PREVENTIVE EFFECT OF CHITOSAN GEL CONTAINING CANECPI-5 AGAINST ENAMEL EROSIVE WEAR *IN SITU*

Vinícius Taioqui Pelá[a], Leonardo Brito[b], Even Akemi Taira[b], Flávio Henrique-Silva[a], Joana Claudio Pieretti[c], Amedea Barozzi Seabra[c], Cristiane de Almeida Baldini Cardoso[d], Eduardo Pereira de Souza[a], Sonia Groisman[e], Marcela Charantola Rodrigues[f], Adrian Lussi[g], Thiago Saads Carvalho[h], Marília Afonso Rabelo Buzalaf[b*]

[a]Department of Genetics and Evolution, São Carlos Federal University, São Carlos, São Paulo, Brazil.
[b]Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Bauru, São Paulo, Brazil.
[c]Center for Natural and Human Sciences, Federal University of ABC, Santo André, São Paulo, Brazil.
[d]Postgraduate Department, Cruzeiro do Sul University, São Paulo, São Paulo, Brazil.
[e]Federal University of Rio de Janeiro, Rio de Janeiro, Brazil.
[f]Municipal University of São Caetano do Sul (USCS), São Caetano do Sul, São Paulo, Brazil
[g]Department of Operative Dentistry and Periodontology, University Medical Centre, Freiburg, Germany
[h]Department of Restorative, Preventive and Pediatric Dentistry, School of Dental Medicine, University of Bern, Bern, Switzerland.

***Corresponding author:**
Marília Afonso Rabelo Buzalaf
Present address: Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Bauru, SP. Al. Octávio Pinheiro Brisolla, 9-75. Bauru-SP, 17012-901, Brazil. Tel. + 55 14 32358346 Fax + 55 14 32271486 E-mail: mbuzalaf@fob.usp.br

Author contributions: Conceptualization: Marília Afonso Rabelo Buzalaf; Methodology: Vinícius Taioqui Pelá, Leonardo Brito, Even Akemi Taira, Joana Claudio Pieretti, Eduardo Pereira de Souza; Formal analysis and investigation: Vinícius Taioqui Pelá, Flávio Henrique-Silva, Amedea Barozzi Seabra, Cristiane de Almeida Baldini Cardoso, Sonia Groisman, Marcela Charantola Rodrigues, Adrian Lussi, Thiago Saads Carvalho; Writing - original draft preparation: Vinícius Taioqui Pelá and Marília Afonso Rabelo Buzalaf; All authors read and approved the final manuscript.

Abstract

Objective: This study evaluated the preventive effect of a chitosan gel containing CaneCPI-5 against enamel erosion and erosion+abrasion *in situ*. Methods: Sixteen volunteers participated in a crossover, double-blind protocol, comprising 4 phases: 1) No treatment (Nt); 2) Chitosan gel (Cg); 3) Chitosan gel+12,300ppm NaF (Cg+NaF) and 4) Chitosan gel+0.1mg/mL CaneCPI-5 (Cg+Cane). Volunteers wore an appliance containing 4 specimens. Once/day, they applied the gel (except for Nt) (4 min/specimen). Erosive challenges were performed extra-orally (0.1% citric acid, 90s, 4X/day; ERO). Specimens were also abraded (toothbrush, 15s/specimen, 2X/day; ERO+ABR). Enamel wear was assessed by profilometry and relative surface reflection intensity (%SRI). Two-way RM-ANOVA/Sidak's tests and Spearman´s correlation were used ($p<0.05$). Results: For profilometry, ERO+ABR promoted significantly greater wear when compared with ERO. There was significant difference among all treatments. The lowest enamel loss occurred for Cg+Cane, followed by Cg+NaF, Cg and Nt ($p<0.05$). The %SRI was significantly lower for ERO+ABR when compared to ERO, only for Nt group. The greatest %SRI was found for Cg+NaF and Cg+Cane groups, which did not differ significantly, regardless of the conditions. The lowest %SRI was found for the Nt and Cg groups, which did not differ from each other, regardless of the conditions. The Nt group did not differ significantly from the Cg+NaF (ERO). There was significant correlation between both analyzes. Conclusion: The incorporation of CaneCPI-5 in the chitosan gel prevented erosive wear *in situ*. Clinical relevance: These results open a new perspective for the use of CaneCPI-5 in other application vehicles, such as chitosan gel.

Keywords: Acquired Pellicle; Cystatin; Prevention; Tooth.

INTRODUCTION

Dental erosion is the chemical loss of tooth substance caused by non-bacterial acids [1]. It is a major problem because of its association with multiple factors that are difficult to control, such as nutrition and behavior [2]. Dental abrasion is the physical loss of tooth substance caused by objects [1]. It is often associated with incorrect oral hygiene, preceded or not by dental erosion [3]. In both conditions, the loss of tooth mineral is initially superficial, but progressive [4], and leads to erosive tooth wear. The prevalence of erosive tooth wear in permanent teeth is reported to be around 30% [5, 6], being associated with lifestyle, diet, and sociodemographic and economic characteristics [7].

Given this fact, the search for components that can act against these types of wear is increasing. These components might be employed in different forms, such as inorganic actives [8], organic actives [9], different combinations between these actives [10] as well as natural products [11]. Currently, our group has focused on organic components, through the development of a rinse solution containing a sugarcane-derived cystatin (CaneCPI-5) [12, 13]. This recombinant protein protected enamel against erosive challenges *in vitro*, *in situ* and *in vivo*, when a 1-min rinse with a solution containing 0.1 mg/mL of CaneCPI-5 was employed, and its main mode of action was through "acquired pellicle engineering" [13-16]. It is known that the acquired pellicle (AP) acts as a barrier, protecting the dental surface against the acids [17]. In addition, the application of CaneCPI-5 before the AP formation allowed the strengthening of this protein layer due to the high binding strength of CaneCPI-5 to hydroxyapatite [12] and also increased important acid-resistant proteins within the AP, which are favorable against erosive wear [13].

Despite the use of the CaneCPI-5 seems to be well established in rinse solutions, its application in other vehicles should still be investigated. Currently, gels and toothpastes are being extensively studied against dental erosion and abrasion [18, 19]. However, several toothpastes may have components with abrasive potential, such as hydrated silica, calcium carbonate and alumina [20]. On the other hand, most gel formulations consist of non-abrasive components, such as hydroxyethylcellulose and propyleneglycol [21]. Moreover, a new gel formulation composed of chitosan and Sn^{+2} opened a new path of investigation [22, 23] with potential prevention against erosive wear [24]. With these promising findings, our research group recently demonstrated that the incorporation of CaneCPI-5 in a chitosan gel was able to reduce enamel and dentin erosion in an *in vitro* prolonged erosion model [16] due to adsorption of the chitosan gel to hydroxyapatite-coated crystals and its interaction with AP proteins [24, 25] and also by protection mechanism of CaneCPI-5 [12, 13].

However, the ability of a chitosan gel containing CaneCPI-5 was never evaluated under more clinically relevant conditions. Neither its protective potential against abrasive challenges. Thus, we aimed to evaluate the preventive effect of a chitosan gel containing CaneCPI-5 against enamel erosion and erosion plus abrasion *in situ*. The null hypothesis tested was that the chitosan gel containing CaneCPI-5 does not prevent enamel erosion or erosion plus abrasion.

METHODOLOGY

Ethical Issues and recruitment of volunteers

This study was approved by the Ethics Committee for Human Research (CAAE: 86783418.8.0000.5417) of the Bauru School of Dentistry, University of São Paulo, SP, Brazil. All volunteers participated after signing the informed consent form, following the guidelines of the Declaration of Helsinki.

Sixteen volunteers (eight women and eight men) with age between 27 to 32 years were selected, according to the following general inclusion criteria: non-pregnant women, non-smokers, not under constant use of medications and free of systemic diseases. In addition, the following oral health inclusion criteria were adopted: not having active caries or periodontal disease, not under use of orthodontic appliances, not having had professional fluoride application near the beginning of the study and with a normal salivary flow, considering for stimulated saliva flow rate > 1.0 mL/min and for unstimulated saliva flow rate > 0.3 mL/min.

Cut and polish of bovine enamel specimens

The use of bovine teeth for this research was also approved by the Ethics Committee on Animal Use of the Bauru School of Dentistry, University of São Paulo (Protocol: 005/2018). All permanent bovine teeth were stored in 0.1% buffered thymol solution (pH 7.0) at 4 °C. One hundred and twenty-eight bovine teeth were used for the study. From this amount, two hundred and fifty-six bovine enamel specimens (4 mm × 4 mm × 3 mm) were obtained, using a cutting machine with two diamond discs (ISOMET Low Speed Saw Buehler, Lake Bluff, IL, USA). Afterwards, the specimens were subjected to polishing, removing approximately 130 µm from the surface of the enamel bovine. For that, 350, 600 and 1200 granulation silicon carbide sandpapers were used (Extec Corp. Papers; Buehler, Lake Bluff, IL, USA). To finalize the polishing, a felt polishing cloth (Extec Corp. Polishing cloth; Buehler, Lake Bluff, IL, USA), moistened with a 1-µm diamond solution (Extec Corp. Buehler, Lake Bluff, IL, USA) was used on the surface of interest. Then, the specimens were submitted to visual and microscopic analysis to investigate possible stains and cracks, in which case they were excluded from the study. Finally, the specimens were cleaned by ultrasonication (T7 Thornton, Unique Ind. E Com. Ltda., São Paulo, SP, BR) with deionized water for 7 min at 25 °C and stored under gauze and cotton, moistened (with deionized water) in a cold chamber at 4 °C.

Palatal appliance preparation

Plaster casts of the volunteers' upper arch were used to make four intraoral palatal appliances (with acrylic resin) for each volunteer (one appliance for each *in situ* phase). Then, each appliance held four bovine enamel specimens, which were fixed with wax (Asfer Indústria Química Ltda., São Caetano do Sul, SP, BR) at the same level of the acrylic resin.

After initial analysis by profilometry and reflectometer (described in the subsection below), the selection and distribution of specimens in the appliance occurred through previous randomization with Reflectometer Optipen, which assigned two specimens to the erosion procedure (right side) and two specimens to the erosion plus abrasion procedure (left side). Until the beginning of the experimental phase, the appliances were kept humid with gauze moistened with tap water and stored at 4 °C [26].

Gels formulation and recombinant production of CaneCPI-5

For the formulation of the gels, chitosan (75% deacetylation, medium molecular weight, Sigma-Aldrich, St. Louis, MO, USA) was added in 1% acetic acid solution (Synth, Diadema, SP, BR). The concentration used was 30 mg of chitosan for 1 mL of 1% acetic acid. Afterwards, it was homogenized during 2 hours, at 25 °C. With respect to gels containing the actives (12.300 ppm NaF or 0.1 mg/mL CaneCPI-5), both were incorporated during the chitosan dissolution. In this case, the mixture was also homogenized for 2 hours at 25 °C. Finally, the pH of all gels was analyzed and remained stable at 5.5 at 5 °C until the beginning of the experimental phase [16]. The gels were prepared at Federal University of ABC, São Paulo, Brazil.

The recombinante CaneCPI-5 was produced in Escherichia coli Rosetta (DE3) using the pET28a vector, as previously described [12, 27]. Briefly, a culture transformed with the vector pET28aCaneCPI-5 was induced with 0,4 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside, Sigma-Aldrich, St. Louis, MO, USA), centrifuged, sonicated, and the expressed protein was purified from the soluble fraction by affinity chromatography, using columns containing nickel resin Ni-NTA Superflow (Qiagen). CaneCPI-5 was produced at Federal University of São Carlos, São Carlos, Brazil.

Guidance for volunteers to carry out the study practices

All volunteers received the following guidance: 1) Use exclusively the kit provided by the researchers, containing toothbrush (Oral B®, The Procter & Gamble Company, Cincinnati, OH, USA), dental floss (Oral B®, The Procter & Gamble Company, Cincinnati, OH, USA) and fluoride toothpaste (1100ppmF, NaF; Oral B®, The Procter & Gamble Company, Cincinnati, OH, USA) throughout the study; 2) Remove the appliance only to drink water (1 min), during the meals (20 min), and overnight (8 h); 3) Store the appliance wrapped in gauze moistened with tap water during the meal periods and overnight; 4) Perform oral hygiene after each meal and upon waking up; 5) Perform hygiene of the palatal appliance, brushing only the palatal surface (side without specimens); 6) Remain vigilant about any detachment of specimens, 7) Have breakfast before oral cavity hygiene; 8) Do not have extra meals during the day and 9) Perform the abrasion procedure with a standardized force, as previously trained by the researchers [15].

In situ experimental protocol

The volunteers participated in 4 crossover and double-blind phases. For each phase, 4 volunteers were destined to one of the four groups (determined by computerized random numbers) as follows: 1) No treatment (Negative control; Nt); 2) Chitosan gel (Placebo gel; Cg); 3) Chitosan gel containing 12.300 ppm NaF (Positive control; Cg+NaF) e 4) Chitosan gel containing 0.1 mg/mL CaneCPI-5 (Cg+Cane) [16]. During each phase, volunteers used one of the palatal appliances during 5 days (from Monday to Friday, 8 am to 7 pm). For each phase, a new appliance was used. Between each phase, a wash-out period of ten days was established [15].

During the experimental phase, the volunteers performed the following daily procedure: oral cavity hygiene (7:55 am) and then they applied the treatment gel (once/day at 8:00 am) according to the respective group (except for the specimens on the Nt phase that received no treatment). For this, the gel was individually applied with a *microbrush* (approx. 20 µl per specimen). The gel remained in place for 4 min, and it was then removed with a cotton swab. Immediately, the appliance was placed in the mouth for the formation of the AP for two hours.

All enamel specimens underwent four extra-oral erosive challenges (ERO) per day (10:00 am; 2:00 pm; 4:00 pm; 6:00 pm). Herewith, the volunteers submerged the appliance in a cup with 150 mL of 0.1% citric acid solution (pH 2.5), at room temperature, for 90 s (without agitation). After that, they rinsed the appliances with tap water for 5 s and replaced them in the mouth. For every ERO, a new aliquot of citric acid was used [15].

For the ERO group, the enamel specimens underwent erosion only, but for the ERO+ABR group, the specimens also underwent abrasion procedure (only for specimens on the left side of the device). The abrasion was conducted by the volunteers twice a day, 30 min after the first and the last erosive challenges (at 10:30 am and 6:30 pm, respectively) throughout the entire experimental phase. For this, the volunteers brushed individually each specimen, using an electric toothbrush (OralB® Vitality Precision Clean - Electric Toothbrush, Kronberg, HE, DE) and slurry (1 g of toothpaste 1100ppmF, NaF, Oral B®, The Procter & Gamble Company, Cincinnati, OH, USA: 3 mL of deionized water) for 15 s. Then, the volunteers washed the appliance (with tap water for 5 s) and replaced them in their mouths. The volunteers received prior training to perform the correct brushing (force around 1.5 N) [15].

Enamel wear measurement by Profilometry

Before palatal appliance preparation, the baseline profiles of the enamel specimens were performed using a contact profilometer (MarSurfe XCR20, Göttingen, NI, DE). Initially, a small drilling was done with a ¼ drill (in the upper and left corner of each specimen) as an indicator and standardization for the beginning of the readings.

Regarding the parameters, five scans of each specimen (3 mm of reading and 250 µm apart from each other) were carried out. After this initial analysis, the enamel surface was divided into two-thirds to obtain the control areas. Outer portions (ends) were covered with nail polish (Risque®, São Paulo, SP, BR), while in the central portion the bovine enamel surface remained exposed. Afterwards, all specimens were subjected to sterilization with ethylene oxide [(30% ETO/70% CO_2) for 5 h under a pressure of 0.5 ± 0.1 kgF/cm^2] [26].

After the *in situ* experimental protocol, the nail polish was carefully removed (1:1 acetone:water) and the final profile was performed, as described above. For the final reading to be carried out in the same place where the baseline was performed, three conditions were needed: 1) a metal device was used, which served as a support for fixing the specimens; 2) the correct repositioning of the x and y axis of the profilometer and 3) follow the indication made with ¼ drill. The graphs of each reading (baseline and final profile) were overlaid and compared through the MarhSurf XCR20 software (Mahr, Göttingen, NI,

DE). Finally, the enamel wear was measured (μm), considering the minimum detection limit of 0.5 μm [15].

Enamel wear measurement by Reflectometer Optipen

The Surface Reflection Intensity (SRI) was performed by hand-held reflectometer Optipen. The baseline (SRI_b) was made before covering the enamel with nail polish and the final analysis (SRI_f) was made after the final profilometry. For both analyses (baseline and final), all specimens were initially dried for 5 s and the tip of the reflectometer was touched on the bovine enamel surface. Then, the portable equipment was inclined at various angles to obtain the highest reflection record of each specimen.
The values presented by the software were tabulated and calculated as follows: %SRI = $(SRI_f / SRI_b) \times 100$ [14, 28].

Statistical analysis

The results obtained from the analysis were statistically verified using the software GraphPad Prism software (version 6.0 for Windows, GraphPad Software Inc., La Jolla, CA, USA), after checking for normality (Kolmogorov-Smirnov test) and homogeneity (Bartlett test). Data were analyzed by two-way (treatments and conditions) repeated-measures (by both factors) ANOVA and by Sidak's tests for individual comparison. Spearman's correlation coefficient was calculated between contact profilometer (μm) and Reflectometer Optipen (%SRI). The significance level was set at 0.05.

RESULTS

For the profilometry analysis **(Figure 1)**, there was a significant difference between the treatments ($F=206.9$, $p<0.0001$) and the conditions (ERO and ERO+ABR; $F=106.1$, $p<0.0001$), without significant interaction between them ($F=2.295$, $p=0.0808$). There was a significant difference between all the treatments, with the lowest enamel loss for the Cg+Cane, followed by the Cg+NaF and the Cg. The greatest wear was found for the Nt group ($p<0.05$). ERO+ABR condition promoted significantly greater wear when compared to ERO, regardless of the treatment ($p<0.05$).

Regarding the %SRI analysis **(Figure 2)**, there was also a significant difference between the treatments ($F=44.48$, $p<0.0001$) and the conditions ($F=12.99$, $p=0.0032$), without significant interaction between them ($F=0.829$, $p=0.486$). With respect to the difference between the treatments, the greatest %SRI was found for Cg+Cane and Cg+NaF groups, both showing significant protection when compared to Cg and Nt groups for the ERO+ABR condition, but for the ERO condition, the Cg+NaF did not differ significantly from the Nt. The lowest %SRI was found for Nt and Cg groups, with no significant difference between them. With respect to the conditions, there were generally no differences, except for the Nt group, that presented significantly lower %SRI for the ERO+ABR condition compared to ERO.

There was a significant correlation ($r = -0.5168$, $p<0.0001$) between contact profilometer (μm) and Reflectometer Optipen (%SRI) for erosion ($y = -0.0149x + 2.1231$) and erosion + abrasion ($y = -0.0163x + 2.2887$) **(Figure 3)**.

DISCUSSION

This is the first time that a chitosan gel containing CaneCPI-5 was evaluated for the prevention of erosive enamel wear under clinically relevant conditions. In our *in situ* protocol, we had participation of all volunteers throughout the study, no complaints about the use of the appliance and the procedures performed by them, such as gel application, erosive and abrasive challenges. The appliance was not worn overnight since the salivary flow during this period is low and is not expected to provide additional enamel rehardening [29]. Moreover, we chose not to use the device overnight to ensure the comfort and quality of sleep of the volunteers and increase compliance with the study. In addition, we used sterilized bovine enamel due to the high amount of specimens needed and the good acceptance of them in researches involving erosive wear and adhesion of salivary proteins [30,31]. Moreover, it was recently shown that the proteomic profile of the AP formed *in situ* and *in vivo* is similar, especially considering acid-resistant proteins such as cystatins [32].

A relevant aspect of our protocol, with implications from the clinical point of view, is that the gel was applied only once/day (for 4 min, before the formation of the AP). This is important because in our previous *in situ* study in which a solution containing CaneCPI-5 was evaluated, the volunteers applied the solution on the specimens for 1 min, 4 times per day, before each erosive challenge [15]. One may argue that this is not practical, and this was one of the reasons why we decided to test another vehicle for the use of CaneCPI-5. Regarding the time of gel application (4 min), it is widely employed in dentistry for caries prevention when fluoride gels are used [33].

The present study is a step towards the clinical application of the chitosan gel containing CaneCPI-5. In our first *in vitro* study, bovine enamel specimens were submitted to 0.1% citric acid pH 2.5 for 90 s, 4 times per day, for 7 days. The chitosan gels were applied during pH cycling with artificial saliva, 2 times per day for 4 min, after the first and last erosive challenges [16]. In the present *in situ* study, bovine enamel specimens were also submitted to 0.1% citric acid pH 2.5 for 90s, 4 times per day, followed or not by abrasive procedures (2 times per day), for 5 days. However, in this protocol, the chitosan gels were applied only once/day for 4 min, before the AP formation. The response variable for both studies was contact profilometry. The enamel loss for the condition erosion only, was 6.3, 4.1, 1 and 1.2 times higher for the groups that were not treated, or that were treated with Chitosan gel, Chitosan gel + NaF or Chitosan gel + CaneCPI-5, respectively, in our *in vitro* study [16] when compared with the present one. In the present study, there was a greater protection of enamel for the groups that were not treated and treated with the placebo gel; this may be due to: 1) the presence of the AP for protection against erosive wear [34]; 2) buffering capacity of natural saliva against acidic challenges [17] and 3) less days under erosive challenges.

Also, regarding the profilometry result (ERO condition), the Cg+Cane showed a significantly better protection when compared to all the other groups; including the positive control (Cg+NaF). It should be highlighted that conventional fluorides are not as effective against erosive demineralization [35] as they are against carious demineralization [36], since the CaF_2-like layer formed on the enamel is

short-lived under the more severe nature of the erosive challenges. So far, the greatest effectiveness is seen for the combination of fluoride and tin [35, 37]. This might be the reason why in our previous *in situ* study, the commercial fluoridated solution (Elmex®, containing tin and fluoride) was as effective as the experimental solution containing CaneCPI-5, which was not observed in the present study for Cg+NaF and Cg+Cane groups. The preventive effect of CaneCPI-5 might be related to its interaction with hydroxyapatite, thus changing the protein profile of the AP [12, 13]. Another noteworthy aspect is the preventive capacity of Cg group (without other active ingredients than chitosan) [16]. This gel demonstrated significant protection for enamel when compared to the Nt group. This might be due to the ability of chitosan to adsorb to hydroxyapatite and to possibly bind AP proteins, preventing erosion [16, 24, 25]. Although this group provided a certain prevention for enamel wear, it can be considered as a placebo gel when compared with the other groups, which had the presence of additional active agents (CaneCPI-5 or NaF).

Another novelty of the study was to evaluate the preventive effect of gels against abrasive challenges. This was done through individually brushing the specimens brushing for 15 s, two times per day. The evaluation of this type of wear becomes important due to high prevalence of inadequate brushing [38] and to the high degree of abrasivity of certain toothpastes, especially those with whitening properties [39]. This becomes even more harmful when abrasion is followed by an erosive challenge [40]. However, the present protocol allocated a time of 30 minutes between the erosive challenge and the abrasive challenge. This time was important to allow some degree of rehardening of enamel, since during the erosive challenge there is removal of a superficial layer of enamel and the remaining layer becomes softened [4]. Our results from profilometry showed that the ERO+ABR condition was able to promote a significantly greater wear compared to the ERO condition. This result demonstrates the standardization and good performance of all volunteers in the abrasion methodology. Moreover, the Cg+Cane also led to lowest enamel loss compared to all the other groups. Thereafter, the degree of protection significantly decreased for the Cg+NaF, Cg and Nt, i.e., the results for the ERO+ABR condition followed the same pattern as those in the ERO condition.

Remarkably, this study also presented a different analysis tool for erosive tooth wear, namely the surface reflection intensity assessed with the hand-held reflectometer Optipen. This pen-like device was successfully employed in erosion studies *in vitro* [28, 41] and particularly for a study involving the protection of CaneCPI-5 against dental erosion *in vitro* [14]. This is the first study in which the device is employed under an *in situ* protocol. The Cg+Cane group obtained greater reflection (which means significant protection against enamel wear) in ERO condition, compared to the other groups. However, it did not significantly differ from the Cg+NaF in the ERO+ABR condition, as well as there was no difference between the Nt and the Cg groups (in both conditions). Only the Nt group showed a significant difference between ERO and ERO+ABR. When the results found for the reflectometer are compared to those obtained with the profilometer, it is evident that the latter can discriminate better among distinct treatments. The lack of difference shown by the reflectometer can be explained in two ways: 1) Due to the high amount of erosive challenge (30 min) on the enamel surface, the reflection analysis might have lost sensitivity to detect small differences between conditions and groups [41, 42]. In addition, this aspect may

have been more prominent in the ERO+ABR condition, since in the profilometric analysis there was greater enamel wear; and 2) by the AP engineering, which may have influenced (with increased reflection) [14] due to the strong binding of CaneCPI-5 [12] and other AP proteins on the enamel surface, especially in the ERO condition, in which there was no mechanical wear caused by brushing, keeping the proteins present on the enamel surface and consequently, causing greater reflection [14, 42].

Due to these small inconsistencies between the reflectometer and the profilometer data, it became important to verify the correlation between the methods. An *in vitro* study demonstrated that the hand-held reflectometer correlates well with surface hardness and calcium release measurements when erosion was superficially assessed [28]. However, the lack of correlation of the reflectometer with the profilometer, which assesses more severe erosion (with loss of enamel structure) was unknown so far. Our results showed a significant negative moderate correlation between the reflectometer and the profilometer. While the reflectometer shows increasing figures indicating a protection, the profilometer shows decreasing figures that also indicate protection (less enamel loss). This negative correlation thus enhance the potential for clinical use of the Reflectometer to assess enamel wear *in vivo*, ranging from early erosive stages (with correlation to microhardness and calcium analysis) [28] to more severe stages, with correlation to profilometry (as shown in this study). It is important to highlight that the limitations presented here by the reflectometer (loss of sensitivity) will hardly be translated to *in vivo* studies, due to the great wear caused in the present *in situ* study, which might not occur *in vivo*, due to the slow progression of erosive wear. Furthermore, the values obtained by the reflectometer are not easily translated into exactly how much wear (in µm) occurs on the tooth surface, since the reflectometer only evaluates the exposed surface, without considering the layers lost by the erosive challenge. The logical and future sequence will be to develop an *in vivo* study to evaluate the protective role of the chitosan gel containing CaneCPI-5 against erosive wear, using the Reflectometer Optipen as a response variable. It would also be interesting to shed light onto the mechanism of action between chitosan gel and CaneCPI-5 on enamel.

Several points can be highlighted from the present study: 1) the potential of a chitosan gel to protect against erosive enamel wear; 2) the satisfactory results of a chitosan gel containing NaF and, particularly, CaneCPI-5 to reduce erosive enamel wear; 3) prevention of erosive enamel wear by acquired pellicle engineering with only one daily application of gel (product for professional application); 4) the novelty of the protective effect of gels on enamel in the ERO+ABR condition 5) the use of an equipment capable of evaluating wear *in situ* and its correlation with profilometric analysis.

Based on our results, the null hypothesis was rejected. In conclusion, the chitosan gel containing CaneCPI-5 was able to prevent enamel ERO and ERO+ABR *in situ*. These results open a new perspective for the development of an innovative professional dental product, aiming at the prevention of enamel erosive wear.

Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

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Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This work was approved by the Research Ethics Committee of Bauru School of Dentistry, University of São Paulo, SP, Brazil (CAAE: 86783418.8.0000.5417). The use of bovine teeth for this research was also approved by the Ethics Committee on Animal Use of the Bauru School of Dentistry, University of São Paulo (Protocol: 005/2018).

Informed consent: Informed consent was obtained from all individual participants included in the study. Sixteen volunteers (eight women and eight men) took part in the study, after signing an informed consent document. The volunteers participated in 4 crossover and double-blind phases.

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Fig 1. Mean enamel loss of bovine enamel specimens by contact profilometry. Light gray columns represent the groups with the erosion procedure. Dark gray columns represent the groups with erosion and abrasion procedures. Upper case letters denote significant differences between the conditions. Lower case letters show significant differences among the treatments (2-way RM ANOVA and Sidak´s test, $p<0.05$). n=16. Bars denote SD.

Fig 2. Relative surface reflection intensity (%SRI) of bovine enamel specimens. Light gray columns represent the groups with the erosion procedure. Dark gray columns represent the groups with erosion and abrasion procedures. Upper case letters denote significant differences between the conditions. Lower case letters show significant differences among the treatments (2-way RM ANOVA and Sidak´s test, $p<0.05$). n=16. Bars denote SD.

Fig 3. Correlation between contact profilometry (μm) and reflectometer Optipen (%SRI) for erosion (Solid line; $y = -0.0149x + 2.1231$) and erosion + abrasion (Dashed line; $y = -0.0163x + 2.2887$).