free end can also chelate with metal cations (e g, Ca^{2+}, Mg^{2+}, and Au^{2+}), thereby potentially accelerating ACP nanoparticle deposition and HA formation.[19–22]

PA has been shown to have positive effects on dentin remineralization.[23–25] In the past 3 years, the clinical value of myricetin, resveratrol, and kaempferol has gradually increased, and these polyphenols have been proven to improve the

resin-dentin bonding durability by modifying collagen and inhibiting MMP activity;[16–18] however, their contribution to biomimetic remineralization remains unclear.

In this study, we evaluated and compared the effects of four polyphenols (PA, myricetin, resveratrol, and resveratrol) on the promotion of biomimetic remineralization and the bonding performance of resin-dentin. Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) and in situ zymography were employed to confirm dentin collagen modification and MMP activity inhibition, respectively. Biomimetic remineralization was observed via scanning electron microscopy/energy dispersive spectrometry (SEM/EDS), X-ray diffraction (XRD), ATR-FTIR, and Vickers hardness numbers (VHN), and quantitatively analyzed by micro-computed tomography (micro-CT). Furthermore, the resin-dentin bonding durability was examined by microtensile bond strength (μTBS) and nanoleakage analyses. The null hypotheses were as follows: These four polyphenols (i) cannot modify collagen, (ii) do not contribute to MMP activity inhibition; (iii) have no effect on promoting biomimetic remineralization of demineralized dentin; and (iv) contribute to improving the dentin bond durability little.

Materials and Methods

Preparation of Dentin Slices

This study was conducted in accordance with the Declaration of Helsinki. One hundred and seventeen no-carious human third molars were obtained for in vitro research with the patients' informed consents according to the protocol approved by the Ethics Committee of Nanjing Medical University, China (No. [2019] 277). The dentin specimens were sectioned into dentin slices with different thicknesses (0.5 ± 0.1 mm for analyzing the interaction between polyphenols and dentin collagen as well as the quantitative analysis of dentin remineralization; 1 ± 0.1 mm for the characterization of dentin remineralization and the analysis of MMP activity; and 3 ± 0.1 mm for μTBS and nanoleakage tests), using a slow speed saw under water irrigation (Isomet 1000, Buehler Ltd., Lake Bluff, IL, USA).[26] These teeth were obtained and stored in 0.01% sodium azide solution at 4°C less than 1 month before use.

Interaction Between Polyphenols and Dentin Collagen

In this stage, 55 mg/mL PA/ethanol, 0.2 mg/mL myricetin/ethanol, 10 mg/mL resveratrol/ethanol, and 10 mg/mL kaempferol/ethanol primers were prepared and stored at 4°C before use.[16,17,25,27] All the chemical reagents were purchased from Macklin, Shanghai, China. Dentin slices were immersed in 1 M/L hydrochloric acid (HCl) for 12 h for complete demineralization (examined using an intraoral dental X-ray device [Focus 50540-IMG, KAVO, California, USA]),[28] rinsed completely with double distilled water, and blotted dry. Thereafter, the slices were divided into five groups and subjected to pretreatment for 30 min (n=1): (1) Ctr1, polyphenol-free/ethanol solution pretreatment as a blank control group; (2) PAs, PA/ethanol solution; (3) Myr, myricetin/ethanol solution; (4) Res, resveratrol/ethanol solution; and (5) Kae, kaempferol/ethanol solution.

The pretreated slices of each group were also thoroughly rinsed and dried, and then examined by ATR-FTIR (Thermo Fisher Scientific, USA) with 32 scans in the range of 500–4000 cm^{-1} with a resolution of 4.0 cm^{-1}.

Analysis of MMP Activity

The dentin surface was acid-etched with 35% phosphate gel for 15s, rinsed for 30s, and then grouped and pretreated separately as described above (n=5). A layer of adhesive (Single Bond 2, 3M ESPE, St. Paul, MN, USA) was immediately applied according to the manufacturer's instructions. Subsequently, two 1-mm-thick layers of composite resin (Filtek Z250; 3M ESPE, St. Paul, MN, USA) were placed over the bonded surface with 20s light-curing for each layer using an LED unit (EliparTM S10, 3M ESPE, São Paulo, MN, USA).

Three bonded slabs from each group were wet-polished with #600, #1200, and #2000 silicon-carbide papers to thicknesses of approximately 500 μm, ultrasonically cleaned for 10 min, and then glued to glass slides using cyanoacrylate glue. 50 μL of freshly quenched fluorescein-conjugated gelatin mixture (E-12055; Molecular Probes, Eugene, OR, USA) was applied on the top of each resin-dentin slab and covered with a coverslip.[29] After the microscope slides were incubated in the dark at 37 ◦C for 24 h, they were examined with a confocal microscope (Zeiss LSM880 with NLO & Airyscan, Germany).

Characterization of Dentin Remineralization

The dentin slices were subjected to acid etching and grouping pretreatment. Thereafter, the slices were immersed in a 10-mL remineralization solution (12 mM Na_2HPO_4, 20 mM $CaCl_2{\cdot}2H_2O$, 18 mg/mL NaCl, 14 mg/mL Tris, 60 mM HCl, 700 μg/mL polyacrylic acid, pH =7.4±0.1)[30] for 7 days and incubated on a shaker (Minquan MQT-60R, Shanghai, China) at 37°C and 100 rpm/min. Figure 1 shows the preparation process of dentin remineralization.

Color changes of the dentin surfaces before and after remineralization were observed visually and photographed with a digital camera (D7500, Nikon, Tokyo, Japan) (n=1).

After remineralization, the samples of each group were successively dehydrated for 30 min in solutions with an increasing concentration of ethanol (25 wt%, 50 wt%, 75 wt%, 90 wt%, 95 wt%, and 100 wt%). The untreated sample was used as the control group (labeled as Ctr0). All groups were submitted to SEM/EDS (TESCAN MAIA3, Kohoutovice, Czech Republic), XRD (D8 ADVANCE, Bruker AXS, Germany), ATR-FTIR (Thermo Fisher Scientific, USA), and VHN (TUKON 1102, Wilson, Norwood, USA) testing to observe remineralization.

Three dentin slices were coated with Pt and observed using SEM in the secondary electronic mode with an accelerating voltage of 20 kV and a working distance of 5 mm.

XRD was performed using Ni-filtered Cu K (λ = 1.5418 Å) radiation at ambient temperature, with a 2θ step size, angular range, and scan rate of 0.02°, 20°–80°, and 2° /min, respectively (n=1).

The ATR-FTIR spectra were acquired with 32 scans in the range of 800–4000 cm^{-1} with a resolution of 4.0 cm^{-1} (n=1).

The VHN test of dentin surfaces was performed under a load of 100 g for 15s (n=5). One-way analysis of variance (ANOVA) and Tukey's post hoc least significant difference (LSD) tests were performed using the SPSS 21.0 statistical software package (IBM SPSS, Inc., Chicago, IL, USA) to evaluate the hardness of the dentin surfaces (α = 0.05).

Quantitative Analysis of Dentin Remineralization

Dentin slices were immersed in 1 M/L HCL for 12 h for complete demineralization; further, the slices were divided into six groups to be treated separately. The zones of interest in the dentin slices were divided (from superficial to deep) into two zones: the external (0–21 μm) and internal (remaining central area) zones of interest. The mineral content of dentin after remineralization for 7 days was detected using a micro-CT (viva CT 80, SCANCO Medical AG, Switzerland) system with a current of 72 mA and a voltage of 55 kV. SkyScan CTAn software (version 1.15.4.0+) was used for data analysis.[31]

After recording the mineral volume percentage (V%, equivalent to bone volume percentage in bone micro-CT measurements) and mineral separation (Tb. Sp, equivalent to trabecular separation in bone micro-CT measurements), the two-way ANOVA followed by Tukey's post-hoc LSD tests were performed to evaluate the effects of the primer and zone of interest factors on mineral content (α = 0.05).

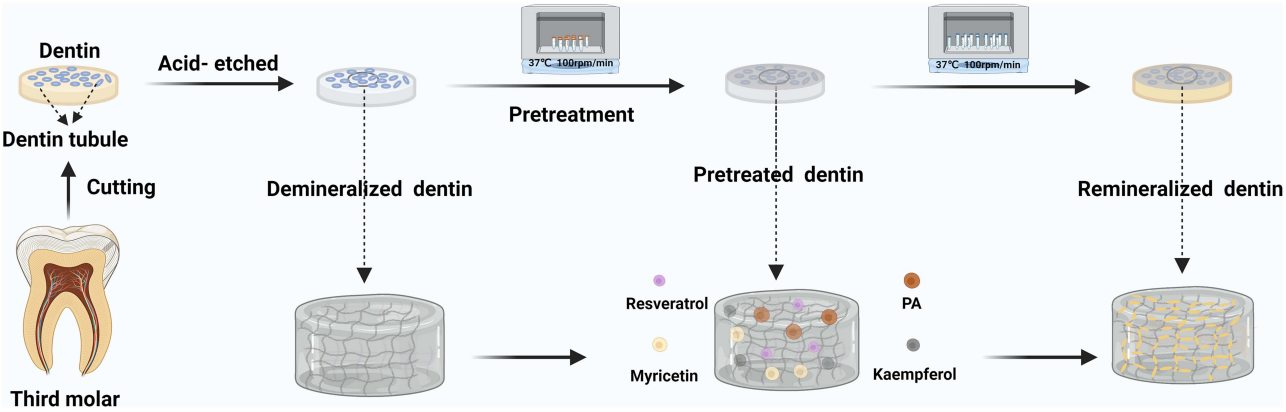


Figure 1 The process of preparing remineralization samples.

μTBS Testing and Nanoleakage

Remineralized dentin specimens were divided into six groups and prepared as described above. A layer of adhesive (Single Bond 2) was applied to the bonding surfaces according to the manufacturer's instructions. Further, a 4-mm composite resin was placed in layers, with 20s light-curing for each layer that was less than 2 mm. After 24 h of water storage or 3 months of aging plus 10,000 thermocycles (TC-501F, Suzhou, China) for 30s at 5°C and 30s at 55 °C, with a transition time of 3s, the bonded specimens were further sectioned into pieces with dimensions of 1 mm × 1 mm × 7 mm. Each beam was subjected to μTBS testing using a universal testing machine (Instron 3365 ElectroPuls, Boston, MA, USA) at a crosshead speed of 1 mm/min until fracture occurred (n=15). The μTBS values were calculated by dividing the load at failure by the cross-sectional bonding area. The same statistical analysis as that for the micro-CT test was performed to evaluate the effects of the primer and aging factors on μTBS ($\alpha = 0.05$).

Three dentin-bonded slabs (with or without aging) were selected from each group and observed for nanoleakage within the HL using SEM.[29]

Results

Interaction Between Polyphenols and Dentin Collagen

Representative ATR-FTIR spectra of dentin collagen treated with PA, myricetin, resveratrol, or kaempferol are shown in Figure 2. Typical amide I, II, and III bands as well as the scissoring mode of CH_2 groups, which represent dentin collagen, were observed in all groups. There were no obvious changes in the positions of the amide I band in the PAs, Myr, Res, and Kae groups. The AIII/A1454 value for the PAs group was 0.5, whereas those for the Ctr1, Myr, Res, and Kae groups were all approximately equal to 1.0 (Table 1).

Activity Analysis of MMPs

The results of in situ zymography for each group are presented in Figure 3. A strong and wide green fluorescence band was observed in the HL of the Ctr1 group (Figure 3A), indicating that fluorescein-conjugated gelatin was strongly hydrolyzed. Compared with the Ctr1 group, the fluorescence intensities of the PAs, Myr, Res, and Kae groups (Figure 3B–E) were significantly weakened, and the fluorescence band width was narrowed, indicating inhibited enzymatic activity; the weakest and strongest inhibitions were observed in the Myr (Figure 3C) and Kae (Figure 3E) groups, respectively.

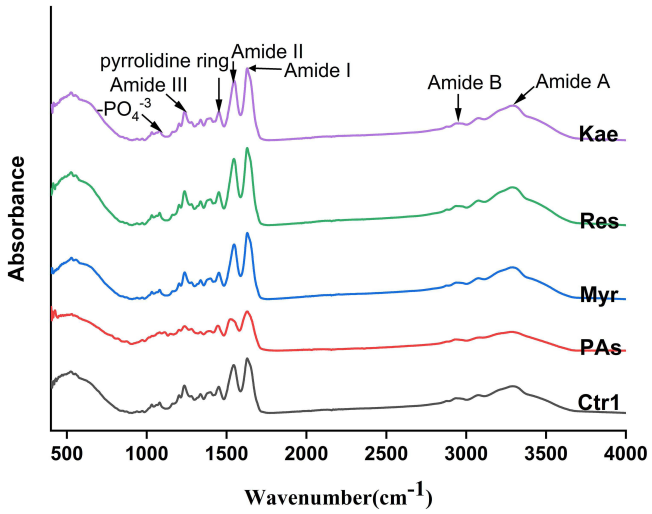


Figure 2 ATR-FTIR spectrum of each group with different pretreatment conditions.

Table 1 AIII/A1451 Value of Each Group with Different Pretreatment Conditions

Group	Parameters	
	Amide I(cm-1)	Amide III/A1450
Ctr1	1629.5	1.07
PAs	1629.7	0.50
Myr	1629.8	1.19
Res	1629.3	1.13
Kae	1629.3	1.06

Characteristics of Dentin Remineralization

Color Change of Dentin Caused by the Four Polyphenols

Before remineralization, compared with the Ctr1 group (Figure 4A1), the dentin surfaces of the PAs, Kae, Myr, and Res groups showed a brownish red color (Figure 4B1), a dark yellow color (Figure 4E1), no obvious staining (Figure 4C1), and no obvious staining (Figure 4D1), respectively. After remineralization, except for the Ctr1 group (Figure 4A2), the color lightened in all polyphenol pretreated groups (Figure 4B2–E2), although the dentin surface color remained most visible in the PAs group (Figure 4B2). Typical images are shown in Figure 4.

SEM/EDS

Figure 5a shows typical SEM images of the remineralized dentin surfaces. The dentin tubules in the Ctr0 group remained completely open (Figure 5A1 and A2), and the collagen fiber morphology on the tubular wall and between dentin tubules was visible. The dentin tubules in the Ctr1 group remained open, but a small number of crystals formed between the collagen fibers and within the dentin tubules; moreover, the collagen morphology became blurred (Figure 5B1 and B2). The PAs, Myr, Res, and Kae groups showed a large increase in crystal formation, by which the collagen morphology was completely covered; even the dentin tubule diameter appeared to be significantly reduced (Figure 5C1–F1 and C2–F2).

Figure 5b shows the results of the mapping of Ca and P contents on each group. The Ca and P contents were lowest in the Ctr0 group, followed by the Ctr1 group; compared with the Ctr1 group, the Ca and P contents were significantly increased in the PAs, Myr, Res, and Kae groups.

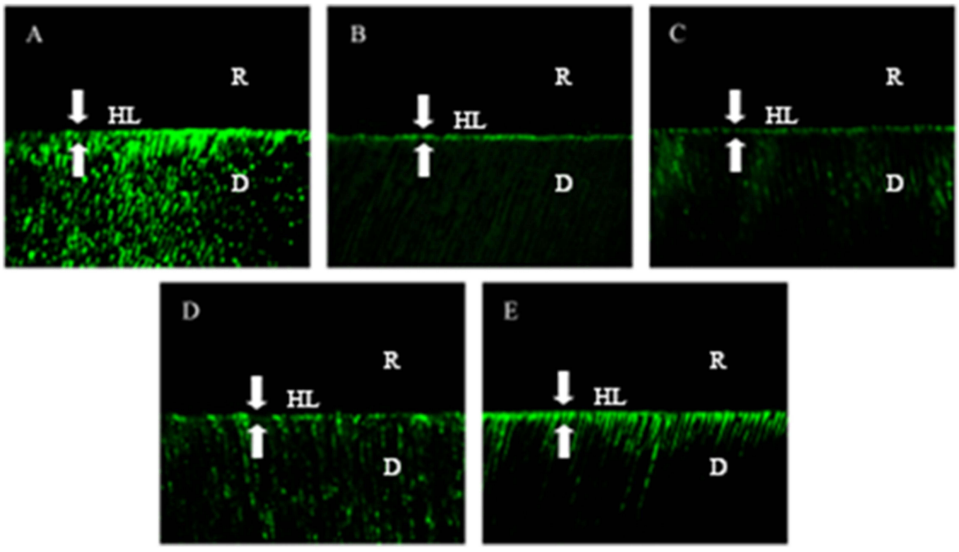


Figure 3 Typical images of quenched fluorescein-conjugated gelatin substrates of each group. (**A**) No pretreatment; (**B**) Pretreatment with PA/ethanol solution; (**C**) Pretreatment with myricetin/ethanol solution; (**D**) Pretreatment with resveratrol/ethanol solution; (**E**) Pretreatment with kaempferol/ethanol solution. R = resin, D = dentin, HL = hybrid layer (between arrowheads).

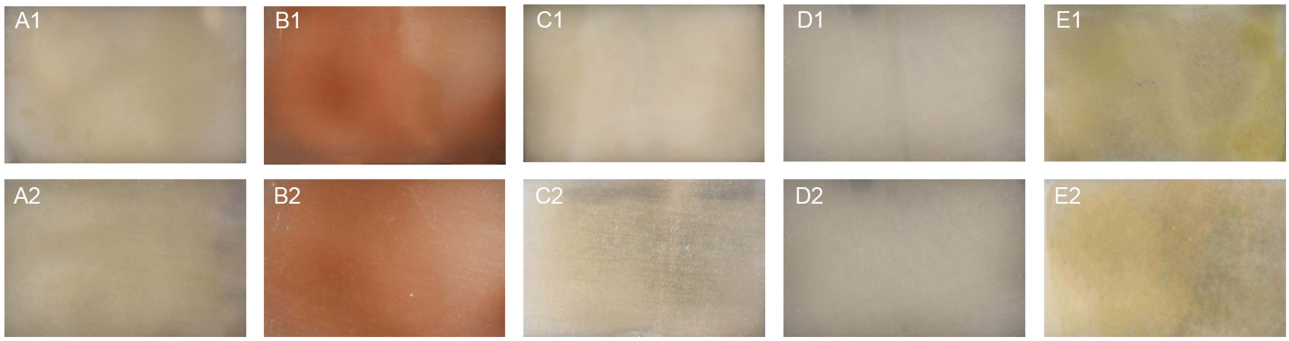


Figure 4 Color changes of dentin surface before (**A1–E1**) and after (**A2–E2**) remineralization caused by four polyphenols primers. (**A1** and **A2**) No pretreatment; (**B1** and **B2**) Pretreatment with PA/ethanol solution; (**C1** and **C2**) Pretreatment with myricetin/ethanol solution; (**D1** and **D2**) Pretreatment with resveratrol/ethanol solution; (**E1** and **E2**) Pretreatment with kaempferol/ethanol solution.

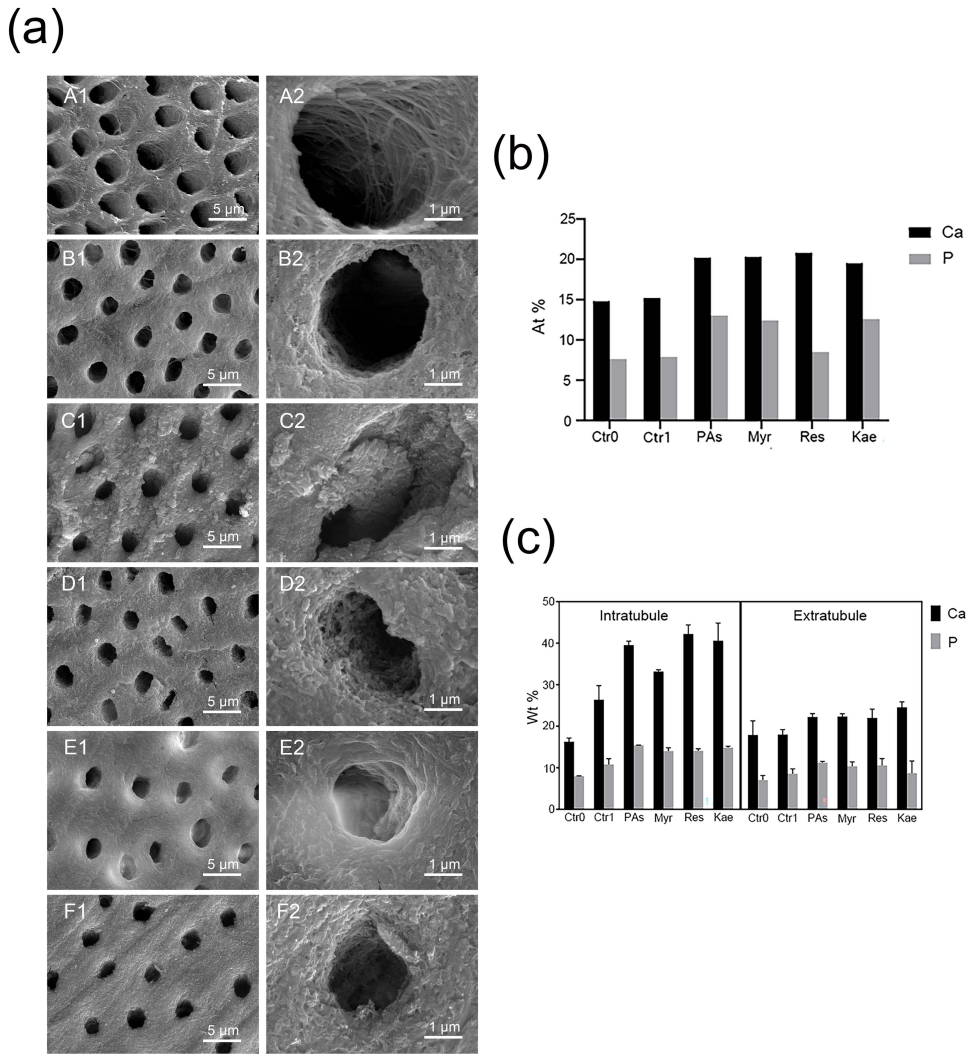


Figure 5 Representative SEM images (**a**) of dentin surfaces after different treatments. (**A1** and **A2**) No treatment; (**B1** and **B2**) Polyphenol-free/ethanol solution pretreatment + remineralization; (**C1** and **C2**) Proanthocyanidins/ethanol solution pretreatment + remineralization; (**D1** and **D2**) Myricetin/ethanol solution pretreatment + remineralization; (**E1** and **E2**) Resveratrol/ethanol solution pretreatment + remineralization; (**F1** and **F2**) Kaempferol/ethanol solution pretreatment + remineralization. Magnification: ×10,000 (**A1–F1**); ×50,000 (**A2–F2**). (**b**) The results of the mapping of each group (At%: Atomic percentage). (**c**) The results of the EDS of each group (Wt%: Weight percentage).

Figure 5c shows the results of the EDS of Ca and P contents on each group. The Ca and P contents within the dentin tubules had the same trend as shown in Figure 5b. However, the Ca and P contents between dentin tubules almost remained unchanged.

XRD and ATR-FTIR

Figure 6 shows XRD and ATR-FTIR spectra of dentin in each group. Typical diffraction peaks representing HA were detected in all groups at 2θ = 25.9 (002), 2θ = 31.8 (112), and 2θ = 32.9 (300). After remineralization, most of the characteristic HA peaks increased. In the ATR-FTIR spectra, the PO_4^{3-}, amide I, and amide II groups were detected from 885 to 1190 cm^{-1}, 1600 to 1700 cm^{-1}, and 1510 to 1580 cm^{-1}, respectively; the amide III and N-H groups were detected from 1220 to 1340 cm^{-1}. The intensities of typical diffraction peaks in the Ctr1 group were nearly the same as those in the Ctr0 group, but lower than those in the four polyphenol groups. Combining with XRD and ATR-FTIR results, the formation of HA was obtained.

VHN

The VHN of the dentin surfaces for each group is shown in Table 2. The type of primer had a significant effect on the VHN ($p<0.001$). The Ctr0 group had the lowest VHN, and the Ctr1 group, which was treated only with remineralization, achieved significantly higher VHN. The VHN of the PAs, Myr, Res, and Kae groups were further significantly increased after treatment with polyphenol/ethanol solution primer compared with those of the Ctr1 group, with the largest VHN in

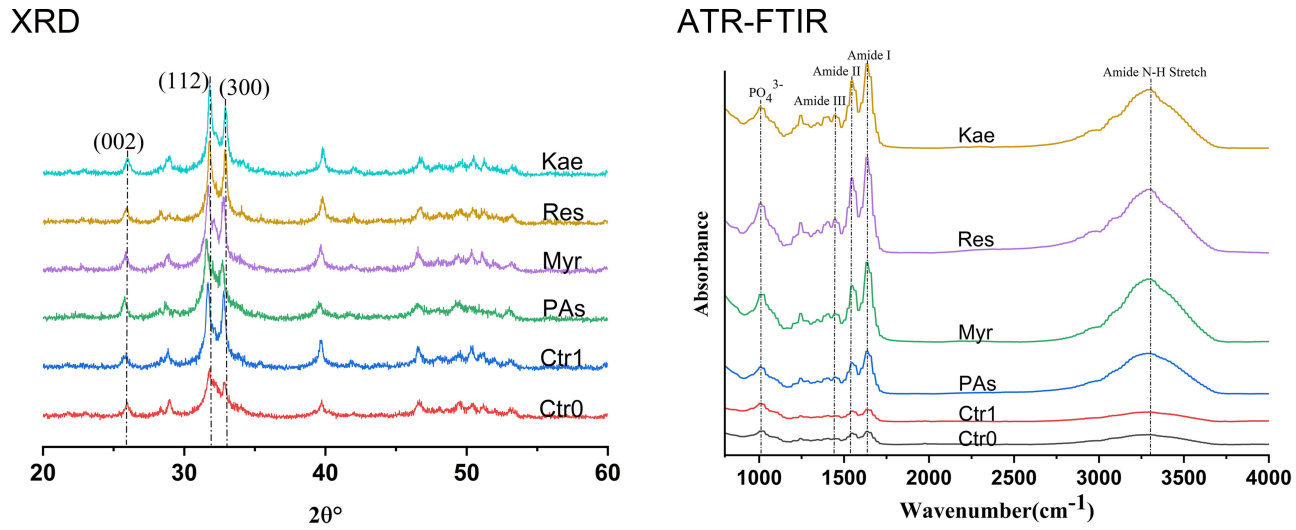


Figure 6 XRD and ATR-FTIR spectra of dentin in each group after different treatments.

Table 2 Vickers Hardness Numbers (VHN, Mean and Standard Deviation) of Dentin After Different Experimental Primer Treatments

Group	Mean ± Standard Deviation (MPa)
Ctr0	20.52 (0.87)[e]
Ctr1	22.79 (0.81)[d]
PAs	36.35 (0.73)[a]
Myr	32.97 (0.39)[b]
Res	27.03 (0.62)[c]
Kae	26.37 (0.96)[c]

[a-e]Different superscript letters indicate statistically significant differences between experimental groups (P < 0.05).

the PAs group ($p < 0.001$), followed by the Myr group ($p < 0.001$); the smallest VHN were found in the Res and Kae groups with no significant difference ($p = 0.178$).

Micro-CT

The reconstructed images of dentin are shown in Figure 7, and the V% results are shown in Table 3. Both primer type and zone of interest had a significant effect on the V% ($p < 0.001$), and there is a significant interaction between these two factors ($p < 0.001$). The V% values of the external and internal zones of interest were lowest in the Ctr0 group, followed by the Ctr1 group ($p < 0.001$). In the external zone of interest, the V% value was greater in the PAs group than in the Myr group ($p < 0.001$), whereas the V% values were smaller in the Res and Kae groups, with no statistically significant difference ($p = 0.647$). In the internal zone of interest, the V% values were largest and smallest in the Myr and PAs groups, respectively.

The Tb. Sp values of the external zone of interest were 2.00 in all groups, whereas the Tb. Sp values of the internal zone of interest were different. The Ctr0 group showed the highest Tb. Sp value (6.54 μm), followed by the Ctr1 group (3.85 μm). A significant decrease in the Tb. Sp value was observed in the PAs (2.76 μm), Myr (2.56 μm), Kae (2.60 μm), and Res (2.50 μm) groups.

μTBS Testing

Table 4 summarizes the μTBS results obtained after 24 h of water storage or after aging. The μTBS was significantly affected by the primer solution type and aging ($p < 0.001$); additionally, a significant interaction was observed between these two factors ($p = 0.024$).

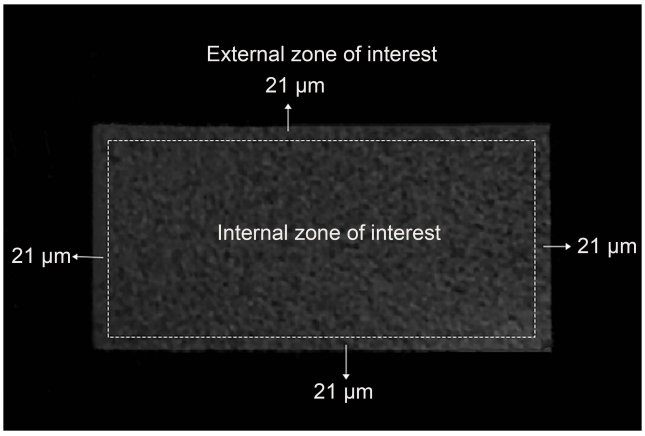


Figure 7 Reconstructed image of dentin and different zones of interest.

Table 3 Mineral Volume Percentage (V%, Mean and Standard Deviation) of Dentin After Different Experimental Treatments

Group	External Zone	Internal Zone
Ctr0	3.66 (2.07)^{e}	6.47 (2.61)^{j}
Ctr1	17.38 (2.22)^{d}	24.22 (1.04)^{i}
PAs	75.81 (3.02)^{a}	53.59 (0.75)^{h}
Myr	52.97 (2.18)^{b}	62.10 (3.66)^{f}
Res	45.35 (1.17)^{c}	57.21 (1.28)^{g,h}
Kae	46.15 (1.14)^{c}	59.47 (3.00)^{f,g}

Notes: $^{a-j}$Different superscript letters indicate statistically significant differences between experimental groups ($P < 0.05$).

Table 4 Mean Values (with Standard Deviations in Parentheses) of Micro-Tensile Bond Strength (MPa) for the Six Groups After 24 h of Water Storage and After Aging for 3 Months in Water at 37°C Plus 10,000 Thermocycles

Group	24 h	3 Months + 10,000 Thermocycles
Ctr0	34.23 (3.85)[b]	29.77 (4.91)[f]
Ctr1	36.11 (4.47)[b]	31.69 (4.10)[e,f]
PAs	44.83 (4.60)[a]	35.20 (5.71)[d,e]
Myr	46.70 (4.82)[a]	40.70 (5.95)[c]
Res	44.63 (5.35)[a]	38.56 (5.87)[c,d]
Kae	45.82 (5.38)[a]	33.54 (5.24)[e,f]

Notes: [a-f]Different superscript letters indicate statistically significant differences between experimental groups ($P < 0.05$).

After 24 h of water storage, the μTBS value was lowest in the Ctr0 group and increased in the Ctr1 group, although there was no statistical difference between the values in these two groups ($p = 0.286$). The μTBS values in the PAs, Myr, Res, and Kae groups were significantly higher than that in the Ctr1 group ($p < 0.001$), although there was no significant difference between the values in these four groups ($p > 0.05$). After aging, the μTBS decreased in all six groups; however, the PAs and Kae groups showed a higher decrease in μTBS values than the Myr and Res groups.

Nanoleakage

The representative SEM images of each group after 24 h of water storage or after aging are presented in Figure 8. After 24 h of water storage, the Ctr0 group showed a large number of dense and continuous depositions with high-electron-density particles within the HL (Figure 8A1 and A2), and the Ctr1 group showed reduced silver deposition with the HL having continuous but sparse depositions (Figure 8B1 and B2). The PAs, Myr, and Kae groups showed only a few discontinuous particles scattered at the bottom of the HL (Figure 8C1, D1, F1; C2, D2 and F2), whereas the Res groups revealed almost no deposition of high-electron-density particles (Figure 8E1 and E2).

Aging aggravated nanoleakage for all the groups. The Ctr0 group demonstrated the greatest nanoleakage (Figure 8A3 and A4). Less silver deposition was observed in the Ctr1 group (Figure 8 B3 and B4) than in the Ctr0 group. Among the four polyphenol/ethanol solution pretreatment groups, the amount of silver deposited was larger in the PAs (Figure 8 C3 and C4) and Kae (Figure 8 F3 and F4) groups than in the Myr (Figure 8 D3 and D4) and Res (Figure 8 E3 and E4) groups.

Discussion

The concentrations of the primers investigated in this study were based on previous studies;[16,17,25] these primers almost had no cytotoxic effects on cells, and could promote collagen cross-linking. Based on the observation of the color of dentin treated with the four polyphenols, only PA caused a significantly darker staining, which is contrary to the concept of esthetic restoration.

The ATR-FTIR results showed that the amide I group was mainly attributed to the stretching vibration of the C-O group; the amide II group was coupled by the N-H bending vibration and the C-N stretching vibration; and the amide III group resulted from the C-N stretching vibration and N-H bending vibration of the amide bond as well as the swinging vibration of the CH_2 group on the glycine backbone and proline side chain, which was attributed to the organic collagen fibrils.[32,33] The position of the amide I group was almost unchanged before and after the interaction of PA, myricetin, resveratrol, and kaempferol with dentin collagen, indicating that none of these four polyphenols disrupt the triple helix structure of collagen fibers.[34] The AIII/A1454 value, another indicator to evaluate the binding of polyphenols to collagen, is about 1.0 for pure collagen fibers. There was a small increase in the AIII/A1454 value (from 1.07 to 1.16) after the interaction of myricetin, resveratrol, and kaempferol with collagen fibers, indicating that these three polyphenols have no effect on the triple helix structure of collagen fibers.[35,36] An AIII/A1454 value of 0.5 after PA interaction is due to the absence of AIII in the ATR-FTIR spectrum of pure PA, which has little effect on its AIII band; nevertheless, the effect is considerable on the band at

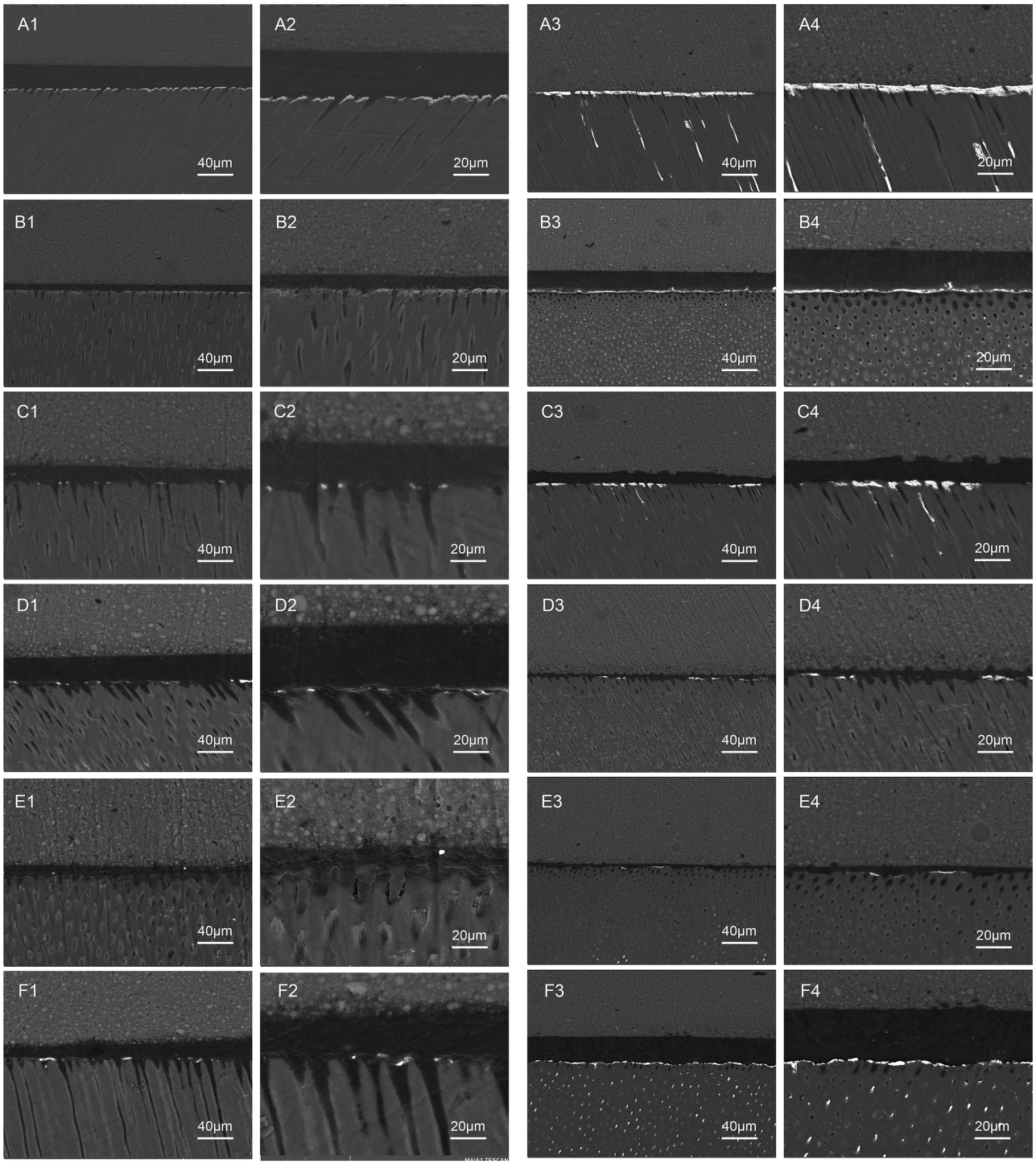


Figure 8 Representative SEM images of the bonded interfaces evaluated after 24 h of water storage (**A1–F1** and **A2–F2**) and after aging for 3 months in water at 37°C plus 10,000 thermocycles (**A3–F3** and **A4–F4**). (**A1–A4**) No treatment; (**B1–B4**) No pretreatment + remineralization; (**C1–C4**) PA/ethanol solution pretreatment + remineralization; (**D1–D4**) Myricetin/ethanol solution pretreatment + remineralization; (**E1–E4**) Resveratrol/ethanol solution pretreatment + remineralization; (**F1–F4**) Kaempferol/ethanol solution pretreatment + remineralization. Magnification: ×1000 (**A1–F1** and **A3–F3**); ×2000 (**A2–F2, A4–F4**).

1451 cm^{-1}.[37] Thus, the current experimental results suggest that the effective interaction of PA, myricetin, resveratrol, and kaempferol with dentin collagen is the result of hydrogen bond formation between the -OH and amide carbonyl groups on the amino acids of collagen. Hence, the null hypothesis 1 is rejected. The hydrogen bond exerts the main force, while covalent, ionic, and hydrophobic interactions play a secondary role.[38–40]

It has been extensively demonstrated that the -OH groups of polyphenols can act as ligands to bind Ca^{2+}, and in particular, the catechol groups seem to have a stronger chelating effect on Ca^{2+}, which more effectively promotes the deposition of minerals to the collagen matrix.[41] In this experiment, the PILP method was adopted to guide the bottom–up assembly of apatite crystallites. Moreover, PA, myricetin, resveratrol, and kaempferol were used as primers to observe the formation of minerals. The typical diffraction peaks of HA and PO_4^{3-} detected by XRD and ATR-FTIR, respectively, suggested that HA was regenerated in the dentin matrix. Importantly, the intensities of $PO_4^{3-}$and amide peaks increased, which is attributed to the positive effects of polyphenols on mineral formation and the protection of collagen fibers.

Dentin biomimetic remineralization can improve the mechanical properties of demineralized dentin,[10] and the differences in dentin remineralization triggered by the four polyphenols resulted in differences in dentin hardness. Concerning the microhardness findings in this experiment, the microhardness value of fresh dentin was 53.82 ± 1.76 MPa; partially demineralized dentin surface hardness caused by 35% phosphate gel was reduced to 38% of the original value, and increased to only 42% after simple remineralization. However, pretreatment with polyphenols for 30 min led to a significant increase in dentin hardness values, resulting in the recovery of 49–68% of fresh dentin, and thus partially compensating for the loss of dentin surface hardness due to demineralization; in this case, the biomimetic remineralization effect was in the following order: PA > myricetin > resveratrol and kaempferol. To analyze the reasons for the differences in the restoration of demineralized dentin hardness using the four polyphenols, we performed SEM/EDS and Micro-CT.

SEM/EDS analysis revealed that a large number of granular substances appeared in the dentin treated with PA, resulting in a more uneven and rough surface. This is because the oligomeric structure of PA molecules limits their permeability, and the large amount of PA molecules bound on the superficial collagen fibers of the HL rapidly transform ACP nanoparticles into HA, which hinders the formation of minerals in deeper collagen fibers to a large extent.[42] Therefore, the higher amount of superficial mineral deposition considerably contributed to the highest surface hardness achieved in the PA-treated dentin. The chemical structure and molar mass analysis of the four polyphenols indicated that myricetin, resveratrol, and kaempferol, which are unimolecular, low-molecular-weight compounds with high permeability, can quickly and freely penetrate all interstices to reach the deeper layers and effectively bind to the collagen fiber surface using the -OH group to exert a bottom-up remineralization effect. In cases where the mineralization time was not long enough, the dentin surface had a relatively lower crystalline material mass and Ca and P contents; therefore, it was relatively flat and smooth, as observed by SEM. Interestingly, the EDS results also revealed that remineralization preferentially occurs within than between the dentin tubules over a certain period. This suggests the potential of the four polyphenols in the treatment of dentin sensitivity, although it is beyond the scope of this study.

The quantitative analysis of dentin remineralization performed by micro-CT showed that only PA-treated dentin mineral deposition was highest in the superficial layer and lower in the deeper layers. This result could not be reversed even after the immersion time was extended to 30 min to reduce the effects of incomplete permeability. This finding is consistent with that of a previous study wherein greater mineral deposition was observed in the superficial layer when PA was applied for root surface dentin remineralization.[23] However, the other three polyphenols promoted dentin remineralization progressively from superficial to deep layers, indicating that these three polyphenols preferentially penetrated the deep layer to promote remineralization. This is more conducive to the rewrapping of the exposed collagen fibers at the bottom of the HL by HA, thereby avoiding the defect of weak mechanical properties of exposed collagen fibers in the HL. The experimental results are consistent with the present SEM and VHN results. Consequently, null hypothesis 3 is rejected.

It is well established that dentin biomimetic remineralization of the HL facilitates the improvement of the resin-dentin bonding performance.[9,43,44] The current μTBS findings revealed that the remineralization treatment alone increased the initial μTBS values by only approximately 5%, whereas the initial μTBS values increased by 30–36% after the pretreatment of the dentin surface with PA, myricetin, resveratrol, and kaempferol. This is because polyphenol-promoted biomimetic remineralization enhances the protection of minerals on collagen fibers.[9] Similar to the nanoleakage experiment findings, the effects of PA, myricetin, resveratrol, and kaempferol pretreatments followed by biomimetic remineralization were extremely significant in reducing silver deposition in initial samples.

Biomimetic remineralization, as a progressive dehydration mechanism of water-rich, resin-sparse collagen matrices, enables resin-dentin adhesive joint to resist degradation for a long period of time.[45] These four polyphenols are reportedly hydrophobic, which contributes to repelling the invasive effect of water molecules on the HL in a long-term aqueous environment, thereby inhibiting the dissolution of the resin matrix.[46] Another function of polyphenols is to protect collagen fibers from enzymatic degradation by inactivating MMPs around them through three mechanisms: down-regulation of endogenous protease expression, protease inactivation/silencing, and protection of cleavage sites within collagen.[47] The current in situ enzyme experiment also confirmed the MMP inhibitory properties of these four polyphenols. Despite the strong MMP inhibitory activity of PA, the present micro-CT and SEM/EDS analyses confirmed the formation of a large amount of shallow agglomerated crystals in the superficial layer of dentin after PA treatment, which, to some extent, hindered the formation of resin-dentin tags (the main functional structure of resin-dentin bonding). The low level of deep permeability reduces the protective effect of remineralization on collagen in the deeper layers. Although kaempferol provided superior remineralization in the deep layer compared with PA, the inhibitory activity of MMP was worse. Therefore, the resin-dentin specimens pretreated with PA and kaempferol after aging showed a greater increase in nanoleakage and a greater decrease in the μTBS value, which was not significantly different from the remineralization-only group findings, indicating that a valuable durability improvement could not be achieved by relying solely on PA and kaempferol to promote remineralization. Myricetin and resveratrol elicited a strong ability to penetrate deeper layers to provide biomimetic remineralization and inhibit the activity of MMP, a smaller increase in nanoleakage, and a smaller decrease in μTBS values after aging, thereby suggesting a potential clinical value for improving the durability of resin-dentin bonding. Therefore, null hypothesis 2 is rejected, and null hypothesis 4 is partially rejected.

Conclusion

Based on the current experimental results and within the limits of this study, all four polyphenols are effective in inhibiting MMP activity, accelerating the deposition of ACP nanoparticles, and promoting biomimetic remineralization of demineralized dentin when they are used as bonding interface primers; nevertheless, not all four polyphenols can improve the durability of resin-dentin bonding. Furthermore, the following conclusions can be drawn:

(I) PA, myricetin, resveratrol, and kaempferol can modify collagen by forming hydrogen bonds, without denaturing its three-dimensional structure.
(II) PA, myricetin, resveratrol, and kaempferol can inhibit MMP activity to protect collagen fibers from enzymatic hydrolysis.
(III) PA, myricetin, resveratrol, and kaempferol can promote the deposition of ACP nanoparticles and biomimetic remineralization of demineralized dentin; the effect of PA was mainly exerted on the dentin surface, whereas the effects of myricetin, resveratrol, and kaempferol were more pronounced in deeper layers.
(IV) Myricetin and resveratrol can improve the bond durability of resin-dentin by inhibiting MMP activity and promoting biomimetic remineralization at the bottom of the HL with no visible color changes.

In summary, myricetin and resveratrol may avoid the aesthetic and shallow remineralization problems of PA, and represent potential approaches to achieve desirable bonding stability and reduce the frequent replacement of composite restorations in clinic.

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Disclosure

The authors report no conflicts of interest in this work.

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