Original Research
Dentistry

Protective effect of green tea catechins on eroded human dentin: an *in vitro/in situ* study

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Abstract: The present study sought to evaluate the protective effect of Epigallocatechin-3-gallate (EGCG) and commercial green tea (GT) on eroded dentin using *in vitro and in situ* experimental models. For the *in vitro* experiment, matrix metalloproteinases (MMPs) were extracted from demineralized human coronary dentin powder (citric acid, pH 2.3) and assessed via a colorimetric assay and electrophoresis in gelatin. The gels were exposed to buffers with: control (no treatment), 0.05% sodium fluoride (NaF), 0.12% chlorhexidine digluconate (CHX), GT infusion, and 0.1% EGCG, and their respective activity was analyzed by zymography. For the *in situ* experiment, 20 healthy volunteers (aged 20-32 years) participated in this single-center, blind, crossover study. The subjects wore upper removable devices containing four human dentin blocks. Erosive challenge (coke-1 min) was performed four times/day/5 days. Blocks were treated for 1 min with: control (No treatment), 0.05% NaF, 0.1% EGCG, and GT. Thereafter, the specimens were subjected to stylus profilometry and SEM. ANOVA was used to evaluate dentin roughness and wear, with a significance level of 5%. In the zymography analysis, 0.12% CHX, GT, and 0.1% EGCG were found to inhibit the action of MMPs; however, in the colorimetric assay, only green tea inhibited the activity of MMPs. There were no significant differences observed in dentin roughness or wear ($p > 0.05$). Herein, EGCG and GT inhibited the activity of endogenous proteases, resulting in protection against erosion-induced dentin damage; however, they could not prevent tooth tissue loss *in situ*.

Keywords: Tooth Erosion; Tooth Wear; Collagen; Matrix Metalloproteinases; *Camellia sinensis*.

Introduction

Dentin erosion is an irreversible chemical loss from hard dental tissue without bacterial involvement. Accordingly, dentin erosion is significantly associated with dentin hypersensitivity.[1-5] A recent systematic review demonstrated that the prevalence of erosive tooth wear varies widely, ranging from 9.1% to 93%. Studies with a low estimated risk of bias revealed that the mean prevalence of dental wear is 40.7%.[6] Further, dental wear was identified to be highly prevalent in adults and adolescents.[3,7] In fact, the incidence of dental erosive wear increases with age and dietary acid intake.[4,8] Preventive

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strategies should thus be established to hinder the formation of early lesions. However, for the advanced stages of dentin exposure, maintaining a demineralized organic matrix (DOM) to prevent ion diffusion and reduce acid damage to dentin has been proposed. The prevention of DOM degradation by endogenous proteases can be achieved by inhibitors, such as sodium fluoride (NaF), chlorhexidine digluconate (CHX) solutions, and green tea catechins, such as epigallocatechin-3-gallate (EGCG). [9-17]

Plant-derived polyphenols have demonstrated their biocompatibility and bioactivity in preventing dental erosion. The main components responsible for the preventive action of green tea are catechins, such as EGCG, which is its most important polyphenol.[11] EGCG can increase collagen crosslinking and prevent the free access of collagenase to the active sites on the collagen chains, thereby reducing dentin biodegradation.[18] Moreover, based on *in vitro* evidence, commercial green tea is a promising agent for controlling dentinal wear.[11,13,17,19,20,21] The catechins in green tea have antioxidant characteristics and can inhibit MMP-2 and MMP-9.[22] The effectiveness of DOM, which acts as a protective layer against further erosion, can be tested by the Erosive challenge. EGCG and green tea can prevent MMPs from degrading this matrix.[23]

Clinical studies to evaluate erosive tooth wear are challenging due to the difficulty in distinguishing this lesion type from other non-carious cervical lesion types. Further, dental erosive wear score systems have not been standardized.[8] However, *in situ* studies could be a useful tool to investigate the protective effect of polyphenols on dentin lesions, as the natural protective effects of the oral cavity mimic an *in vivo* situation. This study was designed to test the protective effect of polyphenols found in commercially available green tea and their isolated, purified form (EGCG) on dental erosion, using *in vitro* and *in situ* experimental models.

Methodology

Test products

The tested products were prepared as described below for the *in vitro* and *in situ* studies. Green tea was freshly prepared according to the manufacturer's instructions. A sachet of 2 g of *Camellia sinensis* leaves (Dr. Oetker, São Paulo, Brazil) was immersed in 200 mL of boiled water for 3 min. The fluoride solution was prepared by mixing 0.05 g of NaF powder (Dinâmica, Diadema, SP, Brazil) with 100 mL of distilled water. EGCG (0.24 g EGCG; Sigma-Aldrich, St Louis, USA) was prepared via dilution in 100 mL of distilled water. For the *in vitro* study, 0.12% CHX was prepared.

In vitro study

Experimental design

This blinded (blinded to the person responsible for treatment application) *in vitro* study had a randomized design with five groups (n = 3). The following MMP inhibitors (five levels (including control)) were under investigation: 0.05% NaF, 0.12% CHX, green tea, 0.1% EGCG, and the control (no treatment). The dependent variables were analyzed via electrophoresis in gelatin, qualitative evaluation by zymography, and quantitative evaluation using a colorimetric assay, with each performed in triplicate. The protocol was reviewed and approved by the local research and ethics committee (#003438/2016).

Statistical analysis of the colorimetric data was performed using repeated two-way ANOVA and Bonferroni's post-test ($\alpha = 0.05$).

Sample preparation and processing

Dentin blocks with dimensions of 4 × 4 × 3 mm were prepared from 35 caries-free human third molars within two months after extraction, as previously described.[17] Briefly, the blocks were frozen in liquid nitrogen and triturated using a Retsch mill (MM400, Retsch GmbH, Haa, Germany) at 30 Hz for 15 min; the obtained powder was stored at -20 °C until use.[24] Mineralized dentin powder was brought into contact with 0.87 M citric acid (1:10, m/v; pH 2.3) for 24 h under moderate agitation in an orbital shaker (AP22, Phoenix Luferco, Araraquara, Brazil) at 4°C. The suspension was then centrifuged at 10,000 × *g* (Hettich Rotina 380R-Tuttlingen, Germany) for 10 min at 4°C, and the supernatant was discarded. The pellet was resuspended in distilled water and centrifuged under the same conditions; this procedure was repeated three times. For the extraction of proteases from dentin, the

pellet was resuspended in 10 mL extraction buffer (0.05 M Tris-HCl buffer, containing 0.005 M CaCl2, 0.1 M NaCl, 0.1% [v/v] Triton X-100, 0.0001 M ZnCl2, 0.02% [m/v] NaN3, pH 7.5), and subjected to moderate agitation in an orbital shaker for 24 h at 4 °C. The extract was subsequently centrifuged at 10,000 × *g* for 30 min at 4 °C.[25] The supernatant, which was rich in MMPs from dentin, was collected and dialyzed through a 12–14 kDa membrane (Sigma-Aldrich Co., St. Louis, USA) against distilled water at 4 °C for 24 h; lyophilized; and stored at -20 °C until use. The quantification of soluble protein was determined following the method described by Bradford (1976),[25] using bovine serum albumin (BSA) as a standard protein.

Colorimetric assay

To evaluate the inhibitory activity of the MMP inhibitors tested in this study, a colorimetric assay was performed in a 96-well microplate using a SensoLyte® Generic MMP Assay kit (#1022, AnaSpec, Freemont, USA), according to the manufacturer's instructions. A pool of proteases extracted from dentin, as previously described, was used as the source of MMPs. Forty microliters of the enzyme solution was mixed with the MMP inhibitors (50 µL of 0.05% [m/v] NaF, 0.12% [v/v] CHX, 20 µL of green tea (EGCG concentration 0.0014%), or 0.1% [m/v] EGCG). Subsequently, component C (assay buffer component C- SensoLyte® Generic MMP Assay, AnaSpec, Freemont, USA) was added to achieve a final volume of 100 µL. The plates were then incubated at 37 °C for 10 min. The reaction began after the addition of 50 µL of 0.2 mM component A (MMP colorimetric substrate-component A- SensoLyte® Generic MMP Assay, AnaSpec, Freemont, USA). The plate was allowed to stand at the same temperature, and absorbance was measured at 412 nm every 10 min for 2 h using a microplate reader (EpochTM, BioTek, Winooski, USA). The experiment was performed in triplicate. Two control groups, no inhibitor solution and another with 10 µL of 20 µM component D (MMP inhibitor -component D- SensoLyte® Generic MMP Assay, AnaSpec, Freemont, USA), were employed herein. Statistical analysis was performed using repeated two-way ANOVA and Bonferroni's post-test ($\alpha = 0.05$).

Zymography

Visualization of the in-gel profile of dentin MMPs in the absence and presence of inhibitors was performed according to Kato *et al.*[26] Initially, samples of the MMP protein extract (40 µL) were applied to a 12.5% (m/v) polyacrylamide gel (8.5 × 8.0 cm) prepared in 0.025 M Tris-HCl buffer (pH 8.9) containing 1% SDS[27] and 0.1% (m/v) gelatin in a vertical system. Electrophoresis was performed at a constant current of 20 mA. After electrophoresis, the gel was incubated twice in renaturation buffer (0.05 M Tris HCl buffer, containing 2.5% [v/v] Triton X-100 and 0.02% [m/v] NaN_3, pH 7.5) for 30 min at 37°C and washed with distilled water. Thereafter, the gel was cut into 2-cm strips and incubated for 3 h at 37 °C in activation buffer (0.05 M Tris-HCl buffer, containing 0.005 M $CaCl_2$, 0.0001 M $ZnCl_2$ and 0.02% [m/v] NaN_3, pH 7.5), in the absence (control group) or presence (experimental groups) of 0.05% (m/v) NaF; 0.12% (v/v) CHX; green tea (used instead of distilled water to prepare the activation buffer), or 0.1% (m/v) EGCG. Staining was performed overnight with a solution containing 0.025% (w/v) Coomassie Brilliant Blue G-250 in 10% (v/v) acetic acid. Excess dye was removed from the gel using a solution of 10% (v/v) acetic acid.

In situ study

Experimental design

The second experiment was a randomized, blinded, split-mouth design (control group included in each phase), crossover *in situ* study for erosive induction by exposure to an acidic solution in three phases, with each phase consisting of 5 days. The following MMP inhibitors (including control) were under investigation (four levels): 0.05% NaF; 0.1% EGCG, Green tea infusion, and control (no treatment).

The sample size was selected according to the data obtained from a previous study.[28] As a result, 20 healthy adult volunteers, ranging from 20 to 32 years old, were recruited. Visual and tactile clinical examination was carried out to select volunteers with teeth that were caries-free as per the International Caries Detection and Assessment System (ICDAS),[29] and free of dental wear as per the Basic Erosive Wear Examination (BEWE) index.[30] Study subjects

from the Dental School were selected according to the following conditions: good oral health, absence of active caries, no evidence of reflux and dental erosion, and absence of an oral device. Subjects taking antibiotics or any medication that could significantly interfere with saliva flow were not enrolled in the study. A computer-generated randomization list (Microsoft Office 2007, EUA) was used to assign patients to the phases as well as the specimen positions on the device. During the intraoral phase, the volunteers received unidentified bottles with appropriate treatments for each stage to fulfill the blinding process. Informed consent was obtained from all participants (#003438/2016).

The dependent variables were quantitatively evaluated by profilometry (roughness and wear) and qualitatively assessed using scanning electron microscopy (SEM). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 17.0 for Windows, SPSS Inc., Chicago, USA). A Kolmogorov-Smirnov test was applied to all groups to test for the normal distribution of errors. Because the values were normally distributed across all groups, ANOVA was used to evaluate dentin roughness and wear. The significance level was 5%.

Sample preparation

The superficial coronal dentin samples (4 × 4 × 2 mm) below the dentinoenamel junction were obtained from extracted human third molars. This coronal dentin sample was obtained on removing the occlusal enamel and root dentin by two perpendicular cuts to the tooth's long axis with a metallographic cutter (Isomet Buehler, Lake Bluff, USA). Four cuts parallel to the tooth's long axis were performed to obtain a coronal dentin specimen.[17] The baseline surface Knoop hardness test (Microhardness Tester FM 100, Future Tech, Fujisaki, Kawasaki-City, Japan) was performed by making five indentations at 10 g for 5 s on the superficial dentin surface; 240 dentin blocks showed surface hardness ranging from 68 to 75 Knoop hardness number (KHN). These blocks were selected and randomly assigned using a computer-generated randomization list (Microsoft Office 2007, EUA).

The coronal dentin samples were individually packaged and marked for ethylene oxide sterilization before the *in situ* phases.[31] Before the experiment, the specimens were coated with adhesive unplasticized polyvinyl chloride (UPVC) tapes, with half (2 × 2 mm) left exposed for subsequent testing.[32]

Intra-oral phase

In each experimental phase, the volunteers used acrylic upper removable devices. The devices were built with acrylic resin using the palate as a retentive area. Further, they were prepared with four cavities (5 × 5 × 3 mm), with four dentin blocks randomly positioned and fixed with wax. The specimens were carefully placed to allow a 1-mm position below the device surface, avoiding contact with the tongue. Three treatment solutions were compared according to their ability to protect the human dentin surface from erosive lesions. In each phase, the control and treatment groups were tested. Accordingly, the volunteers' participation in all stages and testing of all treatments enabled a crossover design (Figure 1).

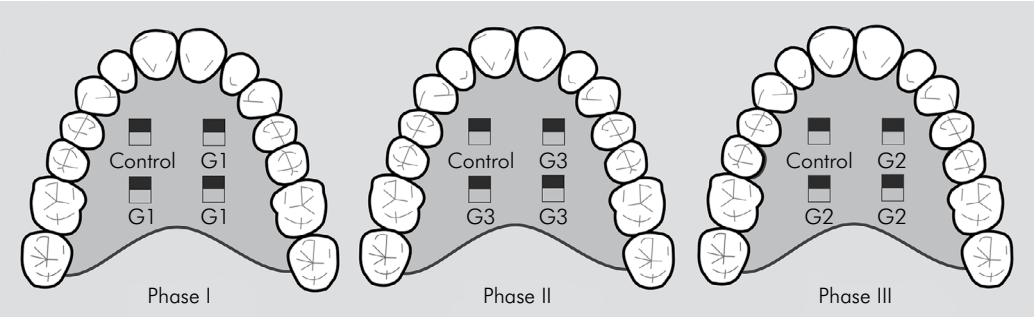

Figure 1. Schematic outline of the three phases included in the experimental design for the clinical study. Control (no treatment), 0.05% NaF, 0.1% EGCG, and Green tea. The control group was present in all phases.

Subjects were required to wear their device for 12 h before the study, to allow mineral equilibrium with saliva, and the formation and maturation of the salivary pellicle.[33] The subjects wore the devices both in the day and night during the three phases for 5 days per phase, and were instructed to avoid eating, drinking, or performing oral hygienic procedures with the intra-oral devices. The volunteers immersed the device in a cup containing 50 mL of Coke (pH 2.6, 0.32 ppm F, Coca-Cola Company, Ceara, Brazil) at room temperature for 1 min, four times per day (8 h, 12 h, 16 h, 20 h). After erosion, the devices were rinsed for 60 s in tap water (fluoridated, 0.7 µg/mL). Thereafter, the excess water was removed with absorbent paper. The volunteers also transferred one drop of the test solutions onto the blocks for 1 min (4 x/day) at room temperature: negative control (no treatment); 0.05% NaF (positive control, 0.01 M, pH 6.8), 0.1% EGCG (0.002 M, pH 5.5), and green tea extract (Dr. Oetker, São Paulo, Brazil, pH 5.45, EGCG concentration 0.0014%), before reinserting the device into the mouth (Figure 1).[9,34]

Over a two-day lead-in period, oral hygiene (modified Bass brushing technique) was standardized across the volunteers. Briefly, each volunteer received a commercial non-fluoridated toothpaste (Bitufo, Itupeva, São Paulo, Brazil; composition: sorbitol, glycerin, cellulose gum, Xanthan gum, PEG-8, methylparaben, propylparaben, sodium saccharin, hydrated silica, sodium lauryl sulfate, xylitol, titanium dioxide, triclosan, calcium disodium EDTA, and alcohol) to brush their teeth and devices during the course of the experiment. The volunteers were also required to brush the devices extra-orally rather than over the specimens. A 2-day washout period between each phase was maintained to avoid a crossover effect.

Surface profile and roughness measurement

Measurements of dentin wear and roughness were performed after five experimental days. Briefly, the adhesive tape attached to the half-block was carefully removed to expose the untreated area. The dentin surface wear and roughness measurements were recorded with a stylus profilometer (Hommel Tester T1000, Hommelwerke GmbH, Germany). At intervals of 0.5 mm, five profile traces (1.5 mm in length) were recorded on each specimen. The wear levels were determined for the reference surfaces. All specimens had a standardized initial roughness of approximately 0.01 µm.

Scanning electron microscopy (SEM)

Two random samples from each group were analyzed using SEM (Quanta FEG 450, FEI, Thermo Fisher Scientific, Hillsboro, USA). The samples were dehydrated in a desiccator for 24 h at room temperature, fixed in stubs, and sprayed with gold. Representative images of the interface area (reference/treated) and morphological changes of the treated surfaces were obtained at 4000x standardized magnification.

Results

In vitro

Figure 2 shows the results of the colorimetric assay. After 80 min, the green tea extract demonstrated the best inhibitory activity against MMPs, similar to the standard inhibitor ($p = 0.417$). In contrast, only a minor inhibitory activity was exhibited by EGCG until 60 min; thereafter, its activity was found to be stabilized. EGCG was not observed to be better than CHX and NaF ($p > 0.05$). Further, 0.12% CHX and 0.05% NaF were not found to exert any inhibitory activity as their behavior was found to be similar to that of the negative control ($p > 0.05$).

Figure 3 shows the results of gelatin zymography. Treatment with 0.12% CHX, green tea, and 0.1% EGCG inhibited the proteolytic activity of MMPs, as revealed by the absence of bright protein bands on the dark blue background. However, as the protein bands representing proteolytic activity were similar to the control, the activity of dentin MMPs was identified to be intact in the presence of 0.05% NaF.

In situ

Table displays the mean and standard deviation of the wear and roughness values found for all treatments evaluated in the five-day experiment. There was no significant difference between the treatments based on wear ($p > 0.05$) and roughness ($p > 0.05$).

SEM photomicrographs of each group are shown in Figures 4 and 5. Figure 4 displays images of the

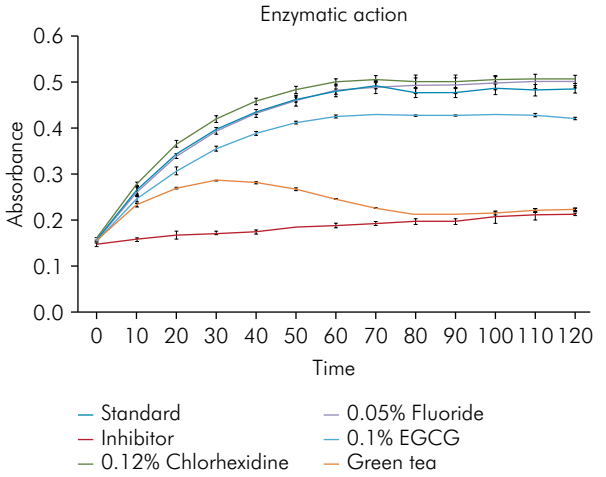

Figure 2. Residual enzymatic activity, in triplicate (*in vitro*), of MMPs (proteases extracted from dentin) treated with the Negative control, Standard inhibitor, 0.12% Chlorhexidine, 0.05% Fluoride, 0.1% EGCG, and Green tea. Absorbance x time (min). Compared to the standard inhibitor, Green tea was found to inhibit enzymatic activity throughout the 80-min incubation period.

Figure 3. Effect of the different treatments tested *in vitro* on the MMPs from the dentin extract. Gelatinase zymography, in triplicate, of (1) no treatment, (2) 0.05% NaF, (3) 0.12% chlorhexidine digluconate, (4) green tea, and (5) 0.1% EGCG, respectively.

Table. Mean and standard deviation of dentin wear (n = 20) and roughness (n = 20) values found for all the treatments evaluated in the five-day experiment.

Group	Analyses	
	Wear (μm)	Roughness (Ra)
Control	0.64 (0.1)	0.19 (0.4)
0.05% NaF	0.65 (0.2)	0.17 (0.6)
0.1 % EGCG	0.66 (0.1)	0.17 (0.6)
Green tea	0.58 (0.2)	0.16 (0.8)

*To both analyses p > 0.05.

interface between the reference and eroded areas, while Figure 5 shows the eroded dentin for each treatment. In both figures, the effectiveness of the erosive cyclic challenge was confirmed. The morphology of the dentinal tubules is shown in Figure 5. Figure 5C and 5D display more obliterated tubules, which differ from the control group (Figure 5A) while Figure 5B (NaF) shows an intermediate obliteration.

Discussion

The current study investigated the efficacy of purified tea polyphenols (EGCG) and commercial green tea in reducing dentin erosion caused by acid, by using an *in vitro/in situ* cyclic erosive model. The simulated experimental design involved the application of treatment on already-eroded surfaces to simulate patients with erosive tooth wear.[20]

Commercial green tea and the isolated catechin (EGCG) were employed herein to precisely verify which compound could exert a more significant inhibitory effect on dentin MMPs and to determine whether the effect was due to one compound or a synergistic effect of polyphenols. The compounds of green tea have a different mechanism of action because of the presence of other polyphenols in the composition. Previous results strongly suggest that the protease inhibitors' preventive effects against dentin erosion are due to their ability to reduce the degradation of the demineralized organic matrix.[14] It can be speculated that these substances present a potential inhibition of MMPs, reducing proteolytic degradation of dentin, as demonstrated by the results of zymography and colorimetric assay. Additionally, polyphenols derived from plants, like green tea polyphenols, modify the collagen matrix improving mechanical properties and resist enzymatic degradation.[16,18,35] According to the present results, green tea infusion and 0.1% EGCG might emerge as powerful tools for preventing the progression of erosive lesions.

SEM was used to obtained information that positively contributed to the observed features of dental wear lesions.[36] Herein, based on SEM analysis, the green tea solution and EGCG (Figure 4) prevented erosive dentin wear, as previously demonstrated.[9,11,20,21] Hydroxyl groups can cause the preventive effect induced by EGCG and other catechins, which have a chelating effect on metallic ions and thus result in the formation of a protective layer (Figure 5C and Figure 5D). This analysis confirmed the qualitative results of

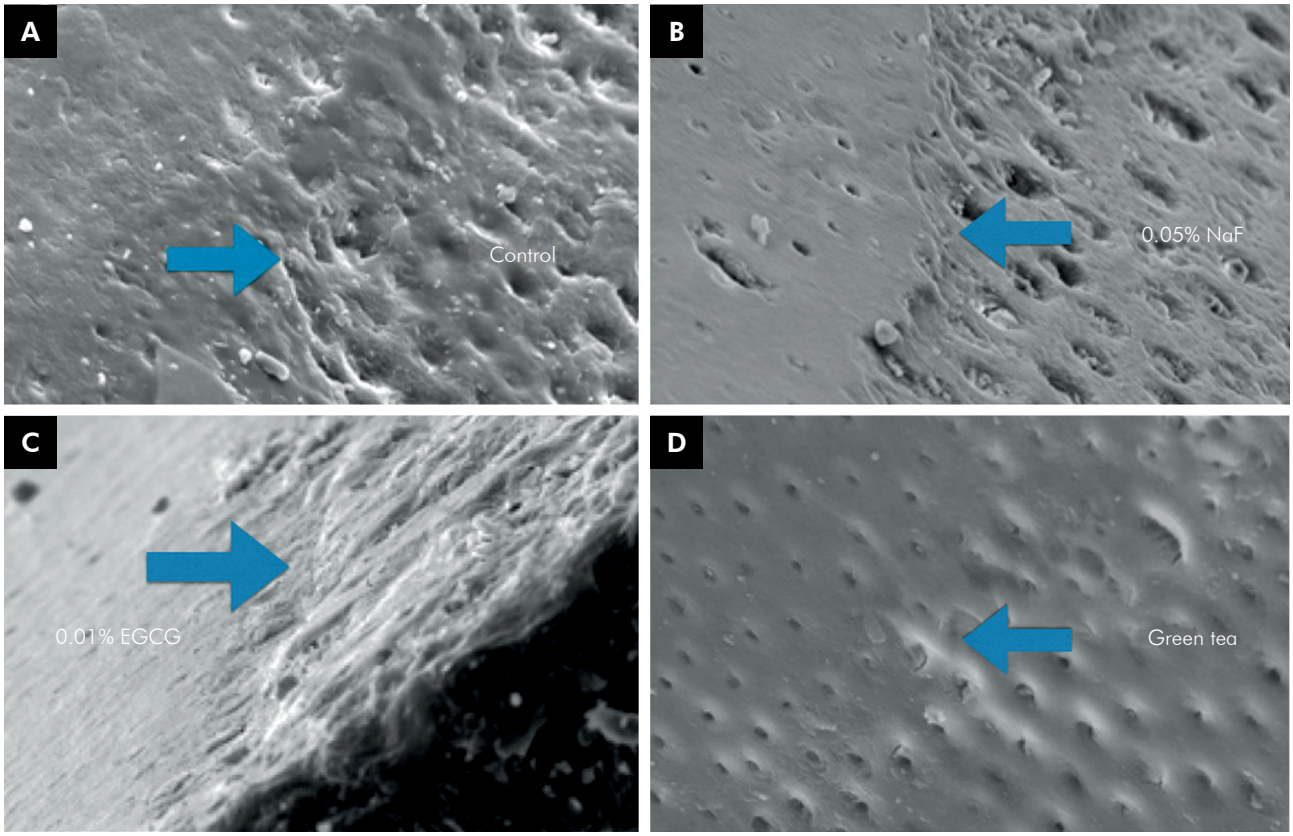

Figure 4. Representative scanning electron microscopy (SEM) (magnification 4000x) images of the interface between the reference (left) and eroded (right) area that were not treated (negative control) (A), or treated with NaF 0.05%(B); EGCG 0.1%(C); and Green tea solution (D). The arrow represents the interface between the reference and eroded area.

this study as EGCG and green tea were demonstrated to protect against dentin wear by inhibiting MMP (Figure 3). In the SEM analysis (Figure 4), rinsing with 0.1% EGCG and the green tea infusion solution led to less wear than that observed with the control and NaF solutions. However, the differences between these solutions were not significantly demonstrated by profilometric analysis. Before the *in situ* study, specimens were subjected to a 12 h-acquisition of salivary pellicle. As a result, the salivary fluid was found to prevent direct contact with acids. Despite the use of a validated methodology herein, a more intense erosion challenge would enable a better assessment of the potential differences between the groups. However, the size of the diamond tip may make it difficult for the profilometer to identify subtle peaks and valleys.[37,38] One possible explanation for the lack of difference between the tested solutions and the negative control is the presence of the control group in each phase of the study. Accordingly, the control could have been contaminated by the treatment solutions. Another limitation of the current *in situ* model is that it does not evaluate the abrasive effect of the dentifrice. Nonetheless, the no abrasive processwas intentionally performed to understand the real actions of each tested product.

An NaF mouthrinse and CHX, which are widely used in oral hygiene, were applied herein. Further, the most appropriate analyses used in studies on erosion were employed.[39] The results of the colorimetric assay were not found to corroborate with those of a zymography study where salivary MMPs were clearly found to be inhibited by fluoride and CHX.[15] This contrasting result may be caused by the use of a different source of MMPs from dentin; most studies were found to use isolated MMP.[10,11,15] Although fluoride solutions are widely employed to prevent dental erosion, NaF was not found to be effective in

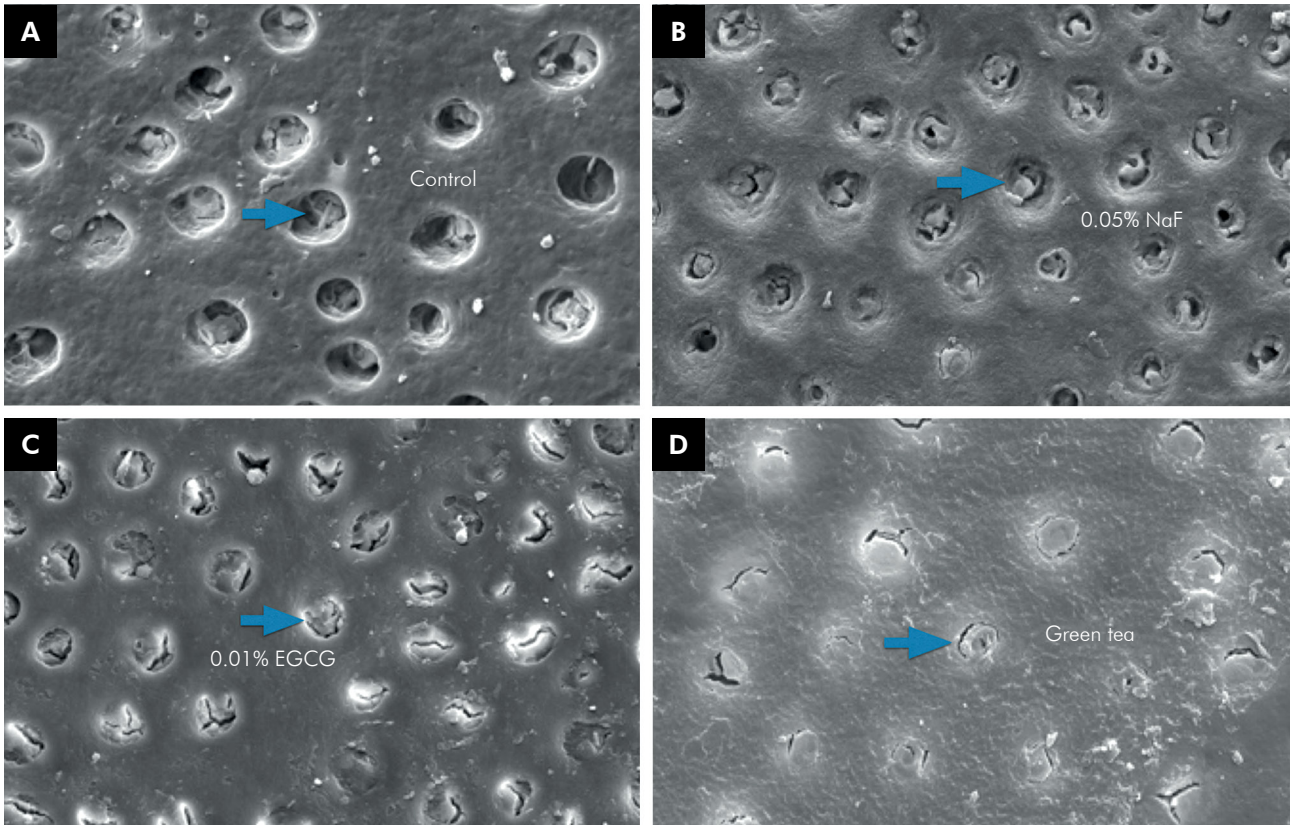

Figure 5. Scanning electron microscopy (SEM) (magnification 4000x) images of eroded dentin that was either not treated (A), or treated with NaF 0.05% (B); EGCG 0.1% (C); and Green tea solution (D). The arrow represents the morphology of the dentinal tubules. Figure 3C and 3D display more obliterated tubules, which differ from the control group (Figure 3A), while figure 3B (NaF) displays an intermediate obliteration.

preventing MMP-mediated collagen degradation.[40] Further, the result obtained with CHX differed from that of another study where loss was assessed by surface measurements.[41] Hannas et al.[41] investigated the erosive/abrasive process, which causes more considerable wear and facilitates the visualization of the difference between the tested groups. The MMP inhibitory effect of CHX in the zymography test was consistent with that observed previously.[13]

Herein, we did not investigate the mechanism employed by the tested substances to exert a direct effect on collagen degradation via the assaying of hydroxyproline or NMR spectroscopy. However, we verified the effect of the inhibitors on MMPs extracted from dentin. Based on the results of the zymography assays, 0.1% EGCG and green tea infusion were sufficient to inhibit MMP activities, as shown previously.[11] Such findings were also confirmed by the colorimetric assay, which revealed that the green tea infusion exerted the best inhibitory action against MMPs, similar to the standard inhibitor. This result can be justified due to the characteristic of bioactivity that has been reported for polyphenols from plants.[18] It is an approach to improve the mechanical properties of collagen and prevent enzymatic degradation.[35] The inhibition of proteolytic activity by green tea catechin provides new insights into its protective effect against dentin erosion. Altogether, our findings can encourage new investigations into the effect of natural agents on erosion-induced demineralized dentin.

Conclusions

Based on the findings obtained herein, the green tea infusion and EGCG could inhibit the activity of endogenous proteases and protect against

erosion-induced dentin damage; however, these treatments could not prevent the loss of tooth tissue.

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