UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

LETHYCIA ALMEIDA SANTOS

Effect of solutions and gels containing a sugarcane-derived cystatin on enamel and dentin erosive wear in vitro

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Dissertation presented to Bauru School of Dentistry, University of São Paulo, to obtain the degree of Master in Sciences in the Applied Dental Sciences Program, Oral Biology, Stomatology, Radiology and Imaginology concentration area.

Supervisor: Prof. Dr. Marília Afonso Rabelo Buzalaf

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"Renda-se, como eu me rendi. Mergulhe no que você não conhece como eu mergulhei. Não se preocupe em entender, viver ultrapassa qualquer entendimento."

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ABSTRACT

The protective effect of solutions and gels containing a sugarcane-derived cystatin (CaneCPI-5) on erosive wear of enamel and dentin was evaluated in vitro. Bovine enamel and dentin specimens were divided into two groups (n = 60 and 68 / group for enamel and dentin, respectively) that were treated with solutions or 3% chitosan-based gels containing 0.1 or 0.25 mg/ml CaneCPI-5. The positive controls for solutions and gels were Elmex Erosion Protection ™ solution (Colgate) and 12,300 ppm F 3% chitosan-based gel (as NaF), respectively. Negative controls were deionized water and 3% chitosan-based placebo gel, respectively. Stimulated saliva was collected from 3 donors to form the acquired pellicle (for 2 h) on the specimens. The specimens were submitted to an erosive pH cycling protocol 4 times/day for 7 days (0.1% citric acid pH 2.5/90s, artificial saliva/2h, artificial saliva overnight). The solutions and gels were applied during pH cycling, 2 times/day for 1 min and 4 min, respectively, after the first and last erosive challenges. Enamel and dentin wear (μm) were assessed by contact profilometry. Data were analyzed by 3-way ANOVA and Tukey´s test ($p < 0.05$). All the treatments significantly reduced enamel and dentin loss in comparison with placebo. For enamel, both CaneCPI-5 concentrations were similar to the positive controls, while for dentin, only 0.25 mg/ml CaneCPI-5 was similar to the positive controls. Regarding the vehicles, only the 0.1 mg/ml gel performed worse than the positive control. In conclusion, CaneCPI-5 reduces enamel and dentin wear to a similar extent as the fluoridated vehicles. Moreover, dentin seems to require higher CaneCPI-5 concentrations, especially in the case of gels. Solutions or chitosan-based gels containing CaneCPI-5 might be a new approach to protect against erosive tooth wear.

Keywords: Acquired pellicle; sugarcane; cystatin; erosive tooth wear; in vitro.

RESUMO

O efeito protetor de soluções e géis contendo uma cistatina derivada da cana-de-açúcar (CaneCPI-5) no desgaste dentário erosivo do esmalte e dentina foi avaliado in vitro. Espécimes de esmalte e dentina bovinos foram divididos em dois grupos (n = 60 e 68 / grupo para esmalte e dentina, respectivamente), os quais foram tratados com soluções ou géis à base de quitosana 3% contendo CaneCPI-5 nas concentrações de 0,1 ou 0,25 mg/ml. Os controles positivos para soluções e géis foram solução Elmex Erosion Protection ™ (contendo 800 ppm Sn^{+2} a partir de $SnCl_2$, 500 ppm F a partir de fluoreto de amina e fluoreto de sódio, pH 4,5, Colgate) e gel à base de quitosana 3% contendo 12.300 ppm F (como NaF), respectivamente. Os controles negativos foram água deionizada e gel à base de quitosana 3% placebo, respectivamente. As soluções foram aplicadas nas amostras por 1 min e os géis à base de quitosana 3% por 4 min. A saliva estimulada, utilizada para formar a película adquirida, foi coletada de 3 doadores e adicionada por 2 h nas amostras. No protocolo de ciclagem, os espécimes foram submetidos a uma ciclagem de pH erosiva 4 vezes / dia durante 7 dias (90 s em ácido cítrico 0,1% pH 2,5, 2 h em saliva artificial e saliva artificial durante a noite). As soluções e géis foram aplicados durante a ciclagem de pH, 2 vezes / dia por 1 min e 4 min, respectivamente, após o primeiro e o último desafios erosivos. O desgaste do esmalte e da dentina foi avaliado por meio da perfilometria de contato (µm). Os dados foram analisados por ANOVA a 3 critérios e teste de Tukey ($p < 0,05$). Todos os levaram a uma redução significativa da perda de esmalte e dentina em comparação ao placebo. Para o esmalte, ambas as concentrações de CaneCPI-5 foram semelhantes ao controle positivo, enquanto para a dentina, apenas CaneCPI-5 a 0,25 mg/ml foi semelhante ao controle positivo. Quanto aos veículos, apenas o gel contendo 0,1 mg/ml CaneCPI-5 teve um pior desempenho em comparação ao controle positivo. Em conclusão, a CaneCPI-5 reduz o desgaste do esmalte e da dentina em uma extensão semelhante à dos veículos fluoretados. Em adição, aparentemente a dentina necessita de concentrações mais altas de CaneCPI-5, principalmente no caso de géis. Soluções ou géis à base de quitosana contendo CaneCPI-5 podem ser um novo método para proteger contra o desgaste dentário erosivo.

Palavras-chave: Película adquirida; cana-de-açúcar; cistatina; desgaste dentário erosivo; in vitro.

TABLE OF CONTENTS

1 INTRODUCTION

1 INTRODUCTION

Erosive tooth wear (ETW) is characterized by the loss of dental hard tissue caused by acids of non-bacterial origin, which may come from the diet (extrinsic) (Barbour and Lussi 2014) or from the host's gastric content (intrinsic) (Moazzez and Bartlett 2014). The prevalence of ETW in the permanent teeth of children and adolescents is estimated at 30.4% (Salas, Nascimento, Huysmans, & Demarco, 2015). It must be considered that the teeth gradually wear out along time. Thus, if adequate preventive measures are not taken in time, the prevalence and severity of the condition tend to increase with age (Jaeggi and Lussi 2014), which can lead to tooth loss. Due to the high prevalence of ETW and its sequelae, prevention is very necessary.

Despite being an injury essentially caused by acids of non-bacterial origin, several factors, some related to the patient (eating habits, form of oral hygiene, presence of gastroesophageal reflux, frequent vomiting, use of certain medications and quality of saliva and acquired pellicle) and others associated to nutrition (type of acid, pK, pH, buffering capacity, concentration of calcium, phosphate and fluoride) are implicated in the etiology of ETW, which is also influenced by the educational level, behavior, health conditions and type of occupation of the patients (Lussi and Carvalho 2014).

Due to the multifactorial etiology of ETW, several preventive and therapeutic possibilities have been proposed for the control of these injuries. These strategies are, in the first instance, focused on the etiological factors (Buzalaf et al. 2018; Lussi and Hellwig 2014; Magalhaes et al. 2009b). Among the factors related to the patient, saliva is the most important, due to its buffering capacity, ability to supply calcium and phosphate ions to enhance the processes of de and remineralization, in addition to providing proteins that constitute the acquired enamel pellicle (AEP) (Buzalaf, Hannas, & Kato, 2012; Vukosavljevic, Custodio, Buzalaf, Hara and Siqueira, 2014).

The AEP acts as a mechanical barrier to prevent the direct contact of the acids with the dental surface, thus preventing erosive demineralization (Hara et al., 2006). Studies on the AEP ultrastructure have shown that even after severe erosive

attacks, its basal layer is not removed (Hannig, Berndt, Hoth-Hannig and Hannig, 2009). This suggests that some proteins in this layer may have a strong binding force to enamel. This prompted our group to identity these proteins, because once identified, enriching the basal layer of the AEP with these acid-resistant proteins could be a new approach to increase the resistance against ETW.

Using proteomics tools, we found that the Cystatin B resists to removal by citric and lactic acids (Delecrode et al., 2015), making this protein a natural candidate to be added in dental products to prevent ETW. Due to the high cost of the recombinant human cystatin, to continue our experiments, we cloned a cystatin from sugarcane and expressed this protein in a bacterial system. The product was named CaneCPI-5. Atomic force microscopy (AFM) revealed that CaneCPI-5 has a strong binding force to bovine enamel. Moreover, this protein was shown to reduce initial enamel erosion in vitro, being the best results obtained for the solution containing 0.1 mg/ml CaneCPI-5.

Recently, an in vivo proof-of-concept study showed that rinsing with a solution containing 0.1 mg/ml CaneCPI-5 for 1 minute increases the acid-resistant proteins in AEP, preventing initial erosion (Carvalho et al., 2020). However, the erosion models employed in the previous experiments (Santiago et al., 2017; Carvalho et al., 2020) only simulate the initial challenge that can be assessed by changes in surface hardness (Cheaib and Lussi, 2011) or calcium released from tooth enamel (Carvalho et al., 2020). Experiments with longer periods of erosive challenges are required before CaneCPI-5 can be universally used to protect against ETW. This is one of the objectives of the present study. We employed an in vitro pH cycling protocol to evaluate the ability of CaneCPI-5 to protect against ETW, using profilometry as response variable.

Moreover, in our previous studies (Carvalho et al., 2020; Santiago et al., 2017), CaneCPI-5 was included in solutions. In the present study, we compared the protective potential against ETW of CaneCPI-5 added in solutions, in two different concentrations, with that of the protein added to gels.

Thus, the aims of the present study were to evaluate the protective effect of solutions and gels containing different concentrations of a sugarcane-derived

cystatin (CaneCPI-5) on erosive tooth wear of enamel and dentin in vitro. The null hypothesis evaluated was that CaneCPI-5, regardless the concentration and the vehicle used, does not protect enamel and dentin against ETW.

2 ARTICLE

2 ARTICLE

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Solutions and gels containing a sugarcane-derived cystatin (CaneCPI-5) reduce enamel and dentin erosive wear in vitro

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Short Title: CaneCPI-5 gels and solutions protect against erosive wear

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Abstract

The effect of solutions and gels containing a sugarcane-derived cystatin (CaneCPI-5) on the protection against enamel and dentin erosive tooth wear erosion in vitro was evaluated. Bovine enamel and dentin specimens were divided into two groups (n=60 and 68/group for enamel and dentin, respectively) that were treated with solutions or gels containing 0.1 or 0.25 mg/ml CaneCPI-5. The positive controls (+) for solutions and gels were Elmex Erosion Protection™ solution (Colgate) and 12,300 ppm F (as NaF) 3% chitosan-based gel, respectively. Negative controls were deionized water and 3% chitosan-based placebo gel, respectively. The solutions were applied on the specimens for 1 min and the chitosan-based gels for 4 min. Stimulated saliva was collected from 3 donors to form the acquired pellicle (for 2 h) on the specimens. The specimens were submitted to an erosive pH cycling protocol 4 times/day for 7 days (0.1% citric acid pH 2.5/90s, artificial saliva/2h, artificial saliva overnight). The solutions and gels were applied during pH cycling, 2 times/day for 1 min and 4 min, respectively, after the first and last erosive challenges. Enamel and dentin wear (μm) were assessed by contact profilometry. Data were analyzed by 3-way ANOVA and Tukey´s test ($p < 0.05$). All the treatments significantly reduced enamel and dentin loss in comparison with placebo. For enamel, both CaneCPI-5 concentrations were similar to +, while for dentin, only 0.25 mg/ml CaneCPI-5 was similar to +. Regarding the vehicles, only the 0.1 mg/ml gel performed worse than +. CaneCPI-5 reduces enamel and dentin wear to a similar extent as the fluoridated vehicles. Moreover, dentin seems to require higher CaneCPI-5 concentrations, especially in the case of gels. Solutions or chitosan-based gels containing CaneCPI-5 might be a new approach to protect against erosive tooth wear.

Key words: Acquired pellicle; sugarcane; cystatin; erosive tooth wear; in vitro.

1 Introduction

Erosive tooth wear (ETW) is the loss of hard dental tissue having acids of non-bacterial origin as the primary etiological factor [Schlueter et al., 2020]. The prevalence of ETW on permanent teeth of children and adolescents is estimated at 30.4% [Salas et al., 2015]. Considering the high prevalence at younger ages and the progressive nature of the condition along time, preventive measures are required and can be achieved by fighting the etiological factors.

Despite the primary causative factors of ETW are non-bacterial acids, the condition is multifactorial and its progression in directed by an intricated interplay between nutritional and patient-related factors [Buzalaf et al., 2018; Lussi and Carvalho, 2014]. Among the patient-related factors, saliva is the most important one, due to its buffering capacity, ability to supply calcium and phosphate ions to drive the de- and remineralization processes, as well as to supply proteins that constitute the acquired enamel pellicle (AEP) [Buzalaf et al., 2012; Vukosavljevic et al., 2014]. The AEP functions as a mechanical barrier that avoids the direct contact of the acids with the tooth surface, thus protecting against erosive demineralization [Hara et al., 2006]. Studies on the ultrastructure of the AEP have shown that its basal layer is not removed, even after severe erosive challenges [Hannig et al., 2009], which implies that some proteins within this layer might have a strong binding force to enamel. This prompted us to find out the identity of these proteins, since, once identified, the enrichment of the basal layer of the AEP with these acid-resistant proteins could increase the resistance against ETW. Employing proteomic tools, we observed that cystatin-B is resistant to removal by citric and lactic acids [Delecrode et al., 2015], which turned this protein a natural candidate to be included in dental products to protect against ETW.

Due to the high cost of the human recombinant cystatin, to proceed with the experiments, we cloned a cystatin from sugarcane and expressed this protein in a bacterial system. The protein was named CaneCPI-5. It was shown to have a strong binding force to bovine enamel and to reduce initial enamel erosion in vitro [Santiago et al., 2017]. More recently, an in vivo proof-of-concept study showed that a 1-min rinse with a solution containing 0.1 mg/mL CaneCPI-5 increased acid-resistant proteins in the AEP, which protected against initial erosion [Carvalho et al., 2020]. However, the erosion models that we used in our previous experiment withs CaneCPI-5 only simulated initial challenges that could be evaluated by changes in surface hardness

[Cheaib and Lussi, 2011] or calcium released from enamel [Carvalho et al., 2020].

Experiments employing more prolonged erosive challenges are the next natural step in order that we can progress on the use of CaneCPI-5 to protect against ETW. This was one of the aims of the present study. We employed an in vitro pH cycling protocol to evaluate the ability of CaneCPI-5 to protect against ETW using profilometry as response variable. Moreover, in our previous studies [Carvalho et al., 2020; Santiago et al., 2017], CaneCPI-5 was included in solutions. In the present study, we compared the protective potential against ETW of CaneCPI-5 added in solutions, in two different concentrations, with that of the protein added to gels. Furthermore, so far the protective effect of CaneCPI-5 against ETW was only evaluated using enamel specimens as substrates so far. Dentin has a different composition, which might impact in the binding ability of CaneCPI-5. Thus, in the present study, dentin specimens were also tested. The null hypothesis evaluated was that CaneCPI-5, regardless the concentration and the vehicle used, does not protect enamel and dentin against ETW.

2 Material and methods

2.1 Heterologous expression of CaneCPI-5

The bacterial strain *E. coli* Rosetta, transformed with the plasmid pET28a, was used for the heterologous expression of CaneCPI-5. The expressed protein was purified from the soluble fraction of bacterial cultures induced by IPTG (Isopropyl-beta-D-Thiogalactoside), submitted to centrifugation and sonication. Purification was performed by affinity chromatography, using columns containing Ni-NTA Superflow nickel resin (Qiagen) [Santiago et al., 2017].

2.2 Ethical aspects and subjects

The project was approved by the Animal Research Ethics Committee under the protocol 008/2019 and by the Institutional Ethics Committee (protocol 14969919.0.0000.5417) of Bauru School of Dentistry, University of São Paulo.

Three volunteers (2 females, 1 male; 22-26 years of age) participated after signing an Informed Consent Form. They were non-smokers, had normal salivary flow (> 1 and 0.25 mL/min for stimulated and non-stimulated, respectively) and did not present risk factors for ETW, such as gastric disorders, high consumption of acidic fruits or fruit juices, soft drinks.

2.3 Enamel specimens and groups

The sample size calculation was based on a previous experiment by our group [Magalhaes et al., 2016]. For enamel, it was considered a minimal detectable difference in tissue loss of 1.86 µm and SD of 0.48 µm, and for dentin, a minimal detectable difference of 3.96 µm, and SD of 1.20 µm, considering an α error of 5% and a β error of 20%.

135 enamel blocks and 153 root dentin blocks (4x4x3 mm) were prepared from the buccal surface of bovine incisors (Fig 1a, b, c). For this, a 4-mm thick spacer was placed between two diamond disks (Extec, Enfield, USA) that were coupled to an ISOMET low-speed saw (Buehler, Lake Bluff, USA). The blocks samples were polished using 320, 600 and 1200 grit sandpapers (Buehler, Lake Bluff, IL, USA) and at the end with felt paper moistened with diamond spray (Fig 1d and e). For cleaning purposes, the blocks were immersed in an ultrasonic bath filled with deionized water.

The samples received a mark in the control area using a drill to facilitate the location of the first profile reading (baseline). In addition, two lines were produced using a scalpel on the dental surface to separate the eroded area from the control area, thus allowing the comparison of the baseline and final profiles. The baseline profile (Fig 1f) was then measured as described below, and the control areas were protected with colored nail polish (Risqué, Taboão da Serra, Brazil) (Fig 1g) [Magalhaes et al., 2016].

The blocks were divided into 2 groups. In the first group (Fig 1h), they were further divided into 4 subgroups (n=15/group for enamel and 17/group for dentin) that differed according to the treatment solutions, as follows: negative control (placebo, deionized water), positive control (Elmex Erosion Protection™ mouthwash, containing 800 ppm Sn^{+2} from $SnCl_2$, 500 ppm F from amine fluoride and NaF, pH 4.5, Colgate) and experimental solutions containing 0.1 or 0.25 mg/ml CaneCPI-5. The solutions containing CaneCPI-5 were prepared with deionized water (native pH), without additives.

In the second group (Fig 1i), the blocks were divided into 5 subgroups (n=15/group for enamel and 17/group for dentin), according to the treatment gels, as follows: negative control (placebo gel), positive control (gel containing 12,300 ppm F as NaF), experimental gels 0.1 or 0.25 mg/ml CaneCPI-5. In one additional group, specimens remained untreated, as an additional control for the chitosan gel. All gels had the same 3% chitosan-based composition.

2.4 Preparation of the gels

Firstly, chitosan (75% deacetylation, medium molecular weight, Sigma-Aldrich, MO, USA) was dissolved in 1% acetic acid (Synth, Diadema, SP, Brazil), in a concentration of 30 mg of chitosan for 1 mL of 1% acetic acid. The mixture was homogenized for 2 hours, at room temperature. For the gel formulations containing NaF and CaneCPI-5, these actives were incorporated during the chitosan dissolution. The mixture was homogenized for 2 hours at room temperature and stored at 4 °C.

2.5 Total saliva collection

Total saliva was collected from 3 healthy donors, between 9 and 11 am, under chewing stimulus (Parafilm) (Fig 1j). The samples were centrifuged at 14,000 g for 20 minutes at 4ºC. The supernatants were collected to form a pool of saliva, which was stored in the freezer at -80ºC for use in the experiment.

2.6 Treatment and pH cycling

The solutions (25 µl/specimen) were applied on the specimens with a pipette, for 1 minute, at 37°C, under agitation. The gels (20 µl/specimen) were applied with microbrush for 4 minutes, at 37°C. Then the specimens were incubated with a pool of human saliva (300 µl/) for 2 hours at 37°C, to allow the formation of the AEP [Cheaib and Lussi, 2011], only on the first day of treatment (Fig 1k).

After the formation of the AEP, the specimens were subjected to erosive pH cycling 4 times a day, for 7 days [Magalhaes et al., 2008]. Each cycle consisted of: immersing the specimens in 0.1% citric acid pH 2.5 for 90 seconds (30 ml/specimen) at 25ºC, washing in deionized water for 5 s, rehardening by immersion in artificial saliva [Klimek et al., 1982] for 2 h (pH 6.8, 30 ml/specimen) and washing with deionized water for 5 s (Fig 1l). The solutions (25 µl/specimen) or gels (20 µl/specimen) were applied during pH cycling, twice a day for 1 min or 4 min, respectively after the first and last erosive challenge each day (Fig 1m). The specimens were immersed in artificial saliva overnight, completing every 24 h of the cycle. The loss of enamel was assessed using contact profilometry after 7 days of pH cycling (Fig 1n).

2.7 Contact profilometry

Profiles of the enamel and dentin surfaces were obtained with a contact profilometer (MahrPerthometer, Göttingen, Germany), before (baseline) and after the

experimental period. The control areas (protected with cosmetic nail polish) were marked with a scalpel blade, to allow the exact positioning of the cosmetic nail polish. Additionally, the samples had a mark (small cavitation) made with a 1/4 spherical drill, to allow the exact positioning of the tip of the profilometer in the 1st scan of each reading. At each reading, five scans (3 mm in length) were performed in the center of the sample surface at 250 µm each. To determine the alteration of the sample surface profile, after the experimental phase, the cosmetic nail polish was removed with an acetone solution (1: 1 - acetone: water), and 5 final readings were taken in the same areas as the initial readings. To enable the correct repositioning of the samples during the readings, we use a device by which we standardize the position of the samples on the x, y and z axes. Initial and final profiles were performed and compared using the MarhSurf XCR20 software (Fig 1o). The average wear for each sample was calculated (µm).

2.8 Statistical analysis

The softwares Statistica 10.0 and GraphPad InStat version 3.0 for Windows (GraphPad Software Inc., La Jolla, Ca, USA) were used.

Data were analyzed by Three-way ANOVA and Tukey´s multiple comparison test.

In addition, since in the case of the gels we had an additional group consisting of specimens that were not treated, the groups treated with gels were also additionally analyzed by One-way ANOVA (after logarithmic transformation) and Tukey´s test, in the case of enamel, and Kruskal-Wallis and Dunn´s test, in the case of dentin, after checking for normality (Komogorov-Smirnov test) and homogeneity (Bartlett test).

In all cases, the level of significance was set at 5%.

3 Results

Mean (±SD) enamel and dentin losses after treatment with the different solutions and gels and erosive pH-cycling are shown in Table 1. Three-way ANOVA found significant difference between the substrates ($F=47.398$, $p<0.0001$), among the treatments ($F=473.832$, $p<0.0001$) and between the vehicles ($F=29.101$, $p<0.0001$). There was a significant interaction between substrates X treatments ($F=9.572$, $p<0.0001$; Table 2) and between vehicles X treatments ($F=22.123$, $p<0.0001$; Table3), but not between substrates and vehicles ($F=1.310$, $p=0.254$). Regardless the

substrates and vehicles, all treatments significantly reduced erosive loss in comparison with placebo ($p<0.05$) (Tables 1 and 2). For the interaction between substrates X treatments (Table 2), 0.1 mg/mL CaneCPI-5 when applied on dentin led to significantly higher erosive loss in comparison to all the other treatments, which did not significantly differ from each other. As for the interaction between vehicles X treatments (Table 3), the gel containing 0.1 mg/mL CaneCPI-5 led to the highest erosive loss compared to the other treatments but did not significantly differ from the solutions containing 0.1 and 0.25 mg/mL CaneCPI-5. All treatments, except the gel containing 0.1 mg/mL CaneCPI-5 had a similar performance, leading to the lowest degree of erosive loss.

In the case of the gels, for enamel, One-way ANOVA found a significant difference among the groups ($F=361.6$, $p<0.0001$). Tukey´s test revealed significant differences among all the groups, except for the groups treated with 1.23% F and 0.1 mg/mL CaneCPI-5 that presented the lowest enamel loss and differed from all the other groups. The group that remained untreated presented the highest enamel loss that was significantly different from the placebo group. The group treated with 0.25 mg/mL CaneCPI-5 presented enamel loss significantly lower than placebo untreated groups, but significantly higher than fluoride and 0.1 mg/ml CaneCPI-5 groups (Fig. 1).

For dentin, in the case of gels, Kruskal-Wallis test found a significant difference among the groups ($KW = 68.964$, $p<0.0001$). The highest dentin losses were found for the group that remained untreated and placebo, which did not significantly differ from each other but differed from all the other groups. The group treated with 0.1 mg/mL CaneCPI-5 had significantly higher dentin loss when compared with the groups treated with 0.25 mg/mL CaneCPI-5 and 1.23%F that did not significantly differ from each other (Fig. 2).

4 Discussion

Acquired pellicle engineering with proteins that bind to hydroxyapatite and are not removed upon acidic challenges is a recently suggested approach to prevent erosive demineralization. Rinsing with solutions containing CaneCPI-5, StN15 (statherin-derived peptide) or hemoglobin were able, in a proof-of-concept in vivo study, to reduce initial enamel erosion provoked by a 10 s challenge with 1% citric acid (pH 2.5) [Carvalho et al., 2020]. However, despite being conducted in vivo, the study by Carvalho et al. [Carvalho et al., 2020] employed a very mild erosive challenge. To add more evidence on the feasibility of the addition of CaneCPI-5 in dental products to

protect against erosive demineralization, it is necessary to evaluate the protective potential of this protein upon more prolonged erosive challenges, which was one of the aims of the present study. For this purpose, we employed a well-established 7-day pH cycling protocol using 0.1% citric acid (pH 2.5) comprising in total 42 min of erosive challenge [Magalhaes et al., 2008]. As positive control we employed a commercial fluoridated solution also containing tin. We chose this solution because the degree of protection conferred by conventional fluorides against erosive demineralization is limited. Currently, the best evidence for effectiveness is seen for the combination of fluoride and tin [Huysmans et al., 2014; Lussi et al., 2019]. The experimental solutions evaluated, regardless the concentration of CaneCPI-5, significantly protected enamel against erosive demineralization, to the same extent as the positive control.

Regarding the concentrations of CaneCPI-5 tested, the solution containing the lower concentration (0.1 mg//ml) has been shown to be effective to reduce initial enamel erosion in our previous in vitro [Santiago et al., 2017] and in vivo [Carvalho et al., 2020] studies. In the present study, we decided to also evaluate a higher concentration of CaneCPI-5 (0.25 mg/ml), because due to the prolonged nature of the erosive challenge, the lower concentration employed in the previous studies could not be enough to provide effective protection. However, this was not the case, since the degree of protection conferred to enamel by both concentrations was virtually the same.

A second aim of the present study was to evaluate another vehicle of application of CaneCPI-5 besides solution. The vehicle chosen was a 3% chitosan-based gel. Chitosan is a linear, semi-crystalline, positively charged polysaccharide composed of *N*-acetyl-*D*-glucosamine and *D*-glucosamine, derived from partial deacetylation of chitin [Younes and Rinaudo, 2015]. Chitosan adsorbs to hydroxyapatite and prevents erosion [Lee et al., 2012]. Moreover, chitosan can interact with proteins, such as albumin [Bekale et al., 2015]. For these reasons, we decided to include CaneCPI-5 in a chitosan gel. Considering that chitosan itself when adsorbed onto hydroxyapatite can reduce erosion [Lee et al., 2012], we also included a group that was not treated. For enamel, this group had significantly higher wear when compared with the placebo gel, which could reflect the protection conferred by chitosan (Fig. 1). Regarding the concentrations of CaneCPI-5 in the gels, when One-way ANOVA was performed, the higher concentration led to significantly higher enamel wear. At first glance, this could seem contradictory. However, the probable reason for

the worse performance of the gel containing the higher CaneCPI-5 concentration is protein dimerization. Structural analysis of CaneCPI-1, another sugarcane cystatin, reported protein dimerization through domain swapping, leading to the formation of dimers and even tetramers [Valadares et al., 2013]. This reduces the amounts of free protein to bind to enamel and protect against erosive demineralization. The fact that the lower concentration of the gel containing CaneCPI-5 provided better enamel protection is interesting from the commercial point of view, since the necessity of including lower amounts of protein makes the product cheaper.

On the other hand, for dentin, the opposite was found, i.e., the gel containing the higher CaneCPI-5 concentration protected against erosive demineralization to the same extent as the positive control (1.23% F gel) and performed significantly better than the 0.1 mg/ml CanCPI-5 gel. In fact, for dentin, three-way ANOVA showed that despite providing significant protection in comparison with placebo, treatment with 0.1 mg/ml CaneCPI-5 (regardless the vehicle) conferred lower protection in comparison to the other treatments. These data suggest that dentin requires higher concentrations of CaneCPI-5 to achieve the same degree of protection as enamel (Table 2). Since this is the first study evaluating the use of CaneCPI-5 to protect against dentin erosive demineralization, future studies employing protocols that more closely resemble the clinical condition, must be conducted to confirm these findings.

Another interesting point to be discussed is the comparison between the different vehicles. Analysis of table 3 indicates that solutions might be more appropriate vehicles, since regardless the concentration of CaneCPI-5, they were able to protect against erosive loss to the same extent as the positive controls. In the case of gels, this was the case only for the higher concentration of CaneCPI-5.

According to our results, the null hypothesis was rejected, since CaneCPI-5, at both concentrations tested and regardless the vehicle evaluated, significantly reduced enamel and dentin erosive wear in comparison with placebo. In addition, dentin requires higher concentrations of CaneCPI-5 to achieve the same degree of protection as enamel, especially in the case of gels. Moreover, solutions seem to provide better protection than gels, since they can protect to the same extent as the positive control (F), regardless of the concentration of CaneCPI-5. Preferably solutions, but also chitosan-based gels containing CaneCPI-5 might be a new approach to protect enamel and dentin against erosive wear.

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Authors contributions

Conceived and designed the experiments: MB, FS, AS, CC; Acquired data: LA, TM, FR, AO, GC, FL, PS, JP; Analyzed and interpreted data: FL, LA, HH, MB; Drafted the manuscript: LA, MB, FS. Critically revised the manuscript: AS, CC, FL; Approved the final version to be submitted: All the authors.

Conflict of interest statement: University of São Paulo and São Carlos Federal University hold a patent request at INPI (Brazil) entitled “Sugarcane derived cystatin to protect against dental caries and dental erosion”

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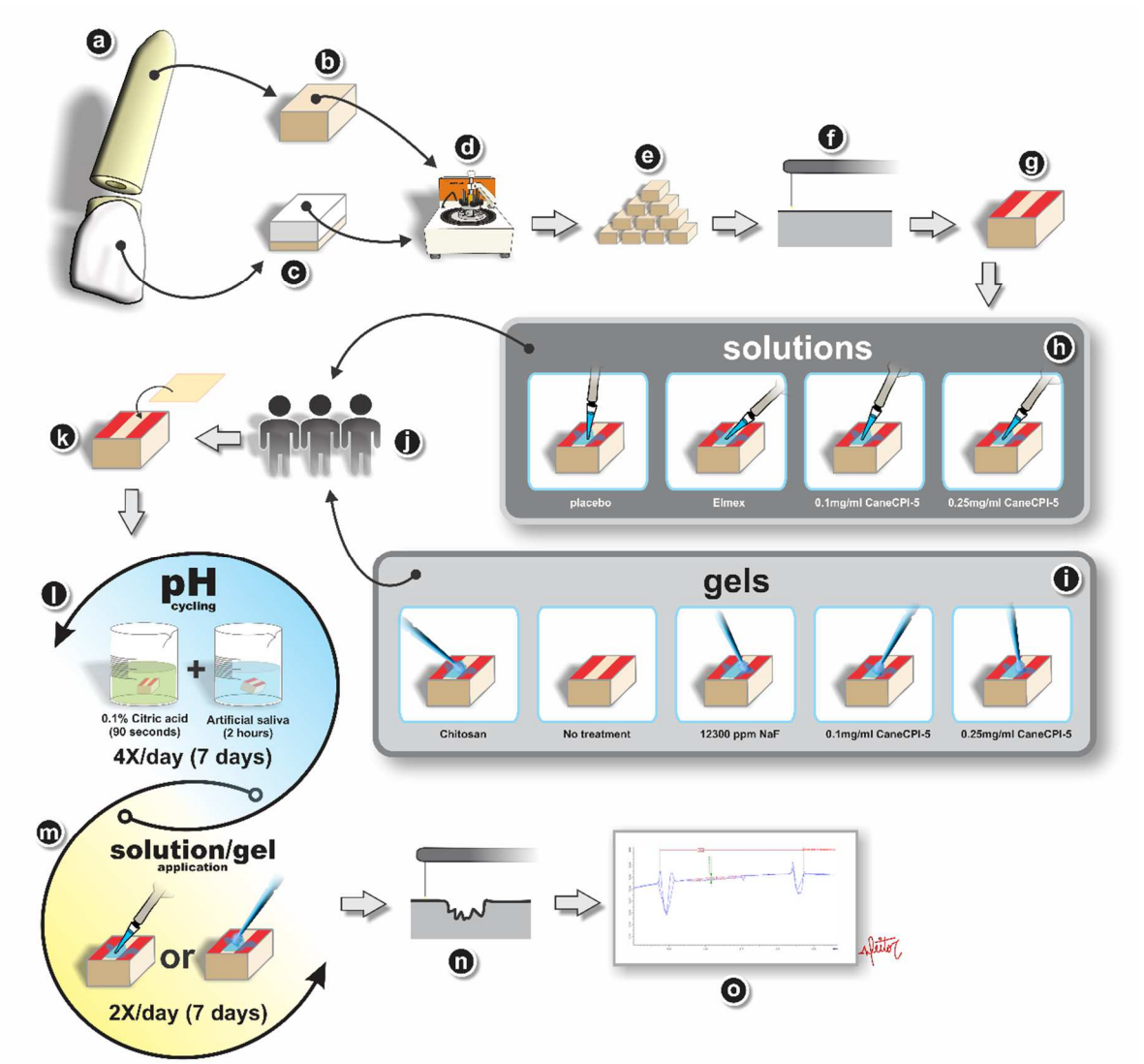

Figure 1. Flowchart displaying the experimental design of the study.

Figure 2. Mean enamel loss after treatment of specimens with chitosan gels containing fluoride, different concentrations of a sugarcane cystatin (CaneCPI-5) or not (placebo) for 4 minutes, followed by formation of acquired pellicle for 2 hours (pooled human saliva) and subsequent erosive challenges (0.1% citric acid pH 2.5 for 90 s) 4 times/day for 7 days. In NT group specimens remained untreated. Data were analyzed by ANOVA (after log transform) and Tukey´s test ($p<0.05$). Distinct letters denote significant differences among the groups. n=15. Bars indicate SDs.

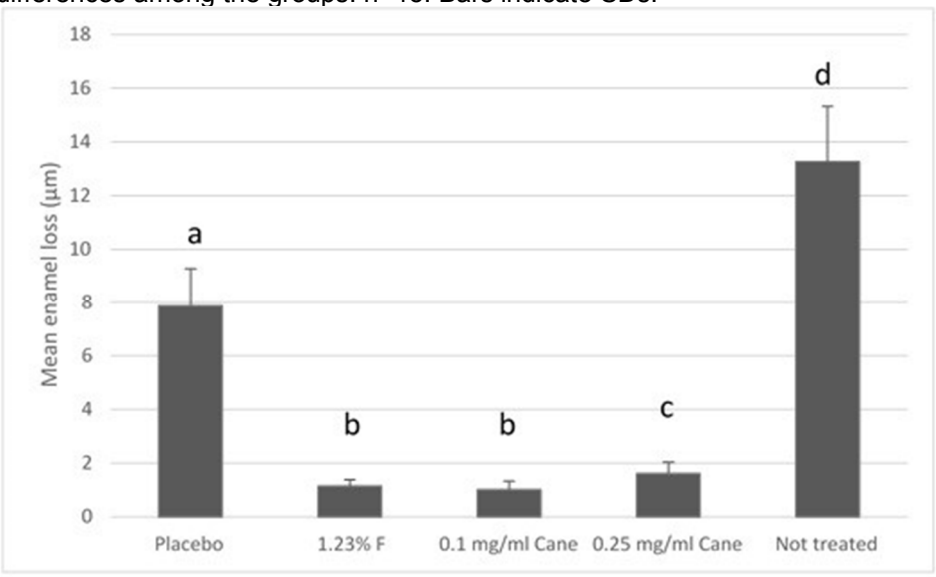

Figure 3. Dentin loss after treatment of specimens with chitosan gels containing fluoride, different concentrations of a sugarcane cystatin (CaneCPI-5) or not (placebo) for 4 minutes, followed by formation of acquired pellicle for 2 hours (pooled human saliva) and subsequent erosive challenges (0.1% citric acid pH 2.5 for 90 s) 4 times/day for 7 days. In NT group specimens remained untreated. Data were analyzed by Kruskal-Wallis and Dunn´s tests ($p<0.05$). Distinct letters denote significant differences among the groups. n=17.

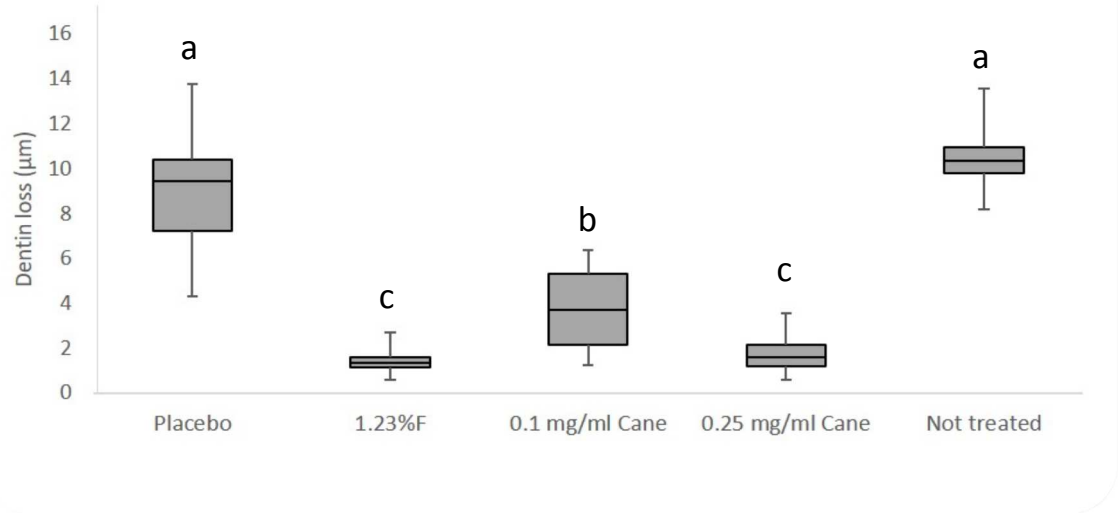

Table 1. Mean (±SD) enamel and dentin losses (µm) after treatment with solutions or chitosan gels containing fluoride, different concentrations of a sugarcane cystatin (CaneCPI-5) or not (placebo) for 1 minute (solutions) or 4 minutes (gels), followed by formation of acquired pellicle for 2 hours (pooled human saliva) and subsequent erosive challenges (0.1% citric acid pH 2.5 for 90 s) 4 times/day for 7 days.

Substrates	Vehicles	Treatments	Erosive loss (µm)
Enamel	Solution	Placebo	5.56 ± 0.89
Enamel	Solution	800 ppm Sn^{+2} + 500 ppm F^{-}	1.17 ± 0.35
Enamel	Solution	0.1 mg/ml CaneCPI-5	1.19 ± 0.31
Enamel	Solution	0.25 mg/ml CaneCPI-5	1.45 ± 0.57
Enamel	Gel	Placebo	7.88 ± 1.40
Enamel	Gel	1.23% F	1.15 ± 0.23
Enamel	Gel	0.1 mg/ml CaneCPI-5	1.01 ± 0.32
Enamel	Gel	0.25 mg/ml CaneCPI-5	1.63 ± 0.41
Dentin	Solution	Placebo	6.74 ± 1.32
Dentin	Solution	1.23% F	1.36 ± 0.48
Dentin	Solution	0.1 mg/ml CaneCPI-5	2.35 ± 0.57
Dentin	Solution	0.25 mg/m CaneCPI-5	2.03 ± 1.04
Dentin	Gel	Placebo	9.55 ± 2.54
Dentin	Gel	800 ppm Sn^{+2} + 500 ppm F^{-}	1.39 ± 0.34
Dentin	Gel	0.1 mg/ml CaneCPI-5	3.57 ± 1.49
Dentin	Gel	0.25 mg/ml CaneCPI-5	1.50 ± 0.59

Treatments were applied twice/day, after the first and last erosive challenges.

Table 2. Mean (±SD) enamel and dentin losses (µm) after treatment with solutions or chitosan gels containing fluoride, different concentrations of a sugarcane cystatin (CaneCPI-5) or not (placebo) for 1 minute (solutions) or 4 minutes (gels), followed by formation of acquired pellicle for 2 hours (pooled human saliva) and subsequent erosive challenges (0.1% citric acid pH 2.5 for 90 s) 4 times/day for 7 days.

Substrates	Treatments	Mean
Dentin	0.1 mg/ml CaneCPI-5	3.03^{b}
Dentin	0.25 mg/ml CaneCPI-5	1.74^{a}
Dentin	F	1.37^{a}
Dentin	Placebo	8.20^{d}
Enamel	0.1 mg/ml CaneCPI-5	1.10^{a}
Enamel	0.25 mg/ml CaneCPI-5	1.54^{a}
Enamel	F	1.58^{a}
Enamel	Placebo	6.72^{c}

Treatments were applied twice/day, after the first and last erosive challenges. Data were analyzed by three-way ANOVA and Tukey´s test. Interaction between substrates *versus* treatments ((p<0.05). n=15 for enamel and n=17 for dentin. Means followed by different letters are significantly different.

Table 3. Mean (±SD) enamel and dentin losses (µm) after treatment with solutions or chitosan gels containing fluoride, different concentrations of a sugarcane cystatin (CaneCPI-5) or not (placebo) for 1 minute (solutions) or 4 minutes (gels), followed by formation of acquired pellicle for 2 hours (pooled human saliva) and subsequent erosive challenges (0.1% citric acid pH 2.5 for 90 s) 4 times/day for 7 days.

Vehicles	Treatments	Mean
Gel	0.1 mg/mL CaneCPI-5	2.37^{b}
Gel	0.25 mg/mL CaneCPI-5	1.56^{a}
Gel	1.23% F	1.27^{a}
Gel	Placebo	8.71^{d}
Solution	0.1 mg/mL CaneCPI-5	1.77^{ab}
Solution	0.25 mg/mL CaneCPI-5	1.74^{ab}
Solution	800 ppm Sn^{+2} + 500 ppm F^{-}	1.26^{a}
Solution	Placebo	6.13^{c}

Treatments were applied twice/day, after the first and last erosive challenges. Data were analyzed by three-way ANOVA and Tukey´s test. Interaction between vehicles *versus* treatments ((p<0.05). n=15 for enamel and n=17 for dentin. Means followed by different letters are significantly different.

3 DISCUSSION

3 DISCUSSION

Among the strategies currently available to protect against erosive demineralization, fluorides are still the most studied [Lussi et al., 2019]. However, the protective ability of conventional fluorides in ETW is much lower than their widely known preventive capacity against caries [Ten Cate and Buzalaf, 2019], with the best results shown for polyvalent metal fluorides such as titanium tetrafluoride and SnF_2 [Huysmans et al., 2014].

Considering the limitations of fluorides to protect against ETW, our group recently proposed a new strategy, based on organic compounds. This strategy, based on concepts of “acquired pellicle engineering”, consists in increasing the amount of acid-resistant proteins in the basal layer of the AEP. The present study fits into this concept. The protein of choice was CaneCPI-5, a sugarcane-derived cystatin that is expressed in *E. coli*, first cloned in 2017 [Santiago et al., 2017]. This innovative protein demonstrated several advantageous features, among them a strong binding force to hydroxyapatite and inhibition of initial enamel erosion *in vitro* [Santiago et al., 2017]. Furthermore, in an *in vivo* proof-of-concept study, rinsing with CaneCPI-5 before the formation of the AEP increased several acid-resistant proteins in the AEP and reduced initial enamel erosion [Carvalho et al., 2020b].

Despite CaneCPI-5 showed promising results in the studies by Santiago et al. [Santiago et al., 2017] and Carvalho et al. [Carvalho et al., 2020a], the conditions employed in those studies involved very mild erosive challenges. In the study by Santiago et al. [Santiago et al., 2017], the erosive challenges consisted of 3 x 1 min 0.65% citric acid pH 3.5, while in the study by Carvalho et al. [Carvalho et al., 2020a], there was only one challenge with 1% citric acid pH 2.5 for 10 s. Moreover, in the Santiago et al [Santiago et al., 2017] study, the CaneCPI-5 solution was incubated with the enamel specimens for 2 h, which is unrealistic.

In order to increase the evidence on the feasibility of adding CaneCPI-5 to dental products to prevent erosive demineralization, in the present study we evaluated the protective potential of CaneCPI-5 against long-term erosive challenges. For this, we used a established 7-day pH cycling protocol employing 0.1% citric acid (pH 2.5),

comprising in total 42 min of erosive challenge [Magalhaes et al., 2008]. As positive control we employed a commercial fluoridated solution also containing tin (Elmex Erosion Protection™). We chose this solution because, as mentioned above, the degree of protection conferred by conventional fluorides against erosive demineralization is limited. Currently, the best evidence for effectiveness is seen for the combination of fluoride and tin [Huysmans et al., 2014; Lussi et al., 2019]. The experimental solutions evaluated, regardless the concentration of CaneCPI-5, significantly protected enamel against erosive demineralization, to the same extent as the positive control.

An important aspect to be highlighted in the experimental procedure is the application of CaneCPI-5 on the dental surfaces before the formation of the AEP. This procedure is part of the concept of “AEP engineering”, which has the aim of redirecting the formation of this integument, increasing the amount of acid-resistant proteins, to improve its protective potential against enamel demineralization. This is important because CaneCPI-5 presents a high binding force to hydroxyapatite, as evaluated by AFM [Santiago et al., 2017]. This allows its direct interaction with the tooth surface, leading to the formation of a basal layer of the AEP containing high amounts of CaneCPI-5. Furthermore, the treatment after the first and last erosive challenge may enhance the quality of the already formed AEP, which may be an appropriate strategy for patients with high risk for ETW.

As for the CaneCPI-5 concentrations evaluated, the solution containing the lower concentration (0.1 mg/ml) was effective to reduce initial enamel erosion in previous in vitro [Santiago et al., 2017] and in vivo [Carvalho et al., 2020] studies by our group. In the present study, we also tested a higher concentration of CaneCPI-5 (0.25 mg/ml), because due to the prolonged nature of the erosive challenge, the lower concentration employed in the previous studies could not be enough to provide effective protection. However, this was not the case, since the degree of protection conferred to enamel by both concentrations was the same.

The second objective of this study was to test another vehicle suitable for CaneCPI-5 appliaction, in addition to the solution. The vehicle of choice wass a chitosan-based gel. Chitosan is a semicrystalline, linear, positively charged polysaccharide derived from partial deacetylation of chitin and composed of N-acetyl-

D-glucosamine and D-glucosamine [Younes and Rinaudo, 2015]. It adsorbs to hydroxyapatite and prevents erosion [Lee et al., 2012]. In addition, chitosan can also interact with proteins, such as albumin [Bekale et al., 2015]. For these reasons, we decided to add CaneCPI-5 in a chitosan gel. Considering that chitosan itself when adsorbed onto hydroxyapatite can reduce erosion [Lee et al., 2012], we also included an untreated group. For enamel, compared to placebo gel, wear in this group was significantly greater, which may reflect the protective effect provided by chitosan (Figure 1).

Regarding the concentrations of CaneCPI-5 in the gels, when one-way ANOVA was performed, it was observed that the higher concentration caused greater enamel wear. At first glance, this seems contradictory. However, the possible reason for the worst performance of the gel containing the highest concentration of CaneCPI-5 might be protein dimerization. Structural analysis of CaneCPI-1, another sugarcane cystatin, revealed that protein dimerize through domain swapping, leading to the formation of dimers and even tetramers [Valadares et al., 2013]. This reduces the amount of free protein attached to enamel that could prevent erosive demineralization. From a commercial point of view, the fact that gels with lower concentration of CaneCPI-5 can provide better enamel protection is interesting, since the need to contain less protein makes the product cheaper.

In contrast, for dentin, the opposite was found, that is, the gel containing the highest concentration of CaneCPI-5 can prevent erosive demineralization in the same extent as the positive control (1.23% F gel), and its performance is significantly better than the 0.1 mg/ml CaneCPI-5 gel. In fact, for dentin, the 3-way ANOVA showed that, while providing significant protection compared to placebo, treatment with 0.1 mg/ml CaneCPI-5 (regardless of vehicle) provided less protection compared to the other treatments. These data indicate that dentin requires a higher concentration of CaneCPI-5 to achieve the same degree of protection as enamel (Table 2). As this is the first study to evaluate the use of CaneCPI-5 to prevent erosive dentin demineralization, further studies should be conducted using a protocol more similar to the clinical situation to confirm these findings.

Another interesting issue to discuss is the comparison between the different vehicles. The analysis of Table 3 indicates that solutions may be more suitable vehicles, as they can prevent erosive loss to the same extent as the positive control regardless of the concentration of CaneCPI-5. Regarding the gels, this was the case only for the higher CaneCPI-5 concentration.

Based on the results of our study, the null hypothesis was rejected because CaneCPI-5 significantly reduced the erosive wear of enamel and dentin compared to placebo, regardless of the concentrations tested and regardless of the vehicle assessed. Furthermore, dentin requires a higher concentration of CaneCPI-5 to achieve the same degree of protection as enamel, especially in the case of gels. In addition, regardless of the concentration of CaneCPI-5, the solutions appear to provide better protection than the gels, as they can protect to the same extent as the positive control (F). Therefore, solutions or gels containing CaneCPI-5 can be a new approach to protect enamel and dentin against erosive tooth wear.

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ANNEX

ANNEX 1

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Uso de Animais

Uso exclusivo da CEUA/FOB/USP
Reg. Nº **008/2019**
Recebido em: 25/04/2019
Maristela Petenuci Ferrari
Secretária da CEUA – SRTE 53052

PROTOCOLO DE RECEBIMENTO DO FORMULÁRIO PARA REGISTRO DE PROTOCOLOS EFETUADOS COM CADÁVERES, OU PARTE DELES, EM ENSINO E/OU PESQUISA

Finalidade:	Pesquisa
Período:	Jun/2019 a Jun/2020
Título da pesquisa:	Efeito de solução e gel contendo uma nova Cistatina derivada da cana-de-açúcar na erosão do esmalte e dentina in vitro
Pesquisador Responsável:	Profa. Dra. Marília Afonso Rabelo Buzalaf
Pesquisador Executor:	Lethycia Almeida Santos
Colaboradores:	Flavia Mauad Levy Abrahão, Vinícius Taioqui Pelá, Flávio Henrique da Silva
Nota Fiscal/Termo de Doação	NF 000.024.679 - José Cláudio Mozardo EPP **Total adquirido:** 500 (doados 66)
Nº Lote / Data do Abate	01, 02, 03 e 04 / 29/09/2018
Nº utilizados / Nº de grupos:	4 (120 blocos de esmalte bovino e 144 blocos de dentina radicular)

ANNEX 2

USP - FACULDADE DE ODONTOLOGIA DE BAURU DA USP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: EFEITO DE SOLUÇÃO E GEL CONTENDO UMA NOVA CISTATINA DERIVADA DA CANA-DE-AÇÚCAR NA EROSÃO DO ESMALTE E DENTINA IN VITRO.

Pesquisador: LETHYCIA ALMEIDA SANTOS

Área Temática:

Versão: 2

CAAE: 14969919.0.0000.5417

Instituição Proponente: Universidade de Sao Paulo

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.518.613

Apresentação do Projeto:

O presente estudo irá avaliar o efeito de soluções ou géis contendo CaneCPI-5, em diferentes concentrações, na proteção contra a erosão do esmalte e dentina in vitro.

Objetivo da Pesquisa:

Objetivo Primário: