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Chlorhexidine and green tea extract reduce dentin erosion and abrasion in situ

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ABSTRACT

Objectives: This in situ/ex vivo study aimed to analyse the impact of possible MMP-inhibitors (chlorhexidine and green tea extract) on dentin wear induced by erosion or erosion plus abrasion.

Methods: Twelve volunteers took part in this cross-over and double-blind study performed in 4 phases of each 5 days. Bovine dentin samples were worn in palatal appliances and subjected to extraoral erosion (4 times/day, Coca-Cola, 5 min) or erosion plus abrasion (2 times/day, fluoride-free toothpaste and electrical toothbrush, 15 s/sample). Immediately after each erosion, the appliances were reinserted in the mouth and the oral cavity was rinsed for 60 s with: 250 ppm F solution (SnF_2/AmF, pH 4.5, Meridol-Gaba, Switzerland), 0.12% chlorhexidine digluconate (0.06% chlorhexidine, pH 6.0, Periogard-Colgate, Brazil), 0.61% green tea extract solution (OM24®, 100% *Camellia Sinensis* leaf extract, catechin concentration: 30 ± 3%, pH 7.0, Omnimedica, Switzerland) or deionized water (pH 6.0, control). Dentin loss was assessed by profilometry (μm). The data were analysed by two-way repeated measures ANOVA and Bonferroni *post hoc* test.

Results: There was a significant difference between the conditions (Ero × Ero + Abr, $p < 0.001$) and among the solutions ($p < 0.001$). All solutions (F: 1.42 ± 0.34; 1.73 ± 0.50, chlorhexidine: 1.15 ± 0.26; 1.59 ± 0.32, green tea: 1.06 ± 0.30; 1.54 ± 0.55) significantly reduced the dentin wear when compared to control (2.00 ± 0.55; 2.41 ± 0.83) for both conditions. There were not significant differences among green tea extract, chlorhexidine and F solutions.

Conclusions: Thus, the possible MMP-inhibitors tested in this study seem to be a promising preventive measure to reduce dentin erosion-abrasion, but their mechanism of action needs to be investigated in further studies.

1. Introduction

Dental erosion is defined as substance loss by exogenous or endogenous acids without bacterial involvement. Enamel erosion is predominantly a surface phenomenon with a centripetal mineral dissolution process until the underlying dentin is exposed. In dentin, the presence of the demineralised organic hampers ionic diffusion and, thus, decreases the progression of erosive substance loss.[1,2] Previous in vitro studies showed that the enzymatic removal of the organic

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matrix by collagenase significantly increased the demineralisation process.[2,3] Considering that proteolytic enzymes like pepsin or trypsin are present in gastric juice, it was assumed that the erosion process in patients suffering from frequent vomiting and chronic reflux disease is at least partly affected by the degradation of the organic matrix.[4,5]

The organic degradation of dentin might also be affected by other host-derived enzymes, such as matrix metalloproteinases (MMPs), which are present in saliva and dental hard tissues.[6] Two recent reviews summarized the role of MMPs in the oral environment and especially in the pathogenesis of dental caries.[7,8] Thus, host-derived MMPs localized in saliva and dentin could be responsible for the matrix degradation in dentinal caries lesions. Especially, collagenase MMP-8 and gelatinases MMP-2 and -9 are related to the degradation of the collagen matrix in dentin. Tjaderhane et al.[9] showed that the latent forms of these enzymes get activated when the pH drops in the presence of acids from cariogenic challenges. The subsequent neutralisation by salivary buffer systems enhances the degrading activity of the organic matrix.

Despite a lack of studies evaluating the role of MMPs in dental erosion, it might be speculated that similar processes to those in caries might affect the degradation of the dentinal organic matrix also under erosive conditions. As the maintenance of the organic matrix is desirable to decrease the progression of erosive dentin lesions,[2] it seems worth to analyse whether the application of potential MMP-inhibitors affects indirectly the progression of erosion by reducing the organic breakdown. Previous studies found that the application of potential MMP-inhibitors reduced the dentin collagen solubilisation[10] and diminished the progression of caries lesions in rats.[11] One potent MMP-inhibitor affecting MMP 2, 8 and 9 is chlorhexidine,[12] which was shown to reduce the degradation of the dentin hybrid layer[13] and the solubilisation of dentinal collagen.[10]

In recent years, the MMP-inhibitory potential of several naturally derived substances gained increasing attention. Green tea polyphenols, especially epigallocatechin-3-gallate (EGCG) were found to have distinct inhibitory activity against MMP-2 and -9.[14] Recently, it was shown that rinsing the oral cavity with green tea reduced dentin wear under erosive/abrasive conditions significantly better than rinsing with water.[15]

Taking into consideration the distinct inhibitory potential against MMPs and the easy application in the oral cavity of chlorhexidine (oral hygiene products) and green tea (food), the present study aimed to analyse and compare the effects of chlorhexidine and green tea extract solutions on the development of erosive dentin lesions in situ. The null hypothesis tested were that 0.12% chlorhexidine digluconate and 0.61% green tea extract solutions are not able to reduce dentin wear and are not as effective as a 250 ppm fluoride solution (positive control).

2. Materials and methods

2.1. Ethical aspects

Twelve healthy adult volunteers (11 female, 1 male, aged 23–34 years) who fulfilled the inclusion criteria (physiological salivary flow rates: stimulated: >1 ml/min, unstimulated: >0.25 ml/min; good oral health: no frank cavities or significant gingivitis/periodontitis) without violating the exclusion criteria (systemic illness, pregnancy or breastfeeding, use of fixed or removable orthodontic appliances, hyposalivation) were enrolled following CONSORT guidelines. Sample size calculation was based on previous in situ study.[15] A sample size of twelve volunteers was calculated considering α-error level of 5% and β-error level of 20% (www.ddsresearch.com).

The study conformed to the Declaration of Helsinki and was performed to the guidelines of good clinical practice. Ethical approval for the study involving human subjects was granted by the local Ethics Committee (Ethics Committee of the Bauru Dental School, University of Sao Paulo, Brazil, no. 011/2008). The study was planned as a prospective, single-center, double blind, and four-cell study in cross-over design with an overall experimental period of 4×5 days (washout period of 10 days). Participants received written instructions and a schedule and were extensively trained for all procedures. Informed consent was obtained from all volunteers prior to the study.

2.2. Sample preparation

Root dentin samples ($n = 192$, 4 mm × 4 mm × 2 mm) were prepared from extracted bovine incisors. The preparation procedures were described elsewhere.[16] Prior to the experiment, nail varnish was applied on half of the surface of each sample to maintain a reference surface for wear determination after the experiment. Each 4 samples were fixed with wax into the recesses of the individual acrylic palatal appliances. The position of the samples in the rows ERO and ERO + ABR was randomly determined for each volunteer (Excel, Microsoft, USA).

2.3. In situ experiment

Ten days prior to and throughout the entire experiment, the volunteers brushed their teeth with fluoride-free toothpaste (Crest, Procter & Gamble, USA). The volunteers wore the appliance for 12 h prior to the start of each phase to allow for the formation of a salivary pellicle. After the 12 h lead-in period, the regimens (erosion and rinse) were performed four times daily (morning, midday, afternoon and evening) with at least 4 h apart. The appliances were worn day and night and were stored in humidity during meals and oral hygiene (4 times daily, 1 h each) procedures. The participants were advised not to eat or to drink while the appliances were in place. A minimum of 30 min elapsed between individual toothbrushing and the experiment.

For erosion, the volunteers were instructed to immerse the appliance in a cup containing 150 ml Coca-Cola (pH 2.6, 0.32 ppm F, Coca-Cola Company, Brazil) at room temperature for 5 min. Immediately after erosion, the appliances were reinserted into the mouth and the volunteers rinsed for 60 s with 10 ml of the test solutions at room temperature: 250 ppm fluoride (positive control, AmF/SnF_2, pH 4.5, Meridol, GABA, Switzerland), 0.12% chlorhexidine digluconate (0.06% chlorhexidine, pH 6.0, Periogard-Colgate, Brazil), 0.61% green tea extract (OM24®, 100% *Camellia Sinensis* leaf extract, catechin concentration: EGCG, EGC, ECG, EC 30 ± 3%, pH 7.0, 0.39 ppm F,

Table 1 – Mean dentin loss (μm) ± S.D. in the different groups.

	Green tea extract solution[a]	0.12% chlorhexidine[a]	250 ppm F[a]	Control[b]
ERO[A]	1.1 ± 0.3	1.2 ± 0.3	1.4 ± 0.3	2.0 ± 0.6
ERO + ABR[B]	1.5 ± 0.6	1.6 ± 0.3	1.7 ± 0.5	2.4 ± 0.8

Distinct upper case letters indicate significant difference between the conditions. Distinct lower case letters indicate significant differences among the solutions.

Omnimedica, Zurich, Switzerland) or deionized water (pH 6.0, negative control). The green tea extract solution was prepared freshly prior to each rinsing by mixing 6.1 mg of the OM24® powder with 10 ml of deionized water. The volunteers received the weighed green tea powder by one of the researchers, and they were kindly asked to mixture with a pre-determined volume of water.

Twice daily, after the first and third erosive challenges and the rinse with one of the solutions, abrasion was performed in one row. Each sample was brushed extraorally with an electrical toothbrush (Colgate® Motions Multi-action, Brazil) for 15 s (166 oscillations/s) using one drop (around 35 μl) of a fluoride-free dentifrice slurry (Crest, Procter & Gamble, USA, Ratio dentifrice:water = 1:3, pH 6.8).

2.4. *Wear analysis*

Dentin loss (μm) was defined as outcome parameter. After 5 days, the samples were removed from the appliances and the nail varnish on the reference surfaces was carefully removed with acetone/water-soaked cotton wool.[16] The dentin samples were stored in humidity until the analysis to avoid shrinkage.

Dentin loss was assessed profilometrically (T1000 Tester, Hommelwerke, Schwenningen, Germany) in relation to the reference area by one trained examiner. Therefore, the diamond stylus moved from the reference to the exposed area (1.5 mm). The differences in the height between reference and exposed areas were quantified in microns. Five profile measurements were performed in the center of each specimen and averaged.

2.5. *Statistical analysis*

The assumptions of equality of variances and normal distribution of errors were checked for all the variables tested using the Bartlett and Kolmogorov–Smirnov tests (GraphPad Instat for Windows version 4.0, San Diego, CA, USA), respectively. Since the assumptions were satisfied, two-way repeated measures ANOVA and Bonferroni *post hoc* test were used (GraphPad Prism 4 version 4.0 for Windows, Graph Pad Software, San Diego, CA, USA). Thereby, the different solutions were considered as dependent and the conditions as independent variables. The significance level was set at 5%.

3. Results

All participants completed the study, and all samples could be measured profilometrically. There was a significant difference between the conditions ERO and ERO + ABR ($F = 19.34$, $p < 0.001$) and among the solutions ($F = 16.32$, $p < 0.001$). All tested solutions reduced the dentin wear significantly when compared to the control (deionized water) for both conditions ($p < 0.001$, Table 1). Although rinsing with chlorhexidine and green tea extract solution led to lesser wear when compared to the 250 ppm F solution, the difference between these solutions was not significant. For all the treatments, the wear was significantly higher when the specimens were also abraded, when compared to the condition erosion only ($p < 0.05$, Table 1).

4. Discussion

The results of this study showed that both chlorhexidine and green tea extract solutions were able to reduce dentin erosion under clinical conditions and were at least as effective as a conventional 250 ppm fluoride solution. Even though the degradation of collagen by MMPs could not be measured directly by this protocol, the results suggest that the inhibition of MMPs by chlorhexidine and green tea extract solution might be a possible mechanism of action leading to a reduction of dentin loss.

It is well known that MMPs become activated at a low pH and that their degrading activity is enhanced by the subsequent neutralisation of the pH.[9] In the present study, erosive demineralisation was performed by Coca-Cola, presenting a pH of 2.6. Due to the low pH of the soft drink, it might be assumed that the dentin-derived MMPs became activated and exhibited their degrading potential in the following remineralisation period. Both chlorhexidine and green tea extract solution were administered in a dose exceeding the inhibitory doses reported for MMP-2, MMP-8 and MMP-9.[12,14] Thereby, chlorhexidine was applied in the form of a commercially available mouthrinse, while the concentration of the green tea extract solution aimed to simulate the catechin content normally found in green tea. Different studies showed that the catechin amount in green tea solutions (1 g leaves/100 ml water) ranged from 20 to 130 mg.[17,18]

Demeule et al.[14] analysed the inhibitory doses of different green tea polyphenols on MMPs. Thereby, the IC_{50} values of MMP-2 amounts to 6 μM (EGCG) and 95 μM (ECG), whereas 0.8 μM (EGCG) and 28 μM (EGC) were reported as IC_{50} values for MMP-9. Chlorhexidine produced a complete inhibition of MMP-2 and MMP-9 at a concentration of 0.03% and of MMP-8 at a concentration of 0.02–0.01%.[12]

On the other hand, it seems worth to mention that the solutions were applied in doses excluding adverse long-term side effects. As shown by Isbrucker et al.,[19] the dietary administration of a highly concentrated EGCG preparation (90%) to rats for 13 weeks was not toxic at doses up to 500 mg/kg/day. In healthy individuals, green tea polyphenol products in amounts equivalent to the EGCG content in 8–16 cups of

green tea/day for a time period of 4 weeks seemed to induce only transient side effects and were considered to be safe.[20]

The LD_{50} value for the oral administration of chlorhexidine in mice amounted to 1800 mg/kg bodyweight.[21] The repeated daily application of 2% chlorhexidine to the oral mucosa of hamsters of up to 12 weeks did not induce histological or enzyme changes of the tissue.[22] Thus, besides from some reversible local side effects (discoloration, mucosal irritation, taste disturbance) chlorhexidine in oral hygiene is considered as safe.[21]

The inhibitory effect of chlorhexidine on MMPs (zinc-activated, calcium-dependent endopeptidase) is attributed to a chelating mechanism, since the inhibition of MMP-2 and MMP-9 could be prevented by the addition of calcium chloride binding chlorhexidine. It was also discussed that chlorhexidine might affect essential sulfhydryl groups and/or cysteine present in the active site of MMPs.[12] At salivary concentrations above 0.2%, the inhibitory action of chlorhexidine might be also related to a protein denaturation.[23]

Different mechanisms are also discussed for the inhibitory efficacy of green tea polyphenols, especially of EGCG, on the expression and the activity of MMPs. By hydrogen bonding and hydrophobic interactions with the collagenase, EGCG might lead to conformational changes or to masking of the catalytic region of MMP-2.[24,25] EGCG can also inhibit the activation of MMP-8,[26] which in turn was shown to affect the remineralisation of artificially demineralised dentin.[27]

In the present study, the chlorhexidine and green tea extract solution were at least as effective as the 250 ppm F solution, whose efficacy to reduce dentin erosive demineralisation was shown previously.[5,28] However, the efficacy of fluoride to inhibit dentin erosion seems to be highly dependent on the organic matrix. Ganss et al.[2] assumed that the organic matrix layer of demineralised dentin exhibit a buffering capacity which might prevent deeper dentin areas from low pH values and reduce further demineralisation in the presence of high amounts of fluoride. Therefore, it seems interesting to evaluate the combined effect of fluoride and MMP-inhibitors on dentin demineralisation in further studies, since it has already been shown that fluoride can alter MMP-20 activity in enamel.[29]

To simulate clinical conditions as closely as possible, the samples were brushed twice daily after the erosive treatment. Eroded dentin is known to exhibit a higher susceptibility to abrasion in situ,[16,30] which is also confirmed by the results of the present study. However, the organic matrix of dentin does not only impede ionic diffusion during demineralisation, but might also reduce the mechanical stress on the demineralised dentin. Ganss et al.[31] showed that an erosively demineralised organic dentin layer of distinct thickness (~50 μm) could not be removed by toothbrushing. Thus, it might be speculated that the inhibition of MMPs affects indirectly also the abrasion of eroded dentin by reducing its degradation.

In conclusion, the null hypotheses of the present study were rejected as the 0.12% chlorhexidine digluconate and 0.61% green tea extract solutions were shown to reduce dentin wear with being equally effective as the 250 ppm fluoride solution. Further studies are necessary to confirm the inhibitory action of these solutions on MMPs present in erosively demineralised dentin.

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