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Stability of dentin matrix treated with caffeic acid phenethyl ester at different concentrations

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Aim: The aim of this study was to investigate the impact of pre-treatment with ethanolic solutions of caffeic acid phenethyl ester (CAPE) at varying concentrations on the dentin collagen matrix, specifically focusing on its biomodification potential. This was assessed through evaluations of the modulus of elasticity and changes in mass. **Methods:** Seventy dentin collagen matrices (demineralized sticks) were prepared to receive treatments with ethanolic solutions of CAPE at concentrations of 0.05%, 0.1%, 0.5%, or 2.5%, or with control treatment solutions (distilled water or ethanol) for one hour. The dentin matrices were evaluated for modulus of elasticity and mass before (baseline), immediately after treatment (immediately), and after storage in Simulated Body Fluid (SBF) for time intervals of 1 and 3 months. **Results:** Generalized linear models for repeated measures over time indicated no significant differences between groups (p=0.7530) or between different time points (p=0.4780) in terms of the modulus of elasticity. Regarding mass variation, no differences were observed in the time interval between 1 month and the immediate time (p=0.0935). However, at the 3-month mark compared to the immediate time, the 0.1% CAPE group exhibited less mass loss compared to the water group (p=0.0134). **Conclusion:** This study concludes that various concentrations of CAPE in an ethanolic solution did not affect the modulus of elasticity of dentin, suggesting that CAPE lacks biomodifying potential in this context. However, it was observed that 0.1% CAPE positively influenced the variation in mass over different evaluation time intervals.

Keywords: Caffeic acids. Collagen. Dentin. Ethanol.

Introduction

The longevity of the restorative interface is affected by processes such as hydrolysis and enzymatic degradation, which impact the hybrid layer[1-4]. Hydrolysis, a chemical process, breaks the covalent bonds within polymers by adding water to the ester bonds, resulting in a loss of resin mass[5]. Enzymatic degradation occurs due to matrix metalloproteinases (MMPs) that contribute to the denaturation of collagen fibrils in the hybrid layer incompletely infiltrated by the adhesive[6,7]. Studies have identified that the acidic properties of adhesive systems can activate MMPs trapped in mineralized dentin[8]. Activation of MMPs leads to collagenolytic activity[9,10], resulting in the degradation of incompletely infiltrated collagen fibrils in the hybrid layer and consequent loss of mechanical properties of the collagen matrix[11,12].

Strategies have been developed to increase the longevity of the hybrid layer by inhibiting the endogenous activity of these enzymes using various synthetic or natural pretreatment agents[3,6,11,13-15]. Among these agents, there are polyphenolic-based compounds that contain secondary metabolites produced by plants, such as those derived from natural plant extracts (grape seed, cocoa, green tea), as part of their defense mechanisms[9,11]. These compounds can act as inhibitors of MMPs or collagen cross-linkers, thereby inhibiting collagenolytic activity or increasing the mechanical strength of collagen as biomodifiers in the degradation process[5,11].

Among the polyphenolic compounds, caffeic acid phenethyl ester (CAPE) is a biologically active ingredient found in bee propolis, exhibiting antioxidant and anti-inflammatory actions[16]. It is an outstanding inhibitor of MMP-2 and MMP-9[17,18] in other tissues, such as in the accelerated healing of alveolar bone defects[19], and has exhibited anti-enzyme activity in dentin[20,21]. Additionally, CAPE has shown anti-enzyme activity in dentin. CAPE might be useful for vital pulp therapy by stimulating the effects of vascular endothelial growth factor production and anti-inflammatory activities[22].

Studies have suggested that the mechanism of action of CAPE is related to the inactivation of the proenzyme, a precursor of proteolysis of MMPs, in addition to stimulating the activity of tissue inhibitors of metalloproteinases (TIMPs)[6]. Due to the inhibitory effect on MMPs, studies have suggested that its use may slow down the degradation of the hybrid layer[14,19,20], but the optimal concentration of CAPE for this effect has not yet been established. When solubilized in dimethyl sulfoxide (DMSO), 5% CAPE reduced nanoleakage in the hybrid layer[14] and also significantly reduced dentin MMP-2 concentration[19] and gelatinolytic activity[20] when using a conventional three-step adhesive, especially when applied prior to acid etching at a 0.1% concentration. However, considering that DMSO is not only a synthetic inhibitor of MMPs[22] but also an apoptotic and polar solvent, its volatilization from the dentin substrate can hinder the process of volatilizing solvents (water and ethanol) present in adhesive systems[22]. Due to its attraction to hydrogen molecules[23], DMSO can absorb water. Therefore, the collagen fibers become even more impregnated with moisture, potentially impeding the effect of CAPE as an antiproteolytic agent.

From this perspective, solubilizing CAPE in ethanol could potentially enhance the infiltration of the bioactive agent through collagen fibrils. This is because when CAPE was solubilized in DMSO at concentrations of 0.05% and 0.1%, no observable influence on the dentin matrix was noted regarding the modulus of elasticity and degradation caused by endogenous proteases[20]. Therefore, the objective of the current study was to assess the stability of the dentin matrix following pretreatment with ethanolic solutions of caffeic acid phenethyl ester at varying concentrations. The null hypothesis under investigation posited that pretreatment with different concentrations of caffeic acid phenethyl ester in ethanolic solution would not affect: 1) the modulus of elasticity; 2) mass change.

Materials and Methods

Preparation of collagen matrices

After obtaining approval from the Research Ethics Committee of the São Leopoldo Mandic School of Dentistry (CAAE number 40008920.4.0000.5374), 25 human molars were selected and stored in a freezer at -20°C until the time of use. Figure 1 depicts the stages of specimen preparation. To prepare the collagen matrices, the occlusal enamel surface of the teeth was removed by using a metallographic cutter with a double-sided diamond disc to expose the superficial dentin. Subsequently, a second section was cut to obtain a 2 mm thick slice of dentin. Sections were then made to produce 70 rectangular sticks, approximately 0.5 to 0.6 mm thick, 1.7 to 1.9 mm wide, and 7.0 mm long.

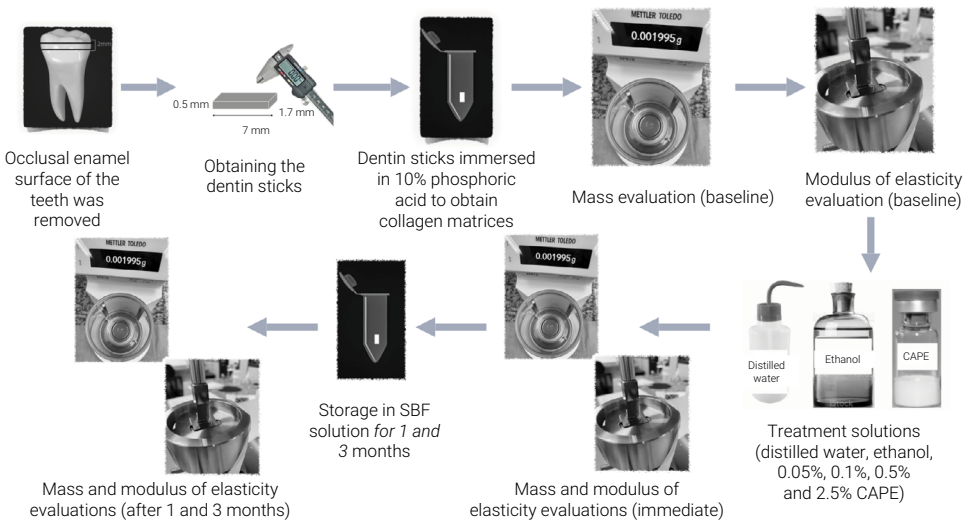

Figure 1. Flowchart of the experiment.

At one extremity of the tooth stick, a dimple was created using a 1/2 spherical carbide bur at high speed, cooled with water, ensuring that modulus of elasticity evaluations were consistently conducted at the same location. The tooth sticks were individually

immersed in Eppendorf tubes containing 2 mL of 10% phosphoric acid (LabChem, Pittsburgh, PA) placed in a rotational solution homogenizer (Phoenix-Luferco, Araraquara, São Paulo, Brazil) for a period of 5 hours. Following this duration, the phosphoric acid was replaced with 2 mL of deionized water at 4ºC, and the tubes were shaken once again for 30 minutes.

Obtaining the treatment solutions, evaluating the pH of the solutions, and treating the collagen matrices

The treatment agents utilized are outlined in Table 1. CAPE was procured in pro-analysis (PA) form and solubilized in ethanol following the manufacturer's instructions. Specifically, 10 mg of CAPE was diluted in 35.17 mL of ethanol under stirring for 60 seconds, resulting in a stock solution with a concentration of 9.29% CAPE[19,20]. This "stock" solution was then fractionated and further diluted in ethanol to achieve concentrations of 0.05%, 0.1%, 0.5%, and 2.5%.

Table 1. Specifications of the materials to be used in the research.

Material/Commercial (brand) name/Manufacturer (city, state, country)	Composition
Absolute ethyl alcohol/Dinâmica Química Contemporânea Ltda (Indaiatuba, SP, Brasil)	Ethyl alcohol P.A (C_2H_6O) 99,5%
Caffeic acid phenethyl ester ($C_{17}H_{16}O_4$)/Tocris Bioscience (Bristol, United Kingdom)	2- Phenylethyl-(2E)-3-(3,4- dihydroxyphenyl) acrylate

The pH values of the solutions were measured in triplicate using a microelectrode (Model 2A14, Analyser Instrumentação Analítica, São Paulo, SP, Brazil) and pH meter (Model MPA 210, MS Tecnopon Instrumentação, Piracicaba, SP, Brazil). Descriptive statistics were employed to present the data, including the mean of the triplicate values.

The collagen matrices were immersed in the treatment solutions for 1 hour. The elastic modulus and mass of the dentin matrix were assessed at various time intervals: immediately after 1 hour of treatment, and subsequently at 1 and 3 months of storage. The collagen matrices were stored in Simulated Body Fluid (SBF) comprising 50 mmol/L HEPES; 5 mmol/L $CaCl_2$-$2H_2O$; 0.001 mmol/L ZnCl2; 150 mmol/L NaCl; and 3 mmol/L sodium azide at pH 7.4[12] in a bacteriological oven set at 37 °C. The SBF solution was refreshed every 2 weeks.

Collagen matrices mass evaluation

The collagen matrices were weighed both before (baseline) and after (immediate) dentin treatment, as well as after 1 and 3 months of storage in the SBF solution. Mass evaluations were conducted with a precision of 0.01 mg using an ultra-microbalance (XPR10, Mettler-Toledo GmbH, Greifensee, Switzerland) after dehydration. Weight mass change assessments were determined as a percentage of gain or loss in mass for each specimen at each time interval.

Modulus of elasticity evaluation

The modulus of elasticity was determined in a three-point bending test with the collagen matrices immersed in distilled water. The load was applied to the center of the specimen using a 5 N load cell mounted on a universal testing machine (EZ Graph, Shimadzu, Kyoto, Japan) at a speed of 0.5 mm/min. During compression, the displacement was evaluated in millimeters and calculated with the maximum deflection of 3% deflection using the following formula[13]: $D = \varepsilon L2 / 6T$ (ε is the displacement, L is the width of the support and T is the specimen thickness). The modulus of elasticity was measured in MPa (Mega Pascal) and calculated using the following formula: $E = PL^3 / 4DbT^3$ (P was the maximum force, L was the support width, D was the displacement, b was the specimen width and T was the specimen thickness).

Statistical analysis

After conducting descriptive and exploratory analyses of the elastic modulus and mass data, generalized linear models for repeated measures over time were employed, considering the effects of treatment, time, and their interaction. As the variations in mass at different time intervals did not conform to a known distribution, they were analyzed using non-parametric Kruskal-Wallis and Dunn tests. All analyses were conducted using the R program (R Core Team, Vienna, Austria, 2022), with a significance level set at 5%.

Results

The mean pH values of the triplicate solutions were as follows: distilled water = 6.90; ethanol = 7.8; CAPE 0.05% = 6.94; CAPE 0.1% = 6.98; CAPE 0.5% = 7.59; CAPE 2.5% = 7.35. Distilled water exhibited the highest acidic pH value among them, whereas ethanol and the different concentrations of CAPE displayed values close to neutrality.

There were no significant differences in the modulus of elasticity observed between treatments (p=0.7530) or between different time points (p=0.4780) (Table 2). Additionally, the interaction between these factors was not found to be significant (p=0.9501).

Table 2. Mean (standard deviation) of modulus of elasticity (MPa) as a function of the treatment and time.

Treatment	Time			
	Baseline	Immediate	After 1 month	After 3 months
Water	4.95 (1.93) Aa	4.70 (3.55) Aa	5.92 (3.12) Aa	4.60 (3.40) Aa
Ethanol	4.75 (2.93) Aa	3.76 (2.64) Aa	4.81 (2.56) Aa	4.82 (2.56) Aa
CAPE 0.05%	5.25 (2.69) Aa	4.59 (2.65) Aa	4.97 (2.81) Aa	5.64 (3.43) Aa
CAPE 0.1%	6.02 (2.50) Aa	5.14 (4.18) Aa	6.35 (5.33) Aa	6.34 (4.95) Aa
CAPE 0.5%	5.54 (2.24) Aa	4.5 (1.77) Aa	3.55 (3.44) Aa	5.79 (4.47) Aa
CAPE 2.5%	5.34 (3.15) Aa	5.44 (2.99) Aa	4.86 (1.95) Aa	5.09 (5.07) Aa

Same capital letters (capital letters horizontally and lower case vertically) indicate no statistically significant difference (p>0.05)

When comparing the mass change values at the one-month interval versus the immediate time, no significant differences were observed between the groups (p=0.0935) (Table 3). However, at the three-month time interval, when compared with the immediate time, the change in mass was less negative (indicating less loss) in the CAPE group (0.1%) compared to the water (control) group. This difference was statistically significant (p=0.0134).

Table 3. Median (minimum and maximum value) of the variations in mass (%) as a function of treatment.

Treatment	Time range		
	Immediate – Baseline	1 month – Immediate	3 months – Immediate
Water	2.46 (1.90-3.19) a	-0.05 (-2.69-5.86) a	-10.72 (-13.96; -4.56) b
Ethanol	2.31 (-2.15-3.34) a	-0.28 (-2.41-7.68) a	-9.47 (-20.84; -5.12) ab
CAPE 0.05%	2.83 (0.10-5.00) a	-2.14 (-3.14-11.52) a	-8.64 (-9.96; -6.75) ab
CAPE 0.1%	2.68 (1.52-3.69) a	-1.46 (-3.35-39.05) a	-7.55 (-11.05; -6.51) a
CAPE 0.5%	2.96 (1.69-3.78) a	-2.12 (-3.51-32.20) a	-9.66 (-21.25; -6.46) ab
CAPE 2.5%	3.05 (0.75-4.02) a	-2.47 (-3.61-1.90) a	-9.41 (-11.48; -8.47) ab
p-value	0.5179	0.0935	0.0134

Different letters in the vertical direction indicate statistically significant differences (p≤0.05).

Discussion

A biomodifier or cross-linker acts on the mechanical properties of dentin in a biomimetic manner by penetrating through the collagen fibrils, thereby locally modifying the biochemical and biomechanical characteristics[11]. This binding occurs within type 1 collagen fibrils through a process of inter- and intramolecular cross-linking, facilitated by reactions with free amino acids (lysine, hydroxylysine or arginine) to form an aromatic monomer, as well as to establish additional covalent and non-covalent bonds.[11,24]. As a result, the collagen structure becomes more rigid[25], leading to enhanced stability of the hybrid layer and increased durability of the restorations[14].

In the present study, it was observed that CAPE did not exhibit properties consistent with those of a collagen cross-linker, as none of the concentrations used resulted in an increase in the modulus of elasticity. Consequently, the first null hypothesis was accepted. Other cross-linking substances that have the ability to promote intra- or intermolecular cross-linking within a collagen molecule or between neighboring collagen molecules include green tea and grape fruit seed extract[13,15,26]. These agents demonstrated significant increases in modulus of elasticity values compared to the initial values and differed from water treatment (control), contrary to the findings reported in the present study. Applying the 6.5% concentrations of several polyphenolic agents, like grape seed, Camellia sinensis, and cocoa, to demineralized dentin for 60 minutes resulted in an 11–15-fold increase in the modulus of elasticity and a nota-

ble increase in mass[9]. Additionally, when 6.5% extracts of cardol, cardanol, and aroeira were applied to demineralized dentin for 60 seconds, Moreira et al.[24] discovered a mean increase in modulus of elasticity of 338.2% along with a considerable increase in mass. When the experiments were conducted with CAPE dissolved in ethanol at various doses, this failed to occur.

The cross-linking effect should demonstrate stability over time[9], as this would enable greater resistance to degradation of the hybrid layer. The present study revealed no initial increase in the modulus of elasticity for treatments with CAPE; nevertheless, stability of this property was observed over the 1- and 3-month intervals, with no significant differences observed compared to the values of the control groups (water and ethanol). Although there were no differences observed regarding the variation in mass between the treatments, the loss of mass after 3 months may be attributed to the degradation of the collagen matrix[9,24]. No significant differences were noted between the groups at the 1-month time interval when compared with the immediate time. Additionally, at the 3-month time interval, the change in mass was less negative (indicating lower loss) in the 0.1% CAPE group compared to the water (control) group when compared with the immediate time. This outcome led to the rejection of the second null hypothesis. In this regard, it could be proposed that the concentration of CAPE utilized may indeed influence its efficacy as a metalloproteinase inhibitor. Pedrosa et al.[19] observed that CAPE in an aqueous solution, at concentrations of 0.05% and 0.1%, diminished the gelatinolytic activity of dentin when used as a pretreatment prior to the application of a conventional adhesive system. Similarly, they found that the concentration of 0.1% reduced gelatinolytic activity when a self-etching adhesive system was applied to dentin[20].

Moreira et al.[24] highlighted that the greater increase in mass value was attributed to the more extensive and rapid penetration of the solutions into the collagen matrix. This phenomenon could be associated with the use of ethanol as the solubilization medium. Considering that ethanol has been shown to decrease the fibrillar diameter of collagen and increase the interfibrillar spaces[27], this likely facilitated better permeation of the active agent through the collagen fibrils.

The mode of action of CAPE is associated with the suppression of MMP-2 and MMP-9[17,18], selectively inhibiting the activity of these enzymes through three mechanisms: modulation of gene transcription levels, inhibition of the enzyme precursor, and activation of tissue inhibitors of metalloproteinases (TIMP)[6]. Indeed, CAPE possesses the ability to inhibit the activity of MMP-2 and MMP-9 enzymes, thereby contributing to the stability of the resin-dentin bond and ultimately enhancing the longevity of restorations[14,19,20]. Moreover, ethanol also exhibits the ability to inhibit the activity of dentin matrix proteases[28]. This inhibition occurs through the formation of covalent bonds between the catalytic zinc sites of MMPs and the oxygen atom of the hydroxyl groups present in alcohols. This mechanism may promote greater resistance to collagen degradation.

The absence of an effect on the modulus of elasticity of dentin by different concentrations of CAPE in an ethanol solution suggests that CAPE does not possess biomodifying potential in this context. However, it is noteworthy that 0.1%

CAPE may influence the variation in mass over different evaluation time intervals. Consequently, it is apparent that 0.1% CAPE demonstrated a tendency to increase the modulus of elasticity throughout the evaluation periods, as it was the only group to significantly minimize the loss of mass. While the findings of the current study did not conclusively demonstrate the effect of CAPE on the stability of the dentin matrix, it is imperative to conduct long-term investigations to further elucidate the impact of this bioactive agent on the inactivation of proteases in the hybrid layer, particularly when utilized in conjunction with a universal adhesive system. Assessments of hydroxyproline release and degradation by collagenase could provide additional support for the results obtained. These analyses would be particularly valuable when CAPE is administered as a dentin pre-treatment before restoration. The feasibility of utilizing the wet adhesion technique with ethanol is underscored, considering both its method of application and its cost-effectiveness. However, further research is warranted to validate the applicability of CAPE in various solutions and concentrations using alternative analytical methodologies.

In conclusion, this study demonstrated that various concentrations of CAPE in an ethanolic solution did not impact the modulus of elasticity of dentin, suggesting that CAPE lacks biomodifying potential. However, it was observed that 0.1% CAPE could influence the variation in mass over different evaluation time intervals.

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Data Availability

Datasets related to this article will be available upon request to the corresponding author.

Conflict of Interest

The authors declare no conflict of interest

Author Contribution

- **Aline Honorato Damázio:** Conceptualization, Methodology, Investigation, Resources Data curation; Writing - Original Draft; Writing – review & editing.
- **Rosanna Tarkany Basting:** Conceptualization; Data curation; Investigation; Methodology; Writing – original draft.
- **Enrico Coser Bridi:** Investigation; Supervision; Validation; Visualization; Writing – original draft.
- **Fabiana Mantovani Gomes França:** Supervision; Visualization; Writing – original draft.
- **Flávia Lucisano Botelho do Amaral:** Supervision; Visualization; Writing – original draft.
- **Cecilia Pedroso Turssi: Supervision;** Validation; Visualization; Writing – original draft.

- **Waldemir Francisco Vieira Junior:** Supervision; Validation; Visualization; Writing – original draft.
- **Roberta Tarkany Basting:** Conceptualization; Funding acquisition; Methodology; Project administration; Writing – review & editing.

The authors have revised and approved the final version of the manuscript.

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