SAFETY AND *IN SITU* ANTIEROSIVE EFFECT OF CANECPI-5 ON DENTAL ENAMEL

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ABSTRACT

The sugarcane cystatin (CaneCPI-5) was recently cloned and showed strong binding force to dental enamel and to protect against initial erosion. However, evaluations on its safety and efficacy in a situation closer to the clinical condition are necessary. In the present study we analyzed: 1) the cytotoxicity of CaneCPI-5 on human gingival fibroblasts; 2) the ability of CaneCPI-5 to reduce enamel erosion and erosion+abrasion *in situ*. In part 1, human gingival fibroblasts were treated with CaneCPI-5 (0.025, 0.05, 0.1, 0.5 or 1.0 mg/ml) or not (control). The cytotoxicity was assessed after 60s and 24h by mitochondrial activity (MTT), confocal microscopy and hematoxylin/eosin staining. In part 2, 15 volunteers participated in a double-blind crossover protocol consisting of 3 phases, according with the treatments: 1) 0.1 mg/ml CaneCPI-5; 2) $SnCl_2$/NaF/AmF (Elmex® - positive control); 3) water (negative control). They wore an appliance containing 4 bovine enamel specimens for 5 days. Each day, the specimens were individually treated with 50µl of the tested solutions per 60s and then subjected to erosive challenges (0.1% citric acid, pH 2.5, 90s, 4 times/day). After the 1st and last erosive challenges each day, two samples were abraded (toothbrushing, 15s). Enamel wear was measured by contact profilometry. One or two-way ANOVA/Tukey's or Sidak's tests ($p<0.05$) were applied. Regardless of the concentration and the experimental time, CaneCPI-5 did not decrease the cell viability compared to the negative control ($p<0.05$). Erosion+abrasion led to significantly greater wear compared to erosion only. For both conditions, the lowest wear was found for $SnCl_2$ and CaneCPI-5, which did not differ significantly from each other, but showed significant protection when compared to the negative control. In conclusion, CaneCPI-5 is safe to human gingival fibroblasts and reduces enamel erosive wear to the same extent as a commercial solution used to control erosive tooth wear.

Keywords: Erosion; Fibroblast; Prevention; Saliva; Tooth wear.

INTRODUCTION

The estimated prevalence of erosive tooth wear (ETW) among children and adolescents is around 30% (Salas et al. 2015). Considering the progressive nature of the condition, measures to prevent and/or intercept it are necessary. Most of the dental products developed so far include inorganic components, especially fluorides (Huysmans et al. 2014), with only a few focusing on organic compounds (Buzalaf et al. 2014; Buzalaf et al. 2015). In view of this gap in the literature, our group turned attention to finding acid-resistant proteins in the acquired enamel pellicle (AEP). Employing proteomic tools, we identified cystatin-B as an acid-resistant protein in the AEP (Delecrode et al. 2015). Later, in view of the economic and technological relevance for inclusion of this type of protein in dental products, we cloned and recombinantly expressed a sugarcane-derived cystatin, named CaneCPI-5 (Santiago et al. 2017).

Cystatins are cysteine protease inhibitors present in almost all organisms that have been extensively studied in the last decades (for review see Barrett 1986; Turk and Bode, 1991; Benchabane et al., 2010; Shibao et al. 2020). As competitive inhibitors, they interact with the active-site cleft of the target cysteine peptidases by three-point interaction between the inhibitor and the enzyme (Turk and Bode 1991; Margis et al. 1998). In plants, cystatins are classified in a special family, called phytocystatins (Margis et al. 1998), wherein CaneCPI-5 is included. 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 (Carvalho et al. 2020). In order that the concept of AEP engineering with this protein can be broadly employed to prevent and/or intercept erosive wear clinically, it is essential to evaluate the performance of this protein in more advanced stages of ETW, involving also abrasive components that are almost always associated with erosive ones. In addition, before CaneCPI-5 is included in dental products, its safety must be evaluated.

These aspects were considered in the present study that had two aims: 1) to analyze the cytotoxicity of CaneCPI-5, in concentrations ranging between 0.025-1.0mg/ml, in human gingival fibroblasts culture; and 2) to evaluate the protective potential of modification of the AEP with a solution containing CaneCPI-5 against erosion associated or not to abrasion *in situ.* The null hypotheses tested were: 1) CaneCPI-5, in concentrations ranging between 0.025-1.0mg/ml, is cytotoxic to human gingival fibroblasts; and 2) The modification of the AEP with a solution containing 0.1mg/ml CaneCPI-5 does not protect enamel against erosion associated or not to abrasion *in situ*.

MATERIAL AND METHODS

Ethical Aspects

The Ethics Committees for Human (86783418.8.0000.5417 and 39327520.2.0000.5417) and Animal (005/2018) Research of Bauru School of Dentistry, University of São Paulo, Brazil approved this study. It followed the guidelines of good clinical practice and conformed to the Declaration of Helsinki.

Safety of CaneCPI-5 in human gingival fibroblasts

Cell Culture

Human Gingival Fibroblast (HGF) cells were obtained from primary culture (explant technique) from the healthy gingival tissue of patients who attended the University of São Paulo. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM- Sigma-Aldrich Co, St. Louis, MO, USA) supplemented with antibiotics (100IU/ml penicillin and 0.1mg/ml streptomycin) and 10% (v/v) fetal bovine serum (FBS-GIBCO Laboratories, Life Technologies Inc., New York, NY, USA). After that, the cells were maintained at 37°C in an atmosphere containing 5% CO_2 with 95% air.

Cytotoxicity Assay

$5x10^3$ cells per well were plated in 96-well plates (Corning Inc., Corning, NY, USA) and upon 48h of cell adhesion, concentrations of 0.025, 0.05, 0.1, 0.5 and 1.0mg/ml of CaneCPI-5 (no color change) were added in culture medium. A group without CaneCPI-5 was used as control. The medium containing or not CaneCPI-5 was maintained for 1min or 24h in contact with the HGF cells.

The cytotoxicity was evaluated by MTT cell viability assay (Sigma-Aldrich Co, St. Louis, MO, USA). 0.5 mg/ml MTT in culture medium was added in each well and after 4h of incubation, the absorbance was evaluated (Synergy H1 microplate reader, BioTek, Winooski, VT, USA), using the software Gen5 2.06 (Oliveira et al., 2016).

For qualitative assay, 10^4 cells were plated on 13mm tissue culture coverslips (Sarstedt, Inc; Newton, NY, USA) in 24-well plate (Corning Inc., Corning, NY, USA). After 48h of cell adhesion, all the groups were added to cultured medium, as described above.

Fluorescence Confocal Microscopy

The cells were fixed in 10%formalin (Sigma-Aldrich Co, St. Louis, MO, USA), permeabilized with 0.5%Triton X-100 solution (Sigma-Aldrich Co, St. Louis, MO, USA). The cytoskeleton was stained with Rhodamine Phalloidin (Molecular Probe, Eugene, OR, USA) and nucleus with DAPI 4',6-Diamidino-2-Phenylindole, Dihydrochloride (Invitrogen, Life

Technologies Inc., New York, NY, USA) (Kihara et al., 2004). Then, the cells were analyzed by confocal fluorescence microscopy (40x, Leica TCS SPE Confocal Laser Scanning Microscope, Mannheim, BW, Germany).

Hematoxylin Eosin (HE) Staining

The cells were fixed in 10%formalin for 30min followed by 3 washes with PBS and stained by Harris Hematoxylin (Si et al., 2015). Coverslips with cells were fixed on slides with entellan (Merck KGaA, Darmstadt, HE, Germany) and each cell morphology was examined by inverted optical microscopy, 10x (BX43F, Olympus Corporation, Tokyo, Japan) (Si et al. 2015).

Statistical analysis

The software GraphPad Prism 6 (GraphPad software Inc., La Jolla, CA, USA) was used. After checking for normality (Kolmogorov-Smirnov test) and homogeneity (Bartlett test), data were analyzed by One-way ANOVA and Tukey´s test ($p<0.05$).

Efficacy of CaneCPI-5 against erosion associated or not to abrasion of the enamel *in situ*

Preparation of enamel specimens

One hundred and eighty enamel specimens were prepared (4mmx4mmx3mm) from permanent bovine teeth stored in 0.1%buffered thymol solution (pH 7.0). Then, the enamel surface of the samples was polished with 600 and 1200 granulation silicon carbide sandpaper (ExtecCorp. papers; Buehler, Lake Bluff, IL, USA). Approximately 150µm of enamel was removed. Also, a felt (Polishing cloth, Buehler, Lake Bluff, IL, USA) moistened with a 1µm diamond solution was used. All samples were stored under gauze moistened with deionized water at 4 °C.

Baseline profilometric measurement

Baseline profiles of the enamel surface were obtained with a contact profilometer (Mahr Perthometer, Göttingen, NI, Germany). Five scans (4mm in length, 250 µm apart from each other) were performed.

After the baseline profile, 2/3 of the surfaces were protected with red nail polish (Risque, São Paulo, SP, Brazil), to obtain control surfaces. Then, the samples were sterilized using ethylene oxide [(30% ETO/70% CO_2) for 4h under a pressure of $0.5 \pm 0.1 kgF/cm^2$].

Selection of volunteers and preparation of palatal appliances

Fifteen 25-30-year-old adults (seven women and eight men) were selected. The exclusion criteria regarding general health were: pregnant women, patients with systemic

diseases, under medication and smoking. The exclusion criteria for oral health were: low salivary flow (unstimulated < 0.3ml/min and stimulated < 1.0ml/min), active caries and periodontal disease.

The palatal intraoral appliance was made using acrylic resin. It contained 4 samples (randomly distributed on the left and right sides), corresponding to the type of wear (2 samples for erosion condition and 2 samples for erosion plus abrasion condition) (de Souza et al. 2018). When being stored and not in use, the device was wrapped in gauze moistened with tap water.

Experimental groups

The volunteers participated in 3 crossover and double-blind phases. In each phase, 5 volunteers were assigned to one of the 3 treatment solutions (determined by computerized random numbers), as follows: experimental solution containing 0.1mg/ml CaneCPI-5 in deionized water (final pH 7.88) (Santiago et al. 2017); commercial solution containing $SnCl_2$/NaF/AmF (800 ppm Sn^{+2}, 500 ppmF^-, pH 4.5, Elmex® − GABA International AG, Therwil, BL, Switzerland, positive control); placebo solution (deionized water, pH 9.4, negative control) (de Souza et al. 2018).

In situ guidelines and procedures

For each phase, a palatal intraoral appliance was used for 5 consecutive days between 8am and 8pm. During the night, the appliance was stored in gauze moistened with tap water. Between the phases, the volunteers had an interval period of 10 days.

During the experimental period (5 days), the intraoral appliance remained in the mouth, being allowed its removal only for drinking water and during the meals with a maximum duration of 30min each. The volunteers were instructed to perform oral hygiene using the materials provided that included toothbrush (Curaprox®, Kriens, LU, Switzerland), dental floss (Jadefrog®, Londrina, PR, Brazil) and fluoride toothpaste (1100ppmF, NaF, Colgate, São Bernardo, SP, Brazil). During the meal periods and overnight, the appliance was kept in gauze moistened with tap water. Oral hygiene was mandatory 5min before placing the appliance into the mouth. The hygiene of the appliance was restricted to its palatal surface (not containing samples).

Treatment, erosive and abrasive challenges

On the first day of each phase (before inserting the appliance in the mouth), an additional treatment was carried out (8:00am) with 50µl of the treatment solution on each sample for 1min. Immediately, the appliance was washed (with tap water) for 5s and placed in the mouth for the formation of the AEP. Four treatments were performed before each erosive challenge (10:00am; 2:00pm; 4:00pm; 6:00pm), totaling 4 treatments/day. For this, 1min before

the acid challenge, the volunteers removed the appliance from the mouth and performed the treatment (50µl on each sample for 1min). Then, they washed the appliance with tap water for 5s and immediately performed the acid challenge extra-orally, as described below.

The erosive challenges were done at pre-established times of the day (10:00am; 2:00pm; 4:00pm; 6:00pm). For this, the volunteers immersed the appliance in 150ml of 0.1%citric acid solution (pH 2.5), at room temperature, for 90s (without agitation). Then, the volunteers washed the appliances with tap water for 5s and replaced them in the mouth. The erosion plus abrasion condition was conducted 30min after the first (10:00am) and the last erosive challenges (6:00pm) each day, to allow some rehardening of enamel. For this, the volunteers added a drop of fluoride toothpaste slurry (1g of toothpaste: 3ml of deionized water) on each sample and brushing was performed using an electric toothbrush (OralB® Vitality Precision Clean - Electric Toothbrush, Kronberg, HE, Germany) during 15s (Levy et al. 2014). Afterwards, the volunteers rinsed the appliance with tap water for 5s and the device was put back in the mouth. All volunteers were trained by the researchers before the beginning of the experiment to perform the correct brushing procedure (force around 1.5N).

Final profilometric analysis

To determine the alteration of the sample surface profile after the *in situ* phase, five final readings were taken in the same areas as the initial readings, using a contact profilometer. The cosmetic nail polish was removed with an acetone solution (1:1-acetone:water) before the final measurements. For the correct reposition of the samples during the readings, they were included in a device and drillings using ¼ drill were performed to standardize the beginning of the readings. Initial and final profiles were compared using the MarhSurf XCR20 software. Average depth of the surface was analyzed to quantify the enamel wear (µm) for each sample, considering 0.5µm as the limit of detection (Levy et al. 2014).

Statistical Analysis

GraphPad Prism software (version 6.0 for Windows, GraphPad Software Inc., La Jolla, CA, USA) was used. Data passed normality (Kolmogorov-Smirnov test) and homogeneity (Bartlett test) and were analyzed by 2-way repeated-measures (for both criteria) ANOVA and Sidak´s test ($p<0.05$).

RESULTS

CaneCPI-5 is safe to human gingival fibroblasts

The MTT reduction demonstrated that the concentrations of CaneCPI-5 employed were not cytotoxic to the HGF. In both experimental times, cell viability did not decrease compared

to the control group. Comparing the treated groups, only the concentrations of 0.1 and 0.5mg/ml presented significant difference between each other at 24h ($p<0.05$) (Figure 1).

Microscopic images confirmed the quantitative results of the MTT assay. None of the tested CaneCPI-5 concentrations affected the viability of the HGF. Regarding the cells stained with HE, it was observed that the treatment did not alter neither the morphology nor the number of cells (Figure 2). Moreover, similar findings were observed in the confocal microscopy images, in which no phenotypic cell alterations were seen upon treatment with CaneCPI-5 (Figure 3).

CaneCPI-5 reduces enamel erosion and abrasion *in situ*

The results showed a significant difference between the two conditions Erosion x Erosion plus Abrasion ($F=33.75$, $p<0.0001$) and among the treatments ($F=62.82$, $p<0.0001$), without interaction between the factors ($F=0.59$, $p=0.558$). Erosion+Abrasion led to significantly greater wear when compared with Erosion, regardless of the treatment ($p<0.05$). Regarding the treatments, the lowest wear was found for the commercial solution containing $SnCl_2$/NaF/AmF and for the solution containing 0.1mg/ml CaneCPI-5, which did not significantly differ from each other ($p>0.05$), but both showed significant greater protection when compared with the negative control (deionized water), regardless the condition ($p<0.05$) (Figure 4).

DISCUSSION

The present study brings together important results that pave the way for the clinical use of CaneCPI-5 for the prevention of ETW. However, before CaneCPI-5 is included in dental products for clinical use, cytotoxicity tests are necessary to ensure its safety for the oral soft tissues. This aspect was evaluated in the first part of the present study. The MTT test is universally accepted by International Standardization Organization, allowing the verification of the activity of succinate dehydrogenase present in mitochondria of live cells (Silva et al. 2013). In the present study, we chose HGFs obtained by primary culture from the gingival tissue of healthy patients. This choice is recommended in the literature, since it better simulates the clinical condition (Geurtsen 2000). Regarding the choice of the experimental times, we opted for a short period (1min) to simulate the treatment time often used during mouth-rinsing treatments (Marinho et al. 2016), such as the one employed in our *in situ* study and also applied in a previous *in vivo* initial erosion study (Carvalho et al. 2020). Also, a longer period (24h) was chosen to make sure that no long-term residual cytotoxic effects would be found.

The cell cytotoxicity has been used to drug screening assays because some molecules display toxic effects on the proliferation and cell viability (Adan et al 2016). Besides the mitochondrial activity, we evaluated possible phenotypic changes in HGF cells when exposed to

CaneCPI-5. These results led to the rejection of the first null hypothesis and assure the safety of the inclusion of CaneCPI-5 in dental products in the tested concentration for enamel wear prevention in second part of the present study.

Our *in situ* study constitutes an important step forward for the clinical use of CaneCPI-5 to protect against ETW, since we employed prolonged erosive challenges that were also associated to abrasive episodes. This experimental protocol simulates much better the clinical condition, where erosive challenges are associated to abrasive ones, what we name ETW (Lussi and Carvalho 2014). In our previous studies, we employed only mild erosive challenges (3x1min 0.65%citric acid pH 3.5) so that the response variable used was percent surface hardness change (Santiago et al. 2017). In the present study, however, a prolonged erosion model was applied (de Souza et al. 2018) with four challenges (90s each) per day, for five days, using 0.1%citric acid (pH 2.5). Even with this prolonged erosive challenge, the mean enamel loss in the group treated with CaneCPI-5 was below the detection limit. In addition, abrasive challenges were also performed in half of the specimens twice/day (Levy et al. 2014). This experimental protocol provoked enamel wear measurable by profilometry (Wiegand and Attin 2011). Regarding the type of sample, the literature has shown that bovine teeth can be used instead of human ones for studies involving ETW (Laurance-Young et al. 2011) and AEP (Pela et al. 2018).

One important aspect to be highlighted in the experimental procedures is the application of CaneCPI-5 on the enamel surface before the formation of the AEP. This procedure is part of the concept of "AEP engineering", which aims at redirecting the formation of this integument, increasing the amount of acid-resistant proteins, to improve its protective potential against enamel demineralization (Carvalho et al. 2020). Moreover, CaneCPI-5 presents high binding force to hydroxyapatite (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 before each erosive challenge may enhance the quality of the AEP already formed, protecting against the immediate acid attack, which may be an appropriate strategy for patients at high risk for ETW. In the present study, the treatments were performed before each erosive challenge. Following this rationale, in the clinical situation, the patients would have to rinse with the product before consuming the acidic beverages. It is possible that the treatment also works under less frequent application, but this needs to be evaluated in further studies.

The most remarkable finding of the present study was the fact that CaneCPI-5 protected enamel against erosion and erosion associated to abrasion to a similar extent as the positive control (commercial solution containing $SnCl_2$/NaF/AmF). It is important to highlight that the commercial solution evaluated is so far one of the most efficient preventive approaches for ETW, due to the combination of tin and fluoride ions (Huysmans et al. 2014). Our results are

consistent with another *in situ* study, showing 35% reduction of erosion *in situ* for the combination of tin and fluoride ions (de Souza et al. 2018). However, tin-containing products may provoke dull feeling on the tooth surface, discoloration, and astringent sensation (West et al., 2012; de Souza et al., 2018), which limits their long-term use in the clinical condition. 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). These results led us to reject the second null hypothesis of the study and show, for the first time, that CaneCPI-5 is also effective for prolonged erosive challenges associated or not to abrasion.

The next logical step would be to perform clinical studies on this topic. Despite some technologies have been recently developed for clinical measurement of ETW, they are not standardized and accessible yet (Rakhmatullina et al. 2013; Mullan et al. 2017). Therefore, most evidence regarding preventive measures for ETW still comes from *in situ* studies, which are the model that more closely resemble the clinical condition. Moreover, a recent study by our group compared the proteomic profile of the AEP formed *in vitro*, *in situ* and *in vivo*. The AEP formed *in situ* was similar to *in vivo* (Pelá et al. 2020). Considering that our preventive strategy is based on AEP engineering, the results obtained *in situ* with this protein might be highly comparable with *in vivo* conditions.

In conclusion, our results show that CaneCPI-5 is safe and does not promote phenotypic alterations in HGF. Furthermore, CaneCPI-5 protects enamel against erosion and erosion+abrasion *in situ*. These results open a new avenue for the development of oral hygiene products aimed to protect against ETW, based on AEP engineering. Additional studies refining the mechanisms of action of CaneCPI-5 must be conducted. Moreover, it is important to evaluate if the same protective effect occurs in dentin, as well as using other vehicles of application of CaneCPI-5, such as gels or dispersible films.

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Author contributions

V.T. Pelá, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; J.G.Q. Lunardelli, C.K. Tokuhara,

C.C. Gironda, N.D.G. da Silva, T.S. Carvalho, A.C. Santiago, B.M. de Souza, S.M. Moraes, data acquisition, and analysis, drafted and critically revised the manuscript; F. Henrique-Silva, A.C. Magalhães, R.C. de Oliveira, M.A.R. Buzalaf, contributed to conception, design, analysis, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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Figure legend

Figure 1. Colorimetric assay by MTT reduction of Human Gingival Fibroblast (HGF) after 1min (A) and 24h (B) treated with different concentrations of CaneCPI-5 (0.025 to 1.0mg/mL) and control group (no CaneCPI-5). * Represents statistical difference, $p<0.05$. Values are represented in percentage and the control group was considered 100%. ANOVA complemented with Tukey test.

Figure 2. Representative H.E. (Hematoxylin Eosin) images of Human Gingival Fibroblast, upon 24h of treatment of CaneCPI-5 (0.025, 0.05, 0.1, 0.5 and 1.0mg/ml) and control group (no CaneCPI-5). Scale bar, 100µm. Olympus BX43.

Figure 3. The organization of cytoskeleton and nucleus of Human Gingival Fibroblast treated or not with CaneCPI-5. The cells were labeled with Rhodamine Phalloidin (red) and Dapi (blue). Scale bar, 20µm. Leica TCS SPE Confocal Laser Scanning Microscope, using a 40x/1.15 oil objective.

Figure 4. Average enamel wear after treatment with deionized water, $SnCl_2$ or 0.1mg/ml CaneCPI-5. Distinct upper-case letters denote significant differences between the conditions, while distinct lower-case letters denote significant differences among the treatments (2-way RM ANOVA and Sidak´s test, $p<0.05$). Mean ± SD. n=15.