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Active compounds and derivatives of *camellia sinensis* responding to erosive attacks on dentin

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Abstract: This research explored the potential of *Camellia sinensis*-derived teas and active compounds to be used as treatments to prevent dentin wear. Human root dentin slabs were randomly assigned to 5 groups (n = 10) as follows: distilled water (DW, control), epigallocatechin-3-gallate (EGCG), theaflavin gallate derivatives (TF), commercial green tea (GT), and commercial black tea (BT). The samples were submitted to a pellicle formation and an erosive cycling model (5x/day, demineralization using 0.01 M hydrochloric acid/60 s) followed by remineralization (human stimulated saliva/60 min) for three days. The samples were treated for 5 min using the test group solutions between the erosive cycles. Dentin changes were assessed with profilometry analysis and FT-Raman spectroscopy. The data regarding wear were analyzed by ANOVA followed by Tukey's test ($p < 0.05$). EGCG, TF derivatives, and both regular teas significantly suppressed erosive dentin loss (38–47%, $p < 0.05$). No obvious changes in the Raman spectra were detected in the specimens; however, the DW group had a minor relationship of 2880/2940 cm^{-1}. The phenolic contents in both green and black tea and the important catechins appear to have protective effects on dentin loss.

Keywords: Dentin; Tooth Erosion, Matrix Metalloproteinases.

Introduction

Camellia sinensis is a species of evergreen shrub or small tree whose leaves and leaf buds are used to produce tea. The resulting tea is consumed by more than two-thirds of the world's population. In addition, the tea has an attractive aroma, good taste, and health-promoting effects. These benefits make tea one of the most popular drinks in the world.[1] The infusion of *C. sinensis* is used in thousands of different teas based on how the leaves are cultivated, collected, prepared, and packaged. However, green and black tea are the most widely used. Tea can be studied in the form of leaves taken directly from the plant or as fully fermented commercial tea.

Compared with black teas, green teas are abundant in catechins, because during the fermentation process of black tea (well-fermented), polyphenol oxidase results in the formation of reddish-orange colored dimeric theaflavins (TFs), and the catechins are degraded. This is in contrast to the amount of catechins in unfermented green teas, which remain unaltered.[2] Unfermented green tea contains the basic tea leaf

polyphenols epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG). Epigallocatechin-3-gallate is the major constituent of green tea, whereas theaflavins are constituents of black tea and are derived from catechins as a result of fermentation. Tea leaves also contain caffeine, theanine, myricetin, quercetin, and kaempferol, which are examples of alkaloids, amino acids, and flavonols.[3] The diverse chemical composition of tea has been explored in different biomedical fields, with a focus on its antioxidant, anticancer, and anti-inflammatory potential.[4,5] In conservative dentistry, the beneficial effects of these products have only recently been suggested for the management of a pertinent contemporary disease.

Erosive tooth surface loss caused by non-bacterial acids, particularly to dentin substrate, which presents reduced mineral content, has been shown to be increasingly prevalent, and its damaging effects are emerging as a serious public health issue. Treatments may include the application of fluoride, which has been found to be particularly beneficial at the early stages of erosion, but not wholly preventive.[6,7] There is a growing body of evidence suggesting that commercial green tea and green tea extract rinses can reduce erosive and erosive/abrasive dentin wear caused by extrinsic acids.[8,9,10] However, few studies have examined the effects of *C. Sinensis* tea derivatives/active compounds on dentin after erosion caused by hydrochloric acid.

In particular, epigallocatechin, theaflavins, and theaflavins digallate have been found to have inhibitory activity against collagen-degradable enzymes;[11,12] this is relevant when considering dentin as the target tissue. An erosive challenge to dentin results in exposure of the organic matrix, which acts as a diffusion barrier with a buffering capacity to retard or stop further erosion. In the absence of a collagen matrix, the acid can easily penetrate dentin, causing severe mineral loss even in the presence of high amounts of fluoride.[13,14] Therefore, matrix metalloproteinase (MMPs) that are present in dentin and saliva can degrade this matrix[15] and subsequently increase dentin erosion.

Intrinsic sources of erosive acids, such as hydrochloric acid, are very frequently observed in patients who suffer from eating disorders, such as recurrent vomiting, regurgitation, gastroesophageal reflux, or chronic alcoholism. These issues have become a significant challenge for the dentist in terms of diagnosing the condition, identifying the etiological factors, executing an adequate treatment, and instituting preventive measures.

Therefore, a precise and straightforward in vitro study was carried out, along with an evaluation of fluoride content, to investigate the protective effects of *C. sinensis* tea derivatives/active compounds on dentin. The null hypothesis was that the polyphenols found in commercially available teas or in their isolated, purified forms, such as EGCG, would not be able to reduce dentin erosion in vitro.

Methodology

Sample preparation

The collection and use of extracted human teeth for all of the experiments conducted in this in vitro study were approved by the local Research and Ethics Committee (protocol #175/10). Root dentin specimens (4 x 4 x 2 mm) were prepared from human third molars that had been stored in 0.01% (w/v) thymol solution at 4°C. Dentin slabs were cut, flattened, and polished, as previously described by Melo et al.[16] The teeth were evaluated for surface microhardness using a Future-Tech FM microhardness tester (Knoop diamond, 25 g, 5 s, FM100, Future-Tech Corp., Tokyo, Japan) prior to randomized distribution. Fifty slabs with a mean hardness of 55.39 ± 6.48 kg/mm^2 (from 40 to 70 kg/mm^2) were selected and allocated to the following five treatment groups (n=10) according to a computer-generated randomized list: (1) distilled water (DW, negative control); (2) 0.40 mg/ml of epigallocatechin gallate (EGCG, positive control); (3) 0.03 mg/ml of theaflavin gallate derivatives (TF); (4) commercial green tea (GT); and (5) commercial black tea (BT).

To maintain the reference surfaces for lesion depth determination by profilometry, two layers of an acid-resistant varnish in a dark color (Colorama, CEIL Coml. Exp. Ind. Ltd., São Paulo, SP, Brazil) were applied on half of the surface of each specimen.

Preparation of teas and isolated polyphenols

During the experiment, green and black teas were prepared according to the manufacturer's instructions. Thus, an infusion of 2 g of *C. sinensis* leaves (Dr. Oetker, São Paulo, Brazil) in a sachet was diluted in 180 ml of boiled water for 5 min.

The green tea extract solution was prepared by mixing 4 mg of EGCG powder (Sigma-Aldrich, St. Louis, USA) with 10 ml of distilled water. To prepare the black tea extract solution, 0.3 mg of theaflavin gallate (Sigma-Aldrich) derivatives were diluted in 10 ml of distilled water.[2]

Experimental design

Before starting the erosive procedures, the slabs were immersed in human saliva for 2 hours to form a salivary pellicle. Over the next 3 days, erosive challenges were performed 5x daily by immersing each slab in 3 ml of hydrochloric acid (0.01 HCl M, pH 1.96) for 60 s. Subsequently, the specimens were rinsed with deionized distilled water, and 5 µl of each product used for treatment was applied under constant agitation for 5 min. The samples were subsequently remineralized in stimulated saliva under constant agitation for 60 min. After completion of the daily cycles, the samples were stored in stimulated saliva at 37°C until the next experimental day. The cycle described above was repeated 5x daily for 3 days at room temperature (Figure 1).

Pellicle formation

Whole saliva was collected daily 1 h after breakfast from 10 healthy volunteers. These volunteers had a normal salivary flow rate, and good general and oral health, with no active carious lesions, no need for periodontal treatments and no fixed or removable orthodontic devices. The subjects chewed paraffin wax for 10 min to stimulate saliva secretion. Next, the saliva was collected and centrifuged according to the methodology described by Melo et al.[16] It was then stored in 5 ml aliquots at −80°C until the time of the daily experiments.

Each slab group was independently immersed in clarified human saliva (5 ml per specimen) and incubated under agitation for 2 hours at room temperature (24 ± 1°C) prior to the erosive/treatment procedures.[16]

Tissue loss measurements

After the experimental period, the nail varnish surface layer was carefully removed with a scalpel blade without touching the dentinal surfaces. The dentin slab treatments and parameters that were used to assess the degree of wear were described by Passos et al.[17]

Dentinal wear was determined in relation to the reference surfaces using a profilometer (Hommel Tester T1000, Hommelwerke GmbH, VS-Schwenningen, Germany). The wear assessments were standardized with parameters of Lm = 1.5 mm and Lc = 0.25 mm, with Lm=extension considered, and Lc = cutoff. The device has an accuracy of 0.01 µm, and the diamond stylus has a radius of 5 µm. The constant speed was 0.15 mm/s under a force of 0.8 mN. Five readings were performed on each slab at intervals of 100 µm. These profilometric traces were obtained by moving the stylus from the reference to the exposed surfaces. For each sample, the mean surface loss was calculated from the values obtained from the 5 traces.

Determination of fluoride concentration

The concentration of fluoride in each test substance was evaluated in order to assess any possible of the influence of fluoride in preventing erosion.

The solutions prepared in the laboratory were added to TISAB II (1.0 M acetate buffer, pH 5.0, containing 1.0 M NaCl, and CDTA at 0.4%) in a 1:1 proportion. The analyses were performed in duplicate. Fluoride (F^-) determination was performed with a specific electrode (ORION 96-06; Thermo Scientific, Inc.) and an ion analyzer (EA 940, Thermo Scientific) that was previously calibrated with standards containing 0.1 to 2 µg of F^-/mL prepared from a 100 ppm F^- standard (ORION 940907, Thermo Scientific).

FT-Raman spectroscopy analysis

The treated dentin was analyzed by FT-Raman. An FT-Raman spectrometer (Bruker VERTEX 70 FTIR/FT-Raman spectrometer, Bruker Optics Inc., Ettlingen, Karlsruhe, Germany) with a liquid nitrogen-cooled Ge detector was used to collect the data. To excite the

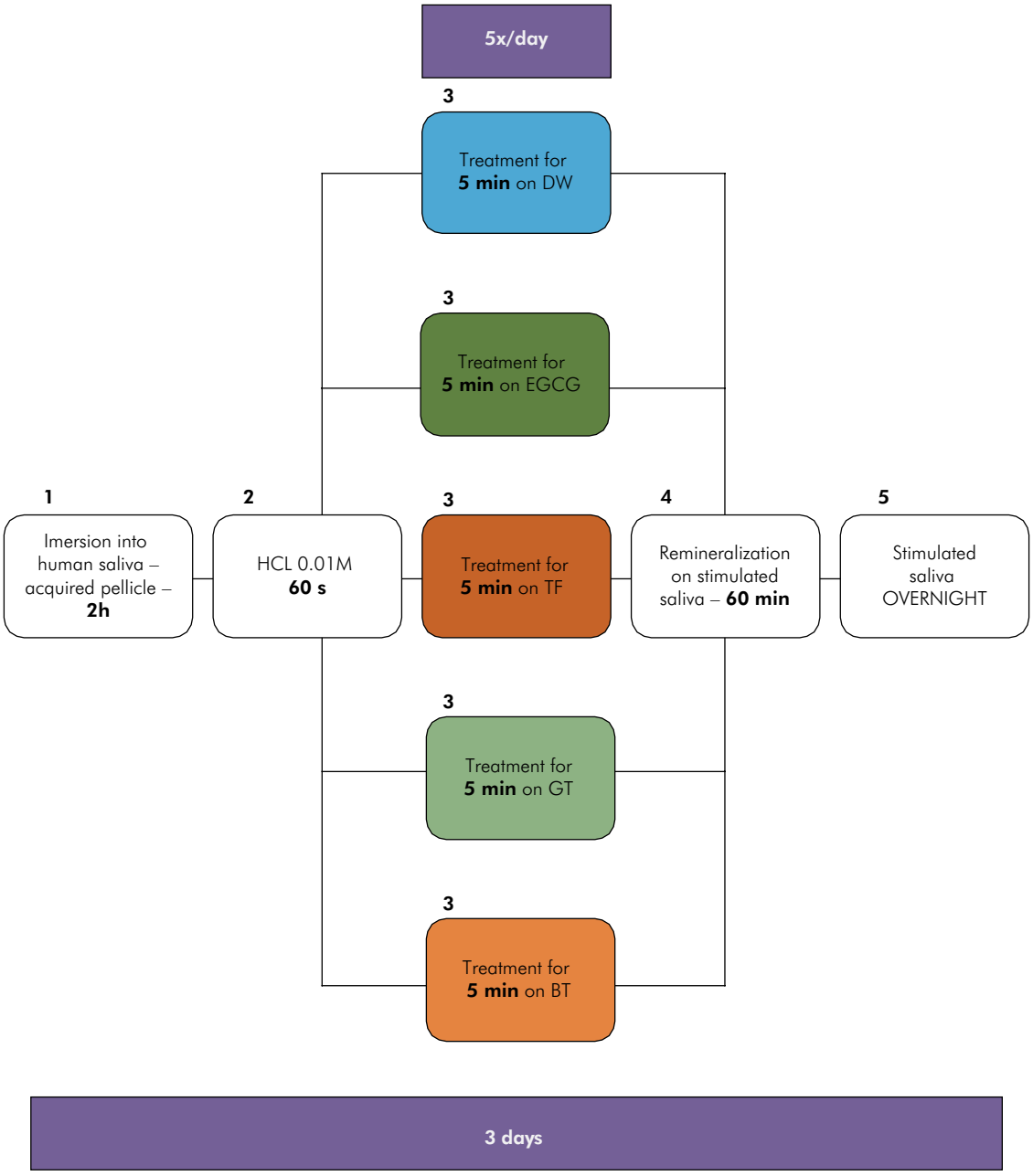

Figure 1. Experimental design: erosive procedures.

spectra, an air-cooled Nd: YAG laser (λ ¼ 1064.1 nm) source was used. The power of the Nd: YAG laser incident was 100 mW at a spectral resolution of 4 cm^{-1}. The Raman system was calibrated with a silicon semiconductor using a Raman peak of 521 cm^{-1}. The spectrum of the specimen was obtained and then analyzed by selecting a range from 400 to 4000 cm^{-1}. Distribution of the organic components was examined using the peak area of the CH stretch band between 2940 and 2880 cm^{-1}, and this band was used for image analysis. A qualitative spectral analysis of the changes in organic content was performed. The spectra in the region of interest, between 2880 and 2940 cm^{-1}, were analyzed with analytical software (Microcal Origin® 5.0 Software, Inc., Westborough, USA). The CH stretching mode (2880 and 2940 cm^{-1}) was used

in this study to detect the collagen component as a signature of organic content in dentin samples.[18]

Statistical analysis

The mean and standard deviation (SD) of wear per group were calculated. Statistical procedures were performed using the Statistical Package for the Social Sciences (SPSS 17.0) for Windows. A Kolmogorov-Smirnov test was applied to all the groups to check for the normal distribution of errors. Since the values were routinely distributed across all the groups, ANOVA and Tukey's post hoc test were used for comparative purposes. The level of significance was set at 5%.

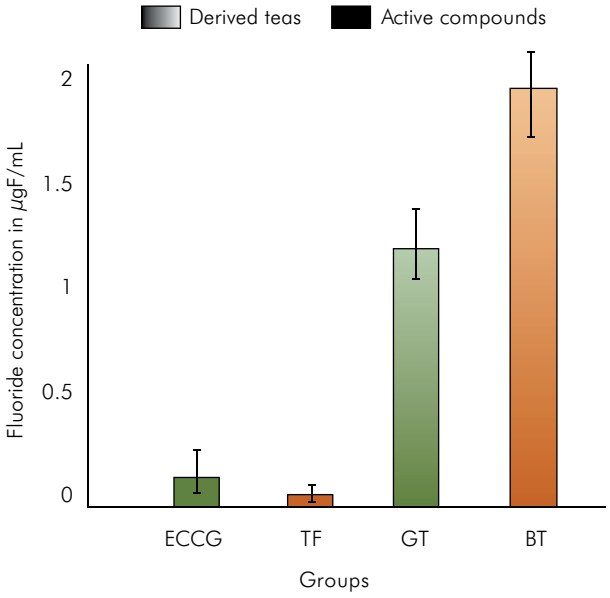

Figure 2. Mean (± standard deviation) fluoride concentrations (µg/mL) found in the different treatment solutions.

Results

The mean fluoride concentrations (µg/mL) found in the treatment solutions were as follows: EGCG (0.14), TF (0.02), GT (1.17), and BT (1.88); these values are presented in Figure 2.

The mean (standard deviation) wear values (µm) for the negative control (DW), EGCG (positive control), TF, GT, and BT were 0.65 (0.19), 0.40 (0.14), 0.39 (0.09), 0.34 (0.13), and 0.37 (0.07), respectively. After 3 days of erosion and treatment cycles, the highest mineral losses occurred in the control group and were significantly different from the other groups ($p < 0.05$). However, no statistically significant difference was found between the commercial teas and the isolated polyphenols. The mean dentinal loss of all the experimental groups is presented in Figure 3.

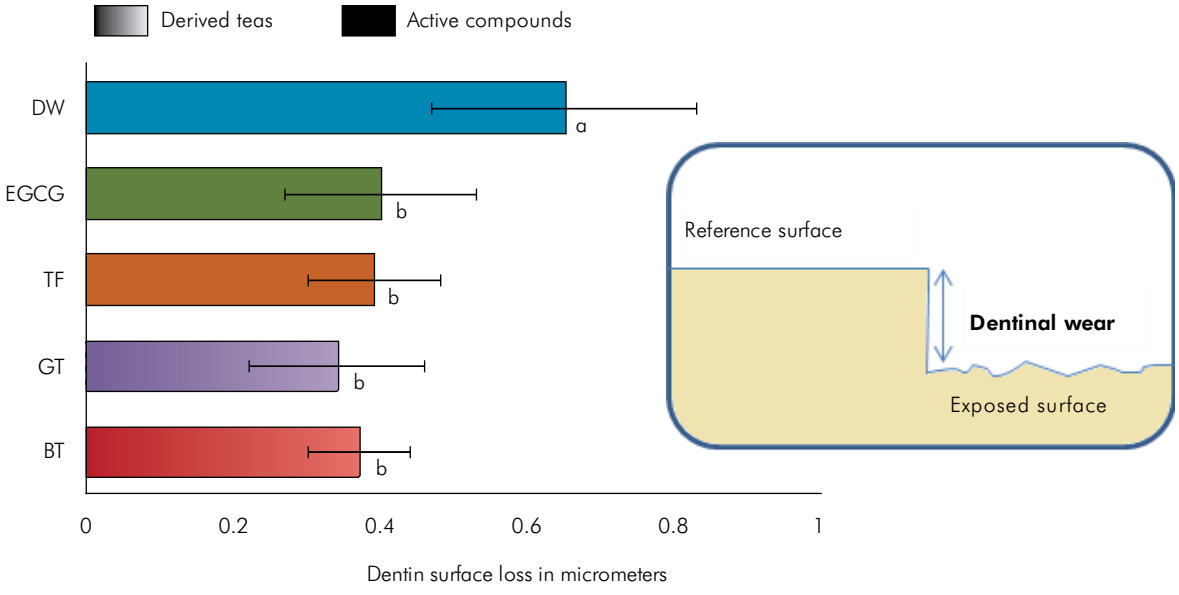

Figure 3. Mean values of wear (µm). Horizontal bars and lines denote wear differences in the groups studied and the standard deviations, respectively. Different letters denote statistically significant differences identified via the Tukey test.

Selected peaks of the Raman spectra from the organic content of the dentin specimens are shown in Figure 4. The CH stretches of collagen are evident in the 2940–2880 cm^{-1} region. This analysis also allowed us to establish a quantitative relationship between mineral content, carbonate distribution, collagen denaturation and carbonate substitution patterns to create a mineral-to-matrix ratio. The 2880/2940 cm^{-1} ratio of all the groups determined by the FT-Raman spectra is shown in Table 1.

Discussion

The current study investigated the efficacy of purified tea polyphenols (epigallocatechin gallate and theaflavin gallate derivatives) and commercial green and black tea for reducing dentin erosion cause by hydrochloric acid using an in vitro cyclic erosive model. The concentrations of tea extract were based on the average levels of epigallocatechin gallate and theaflavin gallate present in commercial teas.[2]

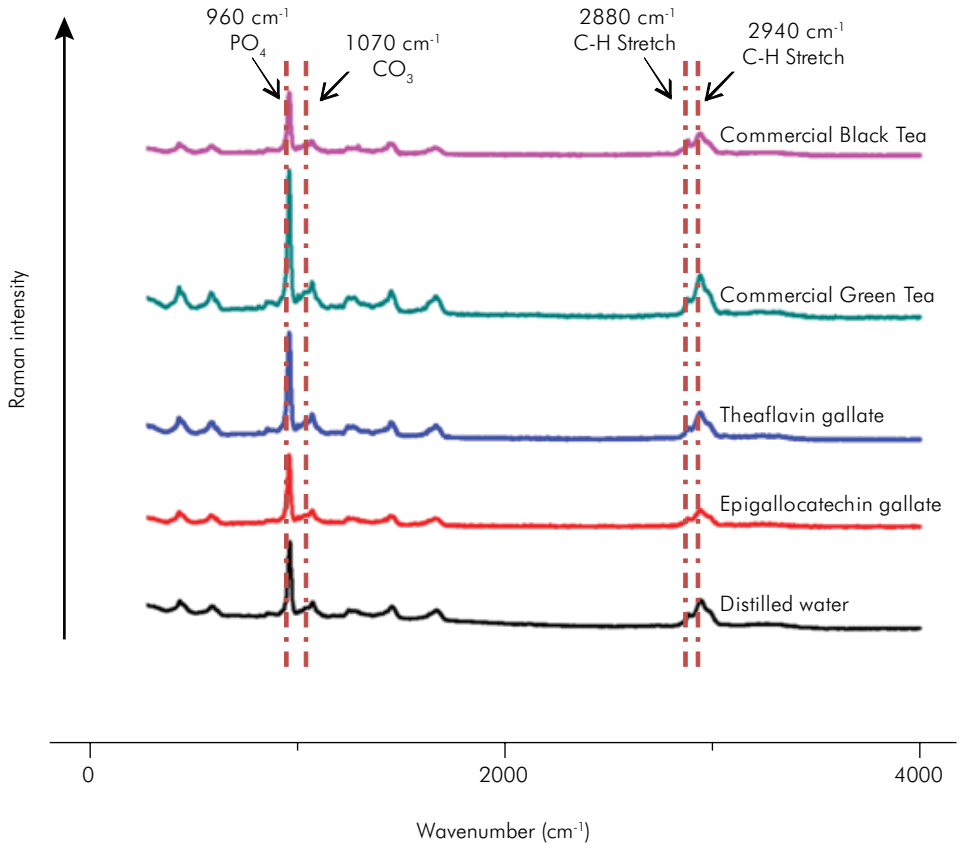

Figure 4. Line scans of representative FT-Raman spectra of dentin after experimental treatment in samples from each group. No obvious changes in the Raman spectra were detected in the specimens.

Table 1. 2880/2940 cm^{-1} ratio of all the groups determined by FT-Raman spectra.

Organic ratio	DW	ECGC	TF	GT	BT
Amide III/CH_2 wag ratio (2880 cm^{-1}/2940 cm^{-1})	0.509	1.423	1.020	1.328	1.160

The use of MMP inhibitors, such epigallocatechin-3-gallate,[12] has been accepted as an effective strategy for reducing dentin mineral loss during erosive challenges,[8,19,20,21] which justifies the choice of EGCG as a positive control. To our knowledge, this is the first study to show the ability of theaflavin gallate derivatives and commercial black tea to reduce dentin erosion.

Regarding the results obtained with respect to dentin wear, the highest degree of tissue loss was found in the negative control group. Therefore, all of the polyphenol groups had reduced tissue loss, and the null hypothesis was rejected.

The results of the GT and EGCG groups were in accordance with previously reported studies that showed protective effects of EGCG.[8,10,19,20,21,22] The protective effects of these products are based on their potent inhibitory activity against MMPs.[12,23] The reduction of enzymatic removal of the organic layer significantly reduces the demineralization process, since the collagen layer serves as a protective barrier. In the present study, other natural products (BT and TF) were able to reduce dentin erosion under experimental conditions. Although the mechanism of action of these products for reducing dentin erosion was not directly analyzed in this study, a correlation was suggested. In the study by Sazuka et al.,[11] black tea theaflavins were found to inhibit matrix metalloproteinases. The results from their study showed that theaflavin and theaflavin digallate inhibited the type IV collagenase activity of MMPs, including MMP-2 and MMP-9. Furthermore, TFs were reported to have antioxidant[24] and antimutagenic activity.[25]

Erosive alterations of dental tissue are mostly investigated in vitro or in situ. In this in vitro study, a profilometer was used to detect mineral loss in prepared specimens. Profilometry is a well-evaluated technique with high precision that is widely used in studies of dentin erosion;[8-,9,100,14,17] however, some studies claim that this measurement underestimates complete substance loss, possibly due to detection of the organic layer surface.[26] This study set out to evaluate only the remaining dentin after erosive attack; profilometry has previously been shown to be the most effective technique for this purpose.[26] To minimize any disparity among the dentin substrates, the specimens were evaluated with respect to the baseline surface microhardness to establish standardization of the dentin substrate. Although this measurement can also be used to determine changes in dental tissue after simulated erosion, this method has proven to be ineffective for this type of erosive evaluation due to the difficulty in taking precise measurements in erosively altered dentin.[26]

Despite the fact that there is no consensus in the literature regarding the best protocol for simulating intra-oral erosion, the cyclical model seems to better reflect acid attacks in the oral cavity due to the critical role of saliva in the remineralization process.[27] To simulate intra-oral erosion, an erosive challenge based on pH cycling was performed, involving an acquired pellicle formed from human saliva prior to the cycles and maintenance of the specimens in artificial saliva in between treatments. In general, in vivo tooth exposure to intrinsic or extrinsic acids is recurrent but lasts no longer than a few minutes;[27] thus, the current study used exposure to an acidic solution for 60 s, 5x per day for three days.

Acid attacks lead to an irreversible loss of the outermost dentin layer and partial demineralization (softening) of the tooth surface.[28] The main difference between enamel and dentin erosion reflects the structural difference between the two tissues. In dentin, acid causes rapid dissolution of mineral, but the organic portion of dentin is not degraded.[28] Beneficially, this resulting demineralized organic layer prevents the diffusion of new acids in dentin and seems to have a protective function,[13,14] even in models associated with abrasion,[29] which is of interest for future studies.

This theory has been associated with low pH values and further reduction of demineralization in the presence of high amounts of fluoride.[13] This study evaluated the fluoride concentrations of the studied solutions, and none of the experimental groups showed a high concentration of fluoride; the highest prevalence was commercial black tea with a concentration of 1.88 ppm. This evaluation, which was previously performed, showed that a 250 ppm fluoride solution reduced the erosive demineralization of dentin.[30]

The effectiveness of fluoride for inhibiting dentin erosion appears to be highly dependent on the organic matrix. The literature has previously demonstrated the ability of F^- to inhibit the human gelatinases MMP-2 and MMP-9 (reversible and irreversible inhibition at 250-1,500 ppm of F^- and 5,000 ppm of F^-, respectively).[31] Considering the low fluoride concentrations used in the tested solutions, this was not a relevant variable in our findings.

Therefore, by attributing the results solely to the solutions tested, we can hypothesize that the polyphenol solutions may have limited the action of MMPs on the collagen fibrils exposed by demineralization, thereby reducing the progression of dentin loss. This shows the potential of naturally derived substances as therapeutic/preventive medical and dental treatment alternatives. Raman spectroscopy is a powerful tool for studying the structural alterations of dental tissues[32]. It is widely applied in the biological field and does not require destructive preparation to obtain chemical composition information[33] thereby allowing the identification of degradation of collagen structure without affecting the samples. According to the results, the FT-Raman experiments were partially supportive of our findings. Type I collagen accounts for 90% of the dentin protein fraction. There was no significant difference in these respective spectra among the groups; there was only was a difference in the amide III/CH2 wag ratio (2880 cm^{-1}/2940 cm^{-1}), and the control group (distilled water) had lower values than the other groups. Most likely, these lower values in the DW group might be related to an imbalance in the amount of organic content in the specimens.

In addition, adverse effects were not observed in rats treated with concentrated solutions of EGCG (90%) for 13 weeks in doses of 500 mg/kg per day.[34] In a human study, green tea polyphenol products with quantities equivalent to the EGCG content in 8 to 16 cups of green tea taken per day for four weeks only seemed to induce transient adverse effects and were considered safe.[35] Although one study evaluated the use of black tea in rats and demonstrated the safety of 2 g/kg tea solution over a follow-up of 90 days,[36] human studies are needed to validate the safety of TF and black tea.

Conclusion

The phenolic contents in green and black tea and their relevant catechins appear to have a protective effect on dentin loss, indicating that the effects of *Camellia sinensis* tea derivatives/active compounds for inhibiting erosive attacks on susceptible hard-tooth substrates are worth exploring; however, further studies still need to be undertaken to elucidate the mechanism of action of this process and to consider other variables, such as the inhibition of MMPs and the effect of the buffer of the organic matrix.

These active ingredients may be promising for the prevention and management of non-carious lesions.

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