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 A laboratory study using a split-tooth design to analyze the micromorphology of the resin-dentin interface when combining the internal/external resin infiltration of proximal caries lesions and the internal tunnel technique V.1

Prof Dr med dent Dr h c Andrej M. Kielbassa¹, Sabrina Summer²,
Julia_batzer³

¹Center for Operative Dentistry, Periodontology, and Endodontology, Department of Dentistry, University School of Medicine and Dentistry, Danube Private University (DPU), Krems, Austria;

²University for Continuing Education, Department for Biomedical Research, Center for Experimental Medicine, University Krems, Krems, Austria;

³Department of Dentistry, University School of Medicine and Dentistry, Danube Private University (DPU), Krems, Austria



Julia_batzer

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ABSTRACT

Introduction

Previously, both the development and the introduction of the resin infiltration technique¹⁻³ have resulted in a non-invasive treatment regimen of proximal enamel caries, aimed to close the gap between preventive and restorative dentistry, and, thus, obviating any sacrifice of dental tissues, and improving oral health⁴. In case the low-viscous resin used with this treatment regimen has been applied on de-proteinized enamel lesions⁵, through capillary forces, major parts of the porous volume of these lesions will be occluded, thereby building a 3D network¹ consisting of the infiltrant resin enwrapping the demineralized enamel remnants, re-hardening and stabilizing the lesion⁶, and paralyzing any progress of the latter. Meanwhile, several independent meta-analyses with acceptable quality⁷⁻¹² (including an umbrella review¹³) have consistently revealed that resin infiltration of enamel lesions will be effective in reducing further clinical increase of white-spot lesions in both dentitions, thus providing prompt and acceptable aesthetic outcomes⁴. However, this refers to lesions restricted to enamel, and it should be highlighted that lesions extending beyond the dentin-enamel junction have never been assessed with the initial developments^{1,2}.

Not astonishingly, some studies have revealed a poor outcome of the infiltration technique when used with treating proximal lesions extending into the outer third of the dentin, with a therapeutic efficacy of resin infiltration considered not significantly different from the controls¹⁰. Consequently, radiographic lesions extending to outer dentin lesions do constitute either a contra-indication of the resin infiltration technique, or would call for a modified treatment regimen. The latter has been introduced recently^{14,15}, and combines the modified (internal) tunnel technique with both internal and external resin infiltration of the carious enamel lesion. By means of an occlusal approach, the dentin caries can be removed, and the enamel caries can be infiltrated both from inside the cavity and from the proximal site, thus obstructing the porous enamel lesion areas by a doubled infiltration through capillary forces. This leads to a stabilization of the weakened proximal enamel^{14,15} and should result in increased success rates. When reflecting on the final restoration of the occlusal access cavity, an adhesively bonded filling would seem recommendable.

However, up to now, no information regarding the morphology of the bonding interface between dentin, dentin bonding agent, caries infiltrant, and resinous restoration is available from the literature. Consequently, the aim of the present laboratory study was to analyse various aspects of the resin-dentin interface after the use of a resin infiltrant (Icon Infiltrant; DMG, Hamburg, Germany) when applied in combination with two commercially available etch-and-rinse adhesive systems (Syntac [Ivoclar Vivadent, Schaan, Liechtenstein] and Adper Scotchbond Multi-Purpose

[3M/ESPE, St. Paul, MN, USA]). Primary aim was to study hybrid layer formation, and our null hypothesis stated that neither the thickness of the hybrid layer nor the homogeneity of the latter would differ between test and control groups when evaluated by means of scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM), respectively. Secondary aims pertained to aspects concerning both adhesive layer thickness and resin tag formation.

MATERIALS

Tables**Table 1** Composition of the caries infiltrant resin and the adhesive systems (information as given by the manufacturers with the respective safety data sheets).

A	B	C
Product	Components [specifications refer to percentages by weight]	Manufacturer
Syntac(unfilled etch-and-rinse adhesive)		Ivoclar Vivadent, Schaan, Liechtenstein
<i>Primer</i>	Triethylene glycol dimethacrylate (TEGDMA) [10 to <20%] Poly(ethylene glycol) dimethacrylate (PEGDMA) [3 to <7%] Maleic acid [3 to 5%] Acetone [30 to <50%] Water	
<i>Adhesive</i>	Poly(ethylene glycol) dimethacrylate (PEGDMA) [25 to 50%] Glutaraldehyde [3 to <10%] Water	
<i>Heliobond</i>	Bisphenol-A diglycidyl methacrylate (Bis-GMA) [50 to 100%] Triethylene glycol dimethacrylate (TEGDMA) [25 to 50%] Stabilizers Camphorquinone	
Adper Scotchbond Multi-Purpose(unfilled etch-and-rinse adhesive)		3M/ESPE, St. Paul, MN, USA
<i>Primer</i>	2-hydroxyethyl methacrylate (HEMA) [35 to 45%], Copolymer of itaconic and acrylic acids [10 to 20%], Water	
<i>Adhesive</i>	Bisphenol-A diglycidyl ether methacrylate (BisGMA) [60 to 70%], 2-hydroxyethyl methacrylate (HEMA) [30 to 40%], Ethyl 4-dimethyl aminobenzoate (EDMAB) [<0.5%], Triphenylantimony [<0.5%], Triphenylphosphine [<0.2%], Hydroquinone [<0.5%]	
Icon-Infiltrant(caries infiltrant resin)	Triethylene glycol dimethacrylate-based resin matrix (TEGDMA), Initiators, Additives	DMG, Hamburg, Germany

Table 2 Adhesive working steps with the various experimental groups.

	A	B	C	D
		Dentin conditioning	Priming/wetting procedure	Bonding procedure
Group 1		37% phosphoric acid gel (60 s) 99% ethanol Icon Dry (60 s)	Syntac Primer (15 s). Syntac Adhesive (10 s)	Hellobond(3 min) Final air-drying (20 s)
Group 2		37% phosphoric acid gel (60 s) 99% ethanol Icon Dry (60 s)	Syntac Primer(15 s) Syntac Adhesive (10 s)	Icon Infiltrant (3 min). Hellobond (20 s). Final air-drying (20 s)
Group 3		37% phosphoric acid gel (60 s) 99% ethanol Icon Dry (60 s)	Scotchbond MP Primer (15 s)	Scotchbond MP Adhesive (3 min). Final air-drying (20 s)
Group 4		37% phosphoric acid gel (60 s) 99% ethanol Icon Dry (60 s)	Scotchbond MP Primer (15 s)	Icon Infiltrant (3 min). Scotchbond MP Adhesive (20 s) Final air-drying (20 s)

Methods

1 Ethical approval, sample size calculation, and tooth selection

With the current report, we will adhere to the modified CONSORT guidelines for reporting pre-clinical studies on dental materials^{15,16}. This study protocol was approved by the Institutional Ethical Review Board of the Danube Private University (DPU) in Krems, Austria (vote number: DPU-EK/025) date of approval: 23 March 2023. Twenty freshly extracted maxillary and mandibular human third molars (numbered consecutively from 1 to 20), without caries, crack, restoration, root canal treatment, and without any other pathological changes (e. g., hypomineralization, fluorosis, and/or erosion), which have at least partially erupted into the oral cavity and show a more or less completed root growth, will be obtained from 20 patients (age range 16–25 years) who will give their written informed consent following a verbal information. Subsequently, the patients' teeth will be included in the present research project. In case of patients below the legal age, a parent or legal guardian will sign the consent form.

With the present study, the well-accepted multi-bottle etch-and-rinse adhesive systems Syntac (Ivoclar Vivadent) and Adper Scotchbond Multi-Purpose (3M/ESPE; from here on called Scotchbond MP), respectively, will serve as positive controls; with these conventional adhesive systems, reliable data are available for the analysis of the micromorphology of the water-wet bonded resin-dentin interface created

on healthy dentin^{17,18}. However, the proprietary adhesive components of these systems (Heliobond [Ivoclar Vivadent] or Scotchbond MP Adhesive [3M/ESPE]) have not yet been supplemented by the resinous caries infiltrant (Icon-Infiltrant; DMG) up to now, and, thus, a sample size calculation will not be possible. Consequently, the estimation of the number of samples and their preparation followed the “Academy of Dental Materials Guidance” on *in vitro* testing of dental composite bonding effectiveness¹⁹. This guideline has recommended 10 teeth per group to study tensile bond strength; to ensure a reliable result with the present study focusing on the resin-dentin interface, the number of samples included per group will be doubled.

Following careful cleaning from all residual adherent external natural deposits (e. g., calculus, biofilm, and/or soft tissues) with a water-cooled dental ultrasonic scaler (Teneo; Dentsply Sirona, Bensheim, Germany), the teeth will be stored immediately in physiological saline (0.9% NaCl solution; Gottlöber Pharmacy, Sandersdorf, Germany) by using hermetically sealed boxes (Labordose; Bürkle, Bad Bellingen, Germany) at room temperature until usage. In accordance with the previously published guideline¹⁹ the time between extraction and experimental procedures will be kept as short as possible (less than 1 month). For the implementation of the split-tooth design used in the present study, each of the four tooth quarters (mesial-buccal, distal-buccal, mesial-oral, and distal-oral, see below) of the 20 teeth ($n = 80$) will be assigned to one of the four bonding strategies to be tested, as described in detail below; thus, the tooth will be the statistical unit. All tooth preparations and the restorative procedures will be conducted using magnifying glasses (opt-on, 2.7× TTL, 350 mm; orangedental, Biberach, Germany).

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Preparation of the teeth

All cutting and preparation operations will be carried out under continuous water cooling. First, the crown of each tooth ($n = 20$) will be separated from the roots 2 mm below the cemento-enamel junction, and perpendicular to the long axis of the tooth by means of a thin double-sided diamond-coated cut-off wheel (#H355SF220, Superdiaflex SF, extra-fine, Ø 22.0 mm, thickness 0.22 mm; Horico Dental, Berlin, Germany), fixed in a straight handpiece (40,000 rpm, T1 Line H 40 L, transmission ratio [TR] 1:1; Dentsply Sirona), thus will be exposing the pulp chamber. The pulp tissue will be removed with a dental scaler (SM239E2 – #23 Gr #9; Hu-Friedy, Chicago, IL, USA).

Subsequently, with each tooth, an occlusal orientation groove of 1 mm depth will be prepared in the region of the deepest pit of the central fissure by using a cylindrical diamond-coated bur (#DM10.314.009, “PrepMarker”, 107 µm, axial cutting depth: 1 mm; Komet Austria, Salzburg, Austria), driven by a red contra-angle handpiece (160,000 rpm, T1 Line C 200 L, TR 1:5; Dentsply Sirona). An occlusal Class-I cavity, central to the marginal ridges, with a maximum dentin depth of 1 mm (proceeding from the deepest pit of the fissure system), and with a box-shaped extension to the maximum extent of the dental crown will be prepared with medium diamond-coated drills (#959KRD.314.018 and #845KRD.314.025, 107 µm; Komet Austria; 160,000 rpm, T1 Line C 200 L [red contra-angle handpiece], TR 1:5; Dentsply Sirona).

The exposed dentin surface at the cavity floor will be checked carefully, to ensure that the pulp chamber has not been opened during the preparation. Then, each dental crown will be sectioned (both in mesial-distal and in lingual-buccal direction (perpendicular to the occlusal tooth surface), thus dividing

the Class-I cavity and the underlying tooth substance into four more or less equally sized parts using a double-sided diamond-coated cut-off wheel (#H355SF220, Superdiaflex SF, extra-fine, Ø 22.0 mm, thickness: 0.22 mm; Horico Dental; 40,000 rpm, T1 Line H 40 L [straight handpiece], TR 1:1; Dentsply Sirona). The diamond-coated burs and the cut-off wheels will be replaced after every fifth tooth¹⁹.

3

Bonding procedures

The two quarters of each cavity assigned to Group 1 and Group 3 (control groups using Syntac or Scotchbond MP), respectively, were treated by means of a modified adhesive protocol (as given below). The other two quarters of each tooth were assigned to Group 2 and Group 4, respectively; with these experimental groups, a modified bonding process using the infiltration resin (Icon) was introduced. The ingredients of the two conventional adhesive systems as well as those of the resinous caries infiltrant are listed in Table 1 (information according to the respective manufacturers), while Table 2 provides a brief overview of the application procedures of the four bonding strategies used with the present set-up. Before starting the adhesive restoration of each tooth quarter, the dissected dentin surfaces were sealed (using a die/colour spacer stump varnish, 7 µm; Yeti Dental, Engen, Germany), thus avoiding any daubing, and allowing the penetration of the bonding agents into the dentin structures via the cavity surface only.

Prior to application, 2 drops of Syntac Adhesive (Group 1), were labelled with 10 µl of a green fluorescing agent (0.1 mmol fluorescein isothiocyanate [FITC]; Gottlöber Pharmacy), while 2 drops of Heliobond (Group 1) and Icon (Group 2), respectively, were marked with 10 µl of a red fluorescing agent (0.1 mmol rhodamine B isothiocyanate [RITC]; Gottlöber Pharmacy). For this reason, 10 µl of the respective fluorescing agent were pipetted (Pipette Research plus; and Pipette tips epT.I.P.S, 100 µl; Eppendorf, Hamburg, Germany) into the hollow of a small mixing palette (#2315, mixing palette; VOCO, Cuxhaven, Germany), and mixed together with Syntac Adhesive or Heliobond (and Icon, respectively). Similarly, 2 drops of Scotchbond MP Primer (Group 3) were labelled with 10 µl of FITC (0.1 mmol fluorescein isothiocyanate [FITC]) and 2 drops of Scotchbond MP Adhesive (Group 3) were remixed with 10 µl of RITC (0.1 mmol rhodamine B isothiocyanate [RITC]). In contrast, in Group 4, 2 drops of Icon were labelled with 10 µl of FITC (0.1 mmol fluorescein isothiocyanate [FITC]) and 2 drops of Scotchbond MP Adhesive were labelled with 10 µl of RITC (0.1 mmol rhodamine B isothiocyanate [RITC]). It was ensured that before mixing in the pigment, the alcohol content (96% ethanol) had completely evaporated and only the dry pigment was still present.

With reference to the split-tooth design ($n = 20$ teeth, with four groups per tooth; $n = 20$ specimens per experimental group), each of the four dentin-cavity quarters was adhesively restored after cavity preparation. After preparation and prior to restoration, the cavities were carefully rinsed and dried using a dental air/water sprayer (Sprayvit, Teneo, Dentsply Sirona; 30 s each). The dentin surfaces of the cavities (all 80 samples) were first etched with phosphoric acid gel (Total Etch Gel, 37%; Ivoclar Vivadent) for 60 s. The etchant was then washed off with water spray (Sprayvit) for 30 s, and the cavity was again thoroughly dried with compressed air (Sprayvit) for 15 s. It should be noted that, subsequently, an ethanol-wet bonding technique was used with both the control groups and the experimental groups. For this purpose, all cavities were additionally treated with pure ethanol (<100%, Icon Dry; DMG, Hamburg, Germany), and the latter was actively applied using a microbrush (Microbrush Plus, superfine white, Ø 1.0 mm; Microbrush International, Grafton, WI, USA) for 1 min. Excess ethanol was gently evaporated

with a stream of air (Sprayvit) for 10 s, and it was ensured that the surface was kept slightly wet and had a moist gloss.

Subsequently, by using a micro-brush, Syntac Primer (Tables 1 and 2) was actively rubbed onto the ethanol-wet dentinal surfaces of the specimens of Group 1 for 15 s, and excess Syntac Primer was dispersed and slightly dried for 5 s with a dental air/water syringe (Sprayvit), thus ensuring a slight excess of the primer. This was followed by the application of Syntac Adhesive (Tables 1 and 2) by means of a micro-brush (\varnothing 1.0 mm). Ten seconds after massaging in and mixing well, Syntac Adhesive was carefully blown with a compressed air stream (Sprayvit; 5 s), just until a shiny surface could still be determined. In the third step, Heliobond (Tables 1 and 2) was applied to the dentin surfaces and massaged in for 3 min using a micro-brush (\varnothing 1.0 mm), thus allowing the bonding agent to extensively penetrate the demineralised collagen. Subsequently, any excess was carefully dabbed off with a new micro-brush, which was wiped over the cavity floor without pressure, and air-blasting to evaporate any solvents from the adhesives was performed for 20 s (Sprayvit)²¹; light-curing (>1,250 mW/cm², Mini LED Curing Light; Satelec/Acteon Group, Mérignac, France) followed for 40 s²², while a 3 mm working distance between the light source and the cavity floor was ensured. The etched, rinsed, and dried (but ethanol-wet) dentin cavities of Group 2 were conditioned with the non-labelled Syntac Primer, followed by application of the FITC-labelled Syntac Adhesive as described above. In contrast to Group 1, the caries infiltration resin (Icon; see Tables 1 and 2) was used. By ensuring a penetration time of 3 min, the infiltration resin was massaged in (finally followed by active application of Heliobond for 20 s). Subsequently, air-drying followed for 20 s (Sprayvit), and all components were polymerized for 40 s (>1,250 mW/cm², 3 mm working distance, Mini LED Curing Light; Satelec).

With the specimens of Group 3, the etch-and-rinse procedure was first performed on the dentin cavities as described for Group 1. Subsequently, Scotchbond MP Primer (Tables 1 and 2) was applied to the ethanol-wet dentin surface for 15 s with a Microbrush Plus (\varnothing 1.0 mm) to create a glossy, moist dentin surface, followed by an air-driven distribution for 5 s (Sprayvit). Then, Scotchbond MP Adhesive (Tables 1 and 2) was actively massaged into the dentin surface for 3 min using a micro-brush (\varnothing 1.0 mm), air-blown to a thin layer (Sprayvit; 20 s) and light-cured with a 3 mm working distance for 40 s (>1,250 mW/cm², Mini LED Curing Light; Satelec).

In Group 4, the etched, rinsed, dried, and ethanol-wetted dentin cavities were conditioned with Scotchbond MP Primer as in Group 3 (15 s). Icon (Tables 1 and 2) was then applied to the dentin surface of the cavities and actively massaged in (3 min), but not light cured. Scotchbond MP Adhesive was then actively applied onto the dentin surface by means of a microbrush (\varnothing 1.0 mm) for 10 s, air-dried to a thin layer (Sprayvit; 20 s), and polymerized as described above (>1,250 mW/cm², 3 mm working distance, Mini LED Curing Light; Satelec).

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Restoration placement

Subsequently, each cavity quarter will be restored with a pure light-curing, high-viscosity, flowable composite restoration (shade P-A3, G-ænial Flo X; GC Europe, Leuven, Belgium). To avoid air bubbles in the restorative material and at the cavity margins, the composite resin will be carefully applied to both the cavity floor and the walls with the spherical end of a WHO probe (UNC15/11.5B; Hu-Friedy). The

restoration will be polymerized for 30 s ($>1,250\text{ mW/cm}^2$, Mini LED Curing Light; Satelec), and with a working distance of 3 mm between the light source and the restorative material. Until the microscopic analyses, the eighty restored tooth quarters will be placed in membrane boxes (Hager & Werken, Duisburg, Germany) containing cotton balls (cotton balls, size 0; Omnident, Rodgau, Germany) soaked in 0.9% saline (Gottlöber Pharmacy), to ensure that the specimens will be kept in a moist, but not wet, environment. Attention will be paid to further proceeding and microscopic examination of all restored specimens within 3 days.

5 *Specimen preparation for microscopy*

Prior to the microscopic examinations, the four adhesively restored segments (mesial-buccal, mesial-lingual, distal-lingual, and distal-buccal) of each dental crown ($n = 80$) will be cleaned with a small spatula (TNPF18A – #8A GR #6S XTS; Hu-Friedy) to remove the previously applied stump varnish (thus avoiding any contamination of the polishing wheel). The polishing of the resin-dentin composite surface will be performed with a thin diamond cutting disc (#H355SF220, Superdiaflex SF, extra-fine, Ø 22.0 mm, thickness 0.22 mm; Horico Dental) under constant water cooling (parallel to the surface to be microscopically assessed) for 30 s. The polishing disc will be fixed in a straight handpiece (T1 Line H 40 L, Dentsply Sirona). After polishing, the surfaces will be first etched with 15% hydrochloric acid (Icon Etch, HCl 15%; DMG, Hamburg, Germany) for 10 s, to visualise resin tag formation in the dentinal tubules; prior to the microscopic analysis, the acid (HCl) will be gently removed using a dental air/water sprayer for 10 s (Sprayvit, Teneo, Dentsply Sirona). This will be followed by both the SEM analysis and the CLSM analysis.

6 *Scanning electron microscopy (SEM) and image analysis*

For microscopic analysis, with each adhesively restored cavity quarter ($n = 80$), the resin-dentin interface morphology in the central part of the mesio-distal interface directly adjacent to the two cut surfaces was examined by means of a scanning electron microscope (SEM, FlexSem 1000 II; Hitachi High Technologies, Tokyo, Japan) working in low-vacuum mode at $650\times$ magnification at 20 kV. Prior to imaging, the samples were coated with gold at 30 mA for 15 s using a Q150T ES Plus sample sputter coater (Quorum Technologies, Laughton, UK). To ensure that the same areas were analysed with both SEM and CLSM, a small indentation was prepared in each sample; for this purpose, the diamond cut-off wheel was used to scratch vertically 2 mm from the end point of the specimen. Images were stored on a suitable storage medium (WD Elements Portable external hard disc; Western Digital, San José, CA, USA), and were analysed using an open-source image processing and analysis program (Image J 1.35 S software, National Institute of Health [NIH]; Bethesda, MD, USA). For accurate measurement of the thickness of the hybrid layer and adhesive layer, measurements at 3 different areas per image were performed (these were located 2 mm to the right and left of the image border and in the centre of the image, respectively). All data were collected in an Excel-file (Microsoft Excel; Microsoft, Redmond, WA, USA; available at: <https://office.microsoft.com/excel>) for statistical analysis. For the following CLSM examination, the sputtered gold was removed again with water. Examination of open dentin tubules and resin tags was limited to the presence of the tubules or whether tag formation had occurred.

7*Confocal laser scanning microscopy (CLSM) and image analysis*

In addition, the samples will be analysed by confocal laser scanning microscopy (CLSM, Leica TCS SP8 DMi8; Leica Microsystems, Wetzlar, Germany). For the CLSM analysis, the first and clearly visible layer of the CLSM tomogram will be selected just below the surface. The confocal micrographs will be recorded in a dual fluorescence mode referring to RITC and FITC, and using an Apochromat 63 \times oil immersion objective with a resolution of 2048 \times 2048 pixels by means of the LASX-software (Version 3.1.5-16308 (Leica Microsystems). The homogeneity of the hybrid layer and the adhesive layer will be visually evaluated. Here, with reference to the distribution of the fluorescence dyes, the homogeneity could be determined as either homogeneous (well mixed, complete commingling of red and green), heterogeneous (not well mixed, separate red and green areas), or inhomogeneous (not mixed, unevenly distributed).

Both SEM and CLSM images were analysed by one investigator at two-week intervals using Image J 1.35 S software (National Institute of Health [NIH], Bethesda, MD, USA) to control measurements (J.-S.B.). The operator had knowledge about the above-mentioned group assignment of the eighty tooth quarters, and the captured confocal micrographs were analysed regarding both thickness and homogeneity of the hybrid- and adhesive layers. In case of any differences between the first and the repeated measurements, a second evaluator was consulted (A.M.K.) to reach a consented value. The determined data were collected in an Excel file (Excel 2023; Microsoft) for statistical analysis.

8*Statistical analysis*

With the current study, we will used a complete block design, with the tooth as experimental unit, and with no replicates within a block. By using the permuted block randomization technique, each tooth quarter (mesial-buccal, mesial-lingual, distal-lingual, and distal-buccal) will be randomly assigned to one treatment strategy (Syntac, Icon/Syntac, Scotchbond MP, Icon/Scotchbond MP) within a block, thus eliminating any within-tooth heterogeneity of experimental units, and ensuring an equal frequency of the four bonding strategies and the respective tooth quarters.

Statistical analyses will be performed using the SPSS 28.0 statistical software package (IBM Analytics, Armonk, NY, USA). The thickness of both the hybrid layer and the adhesive layer will be statistically evaluated. For this purpose, the calculated mean values (\pm standard deviation) of the three-point measurements of all samples will be compared. The visual assessment of homogeneity will be determined by variables, and will be statistically compared as well (homogeneous = variable a, heterogeneous = variable b, inhomogeneous = variable c). This evaluation will be done by means of the chi-squared (χ^2) distribution test. With the current analysis, it should be emphasized that bioequivalence between the four groups will be tested; absence of a significant difference in the rate and extent of both the thickness of the hybrid layer and the adhesive layer of the latter at the site of action will be calculated between the test and the reference groups. For this purpose, and with reference to the analysis of the homogeneity, the modified t-test will be applied. The dentinal tubules will be only visually examined for presence; no statistical analysis followed with this outcome.

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