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

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**Short Title:** CaneCPI-5 gels and solutions protect against erosion

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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 erosion in vitro was evaluated. Bovine enamel and dentin specimens were divided into two groups (n=135 and 153/group for enamel and dentin, respectively) that were treated with solutions or chitosan gels containing 0.1 or 0.25 mg/ml CaneCPI-5. The positive controls for solutions and gels were Elmex Erosion Protection™ solution and NaF gel (12,300 ppm F), respectively. Deionized water and chitosan gel served as controls, respectively. The solutions were first applied on the specimens for 1 min and the gels for 4 min. Stimulated saliva was collected from 3 donors and used to form a 2 h acquired pellicle on the specimens. Then, 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 again during pH cycling, 2 times/day for 1 min and 4 min, respectively, after the first and last erosive challenges. Enamel and dentin losses (μm) were assessed by contact profilometry. Data were analyzed by 2-way ANOVA and Tukey´s test ($p < 0.05$). All the treatments significantly reduced enamel and dentin loss in comparison with controls. Both CaneCPI-5 concentrations had a similar protective effect against enamel erosion, but only the higher concentration was as effective against dentin erosion as the positive control. Regarding the vehicles, only the 0.1 mg/ml gel performed worse than the positive control for dentin. CaneCPI-5 reduced enamel and dentin erosion to a similar extent as the fluoride-containing vehicles. However, dentin requires higher CaneCPI-5 concentrations, in the case of gels. Solutions or gels containing CaneCPI-5 might be a new approach to protect against dental erosion.

## Introduction

The prevalence of erosive tooth wear on permanent teeth of children and adolescents is around 30% [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 targeting the etiological factors.

Despite the primary causative factors of erosive tooth wear are non-bacterial acids, the condition is multifactorial and its progression is directed by a complex 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 remineralize the teeth, 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 helps prevent 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. With this in mind, in a previous study, our research group attempted to identify these proteins, since, once identified, the enrichment of the basal layer of the AEP with them could increase the resistance against dental erosion. Employing proteomic tools, we found cystatin-B as a protein 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 erosion.

Due to the high cost of the human recombinant cystatin, to proceed with the experiments, our research group cloned a cystatin from sugarcane and expressed this protein in a bacterial system. The protein, named CaneCPI-5, was shown to strongly bind 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 with CaneCPI-5 only simulated initial challenges that could be evaluated by changes in surface hardness [Santiago et al., 2017] or calcium released from enamel [Carvalho et al., 2020]. Experiments employing more prolonged erosive challenges are the next natural step to progress on the use of CaneCPI-

5 to protect against erosion. This was one of the aims of the present study. Here, we employ an in vitro pH cycling protocol to evaluate the ability of CaneCPI-5 to protect against erosion using profilometry as response variable. Moreover, in the previous studies by our research group [Carvalho et al., 2020; Santiago et al., 2017], CaneCPI-5 was included in solutions. In previous studies, the inclusion of protease inhibitors in gel formulations [Kato et al., 2010] provided better protection against dentin erosion in comparison to their inclusion in solutions [Magalhaes et al., 2009], which could be due to the prolonged contact time with the tooth surface due to the viscosity of the gels. The same could be the case for CaneCPI-5. In the present study, we compare the protective potential against erosion 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 erosion was only evaluated using enamel specimens as substrates. 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 erosion.

## Material and methods

### *Enamel specimens and groups*

A total of 135 enamel blocks and 153 root dentin blocks (4x4x3 mm) were prepared from the buccal surface of bovine incisors. 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, USA) and at the end with felt paper moistened with diamond spray. 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 was then measured as described below, and the control areas were protected with colored nail polish (Risqué, Taboão da Serra, Brazil) [Magalhaes et al., 2016].

The blocks were randomly allocated for 2 distinct experiments. In the first experiment, they were further assigned to 4 groups (n=15/group for enamel and 17/group for dentin) that differed according to the treatment solutions, as follows: control (deionized water), positive control (Elmex Erosion Protection™ mouthwash, GABA GmbH; Hamburg, Germany 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. In the second experiment, the blocks were randomly assigned to 5 groups (n=15/group for enamel and 17/group for dentin), according to the treatment gels, as follows: control (chitosan gel), positive control (chitosan gel containing 12,300 ppm F as NaF), experimental chitosan gels containing 0.1 or 0.25 mg/ml CaneCPI-5. In one additional group, specimens remained untreated, as an additional control for the chitosan gel.

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 od 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%.

***Preparation of solutions and chitosan gels***

CaneCPI-5 was cloned from sugarcane and recombinantly expressed in *E. coli* Rosetta, transformed with the plasmid pET28a, exactly as previously described [Santiago et al., 2017].

The solutions containing CaneCPI-5 were prepared with deionized water (native pH = 7.9, measured at 25 ºC), without additives.

The chitosan gels were prepared at the Federal University of ABC. Firstly, chitosan (75% deacetylation, medium molecular weight, Sigma-Aldrich, MO, USA) was dissolved in 1% acetic acid (Synth, Diadema, 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 chitosan 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. The pH of the gels was 4.7, measured at 25 ºC.

***Whole saliva collection***

Three volunteers (2 females, 1 male; 22-26 years of age) donated saliva 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 erosive tooth wear, such as gastric disorders, high consumption of acidic fruits, fruit juices or soft drinks.

Whole saliva was collected between 9 and 11 am, under chewing stimulus (Parafilm), in tubes immersed in ice. 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.

***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 in pooled 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.

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, remineralization 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. The solutions (25 µl/specimen) or gels 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. The specimens were immersed in artificial saliva overnight, completing every 24 h of the cycle. The loss of enamel and dentin was assessed using contact profilometry after 7 days of pH cycling. The dentin specimens were kept moist up to and during analysis to avoid shrinkage of the organic material. One drop of deionized water was added before each profilometric reading.

***Contact profilometry***

Profiles of the enamel and dentin surfaces were obtained with a contact profilometer (Perthometer, Mahr, Göttingen, Germany), before (baseline) and after the experimental period, just after the last overnight cycle in artificial saliva. The samples had a mark (small cavitation) made with a FG 1014 spherical diamond drill (KG Sorensen, Cotia, Brasil), 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, 250 µm apart from each other. 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, a device was used to 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 (Mahr, Göttingen, Germany). The average wear for each sample was calculated (µm). The minimum limit of detection was 0.5 µm.

The experimental design is shown in Figure 1.

***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 two-way ANOVA (for enamel and dentin, separately) and Tukey´s multiple comparison test. The factors were vehicles at two levels (solutions and chitosan gels) and treatments at fours levels (deionized water/chitosan gel, Elmex solution/NaF gel, 0.1 mg/ml CaneCPI-5 and 0.25 mg/ml CaneCPI-5).

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 (Kolmogorov-Smirnov test) and homogeneity (Bartlett test).

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

**Results**

According to the two-way ANOVA, for enamel significant difference was found among the treatments ($F=471.9$, $p<0.0001$) and between the vehicles ($F=20.1$, $p<0.0001$) as well as for the interaction between these factors ($F=21.6$, $p<0.0001$). Regardless of the vehicle, all the treatments significantly reduced enamel loss in comparison with control ($p<0.05$), without significant differences among them. Considering the vehicles, no significant differences between solutions and gels were observed for any of the treatments ($p>0.05$). However, the control gel led to significantly higher enamel loss when compared with the control solution ($p<0.05$) (Table 1).

Also, for dentin, according to the two-way ANOVA a significant difference was detected among the treatments ($F=171.2$, $p<0.0001$) and between the vehicles ($F=13.1$, $p<0.001$) as well as for the interaction between these factors ($F=9.7$, $p<0.0001$). For the solutions, all the treatments significantly reduced dentin loss in comparison with control ($p<0.05$), without significant differences among them. These also did not significantly differ from the fluoridated gel and from the gel containing 0.25 mg/ml CaneCPI-5. The 0.1 mg/ml CaneCPI-5 gel led to higher dentin loss when compared to the other treatment gels and solutions (except for the 0.1 mg/ml CaneCPI-5 solution) but performed significantly better than the control solution and gel. Considering the vehicles, no significant differences between solutions and gels were observed for any of the treatments ($p>0.05$). However, the control gel led to significantly higher dentin loss when compared with the control solution ($p<0.05$), similarly to what was observed for enamel (Table 2).

In the case of the gels, for enamel, according to the One-way ANOVA, a significant difference was found 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 the other enamel groups. The group that remained untreated presented the highest enamel loss that was significantly different from the control group. The group treated with 0.25 mg/ml CaneCPI-5 presented enamel loss significantly lower than untreated groups, but significantly higher than fluoride and 0.1 mg/ml CaneCPI-5 groups (Fig. 2).

For dentin, in the case of gels, according to the Kruskal-Wallis test, a significant difference was found among the groups ($KW = 68.964$, $p<0.0001$). The highest dentin losses were found for the group that remained untreated and control, 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. 3).

## 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, the study by Carvalho et al. [Carvalho et al., 2020] employed a very mild erosive challenge, with short acidic exposure. 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 fluoride-containing mouthrinse 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]. However, tin-containing products may provoke discoloration of the tooth surface and astringent sensation [Magalhaes et al., 2011], which limits their clinical use. In previous studies, the experimental CaneCPI-5 solution, on the other hand, was well tolerated by the volunteers, with no complaints regarding taste, staining and sensation during rinsing [Carvalho et al., 2020; Pela et al., 2021]. The experimental solutions evaluated, regardless the concentration of CaneCPI-5, significantly protected enamel against erosive demineralization, to the same extent as the positive control. Thus, they might be a better alternative to protect against erosive wear.

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. The erosive challenges in the studies by Santiago et al. [Santiago et al., 2017] and Carvalho et al. [Carvalho et al., 2020] lasted 3 min and 10 s only, respectively. 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 (28 min), the lower concentration employed in the previous studies could not be enough to provide effective protection. However, this was not the case since there was no significant difference between the effect on enamel of the two different CaneCPI-5 concentrations.

A second aim of the present study was to evaluate another vehicle of application of CaneCPI-5 besides solution. The vehicle chosen was a 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]. An in vitro study showed that chitosan adsorbs to hydroxyapatite-coated crystals 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 loss when compared with the control gel, which could reflect the protection conferred by chitosan (Fig. 2), but the same did not happen for dentin (Fig. 3). This difference might be because enamel has a strong negative zeta potential, which makes easier the adsorption of chitosan [Claesson and Ninham, 1992], while the zeta potential of dentin is as strong as that of enamel [Weerkamp et al., 1988]. Interestingly, the surface loss of the control chitosan gel was significantly higher than that of the control solution, for both substrates (Figs 2 and 3), which might be due to its lower pH (4.7) when compared with the pH of the control solution (7.9). This suggests that the control gel formulation used in the present study seemed to favor the tissue loss. It is known that different factors, such as the viscosity [de Souza et al., 2020] and the presence of phosphorylation [Beltrame et al., 2018] affect the ability of chitosan to protect against erosion. Thus, different chitosan gels formulations should be evaluated in further studies. Regarding the concentrations of CaneCPI-5 in the gels, when One-way ANOVA was performed, the higher concentration led to significantly higher enamel loss. At first glance, this could seem contradictory. However, the probable reason for the worse performance of the gel containing the higher CaneCPI-5 concentration might be 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.

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, despite providing significant protection in comparison with control, 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.

The main difference regarding enamel and dentin is the presence of the organic matrix in the latter. In dentin, an acid impact exposes the organic fraction that acts as a diffusion barrier, slowing down the progression of erosion [Ganss et al., 2004]. However, the demineralized organic matrix can be degraded by matrix metalloproteinases (MMPs) and cysteine cathepsins (CCs), thus allowing erosion to progress [Buzalaf et al., 2015]. NaF [Kato et al., 2014] and $SnF_2$ [Cvikl et al., 2018] inhibit MMPs, while CaneCPI-5 inhibits CCs [Santiago et al., 2017], which means that these compounds were expected to have an additional effect on the reduction of erosion in dentin, in comparison to enamel. However, this does not seem to have occurred, since dentin required higher concentrations of CaneCPI-5 to achieve the same degree of protection as enamel, especially in the case of gels (Tables 1 and 2).

Another interesting point to be discussed is the comparison between the different vehicles. Analysis of tables 1 and 2 indicates that solutions might be more appropriate vehicles, since regardless the substrate and concentration of CaneCPI-5, they were able to protect against erosive loss to the same extent as the positive controls. In the case of the chitosan gels, for dentin, this was the case only for the higher concentration of CaneCPI-5. It should be highlighted, however, that the control chitosan gel formulation employed in the present study seemed to favor the tissue loss, since it led to higher erosion

than the control solution, regardless the substrate. In future studies, it would be interesting to evaluate chitosan gels with different formulations.

Despite we employed a well-established pH-cycling protocol and attempted to mimic as close as possible the clinical condition, this study had limitations: a) bovine teeth were used instead of human teeth; b) artificial saliva was used during pH-cycling instead of human saliva; c) no abrasive challenge was performed; d) the control chitosan gel led to higher tissue loss than the control solution, which might be due to the lower pH of the first, but deserves further investigation. In future studies, these limitations should be addressed.

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 erosion in comparison with control. In addition, dentin requires higher concentrations of CaneCPI-5 to achieve the same degree of protection as enamel, 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. Thus, solutions or gels containing CaneCPI-5 might be a new approach to protect enamel and dentin against erosion.

## Statement of Ethics

The study was conducted in accordance with World Medical Association Declaration of Helsinki. The project was approved by the Animal Research Ethics Committee under the protocol 008/2019 and by the Institutional Human Ethics Committee (protocol 14969919.0.3001.5504) of Bauru School of Dentistry, University of São Paulo. Saliva donors signed an informed consent.

**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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## 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.

## Data Availability Statement

All data generated or analysed during this study are included in this article [and/or] its supplementary material files. Further enquiries can be directed to the corresponding author.

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**Table 1.** Mean (±SD) enamel loss (µm) after treatment with solutions or chitosan gels containing fluoride, different concentrations of a sugarcane cystatin (CaneCPI-5) or not (control) 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** | **Erosive loss (µm)** |
|---|---|---|
| Solution | Control (deionized water) | $5.56 \pm 0.89^{b}$ |
| Solution | 800 ppm $Sn^{+2}$ + 500 ppm $F^{-}$ | $1.17 \pm 0.34^{a}$ |
| Solution | 0.1 mg/mL CaneCPI-5 | $1.19 \pm 0.31^{a}$ |
| Solution | 0.25 mg/mL CaneCPI-5 | $1.45 \pm 0.57^{a}$ |
| Gel | Control (chitosan) | $7.88 \pm 1.40^{c}$ |
| Gel | 1.23% F | $1.15 \pm 0.23^{a}$ |
| Gel | 0.1 mg/mL CaneCPI-5 | $1.01 \pm 0.32^{a}$ |
| Gel | 0.25 mg/mL CaneCPI-5 | $1.63 \pm 0.41^{a}$ |

Treatments were applied twice/day, after the first and last erosive challenges. Data were analyzed by two-way ANOVA and Tukey´s test. ($p<0.05$). n=15. Means followed by different letters are significantly different.

**Table 2.** Mean (±SD) dentin loss (µm) after treatment with solutions or chitosan gels containing fluoride, different concentrations of a sugarcane cystatin (CaneCPI-5) or not (control) 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** | **Erosive loss (µm)** |
|---|---|---|
| Solution | Control (deionized water) | $6.74 \pm 1.32^{c}$ |
| Solution | 800 ppm $Sn^{+2}$ + 500 ppm $F^{-}$ | $1.36 \pm 0.48^{a}$ |
| Solution | 0.1 mg/mL CaneCPI-5 | $2.35 \pm 0.57^{ab}$ |
| Solution | 0.25 mg/mL CaneCPI-5 | $2.03 \pm 1.04^{a}$ |
| Gel | Control (chitosan) | $9.55 \pm 2.54^{d}$ |
| Gel | 1.23% F | $1.39 \pm 0.34^{a}$ |
| Gel | 0.1 mg/mL CaneCPI-5 | $3.57 \pm 1.49^{b}$ |
| Gel | 0.25 mg/mL CaneCPI-5 | $1.50 \pm 0.59^{a}$ |

Treatments were applied twice/day, after the first and last erosive challenges. Data were analyzed by two-way ANOVA and Tukey´s test. ($p<0.05$). n=17. Means followed by different letters are significantly different.

## Figures

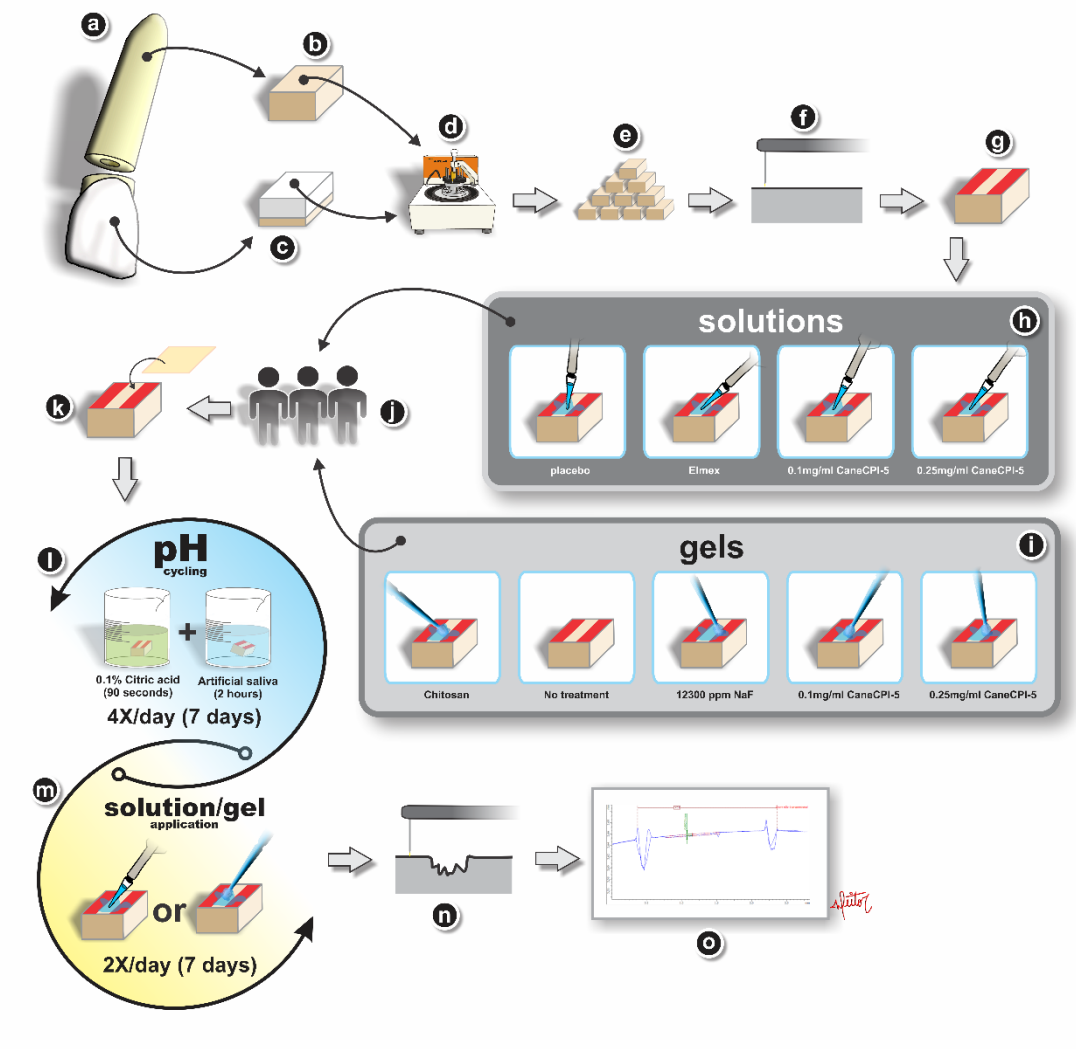


**Figure 1.** Experimental design of the study. (a) Bovine incisors had the crowns separated from the roots. (b, c) 4 x 4 mm (b) dentin and (c) enamel blocks were obtained, (d, e) polished and cleaned. (f) Baseline profile scans were performed and (g) 2/3 of the surfaces were protected with nail varnish (red zones). (h, i) Solutions or gels were applied on the blocks, (j) whole saliva was collected from 3 volunteers and (k) the acquired pellicle was formed on the blocks for 2 h. (l) The erosive pH cycling protocol was conducted 4 times/day for 7 days (0.1% citric acid pH 2.5/90s, artificial saliva/2h, artificial saliva overnight). (m) 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. (n, o) Enamel and dentin mineral loss were assessed by contact profilometry.

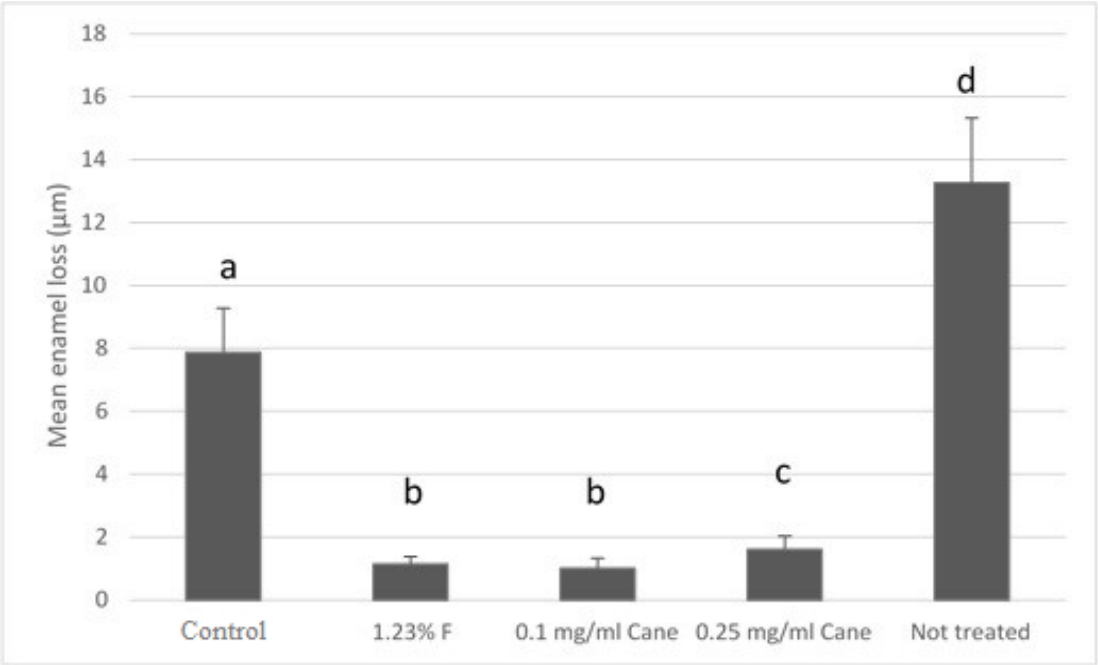


**Figure 2**. Mean (SD) enamel loss after treatment of specimens with chitosan gels containing fluoride, different concentrations of a sugarcane cystatin (CaneCPI-5) or not (control) for 4 minutes, followed by formation of acquired pellicle for 2 hours (pooled human saliva) and subsequent erosive challenges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

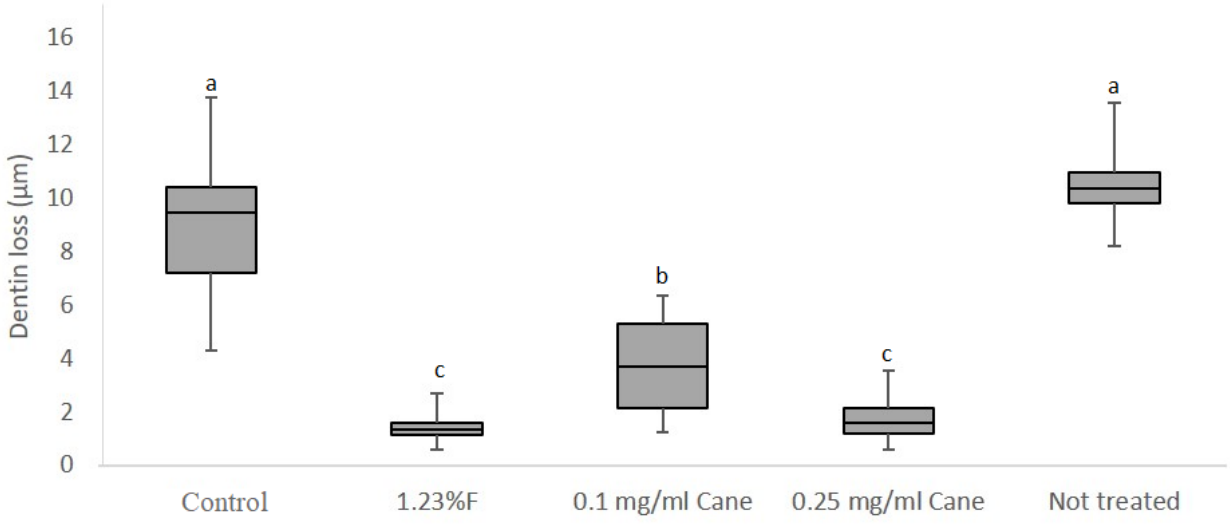


**Figure 3.** Median dentin loss after treatment of specimens with chitosan gels containing fluoride, different concentrations of a sugarcane cystatin (CaneCPI-5) or not (control) for 4 minutes, followed by formation of acquired pellicle for 2 hours (pooled human saliva) and subsequent erosive challenges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