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Enhancing bond strength on demineralized dentin by pre-treatment with selective remineralising agents

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ABSTRACT

Bonding to demineralized dentin of a diseased tooth has shown to be a significant clinical issue. This study evaluated the effect of 0.2% NaF-(NaF), MI Paste™-(CPPACP) and the self-assembling peptide 'P11-4' (Ace-QQRFEWEFEQQ-NH2) contained in Curodont™ Repair, have on microtensile bond strength-(μTBS) of two different adhesive systems (Adper™ Single Bond-(SB) or Clearfil™ SE Bond (CSE)) and wettability of demineralized dentin slices after remineralising agents were applied. The highest μTBS were found for the demineralized dentin-(DD) treated with CPP-ACP; both adhesives systems ($p<0.05$) did not significantly difference from P11-4 treatment associated with SB, and also presented higher values than sound dentin-(SD/SB) ($p<0.01$). DD treated with P11-4 associated with CSE did not differ from DD/CSE ($p>0.05$). The NaF treatment associated with CSE recovered the bond strength values of SD/CSE and associated with CSE demonstrated lower μTBS than other groups, although significantly higher than DD ($p<0.05$). P11-4 and CPP-ACP increased significantly the wettability of demineralized dentin ($p<0.05$); etching acid improved wettability for all groups ($p<0.05$), whilst NaF did not affect the wettability of demineralized dentin ($p>0.05$). Morphological analysis of the dentin surface and dentinresin interface revealed unique features of the applied remineralizing agent. The results indicated that self-assembling peptide P11-4 associated with SB and CPP-ACP associated with SB or CSE significantly enhanced the bond strength to demineralized dentin ($p<0.05$). We conclude that by modifying the dentine surface and restoring conditions found on sound dentin, this can enhance the interfacial bonding.

Key Words: Dentine surface, Remineralization, Adhesives, Biomimetic Materials,

1. Introduction

The evolution of adhesive restorative materials and knowledge of caries lesion progression have led to the development of minimal intervention approaches, with the aim of preserving as much natural tooth structure as possible. This often involves remnants of the inner-layer of carious dentin, known as caries-affected dentin (CAD) (Fusayama, 1979; Lenzi et al., 2015; Yoshiyama et al., 2002). The adhesive procedures in caries-affected dentin have limited durability as the dentin is partially demineralized, affecting bond strength of resin/dentin interfaces (de-Melo et al., 2013; Pinna et al., 2015; Shibata et al., 2016). The irregular demineralization of CAD hinders the permeation of exposed collagen fibrils with resin monomers, resulting in water accumulation along the bonded interface, susceptible to hydrolytic degradation (Tjäderhane et al., 2013; Yoshiyama et al., 2002).

Based on this, new strategies have been employed that focus on the remineralization of the partially demineralized dentin to improve its mechanical/bonding properties (Liu et al., 2011). Reported remineralizing approaches include several types of bioactive materials (Zhong et al., 2015), sodium fluoride (NaF) (Correa et al., 2016), casein phosphopeptide with amorphous calcium phosphate (CPPACP) (Cao et al., 2013; Reynolds, 2008) and, more recently, biomimetics of enamel matrixproteins (NCP) and synthetic peptides, all attempting to remineralize dentin substrate (Cao et al., 2013; Zhong et al., 2015).

NaF, for instance, is widely used for caries control due to its ability to prevent demineralization by the formation of low-soluble Fluorapatite and promoting emineralization by calcium fluoride precipitation on the dental surface (Mukai and ten Cate, 2002). At present, the effect of fluoride application methods prior to bonding procedures remains controversial. Moreover, recent research has shown the inhibitory effect of NaF on endogenous proteases involved in the degradation of dentin collagenous matrix (Altinci et al., 2016; Brackett et al., 2015; Kind et al., 2017), leading to the overall stability of the bonding interfaces.

Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) has been reported for the prevention of demineralization and enhancement of remineralization of enamel and dentin lesions (Rahiotis and Vougiouklakis, 2007; Reynolds, 2008). The mechanism of action of CPP-ACP involves the ability to stabilize calcium phosphate under neutral or alkaline pH solutions, promoting their incorporation in hydroxyapatite of the tooth (Gupta and Prakash, 2011). According to the literature, CPP-ACP would be able to replace the minerals of the intra- and interfibrilar collagen spaces, after demineralization occupied by water, and thus reinforcing the bond strength at the resin/dentin interface (Cao et al., 2013; Sattabanasuk et al., 2014).

Likewise, another strategy of remineralization was developed using an self-assembling peptide P_{11}-4 (CH_3CO-Q-Q-R-F-E-W-E-F-E-Q-Q-NH_2), which has recently received more attention, as it has shown to regenerate enamel (Brunton et al., 2013; Ceci et al., 2016; Jablonski-Momeni and Heinzel-Gutenbrunner, 2014; Kind et al., 2017; Kirkham et al., 2007; Silvertown et al., 2017; Soares et al., 2017). P_{11}-4, a self-assembling biomimetic peptide, it changes from a monomeric state (a non-newtonian fluid) and assembles into a fibrillar hydrogel in response to specific environmental triggers, such as a pH<7 and a variation in ionic strength (Davies and Aggeli, 2011). Then, P_{11}-4 hydrogel attracts calcium ions, inducing “de novo" crystallisation of hydroxyapatite from supersaturated solutions such as saliva (Aggeli et al., 2003; Kirkham et al., 2007; Kind et al., 2017). Although the self-assembled P_{11}-4 matrix could control the deposition and growth of hydroxyapatite crystals on

enamel, the behaviour of self-assembling peptide P_{11}-4 on dentin surfaces remains yet unstudied.

Furthermore, the bonding approaches can be regulated by the surface features of the substrate. The higher surface energy, the higher wettability and the higher penetration of a liquid in this surface can be achieved. Toledano et al demonstrated that the demineralization of dentin resulted in a higher contact angle and the immersion of demineralized dentin into remineralization solutions can improve the dentin wettability (Toledano et al., 2014).

Considering the bonding approaches for different adhesive systems, etchand- rinse or self-etch, and the modified substrate by carious process, the remineralization of demineralized dentin can be a reasonable strategy to improve the adhesion to caries-affected dentin and facilitate the preservation of the inner layer of intact, bacteria-free remineralizable caries-affected dentin, preventing caries lesion progression and secondary caries formation (Niu et al., 2014).

Therefore, the aim of this study was to determine whether NaF, CPP-ACP and P_{11}-4 pre-treatments, influence the wettability of the demineralized dentin and the micro-tensile bond strengths of composite resin adhesive systems to the surface of demineralized dentin, when using an etch-and-rinse or a self-etch system. The tested hypothesis was that the pre-treatment of demineralized dentin with remineralizing agents improves the wettability of demineralized dentin and the microtensile bond strength, regardless of the adhesive strategy.

2. Materials and Methods

2.1. Experimental design

This in vitro study involved a 4×2 factorial design, where the factors under evaluation included (1) substrate condition (4 variables): demineralized dentin, demineralized dentin + 0.2% of NaF (900 ppm of fluoride), demineralized dentin + CPP-ACP (MI Paste™), and demineralized dentin + self-assembling peptide P_{11}-4 (Curodont™ Repair); and (2) adhesive systems (2 variables): Adper™ Single Bond 2 (etch-and-rinse), and Clearfil™ SE Bond (self-etch). Sound dentin was used as a positive control for both groups of adhesives (Figure 1). Experimental units were accomplished by 135 sound humans third molars randomly assigned into groups. The response variables were: microtensile bond strength (μTBS) (n=6), wettability measured by the contact angle formed between dentin and the water (n=6), analysis of bonded interfaces (n=3), dentin surface morphology (n=3) by SEM, according to the factors under study. The design of the present study is presented in Figure 1. The changes will be available in the text." The composition of materials, manufacturers, batch number, pH and application methods are described in Table 1.

2.2. Specimen preparation

For this in vitro study, 135 sound humans third molars were obtained after informed consent was obtained from patients of the Piracicaba Dental School, University State of Campinas and the local Research Ethics Committee provided approval (protocol #088/2011). The teeth were stored in 0.1% thymol solution at 4ºC for no more than two months after extraction. Tooth slices (4 mm thick) were produced from each tooth using a diamond-impregnated disc (Buehler, Lake Bluff, IL, USA) under water cooling in a specific cutter machine (Isomet 1000, Buehler, Lake Bluff, IL, USA). The tooth slices were ground with 600-grit silicon carbide sandpaper to create a smear layer. All surfaces except the occlusal flat surface area were coated with a red acid-resistant nail varnish (Colorama, CEIL; São Paulo, SP, Brazil) (Sacramento et al.,2012).

2.3. Randomization procedure

The tooth slices were randomly allocated into five groups (n=27) according to the remineralization treatment and control groups (sound and demineralized dentin). Three specimens from each group were randomly allocated to the dentin surface morphology (n=3) and wettability evaluation (n=6). The remaining tooth slices from each group were randomly assigned to the two-adhesive system and subjected to microtensile bond strength test (μTBS) and failure mode evaluation (n=6) as well as to the dentin/resin interface morphology analysis (n=3) (Figure 1).

2.4. Dentin demineralization and surface pretreatment

2.4.1. Demineralization procedures

Tooth slices (N=108) were immersed in 5 mL of 6% carboxymethylcellulose acid gel (Proderma Pharmacy; Piracicaba, SP, Brazil) containing 1 M lactic acid titrated with a concentrated KOH solution at 37ºC, pH 5.0, for 48 h (de Carvalho et al., 2008; Pacheco et al., 2013). Tooth slices were rinsed twice with deionized water in an ultrasonic bath for 15 min and slightly dried with tissue paper (Sacramento et al., 2012). The morphology and depth of the demineralized dentin were previously evaluated in a pilot study by polarized light and scanning electronic microscopies and demonstrated ~12 μm depth of demineralized dentin (data not shown).

2.4.2. Dentin Surface Treatment

Twenty-seven teeth were assigned to 5 groups according to the remineralization treatment: sound dentin (without treatment); demineralized dentin (without treatment); demineralized dentin + treated with 0.1 mL of the NaF solution (1min); demineralized dentin + treated with 0.1 mL of the MI Paste™ (1 min); and demineralized dentin + treated with 50 μL of the Curodont™ Repair was applied and left for 5 min, then, a Ca^{2+} and PO_4^{3-} solution for 1 min. For all treatments, the solution excess was removed by with absorbent paper.

2.5. Bonding procedures and microtensile bond strength (μTBS) test

All tooth slices were bonded with adhesive systems by a single operator according to the manufacturer's instructions, except for the dentin specimens that were submitted to morphological surface evaluation (Table 1). Resin composite blocks 4 mm thick (Filtek Z250; 3M ESPE, St. Paul, MN, USA; batch #51202) were built on the dentin surface in 2-mm increments, which were light-cured for 20 secs using an LED Curing Light Valo (Ultradent Products Inc., Indaiatuba, SP, Brazil) at 1000 mW/cm^2. The resin/dentin sets were stored in deionized water at 37°C for 24 h (Muñoz et al.,2015).

Bonded specimens were sectioned in the mesial-distal and buccal-lingual directions with a low speed diamond observed (Isomet, Buehler Ltd, Lake Bluff, IL,USA), resulting in 50 specimens (beams) per group with a cross-sectional area of approximately 1 mm^2 measured with a digital caliper (CD-6" BS, Mitutoyo Corporation, Tokyo, Japan). Specimens were individually fixed with cyanoacrylate based adhesive (Super Bonder Power Flex-Gel Control, Loctite, Henkel Ltda, Itapevi, São Paulo, Brazil) to a custom-made apparatus and then to the grips of a universal testing machine (EMIC DL 500, EMIC - Equipamentos e Sistemas de Ensaio, São José dos Pinhais, PR, Brazil). They were tested under tension (50 N load) at a 1 mm/min crosshead speed until failure. The microtensile bond strength was calculated in MPa according to the formula: R = F (kgF) x 0.098/A, where A = bonding

surface area (incm^2), F = value of force obtained at the failure, and R = resistance value (in MPa).

2.6. Scanning Electron Microscopy (SEM)

2.6.1. Dentin surface morphology

For dentin surface morphology evaluation, the surface treatments were performed as previously described (dentin demineralization and surface pretreatment section). Specimens were dried for 24 h at room temperature. Specimens were gold sputter-coated for 120 secs at 40 mA and examined using a scanning electronic microscope (JEOL, JSM 5600LV, Tokyo, Japan) at a standardized magnification (X3000). The dentin surface morphology was qualitatively analysed considering the parameters: surface homogeneity; dentin tubules; smear layer;demineralization/remineralization features.

2.6.2. Failure mode analysis

All the fractured specimens from the microtensile bond strength analysis were assessed to determine the failure mode using SEM at ×50 and ×150 magnifications. The fractured surfaces of the beams were paired, air dried, mounted on aluminum stubs, gold coated, and examined by SEM (JSM-5600LV, JEOL; Tokyo, Japan), operated at 15 kV. The failure patterns were classified according to the following categories: adhesive, mixed (involving resin composite, adhesive and/or dentin), cohesive failure in the resin composite, and cohesive failure in dentin (Bacchi et al., 2015).

2.6.3. Dentin-resin interface evaluation

The dentin specimens were prepared as described above. After resin composite build-up and overnight storage in deionized water at 37°C, the specimens were perpendicularly sectioned to the interface to produce resin/dentin slices. Specimens were polished using wet silicon carbide sandpaper (#600 to #4000 series) and 3 μm and 1μm diamond pastes (Buehler, Lake Buff, IL, USA). Specimens were ultrasonically cleaned for 20 min after each abrasive paper and polishing paste. Slices were then demineralized with 37% phosphoric acid for 5 secs, rinsed with deionized water for 30 secs, and dried with tissue paper. Subsequently, they were deproteinized with 10% NaOCl for 5 min, rinsed in an ultrasonic bath, and left to dry for 24 h at room temperature. Finally, the slices were gold sputter-coated and observed using SEM at 15kV by a single operator at a standardized magnification (x1500).

2.7. Wettability analysis by contact angle measurements

For dentin surface wettability analysis, thirty flat dentin blocks were submitted to surface treatments performed as previously described (dentin demineralization and surface pre-treatment section). Flat dentin surface were randomly divided according treatment (n=6): Sound dentin (positive control); Demineralized Dentin (negative control); Demineralized dentin + 0.2% NaF; Demineralized dentin + CPP-ACP; Demineralized dentin + P_{11}-4. Specimens were placed in the table of the Digidrop goniometer (Labometric Lda, Leiria, Portugal), at room temperature, which were guided against a water drop. Water contact

angles were measured with a goniometer equipped with a special optical system and a Charge-Coupled Device (CCD) camera. A drop of water (approximately 0.5 μL) was placed on contact against dentin surface and the image was immediately sent via the CCD camera to the computer for analysis, using the software GBX Digidrop (GBX Company, Bourg de Péage, França). Means and standard deviation of water contact angles were measured to assess surface hydrophilicity change by demineralized dentin treatment, including negative and positive control groups. Then, etching acid was performed on each specimen using Scotchbond™ Universal Etchant (3M ESPE; St Paul, MN, USA), for all groups and contact angle was measured again using the same method described above (Ururahy et al., 2017).

2.8. Statistical analysis

Data from μTBS were submitted to the Shapiro-Wilk test, which was followed by factorial ANOVA and Tukey post hoc tests, considering the tooth as an experimental unit. Dunnett test was used to compare the μTBS from each demineralized dentin/treated group to sound dentin, while unpaired t-test was chosen for adhesive system comparisons on sound dentin. The dentin surface morphology and failure mode data were submitted to descriptive analysis. Data from contact angle measurement of experimental groups were submitted to Shapiro-Wilk test and then, to one-way ANOVA and Tukey tests. In order to compare before and after etching acid, t-test for paired samples was used and Dunnett test to compare the experimental groups with sound dentin. All tests were performed considering α=5%.

3. Results

3.1. Microtensile bond strength (μTBS)

The mean μTBS values and standard deviation of demineralized dentin treated with different remineralizing agents and adhesive systems are described in Table 2. A significant interaction was observed between the substrate conditions and adhesive systems ($p<0.01$). For SB adhesive system the highest μTBS averages were found for the demineralized dentin treated with P_{11}-4 and CPP-ACP ($p<0.05$), and they did not significantly differ from each other ($p>0.05$). The μTBS values of CPP-ACP and P_{11}-4 were statistically different with sound dentin ($p<0.05$), presenting values of μTBS higher ($p<0.05$). Demineralized dentin treated with NaF showed averages higher than those found in demineralized dentin ($p<0.05$). The lowest μTBS averages were found for the demineralized dentin ($p<0.05$). For de CSE adhesive system the demineralized dentin treated with CPP-ACP had the highest bond strength value ($p<0.05$). The lowest μTBS averages were found for the demineralized dentin and treated with P11-4, and did not differ from each other ($p>0.05$). The NaF treatement did not significantly differ from to sound dentin ($p>0.05$). The Dunnett test demonstrated a significant difference between the sound dentin and demineralized/treated dentin groups ($p<0.05$)

3.2. SEM analysis

SEM micrographs of the dentin morphology according to the tested substrate are presented in Figure 2. The sound dentin group showed that intertubular and peritubular dentin had a homogeneous surface with noticeable dentinal tubules and no signs of demineralization (Figure 2 – A1). The artificial demineralized dentin demonstrated a heterogeneous surface with decreased amount of intertubular dentin, and some dentinal tubules obliterated by the apparently released minerals (circle in Figure 2 – B1). Figure 2 – C1 shows a surface treated with fluoride solution, which presented quite similar to the non-treated demineralized dentin.

The CPP-ACP treatment showed a residual layer of CPP-ACP-containing paste covering the dentin surface and partially occlude the dentinal tubules (circle in Figure 2 – D1). Figure 2 – E1 illustrates a demineralized dentin coated by P_{11}-4 hydrogel saturated with Ca^{2+} and PO_4^{3-} (circle), displaying a homogenous surface resembling the sound dentin, but with occluded tubules.

The prevalence values of the failure modes are shown in Figure 3. Overall, adhesive and mixed failures were the most frequent. The cohesive type on composite was the most prevalent failure found in sound dentin when the SB (56%) or CSE (58%) systems were used (Figure 2 – A2 and A4). More than 75% and 55% of the failure observed in demineralized dentin corresponded to adhesive and mixed patterns for SB (Figure 2 – B2) and CSE (Figure 2 – B4), respectively. The dentin treatment with NaF promoted a higher frequency of mixed failure (>80%), regardless of the chosen adhesive (Figure 2 – C2 and C4). Most of the failures observed in demineralized dentin pre-treated with CPP-ACP or P_{11}-4 and bonded with SB was the mixed type with values of 54% and 68%, respectively (Figure 2 – D2 and E2). CPP-ACP associated with CSE adhesive caused a higher frequency of cohesive on the dentin failure mode (50%), whereas 93% of failures observed in the P_{11}-4/CSE association were the adhesive type (Figure 2 – D4 and E4).
The formation of a resin-dentin interdiffusion zone created by SB and CSE adhesives, associated with the different dentin substrates, is described in Figure 2 (A3- E3 and A5-E5). For CPP-ACP, it seems that the paste penetrated inside the dentinal tubules and precipitated on the tubule walls (Figure 2 – D1). Therefore, when SB was applied to the CPP-ACP coated dentin, it was observed that the adhesive penetrated the tubules, creating long resin tags covered by a mineralized layer (asterisk in Figure 2 – D3). Interestingly, when SB was used, CPP-ACP appeared to be included into the adhesive, and the resin-tags characteristics differed from sound and demineralized dentin, with numerous and wider resin-tags. Fluoride had a very similar structure as for demineralized dentin when SB was applied. For CSE resin tags appeared numerous and wider than in demineralized dentin. P_{11}-4 provided a similar resin/dentin interface than sound dentin, regardless of the adhesive system.

3.3. Wettability analysis of demineralized dentin treated with remineralizing agent by contact angle measurements

Results of the contact angle experiments (Figure 4, Table 3) showed that treatment with CPP-ACP provided the highest increase of wettability (degree) on demineralized dentin. After acid etching was carried out, all groups indicated an increase in wettability, which is characteristic of a lower contact angle, with the exception of the remineralising agents CPP-ACP and P_{11}-4. The contact angle for CPPACP remained unaltered. Whilst after demineralised dentine was treated with P_{11}-4 its contact angle was similar to that found in sound dentine (with or without acid etching).

4. Discussion

A range of factors, including the structural, physical and chemical characteristics of substrate, might affect the bonding effectiveness of an adhesive system (Bahari et al., 2014; Marshall et al., 1997; Pinna et al., 2015; Shibata et al., 2016). Minimally invasive treatment of cavitated dentin lesions is based on preservation of caries-free tissue but might leave limited caries-affected tissue. The present study investigated whether remineralization pre-treatment of an artificially demineralized dentin would influence the wettability of the dentin surface and microtensile bond strength of two adhesive systems. The null hypothesis rejected because a

significant interaction between remineralization pre-treatment and material bonding was observed.

In an attempt to simulate and standardize the conditions found in natural caries-affected dentin, several methods of producing caries-like lesions in vitro have been developed, such as chemical and biological models (Lenzi et al., 2015; Pacheco et al., 2013). The present study submitted the sound dentin specimens to a chemical demineralizing challenge by applying an acid gel containing lactic acid, a by-product of bacterial metabolism (de Carvalho et al., 2008; Pacheco et al., 2013). It was verified that this method was effective in impairing the μTBS values of dentin; furthermore, SEM analysis presented a emineralized dentin exhibiting a heterogeneous and porous surface with some lumens of dentinal bliterated (Figure 2 - B1).

Considering the demineralized dentin group, the current investigation found similar results of microtensile bond strength for both, SB and CSE adhesive systems, which were significantly lower than those found in the sound substrate (Ceballos et al., 2003; Pereira et al., 2006; Yoshiyama et al., 2002). This is to be considered as mineral loss which may be detrimental for the infiltration of resin monomers into the demineralized dentin, due to the residual water present in demineralized dentin (Lenzi et al., 2015). Changes in the structure and composition of dentin by caries can also be responsible for the formation of a disorganized, acid-resistant crystal layer (whitlockite tricalcium phosphate) that obliterates dentine tubules and disturbs the formation of resin tags (Pinna et al., 2015). In addition, the results by contact angle analysis also indicated a significant wettability decrease for demineralized dentin. The decrease in wettability can reduce the bonding agents ability to wet and spread on dentin surface, thus hampering the resin monomers ability to interlock due to the high hydrophilicity of those substrates.

To minimize the effects caused by caries process on the bonding, remineralizing approaches have been demonstrated as promising alternatives to restructure the chemical-mechanical properties of the damaged dentin and to maintain the integrity of bonding interface (Liu et al., 2011). Of note, NaF has well-established efficacy on caries control (Prabhakar et al., 2013). Generally, mineral deposition of fluoride ions occurs on the surface of caries lesions, causing hypermineralization of dentin and impregnation of calcium fluoride precipitates into the tubules (Arends et al., 1989). In addition, the acid etching used in the etch-and-rinse procedure for SB adhesive system may remove the mineral ions from the surface, interfering with the hybridization in dentin collagen network from demineralized dentin. This process might have occurred when the SB adhesive system was applied after 0.2% NaF pre-treatment of demineralized dentin, as the μTBS values were considerably lower than those observed in the sound dentin or in another remineralizing group. Nevertheless, the application of 0.2% NaF prior to CSE were comparable to the dentin bond strength of sound dentin, suggesting a possible chemical interaction between the functional 10- MDP monomer (10-methacryloxydecyl di-hydrogenphosphate) of CSE and CaF_2 precipitates on the demineralized surface (Pinna et al., 2015). Moreover, the surface created by 0.2% NaF application provided similar wettability than demineralized dentin reinforced the idea that surface characteristics is the main factor affecting the bond strength.

CPP-ACP is based on a complex of the milk protein casein-phosphopeptide (CPP) and amorphous calcium phosphate (ACP). It releases, deposits and stabilizes high concentrations of calcium and phosphate ions on the tooth surface, inhibiting demineralization and enhancing remineralization (Rahiotis and Vougiouklakis, 2007;Reynolds, 2008).

In the current analysis, CPP-ACP pre-treatment of demineralized dentin associated with SB and CSE systems showed promising results, exceeding the bond strength observed for sound dentin. The first hypothesis for these results can be based on the surface

mechanisms provided by the CPP-ACP on the demineralized dentin surface. This result showed a highly significant decrease on contact angle when CPPACP was used for treating demineralized dentin. Consequently, a higher wettability can be found when demineralized dentin is treated with CPP-ACP. The shorter period of CPP-ACP application proposed in the present investigation might have also contributed to the improvement of adhesion by the formation of a thinner residual layer of CPPACP that might not have interfered with the etching and hybridization of demineralized substrate (Sattabanasuk et al., 2014).

Encouraged by the potential CPP-ACP effect, some studies have been performed to assess its influence in the bonding performance of adhesive systems (Bahari et al., 2014; Borges et al., 2012; Sattabanasuk et al., 2014). Sattabanasuk et al, observed that the treatment of sound dentin with CPP-ACP for 5 minutes did not influence the microtensile bond strength of the CSE system, while it decreased the μTBS of a three-step, etch-and-rinse adhesive (Sattabanasuk et al., 2014). Another investigation, performing 15 minutes of CPP-ACP application for five consecutive days, also verified a lack of improvement of the microtensile bond strength of the SB system when natural caries-affected dentin was coated with MI Paste™ (Bahari et al., 2014). These results can be attributed the formation of a CPP-ACP residual layer could reduce the etching effect of phosphoric acid, blocking the micromechanical interlocking of resin monomers from etch-and-rinse adhesives (Bahari et al., 2014; Sattabanasuk etal., 2014).

With respect to the use of CSE, previous studies have indicated that its functional monomer, 10-MDP, has strong affinity to the hydroxyapatite crystals attached around the demineralized collagen (Borges et al., 2012; Sattabanasuk et al., 2014). This chemical interaction induces the formation of an insoluble and resistant Ca-10-MDP monomer salt, which is supposed to enhance the mechanical properties of bonding interfaces (Pinna et al., 2015).

The self-assembling peptide P_{11}-4 was rationally designed as a 3D scaffold for tissue engineering. P_{11}-4 assembles in response to environmental triggers such as pH or variations in ionic strength (Aggeli et al., 2003; Kind et al., 2017; Kirkham et al., 2007; Kyle et al., 2010). This prepositions them for nucleation of hydroxyapatite (Aggeli et al., 2003; Kind et al., 2017; Kirkham et al., 2007). Several approaches, in vitro and in vivo, have recently demonstrated the effectiveness of self-assembling peptide P_{11}-4 in remineralizing early carious lesions as well as in preventing demineralization of enamel (Brunton et al., 2013; Ceci et al., 2016; Jablonski-Momeni and Heinzel-Gutenbrunner, 2014; Silvertown et al., 2017; Soares et al., 2017); however, none have investigated P_{11}-4 on dentin surfaces. Thus, the current analysis was the first to evaluate the use of self-assembling peptide P_{11}-4 as a pre-treatment for demineralized dentin and its influence on the microtensile bond strength of two different adhesive systems.

P_{11}-4 application prior to SB, considerably increased the μTBS of demineralized dentin compared to DD (Figure 2 – E1 and E3), and were similar to the values observed for CPP-ACP pre-treatment followed by SB or CSE bonding. Considering P_{11}-4 self-assembly is dependent on pH (Aggeli et al., 2003; Kind et al., 2017; Kirkham et al., 2007), it is possible that the low pH provided by etching with 37% phosphoric acid (<1.0) (Pashley et al., 2011) enhanced the self-assembly of P_{11}-4. The fibre scaffold formed then binds the available calcium and phosphate ions on the dentin surface as well as chemically reinforced the structure of dentin. Furthermore, it might be hypothesised that the mechanism by which the P_{11}-4 fibres acts may be similar to the dentin matrix phosphoprotein DMP-1; in other words, P_{11}-4 would promote the complete remineralization of collagen fibrils via the stabilization and hierarchical organization of apatite, protecting the fibrils from hydrolytic and proteolytic degradation (Kind et al., 2017; Kirkham et al., 2007).

However, no changes in the bond strength to demineralized dentin were observed when P_{11}-4 was applied prior to a self-etch adhesive. CSE is a two-step, selfetch adhesive system that includes hydrophilic acidic monomers, which simultaneously etch and prime the tooth substrate (Giannini et al., 2015). The results of this study concerning surface properties of a demineralized dentin surface showed that a hydrophobic surface had been formed after the application of P_{11}-4, which probably hampered the primer conditioning by CSE, leading to poor adhesion to demineralized dentin. In addition, missing etching step can lead to smaller number of fibres formed due to the self-assembly being slower and less stable, and thus a lower effect can be observed on remineralization process.

It can be pointed out as a limitation of the current study the fact a long-term assessment was not applied to evaluate the interaction of remineralizing agents and adhesive systems upon the μTBS. In addition, a water-storage medium was chosen, which might have not mimic accurately the influence of dentinal fluid on the degradation of resin composites. Supplementary analysis as the quantification of mineral contents as well as the permeability of the hybrid layer could have been applied to support the outcomes achieved in the present investigation.

Therefore, we emphasize that the treatment of caries-affected dentin with remineralizing agents, such as CPP-ACP and P_{11}-4, prior to the bonding procedures seems to be a promising approach for improving the stability of hybrid layers. However, this approach depends on the adhesive system and remineralizing agent. Furthermore, the protocols of remineralizing agent application used in this investigation could be considered applicable for clinicians as a novel step for adhesive procedures. Finally, we suggest that in situ and clinical studies should be performed to confirm the current outcomes and elucidate the relationship between adhesive systems and demineralizing dentin treatment further.

5. CONCLUSION

Under the limitations of this in vitro study, the pre-treatment of demineralized dentin with remineralizing agents demonstrated to be a good, viable option to improve bonding. Adper™ Single Bond 2 associated with self-assembling peptide P_{11}-4 or CPPACP and Clearfil™ SE Bond associated with CPP-ACP showed promising results by increasing the bonding strength.

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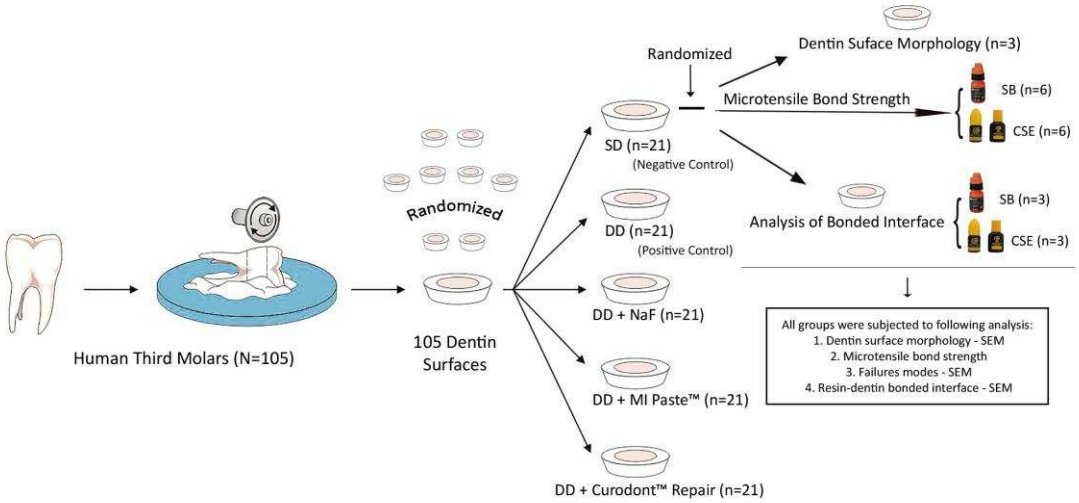
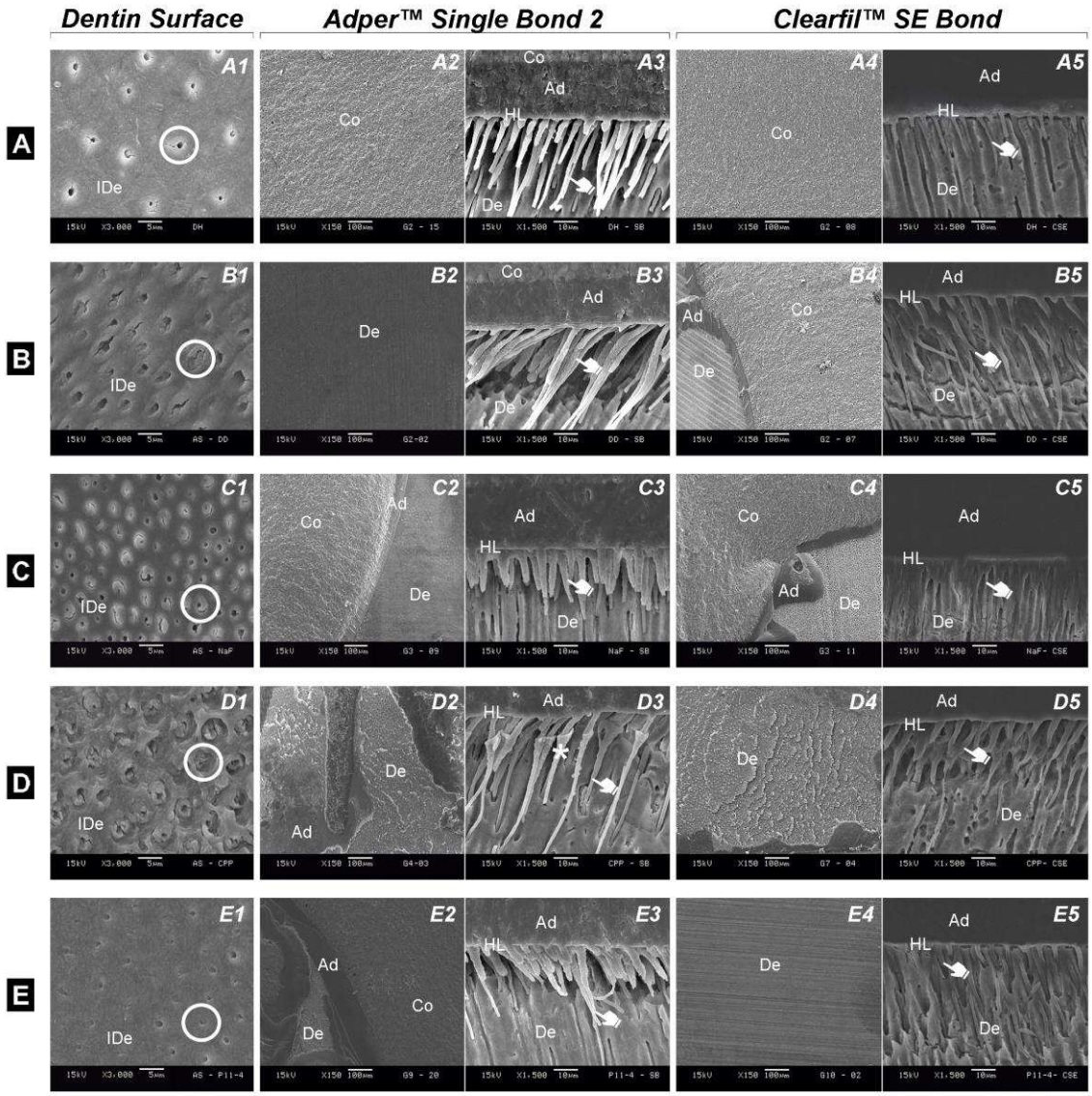
Figure 1



Representation of the experimental design used in this study. Dentin substrate:SD - Sound Dentin (negative control); DD - Demineralized Dentin (positive control); NaF- Sodium fluoride; MI Paste™ containing CPP-ACP), and Curodont™ Repair containingP_{11}-4 self-assembly peptide. Adhesive Systems: SB - Adper™ Single Bond 2 and CSE - Clearfill™ SE Bond.

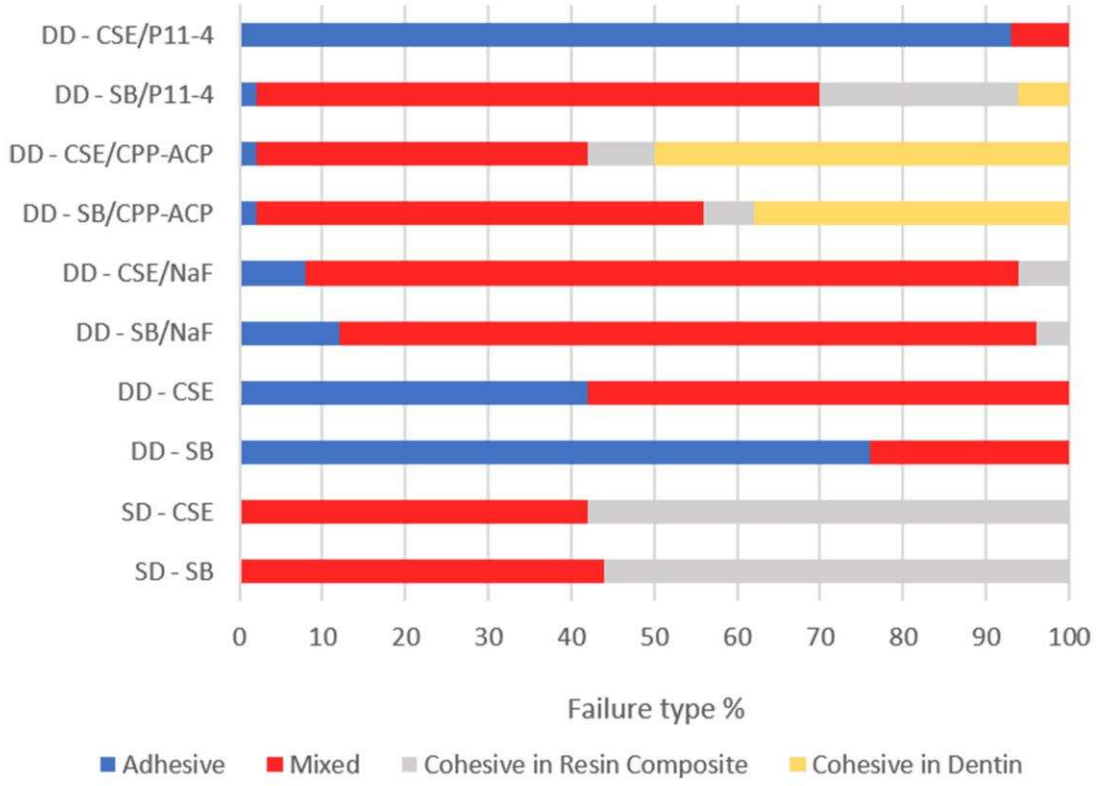
Figure 2



Representative SEM micrographs of specimens. Dentin Surface Features - Magnification micrograph (Å~3000) - Sound Dentin (SD) without treatment (positive control) (A1); Demineralized dentin (DD) without treatment (negative control) (B1); Demineralized dentin treated with NaF (DD + NaF) (C1); Demineralized dentin treated with CPP-ACP containing MI Paste™ (DD + CPP-ACP (D1); and Demineralized dentin treated with $P_{11\text{-}4}$ containing in Curodont™ Repair (DD + P_{11}-4) (E1). Failure Mode (A2, A4, B2, B4, C2, C4, D2, D4, E2 and E4) –Magnification micrograph (Å~150): Cohesive failure on composite observed on SD (positive control) bonded with both adhesive systems (A2 and A4). Adhesive failure observed in the DD group (negative control) (B2 and B4). DD + NaF showed mixed failure (C2) and (C4). Mixed failure was often observed for DD + CPP-ACP bonded with Adper™ Single Bond 2 (D2); cohesive failure on dentin observed by DD + CPP-ACP bonded with Clearfil™ SE Bond (D4). Mixed failure observed by DD + P_{11}-4 bonded with Adper™ Single

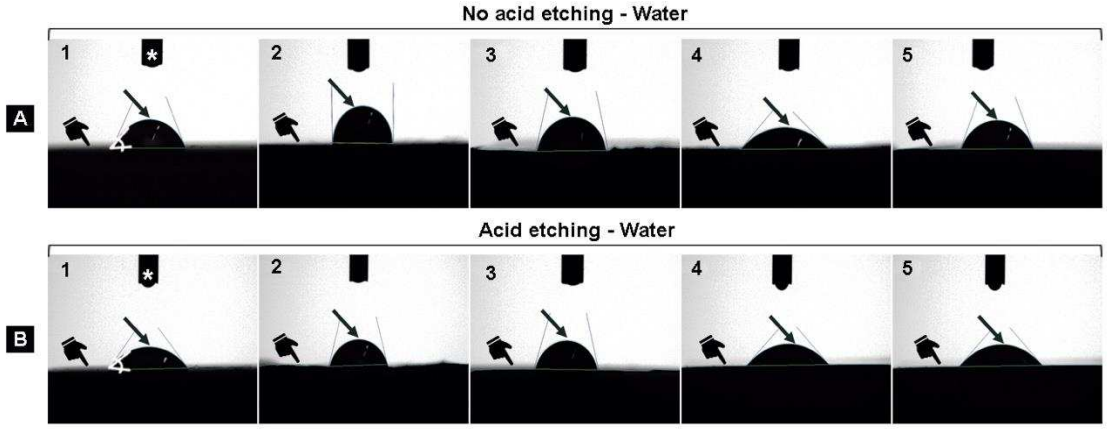
Bond 2 (E2). Adhesive failure observed by DD+ P_{11}-4 bonded with Clearfil™ SE Bond (D4). Adper™ Single Bond 2 Bonded Interfaces: Magnification micrograph (Å~1500) resin/dentin interface created by Adper™ Single Bond 2 for the SD (A3); DD (B3); DD + NaF (C3); DD + CPP-ACP (D3); and DD + P_{11}-4 (E3). Clearfil SE™ Bond Bonded Interfaces: Magnification micrograph (Å~1500) resin/dentin interface created by Clearfil™ SE Bond for SD (A5); DD (B5); DD + NaF (C5); DD + CPP-ACP (D5); and DD + P_{11}-4 (E5). Co – Composite; Ad – Adhesive; De – Dentin; Pointer – Tags; IDe – Intertubular dentin; Circule – Dentinal Tubule; and Asterisk – mineral content from MI Paste™ incorporated to resin tags.

Figure 3



Failure mode (%) regarding the remineralizing treatment and adhesive system. SB: Adper™ Single Bond 2, CSE: Clearfil™ SE Bond. SD: Sound Dentin, DD: Demineralized Dentin, NaF: Sodium Fluoride, CPP-ACP: Casein Phosphopeptide - Amorphous Calcium Phosphate containing in MI Paste™, and P_{11}-4: Self-assembling peptide P_{11}-4 containing in Curodont Repair.

Figure 4



Contact angle between dentin surfaces and water drop observed in the goniometer. 1- Sound dentin; 2- Demineralized dentin-DD; 3-DD+NaF; 4-DD+CPPACP; 5-DD+P_{11}-4. Pointer – dentin surface; black arrow – water drop; *dispenser syringe tip; - contact angle. A- without etching; B – acid etching.

Materials (manufactures)	Main componentes	Batch number	Application mode
0.2% NaF Solution	0.2g of NaF in 100 ml deionized water	Made in Lab*	1. Apply 1.0 mL of 0.2% NaF Solution
Ca^{++} and PO_4^{-3} Solution	Saturated solution of Ca^{++} and PO_4^{-3}	Made in Lab	1. Apply 0.1mL of Ca^{++} and PO_4^{-3} Solution
MI Paste™ - GC Internacional, Itabashi-ku, Tóquio, Japão	Glycerol, CPP-ACP, D-Sorbitol, Propylene glycol, Titanium dioxide and silicon	N100723l	1. Apply 0.1mL of MI Paste™
Curodont™ Repair - Credentis AG, Dorfstrasse, Windisch, Switzerland	P_{11}-4 peptide – amino acid sequence - (Ace-Gln-Gln-Arg-Phe-Glu-Trp-Glu-Phe-Glu-Gln-Gln-NH2)	N203X	1. Apply 50µL of Curodont™ Repair for 5 min 2. Apply 0.1 mL of Ca^{++} and PO_4^{-3} Solution
Scotchbond™ Universal Etchant - 3M ESPE; St Paul, MN, USA	32 % phosphoric acid	N377	1. Apply etchant for 15 s 2. Rinse for 10 s
Adper™ Single Bond 2 – 3M ESPE, St Paul, MN, USA	HEMA, water, etanol, Bis-GMA, dimethacrylates, amines, metacrylate functional copolymer of polyacrylic and polyitaconic acids, 10% by weight of 5 nanometer-diameter spherical sílica particles	N429952	3. Blot excess water 4. Apply 2 consecutive coats of adhesive for 15 s with gentle agitation 5. Gently air dry for 5 s 6. Light-cure for 10 s
Clearfil™ SE Bond – KURARAY Medical INC, 1621, Sakazu, Kurashiki, Okayama, Japan	**Primer:** Water, MDP, HEMA, hydrophilic dimethacrylates, camphoroquinone. **Bond:** MDP, Bis-GMA, HEMA, camphoroquinone hydrophilic dimethacrylates, N/N-diethanol p-toluidine, colloidal silica.	**Primer**: 00861A **Bond**: 01262A	3. Blot excesso water 4. Apply primer for 15 s with gentle agitation for 20 s 5. Gently air dry 6. Apply adhesive and gently air dry
Filtek™ Z350 XT - 3M ESPE, St Paul, MN, USA	BIS-GMA, Bis-EMA, UDMA, TEG-DMA, camphorquinone, non-agglomerated silica nanoparticles	N379474	7. Incremental insertion 2mm 8. Light-cure for 20 s

*Pediatric Dentistry Laboratory

Materials, manufactures, components, batch numbers and application mode of tested materials.

Table 2

Groups	Adhesive Systems	
	Adper™ Single Bond 2 (SB)	**Clearfil™ SE Bond (CSE)**
SD	40.85 ± 5.99 $^{\alpha}$	32.95 ± 7.41
DD	26.38 ± 8.64 Ca *	25.38 ± 8.58 Ca *
DD + NaF	33.43 ± 10.41 Ba *	35.59 ± 9.18 Ba
DD + CPP-ACP	45.25 ± 8.82 Aa *	48.11 ± 11.71 Aa *
DD + P_{11}-4	46.42 ± 12.03 Aa *	25.70 ± 8.95 Cb *

SB: Adper™ Single Bond 2, CSE: Clearfil™ SE Bond. Different capital letters indicate statistically differences between values in the same column. Different small letters indicate significant differences between values in the same row (Factorial ANOVA; Tukey's test). * indicates a significant difference between the sound dentin and demineralized/treated groups ($p<0.05$) by additional Dunnett's test. α indicates significant differences between the adhesive systems for sound dentin by the unpaired test t ($p=0.001$).

Mean and Standard Deviation of μTBS (MPa) regarding the remineralizing treatment and adhesive system.

Table 3

Dentin Substrate	Without Etching	With Etching
SD	67.1 ± 6.36 $^{\alpha}$	44.9±3.01
DD	85.8±4.46 **Aa***	55.4±6.85 **Ab***
DD + NaF	78.2±9.2 **Aa***	52.8±3.16 **Ab***
DD + CPP-ACP	36.1±6.7 **Ca***	34.4±3.82 **Ca***
DD + P_{11}-4	67.8±5.55 **Ba**	44.5±3.34 **Bb**

Different small letters in rows indicate significant differences by t-test ($p<0.001$); Different capital letters in column indicate significant difference on average by ANOVA and Tukey tests ($p<0.05$). * indicate significant difference between control (DH) by Dunnett test ($p<0.05$). α indicates significant differences between the adhesive systems for sound dentin by the unpaired test t ($p=0.001$).

Means and standard deviation of contact angle (grade) between water drop and dentin substrate.