The purpose was to evaluate the effect that deviations from the recommended protocol of a two-step etch-and-rinse adhesive system has on permeability and nanoleakage. One hundred and twenty dentin disks were treated with a two-step etch-and-rinse adhesive system, according to the manufacturer's instructions, or using five simulated deviations from the recommended protocol: applying potassium oxalate, reducing the application time of the adhesive, avoiding adhesive drying, aggressively drying the adhesive, and double application of adhesive. Kruskal–Wallis and Tukey's post hoc comparisons were used to evaluate the permeability reduction ($\alpha=0.05$). Twelve additional dentin disks were prepared for transmission electron microscope (TEM) analysis of nanoleakage. Aggressive drying, adding additional layers of adhesive or using oxalate reduced dentin permeability and yielded a better infiltration of the hybrid layer, whereas reducing the application time or less drying the adhesive did not reduce dentin permeability, caused extensive nanoleakage, showing immediate compromised dentin sealing.

**Keywords**: Dental adhesives, Dentin bonding, Nanoleakage, Permeability, TEM
hypotheses are as follows: 1) no significant differences will be observed in dentin permeability reduction among the treatment groups and 2) no differences will be noted in the nanoleakage analysis among the treatment groups.

MATERIALS AND METHODS

Specimen preparation for permeability study

One hundred and twenty intact human third molar teeth, without restorations or macroscopic carious lesions, were used in this study. The teeth were gathered after obtaining informed consent under a protocol reviewed and approved by the Ethics Committee, College of Dentistry, Universidade de Lisboa (approval number: 201931). Before their preparation, the teeth were randomly selected from a group of teeth stored in 0.5% Chloramine T (Sigma Chemical, St Louis, MO, USA) at 4°C, according to the ISO TR 11405 standard developed by the International Organization for Standardization (ISO).

From each tooth, a coronary dentin disk having a thickness of 0.70 (±0.01) mm was obtained by a section parallel to the occlusal surface within 1–2 mm from the tip of the pulp horns (first cut) and then again about 1 mm more occlusally (second cut), using a diamond disk at low speed (IsoMet, Buehler, Lake Bluff, IL, USA) under plentiful water irrigation. The specimens’ thicknesses were measured with a digital micrometer (Digimatic Caliper Seriates 500, Mitutoyo America, Dawn, IL, USA) to obtain the thickness of 0.70 (±0.01) mm.

The pulpal surfaces of the specimens were etched with 32% phosphoric acid gel (Uni-etch, Bisco, Schaumburg, IL, USA) for 1 min to completely remove the smear layer and smear plugs created by the preparation of the dentin discs, opening up all the tubules and allowing the fluid to freely flow within the dentin tubules.

With the purpose of creating a uniform smear layer obtained in similar conditions to those occurring in the clinic, the occlusal surface of the dentin disks were grinded with 600 grit-silicon-carbide sandpaper (Carbimet Grit 600/P1200, Buehler) in a mechanical grinder (Ecomet 3, Buehler) for 60 s under water irrigation, in accordance with the ISO TR 11405 standard.

Dentin permeability (P) of each specimen was measured at two time points: 1) after the application and rinsing of the etchant (PB), serving as the baseline measurement, and 2) after the application and polymerization of the adhesive (PA).

The 120 specimens were randomly assigned to six groups, with each group consisting of 20 specimens. The order in which the specimens were treated was randomized to avoid possible bias due to any particular sequence of treatment. One-Step Plus (Bisco, Schaumburg, IL, USA) was used for all groups; the composition of the adhesive is described in Table 1.

| Material       | Manufacturer                  | Components                                                                 |
|----------------|-------------------------------|-----------------------------------------------------------------------------|
| Bisblock       | Bisco, Schaumburg, IL, USA    | Oxalic acid, Biphenyl dimethacrylate (BPDM), Photo-initiator,               |
|                |                               | 15–40 vol% 2-hydroxyethyl methacrylate (HEMA), 15–40 vol% bisphenol A glycerolate dimethacrylate (Bis-GMA), 40–70 vol% Acetone, 8.5 wt% Fluoraluminosilicate, glass fillers (1 µm) |
| One-Step Plus  | Bisco                         |                                                                             |

1. Experimental groups

(1) Group 1: Application according to manufacturer’s instructions (Per mfr) —Control group

The occlusal surface of the dentin was conditioned with 32% phosphoric acid gel (Uni-etch) for 15 s. The disk was rinsed with an air/water spray for 10 s, and the excess water was removed in the end using a moist cotton pellet. The bottle of the primer/adhesive was shaken for 3–5 s after the impact of the mixture element was audible in its interior. The primer/adhesive was applied with a brush in two consecutive layers, with scrubbing for 15 s. The solvent was then evaporated by gently but thoroughly air drying for at least 10 s, beginning with a gentle stream initially and increasing to a fairly strong stream of air, leaving the surface shiny.

Finally, the primer/adhesive was polymerized for 10 s, with a light intensity of 600 mW/cm² (QTH, VIP JR, Bisco). The output of the curing light was periodically verified at 600 mW/cm² with a radiometer (Curing Radiometer P/N 10503, Kerr, Orange, CA, USA) throughout the study.

(2) Group 2: Potassium oxalate application (Oxalate appl)

After rinsing and drying the dentin, it was conditioned with phosphoric acid according to the manufacturer’s instructions and a solution of potassium oxalate (BisBlock, Bisco) was applied for 30 s. The dentin surface was abundantly rinsed and then dried using a moist cotton pellet so that the surface stayed shiny and visibly moist. The subsequent steps of the adhesive technique were executed in accordance with the manufacturer’s instructions.

(3) Group 3: Reduced application time of the adhesive (Reduced appl time)

After rinsing and drying the dentin, it was conditioned with phosphoric acid according to the manufacturer’s instructions, and the primer/adhesive was applied
in a single layer, with scrubbing for 5 s and then immediately starting drying. All the subsequent steps of the adhesive technique were executed in agreement with the manufacturer’s instructions.

(4) Group 4: No drying of the adhesive (No drying)
All the adhesive technique steps were executed in agreement with the manufacturer’s instructions. However, the primer/adhesive was not dried; that is, the solvent was not evaporated, the primer/adhesive excess was removed with a paintbrush (Bisco Brush applicator, Bisco) prior to light polymerization.

(5) Group 5: Aggressive drying of the adhesive (Agg drying)
All the adhesive technique steps were executed in agreement with the manufacturer’s instructions except for the drying of the primer/adhesive, which was aggressively dried for 15 s with maximum pressure of the air syringe 1 cm from the dentin surface.

(6) Group 6: Double application of adhesive (Double appl)
After executing all the adhesive technique steps according to the manufacturer’s instructions, the primer/adhesive was reapplied with a brush in two more consecutive layers, with scrubbing of each layer for 15 s. The solvent was evaporated again beginning with a soft blow of air, leaving the surface shiny, and, finally, the primer/adhesive was again polymerized for 10 s, with a light of intensity of 600 mW cm\(^{-2}\) (VIP JR, Bisco).

Dentin permeability measurements
For the measurement of dentin permeability (P), a Pashley apparatus (Kenward Industries, North Augusta, SC, USA) was used with a saline solution (PBS, Gibco, Grand Island, NY, USA), as described by Outhwaite et al.\(^{26}\) and Pashley\(^{27}\) (Fig. 1), with 37 cm H\(_2\)O hydraulic pressure, which is close to the normal pulpal pressure\(^{6,28}\). The dentin disks were placed between two “O” rings in opposition on each side of the specimen surface and then tightly closed inside the Pashley apparatus.

Before the exposure of the occlusal dentin, the absence of fluid conductance was confirmed by separately attaching five intact crown segments to the testing apparatus (as described above) and observing the (absence of) fluid movement for 2 h\(^{28,29}\). To avoid any interference with the effectiveness of the adhesive system, the pressure was interrupted during the application of the adhesive system\(^{6,19}\). The progression of the air bubble was measured every 2 min over a 6-min interval to determine the rate of saline solution flow in millimeters per minute.

Calculations to determine dentin permeability
The dentin permeability (P) of each specimen was measured at two time points: 1) after conditioning with the etchant (PB), serving as the baseline measurement and 2) after the application and polymerization of the adhesive (PA). These two measurements were used to calculate, as a ratio, the reduction in dentin permeability. The initial value of dentin permeability, measured after the conditioning by the acid (PB) (baseline), was assigned a value of 100%. Considering that permeability was determined for each specimen before the application of the adhesive system and it was applied with the specimen placed in the Pashley apparatus, the reduction in dentin permeability after the polymerization of the adhesive (PA) was expressed as a percentage of this maximum value \([100 – (PA/PB \times 100)] \). Thus, each specimen served as its own control.

Statistical analysis
Sample size calculations were performed using the G*Power Program Statistical Analysis\(^{30,31}\), with \(\alpha=0.05\), a desired power of 80%, and data from the pilot study.

The statistical analysis was performed using a rank-based Kruskal–Wallis procedure. Pairwise comparisons among the six groups were made according to the method given by Conover\(^{32}\), using Tukey adjustment for multiple comparisons in conjunction with an overall 5% level of Type I error. The homogeneity of variances was assessed using the method used by Levene\(^{33}\).

Specimen preparation for nanoleakage study
Twelve additional 0.7-mm-thick dentin disks were prepared similar to those for the permeability study. Two dentin disks were used for each of the six adhesive groups, and the adhesive was applied as in the permeability study. After storage in distilled water at 37°C for 24 h, three slabs approximately 0.5 mm thick were sectioned perpendicular to the plain surface using a diamond copper saw (Isomet 1000, Buehler) under plentiful irrigation. The slabs were immersed in an ammoniacal silver nitrate solution in the dark for 24 h, without allowing them to dehydrate, and prepared for nanoleakage\(^{5,34}\). Ninety nanometer-thick epoxy resin-embedded sections were prepared and examined using a transmission electron microscope (TEM; Zeiss EM10,
RESULTS

Dentin permeability
The data on percent permeability reduction are summarized in Table 2 by adhesive types and application modes as mean and standard deviation (SD); the median, range, minimum and maximum, are also presented.

The results showed that the percentage distribution of permeability reduction was different among the six experimental groups ($p<0.0001$). The highest permeability reduction was observed in the double-adhesive application group (88.85±4.59) and the lowest was observed in the no adhesive drying (54.95±20.60) group (Fig. 2).

The distribution of the percentage of permeability reduction did not statistically differ among the double application, oxalate application, and aggressive drying groups ($p=0.21$).

In addition, the data provided evidence that the percentage of permeability reduction in the highest group (that associated with double application) was significantly greater than that obtained by following the manufacturer’s instructions ($p=0.0014$). There was also significant difference between the manufacturer’s instruction group and the oxalate application group ($p=0.0073$), with higher permeability reduction in the oxalate application group.

Nanoleakage
The representative micrographs in this study are shown in Figs. 3–8. At high magnification for all groups and specimens, evidence of spotted silver deposits (black arrows) that are randomly distributed through the entire thickness of the hybrid layer can be seen. The spotted type consisted of isolated spots of silver grains that were observed in varying degrees of intensity within the

| Group                        | $n$ | Mean  | SD   | Median | Minimum | Maximum | Range |
|------------------------------|-----|-------|------|--------|---------|---------|-------|
| Double application (Appl)    | 20  | 88.85 | 4.59 | 88.5   | 80      | 97      | 17    |
| Oxalate appl                 | 20  | 86.70 | 5.57 | 86.5   | 75      | 97      | 22    |
| Aggressive (Agg) drying      | 20  | 83.7  | 7.15 | 83.5   | 67      | 95      | 28    |
| Per manufacturer (mfr)       | 20  | 80.3  | 9.94 | 83     | 62      | 95      | 33    |
| Reduced appl time            | 20  | 72.25 | 12.63| 75.5   | 37      | 86      | 49    |
| No drying                    | 20  | 54.95 | 20.60| 57.5   | 20      | 85      | 65    |

Fig. 2  Box-and-whisker plots of percentage permeability reduction (in descending order by median, with Tukey groupings indicated graphically by the four colored rectangles. Groups within the same rectangle are not significantly different).

Fig. 3  TEM micrograph of representative area of unstained, undemineralized, silver-impregnated sections of the manufacturer’s instructions group. C: resin composite layer, A: adhesive layer, H: hybrid layer, D: mineralized dentin. 5,000×.
hybrid layers and in the adhesive layers.

In the three groups comprising the manufacturer's instructions (Fig. 3), reduced application time (Fig. 4), and no drying (Fig. 5) groups, the reticular nanoleakage pattern (gray arrows) was also observed within the resin-dentin interfaces. The reticular type consisted of discontinuous islands of silver deposits exclusively observed in the hybrid layers or in the hybridized areas.
Fig. 8 TEM micrograph of representative area of unstained, undemineralized, silver-impregnated sections of the aggressive drying of adhesive group. C: resin composite layer, H: hybrid layer, D: mineralized dentin, T: dentin tubule. 3,150×

of the resin tags. In addition, in the no-drying group, a layer of dendritic silver-impregnated water channels known as “water trees” was observed. In the double application (Fig. 6), oxalate application (Fig. 7), and aggressive drying groups (Fig. 8), no reticular type of nanoleakage was noted in the complete extension of the resin-dentin interface or in the adhesive layer.

The manufacturer’s instructions group exhibited minimal but distinctive nanoleakage throughout the hybrid layer, and reticular silver deposits could be identified along the hybrid layer only sporadically. In high resolution (black arrows, Fig. 3), a spotted type of nanoleakage was observed in the hybrid layer, which was slightly more intense in the top and bottom parts of this layer.

Significant nanoleakage was observed in the reduced application time (Fig. 4) group, including both reticular and spotted types of nanoleakage. The hybrid layer consistently showed the presence of silver deposits. Some tubules showed silver deposition between the resin tags and the tubule walls.

The no-drying (Fig. 5) group showed the highest quantity of nanoleakage of all the groups. The severity of nanoleakage in this group was so high that the reticular silver deposits not only occurred continuously along the entire length of the hybrid layer but also extended to its entire thickness. In addition, the extensive silver deposits were also observed within the dentin tubules. The special water channels called “water trees” mentioned above could be seen arising from the surface of the hybrid layer and extending into the adhesive layer.

DISCUSSION

Studies of the permeability characteristics of dentin and their interactions with dentin adhesive systems are of considerable physiopathological and clinical interest because they may help explain some of the reasons for restorative failure and postoperative dentin sensitivity.

The permeability characteristics of the resin-dentin interface, or, more specifically, the hybrid layer or resin-dentin interdiffusion zone, can be separately studied by quantitative or qualitative methods. In this study, dentin permeability was studied quantitatively, using the method described by Outhwaite et al. and Pashley, while nanoleakage was studied qualitatively, using transmission electron microscopy.

For these types of studies, the applied pressure mentioned in the literature has varied greatly. In this study, a low hydraulic pressure (37 cm H₂O) was used to avoid any disturbance of the intratubular content, the resin tags, or the hybrid layer. The use of this hydraulic pressure should result in hydraulic conductance measurements comparable to reality (14 cm H₂O).

The dentin permeability reduction levels achieved among the six different groups were extremely different, with larger-than-expected range of values of dentin permeability reduction for the same adhesive system, where the only difference among the groups was a deviation from a single step of the application protocol. Furthermore, the groups with lower dentin permeability reduction values are also the ones with higher variability and standard deviations. This may denote that the adhesive and its application protocol performance are more prone to be influenced by factors otherwise less relevant, when a deleterious deviation is made on that adhesive protocol. Those factors may be the degree of mineralization of the substrate, the number and size of the dentine tubules or the simulated pulpal pressure. For instance, an alteration in the degree of surface moisture caused by desiccation or blotting yielded different dentin permeability reduction results (Agg drying: 83.7%; Per mfr: 80.3%; No drying: 54.95%).

The composition of adhesive systems and solvent type also affect their behavior. Ethanol/water based systems are less moisture sensitive and good at re-expanding collagen matrix, producing higher bond strengths in dried dentin. Acetone-based systems, such as One-Step Plus, evaporate much more residual water than ethanol/water-based systems; nevertheless, they are more sensitive to air drying as they cannot re-expand the shrunken collagen fibrils. Adoption of the same bonding protocol for different adhesives with different volatilities and capability of water displacement may result in undesirable consequences.

The results suggest that the bonding efficacy of the two-step etch-and-rinse adhesives is strongly affected by all the operator-dependent variables studied.

In the manufacturer’s instructions group, as in
other studies\textsuperscript{4,24,39}, the results were not the best among all the experimental groups. This may suggest that the adhesive system used in this study has the potential for improvement, especially in terms of the instructions for use. As suggested by some authors, changing the application protocol of some adhesives may sometimes improve their bonding capacity\textsuperscript{40,41}.

In this study, longer and more aggressive air drying for the uncured adhesives, which may improve the evaporation of the adhesive solvents and water derived from the dentinal fluids, yielded results that are among the best of the experimental groups (Agg drying: 83.7\%). These results agree with those reported by Barkmeier and Erickson\textsuperscript{42}, who found that severe air drying of the primer only slightly reduced the shear bond strength. Moreover, Frankenberger et al.\textsuperscript{9} showed that drying the primer for 60 s yielded higher bond strengths than other deviations. Furthermore, Hiraishi et al.\textsuperscript{43} found that it is not possible to completely eliminate the presence of water from the hybrid layer even by aggressively air drying the primed dentin surface, allowing nanoleakage to occur along the interface. Thus, bonding to dentin is definitely technique dependent\textsuperscript{44}. To obtain the most reliable clinical results, the manufacturers’ instructions should meticulously describe how to dry the remaining primer/adhesive. Although it is known that clinicians mostly deviate from the protocol to save time\textsuperscript{15}, it should be emphasized that if one has to make a deviation from the recommended protocol, thorough air drying is preferable to leaving any water/solvent remains on the primed dentin before polymerization.

Some studies\textsuperscript{45,46} have shown that longer application time enhanced solvent evaporation in simplified adhesives, resulting in better integrity of the adhesive layer. However, in this study, it was decided not to include a longer application time group because a change in the protocol that increases the adhesive application time is not likely to happen clinically in daily practice because clinicians want to save application time.

In the oxalate application group (Oxalate appl: 86.7\%), the results did not show a statistically significant difference in the dentin permeability reductions between this group and the manufacturer’s instructions group. These results matched well with those reported in other studies\textsuperscript{24,47} and may signify that the use of oxalate—a deviation from the recommended protocol—resulted in a less “technique sensitive” application for this adhesive. In other words, more consistent results with this approach can be expected, suggesting that it may be an improvement in the adhesive technique. Nevertheless, the adhesive used in the study had low levels of fluoride (70 ppm) and was not very acidic (pH=4.6), and these factors may have contributed to the good results because it has been reported\textsuperscript{24,48} that the bond strength and the hydraulic conductance reduction capacity of the simplified adhesives for oxalate-treated, acid-etched dentin may be compromised by the fluoride content and acidity of some etch-and-rinse adhesives. Oxalate desensitizers are effective in reducing the hydraulic conductance of dentin with exposed tubules because they react with calcium ions on dentin and in dentinal fluid to form sparingly soluble calcium oxalate crystals\textsuperscript{49}. Thus, the reduction of dentin permeability is achieved via subsurface tubular occlusion, which should not interfere with the subsequent resin infiltration\textsuperscript{21}. However, the solubility of calcium oxalate is affected by pH because the anion is the conjugate base of a weak acid. The low pH values of some adhesives may increase the solubility of calcium oxalates in the dentinal tubules and may negatively interfere with adhesion\textsuperscript{40}. More long term studies are needed to confirm these observations.

The results obtained in the double-application group (Double appl: 88.85\%) were the best among all the experimental groups, with high dentin permeability reduction and minor nanoleakage mainly of the spotted type. These results are in agreement with several studies that demonstrated that multiple consecutive coats\textsuperscript{50,51} increased the resin-dentin bond strengths for etch-and-rinse adhesives. It appears that when each layer is light cured, the adhesive layer becomes thicker without changing the quality of the hybrid layer and that the increase in bond strength is mainly due to the improved stress distribution via the increased elasticity of the thicker adhesive layer\textsuperscript{50}. The adhesive layer is thicker and more hydrophobic in the double-application group. A hydrophobic adhesive layer has better mechanical properties, and it is thought to better tolerate degradation factors and fatigue stress\textsuperscript{51}. The manufacturers of One-Step Plus recommend this optional double application protocol as an option for Class V restorations but, according to the results of this study, it should be investigated whether it will not be beneficial to use double application in all types of restorations.

In the reduced application time group, the resins did not fully infiltrate the demineralized dentin allowing the existence of interfibrillar spaces filled with water rather than resin, which could be observed by the presence of both reticular and spotted patterns of nanoleakage. The results of this study are in agreement with other studies\textsuperscript{6,9,10} wherein it was seen that whenever the infiltration time of the primer was reduced, the primer was not able to serve its function, proving that primer application is a time-dependent process. Therefore, according to the results of this study, it is recommended to carefully follow the manufacturer’s instructions regarding the application time of the adhesive system, which must be applied for at least 10 s.

Among all the experimental groups, the worst results of dentin permeability reduction were obtained in the no drying group (No drying: 54.95\%). The nanoleakage results are consistent with the results of dentin permeability where extensive nanoleakage could be observed. The nanoleakage was so extensive that the silver deposits impregnated the entire thickness of the hybrid layer and inside the dentin tubules, precluding the dentin tubules from sealing and explaining why the dentin permeability reduction results were so poor. In fact, other studies\textsuperscript{4,52,53} have demonstrated that it is not possible to properly seal a resin-dentin interface without the careful and complete evaporation of the
water/solvents before the polymerization of the resins. The results of this study suggest that not drying the primer/adhesive-impregnated dentin surface before polymerizing is probably the worst bonding mistake that a clinician can make when applying the etch-and-rinse system. It creates additional channels for rapid water movement, which may enhance water sorption within the hydrophilic adhesive layer. This may result in rapid deterioration of the mechanical properties of the resin-dentin bonds and make the resin-dentin interface readily susceptible to long-term bond degradation.

Future research should clarify the effect of other mistakes related to the clinical application of an adhesive system, apart from the ones tested in this study. Furthermore, the effect of isolated mistakes and different combinations of two or more adhesive application mistakes and their interactions should also be examined.

Finally, further studies should be conducted to better understand the impact of the adhesive application mistakes on the hydrolytic degradation rates of the resin-dentin bond over time and the challenge that this degradation causes to the longevity of the resin-bonded restorations.

CONCLUSION

The results of this study require the rejection of the null hypotheses. The deviations in the adhesive protocol that turned the hybrid layer into a more hydrophobic zone —whether by aggressively drying the adhesive, applying an additional layer of adhesive, or blocking the water contamination with oxalate— showed immediate improvement in terms of the dentin permeability reduction and less nanoleakage, resulting in a less technique-sensitive application protocol for this adhesive. However, the deviations that increased the wetness of the hybrid layer and/or decreased the application time immediately compromised the sealing of the dentin and caused extensive nanoleakage.

The inadequate clinical application of the total-etch, two step adhesive system creates imperfect dentin tubule sealing, which may explain clinical reports of postoperative sensitivity associated with adhesive restorations.

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