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In Vitro Secondary Caries Inhibition by Adhesive Systems in Enamel Around Composite Restorations

CF Pinto • AF Paes-Leme
GMB Ambrosano • M Giannini

Clinical Relevance

The ability of the adhesive system to inhibit secondary caries was similar among the materials tested, according to the enamel mineral content around restorations.

SUMMARY

This study compared the ability of adhesive systems to inhibit *in vitro* caries lesions in enamel under high cariogenic challenge. Bovine enamel blocks with cavity preparations were restored with AP-X resin composite (Kuraray Med) using four adhesives systems: Clearfil SE Bond (Kuraray Med); Clearfil Protect Bond (Kuraray Med); One-Up Bond F (Tokuyama) and Single Bond (3M ESPE). The specimens were submitted to an eight-day pH-cycling regimen. After cariogenic challenge, the enamel was evaluated to detect caries lesions using cross-sectional microhardness, polarized light microscopy and scanning electronic microscopy. Data from cross-sectional microhardness and polarized light microscopy evaluations were statistically analyzed by ANOVA and Tukey's test. The mineral % volume showed no statistical difference among adhesives ($p>0.05$); however, polarized light microscopy analysis showed lower caries lesions with Clearfil Protect Bond ($p<0.05$). The scanning electron microscopy images showed greater caries lesions and demineralization areas close to restorations for Clearfil SE Bond, One-Up Bond F and Single Bond compared to Clearfil Protect Bond. The pH-cycling regimen promoted subsurface enamel demineralization in all specimens treated. The polarized light microscopy and scanning electronic microscopy analyses showed that Clearfil Protect Bond seems to produce lower enamel demineralization around restorations;

Cristiane Franco Pinto, DDS, MS, doctoral student, University of Campinas, Department of Restorative Dentistry, Piracicaba, SP, Brazil

Adriana Franco Paes-Leme, DDS, MS, PhD, researcher at the Center for Structural Molecular Biology, Brazilian Synchroton Light Laboratory, Campinas, SP, Brazil

Gláucia Maria Bovi Ambrosano, DDS, MS, PhD, associate professor, State University of Campinas, Department of Social Dentistry, Piracicaba, SP, Brazil

*Marcelo Giannini, DDS, MS, PhD, associate professor, State University of Campinas, Department of Restorative Dentistry, Piracicaba, SP, Brazil

*Reprint request: Av Limeira, 901, Piracicaba, SP 13414-903, Brazil; e-mail: giannini@fop.unicamp.br

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however, cross-sectional microhardness did not demonstrate differences among the adhesives.

INTRODUCTION

Adhesive systems play a crucial role in operative dentistry and dental prostheses, because they are used to seal dental surfaces and bond restorative materials to teeth. Two categories of adhesives are available to dentists, etch&rinse and self-etching systems; however, the bonding mechanism to enamel and dentin is similar and based on micromechanical retention with the hybridization process.[1]

The lack of marginal integrity and sealing increases the risk of enamel and dentin secondary or recurrent caries over time, both of which are the most commonly reported reasons for the replacement of composite restorations. Secondary caries can affect the dental structure around a restoration, forming an outer lesion on the surface and/or a lesion along the cavity preparation wall. Thus, as the adhesive systems are strategically positioned in a cavity preparation, their effectiveness is directly related to durability of the restoration.[2-3]

Silica microfillers, anticariogenic and antibacterial components, such as fluoride and bromide, have been introduced to adhesive systems in order to improve the clinical longevity of composite restorations.[4-7] Regarding fluoride-releasing restorative materials, studies have reported that they are effective for secondary caries inhibition *in vitro*.[3,8-9] Thus, the new fluoride-containing adhesive systems are expected to promote adhesion to tooth substrates and inhibit secondary caries, even in a high cariogenic challenge, wherein the performance of these materials needs more information.

The current study evaluated the effects of different adhesive systems on the inhibition of enamel secondary caries *in vitro* under high cariogenic challenge. The hypothesis tested was that enamel mineralization around composite restorations is affected differently by artificial cariogenic challenge, depending on the type of adhesive used.

METHODS AND MATERIALS

Preparation and Restoration of Enamel Block

Forty sound bovine incisors free from structural cracks and defects were selected for this study. After pumicing, the incisors were stored in 0.1% thymol solution at 4°C for 30 days. Bovine enamel blocks (6 x 6 x 4 mm) were obtained from the buccal surface with the use of double-faced diamond discs (KG Sorensen, Barueri, SP, Brazil). Cylindrical cavities (2 x 2 x 2 mm) were prepared on the buccal surface (in the center of the surface) using a high-speed diamond bur (#3098, KG Sorensen, Barueri, SP, Brazil) with a standardized cavity machine under constant water-cooling (Marcelo Nucci #99/10339-6, Araraquara, SP, Brazil). All the teeth were sterilized in a gamma irradiation chamber (Gammacell 220 Excell, MDS Nordion, Ottawa, ON, Canada) after cavity preparation.

The enamel blocks with cavity preparations were randomly divided into four groups (n=10): Clearfil SE Bond (Kuraray Medical Inc, Kurashiki, Japan); Clearfil Protect Bond (Kuraray Medical Inc); One-Up Bond F (Tokuyama Dental Corp, Taitou-ku, Tokyo, Japan) and Single Bond (3M ESPE, St Paul, MN, USA). The composition of each bonding agent is presented in Table 1. The tooth surfaces were treated with the adhesive systems according to the manufacturer's instructions and Clearfil APX resin composite (Kuraray Medical Inc) was placed incrementally into the cavities.[10] For the Single Bond adhesive, the cavity was previously etched with 35% phosphoric acid for 15 seconds and rinsed for 10 seconds. Each layer was light-cured for 40 seconds (XL 3000, 3M ESPE) and all restored blocks were kept in a 100% humid environment at 37°C for 24 hours. Subsequently, the restorations were serially polished with the use of aluminum-

Table 1: *Compositions of Adhesive Systems Used in This Study*

Adhesive System	Composition
Clearfil SE Bond	Primer: 2-hydroxyethyl methacrylate, 10-methacryloyloxydecyl dihydrogen phosphate, hydrophilic aliphatic dimethacrylate, dl-camphorquinone, water, accelerators, dyes, others. Bond: bisphenol a diglycidylmethacrylate, 2-hydroxyethyl methacrylate, 10-methacryloyloxydecyl dihydrogen phosphate, hydrophobic aliphatic dimethacrylate, colloidal silica, dl-camphorquinone, initiators, accelerators, others.
Clearfil Protect Bond	Primer: 10-methacryloyloxydecyl dihydrogen phosphate, 12-methacryloyloxydodecylpyridinium bromide, hydrophilic aliphatic dimethacrylate, water, initiators, accelerators, dyes, others. Bond: bisphenol a diglycidyl methacrylate, 2-hydroxyethyl methacrylate, sodium fluoride, 10-methacryloyloxydecyl dihydrogen phosphate, hydrophobic aliphatic dimethacrylate, colloidal silica, dl-camphorquinone, initiators, accelerators, others.
One-Up Bond F	Bonding A: Water, methyl methacrylate, 2-hydroxyethyl methacrylate, coumarin dye, metacryloyloxyalkyl acid phosphate, methacryloxyundecane dicarboxylic acid. Bonding B: multifuntional methacrylic monomer, fluoraluminosilicate glass, photoinitiator (arylborate catalyst).
Single Bond	Etchant: 35% phosphoric acid. Primer & Adhesive resin: bisphenol A diglycidyl methacrylate, 2-hydroxyethyl methacrylate, ethanol, water, urethane dimethacrylate, bisphenol A glycerolate, polyalkenoic acid copolymer, dimethacrylate, camphorquinone.

Table 2: *Percentage of Volume Mineral (average of the three locations: 60, 160 and 260 µm from the restorations) According to the Adhesive Systems and Depth From Enamel Surface (mean [SD])*

Depth (µm)	Clearfil SE Bond	Clearfil Protect Bond	One-Up Bond F	Single Bond
20	39.2 (13.7) Ad	33.7 (21.7) Ae	32.4 (23.8) Ab	26.1 (15.5) Ade
40	27.1 (22.6) Ad	20.2 (17.6) Ae	20.3 (16.7) Ab	13.5 (7.0) Ae
60	35.2 (23.9) Ad	23.5 (17.5) Ae	28.3 (23.8) Ab	14.8 (11.4) Ae
80	57.0 (17.7) Ac	48.2 (23.5) Ad	55.4 (25.6) Aa	34.4 (28.9) Ad
100	72.5 (18.6) Ab	64.1 (23.7) Ac	69.7 (21.4) Aa	64.4 (25.6) Ac
120	82.6 (14.5) Aab	77.9 (22.9) Abc	80.1 (17.4) Aa	83.8 (14.8) Ab
140	87.6 (12.8) Aa	83.8 (20.1) Aabc	86.2 (15.2) Aa	89.5 (12.0) Aab
160	92.0 (12.5) Aa	90.1 (16.5) Aab	91.4 (14.2) Aa	95.2 (10.2) Aab
180	94.1 (12.3) Aa	91.9 (16.0) Aab	93.6 (12.7) Aa	98.8 (8.5) Aa
200	95.4 (12.3) Aa	94.9 (14.5) Aa	95.2 (12.0) Aa	99.4 (8.1) Aa

Capital letters show difference among adhesive systems and lower cases show differences among depth from enamel surface ($p<0.05$).

oxide abrasive disks (Sof-Lex, 3M ESPE). Each specimen was analyzed in a stereomicroscope (EMZ 2000, Meiji Techno, Saitama, Japan) at 40x magnification to ensure the absence of an overhang at the composite-enamel interface.

Demineralization-remineralization Cycling (pH-cycling)

The surfaces of all dental blocks were coated with acid-resistant nail varnish and sticky wax except for a circular window including the restorations and a 0.5-mm ring of sound tooth structure surrounding the restoration. The groups were submitted to eight demineralization-remineralization cycles at 37°C for eight days, based on pH-cycling proposed by Shinkai and others.[11] Each cycle was composed of a seven-hour immersion in demineralization solution, followed by 17-hour immersion in remineralizing solution. The demineralizing solution contained 2.2 mM of calcium ($CaCl_2$), 2.2 mM of phosphate ($NaH_2 PO_4$) and 1 ppm of fluoride in 0.05 M acetic acid, pH 4.5 (6.25 mL of solution/mm^2 of exposed dental area). The chemical composition of the remineralizing solution was 1.5 mM of calcium, 0.9 mM of phosphate, 0.15M KCl, pH 7.0 (3.125 mL/mm^2).

After treatment, all the blocks were longitudinally sectioned with a diamond saw (Isomet 1000, Buehler, Lake Bluff, IL, USA) through the center and one-half was used to evaluate the enamel around the restorations and enamel-composite interfaces by SEM and the other half was used to determine the enamel mineral loss by cross-sectional microhardness (CSMH) and to evaluate the lesion depth by polarized light microscopy (PLM).

Cross-sectional Microhardness Analysis

Sectioned surfaces were polished with 1 µm and ¼ µm-grit diamond pastes (APL 4, Arotec, Cotia, SP, Brazil). The indentations were made at 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 µm from the outer enamel surface and at 60, 160 and 260 µm from the restorations, using a microhardness tester (FM 1E, Future Tech, Tokyo, Japan). A Knoop indenter was used with a 25-g load for five seconds. CSMH values were converted to mineral % volume according to Featherstone and others.[12] The values of mineral % volume were analyzed by three-way analysis of variance (split-plot ANOVA), followed by the Tukey test. The SAS software system (SAS Institute Inc, Cary, NC, USA) was used and the significance limit was set at 5%.

Polarized Light Microscopy Analysis

Sections of 100 ± 10 µm from the same specimens were obtained after microhardness determination. The sections were polished using 600 and 1200 grit aluminum oxide disks under water refrigeration to a thickness of 100 ± 10 µm. The slabs were embedded in distilled and deionized water, mounted in glass slides and the caries lesion depth was analyzed in a polarized light microscope (DM LSP, Leica Microsystems, Heerburg, Switzerland).

The images were taken at 200x magnification and transferred to the computer via digital camera. The measurement of lesion depth around the restoration was made at five points (50, 100, 150, 200 and 250 µm from the restorations) using computer software (Image-Pro Plus, 4.1 version for Windows, Media Cybernetics, Silver Spring, MD, USA). The values of caries lesion depth were expressed in micrometers (µm) and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test (α=5%).

Scanning Electron Microscopy Observation

The sectioned surfaces (n=10) from the remaining halves were polished with 600, 1200 and 2000-grit SiC papers, followed by diamond pastes (6, 3, 1 and ¼ µm) and dehydrated in ascending ethanol concentrations (30%, 50%, 70%, 90% and 100%). The samples were submitted to argon-ion beam etching for several minutes at 100 mtorr and 100 W (Anatech LTD–Plasma Series,

LNLS, Campinas, SP, Brazil). They were then sputter-coated with gold (MED 010, Baltec, Balzers, Leichtenstein) and observed under SEM (JSM-5600, JEOL Inc, Peabody, MA, USA). SEM analysis was done under blind conditions as well as CSMH or PLM. Representative areas of enamel around the restorations were photographed at 150x, 1,000x, 2,000x, 2,500x and 3,500x magnifications. Photomicrographs were taken in all the prepared samples in order to identify the demineralization areas around the composite restorations with enamel erosion.

RESULTS

The mean (standard deviation) values of mineral % volume in each depth from enamel for the adhesives tested are displayed in Table 2. Three-way ANOVA revealed that there were statistically significant differences for the factor "caries lesion depth" (F=533.08 and $p<0.0001$); however, it did not detect statistically significant differences for the factor "adhesive system" (F=0.68 and $p=0.5682$) and for the factor "distance of caries lesion from the cavity wall" (F=0.47 and $p=0.6277$). Also, a statistically significant difference was identified for interaction between "adhesive" and "lesion depth" factors (F=3.39; $p<0.0001$) and for interaction between "adhesive" and "distance of caries lesions from the cavity wall" (F=3.21; $p=0.0040$).

The results showed that the enamel mineral % volume increased as a function of enamel depth from the surface with no influence related to type of adhesive system, which had the same performance regarding CSMH analysis (Table 2 and Figure 1). Also, the enamel mineral content was similar when the CSMH was performed at different distances (60, 160 and 260 µm) from the cavity wall.

PLM values showed subsurface demineralization areas in enamel for all the adhesives tested (Figures 2a, 2b, 2c and 2d). One-way ANOVA revealed statistically significant differences among the adhesive systems (F=4.72 and $p=0.0067$). The pH cycling resulted in lower thickness of enamel caries lesions for Clearfil Protect Bond than for Single Bond and One-Up Bond F but it was not significantly different from Clearfil SE Bond (Table 3).

Table 3: *Depth of Enamel Caries Lesions (µm) Around Bonded Restorations with Adhesive Systems by Polarized Light Microscopy (n=10)*

Adhesive System	Depth (SD)
Clearfil SE Bond	53.3 (10.1) AB
Clearfil Protect Bond	42.2 (4.3) B
One-Up Bond F	63.7 (21.9) A
Single Bond	65.9 (26.6) A

Different letters show difference among adhesive systems (p<0.05).

SEM micrographs revealed that pH-cycling produced demineralization areas located at the subsurface region of enamel (Figures 3, 4, 5 and 6), as demonstrated in PLM measures. Demineralization seemed to be milder in the enamel of specimens restored with Clearfil Protect Bond (Figure 4) than those bonded with other systems (Figures 3, 5 and 6). Figures 3d-6d showed bonded interfaces (composite-enamel) with different thicknesses of adhesive layers. One-Up Bond F formed the thinnest adhesive layer, at approximately 1 µm to 5 µm (Figure 5d), while the two self-etching primers (Clearfil SE Bond and Clearfil Protect Bond) formed the thickest layer (15 µm to 25 µm) (Figures 3d and 4d). The adhesive layer formed by Single Bond was uniform, with approximately a 10 µm thickness (Figure 6d). Sodium fluoride particles can be seen at the interfaces with Clearfil Protect Bond (Figure 4d).

DISCUSSION

In the current study, four adhesive systems representing simplified bonding agents were used. The "etch&rinse" adhesive (Single Bond) was applied after phosphoric acid etching, forming a micromechanical

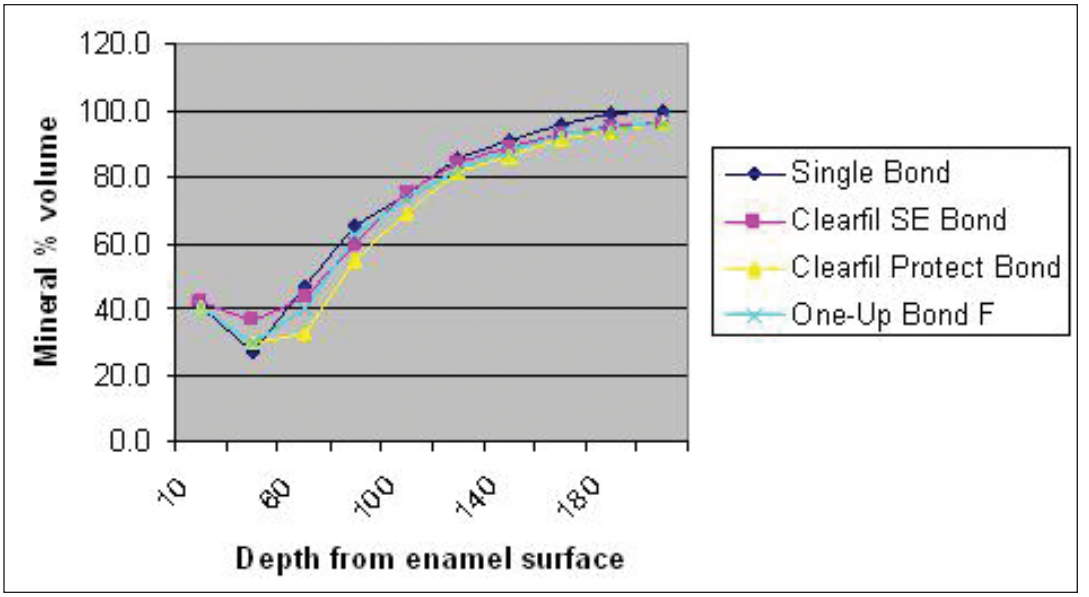

Figure 1. *Graph of enamel mineral content profiles in each group. Values are expressed in mineral % volume as a function of depth (µm).*

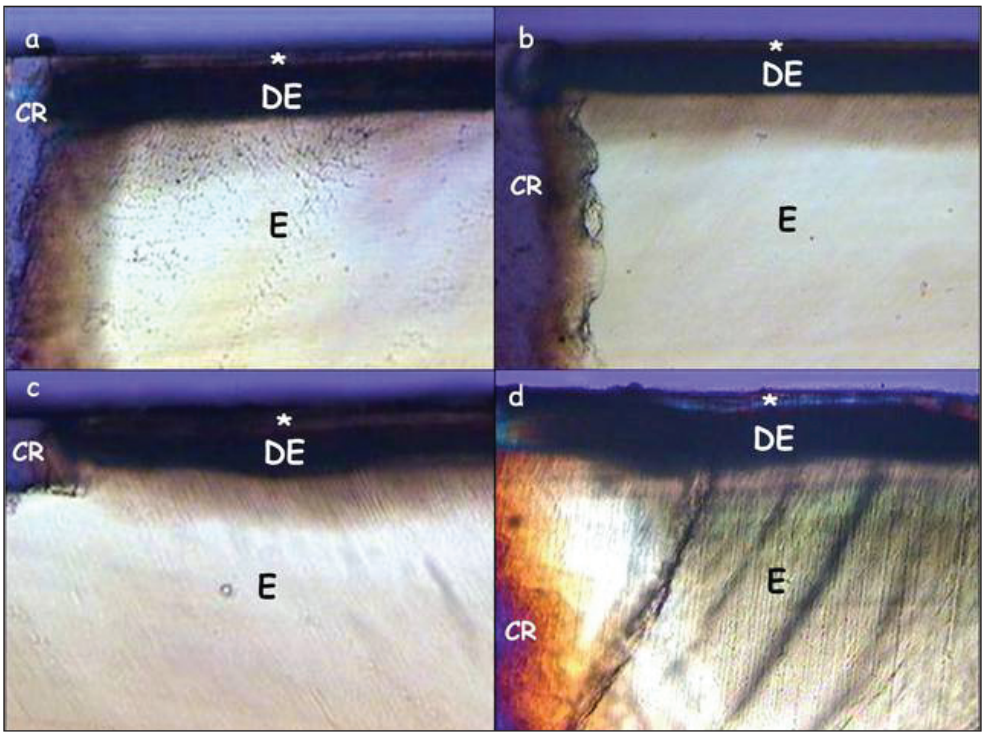

Figure 2. *Polarized transmitted light micrographs (200x): Clearfil SE Bond: Figure 2a) Clearfil Protect Bond (Figure 2b); One-Up Bond F (Figure 2c) and Single Bond (Figure 2d). Demineralization areas (DE) are seen below the enamel surface (asterisks) for all specimens (E- enamel; CR- resin composite).*

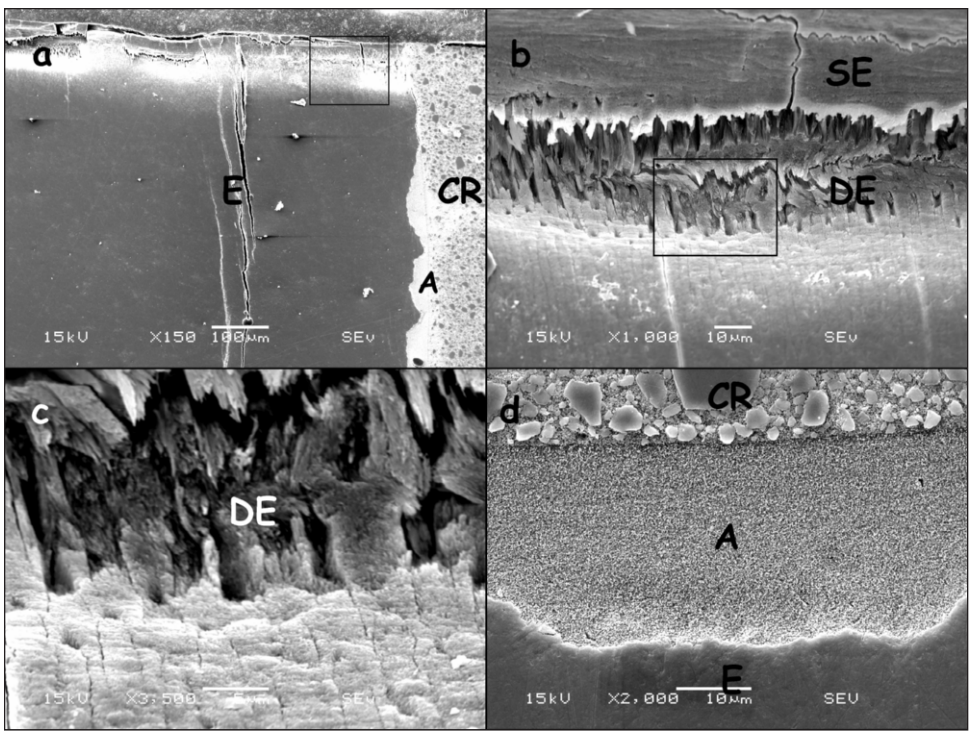

Figure 3. *SEM images of enamel around the restoration with Clearfil SE Bond. A subsurface demineralization can be observed (Figures 3a and 3b). Higher magnification of the demarcated area from Figure 3b shows severe subsurface demineralization (Figure 3c). Composite-enamel bonded interface (Figure 3d). (E–enamel; CR–resin composite; SE–sound enamel; DE–demineralized enamel; A–adhesive layer).*

retention after polymerization *in situ*.[13-14] The two layers of Single Bond solution produced a uniform adhesive layer with an approximate 10 µm thickness (Figure 6d). One-Up Bond F is the single-step "all-in-one" self-etching adhesive. As its application consists of only one adhesive solution, this bonding agent produced the thinnest adhesive layer among the materials tested (1 µm to 5 µm). On the other hand, the two self-etching primers (Clearfil SE Bond and Protect Bond) formed the thickest adhesive layer (15 µm to 25 µm), because of the more hydrophobic second layer applied over primed dentin.[15-16] The morphological features of the resin-enamel bonds (Figures 3d, 4d,e 5d) with self-etching systems are not very different from that formed with the "etch&rinse" adhesive systems (Figure 6d).

Clearfil Protect Bond contains sodium fluoride particles, which can be observed at resin-enamel interfaces under electron microscopy (Figure 4d).[1] The fluoride ions are released from the adhesive and can penetrate into the tooth structure to enhance mineralization and reduce demineralization.[17-18] Studies have shown that some adhesive systems and restorative materials can release fluoride[7,19] and control demineralization during artificial caries formation;[2-3,20-22] however, its ability to inhibit secondary caries is still questionable.[7,23]

One-Up Bond F contains fluoraluminosilicate glass, and studies indicate its capacity to release fluoride in cariogenic conditions.[7] However, the current study did not show the same results obtained with Clearfil Protect Bond. Hara and others[7] related extensive areas of demineralization in dentin around the restoration, using polarized light microscopy, as observed in the

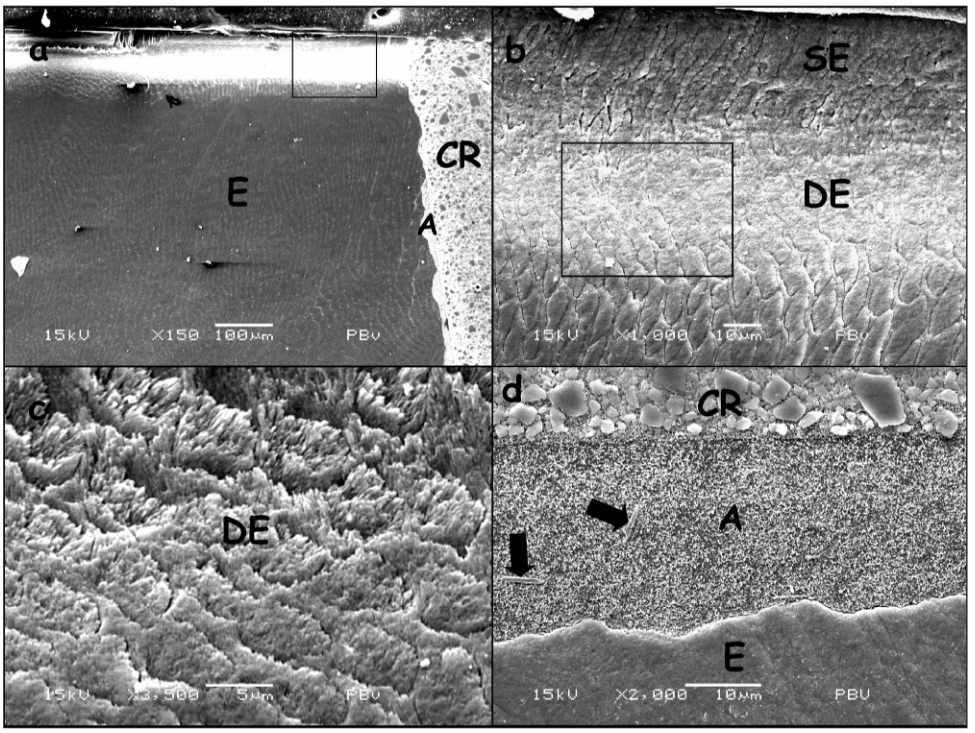

Figure 4. *SEM images of enamel around the restoration with Clearfil Protect Bond. A subsurface demineralization can be observed (Figures 4a and 4b). Higher magnification of the demarcated area from Figure 4b shows the interprismatic matrix partially removed, exhibiting the prism cores (Figure 4c). NaF particles (arrows) can be observed at the composite-enamel bonded interface (Figure 3d).(E–enamel; CR- resin composite; SE- sound enamel; DE- demineralized enamel; A–adhesive layer).*

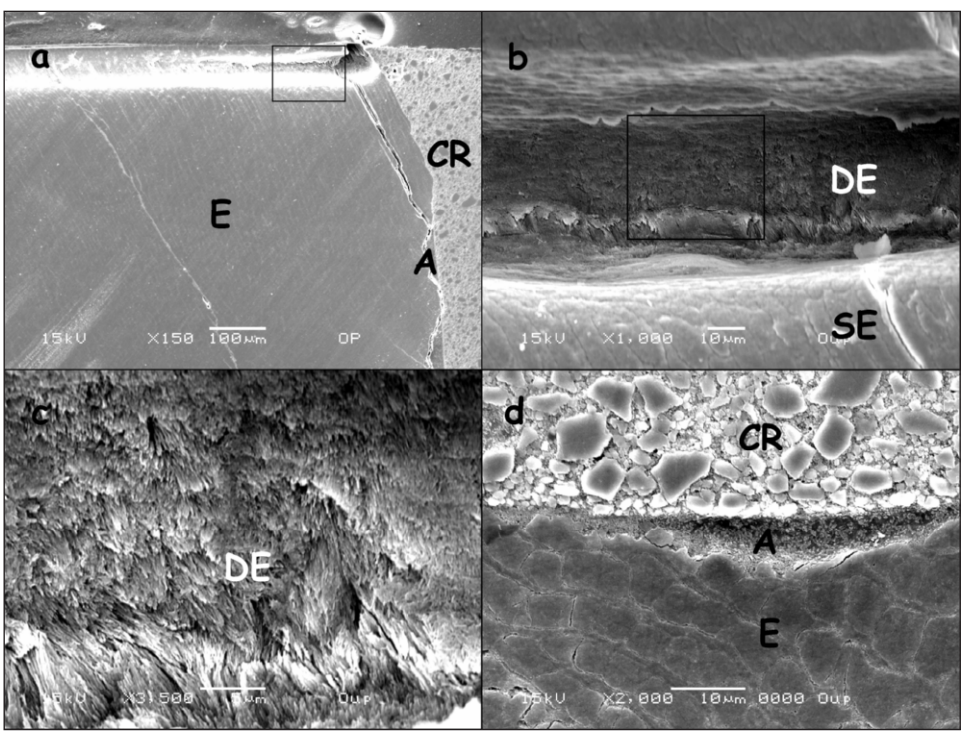

Figure 5. *SEM images of enamel around the restoration with One-Up Bond F. A subsurface demineralization can be observed (Figures 5a and 5b). Higher magnification of the demarcated area from Figure 5b shows a severe subsurface demineralization (Figure 5c). The composite-enamel bonded interface (Figure 5d). (E–enamel; CR–resin composite; SE–sound enamel; DE–demineralized enamel; A–adhesive layer).*

current study. On the other hand, Itota and others[2] observed an acid-resistant layer in the lesions and reported inhibition of secondary caries adjacent to the restorations with One-Up Bond F. Itota and others incubated the specimens in a bacterial medium containing sucrose with Streptococcus mutans for 14 days, and demineralization-remineralization cycling for eight days, which is considered a high cariogenic challenge.[11] Analyzing the percentage of volume mineral according to the adhesive systems and depth from the enamel surface, it was noted that specimens restored with One-Up Bond F presented sound enamel at a depth of 80 µm. For the remaining adhesives, sound enamel was found close to a depth of 120 µm (Table 2). Microradiographs of the caries lesions in root dentin margins demonstrated inhibition of wall lesion formation using One-Up Bond F.[2]

The adhesive systems evaluated showed similar data for CSMH analysis but different results for polarized light and scanning electron microscopy observations. Less demineralization around restoration and lower caries lesion depth were obtained by Clearfil Protect Bond application using PLM and SEM, suggesting some potential to inhibit secondary caries. Conversely, other systems show no protective effect against cariogenic challenge, probably due to a lack of fluoride release or deficient marginal sealing.[3] Thus, the hypothesis tested—that enamel marginal mineralization around restorations is affected in a different way by cariogenic challenge, depending on the type of adhesive used—was not confirmed, since the mineral % volume did not show any statistical differences among the adhesives. The discussion about the diverse methods used here is beyond the objective of the current study; however, the

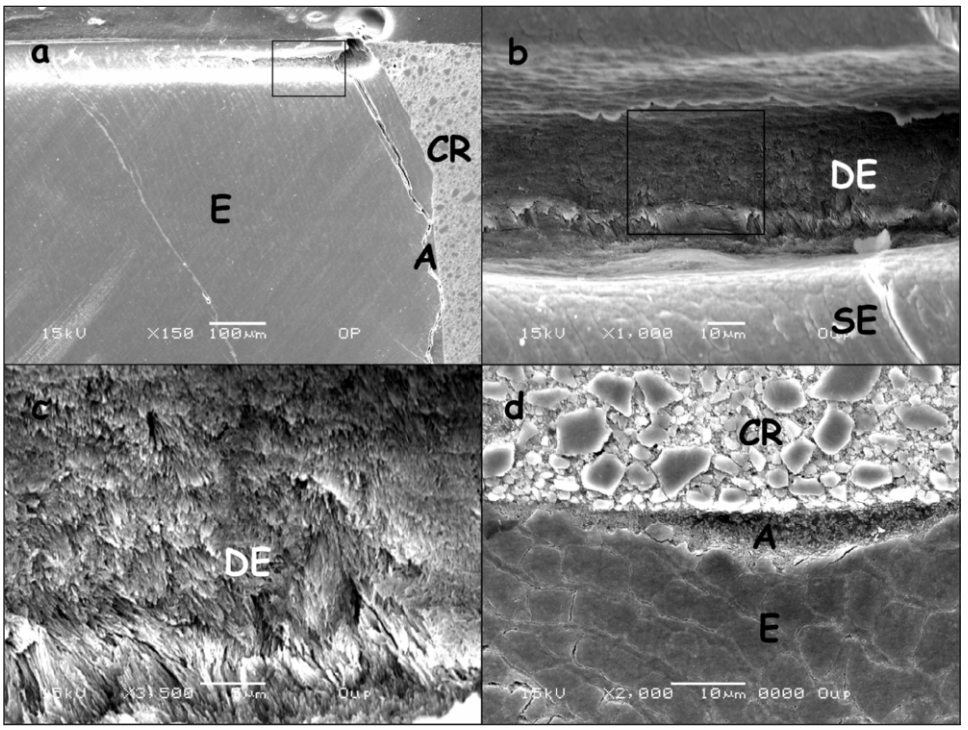

Figure 6. *SEM images of enamel around the restoration with Single Bond. A subsurface demineralization can be observed (Figures 6a and 6b). Higher magnification of the demarcated area from Figure 6b shows a severe subsurface demineralization (Figure 6c). The composite-enamel bonded interface (Figure 6d). (E–enamel; CR–resin composite; SE–sound enamel; DE–demineralized enamel; A–adhesive layer).*

lack of differences in CSMH can be explained by the intense subsurface demineralization.

The current study was conducted using very aggressive pH-cycling that favors demineralization.[11] Specimens were not thermocycled or aged; conditions that have been shown to affect the dentin-bonded interface, reducing bond strength and increasing nanoleakage.[24] These changes at the interface might also affect caries lesion development at the interface, and how they might have affected the results of this study is not known. After eight days, pH-cycling promoted the formation of subsurface caries lesions or demineralization areas in all specimens tested and no adhesive systems completely inhibited the caries formation and its progression. This strong demineralization may lead to equaling CSMH results among the adhesive systems, since the adhesives provided similar effects against cariogenic challenge. Previous findings have shown that demineralization-remineralization solutions and pH-cycling methodologies are different and are reflected in different results for adhesive systems.[2-3,7,20-23] Moreover, other factors, such as the type of fluoride source in the adhesive composition,[8,19] which could interfere in the fluoride release, could also influence the results.

Due to strong demineralization and irregularities created in the subsurface region, it is impossible to measure the CSMH at 15 µm to 35 µm of depth from the enamel surface. This region can be observed in Figures 3a, 4a, 5a and 6a, and at this location, the enamel prism was very fragile and demineralized (Figures 3c, 4c, 5c and 6c). As the lesion created was subsurface at 80 µm to 120 µm of depth from the surface, the enamel had a mineral % volume that was considered sound (Figure 1).

The adhesive systems alone were not able to completely inhibit demineralization around the restoration under the high cariogenic conditions used in this study. Therefore, the association of fluoridation methods, such as fluoridated water and dentifrices, which have been related to caries prevalence reduction,[25] must be used in order to control caries development.

CONCLUSIONS

The demineralization-remineralization cycling regimen promoted subsurface enamel demineralization in all specimens, independent of the adhesive system used. Although the microscopy analyses (SEM and PLM) suggested that Clearfil Protect Bond self-etching primer may result in lower enamel demineralization around restoration, the cross-sectional microhardness analysis did not support these observations.

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