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Methacrylated epigallocatechin gallate functionalized dental adhesives: Antiproteolytic activity and dentin bonding studies

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ABSTRACT

Objectives: To assess the antiproteolytic effect of EGCG-methacrylate monomers and its inhibitory effect on gelatinolytic activity in the hybrid layer. Also, to investigate the effect of an adhesive material functionalized with EGCG-methacrylate monomers on immediate and long-term dentin-resin bond strength. Methods: Neat EGCG (E0) was reacted with three different ratios of methacryloyl ester and dissolved in ethyl acetate to obtain EGCG-methacrylates with hydroxyl functionalization at 33 % (M-E33), 67 % (M-E67) and 100 % (M-E100) levels. Resin composite blocks were built on human dentin surfaces using self-etching adhesive containing E0, M-E33, M-E67, and M-E100 at 1 wt%. Demineralized human dentin disks were immersed in deionized water (DW) or lactic acid (LA) and subsequently treated with DW, acetone (as controls), E0, M-E33, M-E67 and M-E100 diluted in acetone. Concentrations of solubilized type I collagen C-terminal (CTX and ICTP) and N-terminal (NTX) telopeptides were determined from 7-day extracts of dentin matrix specimens by ELISA assays. *In situ* zymography of adhesive-dentin interface slices from restored teeth was performed by confocal microscope after 24 h dentin treatment. Microtensile bond strength (μTBS) and failure pattern were evaluated after 24 h and 6 months. Data were analyzed using two-way ANOVA and Tukey *post hoc* test ($p < 0.05$). Results: All experimental groups statistically reduced the release of solubilized telopeptides from dentin samples in DW and LA. E0 and M-E100 incorporated into the adhesive system reduced the gelatinolytic activity within the hybrid layer. The lowest μTBS values for restored teeth were observed for E0 and M-E100 groups, after 24 h and 6 months, respectively. The most prevalent failure observed was classified as type 4, except for M-E100. Significance: EGCG-methacrylate monomers effectively protected collagen from degradation. When incorporated into adhesive systems, EGCG-methacrylates reduced gelatinolytic activity within the hybrid layer, and did not affect immediate and long-term bond strength values of restorations.

1. Introduction

The popularity of resin-based restorative materials with inorganic fillers has steadily increased since their initial development in the seventies. This is primarily due to the benefits of minimally invasive dental treatment, advancements in resin/adhesive composition and restorative techniques, as well as the demand for aesthetics [1].

During restorative procedure, the process of acid etching performed before or simultaneously with the application of an adhesive system, is critical to facilitate resin infiltration into dentin and promote bonding between the tooth and resin composite [2]. Regardless of whether one employs etch-and-rinse or self-etch bonding agents, both acidic solutions and acidic monomers interact with dentin mineral content, either by removing it or altering its composition. The resulting exposed dentin matrix is then infused with adhesive monomers to create the hybrid layer [3]. Therefore, for the adhesive-dentin interface to remain durable, both polymer and mineral-depleted dentin collagen must remain stable. Over time, their degradation can weaken adhesion, leading to the

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formation of gaps between the tooth and the restoration [2]. In resin composite restorations, the marginal areas, particularly the gingival margins [4], are most susceptible to degradation due to additional factors like polymerization shrinkage, marginal leakage, and inadequate bonding. These issues can lead to secondary caries, staining, and sensitivity, often necessitating clinical intervention.

Cavity wall lesions and/or defective margins promote bacterial infiltration towards the hybrid layer which causes monomer hydrolytic degradation and collagen degradation by bacterial proteases and acids [5]. The low pH originated from bacterial biofilm at defective margins of restorations or etching during bonding procedure, also activates latent endogenous dentin proteolytic enzymes, such as matrix metalloproteinases (MMPs) and cysteine cathepsins (CTs) [6,7]. MMPs, which include collagenases, gelatinases, and stromelysins, degrade native collagen at specific sites. This degradation results in the production of a 3/4 N-terminal fragment and a 1/4C-terminal fragment of the collagen molecule [8], that can be effectively quantified *in situ* using ICTP (cross-linked carboxyterminal telopeptide of type I collagen) ELISA test [9]. Type I collagen can also be cleaved at the C-terminal and N-terminal sites by CT-K releasing CTX (C-terminal telopeptide of type I collagen) and NTX (N-terminal telopeptide of type I collagen) telopeptides [10,11], also quantifiable by ELISA. The involvement of MMPs and CTs in collagen degradation within hybrid layers has been thoroughly described in the literature [12–16].

Numerous antiproteolytic therapies, including chlorhexidine [17] and dimethyl sulfoxide [18], have been evaluated to minimize the degradation of resin-dentin bonding and enhance the overall effectiveness and durability of composite restorations. *In vitro* and *in vivo* studies have shown that both chlorhexidine [12,17] and dimethyl sulfoxide [18] can improve durability of resin composite restorations by inhibiting endogenous proteases such as MMPs and cysteine cathepsins. However, several studies [19,20] have demonstrated and raised concerns about their cytotoxicity, which underscores the need to explore natural and less cytotoxic alternatives to ensure safer and more effective dental treatments.

Epigallocatechin-3-gallate (EGCG), a natural polyphenol catechin extracted from green tea, is being studied in Dentistry for its low cytotoxicity, antioxidant, antimicrobial, and anti-inflammatory properties [21,22]. Moreover, EGCG also has the ability to crosslink with collagen fibrils and inhibit collagenolytic and gelatinolytic activities [23]. Pre-treatment of dentin with EGCG solutions and passive incorporation of EGCG into dental copolymers have been effective in improving bond strength and maintaining original biological properties, such as antimicrobial activity [24–26]. However, aqueous saturation of polymerized material leads to hydration of water-soluble compounds like EGCG and its diffusion mediated release [27]. This depletes and limits sustainability of the beneficial biological properties of materials passively incorporating EGCG.

EGCG-methacrylate monomers have been synthesized and shown to covalently attach to methacrylate-based resins such as triethylene glycol dimethacrylate (TEGDMA). EGCG methacrylation was shown not to affect the inherited antibacterial activity, and interfered only at very high ratios with the mechanical properties of the new materials [28]. This study evaluated for the first time, the antiproteolytic activity of EGCG-methacrylates and EGCG-methacrylate-based adhesives, as well as the effect on immediate and long-term dentin-resin bond strength. The hypotheses of this study are: 1) EGCG-methacrylate monomers will decrease collagen degradation and preserve the hybrid layer by inhibiting dentin proteolytic enzymes; 2) EGCG-methacrylate-based adhesive systems will preserve immediate and long-term dentin bond strength values.

2. Materials and methods

Unless otherwise specified, chemicals were purchased from Sigma-Aldrich (Mississauga, ON, Canada), and reaction solvents from Fisher Scientific (Ottawa, ON, Canada). This study was approved by local Research Ethics Board committee (CAAE: # 54462721.0.0000.5420). EGCG-methacrylates were synthesized and characterized as previously described by Bortolatto *et al.* [28]. Briefly, to generate monomers with varying degrees of methacrylation, EGCG was reacted with 1/3, 2/3, or 1 mol/L equivalents of methacryloyl chloride and triethylamine to produce monomers with hydroxyl functionalization at 33 % (M-E33), 67 % (M-E67), and 100 % (M-E100) levels.

2.1. Demineralized dentin samples preparation

This step was performed to expose dentin matrix from dentin discs, allowing MMPs and cysteine cathepsins to degrade collagen and for the release telopeptides, which will be quantified in the next step. Demineralization protocol was carried out according to Bafail *et al.* [29,30]. Extracted sound human third molars were collected, cleaned, and stored in physiologic saline at 4 °C. Before use, the teeth were sterilized with gamma radiation at a dosage of 2.5 MRad [31]. Teeth were horizontally sectioned at the mid-coronal third producing dentin disks of 0.5 ± 0.1 mm thickness using a diamond saw under water cooling (Isomet, Buehler Ltd, Lake Bluff, IL). The dentin disks were demineralized in 10 % H_3PO_4 (LabChem, Zelienople, PA, USA) for 40 h at room temperature under agitation using the hematology chemistry mixer at high-speed rotation. Subsequently, the dentin matrices were rinsed with deionized water for 5 min and randomly divided into the following groups: i) dentin matrices immersed in 1 mL lactic acid (LA) pH 5.5 for 30 min or ii) dentin matrices immersed in deionized water (DW) for 30 min as a control. Dentin matrices were washed and centrifuged and each group was subdivided into six subgroups and treated with the following solutions (n = 6, 1 mL; 30 min): water, acetone, neat EGCG (E0) solution, and EGCG-methacrylate solutions (M-E33, M-E67, and M-E100). All EGCG based compounds (E0, M-E33, M-E67, and M-E100) were diluted in acetone at 30 wt%. Afterwards, dentin matrices were centrifuged, and each sample was incubated in 1 mL of zinc- and calcium- containing buffer medium at 37 °C for 7 days [32]. Aliquots of medium-containing released proteins were stored at −4 °C for subsequent analysis.

2.2. Quantitative telopeptide release assays

Aliquots of the medium-containing released proteins were used for total protein quantification and solubilized telopeptide analysis. Total protein concentration was measured using Pierce Bicinchoninic Acid Assay (Thermo Scientific 23225, Waltham, MA, USA) at 562 nm. Dentin collagen degradation (n = 3) (solubilized telopeptide analysis) was determined by measuring the amounts of solubilized ICTP (cross-linked carboxyterminal telopeptide of type I collagen), CTX (C-terminal telopeptide of type I collagen), and NTX (N-terminal telopeptide of type I collagen) from extracts of dentin matrices by ELISA (#MBS040005, #MBS731778, #MBS2700252, MyBioSource, Inc., San Diego, CA, USA). Concentrations of solubilized telopeptides were normalized to total protein (TP) concentration (ng/mg) [30,33].

2.3. Ultimate tensile strength measurement of demineralized dentin disks

For ultimate tensile strength (UTS) test, beams (0.5 mm × 1 mm × 6 mm) obtained from dentin matrices incubated in the solutions described in item 2.1. were tested. The specimens (n = 6) were glued to a jig using a cyanoacrylate adhesive system and mounted on a microtensile testing machine (Micro Tensile Tester, Bisco, Inc, Schaumberg, IL, USA). The samples were subjected to tension until rupture at a crosshead speed of 1 mm/min. The ultimate tensile strength (MPa) was calculated by dividing the force (N) by the surface area (mm^2).

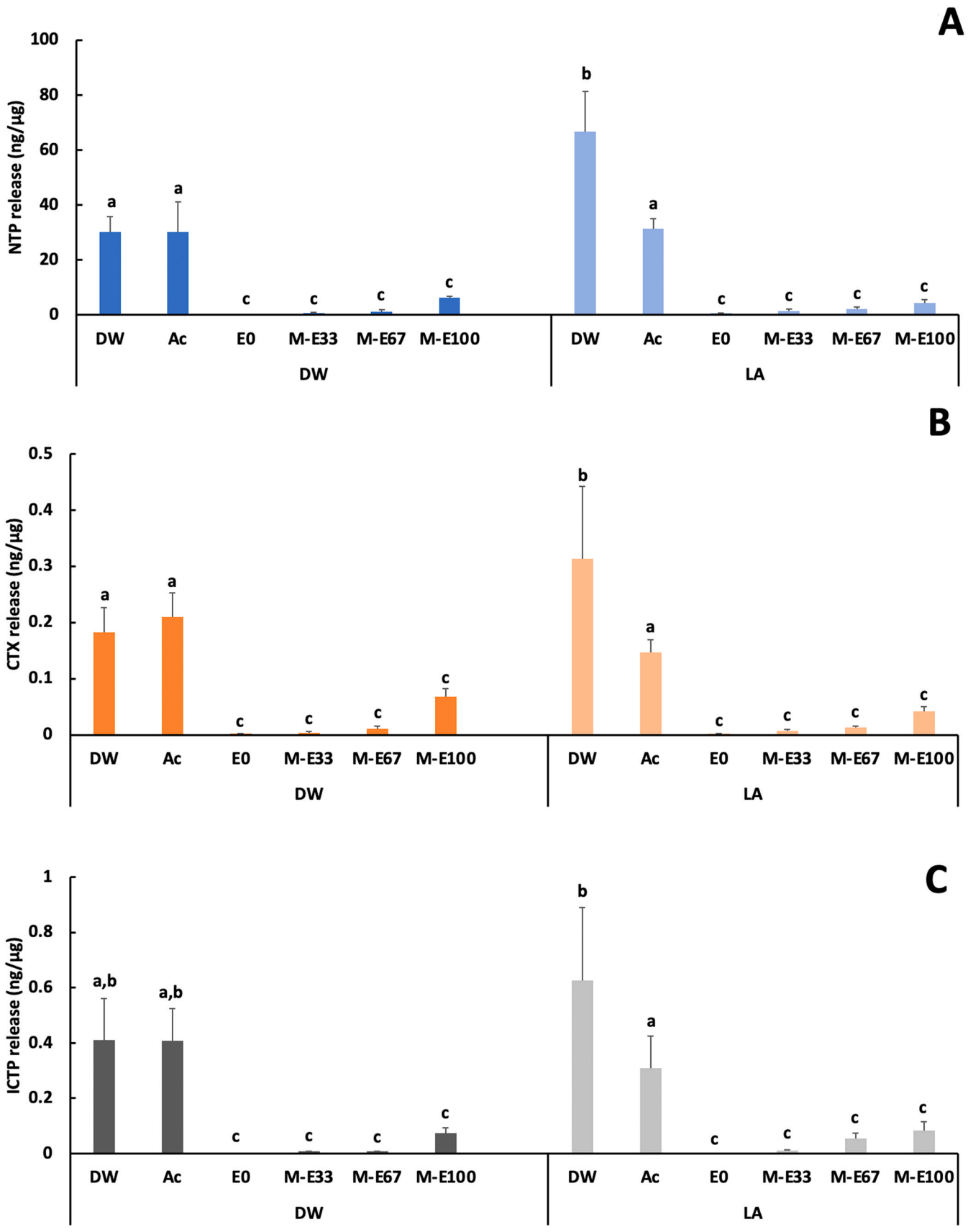

Fig. 1. Mean and standard deviation of the released telopeptides: **(A)** NTX, **(B)** CTX, and **(C)** ICTP. Solubilized telopeptides concentrations were normalized by total protein contents released by each tested sample from demineralized dentin slices stored in deionized water (DW) or 0.1 M lactic acid (LA) for 30 min and 1-week dentin immersion in buffer solution. For each storage condition, different lower-case letters show statistical differences among groups ($p < 0.05$).

2.4. In situ zymography

Extracted sound human third molars were cleaned, stored in saline solution, and sterilized with gamma radiation before use [31]. Teeth were horizontally cut at the mid-coronal third to expose a flat dentin surface, using a diamond saw (Isomet, Buehler Ltd, Lake Bluff, IL), under water cooling. The dentin surfaces were polished with 600-grit aluminum oxide abrasive disks (Extec Corp., CT, USA) to standardize the smear layer. Next, the specimens were etched with 37 % phosphoric acid for 15 s and randomly divided into five groups according to the experimental adhesive system used: Prime&Bond elect (PB) (Dentsply Sirona, Woodbridge, ON, CA), PB+ 1 wt% E0, PB+ 1 wt% M-E33, PB+ 1 wt% M-E67, PB+ 1 wt% M-E100 and photocured for 20 s (Valo Grand, Ultradent Products, South Jordan, UT, USA). Resin composite blocks (Z350, 3 M ESPE, Mississauga, ON, Canada) measuring 4 mm in diameter and 2 mm in height were built onto the pre-treated dentin specimens. Each 2 mm increments were photocured for 30 s. The teeth were wrapped in wet gauze and stored at 37 °C for 24 h.

After 24 h, the 15 restored teeth ($n = 3$) were cut to obtain longitudinal slices of 200 μm thickness using a water-cooled low-speed diamond disk. The slices were immersed in 10 % phosphoric acid for 30 s and then washed with deionized water for 1 min. *In situ* zymography was performed using a quenched fluorescein-conjugated gelatin as substrate (E-12055, Molecular Probes, Eugene, OR, USA), as described by Mazzoni *et al.* [13]. The gelatin stock solution was diluted 1:8 in a NaCl 150 mM, $CaCl_2$ 5 mM, Tris-HCl 50 mM, pH 8.0 buffer, with 10 μL of anti-fading agent (Mounting Medium with Dapi H-1200, Vectashield, Vector Laboratories LTD, Cambridgeshire, UK).

Each dental slice was placed on the glass slide into a small Petri dish and immersed in 80 μL of fluorescent gelatin mixture, prior to being covered with a coverslip sealed with a self-cure nail varnish. Thereafter, all slides were stored at 37 °C and protected from light for 24 h. The enzymatic gelatin activity of each sample was assessed based on the hydrolysis of the quenched fluorescein-conjugated gelatin as the gelatinase substrate (E-12055, Molecular Probes, Eugene, OR, USA). Samples were evaluated under a multi-photon confocal laser scanning microscope (LSM 5 Pa: Carl Zeiss, Oberkochen, Germany) with excitation and emission wavelengths of 488 nm and 530 nm, respectively [34]. Multiple optical sections of 85 μm-thick were acquired from different focal planes from each dental slice, which were stacked, quantified, and processed using ZEN 2010 software (Carl Zeiss, Oberkochen, Germany) before analysis. The enzymatic activity of each group was quantified by fluorescence intensity in the restorations' dentin-adhesive interfaces (HL) region. Three measurements at different sites on each image were recorded using a standardized rectangular selection, and the mean of intensity (pixels/μm^2) of the emitted green fluorescence was determined using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.5. Microtensile bond strength (μTBS)

Sixty teeth ($n = 12$) were prepared, treated with PB, PB+ 1 wt% E0, PB+ 1 wt% M-E33, PB+ 1 wt% M-E67, PB+ 1 wt% M-E100 and restored as previously described in item 2.4. Six specimens from each group were stored in deionized water for 24 h and 6 months. Afterwards, they were cross-sectioned into 1 mm^2 beams, using a slow-speed cutting machine under water cooling. Each beam was mechanically tested using a universal testing machine (ElectroForce 5500, TA Instruments, New Castle, DE) at a crosshead speed of 0.5 mm/min and 10 kgf load cell. The mean value (MPa) for the beams originating from each tooth ($n = 8$, total of 48 per group) was calculated and used for statistical analyses [34].

2.6. Failure pattern

After performing the μTBS, fractured zones of each specimen were stored. Three beams of each group were dehydrated in ethanol at decreasing concentrations. The beams were sputter-coated with gold/palladium (Polaron SC 515 Sputter Coater, Fison Instruments, VG Microtech, Sussex, UK), and the resin-adhesive interfaces were examined in a scanning electron microscope (FlexSEM-1000, Hitach High-Tech, Tokyo, Japan) at 15 kV using 2000 × magnification.

Failure patterns were qualitatively analyzed using an optical microscope (h33 Hund Wetzlar, Helmut Hund GmbH, Wetzlar, Germany) and classified according to the following failure type: Type 1: adhesive failure; Type 2: mixed failure; Type 3: cohesive fracture in composite; Type 4: dentin cohesive fracture. The method's error and the level of agreement within the examiner were assessed by analyzing 20 % of the images with the examiner being blinded, and then re-analyzing them twice by the same calibrated examiner at different times with a one-week interval between the analyses. The results were then subjected to Pearson's correlation test ($\alpha<0.05$), which showed a 98 % level of intra-examiner agreement.

2.7. Statistical analysis

Data analysis was performed with JAMOVI 2.5.7 for MacOS. The assumptions of equality of variances and normal distribution of errors were checked by Levene and Shapiro-Wilks tests, respectively. One or Two-Way ANOVA followed by Tukey *post-hoc* test was used to compare mean values of different groups ($\alpha=0.05$).

3. Results

3.1. Collagen I telopeptide release assay

To evaluate the EGCG-methacrylate potential in protecting the dentinal type I collagen from proteolytic degradation, the release of

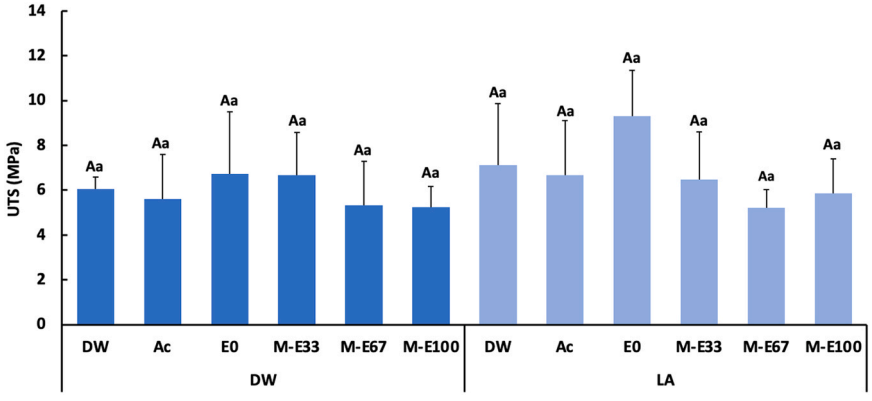

Fig. 2. Means (SD) of ultimate tensile strength (MPa) results for demineralized dentin stored in water - DW (dark blue) and 0.1 M lactic acid - LA (light blue). Different lower-case letters show statistical differences among the groups, independent of the storage, according to two-way ANOVA and Tukey tests ($p < 0.05$).

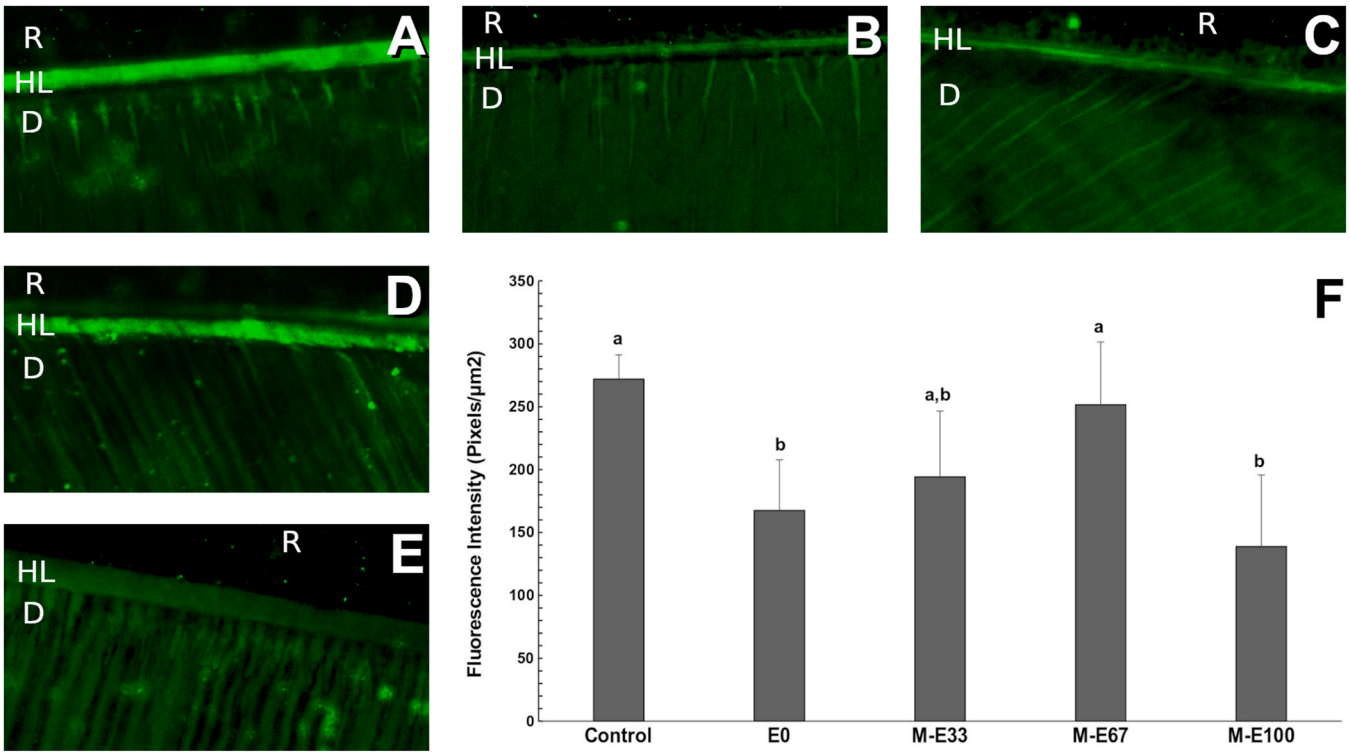

Fig. 3. Representative *in situ* zymography images of the hybrid layer region acquired with the multi-photon confocal microscope for the groups: **(A)** Control, **(B)** E0, **(C)** M-E33, **(D)** M-E67, and **(E)** M-E100, after 24 h of incubation with quenched fluorescein-labeled gelatin. **(F)** Emitted 488 nm fluorescence mean intensity quantification for each tested group sample. Different lower-case letters show statistical differences among the groups ($p < 0.05$). R – restoration, HL – hybrid layer, D – dentin.

different fragments from both N- and C-terminal collagen telopeptides was measured. Fig. 1 presents the solubilized telopeptides, **NTX (Panel A)**, **CTX (Panel B)**, and **ICTP (Panel C)**, released from demineralized dentin slices preincubated in DW or LA in all experimental groups. When pre-incubated in DW, the groups E0, M-E33, and M-E67 almost eliminated the proteolytic activity on N-terminal telopeptide (NTX) with inhibition rates of 99.21 %, 97.82 %, and 95.18 % respectively, while M-E100 showed an inhibition rate of 79.48 %. The pre-incubation in LA presented a similar trend with inhibitory rates of 99.07 % (E0), 97.78 % (M-E33), 96.71 % (M-E67), and 93.34 % (M-E100).

The C-terminal telopeptide (CTX) was also protected from proteolytic hydrolysis in the presence of EGCG-methacrylate. When pre-incubated in DW the groups E0, M-E33, M-E67, and M-E100 presented a protective pattern of type I collagen against proteolytic degradation that allowed a significant decrease in CTX release in the order of 98.94 %, 98.05 %, 94.44 %, and 62.77 % respectively. All experimental groups were also able to protect the CTX degradation with inhibitory rates of 99.22 % (E0), 97.70 % (M-E33), 95.80 % (M-E67), and 86.77 % (M-E100) when pre-incubated with LA.

In the same way, the EGCG-methacrylate presented a protective effect on the release of the cross-linked carboxyterminal telopeptide of type I collagen (ICTP) with proteolysis inhibition rates of 99.90 % (E0), 98.02 % (M-E33), 98.02 % (M-E67), and 82.19 % (M-E100), when pre-incubated in DW, and 99.98 % (E0), 98.38 % (M-E33), 91.29 % (M-E67), and 87.09 % (M-E100), pre-incubated in LA.

3.2. Ultimate tensile strength measurement of demineralized dentin disks

Considering the UTS results obtained after storage of demineralized dentin disks in DW or LA, there was no statistical difference among all the groups tested (experimental or control groups), as shown in Fig. 2.

3.3. In situ zymography

The gelatinolytic activity in the HL region was evaluated by *in situ* zymography. Images of resin-bonding dentin interfaces from control and experimental groups (E0, M-E33, M-E67, and M-E100) can be observed in Fig. 3**,** and the emitted fluorescence intensity means for all groups in **Panel F**. The groups E0 (**Panel B**) and M-E100 (**Panel E**) presented the highest inhibitory potential on the gelatinolytic activity. Compared to the control (**Panel A**), it decreased the hydrolysis of the fluorescent substrate by 38.43 % and 48.95 % respectively. Additionally, M-E33 (28.75 %) and M-E67 (7.50 %) (**Panels C and D**) also showed the ability to inhibit the proteolytic activity, albeit to a lesser extent, in the HL region.

3.4. Microtensile bond strength (μTBS) and fracture pattern

The results in Fig. 4**A** represent the μTBS values for restored specimens after 24 h and 6 months of water storage. The specimens restored with an adhesive containing E0 showed the lowest μTBS values (23.52 MPa) in 24 h of water storage. In comparison, the groups M-E33 (28.15 MPa), M-E67 (32.09 MPa), and M-E100 (30.40 MPa) presented no microtensile bond strength difference from the control group. However, after 6 months, the E0 (30.18 MPa) group did not differ from the other groups, except by M-E100 (25.39 MPa). The μTBS results from groups M-E33, and M-E67 were statistically similar to the control group, in both time points. Fig. 4**B and** Fig. 5 represents the failure mode in 24 h and 6 months. There was an increase in types 1 and 2 failure modes in the experimental groups, E0 (51.6 %), M-E33 (54.54 %), M-E67 (46.1 %), and M-E100 (35.13 %) after 24 hours compared to the control group (25 %). These failures were less recurrent in groups E0 (23.25 %) and M-E67 (29.54 %) compared to the control group (33.32 %) after 6 months.

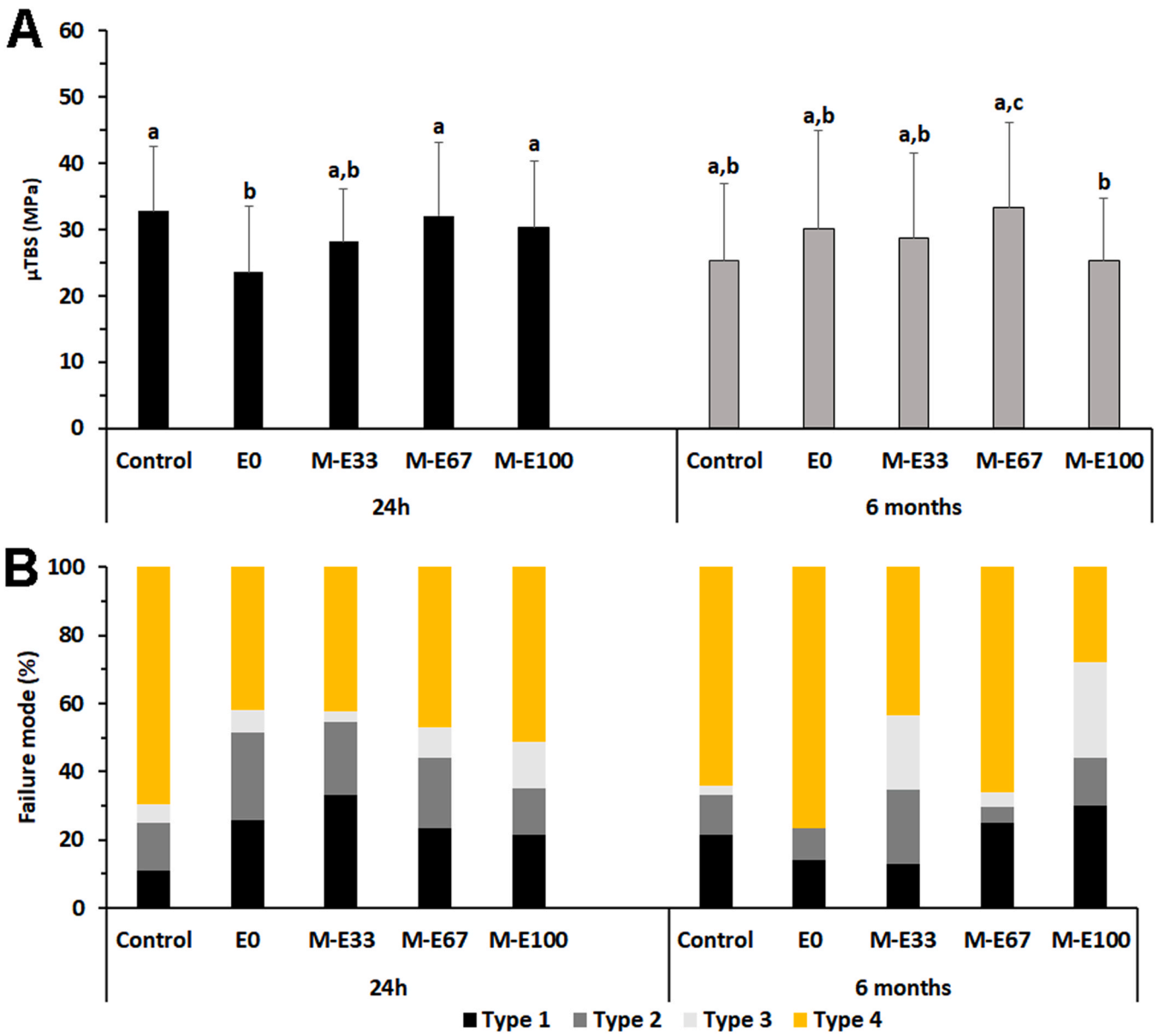

Fig. 4. Mean and standard deviation of the resin/dentin μTBS (MPa) values **(A)**, and distribution of the fracture type according to the dentin surface treatment after 24 h and 6 months **(B),** of the specimen's storage in DW. Type 1: Adhesive; Type 2: Mixed; Type 3: Cohesive in Composite; Type 4: Cohesive in Dentin. Different lower-case letters show statistical differences among the groups, independent of the storage, according to two-way ANOVA and Tukey tests ($p < 0.05$).

4. Discussion

Secondary caries and loss of bond strength are consequences of chronic degradation of resin-bonded interfaces affecting composite restoration longevity. Modern adhesives use comonomer blends to form hydrophilic polymers that absorb 5–12 % water, causing plasticization and weakening the material mechanical properties. Water diffusion triggers leaching and phase separation, limiting polymerization in the hydrophilic domains. In aqueous environments, the poorly polymerized phase degrades quickly, reducing bond strength at the resin-adhesive interface within the hybrid layer. Simultaneously, at the organic portion of dentin tissue the host-derived enzymes, such as matrix metalloproteinases (MMPs) and cysteine cathepsins, play a role in the degradation of collagen matrices at the bonded interface. These enzymes have been identified in dentin and are believed to cleave the collagen fibrils exposed at the adhesive interface. Research on proteolytic degradation at dentin-adhesive interfaces has evolved into a distinct scientific field, leading to increased clinical interest in enhancing the durability of dentin bonds. Consequently, various strategies have been developed to inhibit enzymatic activity and improve the longevity of resin-dentin bonds [35–37].

Among different properties, EGCG is known to promote collagen crosslinking. In this study, UTS tested the cohesive strength of demineralized dentin beams treated with E0, M-E33, M-E67, and M-E100, as increased UTS had been associated with increased collagen crosslinking [38]. Increased crosslinking is in turn associated with lower proteolytic degradation susceptibility of collagen [33]. The present results (Fig. 2) showed that UTS values of 30 min-treated dentin matrices were similar among E0, M-E33, M-E67, and M-E100 treatments, conforming that EGCG methacrylates are able to maintain crosslinking capacity.

Reports predominantly emphasize collagen modifications by polyphenols such as catechin [39], tannic acid [40], and procyanidin [41]. EGCG shares the same functional structure with catechin and tannic acid and exhibits high protein cross-linking activity. The interactions between collagen and EGCG involve hydrophobic interactions and electrostatic interactions. The hydrophobic interface comes from the

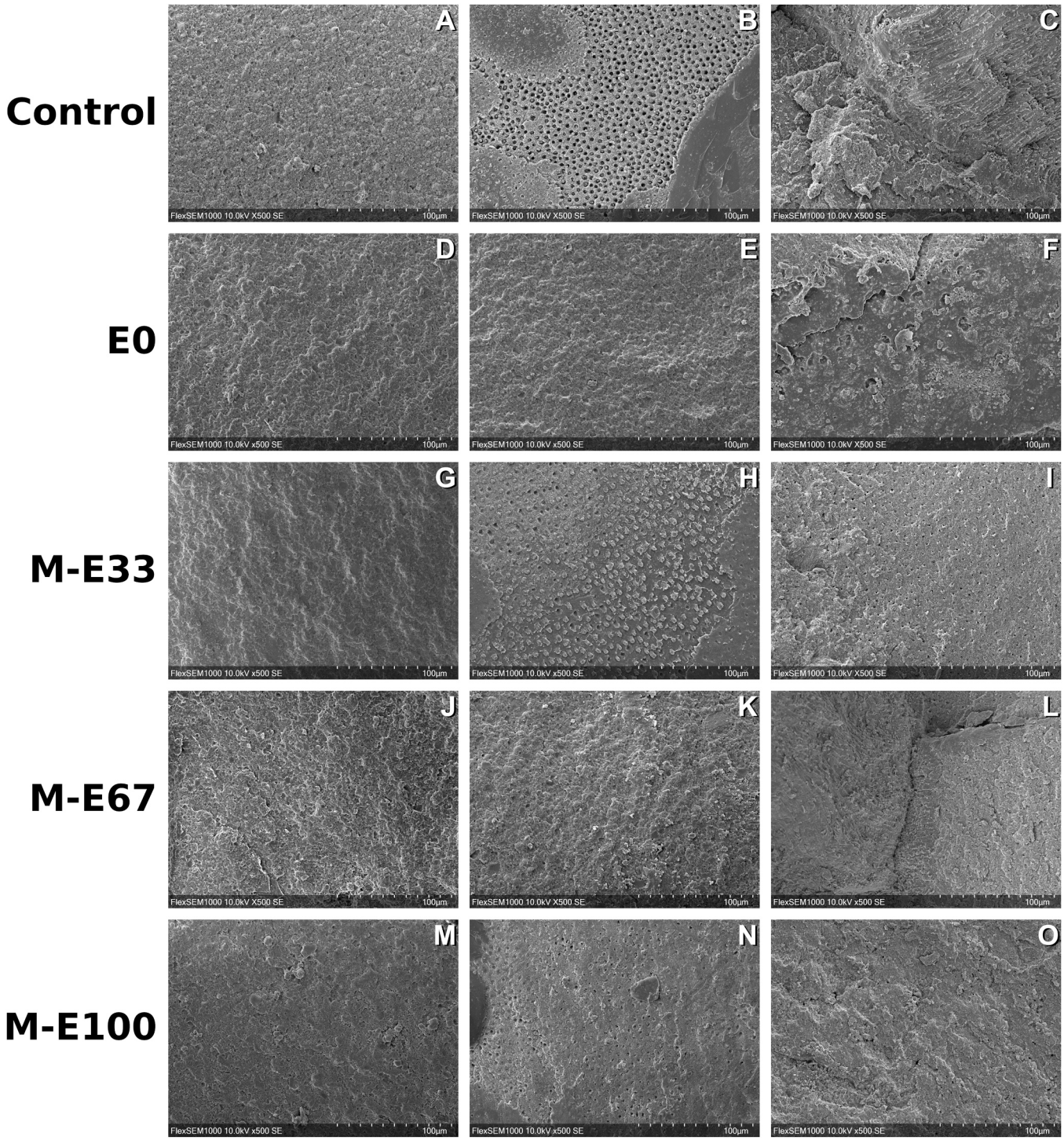

Fig. 5. Scanning electron microscopy (SEM) photographs of resin-bonding dentin interfaces from representative specimens restored with control adhesive - PB (A-D) and adhesive (PB) containing E0 (E-H), M-E33(I-L), M-E67(M-P) and M-E100(Q-T) and stored in water for 6 months. The fracture types are shown in the following figures: Type 1: Adhesive - Figures A, E, G, I, M and Q; Type 2: Mixed Fracture – Figures B, F, J, N and R; Type 3: Cohesive in Composite – Figures C, K, O and S; and Type 4: Cohesive in Dentin – Figures D, H, L, P and T. Magnification: 500 × .

R-Ala-Ala-Leu-Gly sequence in collagen and the benzene rings from EGCG. Additionally, there are two pairs of electrostatic interactions within the collagen-EGCG complex: one involving the EGCG polar oxygen atom surrounded by collagen polar nitrogen or oxygen atoms, and the other involving the EGCG carbon-ring or aromatic ring near the hydrophobic carbon atoms environment from collagen. Furthermore, there is a network of hydrogen bonds among EGCG, the phenolic hydroxyl groups, and the amino groups as well as carboxyl groups from specific amino acids (Glu, Thr, Leu, Ala, Arg, Gly, Asp) in the collagen molecule [23,38]. In this sense, EGCG (E0) and functionalized EGCG-methacrylates were effective in reducing the release of collagen telopeptides from dentin, independent of the ratio of hydroxyl functionalization (M-E33, M-E67 e M-E100). *In vitro* and *in situ* studies have demonstrated the ability of EGCG to inhibit dentinal proteases such as metalloproteinases (MMP) and cysteine cathepsins, reducing proteolytic degradation of dentin [42–44]. It has been known that ICTP and CTX

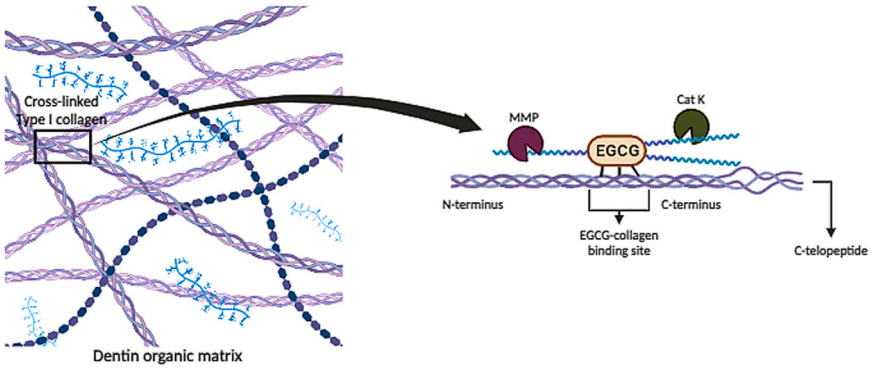

Fig. 6. Representative illustration of the interaction of EGCG-methacrylates with collagen I.

type I collagen fragments are generated by two distinct pathways involving the cleavage of C-terminal region by MMPs and cathepsin-K, respectively [45], while NTX telopeptides are released due to cathepsins activity [30]. As previously suggested, micromolar concentrations of EGCG inhibited both the collagenolytic and gelatinolytic activity of MMP-2 and MMP-9, probably by direct binding of EGCG to the enzymes and formation of a ternary inactive complex among MMP-EGCG-gelatin [46]. However, the inhibitory effect of EGCG on MMPs was also attributed to the synergy between both inhibition of proteolytic enzymes by blocking or changing the conformation of enzymes cleavage sites and changes in collagen structure, forming cross-links between galloyl moieties and proline-rich peptides stabilizing collagen molecule [44, 47]. Our data shows that functionalized EGCG-methacrylates were able to practically eliminate the telopeptide release in all tested conditions.

The emitted fluorescence resulting from hydrolyzed fluorescein-conjugated gelatin in the adhesive/dentin interface was measured by *in situ* zymography. The current study showed that specimens treated with a dental adhesive system containing EGCG (E0) or EGCG-methacrylates (M-E100) presented the lowest fluorescence intensity detected in the hybrid layer, with 38.43 %, and 48.95 % reduction compared to the control group. Considering that gelatin can be hydrolyzed by most human gelatinases and collagenases, and was not protected by EGCG-methacrylates interactions, the lower inhibitory rates compared with the telopeptide release assay strengthens the theory of the importance of the interaction between the folded collagen fiber and EGCG for an effective decrease in proteolytic activity. In this study, intense gelatinolytic activity was observed at the bottom of the hybrid layer created by an etch-and-rinse adhesive in restored human dentin specimens, as observed in a previous study [13]. Previous studies have demonstrated that both acid-etching and subsequent adhesive application increased the MMP [14] and cysteine cathepsin activities within the hybrid layer that can remain active for seven days [48]. Some other investigations also have shown the efficacy of dentin pre-treatment with polyphenols, such as EGCG, proanthocyanidins, and baicalein, before the bonding procedures as a strategy to protect the HL from degradation by adhesive hydrolysis and endogenous enzyme activation [49–51]. To avoid adding additional step to the restoration technique, in this present study, EGCG-methacrylates were incorporated into the adhesive system and reduced the gelatinolytic activity within the hybrid layer. However, this effect was dependent on the methacrylation, since M-E33 and M-E67 results were not different from the control. The higher availability of EGCG is essential to stabilize type I collagen through hydrogen bonds/hydrophobic interactions and inhibit the MMPs [23], and cysteine cathepsins activities (Fig. 6). Studies have reported that some metal ions, such as calcium, can combine with collagen and compete with EGCG by MMP recognition. Besides, the high affinity of EGCG to metal ions can reduce its availability and prevent MMP recognition [52].

Our study also evaluated the dentin-resin bond strength performed with an adhesive system containing E0, M-E33, M-E67, or M-E100 (1 wt %). The lowest μTBS value was observed for E0, at 24 h, which was different from the control. All other groups behaved statistically similarly to the control group, at both time points. A systematic and meta-analysis study suggested that the use of EGCG as dentin pretreatment or when incorporated within the formulation of total-etch or self-etch adhesive systems improved the long-term bond strength of resin composites to dentin [25]. This study did not find change in the immediate bond strength of restorations after the incorporation of EGCG-methacrylates in adhesive systems. It has been reported that higher neat EGCG concentrations (over 1 %) incorporated into the adhesive system have affected the integrity of the HL and bond stability of restorations [24,53]. Based on the results of this study, hypothesis 1 was accepted, as all tested groups efficiently inhibited type I collagen hydrolysis by inhibiting the proteolytic activity. The hypothesis 2 was also accepted, since in all tested groups, the immediate and long-term bond strength of the restorations were not affected when EGCG-methacrylates were incorporated into the adhesive systems.

This is the first time that we are showing that EGCG-methacrylate monomers decrease collagen degradation. When incorporated into an adhesive system, EGCG-methacrylate monomers reduced the gelatinolytic activity within the HL, not affecting the immediate and long-term bond strength of restorations.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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