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Distribution of adhesive layer in class II composite resin restorations before/after interproximal matrix application

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ABSTRACT

Objectives: This study aimed to morphologically investigate the distribution of the adhesive layer when placed prior, or subsequent, to matrix positioning in direct-bonded Class II RBC restorations. Additional aim was to evaluate possible differences when using two-steps (CSE, Clearfil SE Bond2) or one-step adhesive system (CU, Clearfil Universal Bond Quick).

Methods: Standardized mesio-occlusal and disto-occlusal cavities were prepared on 20 human molars. Teeth were randomly allocated to two protocols according to the positioning of contoured sectional metal matrices before (M->A, n = 10), or after adhesive application (A->M, n = 10). Both adhesive systems were additioned with crystal violet dye (CV, 10 vol%). Specimen sections were evaluated using optical and scanning electron microscopy (SEM). Dynamic viscosity, pH, microshear bond strength test (μ-SBS) on enamel and dentin, and three-point bend test (3PB) of polymerized adhesive rods, were performed on both pristine and CV-additioned adhesives.

Results: M->A produced a layer of adhesive both on tooth-restoration interface and on external restoration surfaces in contact with the matrix. A->M produced a thin layer of adhesive on external tooth surfaces, well beyond cavity and RBC restoration margins. In all restorations, excess RBC material with uneven margins was observed protruding over the cervical margin. CV addition slightly increased pH and decreased viscosity. μ-SBS: CU + CV showed a 10-fold reduction in adhesion forces on dentine. 3PB: CSE yielded higher flexural strength values than CU. CV addition reduced flexural strength of CSE.

Conclusions: Both M > A and A > M generated adhesive placement disadvantages with adhesive materials being expressed in difficult to reach locations that may jeopardize complete adhesive polymerization.

Clinical Significance: All cervical margins of RBC restorations should be carefully finished to improve longevity, no matter the clinical protocol adopted. CV addition labelled the tested adhesives without compromising their performances considerably.

1. Introduction

Resin-based composite (RBC) restorations are state of the art in dentistry nowadays due to their aesthetic appearance, mechanical properties, and ability to be bonded to the structure of teeth [1,2]. They show excellent performance and reduce the need for tissue removal, yet their expanded use in a wide range of protocols brings huge demand for a relentless improvement of their properties and performance [1]. Nevertheless, they still face several issues, such as polymerization shrinkage, micro- and nanoleakage and influence on microbial colonization, which cause the deterioration of the interface [1,3] and lead to a decreased longevity of the restoration [2].

Most of the posterior RBCs fail due to secondary caries and fractures [4–7]. When considering class II restorations, secondary caries and leakage primarily originate at the cervical margin of the tooth preparation. Many reasons are provided for this occurrence, mainly that it is

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challenging to ensure a proper application of all clinical steps in a location that is hard to reach. Furthermore, such margins are often placed in dentin where adhesive performances are significantly weaker, and high microbial colonization occurs in this hardly cleanable area [6,8]. It was demonstrated that restorative materials such as RBCs do not possess a buffering capability, as natural dental tissues do. This shortcoming, common to all conventional RBCs, leads to an increased cariogenicity of the overlying biofilm [9].

It is clear from these considerations that the success and durability of a class II restoration depend on many factors, and to a certain extent, they are related to the different steps of the restorative procedure. *In vitro* studies showed that, in class II composite restorations, the cervical margin is the most common location of bonding failures [10,11]. A crucial step in this sense is the placement of an interproximal matrix, which is needed to ensure both a favourable anatomical shape and proper marginal sealing, especially in the cervical area [2]. In the literature, different clinical protocols for class II restorations include positioning of the matrix before the application of the adhesive system, or after having placed and polymerized the adhesive layer [12–14]. The latter option was more recently introduced to ensure better visibility during the application of the adhesive, and also claiming better marginal integrity of the adhesive layer. In particular, Ernst et al. evaluated the marginal integrity of restorations where the adhesive system was applied before or after matrix positioning by a dye penetration method. They found that total-etch adhesives generally achieved better marginal integrity than self-etch adhesives, and they suggested that all adhesive systems should be applied before matrix placement to reduce the influence of the application protocol on marginal integrity [12,13].

The presence of an unnecessary exposed layer of adhesive not covered by the composite on the marginal area of the restoration entails the possibility of microbial overgrowth in a crucial location for the development of secondary lesions. This is particularly important in hard-to-clean areas such as the cervical margin of the Class II restorations. The external part of exposed adhesive is covered by an oxygen-inhibited layer that is rich in non-reacted monomers that particularitly foster higher biofilm formation [15] being detrimental to the longevity of the restoration. Minimizing the presence of such exposed adhesive layer may be beneficial in terms of improving the longevity of a composite restoration. From this point of view, positioning an interproximal matrix before the application of the adhesive system might seem a good clinical protocol. Another problem related to Class II restorations is the presence of excess material protruding over the cervical margin of the cavity preparation (overhang). Indeed, it is known from the literature that, despite all efforts, complete prevention of interproximal overhang is almost impossible [16]. However, no study to date morphologically investigated the effect of adhesive placement prior, or subsequent, to matrix positioning in direct-bonded Class II RBC restorations.

The present study aimed to investigate the distribution of the adhesive layer of direct bonded Class II RBC restorations made using two different clinical protocols, namely performing adhesive procedures before the application of the interproximal matrix or after application of the matrix. An additional aim of the study was to evaluate the behaviour of two different adhesive systems, namely a two-step and a universal one when used with the protocols mentioned above.

2. Materials and methods

The protocol for the present study was approved by the Human Ethics Committee of Near East University, Faculty of Dentistry, Cyprus (No: YDU/2019/70-845). A power calculation was made based on a previously performed pilot study. By assuming an α *value of* 0.05, a mean standard deviation of 19.5, and considering a power of 0.8, the minimum specimen size for each group was determined as 7.

2.1. Specimen preparation

A total of 20 freshly extracted, non-carious, posterior human teeth were used in the present study. Standardized mesio-occlusal (MO) and disto-occlusal (DO) cavities were prepared in all teeth using a diamond bur for inlay preparation (#8959KR-018, Komet, Brasseler GmbH & Co., Lemgo, Germany). The bur was engaged from the interproximal surface toward the centre of the tooth for a total length of 3 mm; the interproximal width of the cavity was 4.0 mm. Cervical margins were located 1.0–1.5 mm coronal to the cementum-enamel junction. The prepared teeth were randomly assigned to two groups. In the first group, teeth (n = 10) were restored using contoured sectional metal matrices held in position by wooden wedges before adhesive application (M->A). In contrast, in the second group, teeth (n = 10) were restored using contoured sectional metal matrices and wooden wedges after adhesive application (A->M). Two different adhesives were used (Table 1), a two-step (CSE, Clearfil SE Bond2, Kuraray Noritake Dental Inc., Kurashiki, Okayama, Japan) or a one-step adhesive system (CU, Clearfil Universal Bond Quick, Kuraray). Both adhesive systems were additioned with crystal violet (CV). A 2 wt% crystal violet hydro-alcoholic solution for Gram staining was used (Merck, Darmstadt, Germany). In the case of the two-step system, CV was additioned to both primer and bond. A pilot study was conducted using 2.5, 5, 10, and 20 vol% CV, and it was ascertained that 10 vol% was the minimum concentration showing clear visibility of the adhesive layer on optical microscopy (final concentration of CV in the adhesive: 0.2 wt%). The influence of CV-addition to the characteristics of the tested adhesive systems were studied in terms of changes in pH, viscosity, bond strength to enamel and dentine and flexural strength, as described below in the following sub-sections.

The enamel surfaces of all specimens were selectively etched with 35 % acid gel (K-ETCHANT Syringe, Kuraray) for 30 s, then rinsed and gently blown with an oil-free air source for 5 s [17]. Then, on one side of each tooth, CSE was applied, while on the opposite side, CU was applied. A LED curing unit (Mini Led Satelec, Acteon Group, Merignac, France, 1250 mW/cm^2) was used for 20 s on each side to light-cure the adhesives. A nanohybrid RBC (G-aenial shade A2, GC Corporation, Tokyo, Japan) was used to fill the cavities using the centripetal technique. Each layer was light-cured for 40 s. The procedures employed using each

Table 1
Composition and application protocol of the adhesives used in the present study.

Material	Composition	Application protocol used in this study
Clearfil SE bond 2 (CSE)	*Primer (CSEP):* 10-MDP, HEMA, hydrophilic dimethacrylate, photo-initiator, water.	Apply phosphoric acid etching gel (37 %) to the enamel, leave it in place for 30 s, then rinse and gently dry.
	Bond (CSEB): 10-MDP, Bis-GMA, HEMA, hydrophobic dimethacrylate, photo-initiator, silanated colloidal silica	Apply primer for 20 s. Dry the cavity wall by blowing mild air for 5 s.
		Apply bond, mild air blow for 5 s, light-cure with 1250 mW/cm^2 LED for 20 s.
Clearfil Universal Bond Quick (CU)	10-MDP, Bis-GMA, HEMA, hydrophilic aliphatic dimethacrylate, colloidal silica, silane coupling agent, dl-CQ, ethanol, water	Apply phosphoric acid etching gel (37 %) to the enamel, leave it in place for 30 s, then rinse and gently dry. Apply bond to the cavity wall with the applicator brush and rub it for 20 s. Dry the cavity wall by blowing mild air for 5 s. Light-cure with 1250 mW/cm^2 LED for 20 s.

Abbreviations: 10-MDP, 10-methacryloxydecyl dihydrogen phosphate; HEMA, 2-hydroxyethyl methacrylate; Bis-GMA, bis-phenol A diglycidylmethacrylate; CQ, camphorquinone.

tested adhesive system before or after matrix placement are shown in Fig. 1. After that, specimens were stored at room temperature in phosphate-buffered solution (PBS), then sectioned at 0.5 mm thickness with a water-cooled, low-speed diamond saw (Isomet 1000, Buehler, Lake Bluff, IL, USA).

2.2. Specimen observation

All sections were assessed with a stereomicroscope at 50x magnification. Afterward, specimens were placed on aluminium stubs, sputter-coated (JEOL FFC-1100, Tokyo, Japan), examined with a scanning electron microscopy (SEM) (JEOL JSM-840A) set at 10 kV accelerating voltage and assessed under secondary electrons emission mode. Microphotographs were taken at a magnification range of 50−500x.

2.3. Viscosity measurements

Dental adhesive systems behave as Newtonian fluids, as long as they are not filled with inorganic particles [18]. A custom-built falling sphere viscometer was used as it can easily determine the absolute shear viscosity of a Newtonian fluid. In this method, a steel bearing was allowed to fall freely a measured distance through the adhesive resin contained inside a 10-ml graduated glass pipette. Knowing the mass, density and diametre of the steel sphere, its measured average velocity was determined by noting the time it took to fall the measured distance. The correction of the velocity to approach terminal velocity was made using Brenner's correlation between the diametre of the sphere and the inner diametre of the tube. Then, the dynamic viscosity was determined at a temperature of 23 °C applying Stoke's formulation for laminar flow (Reynolds Number <1). Results were expressed in mPa*s. A total of six measurements were performed for each resin in a dark room, illuminating the glass pipette with a red light, so as to correctly identify the position of the falling sphere when CV-additioned resins were tested (CV solutions do not absorb high wavelengths) and to avoid polymerization of the bonding systems during tests. The following groups were thus tested:

1 CSE primer(CSEP, n = 6)
2 CSE bond (CSEB, n = 6)
3 CU adhesive (CU, n = 6)
4 CSE primer + CV (CSEP + CV, n = 6)
5 CSE bond + CV(CSEB + CV, n = 6)
6 CU adhesive + CV (CU + CV, n = 6)
7 Crystal violet solution(CV, n = 6)

2.4. pH measurements

A pH Electrode (Orion™ 9110DJWP double junction electrode coupled with a 720A + Advance pH / mV Meter, both from Orion Research Inc., Cambridge, MA, USA) was used to directly measure the pH of the tested adhesives before and after CV addition, as well as the pH of the CV solution. A total of six measurements were performed on each resin, thoroughly rinsing the electrode with 95 % ethanol solution between measurements, and checking the readings against standard pH = 4.0 and pH = 7.0 reference solutions every three measurements. The following groups were thus tested:

1 CSE primer(CSEP, n = 6)
2 CSE bond (CSEB, n = 6)
3 CU adhesive (CU, n = 6)
4 CSE primer + CV (CSEP + CV, n = 6)
5 CSE bond + CV(CSEB + CV, n = 6)
6 CU adhesive + CV (CU + CV, n = 6)
7 Crystal violet solution(CV, n = 6)

2.5. Microshear test

A total of 12 permanent molar teeth were additionally used for this test. The selected teeth crowns were separated from the roots using the diamond saw (Isomet). The surfaces of one group of crowns (n = 6) were additionally cut at the enamel level, while the second group of crowns (n = 6) had the occlusal dentin layer exposed. The surface of each specimen was ground using wet 320-grit silicon carbide abrasive papers to produce a flat surface featuring a smear layer. The flat surfaces thus obtained on top of each specimen were divided into two equal parts by making a thin groove with a diamond fissure bur. Each enamel surface was selectively etched by using the 35 % phosphoric acid gel for 30 s, then rinsed with water spray for 30 s and air-blown for 5 s.

On one half of the surface of every specimen, a CV-additioned adhesive system was applied, while on the other half, the unmodified adhesive system was applied for bond strength comparisons. Therefore, a total of eight different microshear test groups were prepared, as shown below.

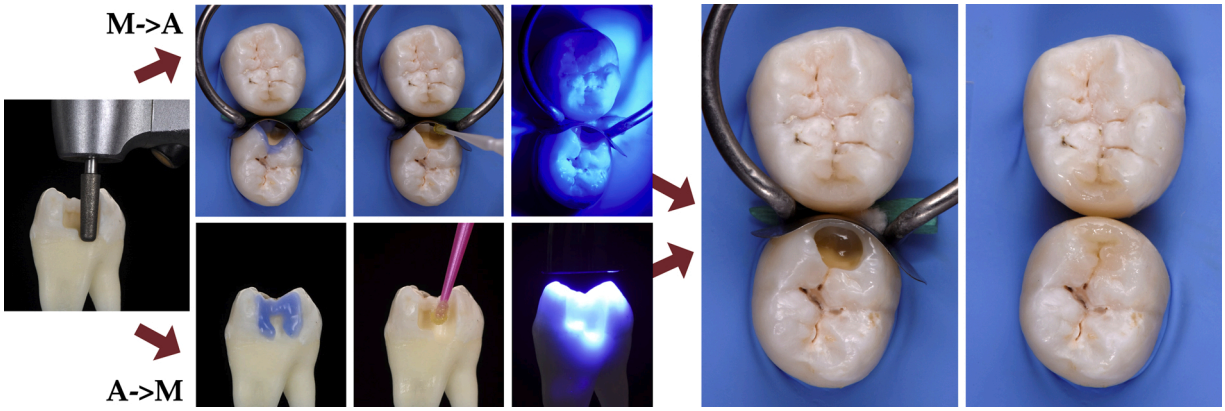

Fig. 1. Diagram depicting the protocols used for the manufacturing of the class II restorations. First, a standardized class II cavity was created on both mesial and distal aspects of molar teeth using inlay preparation and finishing burs. Then, teeth were randomly divided into two groups, following, respectively, (M->A) a protocol that included adhesive procedures performed after interproximal matrix positioning, and (A->M) adhesive procedures performed before interproximal matrix positioning. A setup approaching clinical conditions as close as possible was used, including positioning of the extracted teeth inside a custom carrier made of impression putty, application of dental dam, and wedge and rings to hold the matrix in place. Here an example is given on the application protocol used for the universal adhesive, including the selective etching of enamel. All cavities were filled following the centripetal technique that included the creation of a marginal ridge that reduced the cavity to class I. The creation of a good interproximal contact point can be seen at the end of the procedures. For clarity's sake, no CV-additioned adhesives were used in this diagram to illustrate the procedures.

1 Enamel surface, CSE adhesive **(E-CSE, n = 3)**
2 Enamel surface, CU adhesive **(E-CU, n = 3)**
3 Enamel surface, CSE adhesive + CV **(E-CSE + CV, n = 3)**
4 Enamel surface, CU adhesive + CV **(E-CU + CV, n = 3)**
5 Dentin surface, CSE adhesive **(D-CSE, n = 3)**
6 Dentin surface, CU adhesive **(D-CU, n = 3)**
7 Dentin surface, CSE adhesive + CV **(D-CSE + CV, n = 3)**
8 Dentin surface, CU adhesive + CV **(D-CU + CV, n = 3)**

The adhesive systems were applied to the enamel and dentin surfaces and air-thinned by mild air blow for 5 s. The adhesive was then light-cured for 20 s.

Four cylinders (internal diameter: 0.5 mm, height: 3.0 mm) of Tygon® microbore tubing (R-3603, Norton Performance Plastic Co., Cleveland, OH, USA) were positioned on the flat surfaces totalling two cylinders for each test group (Fig. 2), thus totalling six specimens for each test group.

Each tube was filled with flowable nanofilled composite resin (Clearfil Majesty ES Flow, Kuraray) and then light-cured for 40 s with the tip of the light guide in contact with the top of the tube. A single operator performed all adhesive procedures. The specimens were then immersed in distilled water at 37 °C for 24 h. Before testing, the tube was sectioned and carefully removed to leave a composite rod perpendicular to the tooth surface (Fig. 2).

The specimens were positioned in a jig attached to a universal testing machine (Acquati, Milan, Italy). A stainless steel wire loop (0.2 mm diameter) was placed against the enamel surface, engaging the lower half-circle of one cylinder per time. A shear load was applied at a crosshead speed of 1.0 mm/min until failure occurred. Care was taken to keep the composite cylinder in line with the centre of the load cell. The wire loop was kept parallel to the adhesive interface as well as to the movement direction of the load cell. The maximum load at failure was recorded for each specimen in Newtons (N). The shear bond strength (SBS) was calculated by dividing the maximum load (N) by the bonding surface (mm^2) and expressed in Mega Pascals (MPa).

2.6. Three-point bend test

The influence of CV addition to the flexural strength of the tested adhesives was evaluated on the following groups using the three-point bend test.

1 CSE bond (CSE, n = 6)
2 CU adhesive (CU, n = 6)
3 CSE bond + CV (CSE + CV, n = 6)
4 CU adhesive + CV (CU + CV, n = 6)

For the preparation of test specimens, two microscopy glass slides (1.0 mm thickness) were placed 2.0 mm away from each other between two translucent adhesive papers. In this way, a bar-shaped tunnel was obtained. The tunnel was kept in a vertical position while a total of 100 µl of adhesive was injected (for the CSE adhesive system, only the bond was used), then it was placed in a horizontal position and condensed against a glass plate to remove any excess material. Specimens were light-cured for 10 s with the tip of the light guide in contact with the glass plate, then the glass plate was removed, and the bars were light-cured for additional 10 s (Fig. 3A) with the tip of the light guide placed directly in contact with one of the translucent adhesive papers covering the specimen surface. The ISO 4049/2000 specifications were followed in this test, save for the length of the bars (10 mm) being specifically chosen to be smaller than the diameter of the light guide tip (11 mm) to allow a one-shot polymerization of the specimens (Fig. 3B).

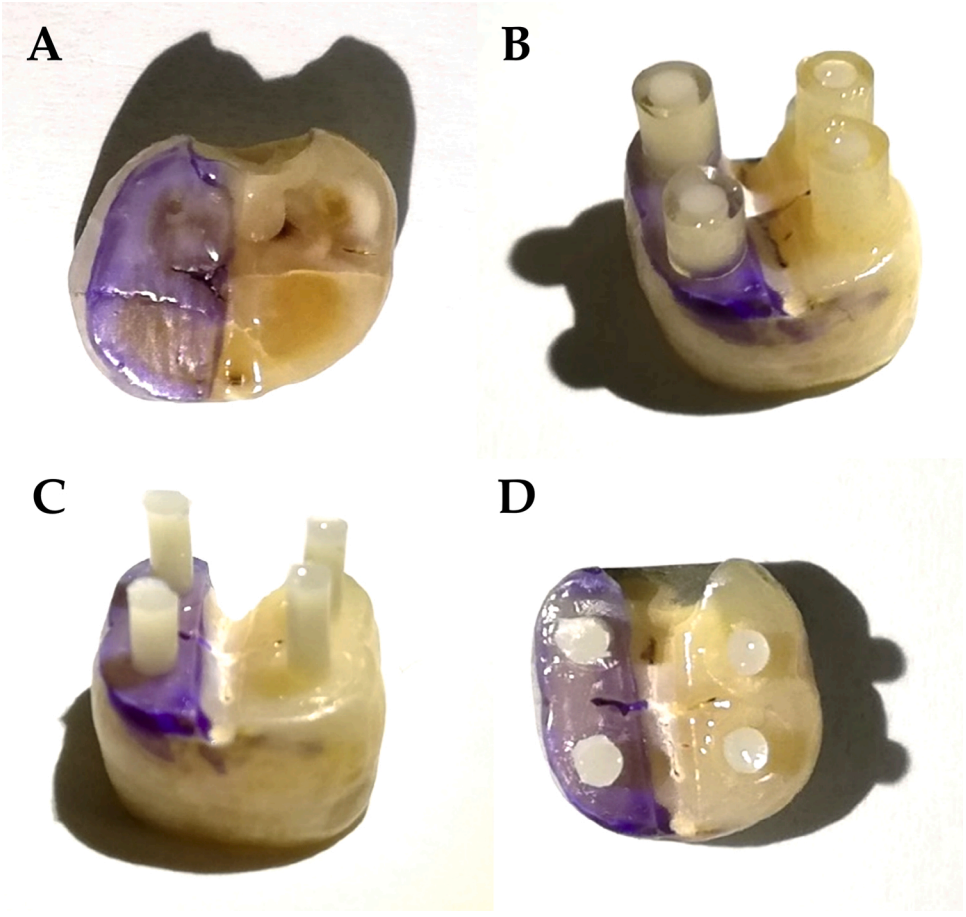

Fig. 2. Fabrication of the µ-SBS test specimens. 2A: A specimen cut to expose dentine is shown, where a groove was made on the top surface to separate the substrate treated with CV-additioned adhesive from dentine treated with conventional adhesive system. 2B: Tygon tubes were used to create standard rods of flowable RBC, two rods for each adhesive type. 2C and D: tubes were gently cut and detached using scalpel and tweezers to expose the rods that, after 24 h storage in distilled water, were one by one transversally pulled until failure of the bonded interface by a wire loop to gather µ-SBS data.

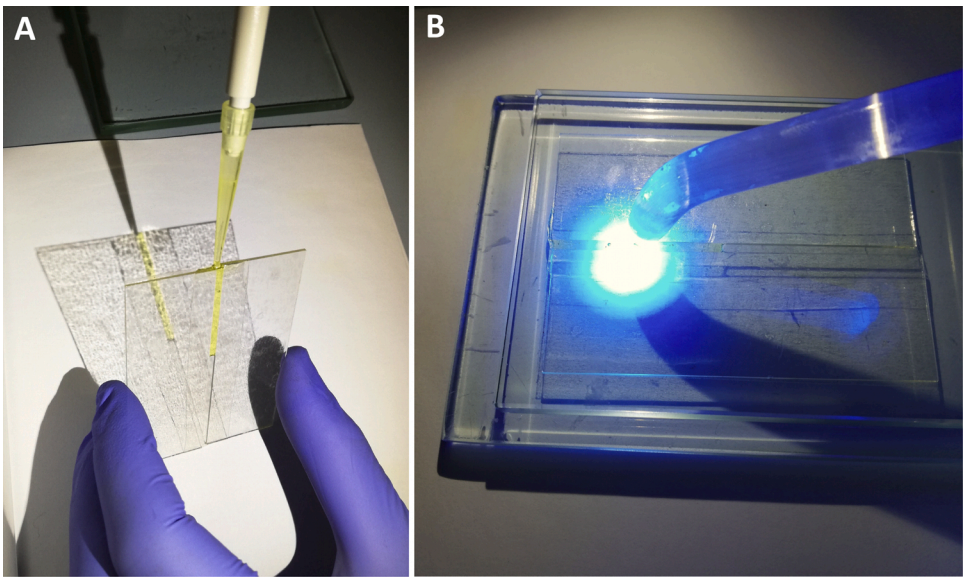

Fig. 3. Preparation of three-point bend test specimens. 3A: the adhesive is injected using a micropipette in a rod-shaped tube obtained by sealing two glass slides with a non-foldable transparent adhesive sheet. 3B: the adhesive rod is condensed against a glass plate, then polymerized for 10 s. After that, the glass plate is removed, and an additional 10 s photo-curing was performed (not shown).

After light curing, the specimens were carefully removed from the moulds, checked for visible surface irregularities and stored at 37 °C in a dark place for 24 h to allow the polymerization reaction to be completed. A total of six bars were produced for each group. The specimens were subjected to a three-point bend test using a universal testing machine (Acquati) with a crosshead speed of 1 mm/min. The bending data were recorded as the load to failure. After fracture, the maximum loads were obtained and the flexural strength *(σ)* was calculated and expressed in MPa by using the following formula:

$$\sigma = 3\,F\,L/(2BH^2)$$

where *F* is the maximum load in Newtons (N), L is the distance between the supports in mm, *B* is the width of the specimen in mm and *H* is the height in mm. The formula was solved by measuring the width and thickness of every prepared bar with a digital micrometer, knowing that the custom-made support for the bars had *L* equal to 8 mm.

2.7. Statistical analysis

All statistical analysis were performed with JMP 14.0 statistical software (SAS Institute, Cary, NC, USA). The normal distribution of the data was checked using Shapiro-Wilk's test, and the homogeneity of the variances was verified using Levene's test. Means and standard errors were calculated from the raw data.

The microshear bond strength test was analyzed using three-way analysis of variance (ANOVA). The data was analyzed considering substrate (enamel, dentin), adhesive type (CSE, CU), and CV addition (normal, additioned with 10 vol% crystal violet) as fixed factors.

The viscosity, pH measurements, and the three-point bend test (3PB), were analyzed using two-way ANOVA. Adhesive type and CV addition were considered as fixed factors. Student's post-hoc *t*-test was used in all tests to highlight significant differences between groups ($p < 0.05$).

3. Results

3.1. Specimen observation

Optical microscopy observations of the sectioned specimens showed that an excess of adhesive layer reached over cervical margins down to the cementum surface in all specimens. Furthermore, in all restorations, excess RBC material with uneven margins was observed protruding over the cervical margin of the cavity preparation (Fig. 4). Adhesive application after matrix positioning produced a layer of adhesive both on tooth surfaces inside the restoration box and on the external restoration surfaces in contact with the matrix. The external interproximal surface of the RBC restoration at the end of the procedures was therefore constituted by a mixture of the RBC material with the adhesive. Matrix positioning after adhesive application procedure resulted in a thin layer of adhesive on the external tooth surfaces, well beyond cavity margins, and over the end of the RBC restorations. Contrarily to CSE adhesive system, CU layer showed impregnation into the RBC material.

Representative SEM microphotographs of the different application procedures from the specimens are given in Fig. 5. SEM observations confirmed optical microscopy findings and additionally showed that matrix positioning after adhesive application produced rougher RBC cervical margins than the opposite procedure.

3.2. Viscosity measurements

The dynamic viscosity results are displayed in Table 2. The bonding agent of CSE had a significantly higher viscosity than CU, while the CSE primer had a very low viscosity, in the range of magnitude of ethanol. CV addition decreased the viscosity of CSE bonding agent and halved the viscosity of CU, while did not significantly influence that of CSE primer.

3.3. pH measurements

The pH measurements are shown in Table 3. It was confirmed that all tested adhesives belong to the "mild self-etch" category, with a pH in the range of 2 (CSE primer) to 2.6 (CU). While not having a considerable effect on CSE primer, CV addition significantly increased pH values of CSE bonding agent and CU.

3.4. Shear bond strength

The results for the SBS test are given in Table 4 and Fig. 6. ANOVA results indicated significantly different SBS values of enamel and dentin ($p = 0.0128$). The two adhesive types were significantly different from each other ($p = 0.0314$), yet there was no significant interaction between the substrate and the adhesive type, meaning that the different

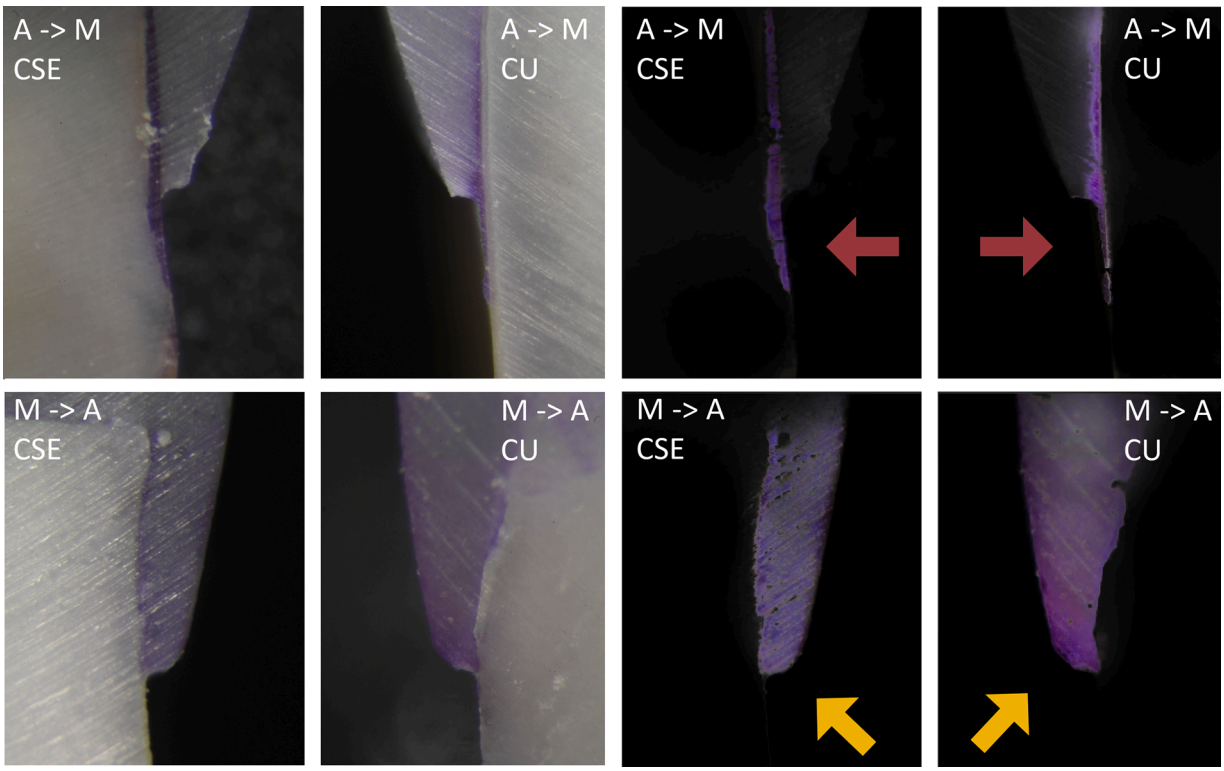

Fig. 4. Optical microscopy observations of the sectioned specimens. A->M, adhesive procedures performed before matrix positioning, leading to exposure of adhesive layer beyond restoration margins (red arrows); M->A, adhesive procedures performed after matrix positioning, leading to esposure of adhesive layer on the RBC restoration surface. The adhesive system impregnated through the RBC (orange arrows) showing both a vertical and horizontal gradient that reached a maximum at the cervical margin. The impregnation was lowest when CSE was used with A->M protocol and was highest when CU was used with M->A. For better visualization, the corresponding sections are additionally displayed in which the violet color is enhanced (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

behaviour of the adhesive systems did not depend on the adherence surface. CV-addition significantly influenced SBS values ($p = 0.0030$), and a significant interaction of CV-addition with the substrate was highlighted ($p = 0.0331$).

The results of the Student's post-hoc *t*-test indicated that enamel yielded significantly higher adhesion forces than dentin ($p = 0.0128$). Also, CSE showed higher adhesion forces than CU ($p = 0.0314$). CV addition significantly reduced adhesion forces to dentin ($p = 0.0015$) in both adhesive systems, but the reduction was particularly high for CU + CV (Fig. 6).

3.5. Three-point bend test

The results of the three-point bend test are given in Table 5 and Fig. 7. The two-way ANOVA showed a highly significant influence of adhesive type and no significant influence of CV addition on flexural strength. Besides, a significant interaction between adhesive type and CV addition was found ($p = 0.0023$). Student's post-hoc *t*-test demonstrated that CSE yielded significantly higher flexural strength values than CU ($p < 0.0001$). CV addition significantly reduced the flexural strength of CSE ($p = 0.0016$), yet it did not have any significant effect on the flexural strength of CU ($p = 0.099$).

4. Discussion

The factors affecting the longevity of direct posterior restorations and the likely reasons for their failures have the highest importance in clinical practice [2,4,5]. In this context, researchers have tried to support clinicians and overcome existing problems by improving material characteristics and application techniques.

In clinical practice, it is commonly thought that placing the matrix before applying the adhesive has the disadvantage that the latter may be accumulated on the matrix-tissue interface after air blowing, leaving a thick layer of adhesive exposed at the interface between RBC and tooth tissues [19]. Another drawback of this technique is that, during the application of the matrix, the formation of narrow gaps between the gingival floor and matrix may be seen, which may be filled with adhesive rather than with RBC [20].

In order to try and overcome such problems, another protocol, including matrix positioning after adhesive application, was proposed to achieve better marginal adaptation of the restoration [12,13]. A disadvantage of the latter technique, however, may be that the adhesive is air-blown on tooth surfaces at significant distances from the restoration margins, instead of being relatively contained inside the cavity. This issue can cause a poorly polymerized layer of adhesive resin to form, with possible consequences ranging from increased cytotoxicity to promotion of biofilm development. To date, no study morphologically investigated the effect of adhesive placement prior, or subsequent, to matrix positioning in direct-bonded Class II RBC restorations.

Our results showed that matrix positioning after adhesive application procedure resulted in a thin layer of adhesive on the external tooth surfaces, well beyond cavity margins, and over the end of the RBC restorations. Adhesive application after matrix positioning caused the external interproximal surface of the RBC restorations to be constituted by an adhesive-rich composite layer whose characteristics depended on the adhesive type. Contrarily to the two-step adhesive system, the universal adhesive showed a much deeper impregnation into the RBC material (Fig. 4). A possible explanation for that is a lower degree of polymerization of the adhesive surface layer of the universal adhesive system compared to the self-etch one, due to the presence of oxygen. Besides, an adequate thickness of the oxygen-inhibited layer is necessary for optimizing the bond strength of RBC restorations [21]. The presence

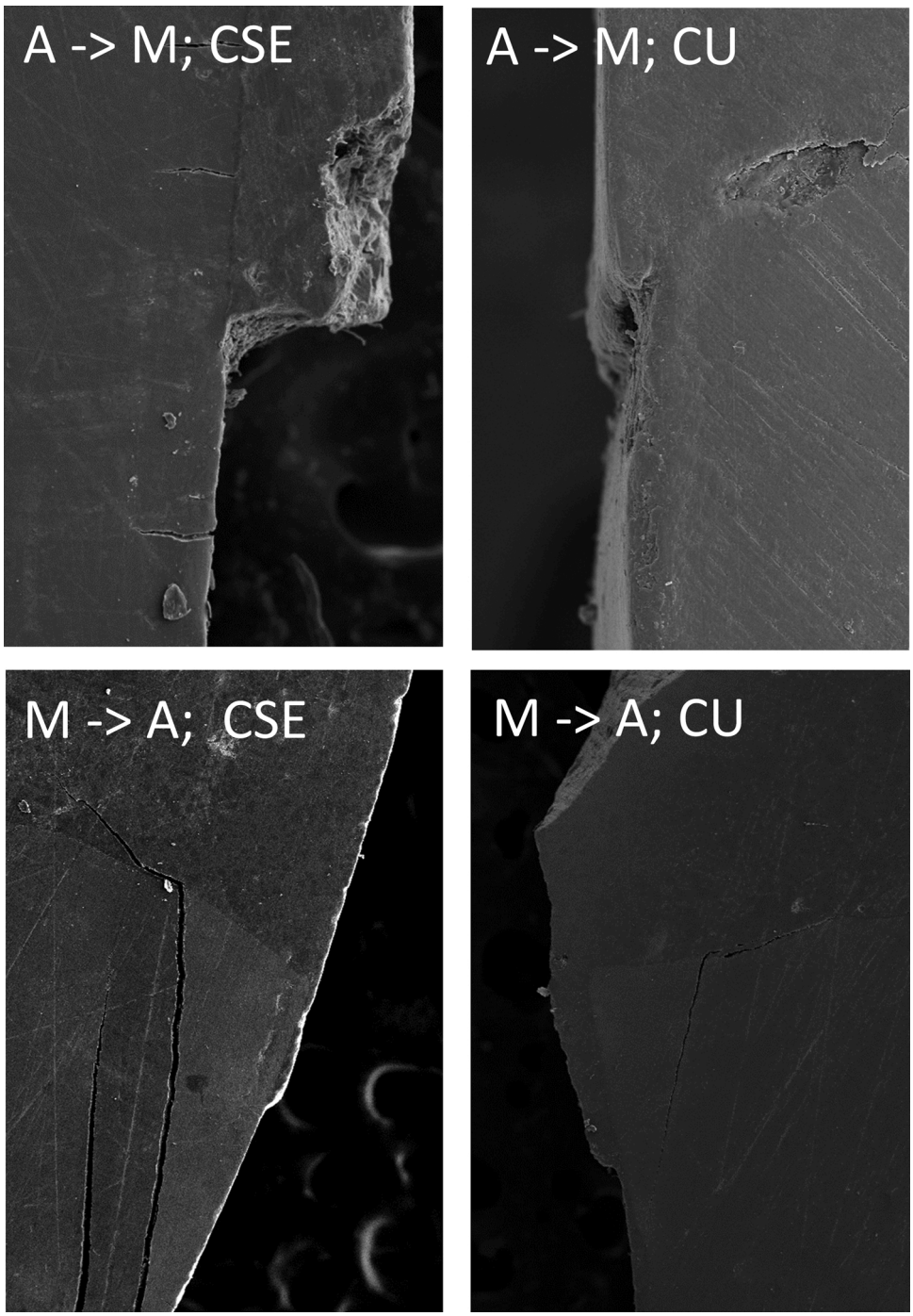

Fig. 5. SEM microscopy observations of the sectioned specimens. A->M, adhesive procedures performed before matrix positioning; M->A, adhesive procedures performed after matrix positioning.

of an ethanol-based solvent in the universal adhesive composition able to elute the RBC resin matrix may also explain such behaviour.

From a microbiological point of view, the presence of an adhesive-rich layer on the external part of the restoration entails higher microbial colonization and degradation of the material and the interface, being detrimental to the longevity of the performed treatment. While research has focused for years on adhesive systems showing antimicrobial activity [22,23], recent efforts are also driven to the development of adhesive systems that are more resistant to degradation, but the latters are still not clinically available [24]. Several other research groups demonstrated that salivary and biofilm-derived esterases could degrade artificial polymeric surfaces of RBCs and adhesives [25,26]. Minimizing the presence of an adhesive layer on restoration and tooth surfaces, especially in hard-to-clean areas, therefore, might improve the restoration longevity. From this point of view, matrix positioning after adhesive application does not seem a good clinical option.

Excess RBC material with uneven margins was observed protruding over the cervical margin of the cavity preparation (overhang) in all restorations independently from the applied protocol or the tested adhesive system. In particular, SEM observations showed that matrix positioning after adhesive application produced rougher RBC cervical margins than the opposite procedure. However, a limitation of this study is that the evaluation of the restoration margins was morphological, therefore the roughness of the margins was not evaluated quantitatively

Table 2
Changes in the dynamic viscosity of the different adhesives after addition of crystal violet dye (CV). CV addition significantly reduced the viscosity of the universal adhesive system and the bonding agent of the self−etch adhesive system tested. CV viscosity was close to that of ethanol (1.00 mPa*s). Different superscript letters indicate significant differences between groups (Student's t−test, $p<0.05$).

Material	Viscosity (mPa*s)
CSEP	6.54 (0.26)^{e}
CSEP + CV	5.66 (0.45)^{e}
CSEB	223.19 (3.91)^{a}
CSEB + CV	151.16(24.30)^{c}
CU	180.04(19.63)^{b}
CU + CV	92.06(13.80)^{d}
CV	1.45 (0.05)^{f}

Table 3
Changes in the pH values of the different adhesives after addition of crystal violet dye (CV). The pH of the 2 wt% CV solution added to the adhesives is also shown. CV addition to the primer of the self−etch adhesive system almost did not alter its pH, while CV addition to its bonding agent or to the universal adhesive system increased pH values. Different superscript letters indicate significant differences between groups (Student's t−test, $p<0.05$).

Material	pH (±1SD)
CSEP	1.95(0.04)^{a}
CSEP + CV	2.03(0.03)^{b}
CSEB	2.47(0.04)^{c}
CSEB + CV	3.44(0.05)^{f}
CU	2.58(0.04)^{d}
CU + CV	3.26(0.03)^{e}
CV	4.40(0.03)^{g}

Table 4
Results of the three-way ANOVA for shear bond strength test.

Factor	DF	Sum of Squares	F Ratio	Prob > F
Substrate	1	268.38021	6.7997	0.0128*
Adhesive type	1	196.42521	4.9766	0.0314*
Substrate*Adhesive type	1	74.25187	1.8813	0.1778
CV addition	1	393.88021	9.9794	0.0030*
Substrate*CV addition	1	192.40021	4.8747	0.0331*
Adhesive type*CV addition	1	77.26687	1.9576	0.1695
Substrate*Adhesive type*CV addition	1	138.38021	3.5060	0.0685

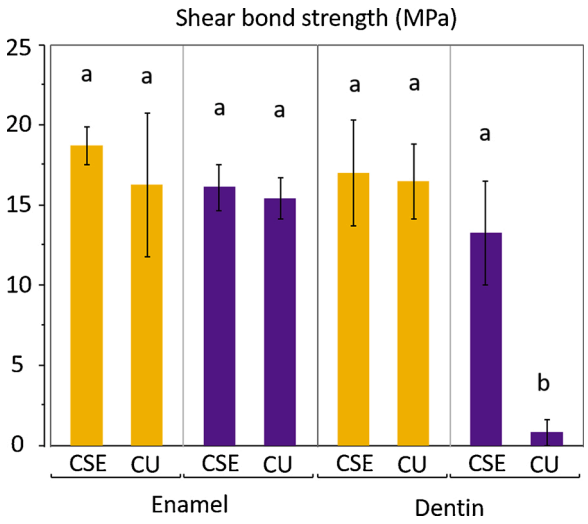

Fig. 6. Mean μ−SBS values (±1SE) of the tested adhesives on the different adherence substrates. Different superscript letters indicate significant differences between groups (Student's t−test, $p<0.05$).

Table 5
Results of the two-way ANOVA for flexural strain test.

Factor	DF	Sum of Squares	F Ratio	Prob > F
Adhesive type	1	43463.096	59.2560	<.0001*
CV addition	1	623.704	0.8503	0.3680
Adhesive type*CV addition	1	9124.822	12.4405	0.0023*

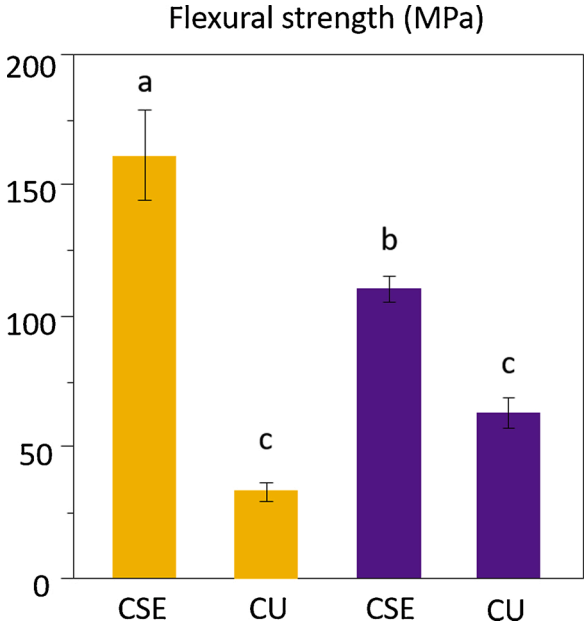

Fig. 7. Mean flexural strength values (±1SE) of the tested adhesives. Different superscript letters indicate significant differences between groups (Student's t−test, $p<0.05$).

or semiquantitatively. Even though all efforts to prevent interproximal overhang, it is known from the literature that complete prevention of this undesired effect is almost impossible [16]. *In vitro* studies showed that, in class II composite restorations, the cervical margin is the most common location of bonding failures [10,11]. Therefore, the stability over time of this particular area seems paramount to obtain a restoration's longevity. It is well established that rough surfaces in the cervical area (caused by caries lesions, poor-quality restorations, or structural defects) restrict adequate biofilm removal, thus leading to secondary caries occurrence [27]. It was demonstrated that the type of interproximal matrix, rather than the type of RBC, influenced the interproximal anatomic shape and the overhang formation, such as the ones observed in the present study. The use of contoured sectional matrices was found to produce the least marginal overhang in Class II cavities [28,29] and is currently considered the best way to reconstruct contact points. From this point of view, given that no matrix seems to be able to entirely avoid marginal overhanging, a clinical procedure in which adhesive is placed after matrix positioning seems to produce more even cervical margins, which may ultimately be linked to increased longevity.

Crystal violet was used for the first time to stain the tested adhesives for microscopic observations, showing good mixing properties and phase stability for both tested adhesive systems. A series of conventional

and fluorescent dyes were tested or screened for this purpose. Wang et al. used the fluorescent dye Rhodamine B (0.10 mg/mL) to label an adhesive system that was also used in this study (CSE), concluding that it could negatively affect the adhesive systems and their bond strength [30]. They cautioned about the dye incorporation into non-simplified dentin adhesive systems as it can interfere with their physical-mechanical properties, leading to bias in the bond integrity analysis. Another publication about the use of fluorescent dyes for the labelling of adhesive systems provided the same caveats [31]. For this reason, a set of additional tests (dynamic viscosity, pH, microshear bond strength, flexural strength) was performed to evaluate the influences that the addition of crystal violet might have caused the experimental setup. An absence of relevant effects of crystal violet addition to the adhesives is a prerequisite to ensure the reliability of the results. For instance, an incomplete polymerization might have caused the displacement of the adhesive during sectioning phases of the tooth specimens, seriously biasing the outcomes. In fact, results indicated that crystal violet addition significantly reduced adhesion forces to dentin in both adhesive systems. The reduction was particularly high when the dye was used to stain the universal adhesive. It might be speculated that the cationic dye interacted with the negatively-charged hydrophilic head of the 10-MPD molecule that is present in both adhesive systems, hampering its adhesive capabilities to the dental substrates, especially when a hydrophilic substrate such as dentine was used. Also, the significant increase in pH values after CV addition to CU may have prevented a good conditioning of the smear layer on dentine surfaces, contrarily to CSE system where CV addition did not alter pH values of the primer in a clinically significant way. In this sense, pH measurements can provide an explanation for the dramatic decrease in adhesion forces to dentine seen when CV was additioned to CU. It has to be noted that all standardized class II restorations performed in this study had cervical margins placed on enamel, where the microshear bond strength test showed no difference of CV addition on adhesion forces.

Shear bond strength testing with bonded cross-sectional areas of one mm^2 or less is considered as 'micro' SBS [32]. The surface area of the cylinder microbore tube was calculated as 0785 mm^2 which justifies the test appellation as microshear bond strength. The microshear bond strength test was performed instead of the microtensile bond strength test since it is most useful for substrates such as glass ionomers or enamel that are particularly vulnerable to the specimen preparation effects and test conditions of micro-TBS tests. In a recent review about micro strength test methods, μ-SBS test revealed better results than μ-TBS for low adhesive forces [16]. In fact, it was *a priori* believed that crystal violet addition to the adhesive system would have resulted in particularly low adhesive forces.

Results also demonstrated that the self-etch adhesive yielded significantly higher flexural strength values than the universal one. Interestingly, crystal violet addition significantly reduced the flexural strength of the self-etch adhesive, yet it did not have any significant effect on the flexural strength of the universal one. It may be assumed that the hydrophobic dye may have interacted with the hydrophobic bonding agent of the self-etch adhesive during polymerization, and these interactions would not have been expressed in the relatively hydrophilic composition of the universal adhesive. Ethanol solvent that is present in the latter may have also played a role in that interaction.

Viscosity measurements were performed to assess possible differences in handling of the CV-additioned systems, for instance during air-blow phases. Dental adhesives can be considered to behave as Newtonian fluids as far as no filler is added to their composition [18]. These measurements showed that CV addition reduced the dynamic viscosity of both the bonding agent in the self-etch adhesive system and the universal adhesive system. One may speculate that this phenomenon might have increased the spread of the adhesives over teeth surfaces, especially when adhesive procedures were performed before matrix positioning. Nevertheless, the primer of the self-etch adhesive system did not have its viscosity changed by CV addition, as it already shows a very low viscosity, even close to that of ethanol. Therefore, it can be assumed that its spreading by air-blow already reached its highest extent. Moreover, the adhesives themselves differed in viscosity, the universal one having a significantly lower viscosity compared to the bonding agent of the self-etch adhesive system. One would expect, therefore, to see the universal adhesive to spread a farther distance on teeth surfaces than the self-etch one. However, this phenomenon was not observed in the present study. Our findings are in keeping with the measurements performed by Faria-e-Silva et al. on the viscosity of the bonding resin of the same self-etch adhesive system tested in the present study [33]. In fact, few studies investigated changes in the viscosity of dental adhesive system, in spite of the informations on their handling that could be thus acquired.

According to a meta-analysis of da Rosa et al., selective enamel etching could be considered the best strategy for optimizing the bond strength of mild universal adhesives [17], which justifies the use of this technique in the present study. Furthermore, the centripetal incremental technique was used to perform the restorations. The ability of such a technique to improve the marginal seal has been confirmed by several laboratory-based studies [34,35]. Besides, Bonilla et al. found no significant difference between placement techniques and fracture resistance of Class II resin composite restorations [36], suggesting that the results of the present study might not have been influenced by the technique used for RBC placement.

Ernst et al. evaluated the marginal integrity of restorations where the adhesive system was applied before or after matrix positioning. They used a dye penetration method, and they found that total-etch adhesives generally achieved better marginal integrity than self-etch adhesives. Therefore, they suggested that all adhesive systems should be applied before matrix placement to reduce the influence of the application protocol on marginal integrity [12,13].

In this study, only two adhesives and one interproximal matrix system were tested. A further study replicating the present experimental design with the main types of adhesive systems and interproximal matrices currently available may provide further insight into the influence of this choice on the adhesive spread and marginal adaptation of the restoration. Future studies may also address the influence of the adhesive system distribution on microbial colonization and secondary caries occurrence. In this sense, the present results have to be proven clinically, even if the design of clinical studies possess several complications (number of patients needed, identification and exclusion of possible confounding factors, follow-up time). Moreover, since dental materials have an extremely high turnover, it would be unsurprising that, from a materials science point of view, such results might be outdated even before the end of the study.

5. Conclusion

The findings of this study, notwithstanding all limitations resulting from an in vitro setup, show that cervical adaptation of the matrix is fundamental. Both $M > A$ and $A > M$ generated adhesive placement disadvantages with adhesive materials being expressed in difficult to reach locations that may jeopardize complete adhesive polymerization. In this sense, any improvement in the polymerization process of the adhesive systems, or the use of adhesives containing antimicrobial compounds, might be seen as beneficial. In this study, it was shown that the adhesive systems were spread on tooth areas well beyond restoration borders when the adhesive was applied before matrix positioning or were embedded on the composite surface when they were applied after matrix positioning. To improve longevity, all cervical margins of RBC restorations should be carefully finished to remove an adhesive-rich layer and to improve marginal adaptation, no matter the clinical restorative protocol adopted. The addition of crystal volet dye to label the tested adhesives generally produced changes in the adhesives' behaviour without compromising their performances considerably, therefore its use may be helpful when performing morphological

observations of the adhesive layers.

Declaration of Competing Interest

None.

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