UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

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Acquired pellicle engineering for the control of erosive tooth wear: in vitro and in situ evaluation of the protective potential of a sugarcane-derived cystatin (CaneCPI-5)

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Acquired pellicle engineering for the control of erosive tooth wear: in vitro and in situ evaluation of the protective potential of a sugarcane-derived cystatin (CaneCPI-5)

Engenharia de película adquirida para o controle do desgaste dentário erosivo: avaliação in vitro e in situ do potencial protetor de uma cistatina derivada da cana-de-açúcar (CaneCPI-5)

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Orientadora: Profa. Dra. Marília Afonso Rabelo Buzalaf

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DEDICATÓRIA

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“Uma vida sem desafios não vale a pena ser vivida, a vida sem ciência é uma espécie de morte,
O início da sabedoria é a admissão da própria ignorância;
todo o meu saber consiste em saber que nada sei.
O homem para ser completo tem que estudar, trabalhar e lutar”.

Sócrates

RESUMO

Engenharia de película adquirida para o controle do desgaste dentário erosivo: avaliação in vitro e in situ do potencial protetor de uma cistatina derivada da cana-de-açúcar (CaneCPI-5)

O desgaste dentário erosivo (DDE) é um problema complexo e multifatorial que requer atenção especial para prevenção e tratamento. A saliva é um fator biológico essencial, que desempenha um papel crucial na proteção contra o desgaste. Grande parte do papel da saliva advém do fato de suas proteínas serem os maiores constituintes da película adquirida, já que algumas proteínas deste integumento são resistentes à remoção por desafios erosivos, sendo, portanto, candidatas à incorporação em produtos odontológicos visando à prevenção do DDE. No presente trabalho, o efeito protetor de uma cistatina derivada da cana-de-açúcar, adicionada a diferentes soluções e géis, foi avaliado *in vitro* e *in situ*. No primeiro capítulo, foi realizado um estudo *in vitro* para avaliar o efeito protetor de géis contendo diferentes concentrações de CaneCPI-5 contra a erosão inicial do esmalte (desafio com ácido cítrico 0,65%, pH 3,4 por 1 min). Os resultados indicaram que o tratamento com géis contendo CaneCPI-5 nas concentrações de 0,1 ou 1,0 mg/mL protegeu o esmalte contra a erosão dentária inicial, provavelmente porque a CaneCPI-5 aumenta o número de sítios doadores de elétrons na superfície do esmalte, o que pode impactar na formação da película adquirida. No segundo capítulo, diferentes veículos contendo CaneCPI-5 foram avaliados *in situ,* utilizando um protocolo cruzado e duplo-cego, conduzido por 5 dias em cada fase. Os voluntários usaram um aparelho contendo 4 espécimes de dentina humana por 5 dias, tratando as amostras com 1 gota das soluções testadas por 60 s antes de serem submetidas a desafios erosivos (ácido cítrico 0,1%, pH 2,5, 90 s, 4X/dia), combinados ou não a desafios abrasivos (escovação por 15 s, 2X/dia). O tratamento com os géis for feito por 4 min, apenas 1 vez a cada dia. Na primeira parte, a solução contendo CaneCPI-5 0,1 mg/mL foi testada em comparação a um controle positivo (Elmex Erosion Protection) e a um controle negativo (água deionizada). Ambos os tratamentos reduziram significativamente a perda de dentina em comparação ao controle negativo, tanto para a erosão quanto para a erosão associada à abrasão. Na segunda parte, os grupos avaliados foram: sem tratamento, gel de quitosana, gel de quitosana contendo 12300 ppm F e gel de quitosana contendo CaneCPI-5 0,1 mg/mL. O menor desgaste foi observado para o gel contendo CaneCPI-5, que apesar de não diferir do gel NaF, foi

o único gel que diferiu do gel de quitosana. Este é o primeiro estudo *in situ* no qual o efeito da CaneCPI-5 para reduzir o DDE da dentina foi avaliado, uma vez que estudos prévios analisaram apenas o esmalte. Nossos dados reforçam os achados, indicando que a adição de CaneCPI-5 tanto a soluções quanto a géis parece ser uma alternativa promissora para reduzir o desgaste dentário erosivo, por meio da engenharia de película adquirida. Protocolos de aplicação clínica deveriam ser testados, combinando aplicação profissional do gel com aplicações caseiras da solução contendo CaneCPI-5.

Palavras-chave: Película adquirida. Erosão dentária. Abrasão dentária. Dentina. Cistatina. Cana de açúcar.

ABSTRACT

Acquired pellicle engineering for the control of erosive tooth wear: in vitro and in situ evaluation of the protective potential of a sugarcane-derived cystatin (CaneCPI-5)

Erosive tooth wear (ETW) is a complex and multifactorial problem that requires special attention for prevention and treatment. Saliva is an essential biological factor that plays a crucial role in protecting against ETW. A large part of saliva's role comes from the fact that its proteins are the major constituents of the acquired pellicle, since some proteins in this integument are resistant to removal due to erosive challenges, and are therefore candidates for incorporation into dental products aimed at preventing ETW. In the present work, the protective effect of a sugarcane-derived cystatin, added to different solutions and gels, was evaluated *in vitro* and *in situ*. In the first chapter, an *in vitro* study was performed to evaluate the protective effect of gels containing different concentrations of CaneCPI-5 against initial enamel erosion (challenge with 0.65% citric acid, pH 3.4, for 1 min). Results indicated that treatment with gels containing CaneCPI-5 at concentrations of 0.1 or 1.0 mg/mL protected enamel against initial erosion, probably because CaneCPI-5 increases the number of electron donor sites on the surface, which can impact the formation of the acquired pellicle. In the second chapter, different vehicles containing CaneCPI-5 were evaluated *in situ*, using a crossover and double-blind protocol, conducted for 5 days in each phase. The volunteers used an apparatus containing 4 specimens of human dentin for 5 days. Specimens were treated with 1 drop of the tested solutions for 60 s before being submitted to erosive challenges (0.1% citric acid, pH 2.5, 90 s, 4X/day), combined or not with abrasive challenges (brushing for 15 s, 2X/day). The treatment with the gels was performed only once/day. In the first part, the solution containing 0.1 mg/mL CaneCPI-5 was tested against a positive control (Elmex™ Erosion Protection) and a negative control (deionized water). Both treatments significantly reduced dentin loss compared to the negative control for both erosion and erosion associated with abrasion. In the second part, the evaluated groups were: no treatment, chitosan gel, chitosan gel containing 12300 ppm F and chitosan gel containing CaneCPI-5 0.1 mg/mL. The lowest wear was observed for the gel containing CaneCPI-5, which despite not differing from the NaF gel, was the only gel that differed from the chitosan gel. This is the first *in situ* study in which the effect of CaneCPI-5 to reduce dentin erosive wear was evaluated, since

previous studies only analyzed enamel. Our data reinforce the findings indicating that the addition of CaneCPI-5 to both solutions and gels seems to be a promising alternative to reduce ETW through acquired pellicle engineering. Clinical application protocols should be tested, combining professional application of the gel with home application of the solution containing CaneCPI-5.

Keywords: Acquired pellicle. Dental erosion. Dental abrasion. Dentine. Cystatin. Sugarcane.

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ARTICLE 1

LIST OF ABREVIATIONS AND ACRONYMS

CaneCPI-5	Sugarcane-derived cystatin
SFE	Surface free energy
%SHC	Percentage Surface hardness change
γs^{LW}	Apolar component or Nonpolar component
γs^{AB}	Polar component
AP	Acquired pellicle
γ^+	Receptor component
γ^-	Donor component
ETW	Erosive tooth wear
DOM	Demineralized organic matrix
MMPs	Matrix metalloproteinases
CCs	Cysteine cathepsins
ELMEXsol	Elmex Erosion Protection™ mouthwash
CaneCPI-5sol	CaneCPI-5, solubilized in deionized water
CaneCPI-5gel	CaneCPI-5, gel

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1 INTRODUCTION

1 INTRODUCTION

Dental erosion is a prevalent oral condition that affects a significant proportion of the adult population, particularly those who consume high amounts of acidic drinks (LUSSI; CARVALHO, 2014). It is characterized by the loss of hard dental tissue due to non-bacterial acids, which can originate from both the diet (extrinsic) (BARBOUR; LUSSI, 2014) and the gastric contents of the host (intrinsic) (CARPENTER et al., 2014). It is important to note that the term "dental erosion" only refers to cases of initial demineralization (softening) caused by acid exposure, while the term "erosive tooth wear" (ETW) encompasses the effects of both chemical and abrasive mechanical forces (SHELLIS et al., 2011). Therefore, using the terms "dental erosion" and "erosive tooth wear" appropriately can help distinguish between chemical and chemical-mechanical processes (LUSSI; CARVALHO, 2014). In addition to causing tooth sensitivity and discoloration, ETW can lead to structural damage to the teeth, such as cracks and chips (MAGALHÃES et al., 2009b). Thus, it is crucial to implement preventive measures, such as reducing the intake of acidic foods and drinks and practicing good oral hygiene, to minimize the risk of ETW (LUSSI; CARVALHO, 2014).

In recent years, there has been a substantial increase in the number of published studies on ETW, indicating its growing importance in current literature. Although epidemiological data on ETW is still limited, it is prevalent in various age groups and can lead to tooth loss if preventive measures are not taken in time (JAEGGI; LUSSI, 2014; SALAS et al., 2015). Factors that contribute to its development include patient-related factors such as dietary habits, oral hygiene, gastroesophageal reflux, and medication use, as well as nutritional factors such as acid type, buffer capacity, and concentrations of calcium, phosphate, and fluoride (LUSSI; CARVALHO, 2014). Other factors such as genetics and environmental exposures have also been found to play a role on its development. Therefore, effective preventive measures, such as reducing the consumption of acidic foods and drinks, are highly necessary to combat dental erosion (LUSSI; JAEGGI, 2008).

The development of erosive lesions is characterized by the softening of dental surfaces and continuous layer-by-layer dissolution of enamel crystals leading to

permanent volume loss, followed by the exposure of dentin (LUSSI; CARVALHO, 2014). This process is influenced by the solubility of the solid and the activity of relevant mineral constituents in the solution, such as calcium, phosphate, and fluoride. Unlike caries, erosive lesions do not have a fixed critical pH. Rather, the critical pH in erosive lesions refers to the pH value at which the erosive solution is exactly saturated with respect to a specific solid, such as dentine (BUZALAF; HANNAS; KATO, 2012). The composition of dentin tissue, specifically the presence of the demineralized organic matrix (DOM), acts as a barrier to ionic diffusion, reducing the progression of erosion (BUZALAF; HANNAS; KATO, 2012). It has been shown that the use of matrix metalloproteinase (MMP) inhibitors, such as chlorhexidine and green tea extract, can be effective in reducing the progression of dentin erosion (BOTEON et al., 2017; BUZALAF; HANNAS; KATO, 2012; KATO et al., 2010; MAGALHÃES et al., 2009a). Further research is needed to explore the potential use of these strategies in preventing dentin erosion in clinical settings.

The formation of the acquired pellicle is a rapid process that starts within a few seconds after enamel exposure to saliva. In the first few minutes, the thickness of the pellicle increases by about 10-20 nm and remains stable for approximately 30 minutes (HANNIG, 1999). The initial step involves the selective binding of precursor proteins, such as PRPs, statherin, and histatins, to the tooth surface through electrostatic interactions with high affinity for hydroxyapatite (HAY, 1973; SIQUEIRA; CUSTODIO; MCDONALD, 2012). Acquired pellicles not only protect teeth from demineralization but also play a crucial role in bacterial adhesion and biofilm formation (HANNIG; JOINER, 2006). The first layer of adhered proteins is electron-dense and seems to provide the most significant protection against dental demineralization. The subsequent layers have a looser arrangement compared to the basal pellicles (HANNIG, 1999).

During the second stage of pellicle formation, known as the maturation stage, the thickness of the pellicle rapidly increases (100-1000 nm). This suggests that protein aggregates, rather than individual proteins, are involved in its development along with the presence of globular structures (HANNIG; BALZ, 2001). The thickness of the pellicle reaches a plateau after 30-90 minutes, and its thickness ranges from 100 to 1000 nm, depending on its location in the oral cavity. The vestibular region of the cavity is thicker compared to the lingual region (HANNIG, 1999). The pellicle can undergo intrinsic and extrinsic maturation, which can influence its solubility. The intrinsic

maturation is mainly caused by enzymatic cross-linking and dephosphorylation, and the presence of transglutaminase and alkaline phosphatase can cause cross-linking between basic PRPs and statherin (HANNIG et al., 2008; YAO; LAMKIN, 2000). Proteolysis plays a lesser role in intrinsic maturation (HANNIG et al., 2008). On the other hand, extrinsic pellicle maturation suffers great influence from salivary proteolysis, which can occur before or after adsorption to hydroxyapatite (MCDONALD et al., 2011), since most of its components are peptide fragments (SIQUEIRA; OPPENHEIM, 2009; VITORINO et al., 2007). The formation and maturation of the acquired pellicle can also be affected by external factors, such as the use of tooth whitening products, abrasive toothpaste, and the intake of acidic foods and beverages (HARA; ZERO, 2010).

Acquired pellicle engineering involves modifying the interactions between the pellicle and the tooth surface to enhance the protective properties of this integument against ETW, dental caries, and other oral conditions. This can be achieved by incorporating specific proteins and/or other biomolecules into the pellicle or by altering its structure or composition through chemical or physical means. Acquired pellicle engineering has the potential to provide a novel approach for the development of preventive dental care strategies and products, such as toothpastes, mouthwashes, and chewing gums, that can effectively protect against oral diseases (ARAUJO et al., 2021; ARAÚJO et al., 2022; CARVALHO et al., 2020; PELÁ et al., 2021, 2019, 2022; VENTURA et al., 2023).

Salivary proteins, including mucin, statherins, and proline-rich proteins (PRPs), play a critical role in the acquired pellicle's ability to protect against erosion (SIQUEIRA; CUSTODIO; MCDONALD, 2012). Research has shown that individuals with erosion have half the amount of proteins in the acquired pellicle compared to the control group, highlighting the importance of these protective proteins (CARPENTER et al., 2014). Our research group recently conducted a study where we identified proteins present in the acquired pellicle formed *in situ* on dentin and *in vivo* on enamel after exposure to citric or lactic acids, simulating erosive or carious processes, respectively. We found acid-resistant proteins, such as mucins and cystatins (DELECRODE et al., 2015a, 2015b). Among them, cystatins appear to be particularly promising, as the relative expression of cystatin B was observed to increase in the acquired enamel pellicle by

20 and 13 times after exposure to citric and lactic acids, respectively (DELECRODE et al., 2015b).

Cystatins are inhibitors of cysteine peptidases, which exert their inhibitory mechanism by blocking proteolytic activity in a competitive manner (ABRAHAMSON 1993). Given that cystatins inhibit cysteine peptidases, it is expected that they may reduce the progression of dentin erosion (DELECRODE et al., 2015a; TJÄDERHANE et al., 2015). However, the high cost of human cystatin B makes it impractical for use in dental products aimed at preventing dental caries and erosion. Therefore, it would be interesting to develop alternatives with better cost-effectiveness. Phytocystatins, a family of inhibitors of plant cysteine peptidases, were first suggested by Kondo et al. in 1991 (KONDO et al., 1991). These proteins have a unique consensus sequence, L-A-R-F-A-V-X (3)-N, which is specific to plant cystatins, thus justifying their inclusion in a new family (MARGIS; REIS; VILLERET, 1998). The protein Canacystatin (CaneCPI-1) was the first sugarcane cystatin to be produced in a heterologous expression system (SOARES-COSTA et al., 2002). This protein has been shown to possess inhibitory activity against cysteine peptidases, including human cathepsins (VILELA OLIVA et al., 2004). Subsequently, four other sugarcane cystatins were produced and studied for their inhibitory activity (GIANOTTI et al., 2006, 2008; MIGUEL, 2014). Among these, the CaneCPI-4 protein exhibited significant inhibitory activity against human cathepsins B and L (GIANOTTI et al., 2008). Recently, a new cystatin from sugarcane, CaneCPI-5, was identified and characterized, which displayed activities very similar to those of CaneCPI-4. It can efficiently inhibit many cysteine peptidases, including cathepsin B, which is resistant to inhibition by cystatins. This inhibition of cathepsin B could be important for preserving the DOM of dentin, as the expression of cathepsin B and the nonspecific activity of CCs in carious dentin have been found to be significantly higher than in healthy dentin (NASCIMENTO et al., 2011). Furthermore, CaneCPI-5 has been found to be much more soluble when produced in a bacterial expression system, which facilitates its production and purification. Additionally, it has shown strong adherence to quartz cuvettes, suggesting that it could bind with greater strength to tooth enamel (SANTIAGO et al., 2017).

In the current project, we aim to test CaneCPI-5 due to its desirable properties in increasing the acid resistance of the acquired pellicle, as well as inhibiting dentin cysteine-cathepsins, which can delay the progression of dentin erosion. As cystatins

from plants and animals exhibit homology (MARGIS; REIS; VILLERET, 1998), and cystatins from plants are cost-effective to produce, they are potential alternatives for inclusion in dental products aimed at preventing caries and dental erosion. Our research group previously reported that CaneCPI-5 has a high bind strength to enamel (6 times higher than the control), as observed by atomic force microscopy (AFM). Moreover, topographic images of enamel samples coated with mucin 2.7 mg/mL, casein 10 mg/mL, and CaneCPI-5 0.086 mg/mL were taken before and after incubation with citric acid (0.65%, pH 3.4 for 1 min), showing that only CaneCPI-5 protected enamel against citric acid-induced damage. Using an *in vitro* initial erosion model (CHEAIB; LUSSI, 2011), we also demonstrated that treating the enamel surface with CaneCPI-5 protected enamel against early erosion, with the best concentration being 0.1 mg/mL (SANTIAGO et al., 2017). However, further studies are needed to test other application methods, such as gels for topical application, and models that better represent the clinical reality, such as *in situ* models, before new products can be marketed. Moreover, most of the studies conducted so far evaluating the role of CaneCPI-5 against erosive wear were performed using enamel samples. Being CaneCPI-5 an inhibitor of cysteine peptidases, this could be an additional mechanism by which the protein might be able to protect against dentin erosive wear, besides its role in strengthening the acquired pellicle.

2 OBJECTIVES

2 OBJECTIVES

This project had the following general objectives:

1) To evaluate the effect of gels containing CaneCPI-5, at different concentrations, in reducing enamel initial erosion *in vitro* and to evaluate the surface free energy (SFE) after treatment of the enamel surface with CaneCPI-5 solution (Article 1);

2) To evaluate the protective potential of CaneCPI-5 in different application vehicles (solution or chitosan gel) against dentin erosive wear *in situ* (Article 2).

3 ARTICLES

3 ARTICLES

The Articles presented in this Thesis were written according to the Journal of Applied Oral Science (Article 1) and Journal of Dentistry (Article 2) instructions and guidelines for articles submission (Annex B).

- Article 1 – New insights into the anti-erosive potential of a sugarcane-derived cystatin: different vehicle of application and mechanism of action.

- Article 2 – Different vehicles containing CaneCPI-5 reduce erosive dentin wear *in situ*.

3.1 ARTICLE I

This article was published in the Journal of Applied Oral Sciences

New insights into the anti-erosive potential of a sugarcane-derived cystatin: different vehicle of application and mechanism of action

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Running title: New insights into the anti-erosive potential of CaneCPI-5
Keywords: Acquired pellicle; Dental erosion; Cystatin.

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ABSTRACT

Introduction: A new sugarcane-derived cystatin (CaneCPI-5) showed protection and a high strength of interaction with enamel when added to solutions. However, the knowledge about its physical properties is still limited. **Aims:** The aims of the present in vitro study were: 1) to evaluate the protective effect of gels containing different concentrations of CaneCPI-5 against initial enamel erosion (experiment 1); and 2) to analyze the surface free energy (γ_S) after treatment of the enamel surface with CaneCPI-5 solution (experiment 2). **Methods:** In experiment 1, 75 bovine enamel specimens (4x4 mm) were divided into 5 groups according with the gel treatments: placebo gel (control); 0.27%mucin+0.5%casein; 0.1mg/mL CaneCPI-5; 1.0mg/mL CaneCPI-5; or 2.0mg/mL CaneCPI-5. The specimens were treated with the gels for 1 min, the AP was formed (pooled human saliva) for 2h at 37°C and the specimens were incubated in 0.65% citric acid (pH=3.4) for 1min. The percentage of surface hardness change (%SHC) was calculated. In experiment 2, measurements were performed by an automatic goniometer using three probing liquids: diiodomethane, water and ethylene glycol. Specimens (n=10/group) remained untreated (control) or were treated with solution containing 0.1mg/mL CaneCPI-5, air-dried for 45min, and 0.5µL of each liquid was dispensed on the surface for the measurement of contact angles. **Results:** The gels containing 0.1 and 1.0mg/mL CaneCPI-5 significantly reduced %SHC when compared to the other treatments ($p<0.05$). Treated enamel presented significantly lower γ_S than control, without changes in the apolar component (γ_S^{LW}), but the polar component (γ_S^{AB}=Lewis acid-base) became more negative ($p<0.01$). Moreover, CaneCPI-5 treatment showed higher γ_S^-(electron-donor) values compared to control ($p<0.01$). **Conclusion:** Treatment with gels containing 0.1mg/mL or 1.0mg/mL of CaneCPI-5 demonstrated protection of enamel against initial dental erosion in vitro, probably because CaneCPI-5 increases the number of electron donor sites at the enamel surface, which may impact on the formation of the AP.

Keywords: Acquired pellicle. Dental erosion. Cystatin.

INTRODUCTION

Dental erosion is the chemical loss of mineralized tooth substance due to exposure to non-bacterial acids (SCHLUETER et al., 2020). The most important patient-related factor interfering in the occurrence of dental erosion is saliva, since it is saturated with respect to apatite, buffers the acids and is the main source of proteins that form the acquired pellicle (AP). This proteinaceous layer acts as a mechanical barrier to the acids, thus reducing erosion (BUZALAF; HANNAS; KATO, 2012).

Not all the proteins found in the AP contribute to the protection of the tooth surface against acid dissolution. It has been suggested that the proteins present in the basal layer have a greater participation in this regard (HANNIG et al., 2009). A series of in vivo studies employed proteomic approaches to identify acid-resistant proteins in the AP that would be natural candidates for inclusion in dental products to reduce erosive demineralization (DELECRODE et al., 2015; MARTINI et al., 2019; TAIRA et al., 2018). Among them, cystatin-B was revealed as a good alternative (DELECRODE et al., 2015), but the cost of the human recombinant protein is prohibitive. With this in mind, our group recently cloned sugarcane-derived cystatin (CaneCPI-5), that was shown to have a strong binding force to hydroxyapatite and to protect against initial erosion in vitro (SANTIAGO et al., 2017) and in vivo (CARVALHO et al., 2020) when added to rinse solutions. Furthermore, the use of the “Acquired pellicle engineering” concept, which involves modifying the AP through the incorporation of molecules, has a strong potential to increase its protective effect on the tooth surface due to the strength of the protein binding to hydroxyapatite (CARVALHO et al., 2020; SANTIAGO et al., 2017). Moreover, the incorporation of molecules in the AP may change the enamel reactivity, provoking changes in the surface free energy (SFE), which might guide protein binding to the AP, thus changing its composition.

Regarding the application vehicle, the use of gels in studies involving the inhibition of matrix metalloproteinases in dentin showed satisfactory results for its protection(KATO *et al.*, 2010; MAGALHÃES *et al.*, 2009). In this way, the use of gels (containing CaneCPI-5) for topical application on enamel can also be a good treatment alternative since the contact of the gel with the enamel surface may be prolonged, due to its structure and density.

Taking the above-mentioned aspects into consideration, the present study was designed to evaluate the protective effect of gels containing different concentrations of CaneCPI-5 against enamel initial erosion in vitro. Since little is known about the mechanisms by which CaneCPI-5 interacts with the enamel surface, we also analyzed the ability of CaneCPI-5 to alter the SFE of enamel by measuring the contact angle using the sessile drop

method. The null hypotheses tested were: 1) gels containing CaneCPI-5 do no protect against initial dental erosion and 2) CaneCPI-5 does not alter the enamel SFE.

MATERIALS AND METHODS

This study comprised 2 experiments: In experiment 1, the effect of CaneCPI-5 (in different concentrations) added in gel on polished enamel specimens was evaluated using surface microhardness analysis. In experiment 2, 0.1 mg/mL CaneCPI-5 (in solution) was applied on polished enamel specimens for SFE analysis.

The use of bovine teeth for this research was approved by the Ethics Committee on Animal Use of Bauru School of Dentistry, University of São Paulo (Protocol: 006/2017 for experiment 1 and Protocol: 010/2021 for experiment 1). Also, this study was approved by the Ethics Committee for Human Research (CAAE: 59786416.9.0000.5417) of Bauru School of Dentistry, University of São Paulo. In addition, saliva donor volunteers signed an informed consent form before the procedures.

Selection of volunteers and saliva collection

Total saliva was collected from 3 healthy volunteers of both genders (aged 24 to 32 years). The exclusion criteria adopted were: smoking, cavitated carious lesions, severe dental wear, use of medications that affect salivary flow, salivary flow under the thresholds for unstimulated (> 0.3 mL/min) and stimulated (> 1.0 mL/min) saliva, xerostomia, type I diabetes, poor nutrition, gastroesophageal problems and regurgitation and vomiting disorders (TENOVOU, J. O., & LAGERLÖF, 1995).

Before collection, all the volunteers performed oral hygiene using a new toothbrush, fluoride toothpaste (CloseUP, 1450 ppm F, Unilever, Brazil) and dental floss. Saliva collections occurred between 9 and 11 a.m. (to avoid circadian effects) under masticatory stimulation using Parafilm. Then, saliva was centrifuged (14,000 g at 4ºC) for 15 min. After that, the supernatants were collected to form a pool of saliva and divided into 13-mL aliquots, which were stored at -80 ° C prior to the experiments (SCHIPPER et al., 2007).

Heterologous expression of CaneCPI-5

The production of CaneCPI-5 was carried out at the Laboratory of Molecular Biology of the Department of Genetics and Evolution of the Federal University of São Carlos, Brazil. For heterologous expression, bacterial strain *Escherichia coli Rosetta* (DE3) transformed with plasmid pET28aCaneCPI-5 was used as previously described(SOARES-COSTA *et al.*, 2002). The expressed protein was purified from the soluble fraction of bacterial cultures induced by

IPTG (Isopropyl-beta-D-thiogalactosidium), subjected to centrifugation and sonication. The purification was done by affinity chromatography using columns containing Ni-NTA Superflow nickel resin (Qiagen), as described previously (SOARES-COSTA et al., 2002).

Preparation of the enamel specimens

Ninety-five bovine enamel specimens were prepared (4 mm × 4 mm × 4 mm), being 75 specimens for “experiment 1” and 20 specimens for “experiment 2”. They were obtained from the buccal-cervical region of bovine incisors and stored in 2% thymol solution (pH 7.0) for 30 days. In addition, the specimens were submitted to visual analysis for the investigation of possible stains and cracks. In these cases, the teeth were excluded. Then, the enamel surface was sequentially polished, using water-cooled silicon carbide paper disks (320, 600, and 1200 grit, Extec, Enfield, CT, USA). To finalize the polishing a felt polishing cloth (Extec Corp. Polishing cloth; Buehler, Lake Bluff, IL, USA), moistened with a 1-µm diamond solution (Extec Corp. Buehler, Lake Bluff, IL, USA) was used on the surface of interest. After polishing, the specimens were immersed in an ultrasonic bath (T7 Thornton, Unique Ind. E Com. Ltda., São Paulo, SP, BR) with deionized water for 7 min at 25°C. Lastly, they were stored (with wet gauze) at 4°C prior to the experiment.

Experiment 1. Effect of gels containing different concentrations of CaneCPI-5 against initial enamel erosion in vitro

Experimental procedures

Seventy-five specimens were divided into 5 groups (n/group = 15, determined by computerized random numbers, after initial surface hardness): 1) placebo gel (negative control), 2) 0.27% mucin plus 0.5% casein (positive control), 3) 0.1 mg/mL CaneCPI-5, 4) 1 mg/mL CaneCPI-5 and 5) 2.0 mg/mL CaneCPI-5. All gels were prepared as described by Kato et al. (KATO et al., 2010) and had exactly the same composition, except for the presence of casein + mucin or CaneCPI-5.

The amount of gel applied was controlled through a dispenser (pipette, 20 µl per specimen), then the gel was added on the microbrush and applied on the enamel surface of each specimen for 1 minute, and the excess was removed with a cotton swab (KATO et al., 2010). The specimens were then incubated in saliva for 2 h at 37°C under agitation to form the AP (CHEAIB; LUSSI, 2011). Then, the specimens were washed in deionized water (10 s) and air-dried (5 s). For the erosive challenge, they were immersed in 0.65% citric acid solution (pH = 3.4) for 1 min at 30°C under agitation, washed in deionized water and air-dried once again (CHEAIB; LUSSI, 2011).

Surface hardness

Surface hardness change (SHC) analyses were performed using a Knoop penetrator, with a load of 50 g for 15 s at baseline ($SHC_{initial}$) and after the experiment (SHC_{final}). Five indentations were made in the central region of each specimen at 50 µm intervals. Control indentations of 2 and 5 g were made to detect possible loss of surface. Specimens with microhardness values 10% lower or 10% higher than the mean of all specimens were excluded from the study. The percentage of surface hardness change (%SHC) was calculated as a measure of enamel softening, according to the following equation: $\%SHC = ([SHC_{initial} - SHC_{final}]/SHC_{initial})*100$ (SANTIAGO et al., 2017).

Experiment 2. Ability of CaneCPI-5 to alter the enamel surface free energy

Twenty enamel specimens were divided into two groups, as follows: Control (untreated) or 0.1 mg/mL CaneCPI-5 (n=10/group determined by computerized random numbers).

Surface free energy measurements

The physical properties of the enamel surface were characterized by contact angle measurements, using the sessile drop method to determine the SFE. Measurements were performed by an automatic goniometer (DSA 100S, Krüss, Hamburg, Germany) using three probing liquids: diiodomethane, water and ethylene glycol. The treated specimens were air dried for 45 min in order to stabilize the pellicle formed (VAN DER MEI et al., 2002). After, 0.5 µL of each liquid was dispensed on the surface of each block, and the contact angles were measured using the images captured by a CCD camera. For each specimen 5 measurements were performed at 20°C and relative air humidity of 47% (HARNETT; ALDERMAN; WOOD, 2007; VAN DER MEI et al., 2002). Different parameters, such as acid (γ^+, receptor component), base (γ^-, donor component) and Lifshiz van der Waals (γ^{LW}, nonpolar component) of surface free energy (mN/m) were calculated according to the model of van Oss, Chaudhery and Good for the determination of the substrates free energy (DELLA VOLPE; SIBONI, 1997; OSS, 1990). The interaction free energy (ΔG_{iwi}) was also calculated to determine the hydrophobicity/hydrophilicity of the enamel surface: $\Delta G_{iwi} > 0$ indicated a hydrophilic surface and $\Delta G_{iwi} < 0$ indicated a hydrophobic surface (HARNETT; ALDERMAN; WOOD, 2007; VAN OSS, 1993).

Statistical Analysis

All the data were analyzed using the softwares GraphPad InStat (version 3.10 for Windows) and GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Data were checked for normality (Kolmogorov-Smirnov test) and homogeneity (Bartlett test) to select the

appropriate statistical test. In the first experiment, the data were analyzed by Kruskall-Wallis and Dunn´s tests. In the second experiment, the data were analyzed by ANOVA and Student-Newman-Keuls's test and by Pearson´s correlation coefficient. The significance levels of both experiments were considered as $p<0.05$.

RESULTS

In the first experiment, only the treatments with CaneCPI-5 at 0.1 and 1.0 mg/mL significantly reduced the %SHC in comparison with control ($p<0.05$). The treatment performed with the higher concentration of CaneCPI-5 did not significantly differ neither from control nor from mucin plus casein ($p>0.05$) (Figure 1).

Regarding the physical properties of the enamel surface after treatment with CaneCPI-5, the SFE (γ_S) was significantly lower with CaneCPI-5 ($p<0.001$) compared with control (Table 1; $p<0.001$). The values of the apolar component (γ_S^{LW}) from enamel surface were not significantly different between the groups ($p=0.161$). The values of the polar component (γ_S^{AB} = Lewis acid-base) became more negative with CaneCPI-5 treatment (Figure 2A; $p<0.001$). Among the parameters from γ_S^{AB}, γ_S^+ = electron-acceptor (Lewis acid) and γ_S^- = electron-donor (Lewis base), the CaneCPI-5 treatment showed higher γ_S^- values compared to control (Figure 2B; $p<0.001$). Significant correlations were observed between γ_S values and γ_S^{AB} (Pearson's $r = 0.987$; $p<0.001$) and γ_S^- (Pearson's $r = -0.942$; $p<0.001$). The interaction free energy (ΔG_{iwi}) was greater than zero for CaneCPI-5 treatment indicating a hydrophilic surface (Table 1).

DISCUSSION

The present study involves the concept of "acquired pellicle engineering" that involves the modification of the AP through the incorporation of molecules or ions that are able to increase its protective effect (VUKOSAVLJEVIC et al., 2014). The modification was done with the use of CaneCPI-5, a sugarcane-derived cystatin that has a strong binding force to hydroxyapatite (SANTIAGO et al., 2017).

In experiment 1, we employed a well-established initial erosion model to evaluate the protective effect of a gel containing CaneCPI-5 against erosion. This model involves one challenge (1 min) with 0.65% citric acid (pH 3.5) (CHEAIB; LUSSI, 2011), causing enamel softening that can be measured by SHC, since enamel loss (detected by profilometry) is not expected at this early stage. Previous studies by our group showed protective effect of the application of solutions containing 0.1 mg/mL CaneCPI-5 against initial enamel erosion when applied *in vitro* for 2 h (SANTIAGO et al., 2017) or *in vivo* for 1 min (CARVALHO et al., 2020). Furthermore, the use of gels containing protease inhibitors (KATO et al., 2010) promoted superior protection against dentine erosion than solutions containing the same inhibitors

(MAGALHÃES et al., 2009), which was attributed to a more intimate contact of the gels with the dental surfaces. By the way, a mixture of mucin (0.27%) and casein (0.5%) was used (in the present study) as a positive control due to the previous results, which demonstrated that the addition of the 2 components in the AP was able to provide the protective effect against initial erosion *in vitro* (CHEAIB; LUSSI, 2011).

Based on the above, in the present study this vehicle was selected for application of CaneCPI-5, at concentrations (ranging between 0.1 and 2.0 mg/mL) based on those employed in solutions (SANTIAGO et al., 2017). The gels containing CaneCPI-5 at 0.1 and 1.0 mg/mL significantly reduced enamel erosion in comparison with the placebo gel, while the product containing 2.0 mg/mL CaneCPI-5 did not (Fig. 1). The overall %SHC reduction promoted by the gels was 30%, while the effects using aqueous solutions at the same concentrations was around 90% (SANTIAGO et al., 2017). It is noteworthy, however, that the solutions remained in contact with the enamel surface for 2 h, while treatment with the gels lasted 1 min only. Based on time-response considerations, it would be interesting to evaluate longer exposure to the gels (e.g., 4 min), given that the application of fluoridated gels for 4 min in the clinical practice has been reported to provide higher caries-protective effects than application for 1 min (VAN RIJKOM et al., 2003; WEYANT et al., 2013). Another interesting finding was that the highest concentration of CaneCPI-5 (2.0 mg/mL) did not protect enamel against initial erosion. This seems to be related to previous studies showing that sugarcane cystatins, at high concentrations, undergo dimerization through domain swapping (CAVINI et al., 2013; VALADARES et al., 2013), which reduces the levels of free protein to bind to enamel.

The experiment 2 had a mechanistic approach. Our objective was to test the enamel reactivity after treatment with CaneCPI-5 using the sessile drop method. This is particularly important for the concept of “acquired pellicle engineering”, since alterations in the SFE upon the treatment with CaneCPI-5 might guide protein binding to the AP, thus changing its composition, especially considering binding of other salivary proteins to CaneCPI-5 and/or to dental surfaces. The untreated enamel was slightly hydrophobic since contact water angle was a little larger than 65° (RÜTTERMANN et al., 2011; VAN DER MEI; WHITE; BUSSCHER, 2004); SFE (γ_s) was lower than 30 mN/m (Table 1) (KNORR et al., 2005; VAN OSS, 1993), ΔG_{iwi} was close to zero, and γ_s^- was lower than 28.5 mN/m (Figure 2) (HARNETT; ALDERMAN; WOOD, 2007; VAN OSS, 1993, 1995), with values of γ_s^+ close to zero. As described in a previous study (NEVES et al., 2018), enamel surface presents characteristics that favor the precipitation of ionic species, such as Ca^{2+} and $CaH_2PO_4^+$, or protein adhesion, both of which are important in reducing the erosive process. Furthermore, it is known that surfaces with lower SFE brings fewer bacteria to its surface than another one with higher SFE. It must be emphasized, however, the acid-base theoretical approach used in this study

(HARNETT; ALDERMAN; WOOD, 2007; VAN OSS, 1993, 1995), involving the decomposition in γ_s^{LW} and γ_s^{AB} (which strongly influence to γ_s), differs from other studies that used different theoretical approaches to calculate γ_s.

In the present study, the reduction of SFE with CaneCPI-5 treatment was related to more negative values of polar energy (γ_s^{AB}), given that no change in nonpolar energy was observed (γ_s^{LW}). Therefore, in these cases, the acid (γ_s^+)/base (γ_s^-) and interaction free energy (ΔG_{iwi}) forces indicate whether a surface is more hydrophobic or hydrophilic, facilitating or not protein adhesion or calcium phosphate precipitation (HARNETT; ALDERMAN; WOOD, 2007; NEVES et al., 2018; VAN OSS, 1995). The theoretical aspects above demonstrate that treatment with CaneCPI-5 increases the hydrophilic character of the surface of the enamel, which makes it prone to water, considering contact angles smaller than 65°, $\gamma_s^- > 28.5$ mN/m and $\Delta G_{iwi} > 0$ (VOGLER, 1998). Also, CaneCPI-5 presented higher γ_s^- values, leading to higher electron-donor sites at the enamel surface which, in turn, favors adsorption of cationic species (Ca^{2+} and $CaH_2PO_4^+$) and cationic acid-resistant proteins from saliva, thus explaining the lower hardness loss after erosion challenge. Consequently, negative surfaces may be partially or fully neutralized by multivalent cations leading to a hydrophobic surface (OLSSON, 1992). Alteration in the SFE explain, at least in part, the changes in acid-resistant proteins of the AP obtained after rinsing for 1 min with 0.1 mg/mL CPI-5 and subsequent challenge with 1% citric acid pH 2.5 for 10 s (increase in keratin, IgG, lactotransferrin, serum albumin, alpha amylase, basic salivary proline-rich protein, carbonic anhydrase) (CARVALHO et al., 2020).

It is essential to recognize the limitations of the present *in vitro* study. Although the protocols are considered suitable for preliminary investigations, they do not accurately simulate the clinical condition due to the absence of oral cavity-specific factors, such as the formation of AP. In experiment 1, limitation of treatment time (with the gels and CaneCPI-5) for 1 min could be extended for longer periods (e.g., 4 min). Regarding experiment 2, the presence of saliva, the most important biological factor involved in the occurrence of dental erosion whose factor is the most determinant for the oral cavity, was not considered. Moreover, in the first experiment CaneCPI-5 was included in gels, while in the second one, it was included in solution, due to the analytical technique employed. In future studies, these limitations need to be addressed.

Based on the results obtained, the two hypotheses were rejected, since: 1) gels containing CaneCPI-5 at 0.1 and 1.0 mg/mL protected enamel against initial dental erosion; and 2) CaneCPI-5 altered the enamel SFE. Moreover, change in SFE of enamel after application of CaneCPI-5 may help to explain, at least in part, alterations in the AP proteome, with consequent change in its protective ability, induced by this phytocystatin.

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Competing interests

The authors have declared no competing interests.

REFERENCES

BUZALAF, M. A.; HANNAS, A. R.; KATO, M. T. Saliva and dental erosion. **J Appl Oral Sci**, v. 20, n. 5, p. 493–502, 2012.

CARVALHO, T. S.; ARAÚJO, T. T.; VENTURA, T. M. O.; DIONIZIO, A.; CÂMARA, J. V. F.; MORAES, S. M.; PELÁ, V. T.; MARTINI, T.; LEME, J. C.; DERBOTOLLI, A. L. B.; GRIZZO, L. T.; CRUSCA, E.; SHIBAO, P. Y. T.; MARCHETTO, R.; HENRIQUE-SILVA, F.; PESSAN, J. P.; BUZALAF, M. A. R. Acquired pellicle protein-based engineering protects against erosive demineralization. **Journal of Dentistry**, v. 102, n. September, 2020.

CAVINI, Í. A.; DE OLIVEIRA-SILVA, R.; DE ALMEIDA MARQUES, I.; KALBITZER, H. R.; MUNTE, C. E. Chemical shift assignments of the canecystatin-1 from Saccharum officinarum. **Biomolecular NMR Assignments**, v. 7, n. 2, p. 163–165, 2013.

CHEAIB, Z.; LUSSI, A. Impact of acquired enamel pellicle modification on initial dental erosion. **Caries Research**, v. 45, n. 2, p. 107–112, 2011.

DELECRODE, T. R.; SIQUEIRA, W. L.; ZAIDAN, F. C.; BELLINI, M. R.; MOFFA, E. B.; MUSSI, M. C. M.; XIAO, Y.; BUZALAF, M. A. R. Identification of acid-resistant proteins in acquired enamel pellicle. **Journal of Dentistry**, v. 43, n. 12, p. 1470–1475, 2015.

DELLA VOLPE, C.; SIBONI, S. Some reflections on acid-base solid surface free energy theories. **Journal of Colloid and Interface Science**, v. 195, n. 1, p. 121–136, 1997.

HANNIG, C.; BERNDT, D.; HOTH-HANNIG, W.; HANNIG, M. The effect of acidic beverages on the ultrastructure of the acquired pellicle-An in situ study. **Archives of Oral Biology**, v. 54, n. 6, p. 518–526, 2009.

HARNETT, E. M.; ALDERMAN, J.; WOOD, T. The surface energy of various biomaterials coated with adhesion molecules used in cell culture. Colloids and Surfaces B: **Biointerfaces**, v. 55, n. 1, p. 90–97, 2007.

KATO, M. T.; LEITE, A. L.; HANNAS, A. R.; BUZALAF, M. A. R. Gels containing MMP inhibitors prevent dental erosion in situ. **Journal of Dental Research**, v. 89, n. 5, p. 468–472, 2010.

KNORR, S. D.; COMBE, E. C.; WOLFF, L. F.; HODGES, J. S. The surface free energy of dental gold-based materials. **Dental Materials**, v. 21, n. 3, p. 272–277, 2005.

MAGALHÃES, A. C.; WIEGAND, A.; RIOS, D.; HANNAS, A.; ATTIN, T.; BUZALAF, M. A. R. Chlorhexidine and green tea extract reduce dentin erosion and abrasion in situ. **Journal of Dentistry**, v. 37, n. 12, p. 994–998, 2009.

MARTINI, T.; RIOS, D.; CASSIANO, L. P. S.; SILVA, C. M. de S.; TAIRA, E. A.; VENTURA, T. M. S.; PEREIRA, H. A. B. S.; MAGALHÃES, A. C.; CARVALHO, T. S.; BAUMANN, T.; LUSSI, A.; OLIVEIRA, R. B.; PALMA-DIBB, R. G.; BUZALAF, M. A. R. Proteomics of acquired pellicle in gastroesophageal reflux disease patients with or without erosive tooth wear. **Journal of Dentistry**, v. 81, n. December 2018, p. 64–69, 2019.

NEVES, J. G.; DANELON, M.; PESSAN, J. P.; FIGUEIREDO, L. R.; CAMARGO, E. R.; DELBEM, A. C. B. Surface free energy of enamel treated with sodium hexametaphosphate, calcium and phosphate. **Archives of Oral Biology**, v. 90, n. March, p. 108–112, 2018.

OLSSON, J. Plaque Formation in vivo and Bacterial Attachment in vitro on permanently hydrophobic and hydrophilic surfaces. 1992.

OSS, van C. J. Aspecific and Specific Intermolecular Interactions in Aqueous Media. **Journal of Molecular Recognition**, v. 3, p. 128–136, 1990.

RÜTTERMANN, S.; TRELLENKAMP, T.; BERGMANN, N.; RAAB, W. H. M.; RITTER, H.; JANDA, R. A new approach to influence contact angle and surface free energy of resin-based dental restorative materials. **Acta Biomaterialia**, v. 7, n. 3, p. 1160–1165, 2011.

SANTIAGO, A. C.; KHAN, Z. N.; MIGUEL, M. C.; GIRONDA, C. C.; SOARES-COSTA, A.; PELÁ, V. T.; LEITE, A. L.; EDWARDSON, J. M.; BUZALAF, M. A. R.; HENRIQUE-SILVA, F.

A New Sugarcane Cystatin Strongly Binds to Dental Enamel and Reduces Erosion. **Journal of Dental Research**, v. 96, n. 9, p. 1051–1057, 2017.

SCHIPPER, R.; LOOF, A.; DE GROOT, J.; HARTHOORN, L.; DRANSFIELD, E.; VAN HEERDE, W. SELDI-TOF-MS of saliva: Methodology and pre-treatment effects. Journal of Chromatography B: **Analytical Technologies in the Biomedical and Life Sciences**, v. 847, n. 1, p. 45–53, 2007.

SCHLUETER, N.; AMAECHI, B. T.; BARTLETT, D.; BUZALAF, M. A. R.; CARVALHO, T. S.; GANSS, C.; HARA, A. T.; HUYSMANS, M. C. D. N. J. M.; LUSSI, A.; MOAZZEZ, R.; VIEIRA, A. R.; WEST, N. X.; WIEGAND, A.; YOUNG, A.; LIPPERT, F. Terminology of Erosive Tooth Wear: Consensus Report of a Workshop Organized by the ORCA and the Cariology Research Group of the IADR. **Caries Research**, v. 54, n. 1, p. 2–6, 2020.

SOARES-COSTA, A.; BELTRAMINI, L. M.; THIEMANN, O. H.; HENRIQUE-SILVA, F. A sugarcane cystatin: Recombinant expression, purification, and antifungal activity. **Biochemical and Biophysical Research Communications**, v. 296, n. 5, p. 1194–1199, 2002.

TAIRA, E. A.; VENTURA, T. M. S.; CASSIANO, L. P. S.; SILVA, C. M. S.; MARTINI, T.; LEITE, A. L.; RIOS, D.; MAGALHÃES, A. C.; BUZALAF, M. A. R. Changes in the Proteomic Profile of Acquired Enamel Pellicles as a Function of Their Time of Formation and Hydrochloric Acid Exposure. **Caries Research**, v. 52, n. 5, p. 367–377, 2018.

TENOVOU, J. O., & LAGERLÖF, F. Cariologia clínica. [s.l: s.n.]17–43 p.

VALADARES, N. F.; DE OLIVEIRA-SILVA, R.; CAVINI, I. A.; DE ALMEIDA MARQUES, I.; D'MUNIZ PEREIRA, H.; SOARES-COSTA, A.; HENRIQUE-SILVA, F.; KALBITZER, H. R.; MUNTE, C. E.; GARRATT, R. C. X-ray crystallography and NMR studies of domain-swapped canecystatin-1. **FEBS Journal**, v. 280, n. 4, p. 1028–1038, 2013.

VAN DER MEI, H. C.; WHITE, D. J.; BUSSCHER, H. J. On the wettability of soft tissues in the human oral cavity. **Archives of Oral Biology**, v. 49, n. 8, p. 671–673, 2004.

VAN DER MEI, H. C.; WHITE, D. J.; KAMMINGA-RASKER, H. J.; KNIGHT, J.; BAIG, A. A.; SMIT, J.; BUSSCHER, H. J. Influence of dentifrices and dietary components in saliva on wettability of pellicle-coated enamel in vitro and in vivo. **European journal of oral sciences**, v. 110, n. 6, p. 434–438, 2002.

VAN OSS, C. J. Acid-base interfacial interactions in aqueous media. Colloids and Surfaces A: BUZALAF, M. a; HANNAS, a R.; KATO, M. T. **Saliva and dental erosion**. J Appl Oral Sci, v. 20, n. 5, p. 493–502, 2012.

CARVALHO, T. S.; ARAÚJO, T. T.; VENTURA, T. M. O.; DIONIZIO, A.; CÂMARA, J. V. F.; MORAES, S. M.; PELÁ, V. T.; MARTINI, T.; LEME, J. C.; DERBOTOLLI, A. L. B.; GRIZZO, L. T.; CRUSCA, E.; SHIBAO, P. Y. T.; MARCHETTO, R.; HENRIQUE-SILVA, F.; PESSAN, J. P.; BUZALAF, M. A. R. Acquired pellicle protein-based engineering protects against erosive demineralization. **Journal of Dentistry**, v. 102, n. September, 2020.

CAVINI, Í. A.; DE OLIVEIRA-SILVA, R.; DE ALMEIDA MARQUES, I.; KALBITZER, H. R.; MUNTE, C. E. Chemical shift assignments of the canecystatin-1 from Saccharum officinarum. **Biomolecular NMR Assignments**, v. 7, n. 2, p. 163–165, 2013.

CHEAIB, Z.; LUSSI, A. Impact of acquired enamel pellicle modification on initial dental erosion. **Caries Research**, v. 45, n. 2, p. 107–112, 2011.

DELECRODE, T. R.; SIQUEIRA, W. L.; ZAIDAN, F. C.; BELLINI, M. R.; MOFFA, E. B.; MUSSI, M. C. M.; XIAO, Y.; BUZALAF, M. A. R. Identification of acid-resistant proteins in acquired enamel pellicle. **Journal of Dentistry**, v. 43, n. 12, p. 1470–1475, 2015.

DELLA VOLPE, C.; SIBONI, S. Some reflections on acid-base solid surface free energy theories. **Journal of Colloid and Interface Science**, v. 195, n. 1, p. 121–136, 1997.

HANNIG, C.; BERNDT, D.; HOTH-HANNIG, W.; HANNIG, M. The effect of acidic beverages on the ultrastructure of the acquired pellicle-An in situ study. **Archives of Oral Biology**, v. 54, n. 6, p. 518–526, 2009.

HARNETT, E. M.; ALDERMAN, J.; WOOD, T. The surface energy of various biomaterials coated with adhesion molecules used in cell culture. **Colloids and Surfaces B: Biointerfaces**, v. 55, n. 1, p. 90–97, 2007.

KATO, M. T.; LEITE, A. L.; HANNAS, A. R.; BUZALAF, M. A. R. Gels containing MMP inhibitors prevent dental erosion in situ. **Journal of Dental Research**, v. 89, n. 5, p. 468–472, 2010.

KNORR, S. D.; COMBE, E. C.; WOLFF, L. F.; HODGES, J. S. The surface free energy of dental gold-based materials. **Dental Materials**, v. 21, n. 3, p. 272–277, 2005.

MAGALHÃES, A. C.; WIEGAND, A.; RIOS, D.; HANNAS, A.; ATTIN, T.; BUZALAF, M. A. R. Chlorhexidine and green tea extract reduce dentin erosion and abrasion in situ. **Journal of Dentistry**, v. 37, n. 12, p. 994–998, 2009.

MARTINI, T.; RIOS, D.; CASSIANO, L. P. S.; SILVA, C. M. de S.; TAIRA, E. A.; VENTURA, T. M. S.; PEREIRA, H. A. B. S.; MAGALHÃES, A. C.; CARVALHO, T. S.; BAUMANN, T.; LUSSI, A.; OLIVEIRA, R. B.; PALMA-DIBB, R. G.; BUZALAF, M. A. R. Proteomics of acquired pellicle in gastroesophageal reflux disease patients with or without erosive tooth wear. **Journal of Dentistry**, v. 81, n. December 2018, p. 64–69, 2019.

NEVES, J. G.; DANELON, M.; PESSAN, J. P.; FIGUEIREDO, L. R.; CAMARGO, E. R.; DELBEM, A. C. B. Surface free energy of enamel treated with sodium hexametaphosphate, calcium and phosphate. **Archives of Oral Biology**, v. 90, n. March, p. 108–112, 2018.

OLSSON, J. Plaque Formation in vivo and Bacterial Attachment in vitro on permanently hydrophobic and hydrophilic surfaces. 1992.

OSS, van C. J. Aspecific and Specific Intermolecular Interactions in Aqueous Media. **Journal of Molecular Recognition**, v. 3, p. 128–136, 1990.

RÜTTERMANN, S.; TRELLENKAMP, T.; BERGMANN, N.; RAAB, W. H. M.; RITTER, H.; JANDA, R. A new approach to influence contact angle and surface free energy of resin-based dental restorative materials. **Acta Biomaterialia**, v. 7, n. 3, p. 1160–1165, 2011.

SANTIAGO, A. C.; KHAN, Z. N.; MIGUEL, M. C.; GIRONDA, C. C.; SOARES-COSTA, A.; PELÁ, V. T.; LEITE, A. L.; EDWARDSON, J. M.; BUZALAF, M. A. R.; HENRIQUE-SILVA, F. A New Sugarcane Cystatin Strongly Binds to Dental Enamel and Reduces Erosion. **Journal of Dental Research**, v. 96, n. 9, p. 1051–1057, 2017.

SCHIPPER, R.; LOOF, A.; DE GROOT, J.; HARTHOORN, L.; DRANSFIELD, E.; VAN HEERDE, W. SELDI-TOF-MS of saliva: Methodology and pre-treatment effects. **Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences**, v. 847, n. 1, p. 45–53, 2007.

SCHLUETER, N.; AMAECHI, B. T.; BARTLETT, D.; BUZALAF, M. A. R.; CARVALHO, T. S.; GANSS, C.; HARA, A. T.; HUYSMANS, M. C. D. N. J. M.; LUSSI, A.; MOAZZEZ, R.; VIEIRA, A. R.; WEST, N. X.; WIEGAND, A.; YOUNG, A.; LIPPERT, F. Terminology of Erosive Tooth

Wear: Consensus Report of a Workshop Organized by the ORCA and the Cariology Research Group of the IADR. **Caries Research**, v. 54, n. 1, p. 2–6, 2020.

SOARES-COSTA, A.; BELTRAMINI, L. M.; THIEMANN, O. H.; HENRIQUE-SILVA, F. A sugarcane cystatin: Recombinant expression, purification, and antifungal activity. **Biochemical and Biophysical Research Communications**, v. 296, n. 5, p. 1194–1199, 2002.

TAIRA, E. A.; VENTURA, T. M. S.; CASSIANO, L. P. S.; SILVA, C. M. S.; MARTINI, T.; LEITE, A. L.; RIOS, D.; MAGALHÃES, A. C.; BUZALAF, M. A. R. Changes in the Proteomic Profile of Acquired Enamel Pellicles as a Function of Their Time of Formation and Hydrochloric Acid Exposure. **Caries Research**, v. 52, n. 5, p. 367–377, 2018.

TENOVOU, J. O., & LAGERLÖF, F. Cariologia clínica. [s.l: s.n.]17–43 p.

VALADARES, N. F.; DE OLIVEIRA-SILVA, R.; CAVINI, I. A.; DE ALMEIDA MARQUES, I.; D'MUNIZ PEREIRA, H.; SOARES-COSTA, A.; HENRIQUE-SILVA, F.; KALBITZER, H. R.; MUNTE, C. E.; GARRATT, R. C. X-ray crystallography and NMR studies of domain-swapped canecystatin-1. **FEBS Journal**, v. 280, n. 4, p. 1028–1038, 2013.

VAN DER MEI, H. C.; WHITE, D. J.; BUSSCHER, H. J. On the wettability of soft tissues in the human oral cavity. **Archives of Oral Biology**, v. 49, n. 8, p. 671–673, 2004.

VAN DER MEI, H. C.; WHITE, D. J.; KAMMINGA-RASKER, H. J.; KNIGHT, J.; BAIG, A. A.; SMIT, J.; BUSSCHER, H. J. Influence of dentifrices and dietary components in saliva on wettability of pellicle-coated enamel in vitro and in vivo. **European journal of oral sciences**, v. 110, n. 6, p. 434–438, 2002.

VAN OSS, C. J. Acid-base interfacial interactions in aqueous media. Colloids and Surfaces A: Physicochemical and Engineering Aspects, v. 78, n. C, p. 1–49, 1993.

VAN OSS, C. J. Hydrophobicity of biosurfaces - Origin, quantitative determination and interaction energies. **Colloids and Surfaces B: Biointerfaces**, v. 5, n. 3–4, p. 91–110, 1995.

VAN RIJKOM, H.; RUBEN, J.; VIEIRA, A.; HUYSMANS, M. C.; TRUIN, G. J.; MULDER, J. Erosion-inhibiting effect of sodium fluoride and titanium tetrafluoride treatment in vitro. **European Journal of Oral Sciences**, v. 111, n. 3, p. 253–257, 2003.

VOGLER, E. A. Structure and reactivity of water at biomaterial surfaces. **Advances in Colloid and Interface Science**, v. 74, n. 1–3, p. 69–117, 1998.

VUKOSAVLJEVIC, D.; CUSTODIO, W.; BUZALAF, M. A. R.; HARA, A. T.; SIQUEIRA, W. L. Acquired pellicle as a modulator for dental erosion. **Archives of Oral Biology**, v. 59, n. 6, p. 631–638, 2014.

WEYANT, R. J.; TRACY, S. L.; ANSELMO, T. T.; BELTRAN-AGUILAR, E. D.; DONLY, K. J.; FRESE, W. A.; HUJOEL, P. P.; IAFOLLA, T.; KOHN, W.; KUMAR, J.; LEVY, S. M.; TINANOFF, N.; WRIGHT, J. T.; ZERO, D.; ARAVAMUDHAN, K.; FRANTSVE-HAWLEY, J.; MEYER, D. M. 11 - Topical fluoride for caries prevention: executive summary of the updated clinical recommendations and supporting systematic review. **Journal of the American Dental Association (1939)**, v. 144, n. 11, p. 1279–1291, 2013.
, v. 78, n. C, p. 1–49, 1993.

VAN OSS, C. J. Hydrophobicity of biosurfaces - Origin, quantitative determination and interaction energies. **Colloids and Surfaces B: Biointerfaces**, v. 5, n. 3–4, p. 91–110, 1995.

VAN RIJKOM, H.; RUBEN, J.; VIEIRA, A.; HUYSMANS, M. C.; TRUIN, G. J.; MULDER, J. Erosion-inhibiting effect of sodium fluoride and titanium tetrafluoride treatment in vitro. **European Journal of Oral Sciences**, v. 111, n. 3, p. 253–257, 2003.

VOGLER, E. A. Structure and reactivity of water at biomaterial surfaces. **Advances in Colloid and Interface Science**, v. 74, n. 1–3, p. 69–117, 1998.

VUKOSAVLJEVIC, D.; CUSTODIO, W.; BUZALAF, M. A. R.; HARA, A. T.; SIQUEIRA, W. L. Acquired pellicle as a modulator for dental erosion. **Archives of Oral Biology**, v. 59, n. 6, p. 631–638, 2014.

WEYANT, R. J.; TRACY, S. L.; ANSELMO, T. T.; BELTRAN-AGUILAR, E. D.; DONLY, K. J.; FRESE, W. A.; HUJOEL, P. P.; IAFOLLA, T.; KOHN, W.; KUMAR, J.; LEVY, S. M.; TINANOFF, N.; WRIGHT, J. T.; ZERO, D.; ARAVAMUDHAN, K.; FRANTSVE-HAWLEY, J.; MEYER, D. M. 11 - Topical fluoride for caries prevention: executive summary of the updated clinical recommendations and supporting systematic review. **Journal of the American Dental Association (1939)**, v. 144, n. 11, p. 1279–1291, 2013.

FIGURE 1

Median enamel loss after short-term erosive challenge. Bovine enamel specimens were treated with gels containing the proteins for 1 min, followed by incubation in pooled human saliva for 2 h for the formation of the acquired pellicle and subsequent challenge with 0.65% citric acid for 1 min. Different letters indicate a significant difference among groups (Kruskal-Wallis and Dunn's tests, $p<0.05$, n=15/group).

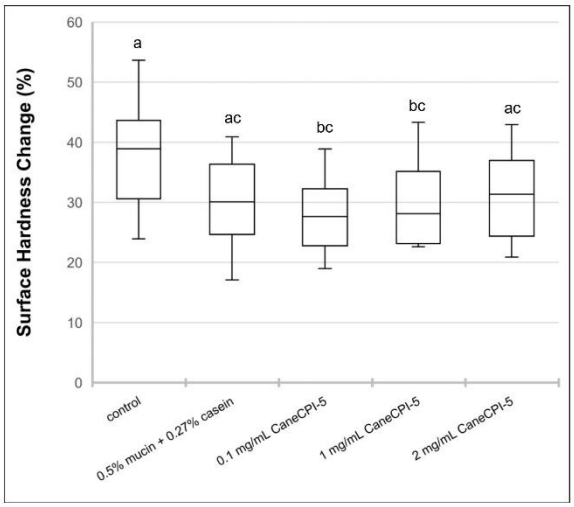

FIGURE 2 (A) Surface free-energy and their components (γ_S^{LW}: Lifshitz-van der Waals surface tension component; γ_S^{AB}: Lewis acid-base interaction) with different enamel-surface treatments. (B) Influence of the treatments on the component polar of surface free energy on enamel surface: Lewis-acid (γ_S^+) and Lewis-base (γ_S^-). Values denote mean and standard deviation (n = 10). Distinct letters show significant differences among means considering treatment (Student-Newman-Keuls, p <0.05).

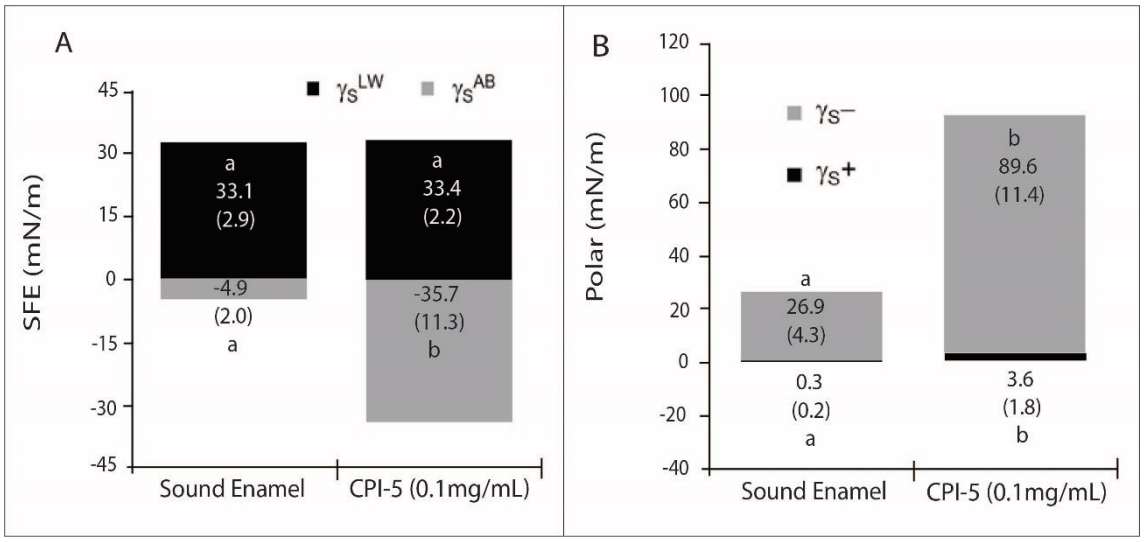

TABLE 1: Means SD of the contact angles of probing liquids, surface free energy (γ_S) and interaction free energy (ΔG_{iwi}) after treatment of enamel surface with 0.1 mg/mL CaneCPI-5 or not (n = 10).

Treatments	Water	Diiodomethane	Ethylene glycol	γS	ΔG_{iwi}
	q (°)	*q* (°)	*q* (°)	(mN/m)	(mN/m)
Sound enamel	67.3[a] (-4.4)	52.6[a] (-5.1)	56.6[a] (-3.6)	28.2[a] (-4.5)	-0.3[a] (-8.2)
CaneCPI-5 (0.1 mg/mL)	37.2[b] (-2.8)	51.6[a] (-3.8)	55.9[a] (-4.3)	-1.7[b] (-10.8)	53.2[b] (-5.6)

Distinct superscript letters indicate significant difference among the groups in each analysis (ANOVA and Student-Newman-Keuls's test, $p < 0.05$, n=10).

3.2 ARTICLE II

This article was submitted to Journal of Dentistry

Different vehicles containing CaneCPI-5 reduce erosive dentin wear *in situ*

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ABSTRACT

Objective: This study evaluated the protective capacity of a sugarcane-derived cystatin (CaneCPI-5) in different application vehicles (1-solution and 2-chitosan gel) against erosive dentin wear *in situ*. Methods: In part-1, 15 volunteers participated in a crossover protocol, with 3 phases (solutions): Water; Elmex™; 0.1mg/mL CaneCPI-5. The volunteers wore an appliance with 4 human dentin samples for 5 days. These samples were treated with a drop of the solutions for 1min (4X/d), then the acquired pellicle (AP) was formed and the samples were subjected to erosive challenges (EROSION: citric acid, for 90s, 4X/day). 2X/day, half of the samples were also abraded for 15s (ABRASION). In part-2, 16 volunteers participated of a double-blind and crossover protocol, consisting of 4 phases (gel): No gel, Chitosan gel, Chitosan gel+12,300ppm NaF and Chitosan gel+0.1mg/mL CaneCPI-5. The volunteers also wore an appliance, as described above. The samples were treated once/day with the gel or not for 4min, then the AP was formed and the samples were subjected to erosive and abrasive challenges, as report in part-1. Dentin wear was measured by profilometry. Data were analyzed by two-way RM-ANOVA and Sidak's tests (p<0.05). Results: Part-1: Elmex™ and CaneCPI-5 significantly reduced dentin loss in comparison with Water for the EROSION/ABRASION conditions (p<0.05). Part-2, all the treated groups significantly reduced the dentin loss in comparison to the No gel. The greatest reduction was found for the CANE group for the EROSION/ABRASION condition (p<0.05). **Conclusion:** The solution and chitosan gel containing CaneCPI-5 protected against erosive dentin wear *in situ*.

Clinical significance: Solution and Chitosan gel containing CaneCPI-5 protect against erosion/abrasion *in situ*, which is probably sufficient for protecting people at high risk for developing erosive dentin wear.

Keywords: Acquired pellicle; Cystatin; Dentin; Gel; Sugarcane.

1. INTRODUCTION

Erosive tooth wear (ETW) is a term used to describe the loss of mineralized tooth surfaces having erosion as the main causal factor. This process may also be associated with attrition and abrasion conditions [1,2]. In addition, ETW can be influenced by chemical, biological, and behavioral factors [3-5].

When the ETW reaches the dentin structure, the initial dissolution of the mineral exposes the organic matrix [6]. At this time, 3 zones can be observed: the demineralized organic matrix at the surface (DOM), followed by a partially demineralized zone and a zone of healthy internal dentin [6]. The DOM hampers ionic diffusion and buffers the acids, protecting against further erosion. However, dentin proteolytic enzymes such as matrix metalloproteinases (MMPs) and cysteine cathepsins (CCs) can accelerate the progress of erosion by degrading the exposed demineralized matrix [7].

Sufficient evidence supports that saliva and acquired pellicle (AP) are essential biological factors influencing ETW [8-10]. Therefore, a new perspective for preventing this process is the concept of "acquired pellicle engineering", that is the modification of the AP adding new components such as proteins/peptides, polyphenols, or lipids, which can be incorporated into the AP, increasing its protective potential against ETW [11,12].

In the last few years, a sugarcane-derived cystatin (named CaneCPI-5) was cloned, recombinantly expressed and shown to have a strong affinity to enamel by atomic force microscopy [13]. This protein, when added to solutions, was shown to reduce initial enamel erosion *in vitro* [13,14] and *in vivo* [12,15], as well as prolonged enamel erosion *in vitro* [16] and enamel erosion associated to abrasion *in situ* [17]. However, solutions must be applied 4 times/day in order to assure protection, which may be a problem in terms of compliance. Having this in mind, another vehicle of application was evaluated. CaneCPI-5 was added to gels, which were applied only once/day for 4 min, showing good protection against prolonged enamel and dentin erosion *in vitro* [16] and against enamel erosion and erosion associated to abrasion *in situ* [18].

However, to the best of our knowledge, there are no *in situ* studies evaluating the performance of CaneCPI-5-containing solutions and gels against dentin erosion and erosion associated to abrasion. It is important to highlight that CaneCPI-5 is a phytocystatin and has inhibitory activity against human cathepsins [13], which might increase its antierosive properties, due to the possible inhibition of dentin CCs. In addition, to increase the efficacy of the gels, the biopolymer chitosan was used as an additive, since it electrostatically interacts with the tooth structure and with the AP proteins, favoring its protective mechanism [16,18,19-21].

Thus, this *in situ* study evaluated the protective potential of CaneCPI-5 added in solution and chitosan gel against erosion and erosion plus abrasion on dentin surfaces.

The null hypotheses tested were: 1) the presence of CaneCPI-5 in solution and 2) in chitosan gel does not prevent dentin loss caused by erosive and erosive plus abrasive challenges *in situ*.

2. MATERIAL AND METHODS

Experimental design and ethical records

The methodology was divided into two studies, carried out in different periods, using two application modalities for dentistry. In Study 1, a solution containing CaneCPI-5 was tested. In Study 2, a chitosan gel containing CaneCPI-5 was evaluated. Both treatments were evaluated in *in situ* erosion and erosion associated with abrasion protocols, using dentin samples. For Study 1, the protocol was performed in 3 experimental phases, including 15 volunteers. As for study 2, the protocol was performed in 4 experimental phases, including 16 volunteers. In both studies, the protocol was crossover and double-blind. Also, each phase consisted of 5 consecutive days, with a washout of 10 days. The dentin samples were individually treated with solutions or gels, followed by AP formation, then submitted to citric acid (for erosive challenge) and to brushing (for abrasive challenge). The alterations in dentin surfaces were determined using profilometry (dentin loss).

All the studies were conducted after approval of the Ethics Committee for Human Research (CAAE:14973519.0.0000.5417) of Bauru School of Dentistry, University of São Paulo, Brazil, following clinical practice guidelines and complied with the Declaration of Helsinki. All volunteers participated after signing the informed consent form.

Cutting and polishing dentin samples

Recently extracted non-carious human third molars (average age of 33 years) were used in this study to prepare four hundred and thirty-six dentin samples (4 mm × 4 mm × 3 mm), being 180 samples for study 1 and 256 samples for study 2. Initially, the teeth were stored in 0.1% buffered thymol solution (pH 7.0). Then, they were visually inspected and cleaned to remove the gingival tissue. Afterwards, the crowns were sectioned from the roots with a diamond saw (Isomet 1000; Buehler, Lake Bluff, IL, USA) and the dentin samples were cut from the cervical third of the root under water cooling.

After cutting, the samples were polished using different grains of sandpaper with water-cooling (Carborundum discs 320, 600, and 1200 grades of Al_2O_3 papers; Buehler, Lake Bluff, Illinois, USA). To complete this step, the samples were polished with felt paper wet by 1 µm diamond spray (1 mm; Buehler, Lake Bluff, Illinois, USA). Approximately 110 µm of dentin was removed. Between each sandpaper polishing and after felt paper, the samples were cleaned in an ultrasound bath (T7 Thornton, Unique Ind. e Com. Ltda., São Paulo, SP, Brazil) for 6 min and stored under humidity control (gauze moistened with deionized water) and temperature control (4º C) [22].

Initial profilometric analysis

For standardization purposes, a small drilling was done with a 1/4 drill (in the upper and left corner of each dentin surface) for the beginning of the readings. Also, two parallel markings were also made with the aid of a scalpel blade, dividing the samples into three portions. Subsequently, the initial analysis was performed by five scans of each sample (3 mm of reading and 250 µm apart from each other), using a contact profilometer (MarSurfe XCR20, Gottingen, NI, DE). After this analysis, the two lateral portions of dentin were covered with nail polish (to determine control areas), with only the central area of the sample remaining exposed. Later, all samples were sterilized with ethylene oxide (4 h; pressure of 0.5±0.1 kg F/cm^2) [23].

Volunteer screening and preparation of the intraoral appliance

For Study 1, fifteen volunteers (7 women and 8 men) were selected. For Study 2, sixteen volunteers (8 women and 8 men) were selected. The participants were aged between 29 and 33 years and complied with the following general health inclusion criteria: non-pregnant women, non-smokers, without systemic diseases and without frequent use of medication. In addition, the following inclusion criteria for oral health were also used: adequate salivary flow (stimulated salivary flow >1 mL/min and unstimulated salivary flow > 0.3 mL/min), intact permanent dentition, no open cavities or deficient restorations, no gingivitis or periodontal disease, no ETW [24].

For the preparation of the intraoral palatal appliances, all volunteers had the upper arch molded with plaster casts. With this mold, an acrylic resin appliance was made for each phase/volunteer. It contained four dentin samples in the concave central region, spaced 3 mm apart and at the same level of the acrylic resin. The samples were randomly allocated and distributed to the two types of procedures (2 samples for erosion and 2 samples for erosion and abrasion), which were destined for one of the sides of the device (right and left), in a

crossed way between the volunteers. Lastly, the appliances were hygienically stored with gauze moistened with tap water at 4 ºC until the start of each experimental phase [25].

Experimental instruction for volunteers

All volunteers received important guidance on experimental procedures and oral hygiene, such as the use of fluoride toothpaste (1100 ppm F, Oral-B, The Procter & Gamble Company, Cincinnati, OH, USA), toothbrush (Curaprox, Kriens, LU, Switzerland), and dental floss (Oral-B, The Procter & Gamble Company, Cincinnati, OH, USA), which were provided by the researchers throughout the study. Moreover, the buccal hygiene was performed 5 min before inserting the appliance into the oral cavity (after each meal and upon awakening), together with the hygiene of the appliance (only on the side where there were no samples). Also, the appliance was allowed to be removed for drinking water (1 min), during the meals (20 min) and overnight (12 h). During these moments (without using the appliance), it was stored with gauze moistened with tap water. Furthermore, the volunteers remained attentive to any problem with the appliance (samples detachment and/or discomfort in use) and they were also previously trained on the correct brushing force for the abrasive procedure (1.5 N). All volunteers received messages (via cell phone) at the time of each experimental procedure and reminders about the guidelines mentioned above [17].

Acquisition of treatments (CaneCPI-5 and Chitosan gel)

The CaneCPI-5 was produced at Federal University of São Carlos, São Carlos, Brazil. The protein was cloned in the expression vector pET28a (Novagen) and recombinantly produced in *Escherichia coli* Rosetta (DE3). The expressed protein was purified from the soluble fraction of bacterial cultures, centrifuged and sonicated. Finally, it was purified by affinity chromatography, using columns containing nickel resin Ni-NTA Superflow (Qiagen) [13].

The preparation of the chitosan gels was carried out at the Federal University of ABC. Initially, chitosan (75% deacetylation, average molecular weight, Sigma-Aldrich, MO, USA) was dissolved in 1% acetic acid (Synth, Diadema, Brazil), at a concentration of 30 mg chitosan per 1 mL of 1% acetic acid. Then, the mixture was homogenized (for 2 h) at room temperature (25 °C). For the preparation of the chitosan gels containing NaF and CaneCPI-5, the active ingredients were added during the chitosan dissolution. Finally, the mixture was homogenized for 2 hours and stored at 4°C. The pH of the gels was 4.7 [16,18].

Study 1: Experimental procedure of treatment with CaneCPI-5 solution against erosive dentin wear in situ

During the experimental period, the intraoral appliance remained in the mouth from 8:00 AM to 8:00 PM. Fifteen volunteers participated in three crossover and double-blind phases. In each phase, 5 volunteers were allocated to one of three treatments (decided by random computer numbers): 1- Deionized water, pH 7,9, negative control (WATER); 2- Commercial solution with 800 ppm Sn^{+2} from $SnCl_2$, 500 ppm F from amine fluoride and NaF, pH 4.5, Elmex Erosion Protection™ mouthwash (GABA GmbH, Hamburg, Germany) (ELMEXsol) [25]; and 3- Experimental solution containing 0.1 mg/mL CaneCPI-5, solubilized in deionized water, pH 7.88 (CaneCPI-5sol) [13].

An additional treatment was performed only on the first day (Monday) of each phase (before the volunteer placed the appliance in the oral cavity). In this way, a drop (approximately 50 µL) of the respective treatment solution was added individually on the dentin samples for 1 min at 7:59 AM. Then, the appliance was washed with tap water and inserted into the oral cavity (after the first oral hygiene of the day). From this moment on (8:00 AM), the AP was formed. Also, four treatments per day were conducted (9:59 AM, 1:59 PM, 3:59 PM and 5:59 PM), as mentioned above. From Tuesday to Friday, only these four treatments were performed at the same times [17].

Immediately, after each treatment (still with the appliance outside the oral cavity), except the additional treatment, the volunteers performed the erosive challenge (EROSION: 10:00 AM, 2:00 PM, 4:00 PM, 6:00 PM). For this, the volunteers immersed the appliance in a cup containing 150 mL of 0.1% citric acid solution (pH 2.5, room temperature, 90 s and without agitation). After this time, the volunteers again washed the appliances and replaced them in the mouth [17].

In half of the samples allocated on the appliance, the abrasive procedure was performed on all days of the experimental phase (ABRASION: 10:30 AM and 6:30 PM). The volunteers dripped (1 drop) fluoride toothpaste slurry (1 g of toothpaste: 3 mL of deionized water) and individually brushed each sample for 15 s, using an electric toothbrush (Oral-B Vitality Precision Clean Electric Toothbrush, Cincinnati, OH, USA). Afterwards, they washed the appliance with tap water for 5 s and put it back in the mouth [26].

Study 2: Experimental procedure of treatment with chitosan gel containing CaneCPI-5 against erosive dentin wear in situ

The experimental period was carried out in the same number of days and time of use of the appliance, as mentioned in study 1. The volunteers participated in four crossover and

double-blind phases. In each phase, 4 volunteers were assigned to one of the following treatment gels or not (determined by random computer numbers): 1- No gel application, negative control (NOgel); 2- Chitosan gel without active principle, placebo group (PLAgel); 3- Chitosan gel containing 500 ppm NaF, positive control (NAFgel) and; 4- Chitosan gel containing 0.1 mg/mL CaneCPI-5, experimental group (CaneCPI-5gel) [16].

Initially, the volunteers performed the hygiene of the oral cavity (7:56 am). Then, they applied the treatment gel, (except for the NOgel phase) once/day at 8:00 am, according to the respective treatment above. The gels were individually applied (approximately 20 µl per sample), using a microbrush, for 4 min. Then, the gels were carefully removed with a cotton swab and the appliance was inserted in the mouth for 2 h, for the formation of the AP [18].

After this time, the volunteers performed the extra-oral erosive challenge (EROSION: 10:00 AM). Also, three more challenges were performed throughout the day (EROSION: 2:00 PM; 4:00 PM; 6:00 PM), as mentioned in Study 1 [17,18].

In half of the samples allocated on the appliance, the abrasive procedure was performed on all days of the experimental phase (ABRASION: 10:30 AM and 6:30 PM), as described in Study 1 [17,18].

Final profilometric analysis

For the final measurements, the cosmetic nail polish was removed with an acetone solution (1:1 acetone: water). To avoid the contraction of the collagen fibrils, all the samples were kept moist with water during the measurements. However, the samples were dried (with filter paper) for analysis. Five final readings (3 mm of reading, 250 µm apart from each other) were performed in the same areas as the initial readings, using the same profilometer. The samples were appropriately repositioned during the readings. For this, they were included in a support to fix them. Moreover, the minor drilling helped to standardize the beginning of the readings, as well as the x and y axis of the profilometer. Finally, initial and final profiles were superimposed, using the MarhSurf XCR20 software (Mahr). The average depth of the surface was analyzed to quantify the dentin wear (µm) for each sample, with limit of detection of 0.5 µm [23].

Statistical Analysis

For both studies, the software GraphPad Prism (version 6.0 for Windows, GraphPad Software Inc., La Jolla, CA, USA) was used. After checking for normality (Kolmogorov-Smirnov test) and homogeneity (Bartlett test), all data were analyzed by two-way repeated-measures

(by both factors) ANOVA. Sidak's test was used as post-hoc for ANOVA. The significance level was set at 5%.

RESULTS

Study 1: Treatment with CaneCPI-5 solution against erosive dentin wear in situ

There was a significant difference among the treatments (F=6.319, p=0.0058) and between the conditions (F=5.029, p= 0.043), without interaction between these factors (F=2.976, p=0.0686) (p<0.05). The samples submitted to the EROSION+ABRASION condition had a significant increase in dentin loss in comparison to the EROSION condition for the WATER and CaneCPI-5sol groups. For the ELMEXsol group, no significant difference was observed between the conditions (p<0.05) (Figure 1).

Regarding the treatments, the CaneCPI-5sol and ELMEXsol groups led to significantly lower dentin loss compared with the WATER (negative control), without significant differences between them (p<0.05) (Figure 1).

Study 2: Treatment with chitosan gel containing CaneCPI-5 against erosive dentin wear in situ

There was a significant difference among the treatments (F=23.15, p<0.0001), but not between the conditions (EROSION and EROSION+ABRASION, F=0.9684, p=0.3274) or for the interaction between these factors (F=1.888, p=0.1363).

All the treated groups significantly reduced the dentin loss in comparison with the NOgel group (p<0.05). The greatest reduction was found for the CaneCPI-5gel group that significantly differed from the PLAgel group, but not from the NaFgel group. In addition, the latter did not significantly differ from the PLAgel group (p<0.05) (Figure 2).

DISCUSSION

ETW is a condition that affects both enamel and dentin. However, the progression is distinct in these tissues, due to the organic content of the dentin. When the erosive process reaches the dentin, the initial dissolution of the mineral exposes the DOM [6]. At the same time, at low pH, matrix metalloproteinases (MMPs) are activated and, together with cysteine cathepsins (CCs), degrade the exposed organic matrix, allowing the erosion progression [27]. Moreover, CCs can be activated by active MMPs [28], and conversely, CCs are responsible for the activation of MMPs [29]. In this sense, protease inhibitors have been studied to maintain

the DOM and to reduce the occurrence of dentin lesions [30]. In the present study, we used CaneCPI-5, a sugarcane-derived cystatin that showed inhibitory activity against human cathepsins [13].

In study 1, the application vehicle was a solution containing 0.1 mg/mL CaneCPI-5. The concentration was based on a previous *in vitro* study, in which a 0.1 mg/mL CaneCPI-5 solution showed a significant a reduction in dentin loss [16]. The protective effect of the CaneCPI-5 solution against dentin erosion and erosion associated to abrasion was similar to the one provided by the commercial product Elmex™. This is important, since so far Elmex™ is the product that presents the best results against ETW [31]. However, its broad use is impaired due to the possibility of tooth staining and sensation of astringency, which did not happen in the case of the CaneCPI-5 that was well tolerated by the volunteers. It is also noteworthy that in the study by Santos [16], which was conducted *in vitro*, only erosive challenges were employed. The present study represents a step forward on the use of CaneCPI-5 rinse solutions to prevent dentin erosive wear, since it was performed *in situ* and involved both erosive and abrasive challenges.

In addition, there are studies about the protective effect of solutions containing CaneCPI-5 against enamel erosive wear using *in vitro*, *in situ* and *in vivo* protocols [14,15,17,24]. The present results showed a significant protective effect of the CaneCPI-5sol and ELMEXsol on dentin, reducing EROSION and EROSION+ABRASION when compared to the negative control. Also, there was a significant difference between EROSION and EROSION+ABRASION conditions, confirming the concept that the erosive challenge softens the surface layer, making it more susceptible to the abrasion process [32]. Thus, the abrasion condition demonstrated greater loss of dentine. Another important result was a significant difference between the groups containing the active principles (CaneCPI-5sol and ELMEXsol) when compared with the placebo group (in the EROSION+ABRASION condition), thus demonstrating that CaneCPI-5 also protects against more severe challenges, probably by protecting the DOM, which is resistant to removal by mechanical forces, such as brushing [33]. Therefore, the CaneCPI-5 solution prevents dentin loss caused by *in situ* erosive and abrasive challenges, rejecting the first null hypothesis.

In study 2, the protein was added together with chitosan in a gel formulation. It has been reported that the addition of biopolymers is able to reduce the erosive wear between 15% to nearly 100% in *in vitro* studies [34]. In the present study, an erosion model was applied with 4 challenges (90 s each) per day for 5 days, using 0.1% citric acid (pH 2.5). Additionally, abrasive challenges were performed twice daily on half of the samples [18].

Chitosan is a polysaccharide obtained by deacetylation of chitin, mainly from the shells of shrimp and other crustaceans. It is a positively charged biopolymer and binds strongly with its amino groups to surfaces with negative zeta potential, such as enamel and dentin. When the chitosan molecule is reactive, it reacts with the dentin surface and leads to the coverage of at least a thin and ubiquitous layer of chitosan. Chitosan-based layers are only a few nanometers thick and precipitated in their composition (with approximately 20 wt% calcium and 10 wt% phosphorus) [35]. A study revealed that chitosan forms layers on the surface, which are differently structured according to the environment: changes in pH, chitosan concentration and application time can vary the type of layers formed [36]. In dental erosion, chitosan increases the number of cross-links between collagen fibers and neutralizes MMPs, preventing collagen degradation [37]. Furthermore, under acidic conditions, the amino groups of chitosan capture hydrogen ions, resulting in an overall positive charge that makes the molecule adhesive by electrostatic forces to negatively charged surfaces, such as enamel or dentin [38]. It has also been shown that chitosan potentially follows a multilayer adsorption behavior, which allows it to be resistant to acids [39].

In the samples from NAFgel group, the protection against erosion was observed when compared to the NOgel and PLAgel groups. The protection conferred may be due to the mineralization properties of dentin through the association of chitosan with fluoride tested with a microparticle bioadhesive [21]. In addition, another protection factor is the increase in viscosity and the control of fluoride release due to chitosan [16]. The reduction in demineralization can be explained by the interaction of the chitosan/fluoride gel with the dentin surface and with some of the material's adhesion promoters, which form a poor covering layer that occluded and partially occluded dentin tubules. Finally, a cover that protects against erosive challenges [37].

Notably, the EROSION+ABRASION condition showed no significant difference to the EROSION condition. The explanation for this fact may be related to two issues: 1) Low performance of gel protection when the two conditions were associated; and 2) A greater amount of erosive challenge (4X) as compared to abrasive challenge (2X) per day. Future studies testing different times of conditions should be carried out. With respect to the treatments, the group that presented the greatest demineralization was the one that did not receive any treatment even though, in the oral cavity, the AP was formed, which has a degree of protection against dentin erosion [40]. The group that showed the most significant protection in this study was the CaneCPI-5gel group, however, it was not significantly different from the NAFgel group.

To understand the probable mechanism of action of the CaneCPI-5gel and CaneCPI-5sol, we will divide it into two parts: the first part is before the erosive challenges, in the application of the chitosan gel and CaneCPI-5, and the second part is after the erosive challenges. Before the erosive challenges, chitosan, as a positively charged biopolymer, binds to dentin due to its negative zeta potential, forming layers on the surface. On the other hand, it was demonstrated by surface free energy (SFE) experiment, that when CaneCPI-5 interacts with hydroxyapatite, the number of electron donor sites on the surface increases (the negative zeta potential increases), possibly affecting the formation and composition of the AP, as well as the layers formed by chitosan [22]. In the second part, similar to what was already mentioned in study 1, when the erosion reaches the dentin, the dissolution of the mineral begins, exposing the organic matrix. Then, proteolytic enzymes such as MMPs and CCs are activated, degrading the exposed matrix and thus accelerating the progression of erosion. Once the proteolytic enzymes are active, CaneCPI-5 might inhibit cathepsins B and K present in dentin [13]. CC inhibition also activates MMP [29,41] and thus reduces dentin demineralization. Another possibility is the binding of CaneCPI-5 to hydroxyapatite, due to its high affinity to this mineral [13], thus changing the proteomic composition of the acquired pellicle and making this integument more resistant to future acidic challenges, as has been shown to occur for enamel [12]. Therefore, the presence of CaneCPI-5 (as a protease inhibitor or a pellicle modulator) in gels protected the loss of dentin caused by *in situ* erosive and abrasive challenges, rejecting the second hypothesis.

Although the two studies were carried out at different times, it is important to compare the two application vehicles. We can observe that the CaneCPI-5sol treatment demonstrated a similar protection when compared with the CaneCPI-5gel treatment. Both vehicles showed approximately 1.0-1.5 µm of dentin wear for the EROSION condition. It should be noted that the solutions were applied 4X/day for 1 min, while the gels were applied only once/day, for 4 min. However, the application of the gels depends on the professional. Thus, a good measure could be that the first application of gel occurred in-office, after dental prophylaxis, while the subsequent ones could be applied by the patient at home, using the solution.

In conclusion, solution and chitosan gel containing CaneCPI-5 protect dentin against erosion and erosion associated to abrasion at the same extent as commercial fluoride solution and chitosan NaF gel *in situ*.

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Competing interests

The authors have declared no competing interests.

REFERENCES

[1] Lussi A, Carvalho TS. Erosive tooth wear: A Multifactorial condition of growing concern and increasing knowledge. Monogr Oral Sci. 2014;25:1–15. https://doi.org/10.1159/000360380.

[2] Schlueter N, Amaechi BT, Bartlett D, Buzalaf MAR, Carvalho TS, Ganss C, et al. Terminology of Erosive Tooth Wear: Consensus Report of a Workshop Organized by the ORCA and the Cariology Research Group of the IADR. Caries Res. 2020;54(1):2–6. https://doi.org/10.1159/000503308.

[3] Bartlett DW. The role of erosion in tooth wear: Aetiology, prevention and management. Int Dent J. 2005;55(4 SUPPL. 1):277–84. https://doi.org/10.1111/j.1875-595X.2005.tb00065.x.

[4] Lussi A, Jaeggi T. Erosion - Diagnosis and risk factors. Clin Oral Investig. 2008;12(SUPPL.1):5–13. https://doi.org/10.1007/s00784-007-0179-z.

[5] Buzalaf MAR, Magalhães AC, Rios D. Prevention of erosive tooth wear: targeting nutritional and patient-related risks factors. Br Dent J. 2018 Mar 9;224(5):371-378. doi: 10.1038/sj.bdj.2018.173.

[6] Buzalaf M a, Hannas a R, Kato MT. Saliva and dental erosion. J Appl Oral Sci. 2012;20(5):493–502. https://doi.org/10.1590/S1678-77572012000500001.

[7] Buzalaf MAR, Charone S, Tjäderhane L. Role of host-derived proteinases in dentin caries and erosion. Caries Res. 2015;49(suppl 1):30–7. https://doi.org/10.1159/000380885.

[8] Wetton S, Hughes J, West N, Addy M. Exposure time of enamel and dentin to saliva for protection against erosion: A study in vitro. Caries Res. 2006;40(3):213–7. https://doi.org/10.1159/000092228.

[9] Wetton S, Hughes J, Newcombe RG, Addy M. The effect of saliva derived from different individuals on the erosion of enamel and dentin: A study in vitro. Caries Res. 2007;41(5):423–6. https://doi.org/10.1159/000104802.

[10] Hellwig E, Lussi A, Goetz F. Influence of Human Saliva on the Development of Artificial Erosions. 2013;553–8. https://doi.org/10.1159/000351634.

[11] Vukosavljevic D, Custodio W, Buzalaf MAR, Hara AT, Siqueira WL. Acquired pellicle as a modulator for dental erosion. Arch Oral Biol [Internet]. 2014;59(6):631–8. Available from: http://dx.doi.org/10.1016/j.archoralbio.2014.02.002.

[12] T.S. Carvalho, T.T. Araújo, T.M.O. Ventura, A. Dionizio, J.V.F. Câmara, S.M. Moraes, V.T. Pelá, T. Martini, J.C. Leme, A.L.B. Derbotolli, L.T. Grizzo, E. Crusca, P.Y.T. Shibao, R. Marchetto, F. Henrique-Silva, J.P. Pessan, M.A.R. Buzalaf, Acquired pellicle protein-based engineering protects against erosive demineralization, J Dent. 102 (2020) 103478. https://doi.org/10.1016/j.jdent.2020.103478.

[13] Santiago AC, Khan ZN, Miguel MC, Gironda CC, Soares-Costa A, Pelá VT, et al. A New Sugarcane Cystatin Strongly Binds to Dental Enamel and Reduces Erosion. J Dent Res. 2017;96(9):1051–7. https://doi.org/10.1177/0022034517712981.

[14] Pela VT, Buzalaf MAR, Niemeyer SH, Baumann T, Henrique-Silva F, Toyama D, Crusca E, Marchetto R, Lussi A, Carvalho TS. 2021a. Acquired pellicle engineering with proteins/peptides: Mechanism of action on native human enamel surface. J Dent. 107:103612.

[15] Pelá VT, Ventura TMO, Taira EA, Thomassian LTG, Brito L, Matuhara YE, Henrique-Silva F, Groisman S, Carvalho TS, Lussi A, Buzalaf MAR. Use of Reflectometer Optipen to assess the preventive effect of a sugarcane cystatin on initial dental erosion in vivo. J Mech Behav Biomed Mater. 2023 May;141:105782. doi: 10.1016/j.jmbbm.2023.105782.

[16] Santos LA, Martini T, Câmara JVF, Reis FN, Ortiz ADC, Camiloti GD, et al. Solutions and Gels Containing a Sugarcane-Derived Cystatin (CaneCPI-5) Reduce Enamel and Dentin Erosion in vitro. Caries Res. 2021;55(6):594–602. https://doi.org/10.1159/000520261.

[17] Pela VT, Lunardelli JGQ, Tokuhara CK, Gironda CC, Silva NDG, Carvalho TS, Santiago AC, Souza BM, Moraes SM, Henrique-Silva F et al. 2021b. Safety and in situ antierosive effect of canecpi-5 on dental enamel. J Dent Res. 100(12):1344-1350.

[18] Pela VT, Brito L, Taira EA, Henrique-Silva F, Pieretti JC, Seabra AB, de Almeida Baldini Cardoso C, de Souza EP, Groisman S, Rodrigues MC et al. 2022. Preventive effect of chitosan gel containing canecpi-5 against enamel erosive wear in situ. Clin Oral Investig. 26(11):6511-6519.

[19] Svensson O, Lindh L, Cárdenas M, Arnebrant T. Layer-by-layer assembly of mucin and chitosan-Influence of surface properties, concentration and type of mucin. J Colloid Interface Sci. 2006;299(2):608–16. https://doi.org/10.1016/j.jcis.2006.02.027.

[20] Van Der Mei HC, Engels E, De Vries J, Dijkstra RJB, Busscher HJ. Chitosan adsorption to salivary pellicles. Eur J Oral Sci. 2007;115(4):303–7. https://doi.org/10.1111/j.1600-0722.2007.00454.x.

[21] Keegan GM, Smart JD, Ingram MJ, Barnes LM, Burnett GR, Rees GD. Chitosan microparticles for the controlled delivery of fluoride. J Dent [Internet]. 2012;40(3):229–40. Available from: http://dx.doi.org/10.1016/j.jdent.2011.12.012.

[22] Gironda CC, Pelá VT, Henrique-Silva F, Delbem ACB, Pessan JP, Buzalaf MAR. New insights into the anti-erosive property of a sugarcane-derived cystatin: different vehicle of application and potential mechanism of action. J Appl Oral Sci. 2022 Jul 29;30:e20210698. doi: 10.1590/1678-7757-2021-0698.

[23] Magalhães AC, Wiegand A, Rios D, Hannas A, Attin T, Buzalaf MAR. Chlorhexidine and green tea extract reduce dentin erosion and abrasion in situ. J Dent. 2009;37(12):994–8. https://doi.org/10.1016/j.jdent.2009.08.007.

[24] Pelá VT, Niemeyer SH, Baumann T, Levy FM, Henrique-Silva F, Lussi A, Carvalho TS, Buzalaf MAR. 2022. Acquired pellicle engineering using a combination of organic (sugarcane cystatin) and inorganic (sodium fluoride) components against dental erosion. Caries Res. 56(2):138-145.

[25] de Souza BM, Santi LRP, de Souza Silva M, Buzalaf MAR, Magalhães AC. Effect of an experimental mouth rinse containing NaF and TiF4 on tooth erosion and abrasion in situ. J Dent. 2018;73(April):45–9. https://doi.org/10.1016/j.jdent.2018.04.001.

[26] Levy FM, Rios D, Buzalaf MAR, Magalhães AC. Efficacy of TiF4 and NaF varnish and solution: A randomized in situ study on enamel erosive-abrasive wear. Clin Oral Investig. 2014;18(4):1097–102. https://doi.org/10.1007/s00784-013-1096-y.

[27] Tjäderhane L, Buzalaf MAR, Carrilho M, Chaussain C. Matrix metalloproteinases and other matrix proteinases in relation to cariology: The era of “dentin degradomics.” Caries Res. 2015;49(3):193–208. https://doi.org/10.1159/000363582.

[28] Hara K, Kominami E, Katunuma N. Effect of proteinase inhibitors on intracellular processing of cathepsin B, H and L in rat macrophages. FEBS Lett. 1988;231(1):229–31. https://doi.org/10.1016/0014-5793(88)80737-3.

[29] Cox SW, Eley BM, Kiili M, Asikainen A, Tervahartiala T, Sorsa T. Collagen degradation by interleukin-1 β-stimulated gingival fibroblasts is accompanied by release and activation of multiple matrix metalloproteinases and cysteine proteinases. Oral Dis. 2006;12(1):34–40. https://doi.org/10.1111/j.1601-0825.2005.01153.x.

[30] Kato MT, Leite AL, Hannas AR, Buzalaf MAR. Gels containing MMP inhibitors prevent dental erosion in situ. J Dent Res. 2010;89(5):468–72. https://doi.org/10.1177/0022034510363248.

[31] M.C. Huysmans, A. Young, C. Ganss. The role of fluoride in erosion therapy, Monogr Oral Sci, 25 (2014) 230-243. https://doi.org/10.1159/000360555.

[32] Rios D, Honório HM, Magalhães AC, Buzalaf MA, Palma-Dibb RG, Machado MA, da Silva SM. Influence of toothbrushing on enamel softening and abrasive wear of eroded bovine enamel: an in situ study. Braz Oral Res. 2006 Apr-Jun;20(2):148-54. doi: 10.1590/s1806-83242006000200011.

[33] Ganss C, Hardt M, Blazek D, Klimek J, Schlueter N. Effects of toothbrushing force on the mineral content and demineralized organic matrix of eroded dentin. Eur J Oral Sci. 2009;117:255–60.

[34] Ganss C, Von Hinckeldey J, Tolle A, Schulze K, Klimek J, Schlueter N. Efficacy of the stannous ion and a biopolymer in toothpastes on enamel erosion/abrasion. J Dent [Internet]. 2012;40(12):1036–43. http://dx.doi.org/10.1016/j.jdent.2012.08.005.

[35] Pini NIP, Lima DANL, Lovadino JR, Ganss C, Schlueter N. In vitro Efficacy of Experimental Chitosan-Containing Solutions as Anti-Erosive Agents in Enamel. Caries Res. 2016;50(3):337–45. https://doi.org/10.1159/000445758.

[36] Lee HS, Tsai S, Kuo CC, Bassani AW, Pepe-Mooney B, Miksa D, et al. Chitosan adsorption on hydroxyapatite and its role in preventing acid erosion. J Colloid Interface Sci [Internet]. 2012;385(1):235–43. Available from: http://dx.doi.org/10.1016/j.jcis.2012.06.074.

[37] Nahórny S, de Oliveira IR, Soares LES. Biomineralization induced by chitosan and collagen-based materials with fluoride for dentin coverage: Chemical and morphological analysis. Microsc Res Tech. 2022;85(3):1089–100. https://doi.org/10.1002/jemt.23978.

[38] Esposti LD, Ionescu AC, Brambilla E, Tampieri A, Iafisco M. Characterization of a toothpaste containing bioactive hydroxyapatites and in vitro evaluation of its efficacy to remineralize enamel and to occlude dentinal tubules. Materials (Basel). 2020;13(13):1–13. https://doi.org/10.3390/ma13132928.

[39] Aykut-Yetkiner A, Attin T, Wiegand A. Prevention of dentin erosion by brushing with anti-erosive toothpastes. J Dent [Internet]. 2014;42(7):856–61. Available from: http://dx.doi.org/10.1016/j.jdent.2014.03.011.

[40] Hannig M, Fiebiger M, Güntzer M, Döbert A, Zimehl R, Nekrashevych Y. Protective effect of the in situ formed short-term salivary pellicle. Arch Oral Biol. 2004;49(11):903–10. https://doi.org/10.1016/j.archoralbio.2004.05.008.

[41] Bafail A, Carrilho MR, Kishen A, Prakki A. Effect of protease inhibitor specificity on dentin matrix properties. J Mech Behav Biomed Mater [Internet]. 2020;109(May):103861. Available from: https://doi.org/10.1016/j.jmbbm.2020.103861.

Figure legend

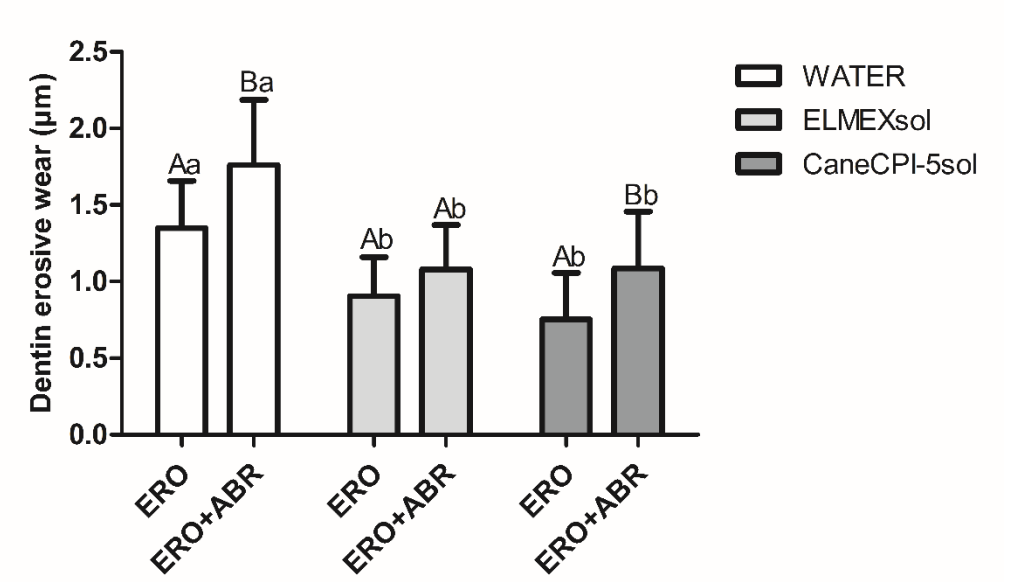

Figure 1. Mean loss of human dentin samples treated for 1 min with one drop of distinct solutions and submitted *in situ* to erosive challenges 4 times/day with 0.1% citric acid pH 2.5 for 90s (ERO) combined or not to abrasion by electric toothbrushing (15 s; ERO+ABR). Each phase was conducted for 5 days. Lower-case letters show significant differences among the treatments. Upper-case letters denote differences between the conditions (2-way-RM ANOVA and Sidak´s test, $p<0.05$). n=15.

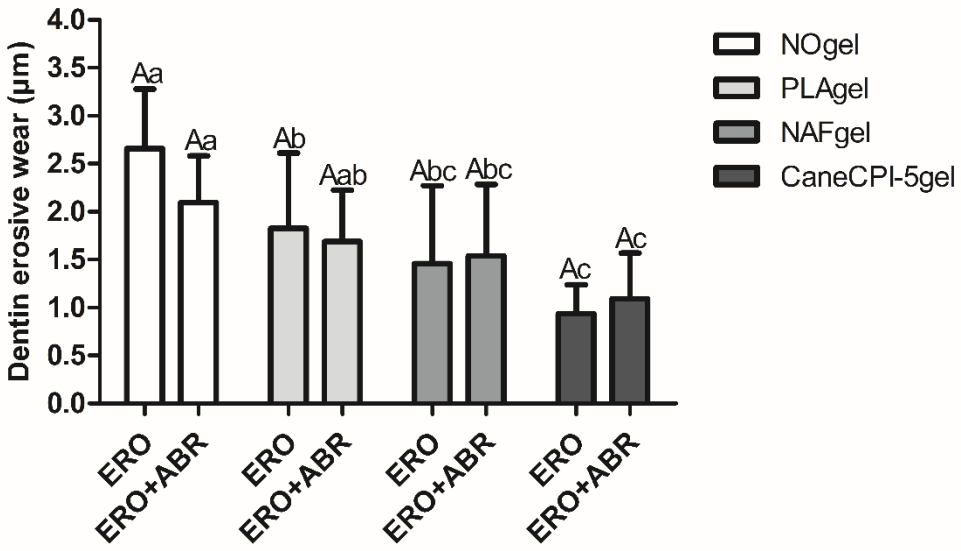

Figure 2. Mean loss of human dentin samples treated for 4 min or not with different chitosan gels and submitted *in situ* to erosive challenges 4 times/day with 0.1% citric acid pH 2.5 for 90s (ERO) combined or not to abrasion by electric toothbrushing (15 s; ERO+ABR). Each phase was conducted for 5 days. Lower-case letters show significant differences among the treatments. Upper-case letters denote differences between the conditions (2-way-RM ANOVA and Sidak´s test, $p<0.05$). n=16.

4 DISCUSSION

4 DISCUSSION

Dental erosion refers to the partial demineralization of tooth surfaces due to repeated exposure to acidic substances, whereas the loss of surface tissue caused by concurrent and/or subsequent exposure to mechanical forces is termed erosive tooth wear (ETW) (SCHLUETER et al., 2020). Excessive consumption of acidic beverages and foods, such as soft drinks and carbonated beverages, is the primary dietary factor associated with ETW. Dissolution of the tooth surface is significantly influenced by various chemical and physical parameters, such as pH, buffer capacity, titratable acidity, viscosity, and concentrations of calcium, phosphate, and fluoride in foods and beverages. Therefore, assessing the erosive potential of different foods and beverages and the frequency of ingestion, as well as evaluating patients' dietary habits, are essential for preventing and managing ETW (SAADS CARVALHO; LUSSI, 2019).

Various preventive and therapeutic approaches have been suggested for managing dental erosion, including dietary advice, stimulation of salivary flow, optimization of fluoride regimens, modification of erosive beverages and adequate oral hygiene measures (MAGALHÃES et al., 2009b). However, these interventions have limitations and may not always be effective. Recently, the significance of salivary proteins that constitute the acquire pellicle in protecting the tooth surface against ETW has been the focus of attention, particularly in the process known as "acquire pellicle engineering". Among these proteins, CaneCPI-5 was found to have a great affinity to hydroxyapatite and also a strong inhibition against human cathepsins B, K, and L (SANTIAGO et al., 2017).

This thesis aimed to evaluate the impact of CaneCPI-5 on the tooth surface using measurements of surface hardness, surface free energy (SFE), and surface profilometry.

Surface hardness measurements have become a crucial tool in determining changes in dental hard tissue caused by erosion. Microhardness measurements can discriminate between different erosive potentials of various substances on dental hard tissue, even after short exposures to acidic agents (SAADS CARVALHO; LUSSI, 2019). In our first Article, the protective effect of a gel containing CaneCPI-5 against

enamel erosion was evaluated erosion using a well-established initial erosion model (CHEAIB; LUSSI, 2011). The results showed that gels containing CaneCPI-5 at 0.1 and 1.0 mg/mL significantly reduced enamel initial erosion, while the product containing 2.0 mg/mL CaneCPI-5 did not (SANTIAGO et al., 2017). The overall %SHC reduction promoted by the gels was 30%, and the effects were less pronounced than those achieved with aqueous solutions. The study also found that the highest concentration of CaneCPI-5 did not protect enamel against initial erosion, likely due to the reduction in free protein available to bind to enamel, probably caused by dimerization. The use of gels containing protease inhibitors was previously shown to promote superior protection against dentine erosion (KATO et al., 2010). The mixture of mucin and casein was used as a positive control due to previous results demonstrating its ability to provide protective effects against initial erosion in vitro (CHEAIB; LUSSI, 2011).

It is important to note the correlation between microhardness and mineral content in healthy human enamel. These two properties are linked, with a higher mineral content generally corresponding to higher microhardness values. This correlation suggests that enamel with a higher mineral content will typically be harder and more resilient to wear and damage compared to enamel with a lower mineral content. Given this relationship, we can infer that CaneCPI-5 may also help preserve mineral content, as suggested in previous studies (AKKUS; KARASIK; ROPERTO, 2017).

In the SFE experiment, the aim was to investigate the effects of CaneCPI-5 on enamel reactivity using the sessile drop method. Previous studies have shown that untreated enamel tends to be slightly hydrophobic (RÜTTERMANN et al., 2011; VAN DER MEI; WHITE; BUSSCHER, 2004), and changes in SFE after CaneCPI-5 treatment could potentially influence protein binding to the AP, ultimately changing its composition. Treatment with CaneCPI-5 reduced SFE, resulting in a more hydrophilic surface, which is more prone to water and makes easier protein adhesion or calcium phosphate precipitation. Hydrophilic surfaces have a strong attraction to water molecules, leading to the formation of a surface hydration layer that can create an environment favorable for the "association" or "entrapment" of proteins near the surface without causing dehydration (VOGLER, 1998). This might help to explain the

proteomic changes found in the AP after treatment with CaneCPI-5 (CARVALHO et al., 2020).

Moreover, CaneCPI-5 was found to have a higher number of electron-donor sites on the enamel surface, which favored the adsorption of cationic ionic species and cationic acid-resistant proteins from saliva. This observation could help explain the lower hardness loss seen after erosion challenge (NEVES et al., 2018). The changes observed in the acid-resistant proteins of the AP after using gel with 0.1 mg/mL of CPI-5 for 1 minute and challenging it with 1% citric acid pH 2.5 for 1 minute can be partially attributed to alterations in SFE. These findings suggest that CaneCPI-5 treatment may have promising applications in protecting enamel against erosion and promoting protein adhesion on enamel surfaces.

Using contact profilometry as a response variable in Article 2, we investigated the efficacy of solution and gel containing CaneCPI-5 in preventing dentin erosion and erosion+abrasion *in situ*. Previous studies have shown that CaneCPI-5 is a potent inhibitor of cathepsins B, K, and L, which, in concert with MMPs, are responsible for the degradation of the collagen matrix in dentin (SANTIAGO et al., 2017). This is likely one of the reasons for the prevention of dentin loss on the surface. The solution and gel containing CaneCPI-5 showed a reduction in dentin loss, which could be due to the inhibition of cathepsins and the maintenance of the DOM. Further mechanistic experiments should be conducted to clarify the mechanism of action of CaneCPI-5 against dentin ETW, i.e., if the protein acts inhibiting CCs, strengthening the AP, or both.

Both the solution and chitosan gel containing CaneCPI-5 were equally effective to Elmex Erosion Protection™ and chitosan gel containing NaF, respectively against dentin erosion and erosion+abrasion, suggesting that the protein-containing vehicles are suitable alternatives to fluoride-containing vehicles. Future studies should evaluate vehicles containing both CaneCPI-5 and fluoride solutions and gels to see if there is a synergistic effect, since at least in vitro, this combination was shown to present synergism against enamel erosion (PELÁ et al., 2022).

The group that showed the most significant protection was the group containing both CaneCPI-5 and chitosan. Chitosan is a biopolymer with a positive charge that can

bind to dentin through its negative zeta potential, forming layers on the surface (PINI et al., 2016). CaneCPI-5 was shown to be a potent inhibitor of cathepsins B and K present in dentin, which are activated during the erosion process (SANTIAGO et al., 2017). Inhibiting cathepsin also activates MMPS, thereby reducing dentin demineralization (COX et al., 2006; HARA; KOMINAMI; KATUNUMA, 1988). In our study, treatment of dentin specimens with chitosan gel effectively protected against erosion compared to the untreated group. Chitosan has been reported to increase the number of cross-links between collagen fibers, neutralize metalloproteinases, and prevent collagen degradation in dental erosion (NAHÓRNY; DE OLIVEIRA; SOARES, 2022). Chitosan also plays a role in increasing the viscosity and controlling the release of fluoride, which further contributes to reducing demineralization (DE SOUZA et al., 2020).

The present study evaluated the efficacy of different treatments on the prevention of dental erosion. The results demonstrated that the combination of Chitosan with CaneCPI-5 and Chitosan with NaF had a statistically significant difference compared to the negative control group (N/T) in both ERO and ERO+ABR outcomes. These findings suggest that the combination of Chitosan with other compounds may have a more pronounced effect on erosion prevention than Chitosan alone. However, further research is needed to confirm these results and investigate the long-term effects of these treatments on dentin erosion.

Despite the two *in situ* studies were carried out at different times, it is interesting to compare the two application vehicles. The CaneCPI-5sol treatment demonstrated a similar protection when compared with the CaneCPI-5gel treatment, since both vehicles showed approximately 1.0-1.5 µm of dentin wear for the EROSION condition. However, the solutions were applied 4X/day for 1 min, while the gels were applied only once/day, for 4 min. On the other hand, application of the gels depends on the professional. Thus, it seems a good measure could be that the first application of gel occurred in-office, after dental prophylaxis, while the subsequent ones could be applied by the patient at home, using the solution.

The limitations of the *in vitro* and *in situ* studies must be carefully considered when interpreting their results. *In vitro* studies are conducted under controlled laboratory conditions and may not fully replicate the natural environment of the oral

cavity, especially the absence of saliva, while *in situ* studies more closely mimic the oral environment but are often conducted with small sample sizes and may not capture the long-term effects of treatments. Additionally, variability in the oral environment can make it difficult to draw definitive conclusions from in situ studies. Thus, it is essential to carefully consider the limitations of both types of studies when interpreting their results and drawing conclusions.

In conclusion, the findings of this work demonstrate the potential of CaneCPI-5 as a novel therapeutic agent for the prevention of dental erosion. The ability of the protein to modify the physical and chemical properties of the tooth surface could make it a highly effective protective agent. Further studies are warranted to evaluate its efficacy *in vivo*. In addition, the mechanisms underlying the protective effect of CaneCPI-5 should be further investigated, as this could provide important insights into the development of new therapies for the prevention and treatment of ETW.

REFERENCES

REFERENCES

ABRAHAMSON, M. Cystatins - Protein inhibitors of papain-like cysteine proteinases**. Journal of the Brazilian Association of the Advancement of Science**, v.45, n.5, p.299-304, 1993.

ARAUJO, Tamara Teodoro et al. A sugarcane cystatin (CaneCPI-5) alters microcosm biofilm formation and reduces dental caries**. Biofouling**, v. 37, n. 1, p. 109–116, 2021. DOI: 10.1080/08927014.2021.1881065.

ARAÚJO, Tamara Teodoro et al. Protein-based engineering of the initial acquired enamel pellicle in vivo: Proteomic evaluation. **Journal of Dentistry**, v. 116, n. October 2021, p. 103874, 2022. DOI: 10.1016/j.jdent.2021.103874.

BARBOUR, Michele E.; LUSSI, Adrian. Erosion in relation to nutrition and the environment. **Monographs in Oral Science**, v. 25, p. 143–154, 2014. DOI: 10.1159/000359941.

BOTEON, Ana Paula; KATO, Melissa Thiemi; BUZALAF, Marília Afonso Rabelo; PRAKKI, Anuradha; WANG, Linda; RIOS, Daniela; HONÓRIO, Heitor Marques. Effect of Proanthocyanidin-enriched extracts on the inhibition of wear and degradation of dentin demineralized organic matrix. **Archives of Oral Biology**, v. 84, n. October 2016, p. 118–124, 2017. DOI: 10.1016/j.archoralbio.2017.09.027.

BUZALAF, M. a; HANNAS, A R.; KATO, M. T. Saliva and dental erosion. **J Appl Oral Sci**, v. 20, n. 5, p. 493–502, 2012. DOI: 10.1590/S1678-77572012000500001.

CARPENTER, G.; COTRONEO, E.; MOAZZEZ, R.; ROJAS-SERRANO, M.; DONALDSON, N.; AUSTIN, R.; ZAIDEL, L.; BARTLETT, D.; PROCTOR, G. Composition of enamel pellicle from dental erosion patients. **Caries Research**, v. 48, n. 5, p. 361–367, 2014. DOI: 10.1159/000356973.

CARVALHO, Thamyris Souza et al. Acquired pellicle protein-based engineering protects against erosive demineralization. **Journal of Dentistry**, v. 102, n. September, 2020. DOI: 10.1016/j.jdent.2020.103478.

CHEAIB, Z.; LUSSI, A. Impact of acquired enamel pellicle modification on initial dental erosion. **Caries Research**, v. 45, n. 2, p. 107–112, 2011. DOI: 10.1159/000324803.

COX, S. W.; ELEY, B. M.; KIILI, M.; ASIKAINEN, A.; TERVAHARTIALA, T.; SORSA, T. Collagen degradation by interleukin-1 β-stimulated gingival fibroblasts is

accompanied by release and activation of multiple matrix metalloproteinases and cysteine proteinases. **Oral Diseases**, v. 12, n. 1, p. 34–40, 2006. DOI: 10.1111/j.1601-0825.2005.01153.x.

DE SOUZA, Beatriz Martines; SANTI, Larissa Rodrigues Pereira; DE SOUZA SILVA, Mayara; BUZALAF, Marília Afonso Rabelo; MAGALHÃES, Ana Carolina. Effect of an experimental mouth rinse containing NaF and TiF4 on tooth erosion and abrasion in situ. **Journal of Dentistry**, v. 73, n. April, p. 45–49, 2018. DOI: 10.1016/j.jdent.2018.04.001.

DE SOUZA, Beatriz Martines; SANTI, Larissa Rodrigues Pereira; JOÃO-SOUZA, Samira Helena; CARVALHO, Thiago Saads; MAGALHÃES, Ana Carolina. Effect of titanium tetrafluoride/sodium fluoride solutions containing chitosan at different viscosities on the protection of enamel erosion in vitro. **Archives of Oral Biology**, v. 120, n. October, 2020. DOI: 10.1016/j.archoralbio.2020.104921.

DELECRODE, Taísa Ribas; SIQUEIRA, Walter Luiz; ZAIDAN, Flavia Cardoso; BELLINI, Melina Rodrigues; MOFFA, Eduardo Buozi; MUSSI, Maria Carolina Martins; XIAO, Yizhi; BUZALAF, Marília Afonso Rabelo. Identification of acid-resistant proteins in acquired enamel pellicle. **Journal of Dentistry**, v. 43, n. 12, p. 1470–1475, 2015. b. DOI: 10.1016/j.jdent.2015.10.009.

DELECRODE, Taísa Ribas; SIQUEIRA, Walter Luiz; ZAIDAN, Flávia Cardoso; BELLINI, Melina Rodrigues; LEITE, Aline Lima; XIAO, Yishi; RIOS, Daniela; MAGALHÃES, Ana Carolina; BUZALAF, Marília Afonso Rabelo. Exposure to acids changes the proteomic of acquired dentine pellicle. **Journal of Dentistry**, v. 43, n. 5, p. 583–588, 2015. a. DOI: 10.1016/j.jdent.2015.02.001.

GIANOTTI, Andréia; RIOS, Wendy M.; SOARES-COSTA, Andrea; NOGAROTO, Viviane; CARMONA, Adriana K.; OLIVA, Maria L. V.; ANDRADE, Sheila S.; HENRIQUE-SILVA, Flávio. Recombinant expression, purification, and functional analysis of two novel cystatins from sugarcane (Saccharum officinarum). **Protein Expression and Purification**, v. 47, n. 2, p. 483–489, 2006. DOI: 10.1016/j.pep.2005.10.026.

GIANOTTI, Andréia; SOMMER, César A.; CARMONA, Adriana K.; HENRIQUE-SILVA, Flávio. Inhibitory effect of the sugarcane cystatin CaneCPI-4 on cathepsins B and L and human breast cancer cell invasion. **Biological Chemistry**, v. 389, n. 4, p. 447–453, 2008. DOI: 10.1515/BC.2008.035.

HANNIG, C.; SPITZMÜLLER, B.; MILLER, M.; HELLWIG, E.; HANNIG, M. Intrinsic enzymatic crosslinking and maturation of the in situ pellicle. **Archives of Oral Biology**, v. 53, n. 5, p. 416–422, 2008. DOI: 10.1016/j.archoralbio.2007.12.003.

HANNIG, M. Ultrastructural investigation of pellicle morphogenesis at two different intraoral sites during a 24-h period. **Clinical oral investigations**, v. 3, n. 2, p. 88–95, 1999. DOI: 10.1007/s007840050084.

HANNIG, M.; BALZ, M. Protective properties of salivary pellicles from two different intraoral sites on enamel erosion. **Caries Research**, v. 35, n. 2, p. 142–148, 2001. DOI: 10.1159/000047446.

HANNIG, M.; JOINER, A. The structure, function and properties of the acquired pellicle. **Monogr Oral Sci**, v. 19, p. 29–64, 2006. DOI: 10.1159/000090585.

HARA, Anderson T.; ZERO, Domenick T. The caries environment: Saliva, pellicle, diet, and hard tissue ultrastructure. **Dental Clinics of North America**, v. 54, n. 3, p. 455–467, 2010. DOI: 10.1016/j.cden.2010.03.008.

HARA, Kenji; KOMINAMI, Eiki; KATUNUMA, Nobuhiko. Effect of proteinase inhibitors on intracellular processing of cathepsin B, H and L in rat macrophages. **FEBS Letters**, v. 231, n. 1, p. 229–231, 1988. DOI: 10.1016/0014-5793(88)80737-3.

HAY, D. I. The interaction of human parotid salivary proteins with hydroxyapatite. **Archives of Oral Biology**, v. 18, n. 12, 1973. DOI: 10.1016/0003-9969(73)90127-1.

JAEGGI, Thomas; LUSSI, Adrian. Prevalence, incidence and distribution of erosion. **Monographs in Oral Science**, v. 25, p. 55–73, 2014. DOI: 10.1159/000360973.

KATO, M. T.; LEITE, A. L.; HANNAS, A. R.; BUZALAF, M. A. R. Gels containing MMP inhibitors prevent dental erosion in situ. **Journal of Dental Research**, v. 89, n. 5, p. 468–472, 2010. DOI: 10.1177/0022034510363248.

KONDO, H.; ABE, K.; EMORI, Y.; ARAI, S. Gene organization of oryzacystatin-II, a new cystatin superfamily member of plant origin, is closely related to that of oryzacystatin-I but different from those of animal cystatins. **FEBS Lett**, v.278, n.1, p.87-90, 1991.

LUSSI, Adrian; CARVALHO, Thiago S. Erosive tooth wear: A Multifactorial condition of growing concern and increasing knowledge. **Monographs in Oral Science**, v. 25, p. 1–15, 2014. DOI: 10.1159/000360380.

LUSSI, Adrian; JAEGGI, T. Erosion - Diagnosis and risk factors. **Clinical Oral Investigations**, v. 12, n. SUPPL.1, p. 5–13, 2008. DOI: 10.1007/s00784-007-0179-z.

MAGALHÃES, Ana Carolina; WIEGAND, Annette; RIOS, Daniela; HANNAS, Angélica; ATTIN, Thomas; BUZALAF, M. A. R. Chlorhexidine and green tea extract reduce dentin erosion and abrasion in situ. **Journal of Dentistry**, v. 37, n. 12, p. 994–998, 2009. a. DOI: 10.1016/j.jdent.2009.08.007.

MAGALHÃES, Ana Carolina; WIEGAND, Annette; RIOS, Daniela; HONÓRIO, Heitor Marques; BUZALAF, Marília Afonso Rabelo. Insights into preventive measures for dental erosion. **Journal of Applied Oral Science**, v. 17, n. 2, p. 75–86, 2009. b. DOI: 10.1590/S1678-77572009000200002.

MARGIS, Rogério; REIS, Emerson M.; VILLERET, Vincent. Structural and phylogenetic relationships among plant and animal cystatins. **Archives of Biochemistry and Biophysics**, v. 359, n. 1, p. 24–30, 1998. DOI: 10.1006/abbi.1998.0875.

MCDONALD, E. E.; GOLDBERG, H. A.; TABBARA, N.; MENDES, F. M.; SIQUEIRA, W. L. Histatin 1 resists proteolytic degradation when adsorbed to hydroxyapatite. **Journal of Dental Research**, v. 90, n. 2, p. 268–272, 2011. DOI: 10.1177/0022034510388653.

MIGUEL, Mariana Cardoso. Mariana Cardoso Miguel. 2014.

NAHÓRNY, Sidnei; DE OLIVEIRA, Ivone Regina; SOARES, Luís Eduardo Silva. Biomineralization induced by chitosan and collagen-based materials with fluoride for dentin coverage: Chemical and morphological analysis. **Microscopy Research and Technique**, v. 85, n. 3, p. 1089–1100, 2022. DOI: 10.1002/jemt.23978.

NASCIMENTO, F. D. et al. Cysteine cathepsins in human carious dentin. **Journal of Dental Research**, v. 90, n. 4, p. 506–511, 2011. DOI: 10.1177/0022034510391906.

NEVES, José Guilherme; DANELON, Marcelle; PESSAN, Juliano Pelim; FIGUEIREDO, Leonardo Raniel; CAMARGO, Emerson Rodrigues; DELBEM, Alberto Carlos Botazzo. Surface free energy of enamel treated with sodium hexametaphosphate, calcium and phosphate. **Archives of Oral Biology**, v. 90, n. March, p. 108–112, 2018. DOI: 10.1016/j.archoralbio.2018.03.008.

PELÁ, V. T. et al. Safety and In Situ Antierosive Effect of CaneCPI-5 on Dental Enamel. **Journal of Dental Research**, v. 100, n. 12, p. 1344–1350, 2021. DOI: 10.1177/00220345211011590.

PELÁ, Vinícius Taioqui; NIEMEYER, Samira Helena; BAUMANN, Tommy; LEVY, Flávia Mauad; HENRIQUE-SILVA, Flávio; LUSSI, Adrian; CARVALHO, Thiago Saads; BUZALAF, Marília Afonso Rabelo. Acquired Pellicle Engineering Using a Combination of Organic (Sugarcane Cystatin) and Inorganic (Sodium Fluoride) Components against

Dental Erosion. **Caries Research**, v. 56, n. 2, p. 138–145, 2022. DOI: 10.1159/000522490.

PELÁ, Vinícius Taioqui; PRAKKI, Anuradha; WANG, Linda; VENTURA, Talita Mendes Silva; DE SOUZA E SILVA, Cíntia Maria; CASSIANO, Luiza Paula Silva; BRIANEZZI, Letícia Ferreira Freitas; LEITE, Aline Lima; BUZALAF, Marília Afonso Rabelo. The influence of fillers and protease inhibitors in experimental resins in the protein profile of the acquired pellicle formed in situ on enamel-resin specimens. **Archives of Oral Biology**, v. 108, n. August, p. 104527, 2019. DOI: 10.1016/j.archoralbio.2019.104527.

PINI, N. I. P.; LIMA, D. A. N. L.; LOVADINO, J. R.; GANSS, C.; SCHLUETER, N. In vitro Efficacy of Experimental Chitosan-Containing Solutions as Anti-Erosive Agents in Enamel. **Caries Research**, v. 50, n. 3, p. 337–345, 2016. DOI: 10.1159/000445758.

RÜTTERMANN, Stefan; TRELLENKAMP, Taina; BERGMANN, Nora; RAAB, Wolfgang H. M.; RITTER, Helmut; JANDA, Ralf. A new approach to influence contact angle and surface free energy of resin-based dental restorative materials. **Acta Biomater**, v. 7, n. 3, p. 1160–1165, 2011. DOI: 10.1016/j.actbio.2010.10.002.

SAADS CARVALHO, Thiago; LUSSI, Adrian. Chapter 9: Acidic beverages and foods associated with dental erosion and erosive tooth wear. **Monographs in Oral Science**, v. 28, p. 91–98, 2019. DOI: 10.1159/000455376.

SALAS, M. M. S.; NASCIMENTO, G. G.; HUYSMANS, M. C.; DEMARCO, F. F. Estimated prevalence of erosive tooth wear in permanent teeth of children and adolescents: An epidemiological systematic review and meta-regression analysis. **Journal of Dentistry**, v. 43, n. 1, p. 42–50, 2015. DOI: 10.1016/j.jdent.2014.10.012.

SANTIAGO, A. C. et al. A New Sugarcane Cystatin Strongly Binds to Dental Enamel and Reduces Erosion. **Journal of Dental Research**, v. 96, n. 9, p. 1051–1057, 2017. a. DOI: 10.1177/0022034517712981.

SCHLUETER, Nadine et al. Terminology of Erosive Tooth Wear: Consensus Report of a Workshop Organized by the ORCA and the Cariology Research Group of the IADR. **Caries Research**, v. 54, n. 1, p. 2–6, 2020. DOI: 10.1159/000503308.

SHELLIS, R. P.; GANSS, C.; REN, Y.; ZERO, D. T.; LUSSI, A. Methodology and models in erosion research: Discussion and conclusions. **Caries Research**, v. 45, n. SUPPL. 1, p. 69–77, 2011. DOI: 10.1159/000325971.

SIQUEIRA, W. L.; CUSTODIO, W.; MCDONALD, E. E. New insights into the composition and functions of the acquired enamel pellicle. **Journal of Dental Research**, v. 91, n. 12, p. 1110–1118, 2012. DOI: 10.1177/0022034512462578.

SIQUEIRA, Walter L.; OPPENHEIM, Frank G. Small molecular weight proteins/peptides present in the in vivo formed human acquired enamel pellicle. **Archives of Oral Biology**, v. 54, n. 5, p. 437–444, 2009. DOI: 10.1016/j.archoralbio.2009.01.011.

SOARES-COSTA, A.; BELTRAMINI, L. M.; THIEMANN, O. H.; HENRIQUE-SILVA, F. A sugarcane cystatin: Recombinant expression, purification, and antifungal activity. **Biochemical and Biophysical Research Communications**, v. 296, n. 5, p. 1194–1199, 2002. DOI: 10.1016/S0006-291X(02)02046-6.

TJÄDERHANE, Leo; BUZALAF, Marília Afonso Rabelo; CARRILHO, Marcela; CHAUSSAIN, Catherine. Matrix metalloproteinases and other matrix proteinases in relation to cariology: The era of “dentin degradomics”. **Caries Research**, v. 49, n. 3, p. 193–208, 2015. DOI: 10.1159/000363582.

VAN DER MEI, Henny C.; WHITE, Don J.; BUSSCHER, Henk J. On the wettability of soft tissues in the human oral cavity. **Archives of Oral Biology**, v. 49, n. 8, p. 671–673, 2004. DOI: 10.1016/j.archoralbio.2004.03.002.

VENTURA, Talita Mendes Oliveira; BUZALAF, Marília Afonso Rabelo; BAUMANN, Tommy; PELÁ, Vinícius Taioqui; NIEMEYER, Samira Helena; CRUSCA, Edson; MARCHETTO, Reinaldo; LUSSI, Adrian; CARVALHO, Thiago Saads. New insights into the protective effect of statherin-derived peptide for different acquired enamel pellicle formation times on the native human enamel surfaces. **Archives of Oral Biology**, v. 148, n. November 2022, 2023. DOI: 10.1016/j.archoralbio.2023.105643.

VILELA OLIVA, Maria Luiza; CARMONA, Adriana K.; ANDRADE, Sheila S.; COTRIN, Simone S.; SOARES-COSTA, Andrea; HENRIQUE-SILVA, Flavio. Inhibitory selectivity of canecystatin: A recombinant cysteine peptidase inhibitor from sugarcane. **Biochemical and Biophysical Research Communications**, v. 320, n. 4, p. 1082–1086, 2004. DOI: 10.1016/j.bbrc.2004.06.053.

VITORINO, R.; CALHEIROS-LOBO, M. J.; WILLIAMS, J.; FERRER-CORREIA, A. J.; TOMER, K. B.; DUARTE, J. A.; DOMINGUES, P. M.; AMADO, F. M. Peptidomic analysis of human acquired enamel pellicle. **Biomed Chromatogr**, v.21, n.11, p.1107-1117, 2007.

VOGLER, Erwin A. Structure and reactivity of water at biomaterial surfaces. **Advances in Colloid and Interface Science**, v. 74, n. 1–3, p. 69–117, 1998. DOI: 10.1016/S0001-8686(97)00040-7.

YAO, Y.; LAMKIN, M. S. Pellicle precursor prolein crosslinking characterizalion of an adduct between acidic proline-ricl protein (PRP-1) and statherin generated by

transglutaminase. **Journal of Dental Research**, 2000. DOI: 10.1177/00220345000790040801.

APPENDIX

APÉNDICE A – **TERMO DE CONSENTIMIENTO LIVRE E ESCLARECIDO**

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Departamento de Ciências Biológicas

Convite ao Participante

Nome do participante da pesquisa:

1. Título do Trabalho Experimental

"Engenharia de película adquirida para o controle da erosão dentária: avaliação *in situ* do potencial protetor de uma nova cistatina derivada da cana-de-açúcar".

2. Objetivo

O objetivo deste trabalho será avaliar o potencial protetor da modificação da PAE com CaneCPI-5 em diferentes veículos de aplicação (solução e gel) contra a erosão dentinária *in situ*.

3. Procedimentos da Fase Experimental
   a. Experimento com solução

Os voluntários participarão de 3 fases com as soluções de tratamento: Fase A: solução experimental CaneCPI-5 0,1 mg/mL; Fase B: solução comercial com $SnCl_2$/NaF/ AmF (800 ppm Sn+2,500 ppm F, pH 4,5, Erosion Protection® – GABA, controle positivo); Fase C: solução placebo (água destilada, controle negativo). As soluções serão inseridas em potes plásticos brancos, para que os voluntários não saibam a origem das mesmas.

Para cada fase, será confeccionado um aparelho palatino contendo as amostras de dente, o qual será utilizado por 5 dias comerciais no horário das 8 às 20 h. No período noturno, o aparelho deverá ser armazenado em gaze umedecida com água da torneira. Entre as fases, os voluntários terão um período de descanso de 10 dias, para evitar contaminação das amostras com o tratamento da fase anterior.

Dez dias antes do início de cada fase *in situ*, os voluntários receberão um kit contendo uma escova dentária convencional (Colgate® Twister Cabeça Compacta), um fio dental sem F e um tubo de dentifrício fluoretado (1.100 ppm F, NaF, Colgate®). No dia anterior ao início da fase *in situ*, os voluntários receberão a solução para bochecho em embalagem não identificada, uma escova elétrica para escovação das amostras (Escova Dental Elétrica Oral-B® Vitality Precision Clean), gaze e uma caixa para aparelho ortodôntico com o aparelho referente à fase experimental.

Durante todo o período experimental, o aparelho deverá permanecer na boca, sendo permitida sua remoção somente para ingestão de água e nas refeições diárias com no máximo 30 min de duração cada (12, 15 e 20h), com exceção da última refeição, que é livre. O intervalo entre as refeições deverá ser de pelo menos 3 h. Durante o período de refeições, o aparelho deverá ser armazenado em gaze umedecida com água, sendo imprescindível a realização da higiene bucal com dentifrício fluoretado 5 minutos antes do aparelho ser recolocado na boca.

Os voluntários serão orientados a manter os hábitos alimentares usuais e a realizar higienização bucal utilizando os materiais fornecidos durante todas as etapas do estudo (incluindo os períodos de descanso). A higienização do aparelho somente será realizada na superfície interna, não sendo permitida a higienização da superfície que contém as amostras dentárias. Não será permitido o uso de nenhum produto fluoretado (com exceção do dentifrício e das soluções testadas) ou antiplaca durante o período experimental.

Os voluntários realizarão o bochecho com 10 mL de solução por 1 minuto e cuspirão o excedente. O bochecho será realizado antes de cada desafio erosivo. Para a realização dos desafios erosivos, em horários pré-estabelecidos (10:00; 14:00; 16:00; 18:00 h), os voluntários deverão imergir o aparelho em 150 mL de solução de ácido cítrico a 0,1% (pH 2,5), à temperatura ambiente, por 90 s. Na sequência, os voluntários deverão recolocar o aparelho na boca. Trinta minutos após a primeira (10h) e a última erosão (18h), os voluntários deverão escovar as amostras referentes à erosão+abrasão utilizando a escova elétrica e uma gota por bloco de *slurry* de dentifrício fluoretado (1 g de dentifrício: 3 mL de água

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deionizada) por 15 s. Os voluntários serão orientados antes do início do experimento sobre como realizar a escovação e sobre a força que deverá ser aplicada durante a escovação. Na sequência, os voluntários enxaguarão o aparelho com água de torneira por 5 s e recolocarão o aparelho na boca para a realização do bochecho. Após a realização do experimento, o aparelho deverá permanecer por mínimo 30 min na boca. Ao final de cada fase experimental, o aparelho será devolvido ao pesquisador para que ele possa fazer a análise do perfil das amostras e calcular o desgaste dentário.

b. Experimento com gel

Os voluntários participarão de 4 fases com os gels de tratamento: Fase A: Um grupo sem tratamento (controle negativo); Fase B: gel contendo quitosana (sem CaneCPI-5);Fase C; gel contendo 500 ppm NaF; Fase D: gel contendo quitosana e 0,1 mg/ml CaneCPI-5. As gels serão inseridas em potes plásticos brancos, para que os voluntários não saibam a origem das mesmas.

Para cada fase, será confeccionado um aparelho palatino contendo as amostras de dente, o qual será utilizado por 5 dias comerciais no horário das 8 às 20 h. No período noturno, o aparelho deverá ser armazenado em gaze umedecida com água da torneira. Entre as fases, os voluntários terão um período de descanso de 10 dias, para evitar contaminação das amostras com o tratamento da fase anterior.

Dez dias antes do início de cada fase *in situ*, os voluntários receberão um kit contendo uma escova dentária convencional (Colgate® Twister Cabeça Compacta), um fio dental sem F e um tubo de dentifrício fluoretado (1.100 ppm F, NaF, Colgate®). No dia anterior ao início da fase *in situ*, os voluntários receberão o gel em embalagem não identificada, uma escova elétrica para escovação das amostras (Escova Dental Elétrica Oral-B® Vitality Precision Clean), gaze e uma caixa para aparelho ortodôntico com o aparelho referente à fase experimental.

Durante todo o período experimental, o aparelho deverá permanecer na boca, sendo permitida sua remoção somente para ingestão de água e nas refeições diárias com no máximo 30 min de duração cada (12, 15 e 20h), com exceção da última refeição, que é livre. O intervalo entre as refeições deverá ser de pelo menos 3 h. Durante o período de refeições, o aparelho deverá ser armazenado em gaze umedecida com água, sendo imprescindível a realização da higiene bucal com dentifrício fluoretado 5 minutos antes do aparelho ser recolocado na boca.

Os voluntários serão orientados a manter os hábitos alimentares usuais e a realizar higienização bucal utilizando os materiais fornecidos durante todas as etapas do estudo (incluindo os períodos de descanso). A higienização do aparelho somente será realizada na superfície interna, não sendo permitida a higienização da superfície que contém as amostras dentárias. Não será permitido o uso de nenhum produto fluoretado (com exceção do dentifrício e das soluções testadas) ou antiplaca durante o período experimental.

Os voluntários realizarão aplicação do gel uma vez por dia por 1 minuto que será realizado antes de cada desafio erosivo. Para a realização dos desafios erosivos, em horários pré-estabelecidos (10:00; 14:00; 16:00; 18:00 h); os voluntários deverão imergir o aparelho em 150 mL de solução de ácido cítrico a 0,1% (pH 2,5), à temperatura ambiente, por 90 s. Na sequência, os voluntários deverão recolocar o aparelho na boca. Trinta minutos após a primeira (10h) e a última erosão (18h), os voluntários deverão escovar as amostras referentes à erosão+abrasão utilizando a escova elétrica e uma gota por bloco de slurry de dentifrício fluoretado (1 g de dentifrício: 3 mL de água deionizada) por 15 s. Os voluntários serão orientados antes do início do experimento sobre como realizar a escovação e sobre a força que deverá ser aplicada durante a escovação. Na sequência, os voluntários enxaguarão o aparelho com água de torneira por 5 s e recolocarão o aparelho na boca para a realização do bochecho. Após a realização do experimento, o aparelho deverá permanecer por, no mínimo, 30 min na boca. Ao final de cada fase experimental, o aparelho será devolvido ao pesquisador para que ele possa fazer a análise do perfil das amostras e calcular o desgaste dentário.

4. Benefícios do Experimento

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Este projeto traz como benefício a importância de uma nova análise em favor da ciência, através da análise da película adquirida (camada de proteína proveniente da saliva), utilizando uma proteína que protege o dente da erosão dentinária, porém não há benefícios imediatos para os participantes da pesquisa. Pensando no produto comercial, o ideal seria que o mesmo fosse colocado em uma embalagem que pudesse ser facilmente levada pelo paciente para que o produto possa ser utilizado antes dos desafios ácidos. A participação será voluntária e entende-se que você poderá fazer qualquer pergunta sobre os procedimentos, sendo que será livre para desistir de participar a qualquer momento da pesquisa, sem nenhum prejuízo de sua parte. Em adição, você terá, também, por parte dos pesquisadores, a garantia do sigilo que assegura a sua privacidade e ainda receberá antes da pesquisa uma via, igualmente válida deste Termo de Consentimento Livre e Esclarecido assinado por ambas as partes (pesquisador e participante), no qual deixa claro seus direitos. Concordando em participar, você entende que este estudo será realizado em benefício das ciências médica e odontológicas, desta forma, concorda com a divulgação dos dados obtidos por meio de publicações científicas.

5. Riscos do Experimento

A sua participação neste trabalho acarretará em risco mínimo, que acontecerá no caso de você ter alguma reação ao material usado no aparelho ou algum dos componentes das soluções, alguns participantes podem eventualmente apresentar algum tipo de reação alérgica. Nestes casos, você será acompanhado até um local apropriado e esperado até o enjoo passar, então, o pesquisador irá acompanhá-lo até um médico. Em caso de reação alérgica, você poderá ser liberado da participação na pesquisa.

Importante ressaltar que não está sendo considerado nenhum pagamento ou recompensa material pela sua participação neste estudo. Em relação a incômodos, podemos afirmar que nada será acarretado, pois a sua participação será apenas na doação da sua saliva. Fica claro que você poderá, a qualquer momento, retirar seu CONSENTIMENTO LIVRE E ESCLARECIDO e deixar de participar do estudo alvo da pesquisa, ficando ciente de que todo trabalho realizado torna-se informação confidencial guardada por força do sigilo profissional (Art. 9º do Código de Ética Odontológica).

Ainda, se caso houver qualquer tipo de despesas tidas pelos participantes da pesquisa e dela decorrentes, serão de responsabilidade do pesquisador os gastos provenientes e/ou o ressarcimento aos participantes. Por fim, você terá garantido o direito à indenização compensatória caso fique comprovado que a sua participação lhe acarretou algum problema.

A liberdade do consentimento deverá ser particularmente garantida para aqueles participantes da pesquisa que, embora plenamente capazes, estejam expostos a condicionamentos específicos, ou à influência de autoridade, caracterizando situações passíveis de limitação da autonomia, como estudantes, assegurando-lhes inteira liberdade de participar ou não da pesquisa, sem quaisquer represálias.

Qualquer dúvida ou maiores esclarecimentos, o sujeito da pesquisa poderá recorrer a qualquer um dos membros da equipe do projeto (Laboratório de Bioquímica 14-3235-8247) ou ao pesquisador responsável Carlos Condarco Gironda (telefone 14-991957131). Caso queira apresentar reclamações em relação a sua participação na pesquisa ou denúncias, poderá entrar em contato com o Comitê de Ética em Pesquisa em Seres Humanos da FOB-USP, pelo endereço da Al. Dr. Octávio Pinheiro Brisolla, 9-75 (sala no prédio da Pós Graduação FOB/USP) ou pelo telefone (14)3235-8356.

Rubrica do Participante da Pesquisa

Rubrica do Pesquisador Responsável

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TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Pelo presente instrumento que atende às exigências legais, o Sr. (a) _____, portador da cédula de identidade _____, após leitura minuciosa das informações constantes neste TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO, devidamente explicada pelos profissionais em seus mínimos detalhes, ciente dos serviços e procedimentos aos quais será submetido, não restando qualquer dúvidas a respeito do lido e explicado, DECLARA e FIRMA seu CONSENTIMENTO LIVRE E ESCLARECIDO concordando em participar da pesquisa proposta em solução _____ em gel _____. Fica claro que o participante da pesquisa, pode a qualquer momento retirar seu CONSENTIMENTO LIVRE E ESCLARECIDO e deixar de participar desta pesquisa e ciente de que todas as informações prestadas tornar-se-ão confidenciais e guardadas por força de sigilo profissional (Art. 9º do Código de Ética Odontológica)

Por fim, como pesquisador(a) responsável pela pesquisa, DECLARO o cumprimento do disposto na Resolução CNS nº 466 de 2012, contidos nos itens IV.3 e IV.4, este último se pertinente, item IV.5.a e na íntegra com a resolução CNS nº 466 de dezembro de 2012.

Por estarmos de acordo com o presente termo o firmamos em duas vias igualmente válidas (uma via para o participante da pesquisa e outra para o pesquisador) que serão rubricadas em todas as suas páginas e assinadas ao seu término, conforme o disposto pela Resolução CNS nº 466 de 2012, itens IV.3.f e IV.5.d.

Bauru, SP, _____ de _____ de _____.

_____ _____

Assinatura do Participante da Pesquisa

Carlos Condarco Gironda
Responsável pela pesquisa

O **Comitê de Ética em Pesquisa – CEP**, organizado e criado pela FOB-USP, em 29/06/98 (**Portaria GD/0888/FOB**), previsto no item VII da Resolução CNS nº 466/12 do Conselho Nacional de Saúde do Ministério da Saúde (publicada no DOU de 13/06/2013), é um Colegiado interdisciplinar e independente, de relevância pública, de caráter consultivo, deliberativo e educativo, criado para defender os interesses dos participantes da pesquisa em sua integridade e dignidade e para contribuir no desenvolvimento da pesquisa dentro de padrões éticos.

Qualquer denúncia e/ou reclamação sobre sua participação na pesquisa poderá ser reportada a este CEP:

Horário e local de funcionamento:
Comitê de Ética em Pesquisa
Faculdade de Odontologia de Bauru-USP - Prédio da Pós-Graduação (bloco E - pavimento superior), de segunda à sexta-feira, no horário das **14hs às 17 horas**, em dias úteis.
Alameda Dr. Octávio Pinheiro Brisolla, 9-75
Vila Universitária – Bauru – SP – CEP 17012-901
Telefone/FAX(14)3235-8356
e-mail: cep@fob.usp.br

ANNEX

ANEXO A – **APROVAÇÃO PELO CEP**

FACULDADE DE ODONTOLOGIA DE BAURU DA UNIVERSIDADE DE SÃO PAULO - USP

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: ENGENHARIA DE PELÍCULA ADQUIRIDA PARA O CONTROLE DA EROSÃO DENTÁRIA

Pesquisador: Carlos Condarco Gironda

Área Temática:

Versão: 3

CAAE: 14973519.0.0000.5417

Instituição Proponente: Universidade de São Paulo - Faculdade de Odontologia de Bauru

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 5.993.139

Apresentação do Projeto:

Estes dados são extremamente importantes para formarem a base para futuros estudos in situ e clínicos que darão respaldo à utilização de produtos à base de canacistatins 5 para prevenção da erosão dentária.

Objetivo da Pesquisa:

Avaliar o efeito de géis contendo CaneCPI-5, em diferentes concentrações, na redução da erosão dentinária in vitro, em comparação a um inibidor específico de CCs (E-64);

Avaliar o potencial protetor da modificação da PAE com CaneCPI-5 (em diferentes veículos de aplicação) contra a erosão dentinária in situ.

Avaliação dos Riscos e Benefícios:

Riscos:

Em os dois experimentos os riscos são mínimos, no primer experimento pode acontecer algum tipo de alergia aos compostos utilizados o algum corte na preparação de amostras, enquanto no segundo experimento pode acontecer enjoos na hora de colocar o aparelho, sejam tomadas as medidas para sua minimização e proteção dos participantes da pesquisa.

Benefícios:

No primer experimento nós esperamos como benefício com a CaneCPI-5 em comparação com um inibidor específico de CCs(E-64), encontrar uma melhora ao tratamento da superfície dentinaria

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FACULDADE DE ODONTOLOGIA DE BAURU DA UNIVERSIDADE DE SÃO PAULO - USP

Continuação do Parecer: 5.993.139

antes da adesão de resinas compostas. Em quanto os benefícios do segundo experimento relacionado ao tratamento com gel e solução nós esperamos encontrar um produto que possa ajudar contra a erosão dental. Também esperamos reduzir o número de aplicações utilizando diferentes veículos.

Comentários e Considerações sobre a Pesquisa:

Grupos em que serão divididos os participantes da pesquisa neste centro

Gel com 16 voluntários nas Fase A, Fase B, Fase C, Fase D

Solução com 15 voluntários nas Fase A, Fase B, Fase C

Considerações sobre os Termos de apresentação obrigatória:

Serão acrescidos de 16 voluntários para avaliação do gel, no total 31.

No presente projeto serão acrescentadas 76 amostras da dentina humana (4x4 mm) para a adição de uma fase de tratamento in situ (totalizando 4 fases) e a adição de um voluntário para toda pesquisa (totalizando 16 voluntários, como mencionado acima).

Conclusões ou Pendências e Lista de Inadequações:

Aprovado

Considerações Finais a critério do CEP:

A emenda apresentada pelo(a) pesquisador(a) foi considerada APROVADA na reunião ordinária do CEP de 04/04/2023, com base nas normas éticas da Resolução CNS 466/12. Ao término da pesquisa o CEP-FOB/USP exige a apresentação de relatório final. Os relatórios parciais deverão estar de acordo com o cronograma e/ou parecer emitido pelo CEP. Alterações na metodologia, título, inclusão ou exclusão de autores, cronograma e quaisquer outras mudanças que sejam significativas deverão ser previamente comunicadas a este CEP sob risco de não aprovação do relatório final. Quando da apresentação deste, deverão ser incluídos todos os TCLEs e/ou termos de doação assinados e rubricados, se pertinentes.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_2041368_E1.pdf	13/03/2023 15:04:36		Aceito
Outros	Oficio_para_emenda.pdf	13/03/2023 12:46:42	Carlos Condarco Gironda	Aceito
Projeto Detalhado	PROJETO_emenda.pdf	13/03/2023	Carlos Condarco	Aceito

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FACULDADE DE ODONTOLOGIA DE BAURU DA UNIVERSIDADE DE SÃO PAULO - USP

Continuação do Parecer: 5.980.139

/ Brochura Investigador	PROJETO_emenda.pdf	12:46:08	Gironda	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCL_emenda.pdf	13/03/2023 12:44:53	Carlos Condarco Gironda	Aceito
Outros	Oficio.pdf	04/07/2019 21:30:58	Carlos Condarco Gironda	Aceito
Outros	Termo_Aquiescencia.pdf	04/07/2019 21:30:44	Carlos Condarco Gironda	Aceito
Folha de Rosto	Folha_de_Rostro.pdf	22/05/2019 18:21:09	Carlos Condarco Gironda	Aceito
Declaração de Pesquisadores	declaracao_de_compromisso.pdf	25/04/2019 02:13:46	Carlos Condarco Gironda	Aceito
Outros	Questionario_Tecnico.pdf	25/04/2019 02:12:54	Carlos Condarco Gironda	Aceito
Declaração de Instituição e Infraestrutura	carta_encaminhamento.pdf	25/04/2019 02:09:23	Carlos Condarco Gironda	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

BAURU, 10 de Abril de 2023

Assinado por:
CASSIA MARIA FISCHER RUBIRA
(Coordenador(a))

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ANEXO B – **ARTIGO PUBLICADO JOURNAL OF APPLIED ORAL SCIENCE**

GIRONDA, Carlos Condarco; PELÁ, Vinícius Taioqui; HENRIQUE-SILVA, Flávio; DELBEM, Alberto Carlos Botazzo; PESSAN, Juliano Pelim; BUZALAF, Marília Afonso Rabelo. New insights into the anti-erosive property of a sugarcane-derived cystatin: different vehicle of application and potential mechanism of action. Journal of Applied Oral Science, v. 30, p. 1–7, 2022. DOI: 10.1590/1678-7757-2021-0698.

Original Article
http://dx.doi.org/10.1590/1678-7757-2021-0698

JAOS
JOURNAL OF APPLIED ORAL SCIENCE

New insights into the anti-erosive property of a sugarcane-derived cystatin: different vehicle of application and potential mechanism of action

Carlos Condarco GIRONDA[1*]
Vinicius Taioqui PELÁ[2*]
Flávio HENRIQUE-SILVA[2]
Alberto Carlos Botazzo DELBEM[3]
Juliano Pelim PESSAN[3]
Marília Afonso Rabelo BUZALAF[1]

Abstract

A new sugarcane-derived cystatin (CaneCPI-5) showed anti-erosive properties when included in solutions and strong binding force to enamel, but the performance of this protein when added to gel formulations and its effect on surface free energy (SFE) requires further studies. Objective: 1) to evaluate the protective effect of gels containing different concentrations of CaneCPI-5 against initial enamel erosion (Experiment 1); and 2) to analyze the SFE (γ_s) after treating the enamel surface with CaneCPI-5 solution (Experiment 2). Methodology: In Experiment 1, 75 bovine enamel specimens were divided into five groups according to the gel treatments: placebo (negative control); 0.27%mucin+0.5%casein (positive control); 0.1 mg/mL CaneCPI-5; 1.0 mg/mL CaneCPI-5; or 2.0 mg/mL CaneCPI-5. Specimens were treated with the gels for 1 min, the AP was formed (human saliva) for 2 h and the specimens were incubated in 0.65% citric acid (pH=3.4) for 1 min. The percentage of surface hardness change (%SHC) was estimated. In Experiment 2, measurements were performed by an automatic goniometer using three probing liquids: diiodomethane, water and ethylene glycol. Specimens (n=10/group) remained untreated (control) or were treated with solution containing 0.1 mg/mL CaneCPI-5, air-dried for 45 min, and 0.5 µL of each liquid was dispensed on the surface to measure contact angles. Results: Gels containing 0.1 and 1.0 mg/mL CaneCPI-5 significantly reduced %SHC compared to the other treatments ($p<0.05$). Treated enamel showed significantly lower γ_s than control, without changes in the apolar component (γ_s^{LW}), but the polar component (γ_s^{AB}=Lewis acid-base) became more negative ($p<0.01$). Moreover, CaneCPI-5 treatment showed higher γ_s^- (electron-donor) values compared to control ($p<0.01$). Conclusions: Gels containing 0.1 mg/mL or 1.0 mg/mL CaneCPI-5 protected enamel against initial dental erosion. CaneCPI-5 increased the number of electron donor sites on the enamel surface, which may affect AP formation and could be a potential mechanism of action to protect from erosion.

Keywords: Acquired pellicle. Tooth erosion. Cystatin.

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* Carlos Condarco Gironda and Vinicius Taioqui Pelá contributed equally to this study.

Introduction

Dental erosion is the chemical loss of mineralized tooth substance due to exposure to non-bacterial acids.[1] Saliva is the main patient-related factor that interferes with dental erosion, since it is saturated regarding apatite, buffers the acids and is the main source of proteins that form the acquired pellicle (AP). This proteinaceous layer acts as a mechanical barrier to the acids, thus reducing erosion.[2]

Not all proteins found in the AP protect the tooth surface from acid dissolution. Studies have suggested that the proteins present in the basal layer have a greater participation in this regard.[3] Thus, the concept of "acquired pellicle engineering", which involves changing the AP by adding molecules, has a strong potential to increase its protective effect on the tooth surface.[4,5] A series of *in vivo* studies used proteomic approaches to identify acid-resistant proteins in the AP that would be candidates for inclusion in dental products to reduce erosive demineralization.[6-8] Among them, cystatin-B is a good alternative,[6] but the cost of the human recombinant protein is prohibitive. Therefore, our group recently cloned sugarcane-derived cystatin (CaneCPI-5) that has a strong binding force to hydroxyapatite and can protect from initial erosion *in vitro*[9] and *in vivo*[8] when added to rinse solutions. Moreover, incorporating molecules in the AP may change the enamel reactivity and the surface free energy (SFE), which might guide protein binding to the AP, thus changing its composition.

Regarding the application vehicle, the use of gels in studies involving the inhibition of matrix metalloproteinases in dentin a offered better protection against dentin erosion when compared with their inclusion in solutions.[9,10] Possibly due to the prolonged contact time of the gel with the tooth surface, due to its viscosity. We hypothesize that the same could happen with CaneCPI-5 gels. If the protection conferred by CaneCPI-5-containing gels is better than that conferred by solutions, the frequency of application of the first can be lower, which is an advantage from the clinical point of view.

Therefore, our study evaluates the protective effect of gels containing different concentrations of CaneCPI-5 against enamel initial erosion *in vitro*. Since little is known about the mechanisms by which CaneCPI-5 interacts with the enamel surface, we also analyzed the ability of CaneCPI-5 to alter the SFE of enamel by measuring the contact angle using the sessile drop method. The null hypotheses tested were: 1) gels containing CaneCPI-5 do not protect from initial dental erosion and 2) CaneCPI-5 does not alter the enamel SFE.

Methodology

This study comprised two experiments: In Experiment 1, the effect of CaneCPI-5 (in different concentrations) added in gel on polished enamel specimens was evaluated using surface microhardness analysis. In Experiment 2, 0.1 mg/mL CaneCPI-5 (in solution) was applied on polished enamel specimens for SFE analysis.

The use of bovine teeth for this research was approved by the Ethics Committee on Animal Use of Bauru School of Dentistry, University of São Paulo (Protocol: 006/2017 for Experiment 1 and Protocol: 010/2021 for Experiment 2). Also, this study was approved by the Ethics Committee for Human Research (CAAE: 59786416.9.0000.5417) of Bauru School of Dentistry, University of São Paulo. Besides, saliva donor volunteers signed an informed consent form before the procedures.

Selection of volunteers and saliva collection

Saliva was collected from three healthy volunteers of both genders (aged 24 to 32 years). The exclusion criteria adopted were: smoking habit, cavitated carious lesions, severe dental wear, use of medications that affect salivary flow, salivary flow under the thresholds for unstimulated (> 0.3 mL/min) and stimulated (> 1.0 mL/min) saliva, xerostomia, type I diabetes, poor nutrition, gastroesophageal problems and regurgitation and vomiting disorders.[11]

All volunteers performed oral hygiene before collection using a new toothbrush, fluoride toothpaste (CloseUP, 1450 ppm F, Unilever, Brazil) and dental floss. Saliva was collected between 9 and 11 a.m. (to avoid circadian effects) under masticatory stimulation using Parafilm. Then, saliva was centrifuged (14,000 g at 4ºC) for 15 min. After that, the supernatants were collected to form a pool of saliva and divided into 13-mL aliquots, which were stored at -80ºC prior to the experiments.[12]

Heterologous expression of CaneCPI-5

CaneCPI-5 was produced at the Laboratory of Molecular Biology of the Department of Genetics and Evolution of the Federal University of São Carlos, Brazil. For heterologous expression, bacterial strain *Escherichia coli Rosetta* (DE3) transformed with plasmid pET28aCaneCPI-5 was used as previously described.[11] The expressed protein was purified from the soluble fraction of bacterial cultures induced by IPTG (isopropyl-beta-D-thiogalactoside), subjected to centrifugation and sonication. The purification was done by affinity chromatography using columns containing Ni-NTA Superflow nickel resin (Qiagen).[11]

Preparation of the enamel specimens

A total of 95 bovine enamel specimens were prepared (4 mm×4 mm×4 mm), being 75 specimens for "Experiment 1" and 20 specimens for "Experiment 2". They were obtained from the buccal-cervical region of bovine incisors and stored in 2% thymol solution (pH 7.0) for 30 days. Besides, the specimens were visually analyzed to assess possible stains and cracks. In these cases, the teeth were excluded. Then, the enamel surface was sequentially polished using water-cooled silicon carbide paper disks (320, 600, and 1200 grit, Extec, Enfield, CT, USA). A felt polishing cloth (Extec Corp. Polishing cloth; Buehler, Lake Bluff, IL, USA), moistened with a 1-µm diamond solution (Extec Corp. Buehler, Lake Bluff, IL, USA), was used on the surface of interest to finalize the polishing. After polishing, the specimens were immersed in an ultrasonic bath (T7 Thornton, Unique Ind. E Com. Ltda., São Paulo, SP, BR) with deionized water for seven min at 25°C. Lastly, they were stored (with wet gauze) at 4°C prior to the experiment.

Experiment 1. Effect of gels containing different concentrations of CaneCPI-5 against initial enamel erosion *in vitro*

Experimental procedures

A total of 75 specimens were divided into five groups (n/group=15, determined by computerized random numbers after initial surface hardness): 1) placebo gel (negative control), 2) 0.27% mucin + 0.5% casein (positive control), 3) 0.1 mg/mL CaneCPI-5, 4) 1 mg/mL CaneCPI-5 and 5) 2.0 mg/mL CaneCPI-5. All gels were prepared as described by Kato, et al.[9] (2010) and had the same composition, except for the presence of casein + mucin or CaneCPI-5.

The amount of gel applied was controlled by a dispenser (pipette, 20 µl per specimen), then the gel was added on the microbrush and applied on the enamel surface of each specimen for 1 minute, and the excess was removed with a cotton swab.[9] The specimens were then incubated in saliva for 2 h at 37°C under agitation to form the AP.[14] Then, the specimens were washed in deionized water (10 s) and air-dried (5 s). For the erosive challenge, they were immersed in 0.65% citric acid solution (pH=3.4) for 1 min at 30°C under agitation, washed in deionized water and air-dried again.[14]

Surface hardness

Surface hardness change (SHC) analyses were performed using a Knoop penetrator, with a load of 50 g for 15 s at baseline ($SHC_{initial}$) and after the experiment (SHC_{final}). Five indentations were made in the central region of each specimen at 50 µm intervals. Control indentations of 2 and 5 g were made to detect possible loss of surface. Specimens with microhardness values 10% lower or 10% higher than the mean of all specimens were excluded from the study. The percentage of surface hardness change (%SHC) was estimated as a measure of enamel softening, according to the following equation: $\%SHC=([SHC_{initial}-SHC_{final}]/SHC_{initial})\times100$[9].

Experiment 2. Ability of CaneCPI-5 to alter the enamel surface free energy

Twenty enamel specimens were divided into two groups, as follows: Negative control (untreated) or 0.1 mg/mL CaneCPI-5 (n=10/group determined by computerized random numbers).

Surface free energy measurements

The surface free energy (SFE) was characterized by contact angle measurements, using the sessile drop method to determine the SFE. Measurements were performed by an automatic goniometer (DSA 100S, Krüss, Hamburg, Germany) using three probing liquids: diiodomethane, water and ethylene glycol. The treated specimens were air dried for 45 min to stabilize the layer formed.[15] Then, 0.5 µL of each liquid was dispensed on the surface of each block and the contact angles were measured using the images captured by a CCD camera. Five measurements were performed at 20°C and relative air humidity of 47% for each specimen.[15,16] Different parameters, such as acid (γ^+, receptor component), base (γ^-, donor component) and Lifshiz van der Waals (γ^{LW},

nonpolar component) of surface free energy (mN/m) were estimated according to the model of van Oss, Chaudhery and Good to determine the substrates free energy.[17,18] The interaction free energy (ΔG_{sl}) was also estimated to determine the hydrophobicity/hydrophilicity of the enamel surface: $\Delta G_{sl} > 0$ indicated a hydrophilic surface and $\Delta G_{sl} < 0$ indicated a hydrophobic surface.[18,19]

Statistical Analysis

All the data were analyzed using the GraphPad InStat (version 3.10 for Windows) and GraphPad Prism (GraphPad Software Inc., La Jolla, CA) software. Data were checked for normality (Kolmogorov-Smirnov test) and homogeneity (Bartlett test) to select the appropriate statistical test. In the first experiment, the data were analyzed using Kruskall-Wallis and Dunn's tests. In the second experiment, the data were analyzed using ANOVA and Student-Newman-Keuls's test and by Pearson's correlation coefficient. The significance levels of both experiments were considered as $p<0.05$.

Results

In the first experiment, only the treatments with CaneCPI-5 at 0.1 and 1.0 mg/mL significantly reduced the SHC compared to control ($p<0.05$). The treatment performed with the higher concentration of CaneCPI-5 did not significantly differ from control or from mucin + casein ($p>0.05$) (Figure 1).

In the second experiment, the SFE (γ_s) was significantly lower with CaneCPI-5 ($p<0.001$) compared to control (Table 1; $p<0.001$). The values of the apolar component (γ_s^{LW}) from enamel surface

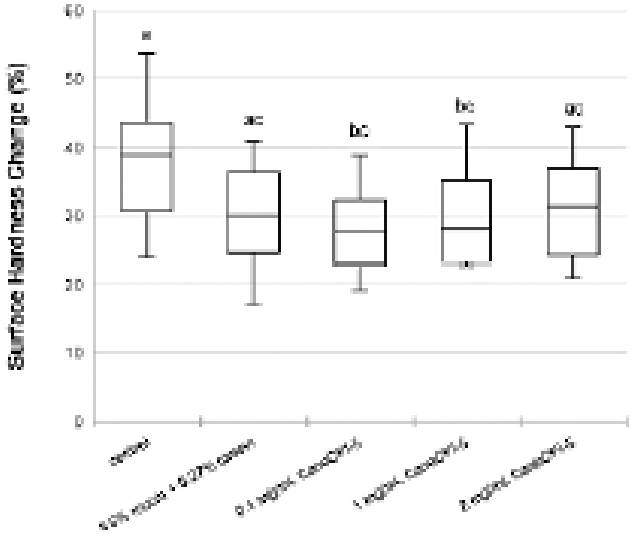

Figure 1- Median enamel loss after short-term erosive challenge. Bovine enamel specimens were treated with gels containing the proteins for 1 min, followed by incubation in pooled human saliva for 2 h to form the acquired pellicle and subsequent challenge with 0.65% citric acid for 1 min. Different letters indicate a significant difference among groups (Kruskal-Wallis and Dunn's tests, $p<0.05$, n=15/group)

Table 1- Means (SD) of the contact angles of probing liquids, surface free energy (γ_s) and interaction free energy (ΔG_{sl}) after treating enamel surface with 0.1 mg/mL CaneCPI-5 or not (n=10).

Treatments	Water	Diiodomethane	Ethylene glycol	γS	ΔG_{sl}
	q (°)	q (°)	q (°)	(mN/m)	(mN/m)
Sound enamel	67.3^a	52.6^a	56.6^a	28.2^a	-0.3^a
	(-4.4)	(-5.1)	(-3.6)	(-4.5)	(-8.2)
CPI-5 (0.1 mg/mL)	37.2^b	51.6^a	55.9^a	-1.7^b	53.2^b
	(-2.8)	(-3.8)	(-4.3)	(-10.8)	(-8.8)

Distinct superscript letters indicate significant difference among the groups in each analysis (ANOVA and Student-Newman-Keuls's test, $p<0.05$, n=10)

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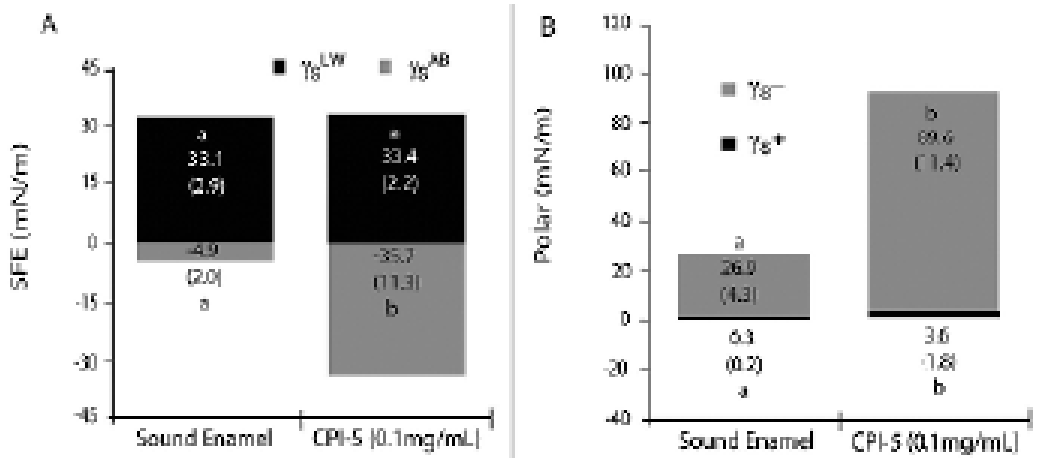

Figure 2- (A) Surface free energy and their components (γS^{LW}: Lifshitz-van der Waals surface tension component; γS^{AB}: Lewis acid-base interaction) with different enamel-surface treatments. (B) Influence of the treatments on the polar component of surface free energy on enamel surface: Lewis-acid (γ_s^+) and Lewis-base (γ_s^-). Values denote mean and standard deviation (n=10). Distinct letters show significant differences among mean considering treatment (Student-Newman-Keuls, p<0.05)

were not significantly different between the groups (p=0.161). The values of the polar component (γ_s^{AB}=Lewis acid-base) became more negative with CaneCPI-5 treatment (Figure 2A; p<0.001). Among the parameters from γ_s^{AB}, γ_s^+=electron-acceptor (Lewis acid) and γ_s^-=electron-donor (Lewis base), the CaneCPI-5 treatment showed higher γ_s^- values compared to control (Figure 2B; p<0.001). We observed significant correlations between γ_s and γ_s^{AB} values (Pearson's r=0.987; p<0.001) and γ_s^- (Pearson's r=-0.942; p<0.001). The interaction free energy (ΔG_{sws}) was > 0 for CaneCPI-5 treatment, indicating a hydrophilic surface (Table 1).

Discussion

Our study involves the concept of "acquired pellicle engineering" that involves changing the AP by adding molecules or ions that can increase its protection against dental erosion.[20] The change was done by using CaneCPI-5, a sugarcane-derived cystatin that has a strong binding force to hydroxyapatite.[4]

In Experiment 1, we used an established initial erosion model to evaluate the protective effect of a gel containing CaneCPI-5 against erosion. This model involves one challenge (1 min) with 0.65% citric acid (pH 3.5),[21] causing enamel softening that can be measured by SHC, since enamel loss (detected by profilometry) is not expected at this early stage. Our previous studies showed protective effect in applying solutions containing 0.1 mg/mL CaneCPI-5 against initial enamel erosion when applied *in vitro* for two h[4] or *in vivo* for 1 min[5]. Furthermore, the use of gels containing protease inhibitors[9] offered better protection against dentine erosion than solutions containing the same inhibitors,[10] due to a more intimate contact of the gels with the dental surfaces. We used a mixture of mucin (0.27%) and casein (0.5%) as a positive control due to the previous results, which showed that adding both components in the AP could offer a protective effect against initial erosion *in vitro*.[14]

Thus, this vehicle was selected for application of CaneCPI-5, at concentrations (ranging between 0.1 and 2.0 mg/mL), based on those used in solutions.[4] The gels containing CaneCPI-5 at 0.1 and 1.0 mg/mL significantly reduced enamel erosion compared to the placebo gel, while the product containing 2.0 mg/mL CaneCPI-5 did not (Figure 1). The gels offered 30%SHC reduction, whereas the effects using aqueous solutions at the same concentrations was around 90%.[4] However, the solutions remained in contact with the enamel surface for 2 h, whereas treatment with the gels lasted only 1 min. Besides, in Atomic Force Microscopy (AFM), enamel samples were incubated in solutions containing CaneCPI-5 for 4 h.[4] Based on time-response considerations, it would be helpful to evaluate longer exposure to the gels (e.g., 4 min), since application of fluoridated gels for 4 min in the clinical practice has been reported to offer higher caries-protective effects than application for 1 min.[21,22] The highest concentration of CaneCPI-5 (2.0 mg/mL)

did not protect enamel from initial erosion. This can be related to previous studies showing that sugarcane cystatins, at high concentrations, undergo dimerization by domain swapping,[25,26] which reduces the levels of free protein to bind to enamel.

Experiment 2 had a mechanistic approach. We aimed to test the enamel reactivity after treatment with CaneCPI-5 using the sessile drop method. This is essential for the concept of "acquired pellicle engineering", since alterations in the SFE upon treatment with CaneCPI-5 might guide protein binding to the AP, thus changing its composition, especially considering binding other salivary proteins to CaneCPI-5 and/or to dental surfaces. The untreated enamel was slightly hydrophobic, since contact water angle was a little larger than 65°;[25,28] SFE (γ_s) was < 30 mN/m (Table 1),[18,27] ΔG_{sws} was close to zero, and γ_s^- was < 28.5 mN/m (Figure 2),[18,19,28] with values of γ_s^+ close to zero. As described in a previous study,[28] enamel surface shows characteristics that favor the precipitation of ionic species, such as Ca^{2+} and $CaH_2PO_4^+$, or protein adhesion, both of which are essential to reduce the erosive process. Furthermore, surfaces with lower SFE brings fewer bacteria to its surface than one with higher SFE. However, we emphasize that the acid-base theoretical approach used in this study,[18,19,28] involving the decomposition in γ_s^{LW} and γ_s^{AB} (which strongly influence to γ_s), differs from other studies that used different theoretical approaches to estimate γ_s.

In our study, the reduction of SFE with CaneCPI-5 treatment was related to more negative values of polar energy (γ_s^{AB}), since the nonpolar energy did not change (γ_s^{LW}). Therefore, the acid (γ_s^+)/base (γ_s^-) and interaction free energy (ΔG_{sws}) forces indicate whether a surface is more hydrophobic or hydrophilic, facilitating or not protein adhesion or calcium phosphate precipitation.[18,28,29] The theoretical aspects above show that treatment with CaneCPI-5 increases the hydrophilic character of the surface of the enamel, which makes it prone to water, considering contact angles < 65°, γ_s^- > 28.5 mN/m and ΔG_{sws} > 0[30]. Also, CaneCPI-5 showed higher γ_s^- values, leading to higher electron-donor sites at the enamel surface that favors adsorption cationic ionic species (Ca^{2+} and $CaH_2PO_4^+$) and cationic acid-resistant proteins from saliva, thus explaining the lower hardness loss after erosion challenge. Consequently, negative surfaces may be partially or fully neutralized by multivalent cations, leading to a hydrophobic surface.[31] Alteration in the SFE partially explains the changes in acid-resistant proteins of the AP obtained after rinsing for 1 min with 0.1 mg/mL CPI-5 and subsequent challenge with 1% citric acid pH 2.5 for 10 s (increase in keratin, IgG, lactotransferrin, serum albumin, alpha amylase, basic salivary proline-rich protein, carbonic anhydrase).[8]

We recognize the limitations of the present *in vitro* study. Although the protocols suit preliminary studies, they do not accurately simulate the clinical condition due to the absence of oral cavity-specific factors, such as the formation of AP. In Experiment 1, limitation of treatment time (with the gels and CaneCPI-5) for 1 min could be extended for longer periods (e.g., 4 min). Regarding Experiment 2, the presence of saliva, which is the main biological factor involved in the occurrence of dental erosion whose factor is the most determinant for the oral cavity, was not considered. Moreover, CaneCPI-5 was included in gels in the first experiment, while it was included in solution in the second one, due to the analytical technique used. These limitations must be addressed in future studies.

We rejected both hypotheses based on the results, since: 1) gels containing CaneCPI-5 at 0.1 and 1.0 mg/mL protected enamel from initial dental erosion; and 2) CaneCPI-5 altered the enamel SFE. Moreover, change in SFE of enamel after applying CaneCPI-5 may help to partially explain alterations in the AP proteome, with consequent change in its protective ability, induced by this phytocystatin.

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Conflict of interest

The authors have declared no competing interests.

Authors' contributions

Gironda, Carlos Condarco: Data curation (Equal); Methodology (Equal); Writing – original draft (Equal); Writing – review & editing (Equal). **Pelá, Vinicius Taioqui:** Data curation (Equal); Methodology (Equal); Writing – original draft (Equal); Writing – review & editing (Equal). **Henrique-Silva, Flávio:** Methodology (Equal); Writing – original draft (Equal); Writing – review & editing (Equal). **Delbem, Alberto Carlos Botazzo:** Methodology (Equal); Writing –

original draft (Equal); Writing – review & editing (Equal). **Pessan, Juliano:** Conceptualization (Equal); Methodology (Equal); Writing – original draft (Equal); Writing – review & editing (Equal). **Buzalaf, Marilia Afonso Rabelo:** Conceptualization (Equal); Data curation (Equal); Investigation (Equal); Methodology (Equal); Supervision (Equal); Validation (Equal); Writing – original draft (Equal); Writing – review & editing (Equal).

References

1- Schlueter N, Amaechi BT, Bartlett D, Buzalaf MA, Carvalho TS, Ganss C, et al. Terminology of erosive tooth wear: consensus report of a workshop organized by the ORCA and the Cariology Research Group of the IADR. Caries Res. 2020;54(1):2-6. doi: 10.1159/000503308.

2- Buzalaf MA, Hannas AR, Kato MT. Saliva and dental erosion. J Appl Oral Sci. 2012;20(5):493-502. doi: 10.1590/s1678-77572012000500001

3- Hannig C, Berndt D, Hoth-Hannig W, Hannig M. The effect of acidic beverages on the ultrastructure of the acquired pellicle: an *in situ* study. Arch Oral Biol. 2009;54(6):518-26. doi: 10.1016/j.archoralbio.2009.02.009.

4- Santiago AC, Khan ZN, Miguel MC, Gironda CC, Soares-Costa A, Pelá VT, et al. A new sugarcane cystatin strongly binds to dental enamel and reduces erosion. J Dent Res. 2017;96(9):1051-7. doi: 10.1177/0022034517712981

5- Carvalho TS, Araújo TT, Ventura TM, Diombio A, Olmara JV, Moraes SM, et al. Acquired pellicle protein-based engineering protects against erosive demineralization. J Dent. 2020;102:103478. doi: 10.1016/j.jdent.2020.103478

6- Delecrode TR, Siqueira WL, Zaidan FC, Bellini MR, Moffa EB, Mussi MC, et al. Identification of acid-resistant proteins in acquired enamel pellicle. J Dent. 2015;43(12):1470-5. doi: 10.1016/j.jdent.2015.10.009

7- Martini T, Rios D, Cassiano LP, Silva CM, Taira EA, Ventura TM, et al. Proteomics of acquired pellicle in gastroesophageal reflux disease patients with or without erosive tooth wear. J Dent. 2019;81:64-9. doi: 10.1016/j.jdent.2018.12.007

8- Taira EA, Ventura TMS, Cassiano LPS, Silva CMS, Martini T, Leite AL, et al. Changes in the proteomic profile of acquired enamel pellicles as a function of their time of formation and hydrochloric acid exposure. Caries Res. 2018;52(5):367-77. doi: 10.1159/000486969

9- Kato MT, Leite AL, Hannas AR, Buzalaf MA. Gels containing MMP inhibitors prevent dental erosion *in situ*. J Dent Res. 2010;89(5):468-72. doi: 10.1177/0022034510363248

10- Magalhães AC, Wiegand A, Rios D, Hannas A, Attin T, Buzalaf MA. Chlorhexidine and green tea extract reduce dentin erosion and abrasion *in situ*. J Dent. 2009;37(12):994-8. doi: 10.1016/j.jdent.2009.08.007

11- Tenovuo J, Lagerlöf F. Saliva. In: Thylstrup A, Fejerskov O. Cariologia clínica [Textbook of Clinical Cariology]. São Paulo: Livraria Santos Editora; 1995. p. 17-43. Portuguese.

12- Schipper R, Loof A, de Groot J, Harthoorn L, Dransfield E, van Heerde W. SELDI-TOF-MS of saliva: methodology and pre-treatment effects. J Chromatogr B Analyt Technol Biomed Life Sci. 2007;847(1):45-53. doi: 10.1016/j.jchromb.2006.10.005

13- Soares-Costa A, Beltramini LM, Thiemann OH, Henrique-Silva F. A sugarcane cystatin: recombinant expression, purification, and antifungal activity. Biochem Biophys Res Commun. 2002;296(5):1194-9. doi: 10.1016/s0006-291x(02)02046-6

14- Cheaib Z, Lussi A. Impact of acquired enamel pellicle modification on initial dental erosion. Caries Res. 2011;45(2):107-12. doi: 10.1159/000324803

15- van der Mei HC, White DJ, Kemmings-Rasker HJ, Knight J, Baig AA, Smit J, et al. Influence of dentifrices and dietary components in saliva on wettability of pellicle-coated enamel *in vitro* and *in vivo*. Eur J Oral Sci. 2003;111(6):434-8. doi: 10.1034/j.1600-0722.2003.21341.x

16- Harnett EM, Alderman J, Wood T. The surface energy of various biomaterials coated with adhesion molecules used in cell culture. Colloids Surf B Biointerfaces. 2007;55(1):90-7. doi: 10.1016/j.colsurfb.2006.11.021

17- van Oss CJ. Aspecific and specific intermolecular interactions in aqueous media. J Mol Recognit. 1990;3(3):128-36. doi: 10.1002/jmr.300030307

18- Volpe CD, Siboni S. Some reflections on acid-base solid surface free energy theories. J Colloid Interface Sci. 1997;195(1):121-36. doi: 10.1006/jcis.1997.5124

19- van Oss CJ. Acid-base interfacial interactions in aqueous media. Colloids Surf A Physicochem Eng Asp. 1993;78:1-49. doi: 10.1016/0927-7757(93)80308-2

20- Vukosavljevic D, Custodio W, Buzalaf MA, Hara AT, Siqueira WL. Acquired pellicle as a modulator for dental erosion. Arch Oral Biol. 2014;59(6):631-8. doi: 10.1016/j.archoralbio.2014.02.002

21- Weyant RJ, Tracy SL, Anselmo TT, Beltrán-Aguilar ED, Donly KJ, Frese WA, et al. Topical fluoride for caries prevention: executive summary of the updated clinical recommendations and supporting systematic review. J Am Dent Assoc. 2013;144(11):1279-91. doi: 10.14219/jada.archive.2013.0057

22- Van Rijkom H, Ruben J, Vieira A, Huysmans MC, Truin GJ, Mulder J. Erosion-inhibiting effect of sodium fluoride and titanium tetrafluoride treatment *in vitro*. Eur J Oral Sci. 2003;111(3):253-7. doi: 10.1034/j.1600-0722.2003.00031.x

23- Cavini IA, Oliveira-Silva R, Marques IA, Kalbitzer HR, Munte CE. Chemical shift assignments of the canecystatin-1 from Saccharum officinarum. Biomol NMR Assign. 2013;7(2):163-5. doi: 10.1007/s12104-012-9401-2

24- Valadares NF, Oliveira-Silva R, Cavini IA, Marques IA, Pereira HD, Soares-Costa A, et al. X-ray crystallography and NMR studies of domain-swapped canecystatin-1. FEBS J. 2013;280(4):1028-38. doi: 10.1111/febs.12095

25- Van der Mei HC, White DJ, Busscher HJ. On the wettability of soft tissues in the human oral cavity. Arch Oral Biol. 2004;49(8):671-3. doi: 10.1016/j.archoralbio.2004.03.002

26- Klüttermann S, Thellenkamp T, Bergmann N, Raab WH, Ritter H, Janda R. A new approach to influence contact angle and surface free energy of resin-based dental restorative materials. Acta Biomater. 2011;7(3):1160-5. doi: 10.1016/j.actbio.2010.10.002

27- Knorr SD, Combe EC, Wolff LF, Hodges JS. The surface free energy of dental gold-based materials. Dent Mater. 2005;21(3):272-7. doi: 10.1016/j.dental.2004.06.002.

28- van Oss CJ. Hydrophobicity of biosurfaces: origin, quantitative determination and interaction energies. Colloids Surf B Biointerfaces. 1995;5(3-4):91-110. doi: 10.1016/0927-7765(95)01217-7

29- Neves JG, Danelon M, Pessan JP, Figueiredo LR, Camargo ER, Delbem AC. Surface free energy of enamel treated with sodium hexametaphosphate, calcium and phosphate. Arch Oral Biol. 2018;90:108-12. doi: 10.1016/j.archoralbio.2018.03.008

30- Vogler EA. Structure and reactivity of water at biomaterial surfaces. Adv Colloid Interface Sci. 1998;74(1-3):69-117. doi: 10.1016/s0001-8686(97)00040-7

31- Olsson J. Plaque formation *in vivo* and bacterial attachment *in vitro* on permanently hydrophobic and hydrophilic surfaces. Caries Res. 1992;26(6):426-33. doi: 10.1159/000261482