Long-term effect of curcuminoid treatment on resin-to-dentin bond strength

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Running title: Effect of curcuminoids on dentin bonding

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Abstract

Endogenous dentin proteases contribute to the degradation of collagen fibrils in the hybrid layer. Recently, inhibition of host-derived proteases by curcuminoids showed promising results. The aim of this study was to evaluate the effect of curcuminoid pretreatment on the microtensile bond strength (μTBS) after 24 h or 12 months of storage. Fifty-four extracted sound human molars were flattened to mid-coronal dentin and divided into 9 groups. After phosphoric acid-etching for 15 s, dentin was treated for 60 s using 100 μM or 200 μM of curcumin, diflourobenzocurcumin or demethoxycurcumin dissolved in 1% or 2% dimethyl sulfoxide (DMSO)/water solution. Untreated or DMSO treated groups served as control. After bonding agent application, each tooth was restored with dental composite. The molars were sectioned into 0.9 x 0.9 x 6 mm^3 beams. The μTBS testing was performed after 24 h and 12 months of storage in artificial saliva. Data were analyzed using regression analyses. Failure patterns were evaluated using scanning electron microscopy. Dentin pretreatment with curcuminoids did not adversely affect 24-h μTBS compared to control. After 12 months, the μTBS of curcuminoid groups was statistically significantly higher than controls. This study indicates the feasibility of using curcuminoids as protease inhibitors.

Keywords: collagen matrix metalloproteinases; cysteine cathepsins; degradation; dentin; curcumin

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INTRODUCTION

Contemporary bonding procedures used in placement of direct or indirect restorations are based on micromechanical retention between resin and tooth structure that results in the formation of a resin-infiltrated gradient interface, the so-called hybrid layer [1, 2]. The acid-treatment of the tooth surface, commonly used during restorative procedures to create microporosities to facilitate bonding, can also cause exposure and activation of endogenous dentin proteases; dentin matrix metalloproteinases (MMPs) [3, 4] and cysteine cathepsins (CCs) [5, 6]. The activation of endogenous proteases causes the gradual destruction of poorly infiltrated collagen fibrils within the hybrid layers. Degradation of collagen can compromise the durability of the resin bonds, resulting in the reduction in bond strength over time [7].

The biomodification of dentin collagen is a promising approach, originally suggested for improving the biomechanical properties of dentin [8-10], but later, used for inhibition of endogenous enzymatic activity [11-13] in dentin. Various synthetic or natural compounds tested for this purpose showed mechanical reinforcement of the collagen scaffold in dentin through additional crosslinks [8, 9, 14, 15] as well as inhibition of collagen degradation [11] resulting in improvement in bond durability over time [16].

Curcumin, extracted from the rhizome of the plant *Curcuma longa L.,* is a natural phenol source [17, 18]. Several modified curcumin analogs, also known as curcuminoids, have been developed and evaluated to improve the solubility, zinc-binding capacity and inhibitory properties [19]. In our previous work, we showed that curcuminoids can preserve the demineralized dentin matrix by inhibiting the degradation generated by matrix-bound MMPs and cathepsin K [20]. Furthermore, the sustainability of the inhibitory effect over an extended period of time was also reported [13]. However, the feasibility of these compounds in combination with the adhesive procedures is not known and the effect of curcuminoids on long-term bond strength has not been studied yet.

Thus, the purpose of this study was to determine the effect of various analogs and concentrations of curcuminoid pretreatment on microtensile bond strength (μTBS) of adhesive resin to dentin using a total-etch adhesive system.

MATERIAL AND METHODS

Microtensile Bond Strength (μTBS) Testing

Fifty-four, intact, non-carious human molar teeth from anonymous donors that are exempt from notification to the Finnish National Research Ethics Committee (According to the Finnish law, Tissue act, section 20), were used in this study. After collection, the teeth were cleaned and stored in a solution of 0.9% sodium chloride (NaCl) containing 0.02% sodium azide (NaN_3) (Sigma) at 4°C to prevent bacterial growth.

Experimental design and bonding procedures

Specimen preparation followed the Academy of Dental Materials guidance of in vitro testing for non-trimmed microtensile bond strength testing [21]. Teeth were coronally sectioned to expose midcoronal dentin surfaces using a low-speed saw (Isomet; Buehler) under water-cooling. The exposed dentin surfaces were treated with 600-grit silicon carbid (SiC) paper (Buehler-MET II; Buehler) for 1 min to create a standard smear layer prior to bonding procedures. The teeth were then randomly allocated to one of nine different treatments (n = 6 teeth per group described below). The dentin surfaces were acid-etched with 32% phosphoric acid (Scotchbond Universal Etchant; 3M ESPE) for 15 s, rinsed with water for 15 s and blot dried. The curcumonoids used as well as bonding materials are listed in Table 1. Dimethyl sulfoxide (DMSO) was diluted in water to prepare 1-2% DMSO/water solutions. Curcuminoid analogs were further dissolved in 1-2% DMSO/water solution to obtain a final concentration 100 μM or 200 μM curcuminoid solutions. Etched teeth were treated with: 100 μM Curcumin (CR100), 200 μM Curcumin (CR200), 100 μM Diflourobenzocurcumin (DC100), 200 μM

Diflourobenzocurcumin (DC200), 100 μM Demethoxycurcumin (MC100), 200 μM Demethoxycurcumin (MC200), 1% dimethyl sulfoxide (1% DMSO) or 2% dimethyl sulfoxide (2% DMSO). One group was untreated (UT), while two other groups were treated with 1% DMSO (DMSO1) or 2% DMSO (DMSO2) and served as controls. After pretreatment with the relevant curcuminoid or DMSO for 60 s, the dentin surface was blot-dried. Adhesive agent (Adper Single Bond Plus; 3M ESPE) was applied to dentin surface for 15 s and dried gently for 5 s, polymerized for 10 s using a LED curing light (Elipar S10; 3M ESPE) at 1200 mW/cm^2. Dental composite (Filtek Supreme XTE; 3M ESPE) was built up in two increments of 2 mm thickness up to 4 mm and each increment was light cured for 20 s. The restored teeth were stored in distilled water at 37°C for 24 h and then were sectioned in the *x* and *y* planes into 0.9 mm x 0.9 mm beams. A minimum of 12 resin-dentin beams were produced per tooth.

Resin-dentin beam storage

Resin-dentin beams were randomly selected within each treatment group for the microtensile test under two conditions: immediate testing (T0), i.e., after 24 h of storage in artificial saliva at 37°C, and at 12 months (T12) after long-term aging in artificial saliva at 37°C. The artificial saliva (AS) (AS containing 5 mM HEPES, 2.5 mM $CaCl_2.H_2O$, 0.02 mM $ZnCl_2$, and 0.3 mM NaN_3, pH:7.2) used in this study to ensure the optimized enzymatic action [22] was changed weekly to prevent pH changes. In order to obtain a research design balanced by tooth dependency, resin-dentin beams from the same tooth were submitted to both testing periods (24 h and 12 months).

Microtensile bond strength (μTBS)

Resin-dentin bond strength evaluation followed the Academy of Dental Materials guidelines for μTBS testing [21]. A minimum of 6 beams per tooth (n = 6 teeth/group) were tested at each storage period. Beams were individually attached to a custom made micro-tensile testing jig using a cyanoacrylate adhesive (Super Bonder Gel, Loctite; Henkel) and tested under tensile

forces using a microtensile testing machine (Bisco) at a cross-head speed of 0.5 mm/min until the failure. The cross-sectional area of each beam was measured with a digital caliper for μTBS calculation in MPa.

Fracture pattern and SEM analysis

After tensile bond testing, debonded surfaces of all specimen were evaluated by SEM and classified according to failure modes; adhesive failure, cohesive failure in composite, cohesive failure in dentin or mixed failures. Following microtensile testing, fractured specimens were dehydrated in ascending ethanol series (50, 70, 80, 90 and 3×100%) and fixed in hexamethyldisilazane and dried under vacuum desiccator. Fracture surfaces of specimens were gold-sputter-coated for examination under scanning electron microscopy (Phenom ProX; PhenomWorld) operated at 10 kV and 4 mm working distance. SEM micrographs were taken sequentially to cover the entire extension of the bonded interface.

Statistical analysis

The number of teeth tested per group followed the Academy of Dental Materials guidance [21]. The tooth was considered the statistical unit, the bond strength average of beams tested at each time period represented the μTBS for each tooth. Premature failures were set to a bond strength of 0 MPa for the statistical analyses.

The data was analyzed using SPSS 27.0 (SPSS). Linear regression modeling was used to describe μTSB values as a function of the pretreatment agent used (dummy coding; DMSO= 0/ each of CR, DC, MC, UT = 1), agent treatment concentration (100 μM/1% = 0 ; 200 μM / 2% = 1) and storage time (24 h=0 /12 months=1). Logistic regression analysis was used to describe the influences of pretreatment agent, agent treatment concentration and storage time (defined and coded as indicated above) on the occurrence of mixed failures. Goodness of fit for the model was checked using Hosmer and Lemeshow test.

RESULTS

Microtensile Bond Strength

The mean values and standard deviation of the μTBS measurements are summarized in Figure 1. Only two occurrences of premature failure were observed, both in the DMSO2 groups. Linear regression analysis revealed that the three curcuminoid pretreatment agents (CR, DC and MC) and storage time were the only variables that affected the μTBS values (Table 2). A statistically significant interaction was observed between the pretreatment agent and time, whereas the agent concentration did not influence the μTBS measurements to any appreciable extent. The estimated μTBS for specimens treated with 1% DMSO at 24 hr was 34.53 MPa, which is very close to the empirical value of 34.56. At 24 hr, the pretreatment agent and the agent concentration exerted only minor and statistically insignificant effects on the μTBS values. This is in stark contrast to 12-month situation, where the bond strength of DMSO was 14.04 MPa lower than seen at 24 hr. The 12-month μTBS values for CR were 5.97 (-14.04+8.07) MPa lower than seen for DMSO at 24 hr, while corresponding values for DC and MC were 3.6 MPa, and 4.46 MPa, respectively. It is thus evident that the pretreatment agents CR, DC and MC alleviated the bond strength reduction that would otherwise result from long-term storage.

Fracture pattern and SEM Analysis

According to the logistic regression analysis, the pre-treatment agent and the concentration were significant factors for the failure probability ($p < 0.05$) (Table 3). The distribution of the fracture pattern as percentages within the experimental groups is shown in Figure 2. Adhesive and mixed failures were the predominant types of failures in all groups, regardless of the incubation period. There was a slight increase in adhesive failure percentages after 12 months of storage compared to immediate results. Figure 3 shows representative SEM images of the

resin/tooth interface. Control groups showed similar fracture pattern that mostly present at the bottom of the hybrid (Figure 3).

DISCUSSION

The longevity of adhesive restorations depends on good bonding between tooth structure and restorative materials. Microtensile bond strength testing has been recognized as a well-accepted method to analyze the performance of dentin adhesive systems in order to evaluate effect of different products and bonding strategies [21].

The results of this study showed that experimental pretreatment of dentin with curcuminoids did not adversely affect the immediate microtensile bond strength compared to control. This finding is also in line with a recent study [23], confirming no adverse effect on immediate dentin bond strength when a curcumin-based cavity disinfectant was used. On the other hand, pretreatment of dentin with curcuminoids before adhesive application resulted in bond strength preservation after 12-month of aging.

The possible role of host-derived endogenous proteases in the progressive loss of resin-dentin bond integrity and the reduction in bond strength has been extensively reported in the last decade [4, 7, 24]. To tackle this problem, use of natural or synthetic crosslinking agents to increase the longevity of resin-dentin bonds has gained increased attention [8-12]. The use of crosslinkers can be considered as a biological tissue engineering approach, where demineralized dentin matrix has been modified through exogenous collagen crosslinking, resulting in the enhanced physicochemical properties [8]. Previous studies investigated the use of crosslinkers such as glutaraldehyde, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), proantocyanidins, green tea extracts as biomodifying agents [8-12]. Among natural crosslinkers, curcuminoids were identified as one of the promising agents, due to antimicrobial

properties and significant inhibitory effect on endogenous enzymatic activity of dentin [25], which was sustainable over an extended period of time [13].

Host-derived enzymatic activity in demineralized dentin matrices was reported to be immediately evident after activation [26], however, a significant loss in the bond strength can only be observed after an extended aging period, such as 6-months of clinical function [4] or 1-2 years of aging in *in vitro* conditions [27]. Therefore, a 12-months water storage in artificial saliva was used in the current study to evaluate if curcuminoids can sustain their activity in dentin over an extended time.

Curcumin is a well-known potent and multi-targeting phytochemicals agent, but its use as a therapeutic agent is limited due to its low bioavailability and rapid metabolism. Curcumin's metabolism is known to result in unstable products and low efficiency [28]. Structural modifications had been reported to overcome the rapid metabolism as well as inhibitory inefficiency. Both demethoxycurcumin (MC) and difluorocurcumin (DC) were reported to exhibit much better chemical stability and produce tighter binding in the active site of enzymes than curcumin (CR) or other analogs [29]. However, previous studies comparing their enzyme inactivation effect on demineralized dentin matrices showed only minor differences between their efficiency, which was slightly increased by increased concentration [20]. However, since the sustainability of the enzyme inactivation was not verified for the analogs of curcumin, this study included different curcuminoid analogs with two different concentrations to determine if these analogs can sustain their dentin preserving effect over time.

Consistent with the previous reports, after 12 months of incubation a range of 38-44% reduction in bond strength was observed in control groups in this study [16]. On the other hand, the pretreatment groups with various curcuminoid analogs showed much less reduction ranging between 5-18% between the groups. Therefore, this results clearly shows that a short pretreatment with curcuminoids can result in significant attenuation of the μTBS reduction

associated with 12 months incubation in artificial saliva. This results also correlates well with our previous findings showing the significant reduction on the degradation of dentin collagen matrices even after aging conditions [20]. The possible mechanism might be explained with the inhibitory effect of curcuminoids on dentin MMPs. Curcuminoids can chelate the catalytic Zn^{2+} ion essential for MMP activity via β-diketone zinc binding site of curcumin similar to the tetracycline based MMP inhibitors [30-32]. This β-diketone form contains an activated carbon in the heptadienone linkage between the two phenolic rings due to the delocalization of the unpaired electron on the adjacent oxygens of this carbon. In acidic and neutral pH (pH between 3 and 7), the C–H bonds are weak on this carbon and this renders curcumin as a potent H-atom donor [33, 34]. In mildly acidic conditions, curcuminoids contribute this process by chelating and removing of metal ions [35] which is already in dentinal fluids. Proteolytic enzymes such as MMPs are calcium- and zinc-dependent endopeptidases [36]. In the present study, we used artificial saliva as a source of zinc and calcium ions, which would ensure the continuing activity of MMPs to simulate clinical conditions.

Since curcumin is practically insoluble in water at acidic and neutral pH, and unstable in alkali solutions, 1-2% DMSO/water solution was used to solubilize the curcuminoids to prepare the pretreatment solutions. DMSO is one of the most common organic solvents in biological science that could help to protect biological materials from damage by freezing [37]. It has been used to decrease the resin-dentin bond degradation and reduce the enzymatic degradation by proteolytic enzymes in the hybrid layer [38-40]. Stape et al. [40] reported a significant reduction of collagen degradation on DMSO-treated demineralized dentin as a result of enzymatic debinding from the collagen matrix solutions. However, DMSO could debind or denaturate proteins only at high concentrations such as over 40w/v DMSO-water concentrations [41]. Thus, it is not surprising to observe similar bond strength reduction after 12 months in 1-2% DMSO pretreated groups compared to UT control group. The

concentrations used in this study to dissolve curcuminoids are obviously too low to interact with endogenous enzymes and preserve the longevity of the restorations.

Beside the inhibitory effect of curcuminoids on host derived endogenous enzymes, it is worth to mention that the interaction between collagen and curcuminoid can also improve the mechanical properties of dentin [42]. The phenolic compound of curcuminoids is responsible for the interaction of the collagen fibril and curcuminoid by forming the hydrogen bonding. However, this interaction is limited with the slight changes without altering the secondary structural features of protein dramatically [42].

When the fractured surfaces were observed under scanning electron microscopy, it was not surprising to see that adhesive and mixed failure modes were the main mode of failure among the fracture modes for all experimental groups. The fracture including the interface is the most common failure mode for resin-bonded restoration [43]. However, the localization of the fracture showed differences between controls and curcuminoid treated groups. The fracture of control groups mostly concentrated in between dentin surface and adhesive layer, deep at the bottom of the hybrid layer, whereas the fractures on curcuminoid treated specimens localized on the very top of the hybrid layer extending inside the adhesive layer. Panchatcharam et al. [44] reported a significant increase in tensile strength and shrinkage temperature on curcumin-treated collagen and also an increase in the aldehyde content which is the indicator of new crosslink formation in collagen. This could explain the differences observed in failures. The interaction between curcuminoid and collagen prior to adhesive application may help to reinforce and stabilize three-dimensional structure of collagen, resulting in increased resistance of anchoring dentinal collagen fibrils to fractures due to the increased number of crosslinking of collagen fibrils. When the collagen fibrils tensile strength increases due to the increased crosslinks, they can resist the stresses better resulting in the failures on the top of the hybrid layer between adhesive and dentin layer rather than at the bottom of the hybrid layer as it was

observed in this study. This may explain the differences between localization of the failure in the hybrid layer.

Like other phenolic compounds such as grape seed extract, one of the concerns for using curcumin as pretreatment on demineralized dentin is the possible discoloration of dentin. Curcuminoid solutions are pale yellow in color, which is more favorable compared to dark-colored grape seed extract solutions, resulting in a slight discoloration in dentin structure. Although one or two shade differences in dentin color could be masked under contemporary tooth-color restoration, it could limit their use for anterior superficial restorations.

Within the limitation of this study, the pretreatment of curcuminoids improved the stability of resin dentin bonds after 12 months storage in artificial saliva. The results of the study suggest that use of corresponding curcuminoids as a pretreatment prior to bonding agent application would help to prevent the degradation of dentin collagen matrices and can increase the longevity of bonding. However, MMPs can be activated *in vivo* by other MMPs and proteases when the inhibition of MMPs by curcumin pretreatment does not continue. Therefore, whether such inactivation is sustainable over an extended time must be verified also in the clinical trials.

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AUTHOR CONTRIBUTION

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CONFLICT OF INTEREST

The authors of this manuscript declare no conflict of interest and certify that they have no proprietary, financial, or other personal interest regarding anything presented in this article.

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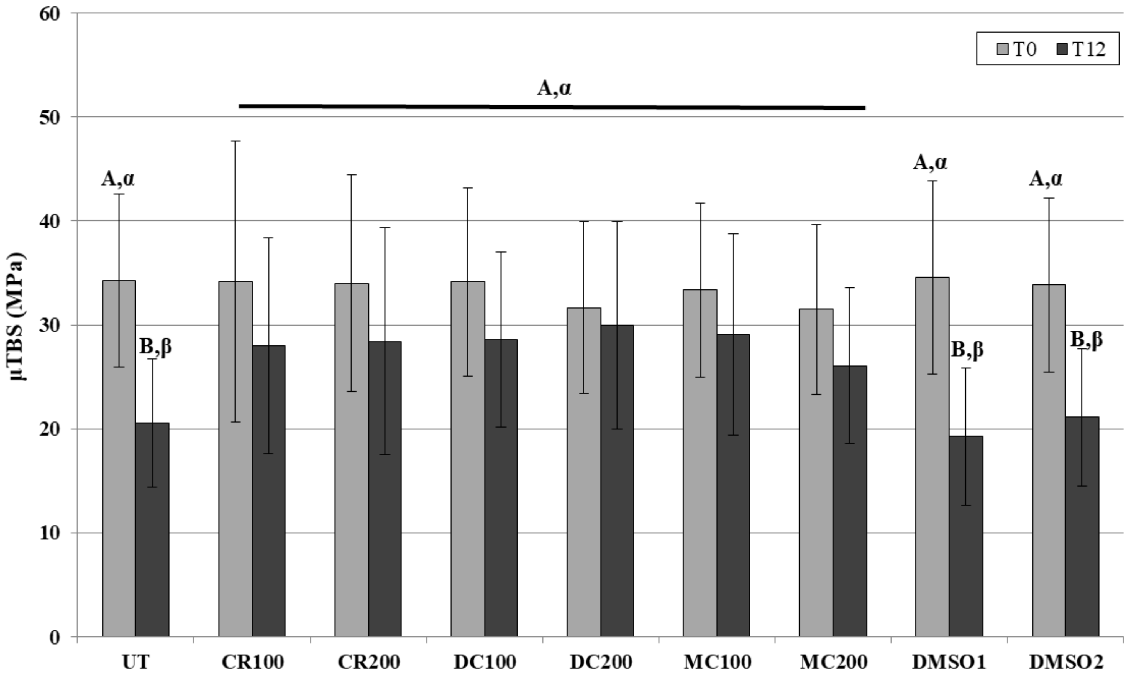
FIGURE LEGENDS

FIGURE 1 The μTBS values (MPa, mean±SD) observed initially (T0) and after 12 months (T12) storage for the experimental groups, following the pretreatment of curcuminoids. Upper letters indicate statistically significant ($p < 0.05$) differences within each treatment, α, β indicate statistical difference between T0 and T12.

FIGURE 2 Distribution of the fracture patterns seen, given as percentages within the experimental groups.

FIGURE 3 Representative micrograps of debonded interfaces of the specimens. (A) UT control showed debonding at the bottom of the hyrid layer (1750x). (B) DMSO1 and (C) DMSO2 treated groups showed similar fracture pattern to UT control (1950x). (D) Most of failures were observed in the adhesive resin layer for group treated with 200μM of curcumin (1850x). (E) and (F) Similar to curcumin, CR100 (1000x) and MC200 also showed predominantly adhesive failure but also including composite resin mixture (1750x). D: Dentin; AS: Adhesive surface; CR: Composite resin.



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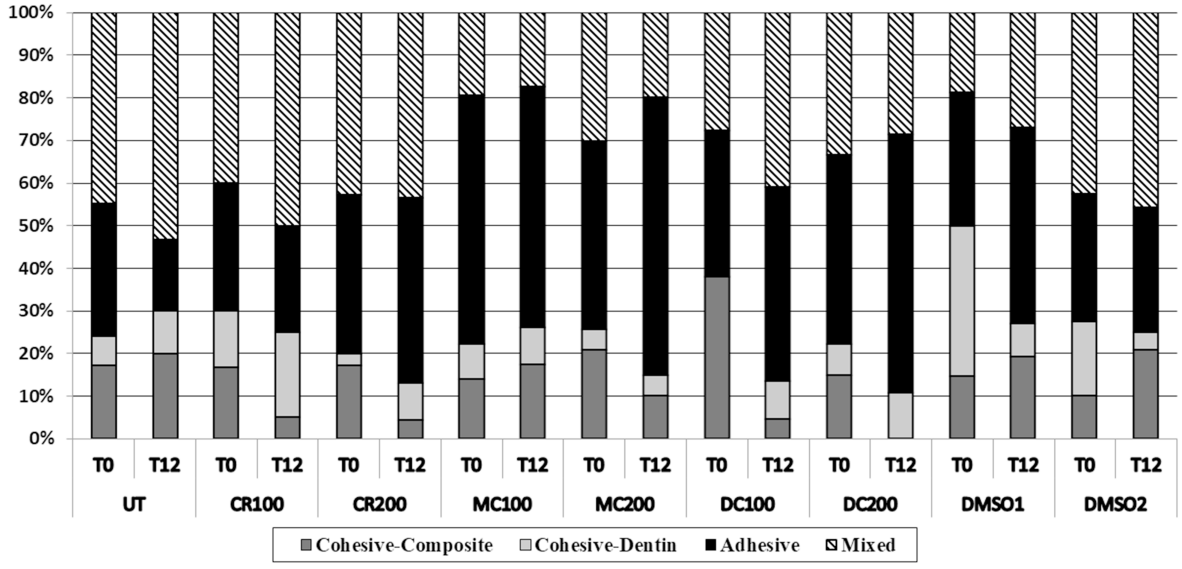

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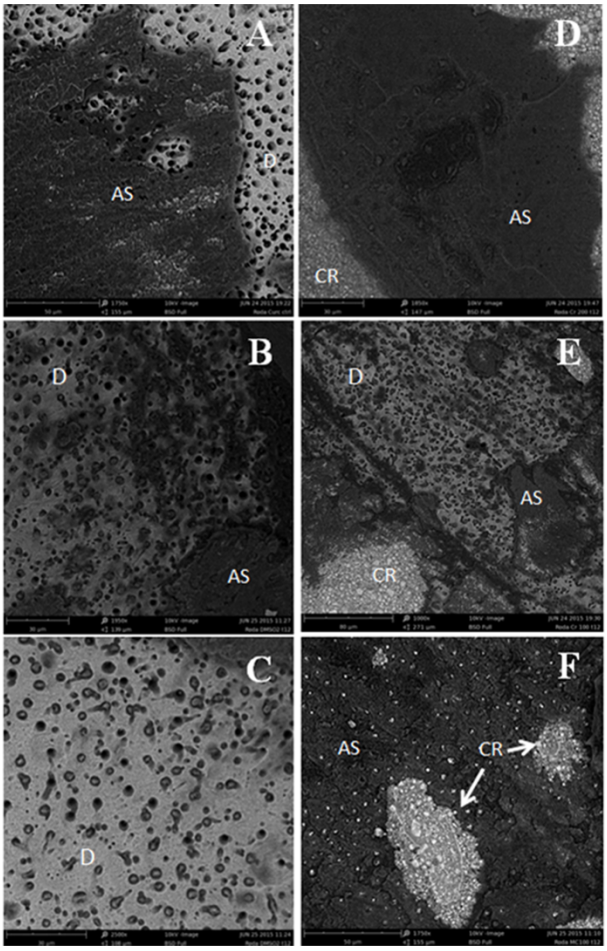

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TABLE 1 Compositions, application procedures, and lot numbers of the restorative and adhesive materials

Material (Manufacturer)	Composition	Application Procedure	Lot No.
Scotchbond™ Universal Etchant (3M ESPE)	32% phosphoric acid (UXT-02/Etch-01)	Etch for 15 s. Rinse for 15 s and blot dry.	466293
			282421
Curcumin (Purity 99%) (LKT Lab.) **Demethoxycurcumin (LKT Lab.)** **3,4 Difluorobenzocurcumin (LKT Lab.)**	100 μM or 200μM of curcuminoid solutions	Pretreatment of curcuminoid solutions or distilled water for 1 min and then blot-drying	282412
			284361
Adper™ Single Bond Plus (3M ESPE)	Filled (5%–10% wt silane-treated colloidal silica [5 nm]): Ethyl alcohol, bis-GMA, HEMA, copolymer of acrylic and itaconic acids, glycerol 1,3-dimethacrylate, water, diurethane dimethacrylate (4BR)	Apply adhesive to tooth surface for 15 s. Gently air dry for 5 s at distance of 10 cm to the surface. Light cure for 10 s.	N417672
Filtek™ Supreme XTE (3M ESPE) Body Shade A3	*Matrix:* bis-GMA, UDMA, TEGDMA, PEGDMA, bis-EMA *Filler:* zirconia/silica cluster (comprised 20 nm silica and 4-10 nm zirconia particles), zircon/silicon nanoclusters (0.6–10 μm), nanofiller 78.5% by wt (63.3% by volume)	Apply in 1-2 mm increments and light cure for 20 s.	N239660

Abbreviations: Bis-GMA, bisphenol A glycidyl methacrylate; HEMA, 2-hydroxyethyl methacrylate; UDMA, urethane dimethacrylate; TEGDMA, triethylene glycol dimethacrylate; PEGDMA, polyethylene glycol dimethacrylate; bis-EMA, ethoxylated bisphenol-A-dimethacrylate.

TABLE 2 Linear regression analysis of the μTSB values (MPa) as a function of pretreatment agent, agent concentration and storage time as the predictor variables.

Predictor variable		Regression coefficient β	Sig.	95% Confidence Interval for β	
				Lower Bound	Upper Bound
Pretreatment agent	DMSO	Ref.	-	-	-
	CR	-0.05	ns	-3.06	2.96
	DC	-1.22	ns	-4.30	1.86
	MC	-1.74	ns	-4.88	1.40
	Untreated	-0.30	ns	-4.39	3.79
Agent Concentration	100 μL/1%	Ref.	-	-	-
	200 μL/2%	-0.67	ns	-2.49	1.15
Storage time	24 h	Ref.	-	-	-
	12 months	-14.04	***<0.001***	-17.76	-10.33
Agent * Time	CR – 12 months	8.07	***0.004***	2.65	13.49
	DC – 12 months	10.44	***<0.001***	5.30	15.58
	MC – 12 months	9.58	***0.001***	4.01	15.15
	Untreated -12 months	0.36	ns	-5.76	6.48
Constant		34.53	***<0.001***	32.32	36.75

Abbreviations: UT: Untreated; CR: Curcumin; DC: Difluorobenzocurcumin; MC: Demethoxycurcumin; DMSO: Dimethyl sulfoxide. ns: not statistically significantly different from 0.

TABLE 3 Results of logistic regression analysis of the outcome mixed failure mode as a function of the pretreatment agent, the agent concentration and the storage time.

	Odds ratio	95% Confidence Interval for OR	
		Lower	Upper
Pretreatment agent			
UT	1 (ref)		
CR	0.61	0.35	1.07
DC	0.65	0.38	1.10
MC	0.39	0.22	0.69
DMSO	1.53	0.78	3.01
Agent concentration			
100 µL/1%	1 (ref)		
200 µL/2%	1.48	1.00	2.19
Storage time			
24 h	1 (ref)		
12 months	1.15	0.79	1.66

Abbreviations: UT: Untreated; CR: Curcumin; DC: Difluorobenzocurcumin; MC: Demethoxycurcumin; DMSO: Dimethyl sulfoxide