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Influence of flavonoids on long-term bonding stability on caries-affected dentin

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ABSTRACT

Objectives. To evaluate the effect of experimental dentin pre-treatment solutions formulated with different flavonoids on microtensile bond strength (μTBS), nanohardness (NH) and ultra-morphological characteristics of artificial caries-affected dentin (CAD) bonded using a universal bonding system.

Methods. A microbiological method was used to create an artificial CAD in 91 human molars. Five experimental pre-treatment solutions were created using the following flavonoids: quercetin (QUE); hesperidin (HES); rutin (RUT); naringin (NAR), or proanthocyanidin (PRO). A placebo solution (PLA) with no flavonoids added was also evaluated. The flavonoids or placebo solutions were applied to the CAD prior to the application and photoactivation of a universal adhesive (Scotchbond Universal, 3M Oral Care). A control group (CON), in which only the bonding agent was applied without any flavonoid solution, was also evaluated. A 3-mm-thick block of resin composite (Opallis, FGM) was built up on the flat bonded CAD surfaces and was light-cured following the manufacturer's instructions. Specimens were sectioned to obtain resin-dentin slices and sticks (cross-sectional area of 0.8 mm^2). The μTBS, NH, and confocal ultramorphology analysis of resin-dentin interface was evaluated at 24 h and after thermo-cycling aging (25,000 cycles). The results were analyzed using 2-way ANOVA followed by Bonferroni's post hoc test (pre-set $\alpha = 0.05$).

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Results. The specimens from groups QUE, NAR, and RUT presented greater μTBS values than those from CON group ($p<0.05$). Specimens from some of these experimental groups presented greater nanomechanical properties ($p<0.05$), and no morphological degradation at the resin-dentin interface after aging.

Significance. The use of exogenous cross-linkers as dentin pre-treatment before bonding procedures may represent a suitable strategy to improve the longevity of universal adhesive systems applied to caries-affected dentin.

1. Introduction

According to the Global Burden of Disease (GBD) study, dental caries is the most prevalent pathological condition worldwide [1]. Indeed, dental caries in primary and permanent teeth continue to be a major problem for the public health system [2]. When cariogenic bacteria reach the dentinal substrate, endogenous metalloproteinases (MMPs) [3–5] and cysteine cathepsins (CP) are activated [6], resulting in denaturation of collagen fibrils. This process compromises the mechanical properties of dentin and accelerates its degradation [7–9].

Considering the philosophy of minimally invasive dentistry, restorative procedures performed on specific substrates such as caries-affected dentin (CAD) [10] have become a feasible option. However, there are still some concerns regarding the bonding on such challenging substrate; the quality and the durability of the bonding to CAD may be considered unreliable [11] when compared to sound dentin [12,13]. Indeed, the degradation of the bonding interface created on CAD is much more evident than that observed on the bonding interface created on sound dentin [9,14].

Despite the immediate effect of chlorhexidine as an MMPs inhibitor, the limited, short-lasting effect of this substance has shifted the attention of researchers towards therapeutic substances that may offer a long-lasting effect. In this context, the use of exogenous cross-linking agents has been proposed as an effective method to inhibit the activity of MMPs, therefore preserving collagen within the dentin-bonded interface [15]. Among these agents, hesperidin (HES), a glycoside vasoactive flavone, has been advocated to reduce the degradation at the bonding interface when incorporated into a two-step self-etching adhesive system [16]; HES was more efficient in preserving the integrity of the resin-dentin interface than proanthocyanidin (PRO) [16,17]. In addition, despite the benefits of PRO as a cross-linker agent, its incorporation within the composition of adhesives may compromise their bonding performance to dentin [16,18,19].

Moreover, glycoside flavones may also have antimicrobial effects on several $Gram^+$ and $Gram^-$ bacterial strains, as well as on *Staphylococcus aureus* [20–22]. The multiple effects of flavonoids, such as HES on sound dentin, have brought a new alternative for the development of therapeutic substances to improve bonding stability on CAD. Among phenolic compounds, there are other molecules with similar structure, such as quercetin (QUE), rutin (RUT), and naringin (NAR) (glycoside flavones). However, to date, no evidence about the potential effects of these substances on long-term bonding on CAD is available.

The aim of this *in vitro* study was to evaluate the influence of experimental dentin pre-treatment solutions containing QUE, HES, RUT, NAR, or PRO on microtensile bond-strength (μTBS), nanohardness (NH), and the morphology of the resin-dentin interface created by a universal adhesive system applied to CAD in etch-and-rinse mode. The first null hypothesis of this study was that there would be no differences in μTBS and NH values when flavonoids are applied to CAD during the bonding procedure. The second null hypothesis was that the μTBS and NH values in experimental groups with flavonoids after ageing would be no different from those before ageing. The third null hypothesis was that there would be no change in the morphology of resin-dentin interface when flavonoids were used.

2. Materials and methods

2.1. Formulation of experimental solutions

Experimental solutions with QUE, HES, RUT, NAR (Sigma Aldrich, St. Louis, MI, USA) and proanthocyanidin (95%) (PRO) (grape seed extract from *Vitis vinifera*. Active Pharmaceutica, Palhoça, SC, Brazil) were made. The physical and chemical properties of the molecules are displayed in Table 1. The primer solutions were determined considering purity, solubility index, hydrophobic nature, and the critical micelle concentration (CMC) of each flavonoid. An exclusive equation was used to the maximum availability of flavonoids in a liquid state without affecting their properties (Table 2) [23]. A placebo solution (PLA), containing only the vehicles (Table 2) used in the solution but with no flavonoid added, was included as a further experimental group. A control group (CON) where only the adhesive was applied to CAD following the manufacturer's instructions was also included.

2.2. Specimen preparation

Ninety-one caries-free extracted human third molars obtained from patients (range: 18 – 35 years old) were used. After approval by the local Ethics Committee (protocol # 41.2017), teeth were collected from several patients, and an informed consent for surgery was obtained through a written document. All the extracted teeth were disinfected in 0.5% chloramine, stored in distilled water and used within three months after extraction. A flat mid-dentin surface was exposed on each tooth using a 180-grit SiC paper under continuous irrigation.

Table 1 – Physical and Chemical properties of the molecules used in this study.

SUBSTANCE	MOLECULAR MASS	NUMBER OF HYDROXYPHENYL RADICALS	NUMBER OF ALCOHOLIC RADICALS	NUMBER OF MOLS (6.5% MASS)	SOLUBILITY IN WATER
HESPERIDIN	610.56 g/mol	2	6	1.06 mM	0.02 mg/mL
NARINGIN	580.53 g/mol	2	6	1.12 mM	1 mg/mL at 40 °C
PROANTHOCIANYDIN	595.55 g/mol	7[a]	2[a]	1.09 mM[a]	0.130 mg/mL[a]
QUERCETIN	302.24 g/mol	5	-	2.15 mM	0.06 mg/mL
RUTIN	610.52 g/mol	4	6	1.06 mM	0.125 mg/mL

[a] Expected properties of the Proanthocianydin mer, which may vary according to the number of mers present in the final molecule (oligomer or polymer), reducing solubility and increasing the molecular mass.

Table 2 – Composition of a hydro-alcoholic solution of flavonoid (6.5% mass).

Component	Compound	Quantity %
Active Compound	Flavonoid	6.5% mass
Vehicle (Pure Ethanol)	Pure Ethanol	30% (3 mL)
Surfactant (Polysorbate 20)	SPAN 20	1% (0.1 g)
Aqueous medium	Distilled Water	QS 10 mL

2.2.1. *Simulated microbiological caries*

Such a method to create a simulated microbiological-based caries lesion on dentin was validated in a previous study [24]. All specimen surfaces were covered with a layer of epoxy resin (Araldite, Brascola Ltda, São Bernardo do Campo, SP, Brazil), followed by a layer of nail varnish, while only the occlusal surface was left exposed. All specimens were sterilized in steam autoclave (Phoenix Ind. Brasileira, Araraquara, SP, Brazil) for 15 min at 121 °C [25], and each tooth was individually immersed in an 8-mL Falcon tube containing an artificial caries solution. Such a solution was composed of 9.25 g of brain heart infusion culture supplemented with 1.25 g of yeast extract, 5.0 g of sucrose, in 250 mL of distilled water and 100 μL of primary culture of *S. mutans* (INCQS 00446), with the pH around 4.0. The specimens were incubated in an anaerobic jar (5% CO_2) at 37 °C. The specimens were transferred to another 8-mL Falcon tube containing a new artificial caries solution every 48 h. After 14 days, all specimens were sterilized again as previously described and washed with deionized water [26].

2.2.2. *Bonding procedures*

The experimental design is presented in Fig. 1. Prior to the bonding procedures, the surrounding enamel of each specimen was removed with a diamond bur (#4137, KG Sorensen, Barueri, SP, Brazil), until the dentin surface was totally exposed. Subsequently, the occlusal dentin surface of each specimen was polished using a 600-grit silicon-carbide paper for 30 s to obtain a standardized smear layer [24]. Afterwards, the teeth were randomly allocated to the following experimental groups: QUE, HES, RUT, NAR, PRO, PLA, and CON.

The occlusal dentin surface was etched with 37% phosphoric acid for 15 s, water-rinsed for 30 s and air-blown dried for 5 s. Each respective experimental solution was actively applied for 1 min to re-wet the dentin surface. These were slightly air-dried for 2 s and the moisture was homogenized with absorbent paper leaving a wet surface. The universal adhesive system Scotchbond Universal (3M Oral Care, Saint Paul, MN, USA) was applied following the manufacturer's instructions (Table 3) and light-cured for 10 s at standard mode using a polywave LED curing system (Valo, Ultradent Products, South Jordan, UT, USA). A 3-mm thick resin composite block (Opallis, FGM Prod. Odont. Ltda, Joinville, SC, Brazil) was built up on the bonded surfaces in three increments of 1-mm thick; each one was individually light-cured for 40 s (Valo, Ultradent Products). A single operator carried out all the bonding and restorative procedures in an environment with controlled temperature and humidity.

The specimens were stored in distilled water at 37 °C for 24 h. After storage, only 21 specimens were longitudinally sectioned in "x" direction across the bonded interface (IsoMet 1000; Buehler, Lake Bluff, USA), under water cooling at 300 rpm to obtain 1.2-mm thick resin-dentin slices for NH analysis. Forty-nine specimens (n = 7) were longitudinally sectioned in both "x" and "y" directions across the bonded interface to obtain resin-dentin sticks with a cross-sectional area of 0.8 mm^2; the exact dimensions were measured using a digital caliper and recorded to determine the μTBS values (Absolute Digimatic, Mitutoyo, Tokyo, Japan). Half of the sticks were evaluated after 24 h, while the other half were submitted to thermocycling (25,000 cycles; dwell time of 30 s from 5 °C to 55 °C; Odeme, Joaçaba, SC, Brazil) prior to μTBS testing [27].

2.3. *Microtensile bond strength testing*

Each stick was attached to a modified device for μTBS test with cyanoacrylate resin (IC-Gel, bSi Inc., Atascadero, CA, USA) and subjected to a tensile force in a universal testing machine (Kratos, São Paulo, SP, Brazil) at 0.5 mm/min. The failure mode was evaluated under an optical microscope (SZH-131, Olympus, Tokyo, Japan) at 40x and classified as cohesive in dentin (failure exclusive within cohesive dentin – CD); cohesive in resin (failure exclusive within cohesive resin – CR); adhesive (failure at resin-dentin interface – A), or mixed (failure at resin-dentin interface that included cohesive failure of the adjacent substrates, M). The number of premature failures (PF) was recorded and was not included in the average μTBS results.

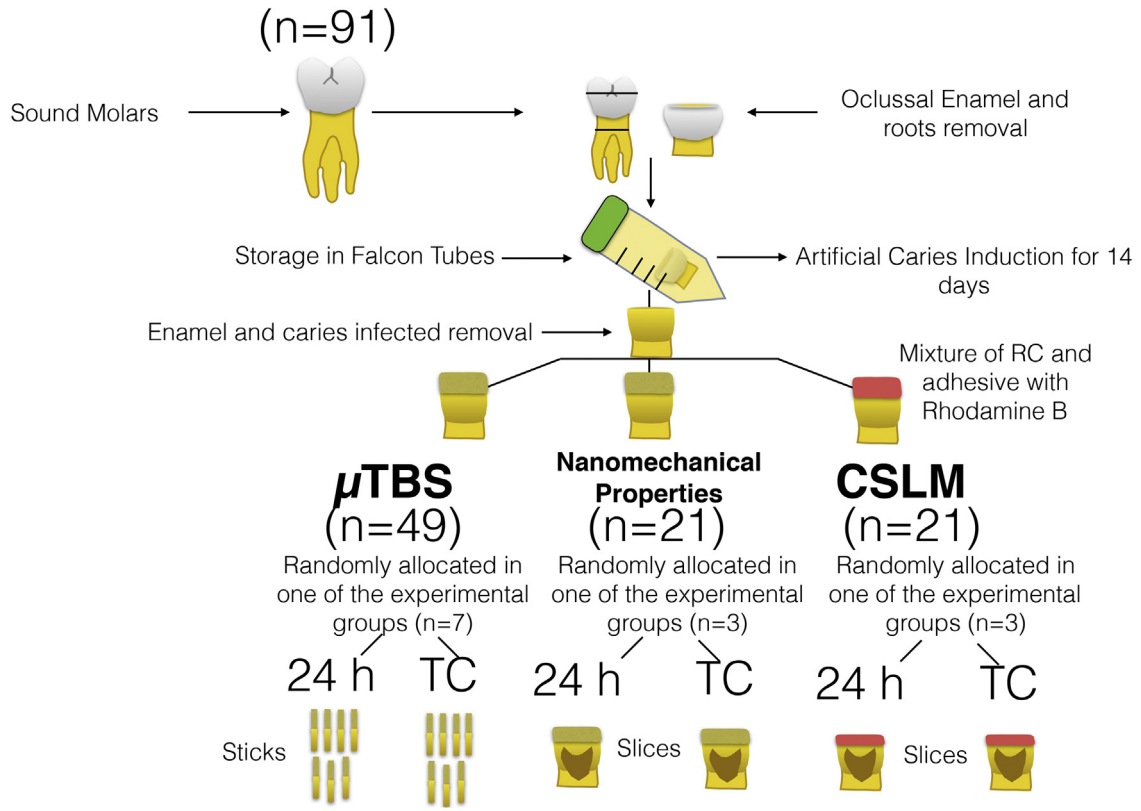

Fig. 1 – Experimental design used in this study.

Table 3 – Adhesive and experimental solutions used in this study.

Substance	Composition	Application
3M Scotch Bond Universal	Ingredients: MDP, Dimetacrilate resins, HEMA, Vitrebond™ copolymer. Filling particles, ethanol, water, initiators, Silane.	Total etch technique – Apply 1 drop of Single Bond Universal over the etched dentin regions for 20 s, dry for 5 s and cure for 10 s
Hesperidin Solution	Hydro alcoholic solution of Hesperidin 6.5%	Actively apply for 1 min over etched dentin to rewet.
Quercetin Solution	Hydro alcoholic solution of Quercetin 6.5%	Actively apply for 1 min over etched dentin to rewet.
Rutin Solution	Hydro alcoholic solution of Rutin 6,5%	Actively apply for 1 min over etched dentin to rewet.
Naringin Solution	Hydro alcoholic solution of Naringin 6.5%	Actively apply for 1 min over etched dentin to rewet.
Proanthocyanidin Solution	Hydro alcoholic solution of GSE 6,5% (95% of PRO)	Actively apply for 1 min over etched dentin to rewet.
Placebo	Hydro alcoholic solution with tensoactive SPAN 20.	Actively apply for 1 min over etched dentin to rewet.

2.4. *Nanohardness within adhesive layer, hybrid layer and dentin*

The resin-dentin slices obtained previously from 21 restored teeth (n = 3) were wet-polished using 1000 to 4000-grit SiC papers for 30 s each and cleaned in an ultrasonic water bath for 5 min. The specimens were attached to a metal stub and placed in a nanoindenter device (UNAT nanoindenter, Asmec, Dresden, Germany), which had a Berkovich indenter (20 nm radius) to evaluate the NH. The adhesive interface was visualized with a microscope, and a net of 24 indentations was created (6 in the "x" axis and 4 in the "y" axis) with a load of 5000 nN and a function time of 10 s starting from the adhesive layer (AL) and moving down towards the resin-dentin interface and dentin. This procedure was done to evaluate the hybrid layer (HL) as well as the dentin at a 50 μm depth. The distance between each indentation was consistently maintained by adjusting the distance range by 100 μm (±10 μm) per range on the "x" axis. The values obtained after the indentation were analyzed in a software to calculate NH (InspectorX, ASMEC GmbH, Dresden, Germany).

2.5. *Confocal ultramorphology evaluation*

Prior to the bonding procedures, the adhesive system was doped with Rhodamine B (83689-1 G, Sigma-Aldrich, Saint Louis, MI, USA) at approximately 0.2 wt.% [28]. The specimens were restored as previously described for μTBS test. Half of the slices (n = 3) were immersed in 0.1 wt.% sodium fluorescein (46960-25G-F, Sigma-Aldrich, Saint Louis, MI, USA) for 4 h [28,29], while the other half (n = 3) were aged by thermocycling, as previously described, and then immersed in Fluorescein.

Specimens were polished with 1000 to 2500-grit SiC for 30 s and ultrasonically cleaned (2 min), air-dried, and the resin-dentine interfaces were analyzed using Confocal Laser

Scanning Microscopy (CLSM) (DMi8 Cell Advanced Leica, Mannheim, Germany) equipped with a 63×/1.4 NA oil immersion lens. The emission fluorescence was recorded at 512–538 nm (Fluorescein) and 585–650 nm (Rhodamine B). Ten images from each slab were randomly captured at 5 and 10 μm and were analyzed with the CLSM image software (LAS X, Leica, Heidelberg, Germany).

2.6. Statistical analysis

The analysis was performed using the tooth as the statistical unit. The μTBS and NH results were averaged to obtain the mean bond strength for each tooth. The values were submitted to a 2-way repeated ANOVA, followed by Bonferroni's post hoc test (pre-set $\alpha = 0.05$). Post-hoc power analysis was performed using the SPSS19 (IBM Company, Armonk, NY, USA). The morphological characteristics of the HL were qualitatively evaluated in CLSM.

3. Results

3.1. Microtensile bond strength and fracture analysis

The study had adequate power for both factors (treatment; time), (> 90%; $\alpha = 0.05$). The μTBS values are displayed in Table 4. After 24 h, the groups QUE, RUT, NAR showed the highest μTBS values, which were significantly higher than those observed in CON (p=0.005, p=0.001, p=0.008, respectively). No significant difference was observed between the other experimental groups; CON showed the lowest values at both periods. After thermocycling, RUT and NAR groups exhibited the highest μTBS values (p<0.001), while the specimens in CON and QUE groups presented the lowest μTBS values after thermocycling. Only specimens from RUT, CON, and QUE groups exhibited a significant drop in μTBS after thermocycling (p=0.013, p=0.012, p<0.01, respectively; Table 4).

Adhesive failures were predominantly found in all groups (Fig. 2). However, RUT showed a higher cohesive/resin pattern than the other groups, while NAR group showed predominantly cohesive/dentin faillure pattern.

3.2. Nanohardness

At the AL, no significant differences in NH values were found over time within each experimental group. Conversely, a significant NH drop was observed after thermocycling within the HL in all groups (p=0.034) (Table 5). The specimens of the QUE group showed the highest values at the AL, while the specimens of the HES and NAR groups had the greatest values at the HL (p<0.05). Only the specimens in the CON group showed significantly lower NH values at the AL compared to those from QUE group (p=0.01). Moreover, at the HL, only PRO and RUT groups showed lower values than NAR and HES groups (p<0.05).

The groups without active compounds in the pre-treatment (CON and PLA) showed the lowest NH values at 50 μm dentin depth. On the other hand, the dentin of the specimens in NAR group showed the highest NH values, but with no significant difference compared to the specimens of the other experimental groups (RUT, QUE, PRO and HES). Overall, NH values were significantly higher at 24 h interval than those after thermocycling.

3.3. Analysis of the hybrid layer using CLSM

Specific morphological aspects were observed at the resin-dentin interface in all groups. For instance, the specimens of the CON group showed a thick HL without long and well-defined resin tags (Fig. 3a). However, some specimens in the control group showed a very thin or no HL, along with long and well-defined resin tags (Fig. 3d). The specimens of the CON group also presented regions with a high number of blisters along the adhesive layer, while the experimental groups showed few or even no blisters. Indeed, RUT, PRO, NAR and PLA groups exhibited almost no blisters at the resin dentine interface at 24 h. The most significant change in morphology observed after thermocycling was the lack of HL in many regions of the interface; this was especially evident in the specimens of the QUE group (Fig. 3).

4. Discussion

This study showed that only some of the tested flavonoids promoted higher μTBS and NH values at the resin-dentin interface compared to the CON group. Thus, the first null hypothesis must be partially rejected. It has been advocated that the potential interaction between crosslinking agents and dentin depends on their ability to form covalent or hydrogen bonds with collagen fibrils [30]. Indeed, previous studies showed a close relationship between the number of reactive hydroxyphenyl groups available in crosslinkers and their ability to react and form ionic or covalent bonding with hydroxyl, carboxyl, amine or amide groups in collagen [31–33].

Some factors may have contributed to improve the effect that crosslinkers have over CAD in improving its mechanical properties. For instance, i) the molecule size: smaller molecules can diffuse more easily into the demineralized dentin; ii) the number of molecules available in the experimental solution; iii) the solubility index of the molecule and its influence on the miscibility of the selected vehicle for its application in dentin; iv) the number and type of reactive sites of the molecule (Table 1); and v) the inherent characteristics of CAD and its limited ability to buffer acids. In this context, even though RUT did not present the highest amount of hydroxyphenyl sites, its smaller size, compared to oligomeric or polymeric molecules of PRO, and its high solubility in water (Table 1), may have contributed to attain the highest μTBS values between all the other tested groups. Although the catechin monomer and RUT may have similar characteristics, the type of molecules in grape seed extracts can vary from monomers to oligomers or even polymers. As a consequence, the diffusion of oligomers with greater molecular weight through the dentin can be compromised, and this can affect their interaction with the collagen [34,35].

Unlike the sound dentin, CAD is a substrate characterized by much more porosities, which may allow molecules to diffuse quickly, especially for the more hydrophilic molecules [36,37]. However, the highly calcified regions such as *whitlockite*

Table 4 – Mean μTBS values (SD) of the experimental groups at two intervals.

	24 h	Thermocycling
CON	14.42 (4.43) Ab	9.43 (4.29) Bb
HES	18.41 (5.30) Aab	15.73 (6.07) Aab
PLA	20.54 (4.88) Aab	17.11 (5.27) Aab
PRO	20.66 (3.92) Aab	17.20 (2.72) Aab
QUE	24.58 (4.90) Aa	12.02 (5.21) Bb
NAR	24.64 (3.70) Aa	22.12 (2.92) Aa
RUT	26.00 (5.51) Aa	21.08 (4.75) Ba

Means folowed by same letter (Upper case letters: within row; lower case letter: within column) are not significantly different (pre-set alpha: 0.05).

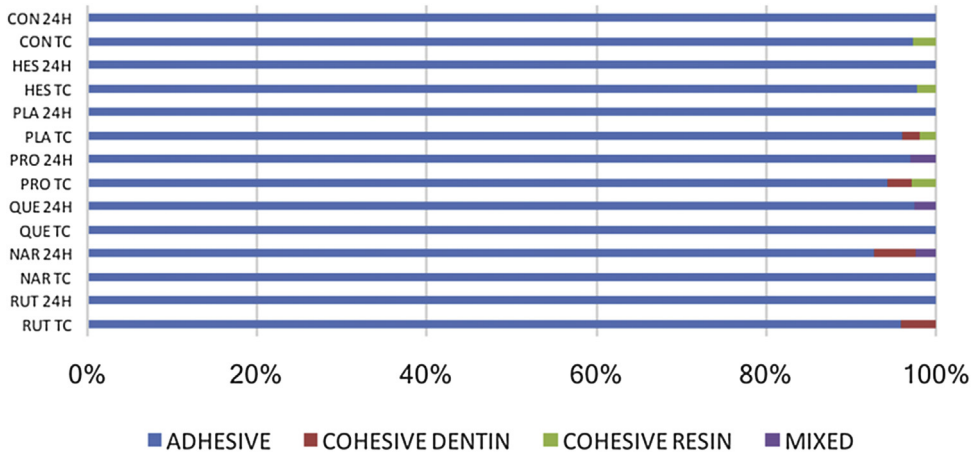

Fig. 2 – Distribution of failure pattern in the experimental groups.

Table 5 – Mean Nanohardness values (SD) of adhesive, hybrid and dentin layers (GPa) created by the different experimental groups at 24 h and after thermocycling.

		CON	HES	PLA	PRO	QUE	NAR	RUT	Average
Adhesive Layer	24 h	0.225 (0.041)	0.328 (0.074)	0.313 (0.087)	0.339 (0.056)	0.455 (0.127)	0.387 (0.092)	0.244 (0.023)	0.327 A
	Thermocycling	0.191 (0.039)	0.306 (0.115)	0.305 (0.013)	0.282 (0.062)	0.338 (0.099)	0.256 (0.025)	0.297 (0.059)	0.282 A
	Average	0.208 b	0.317 ab	0.309 ab	0.311 ab	0.396 a	0.321ab	0.270 ab	
Hybrid Layer	24 h	0.257 (0.156)	0.509 (0.146)	0.208 (0.058)	0.167 (0.076)	0.337 (0.144)	0.489 (0.029)	0.224 (0.018)	0.313 A
	Thermocycling	0.192 (0.069)	0.272 (0.140)	0.222 (0.045)	0.185 (0.093)	0.292 (0.110)	0.306 (0.046)	0.154 (0.061)	0.232 B
	Average	0.225 ab	0.391 a	0.215 ab	0.176 b	0.314 ab	0.397 a	0.189 b	
Dentin 50 μm	24 h	0.482 (0.107)	0.608 (0.018)	0.444 (0.111)	0.454 (0.144)	0.566 (0.098)	0.759 (0.113)	0.571 (0.093)	0.555 A
	Thermocycling	0.370 (0.084)	0.442 (0.063)	0.411 (0.162)	0.470 (0.087)	0.273 (0.072)	0.421 (0.173)	0.461 (0.129)	0.407 B
	Average	0.426 b	0.525 ab	0.428 b	0.462 ab	0.420 b	0.590 a	0.516 ab	

Means folowed by same letter (Upper case letters: within column; lower case letter: within row) are not significantly different (pre-set alpha: 0.05).

areas and sclerotic dentin are missing in artificially-induced CAD [36,38], so the buffering effect of these regions on phosphoric acid may not be expected; the artificially-induced CAD may take longer in re-establishing its original pH due to collagen fibrils that are less capable of counteracting the effect of acid etchants such as phosphoric acid [39]. In an acidic environment, the solubility of organic compounds increases [40], thus allowing flavonoids to migrate and diffuse into dentin. Furthermore, at lower pH, the tridimensional reactive sites of flavonoids can change polarity in their last electron orbit [40]. Indeed, the phenyl hydroxyl reactive sites (cationic) that react with carbonyl and carboxyl may protonate and cause a change in the polarity of the molecule (anionic), turning them reactive with other sites such as amide or amine. Therefore, it is possible that the alcoholic-hydroxyl sites (anionic) protonate and become cationic, so these sites would become reactive with carbonyl and carboxyl sites of collagen. This hypothesis might also explain the high μTBS and NH values observed in the NAR group in both HL and dentin layers, in contrast to previous findings in which NAR promoted lower effect than PRO in sound dentin [41]. This hypothesis may also support the results of RUT, considering that both molecules have a glycoside moiety that could potentially become reactive with collagen in an acidic environment.

The use of some flavonoids also increased the NH values at AL. This effect might be the result of the copolymerization of

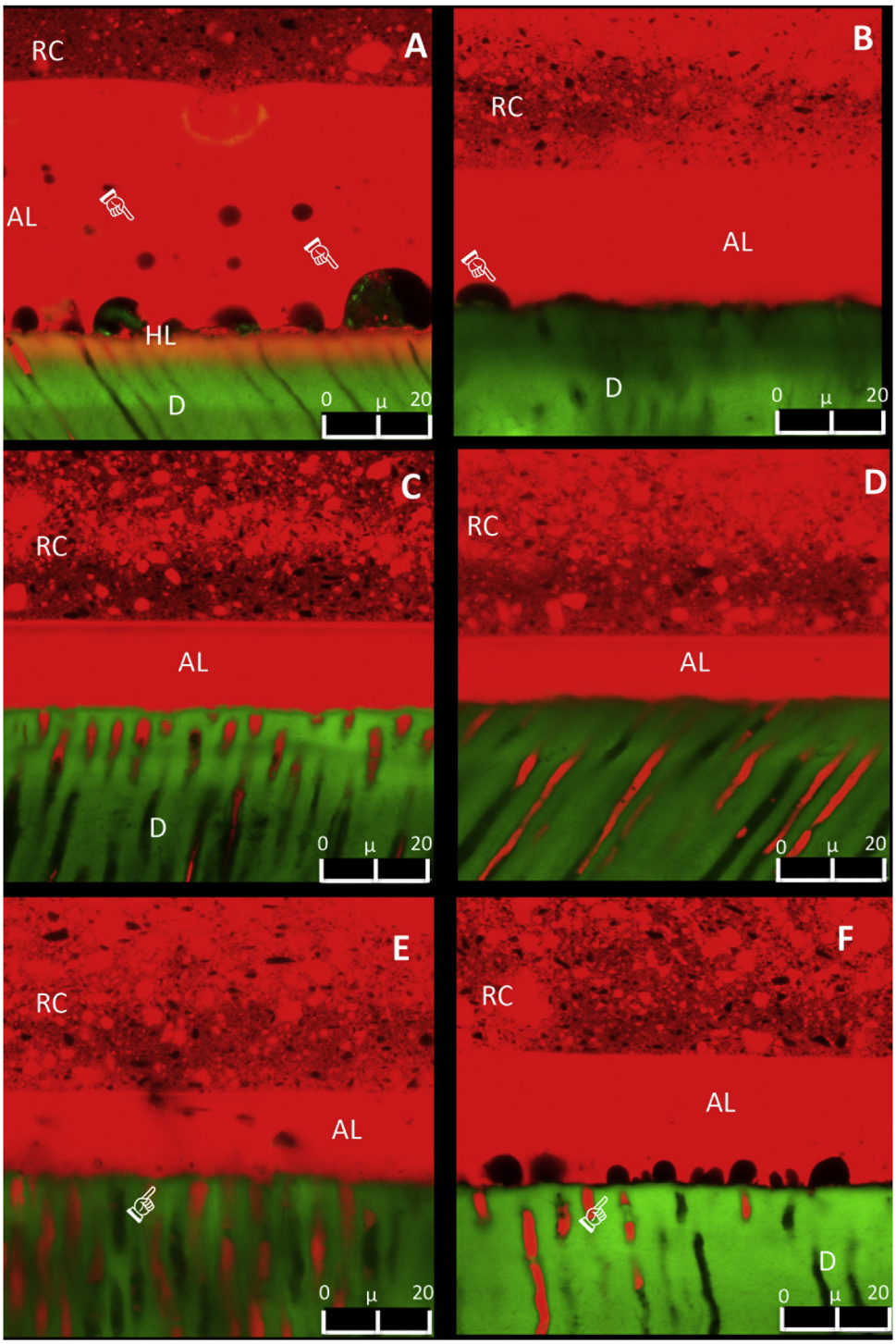

Fig. 3 – Representative CLSM images of adhesive interface created in the CON (a), HES (b), PLA and PRO (c), RUT and NAR (d), QUE (e) groups. Dark circles (white pointer) within the adhesive layer were noted in Control (a) and HES (b) groups. Representative CLSM image (63X, Zoom 2 × . Deep: 10 μm) of QUE group after thermocycling (f). Some regions with missing HL (white pointer) were noted. Resin Composite (RC), Adhesive layer (A), Hybrid layer (HL), Dentin (D). Resin composite (RC).

flavonoids with the bonding agent to create esther-type bonding with the acrylate groups [42,43], which in turn increased the NH values at the AL. Therefore, for some flavonoids, it is reasonable to attribute the increase in NH values at the HL to their ability to not only bind to collagen in an acidic environment, but also to improve the mechanical properties of the adhesive resin within the HL.

The NH values at the dentin layer might somehow indicate the ability of flavonoids to improve the mechanical properties of CAD. However, the variability of the substrate and the

random decalcifying effect of carious lesions made the comparison more difficult to interpret. Nevertheless, the effect of these substances is limited to the collagen fibrils as having only a small effect on the active compounds was encountered, probably due to the reduced mineral content through the tissue. Therefore, the use of flavonoids in combination with mineralizing solutions should be considered to improve the mechanical properties of CAD. Further investigation is required to confirm this latter concept.

The specimens of NAR and RUT groups exhibited the highest μTBS values after thermocycling (TC). In this regard, the influence of flavonoids on the bond strength to CAD after TC may be also useful as an indirect way to evaluate the substantivity of such compounds, which is a relevant aspect of therapeutic biomolecules. In this sense, previous studies using flavonoids have shown promising long-term therapeutic effects [15,16], and this is one of the reasons why the applicability of flavonoids is currently being widely investigated in different biomedical areas. Therefore, it is reasonable to assume that the current outcomes in groups comprising the use of flavonoids may be attributed to the ability of flavonoids to crosslink with the HL structures (natural and synthetic) as well. On the other hand, the QUE group exhibited a significant drop in the μTBS values after ageing (TC). In contrast to RUT or NAR, the QUE molecule does not present a glycoside moiety, so the molecule is susceptible to structural changes when the pH decreases. Therefore, such changes may compromise the effects of QUE molecules over time [44]. Moreover, although the alcoholic hydroxyl endings seem to have an influence over crosslinking and probably act synergistically with the phenyl hydroxyl sites, this effect might be weakened on the polymeric sites of the bonding agent once the NH at the HL values dropped after thermocycling. It is also important to consider that the drop in NH values at the dentin layers may be associated with the ageing method used in this study, because organic compounds such as polyphenols are sensitive to temperature changes over time [44]. Thus, the second null hypothesis must also be partially rejected.

The CLSM images showed the presence of voids in the CON group. This was also previously seen in highly wet environments, such as CAD [8], which affected the performance of simplified adhesive systems [45,46]. Conversely, no voids were observed at the adhesive interfaces when flavonoids were used. These findings can be explained by the ability of crosslinkers to modify the water dynamics of dentin [30,47–49]. In other words, once applied to CAD, flavonoids molecules might decrease bound water levels within the extracellular matrix [15] and thus improve adhesive infiltration and polymerization, which is known to be impaired in excessively wet environments. Therefore, the third null hypothesis must be rejected.

This study showed that the μTBS values of the PLA group were comparable to those of some experimental groups. Since the PLA group has only the vehicle used in the flavonoid solutions, the influence of surfactants present in the experimental solutions on the μTBS values should not be discarded. As a surface modifier, the PLA containing both ethanol and surfactant might have changed the hydrodynamics of the substrate, as such components can increase the surface energy of dentin and decrease the surface tension of water on the dentin surface. Due the amphiphilic nature of the selected surfactant, in presence of organic fluids, SPAN 20 can create hydrogen bonding and Van der Waals forces between the OH- endings in exposed collagen [50] differently from the experimental solutions, where the depends on the hydrophobic and hydrophilic interactions among the components of the solutions. Furthermore, amphiphilic molecules such as SPAN 20 are also capable of increasing the azeotropic effect of ethanol due to the presence of both hydrophilic and hydrophobic endings in their molecular structures. As a result, further hydrodynamic changes on dentin due to the reduction in tension surface of water would be expected as well as a better wettability and greater adhesive infiltration. In this regard, one could state that the increase in bond strength could be attributed exclusively to the effects of the surfactant and ethanol on the dentin surface. However, some experimental groups showed a better effect than the PLA group in all tests either after 24 h or after thermalcycling, as those groups showed significantly higher bond strength values and hardness than the control groups, while no significant difference in outcomes was found between the PLA and CON groups. This means that even though hydrodynamics modification is important to improve bonding, the effect of crosslinkers as bio-modifiers and collagen preservers could be even more significant for therapeutic purposes. Further studies are required to explore more of the potential benefits of surfactants over dentin.

5. Conclusions

Within the limitations of the current study, it is possible to conclude that the use of specific flavonoids (e.g. RUT and NAR) as dentin pretreatment may improve the immediate bonding performance and the longevity of universal bonding system applied to CAD;

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