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Effect of 10% Proanthocyanidin gel on demineralized organic matrix degradation: ELISA method

Efeito do gel de Proantocianidina a 10% na degradação da matriz orgânica desmineralizada: método ELISA

Fabrícia CARDOSO[1], Gabriela Guarda DALLAVILLA[1], Ana Paula BOTEON[1], Angélica Feltrin dos SANTOS[1], Thiago José DIONÍSIO[2], Heitor Marques HONÓRIO[3]

1 - Universidade de São Paulo, Faculdade de Odontologia de Bauru, Departamento de Dentística, Endodontia e Materiais Odontológicos, Bauru, SP, Brazil.
2 - Universidade de São Paulo, Faculdade de Odontologia de Bauru, Departamento de Biologia Oral, Bauru, SP, Brazil.
3 - Universidade de São Paulo, Faculdade de Odontologia de Bauru, Departamento de Odontopediatria, Ortodontia e Saúde Coletiva, Bauru, SP, Brazil.

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ABSTRACT

Objective: This study evaluated Proanthocyanidin protective effect on dentin subjected to erosion and its inhibition on degradation of the demineralized organic matrix (DOM). **Material and Methods:** The tested groups were: G1 - 10% Proanthocyanidin gel (test group), G2 - 1.23% NaF (positive control 1), G3 - 0.012% Chlorhexidine (positive control 2) and G4 – Placebo (negative control with no active compound) and two methodologies were performed: contact profilometry and ICTP ELISA method. To quantify dentin wear, profilometry was performed. Data were submitted to Analysis of Variance followed by Fisher's LSD Test. To assess the collagen degradation, ICTP ELISA method was performed. Data were submitted to the Kruskal-Wallis followed by the Dunn´s test. Simple linear regression and Pearson Correlation test were also performed ($p<0.05$). **Results:** The profilometry showed significantly lower wear of G1 when compared to other groups and G2, G3 and G4, which did not present significant difference among them. In the ICTP ELISA analysis, G1 and G4 did not show significant differences and the same happened between G2 and G3. However, G1 and G4 had lower values of collagen degradation compared to groups G2 and G3. Data showed that degraded DOM is a significant predictor to explain the values obtained through the ICTP ELISA. **Conclusions:** The results allow to verify that 10% proanthocyanidin provided less tooth wear and decreased degradation of the DOM, suggesting a good ability to prevent dentin erosion. The regression analysis also suggests that contact profilometry is a good strategy to quantify dentin wear.

KEYWORDS

Dentin; Tooth erosion; Proanthocyanidins; Dental wear; Preventive health.

RESUMO

Objetivo: Este estudo avaliou o efeito protetor da proantocianidina na dentina submetida à erosão e sua inibição na degradação da matriz orgânica desmineralizada (MOD). **Material e Métodos:** Os grupos testados foram: G1 - gel de Proantocianidina 10% (grupo teste), G2 - NaF 1,23% (controle positivo 1), G3 - Clorexidina 0,012% (controle positivo 2) e G4 - Placebo (controle negativo sem princípio ativo) e duas metodologias foram realizadas: perfilometria de contato e método ICTP ELISA. Para quantificar o desgaste da dentina, a perfilometria foi realizada. Os dados foram submetidos à Análise de Variância seguida do Teste LSD de Fisher. Para avaliar a degradação do colágeno, foi realizado o método ICTP ELISA. **Resultados:** Os dados foram submetidos ao teste de Kruskal-Wallis seguido do teste de Dunn. Regressão linear simples e teste de correlação de Pearson também foram realizados ($p<0,05$). A perfilometria mostrou desgaste significativamente menor do G1 quando comparado aos outros grupos e G2, G3 e G4, que não apresentaram diferença significativa entre si. Na análise ICTP ELISA, G1 e G4 não apresentaram diferenças significativas e o mesmo ocorreu entre G2 e G3. No entanto, G1 e G4 apresentaram valores menores de degradação do colágeno em relação aos grupos G2 e G3. Os dados mostraram que a MOD degradada é um preditor significativo para explicar os valores obtidos pelo ICTP ELISA. **Conclusão:** Os resultados permitem verificar que a proantocianidina a 10% proporcionou menor desgaste dentário e diminuição da degradação da MOD, sugerindo uma boa capacidade de prevenir a erosão dentinária. Também sugere que a perfilometria de contato é uma boa estratégia para quantificar o desgaste da dentina.

PALAVRAS-CHAVE

Dentina; Erosão dentária; Proantocianidinas; Desgaste dentário; Saúde preventiva.

INTRODUCTION

Dentin is composed by 47% inorganic components (apatite), 33% organic components and 20% water. Among the organic components, 90% consists of type I collagen and 10% non-collagenous components: dentin phosphoproteins, proteoglycans and glycosaminaglycans [1-3]. Demineralization by erosion causes histological changes in dentin, starting with an external demineralization [4]. The dentin demineralization rate decreases when the amount of degradable collagen increases, thus, the maintenance of this collagen hinders the diffusion of acids to the tissue, minimizing erosion [5,6]. However, the organic matrix can be chemically degraded due to the presence in dentin enzymes called matrix metalloproteinases (MMPs), which can be activated by a drop in pH below 4.5 [7]. The degradation of the dentin organic matrix is only possible after neutralization of saliva pH, since, although these enzymes are activated at acidic pH, they do not have the capacity to degrade the dentin matrix at the same pH [7,8].

In addition to dentin demineralization, the drop in pH exposes collagen fibrils and activates MMPs that degrade the demineralized organic matrix (DOM). This process causes the progressive loss of dentinal tissue. Thus, the use of MMP inhibitors could reduce this mechanism during subsequent erosion challenges, as the organic matrix would function as a protective layer, reducing erosion progression [5,6]. Several studies have proven this mechanism of inhibition, showing a reduction in the progression of erosion when chlorhexidine [9], green tea [10] and other substances [11] were used as MMP inhibitory agents.

Currently, proanthocyanidin (PA) has drawn the attention of researchers as an alternative MMP inhibitor agent. It is a natural compound derived from fruits, vegetables, nuts, among others [12,13], which has lower toxicity when compared to synthetic products [14,15]. Studies have shown that PA has a great affinity with proline-rich proteins, such as collagen, in addition to being responsible for increasing the capacity of cells to synthesize collagen [12]. Other studies have characterized the behavior of PA on dentin, showing its effects on the resin-dentin bonding interface. In these cases, there was a significant improvement in adhesion and mechanical properties, making adhesive restorations more resistant and more long-lasting [16-18].

A recent *in situ* study demonstrated that PA can play an important role in preventing erosion after evaluating the effect t of a mouthwash on dentin submitted to erosion [19]. The mouthwash provided significantly lower wear values compared to placebo and the control group. This result may be due to the maintenance of a large part of the DOM intact after the erosive challenge. To demonstrate this effect, specific studies are needed to enable the analysis of the post-demineralization organic matrix, such as hydroxyproline dosage, zymography or ICTP Elisa [20].

Analysis by zymography allows visualization of enzymatic activity, but it is not possible to quantify the activity of MMPs on type 1 collagen [20]. The hydroxyproline analysis was used to determine collagen degradation [11,21], although its validity is also discussed due to subjectivity [21]. Another alternative to determine collagen degradation is the use of specific ICTP and CTX ELISA method for enzymes such as MMPs and cysteine cathepsins, respectively. Despite the high cost of the method, the advantage of this indirect approach is that one may assay the total protease activities of the dentin matrix in their natural bound state, and their responses to activators and inhibitors [20,22]. In addition, ICTP level determination is one of the most reliable techniques to quantify the activity of MMPs on type I collagen [20].

Therefore, this study aimed to evaluate the effect of 10% Proanthocyanidin in the form of a topical gel to minimize the wear of dentin submitted to erosion and to inhibit the degradation of the DOM dentin through contact profilometry and the specific ICTP ELISA analysis.

MATERIAL AND METHODS

Methodology 1

Experimental design

This cross-sectional simple-blind randomized study was approved by the Research and Ethics Committee of the Bauru School of Dentistry, University of São Paulo (Process: 94400218.8.0000.5417).

Human molar teeth were used in this study and dentin specimens obtained were randomly allocated into 4 groups: G1 - 10% Proanthocyanidin gel (test group), G2 - 1.23% NaF (positive control 1), G3 - 0.012% Chlorhexidine (positive control 2) and G4 – Placebo (negative control with no active compound).

Before the treatment, samples were demineralized by immersion in 0.87 M citric acid, pH 2.3 (36 h at 4ºC). Then, the studied gels were applied once over dentin for 1 minute. Next, the samples were immersed in artificial saliva containing collagenase obtained from *Clostridium histolyticum* for 5 days. The response variable was depth of dentin loss measured by means of profilometry.

Blocks preparation

In total, 40 specimens were prepared from extracted human teeth. Each dentin specimen (4x4x3 mm) was obtained from a single human tooth and was stored in 0.1% thymol solution (pH 7.0) at 4°C. Each sample was obtained by sectioning the crown longitudinally (Figure 1a) with 2 parallel diamond discs (XLI 2205, Extec Corp., Enfield, CT, USA), that allowed the removal of occlusal enamel, creating a dentin slice. Next, samples were sectioned to obtain dentin blocks (4x4 mm) (Figure 1b), which were stored in 0.1% thymol solution (pH 7.0) at 4ºC. The surface of the specimens was polished (Figure 1c) using water-cooled carborundum discs (320, 600 and 1200 grades of Al_2O_3 papers; Buehler, Lake Bluff, IL, USA) and a 1-μm diamond solution (Buehler). Prior to treatment, marks were made on the surfaces of the samples using a scalpel blade for the precise repositioning on the equipment. Subsequently, three baseline surface profiles were obtained from each wet blocks (only the excess of water was carefully removed with filter paper) using a profilometer (MarSurf GD 25, Göttingen, Germany) at certain distances from the edge: 2.0, 1.75, and 1.5μm (Figure 1d). The marks and external dentin surface were covered with nail varnish (Cosmed *Indústria de Cosméticos e Medicamento*s, S/A, Barueri, São Paulo, Brazil) (Figure 1e) in order to allow reference surfaces for wear analysis.

Treatment

Dentin specimens were demineralized by immersion in 0.87 M citric acid (Figure 1f), pH 2.3 (36 h at 4ºC). Next, samples were thoroughly rinsed in deionized water (30 sec). Excess water was removed with absorbent paper. After demineralization the nail varnish was removed (Figure 1g) and three profilometric analysis was performed again at the same sites as the baseline measurements (2nd measure) (Figure 1h). In sequence, the nail varnish was applied again (Figure 1i) and specimens were randomly allocated into 4 groups, according to the treatment gel (n = 10 per group), as follows: G1 - 10% Proanthocyanidin gel (test group), G2 - 1.23% NaF (positive control 1), G3 - 0.012% Chlorhexidine (positive control 2) and G4 – Placebo (negative control with no active compound). The studied gels were applied once over dentin for 1 minute (Figure 1j). All gels formulations presented essentially the same composition (hydroxyethylcellulose, propyleneglycol, methylparaben, imidazolidinyl urea, and deionized water, pH 7.0) except for the active compounds.

Specimens were subjected to collagen degradation (Figure 1k) by the action of collagenase obtained from Clostridium histolyticum (Type VII, Product No. C0773, Sigma-Aldrich, St. Louis, MO, USA) added in artificial saliva (20 mmol/L HEPES, 0.70 mmol/L $CaCl_2$, 0.20 mmol/L $MgCl_2.6H_2O$, 4 mmol/L KH_2PO_4, 30 mmol/L KCl, 0.30 mmol/L NaN_3) at a concentration of 100 U/mL, for 5 days (37ºC) [23].

Profilometric analysis

The dentin specimens were maintained wet until the analysis to avoid shrinkage of the organic layer. Immediately before the profilometric measurement, only the excess of water was carefully removed with filter paper. After the immersion time, the nail varnish was removed (Figure 1-l) and three profilometric analysis were performed again (Figure 1-m) at the same sites as the baseline measurements (3rd measure). The dentin blocks were precisely repositioned in the wells of the profilometer, enabling baseline profiles to match with the final ones. Then, the dentin loss was quantitatively determined using specific software (MarSurf XCR 20, Göttingen, Germany) by calculating the average depth of the eroded surface relative to the baseline surface profiles. The response variables were the dentin wear (difference between 1st and 3rd measures) and total amount of degradeted DOM (difference between 2nd and 3rd measures) (Figure 2).

Methodology 2

Evaluation of endogenous matrix-linked proteases in demineralized dentin

Dentin specimens measuring 6 mm x 2 mm x 1 mm (Figure 1B) were sectioned from the middle coronal portion. Specimens were completely demineralized by 0.5 M EDTA (pH 7.4; Sigma, St. Louis, USA) for 30 days at 4°C (Figure 1C).

Every four days, the EDTA solution was changed for a new one. Then the specimens were washed with deionized water at 4°C for 72 h for complete removal of EDTA residues, thus avoiding a possible inhibition of MMPs.

In sequence, the specimens were treated according to the treatment gel (n = 10 per group). The studied gels were applied once over dentin for 1 minute (Figure 1D) [11,23].

All the specimens were immersed in 0.5 ml of a buffered medium composed of 5 mM HEPES, 2.5 mM $CaCl_2.H_2O$, 0.05 mM $ZnCl_2$ and 120 mM NaCl adjusted to pH 7.4 (Figure 1E). The sealed tubes were incubated in a shaking water bath at 37°C for 3 days. All 0.5 ml of medium was removed after 3 days. Aliquots of 10 to 20 μL of the incubation medium were used to measure ICTP collagen fragments.

Matrix degradation by MMPs was determined by measuring the amount of solubilized collagen type I C-terminal telopeptides (ICTP) [20,24,25] reconnected over the 3-day incubation periods using the ICTP ELISA method (Human Cross-linked Carboxy-terminal telopeptide of type I collagen, Southern California, San Diego - USA) (Figure 1F). The only source of ICTP telopeptide fragments in mineralized zones is attributed to the telopeptidase activity of MMPs [20,25].

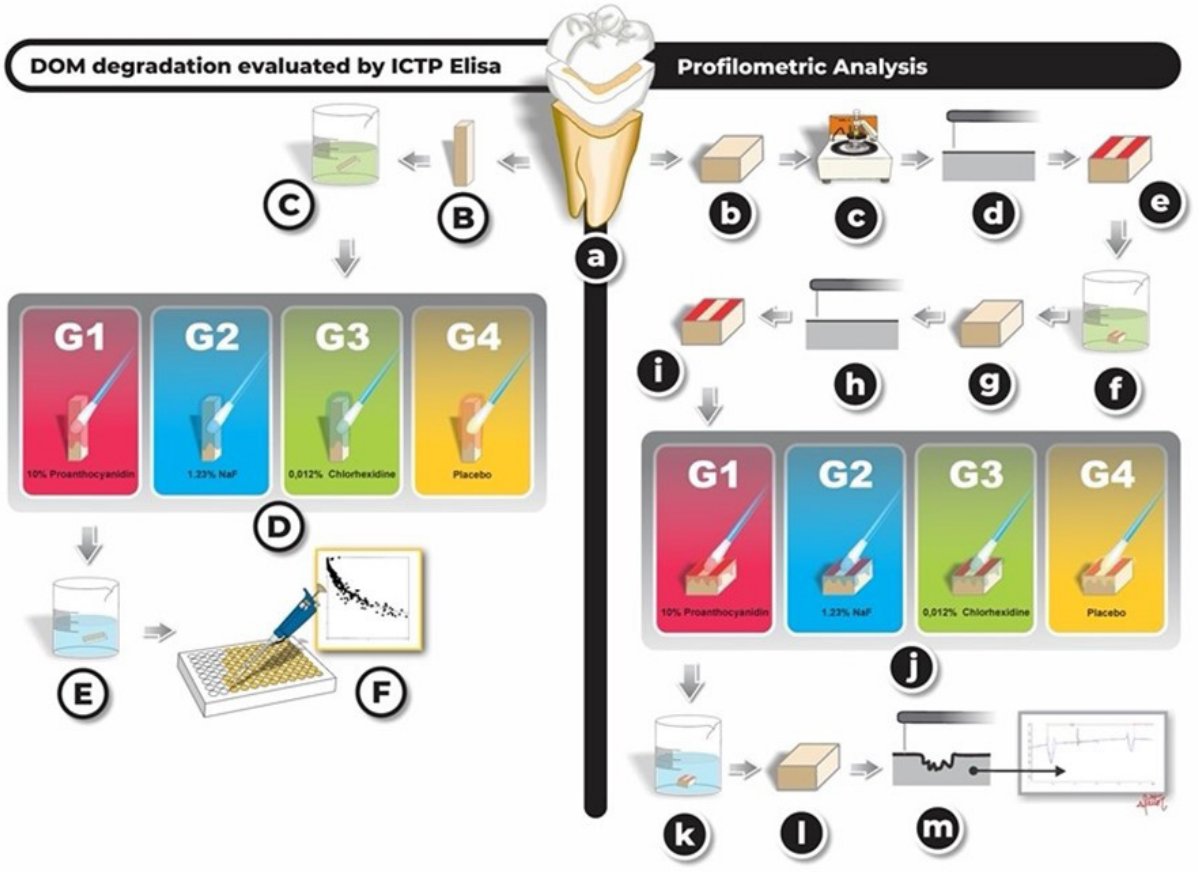

Figure 1 - Experimental step flowchart

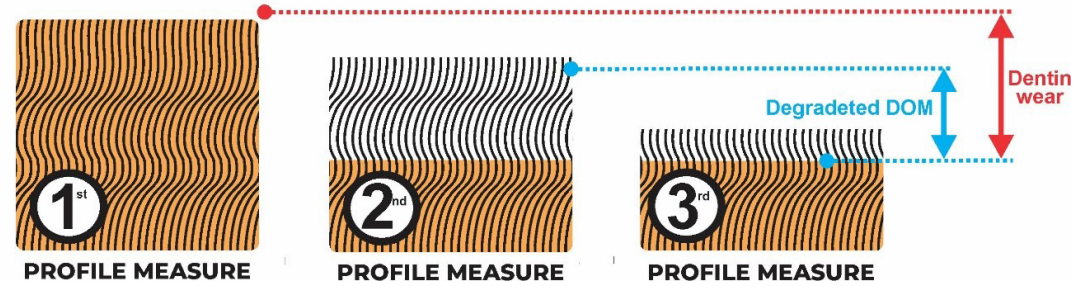

Figure 2 - Illustrative scheme of wear analysis

Statistical analysis

Quantitative data were analyzed by Normality (Shapiro-Wilk test) and variances homogeneity (Levene test). In case they met those assumptions, the data would be compared by a parametric test (ANOVA followed by Fisher's test) and if they did not meet at least one of these assumptions, the test to be used would be the Kruskal-Wallis Test followed by Dunn's test (non-parametric).

Analysis of dentin wear and analysis of the thickness of the demineralized organic matrix (DOM) data were submitted to Analysis of Variance followed by Fisher's LSD test ($p<0.05$).

For the Evaluation of Matrix-Linked Endogenous Proteases in Demineralized Dentin, there was no normal distribution of data and these were submitted to the Kruskal-Wallis Test followed by Dunn's Test ($p<0.05$).

Simple linear regression and Pearson Correlation test were performed to evaluate if the degraded DOM is a good predictor to collagen degradation evaluated by the ICTP ELISA.

Statistical analysis was performed with SigmaPlot 12.0 (Systat Software Inc. San Jose, CA, USA).

RESULTS

The results of dentin wear and DOM degradation are shown in Table I. The data showed that there was no significant difference in dentin wear between groups G2, G3 and G4. However, they showed greater dentin wear when compared to G1.

In the analysis of DOM degradation evaluated by ICTP ELISA, the data showed that there was no significant difference between groups G2 and G4, which had lower values of collagen degradation, showing a statistically significant difference when compared to groups G2 and G3 which did not show significant differences between themselves.

Simple linear regression analysis was performed in which the predictor variable was the degraded DOM evaluated profilometrically and the dependent variable was the collagen degradation evaluated by the ICTP ELISA. For that, the regression equation was: Endogenous Matrix-Linked Proteases in Demineralized Dentin = 0.0497 + 0.000793 x degraded DOM.

Figure 3 represents the scatter plot showing that there is a positive, significant and moderate correlation (0.55) where the degraded DOM is a significant predictor to explain the values obtained through the ICTP ELISA.

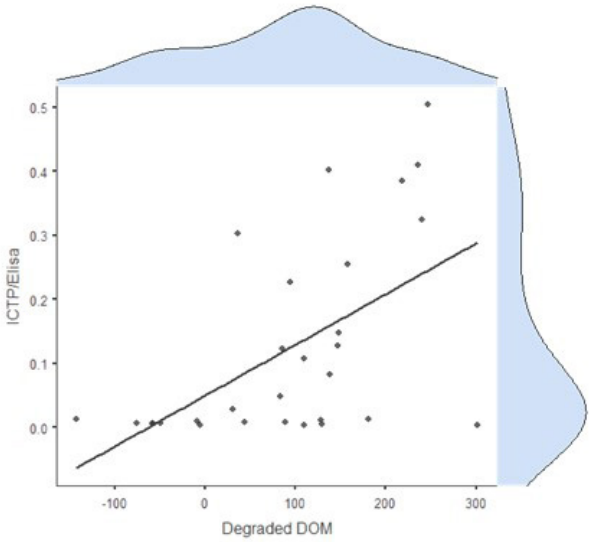

Figure 3 - Scatter Plot representing positive correlation between ICTP/Elisa and Degraded DOM

Table I - Mean (±S.D.) of dentin loss (μm) and DOM degradation for the studied groups. Median (interquartile range) of Endogenous Matrix-Linked Proteases in Demineralized Dentin (ICTP ELISA)

Groups	Dentin Wear (Mean±SD)	Degradeted DOM (Mean±SD)	ICTP ELISA [Median (Interquartile range)]
G1 – PA 10%	156.0 ± 96.8^{a}	4.9 ± 119.0^{a}	7.0 (4.3-8.0)^{A}
G2 - NaF 1.23%	255.0 ± 89.5^{b}	99.0 ± 118.0^{b}	147.0 (16.8-445.0)^{B}
G3 – 0.012% Chlorhexidine	293.0 ± 53.0^{b}	144.0 ± 63.2^{b}	278.0 (126.0-389.0)^{B}
G4 - Placebo	270.0 ± 55.6^{b}	83.9 ± 50.8^{b}	48.0 (11.0-108.0)^{AB}

Different lowercase letters indicate statistically significant difference between the groups for the dentin wear and DOM degradation (One Way ANOVA and Fisher's Test). Different upercase letters indicate statistically significant difference between groups for ICTP ELISA (Kruskal-Wallis and Dunn's Test, $p<0.05$).

DISCUSSION

Several studies have demonstrated effective inhibitory feats of various agents on MMPs [11,26-28]. In this context, chlorhexidine and fluoride have shown the ability to inhibit collagen degradation and inhibit MMPs [9,23,26] and that is the reason why they were used as a positive control. On the other hand, chlorhexidine has unwanted side effects such as taste alteration, numbness in mouth and tongue, pain in mouth and tongue, xerostomia, and subjective discolouration, throughout 21 days of chlorhexidine usage [29,30]. Therefore, it may not be a good strategy to be used in the long term.

Natural products, such as PA, are increasingly intended for the development of products for oral health because, at first, they involve a lower incidence of side effects [31]. The PA purified from grape seed extract was tested because previous studies have demonstrated its protective effect on dentin erosion [19,32,33] and in this study, the group treated with PA also had the best results. Thus, since a PA does not have the side effects of a chlorhexidine, its use might be more advantageous for dentin erosion prevention.

Some studies have shown that agents with a high concentration of fluoride are more effective in preventing dental erosion [11,26,34]. It can form a layer of calcium fluoride that provides temporary protection against erosive challenges [35]. NaF group showed no statistical difference from placebo group. This finding has already founded [11,33] and could be due to the response variable used in these studies, which is dentin wear instead of a direct chemical analysis of the different gels ability to inhibit collagen degradation.

In this study, the results of proanthocyanidin were better than the other groups. Studies show that proanthocyanidin positively affects the demineralization and/or remineralization processes and its remineralizing mechanism seems to be different from fluoride [36] which might be related to the formation of an insoluble complex that remains stable in acid pH [37] that further binds to the Ca^{2+} ions in saliva, thereby enhancing remineralization [36]. However, in the present study, it is most likely to proanthocyanidin have improved the DOM by its ability to induce cross-links in dentin collagen and reinforce the remaining collagen matrix [36,38,39].

In the profilometry analysis, chlorhexidine, fluoride and placebo group showed lower results than the PA group, while in the chemical analysis of collagen degradation performed using the ICTP ELISA, the PA group did not show any significant difference with the placebo group. This can be directly related to the chosen demineralizing agent. Citric acid promotes dentin demineralization and, in this case, caused a great loss of tooth structure, which made it difficult to analyze the exact profilometry of the specimens. Furthermore, in the analysis of collagen degradation performed with the ICTP ELISA, there was a large sample loss. These factors may be related to typing errors in the results or due to the long storage time of the teeth before their use. Despite that, it was noted that both the loss of tooth structure and the difficulty in detecting degraded collagen were equivalent in all groups, which allowed us to maintain the comparison between specimens. Another possible reason for the performance of the proanthocyanidin's performance is the concentration used in this present study (10%). Studies showed good results in concentrations of proanthocyanidin less than 10% and its dose-response effect [39,40].

The selection of a 36-hour demineralization period was a strategic choice aimed at establishing a discernible layer of demineralized organic matrix (DOM) for targeted analysis. This approach, while divergent from the dynamic acid challenges typical of erosive cycling studies, enabled the direct examination of agents' effects on the organic matrix. We acknowledge that the thickness of the resulting DOM layer could potentially lead to an overestimation of the agents' effects, given that clinical scenarios involve frequent acid attacks. It is important to underscore that the study's focus remains on investigating the agents' direct impact on the organic matrix, with mineral loss serving as an ancillary consideration.

Profilometry alone on eroded dentin does not reflect mineral loss, since there is interference of collagen matrix [41]. Furthermore, contact profilometers use a diamond-tip stylus moved across the surface to record the surface profile, which is simple, but this traditional method has the potential risk of affecting the reading or even damaging the sample as a consequence of the contact. However, despite its limited analysis, this method showed a very strong correlation when compared with a non-contact [42].

Profilometer and a confocal laser scanning microscope with the same conclusions being separately drawn from data generated on each instrument [43]. In this study there were a significant difference between PA group gel from other groups showing less dentin wear compared to the other groups. Our findings allow us to understand that there was also a reduction in DOM degradation by profilometry. In addition, a positive, significant and moderate correlation (0.55) was found between the profilometry results with those of the chemical analysis (ICTP ELISA).

Another alternative to evaluate dentin wear and DOM degradation is through collagenolytic activity by measuring the hydroxyproline content in artificial saliva after incubation with collagenase [33]. However, this procedure is prone to error and no statement can be made about the thickness of remaining matrix and reproducibility of the method is limited [21].

In this study, a simple linear regression analysis was performed in which the predictor variable was the degraded DOM evaluated profilometrically and the dependent variable was the collagen degradation evaluated by the ICTP ELISA. It shows that this regression model is significant and that degraded DOM is a significant predictor to explain ICTP ELISA's value. Besides that, the results show us a significant correlation between them ($p<0,002$). This correlation is a positive, significant and moderate (0.55) where the degraded DOM is a significant predictor to explain the values obtained through the ICTP ELISA. For this reason, these results seem to be more reliable.

CONCLUSION

The results of this study showed that 10% proanthocyanidin provided less dentin wear and decreased degradation of the demineralized organic matrix, suggesting a greater capacity to prevent dentin erosion.

Furthermore, the contact profilometry can be an alternative method to measure dentin wear and estimate the degradation of demineralized organic matrix.

Author's Contributions

FC: Experimental design, execution of the experimental stages and writing of the manuscript. GGD: Execution of the experimental stages. APB: Execution of the experimental stages. AFS: Execution of the experimental stages. TJD: Execution of the experimental stages. HMH: Theoretical conceptualization, experimental design, data analysis and article writing.

Conflict of Interest

The authors have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

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Regulatory Statement

This study was conducted in accordance with all the provisions of the local human subjects oversight committee guidelines and policies of Research and Ethics Committee of the Bauru School of Dentistry, University of São Paulo. The approval code for this study is: 94400218.8.0000.5417.

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Heitor Marques Honório
(Corresponding address)
Universidade de São Paulo, Faculdade de Odontologia de Bauru, Departmento de Odontopediatria, Ortodontia e Saúde Coletiva, Bauru, SP, Brasil
Email: heitorhonorio@usp.br

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