UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

JOÃO VICTOR FRAZÃO CÂMARA

Evaluation of the effect of a new sugarcane-derived cystatin (CaneCPI-5) on the profile and viability of microcosm biofilm, as well as on the prevention of dentin demineralization

Avaliação do efeito de uma nova cistatina derivada da cana-de-açúcar (CaneCPI-5) sobre o perfil e viabilidade de biofilme microcosmo, bem como na prevenção da desmineralização da dentina

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Supervisor: Prof. Dr. Marília Afonso Rabelo Buzalaf

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“O sucesso é a soma de pequenos esforços repetidos dia após dia.”

Robert Collier

ABSTRACT

Recently, a new sugarcane-derived cystatin (CaneCPI-5) was produced in a heterologous expression system and demonstrated a high binding capacity to enamel, protecting against enamel erosion in vitro. In addition, matrix proteases are very important for the progression of caries in dentin and cystatins, including CaneCPI-5, are inhibitors of cysteine-catepsins, which may have an additional effect in preventing caries in dentin. The objective of this work was to evaluate the effect of different concentrations of CaneCPI-5 on the bacterial profile and viability of a microcosm biofilm, as well as on the prevention of dentin demineralization. To form the microcosm biofilm, saliva from 10 healthy individuals was collected, diluted (70% saliva and 30% glycerol) and mixed with McBain's artificial saliva (1:50) to form the cariogenic biofilm. Samples (4 mm x 4 mm) of bovine dentin (n=90) were prepared for microcosm biofilm formation for 5 days, treated daily (1x60s/day) with concentrations of CaneCPI-5 0.025 and 0.05 mg/ml, 0.12% chlorhexidine (CHX) (positive control), Fluoride (500 ppm F, as NaF) or PBS (negative control). Evaluation of the profile of the formed biofilm (colony forming units) and the metabolic activity of the biofilm through the addition of resazurin were carried out. The demineralization caused by the biofilm under the conditions studied was measured by transversal microradiography. CXH reduced the metabolic activity of the microcosm biofilm compared to the negative control and treated groups (p 0.01). CHX and F significantly reduced the counts of total microorganisms, mutans streptococci and lactobacilli when compared to the negative control. None of the treatments were able to significantly reduce dentin demineralization compared to the negative control. In the evaluated model, CaneCPI-5 neither changed the profile and viability of the microcosm biofilm nor protected the dentin against demineralization.

Keywords: Antimicrobial agent; dental biofilm; dental caries; dentin.

RESUMO

Recentemente, uma nova cistatina derivada da cana-de-açúcar (CaneCPI-5) foi produzida em sistema de expressão heteróloga que demonstrou uma alta capacidade de ligação ao esmalte, protegendo contra a erosão do esmalte in vitro. Em adição, as proteases da matriz são muito importantes para a progressão da cárie em dentina e as cistatinas, incluindo a CaneCPI-5, são inibidores de cisteíno-catepsinas, o que pode ter um efeito adicional na prevenção da cárie em dentina. O objetivo deste trabalho foi avaliar o efeito de diferentes concentrações da CaneCPI-5 sobre o perfil bacteriano e viabilidade de um biofilme microcosmo, bem como na prevenção da desmineralização da dentina. Para a formação do biofilme microcosmo, a saliva de 10 indivíduos saudáveis foi coletada, diluída (70% saliva e 30% glicerol) e misturada à saliva artificial de McBain (1:50) para formação do biofilme cariogênico. Amostras (4 mm x 4 mm) de dentina bovina (n=90) foram preparadas para a formação do biofilme microcosmo por 5 dias, tratadas diariamente (1x60s/dia) com CaneCPI-5 nas concentrações de 0,025 e 0,05 mg/ml, clorexidina (CHX) 0,12% (controle positivo), Fluoreto (500 ppm F, como NaF) ou PBS (controle negativo). Foram realizadas avaliação do perfil do biofilme formado (Unidades formadoras de colônias) e da atividade metabólica do biofilme por meio da adição da resazurina. A desmineralização provocada pelo biofilme nas condições estudadas foi mensurada por microradiografia transversal. A CHX reduziu a atividade metabólica do biofilme do microcosmo em relação aos grupos controle negativo e tratado ($p < 0,01$). CHX e F reduziram significativamente as contagens de microrganismos totais, estreptococos mutans e lactobacilos quando comparados com o controle negativo. Nenhum dos tratamentos foi capaz de reduzir significativamente a desmineralização da dentina em comparação com o controle negativo. No modelo avaliado, o CaneCPI-5 não alterou o perfil e a viabilidade do biofilme do microcosmo nem protegeu a dentina contra a desmineralização.

Palavras-chave: Agentes antimicrobianos; biofilme dentário; cárie dentária; dentina.

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1 INTRODUCTION

1 INTRODUCTION

Dental caries is one of the most important chronic oral diseases, caused by different species of acidogenic and aciduric microorganisms, organized in a dental biofilm. These microorganisms metabolize dietary sugars, especially sucrose, producing extracellular polysaccharides and acids, which alter the pH of the biofilm and induce tooth demineralization (Keyes, 1960; Marsh et al., 2011; Pitts et al., 2017).

Despite being easily preventable, dental caries is the most prevalent chronic disease worldwide, constituting an important public health problem and consuming considerable resources for its treatment and for the treatment of its sequelae. In the European Union, in 2011, annual expenditure on dental treatment was estimated at 79 billion euros (Rugg-gunn, 2013), while in the United States of America, this cost was 111 billion dollars in 2012. Statistical data on spending on dental treatment in Brazil are not easily available, but due to economic development and the HDI (Human Development Index) of some European countries and the United States of America, it can be speculated that proportionally approximate figures are spent. Worldwide, about 60-90% of children and almost 100% of adults have or have had caries, which often leads to pain or discomfort (Organization, 2012).

Recently, a new sugarcane cystatin, CaneCPI-5, was identified and showed activities very similar to those of CaneCPI-4, being able to efficiently inhibit a large number of cysteine-peptidases, including cathepsin B, which is resistant to inhibition by cystatins. Inhibition of cathepsin B may be important for the preservation of the demineralized organic matrix of dentin, as it has been observed that the expression of cathepsin B, as well as the nonspecific activity of cathepsins in carious dentin are significantly higher when compared to those in healthy dentin (Nascimento et al., 2011). In addition, CaneCPI-5 was much more soluble when produced in a bacterial expression system, which facilitates its production and purification.

Thus, by accumulating the desirable characteristics mentioned above, CaneCPI-5 was evaluated in the present study, in an attempt to increase the acid resistance of the acquired film, as well as to inhibit dentinal cysteine cathepsins, delaying the progression of dentin caries. Due to the existing homology between

plant and animal cystatins (Margis et al., 1998) and the low production cost of plant cystatins, they can be excellent alternatives for insertion in dental products aimed at preventing dental caries. A recent publication by our research group revealed, by atomic force microscopy (AFM), that CaneCPI-5 has a great binding strength to enamel (6 times greater than the control). Topographic images were also taken of enamel samples coated with mucin 2.7 mg/mL, casein 10 mg/mL and CaneCPI-5 0.086 mg/mL, before and after incubation with citric acid (0.65%, pH 3.4 for 1 min). Only CaneCPI-5 protected enamel against citric acid-induced damage. In addition, using an in vitro early erosion model (Cheaib and Lussi, 2011), we demonstrated that treating the enamel surface with CaneCPI-5 protects the enamel against early erosion, with the best concentration being 0.1 mg/mL (Santiago et al., 2017), with no additional protection with the use of higher concentrations in the case of prevention of enamel erosion. However, it is necessary to evaluate the ideal concentration of CaneCPI-5 for preventing dentin caries.

These results make clear the great potential of CaneCPI-5 in preventing tooth decay due to its ability to interact with hydroxyapatite. It is also a protein with low production cost and high thermal stability, which makes it ideal for inclusion in dental products. However, before new products are developed for insertion in the market, more studies are needed, testing the application of this protein for caries prevention. Especially in dentin, a great protective effect is expected, since the protein could be expected to act on 3 fronts: strengthening of the acquired pellicle, antimicrobial action and inhibition of cysteine cathepsins, delaying the degradation of the demineralized organic matrix.

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Effect of a sugarcane cystatin on the profile and viability of microcosm biofilm and on dentin demineralization

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Abstract

To analyze the effect of a sugarcane cystatin (CaneCPI-5) on the microbial profile and viability, as well as on the prevention of dentin demineralization using a microcosm biofilm model. Ninety bovine dentine specimens were divided into 5 experimental groups according with the solution they were treated for 60 s: 1) PBS (negative control), 2) 0.12% chlorhexidine (positive control), 3) Fluoride (500 ppm F, as NaF), 4) 0.025 mg/ml CaneCPI-5 and 5) 0.05 mg/ml CaneCPI-5. Specimens were incubated with inoculum (McBain's saliva plus human saliva) in the first 8 h and from then on, they were exposed to McBain saliva containing sucrose and daily treated (60 s) with the solutions for 5 days. Resazurin and colony-forming unit counting assays were performed. Dentin demineralization was measured by transverse micro-radiography (TMR). 0.12% chlorhexidine significantly reduced the metabolic activity of the microcosm biofilm in relation to the negative control and treated groups ($p < 0.01$). CHX and F significantly reduced the counts of total microorganisms, mutans group streptococci and lactobacillli when compared with the negative control. None of the treatments was able to significantly reduce dentin demineralization in comparison with the negative control. In the model evaluated, CaneCPI-5 neither altered the microcosm biofilm profile and viability nor protected dentin against demineralization.

Keywords: Dental Caries; Dentin; Biofilms; Demineralization; Cystatin B.

Introduction

Dental caries is caused by different species of acidogenic and aciduric microorganisms, organized in a dental biofilm. These microorganisms metabolize sugars in the diet, especially sucrose, producing extracellular polysaccharides and acids, which alter the biofilm pH and induce tooth demineralization (Pitts et al. 2017).

Although dental caries can be easily prevented, it is the most prevalent chronic disease worldwide, constituting an important public health problem and consuming considerable resources for the treatment of its sequelae. Since the prevalence of caries is high and its consequences are undesirable, prevention is highly necessary. Due to its multifactorial etiology, several preventive and therapeutic possibilities have been proposed for the control of caries (Bowen, 2016).

Saliva is the most important host-derived protective factor against dental caries. This fluid is supersaturated with respect to hydroxyapatite and, under favorable conditions, promotes remineralization. Moreover, it buffers the bacterial acids, besides removing them from the oral cavity. Furthermore, saliva is the main source of proteins that constitute the acquired pellicle, a bacteria-free layer that acts as a semipermeable membrane, reducing the contact of the acids with the tooth surface (Buzalaf et al. 2015). Due to its protective ability against acids, acquired pellicle engineering procedures have been recently proposed. These procedures involve mainly the reinforcement of the acquired pellicle with acid-resistant proteins, to improve its protective capacity, which has been successfully employed against initial enamel erosion in vitro (Martini et al. 2020; Santiago et al. 2017; Taira et al. 2020) and in vivo (Carvalho et al. 2020).

Considering that the acquired pellicle in a semipermeable layer that interposes between the tooth surface and the dental biofilm, it might also protect against bacterial acids. In addition, the adhesion of microorganisms to the tooth surface is preceded by the adsorption of proteins (Hannig and Joiner 2006; Lendenmann et al. 2000). This means that the composition of the acquire pellicle drives microbial colonization. With this is mind, we recent showed that acquired enamel pellicle engineering with a sugarcane cystatin (CaneCPI-5) that has a strong affinity to hydroxyapatite reduced the activity of microcosm biofilms, as well as the counts of

total streptococci, mutans group streptococci and lactobacilli, besides reducing the demineralization of the underlying enamel (Araújo et al. 2021).

In addition, CaneCPI-5 efficiently inhibits a large number of cysteine-peptidases, including cathepsin B (Santiago et al. 2017). Inhibition of cathepsin B may be important for the preservation of the demineralized organic matrix of dentin (Buzalaf et al. 2015; Nascimento et al. 2011), which would be an additional mechanism of protection of dentin demineralization by CaneCPI-5.

Thus, we hypothesized that the modification with CaneCPI-5 would be able to change the microbial profile and viability of the microcosm biofilm and to reduce demineralization of the underlying dentin.

Material and Methods

Ethical aspects

The project was approved by the Bauru School of Dentistry (University of São Paulo) Ethics Committee on Human Research under registration CAAE 14953719.1.0000.5417. The volunteers, saliva's donors, participated after signing an Informed Consent Form. Due to the use of bovine teeth, the project was also approved by the Ethics Committee on Use of Animals under registration 009/2019.

Heterologous expression of CaneCPI-5

CaneCPI-5 was expressed in the bacterial strain *E. coli Rosetta* (DE3), transformed with the plasmid pET28aCaneCPI-5, as previously described. The expressed protein was purified from the soluble fraction of bacterial cultures induced by IPTG (Isopropyl-beta-D-Thiogalactosidium), submitted to centrifugation and sonication. Purification was done by affinity chromatography, using columns containing Ni-NTASuperflow nickel resin (Soares-Costa et al. 2002).

Saliva collection and inclusion/exclusion criteria for participants

The saliva of 10 healthy individuals (25-35 years old) was collected. The donors met the following inclusion criteria: 1) adequate salivary flow (stimulated> 1 mL/min and unstimulated > 0.3 mL/min), 2) history of carious lesions but without

active caries lesions (active white spot or/and cavitated lesions), 3) without signs of gingivitis/ periodontitis (presence of bleeding and tooth mobility) and 4) without ingestion of antibiotics in the last 3 months. The exclusion criteria included conditions opposite to those presented above, as well as cases in which the individual had chronic systemic diseases. Smokers, pregnant women, or lactating mothers were also excluded.

On the day of collection, the participants did not brush their teeth for 24 hours and abstained from eating food and liquids in the 2 hours prior to the collection of saliva stimulated by chewing rubber. The saliva collection was carried out in the morning (9-10 h), for 10 min, in a refrigerated and individual container, on ice. The volume of saliva was collected, diluted (70% saliva: 30% glycerol) and frozen.

Preparation of dentin samples and treatment groups

Ninety bovine enamel samples (4 mm x 4 mm) were prepared using a precision cutter (Buehler, USA) and diamond cutting discs. The samples were polished with 600-grit sandpaper (Extec, USA), using a polishing machine (Arotec, Brazil), to remove grooves / cracks in the surface. The roughness (Ra - mean arithmetic roughness) was measured using a contact profilometer (Marh, Germany), for the selection and allocation of samples in the experimental groups so that all groups had samples with similar initial conditions (Ra between 0.200 and 0.500 µm). Then, the samples had 2/3 of the surface protected with cosmetic nail polish to obtain control areas.

The samples were divided into 5 experimental groups (n = 18): 1) PBS (negative control), 2) 0.12% chlorhexidine (positive control), 3) Fluoride (500 ppm, as NaF), 4) 0.025 mg/ml CaneCPI-5 and 5) 0.05 mg/ml CaneCPI-5.

Prior to the beginning of the experiment, the specimens were sterilized by exposure to ethylene oxide gas for 4 h under pressure of 0.5 ± 0.1 Kgf / cm^2 (Acecil Sterilization Central, Campinas, SP).

Formation of microcosm biofilm and treatment

Before exposure to human saliva, the samples were treated with the protein solutions, fluoride, chlorhexidine or PBS, for 1 min (1.5 ml per well), in order to allow

binding of CaneCPI-5 on the dentin surface (Santiago et al. 2017).

The human saliva collected was diluted (1:50) in McBain artificial saliva, containing: 2.5 g l^{-1} of mucin from porcine stomach (type II), 2.0 g l^{-1} of bacteriological peptone, 2.0 g l^{-1} of tryptone, 1.0 g l^{-1} of yeast extract, 0.35 g l^{-1} of NaCl, 0.2 g l^{-1} of KCl, 0.2 g l^{-1} of $CaCl_2$, 0.1 g l^{-1} of cysteine hydrochloride, 0.001 g l^{-1} of hemin and 0.0002 g l^{-1} of vitamin K1, at pH 7.0 (McBain 2009). The reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).

Forty-eight-well plates containing dentin samples (n=9, divided into 3 independent experiments for resazurin and UFC experiments separately) were filled with 1.5 mL of inoculum (human saliva-glycerol + McBain saliva) and incubated with 5% CO_2 at 37 ° C for 8 hours. Only on the first day of the biofilm formation process human saliva-glycerol was used, to allow initial colonization. The other exchanges involved only McBain's artificial saliva supplemented with 0.2% sucrose.

After the initial 8 h, the samples were washed with PBS for 5 s and 1.0 mL of fresh medium (McBain's artificial saliva) supplemented with 0.2% sucrose was added to each well and incubated at 5% CO_2 at 37 °C for 16 h, completing the initial 24 h. Every 24 h, the medium was changed until completing 5 days of cultivation. From the 2nd day of growth of the biofilm, before changing the medium, the biofilm was treated once a day. For this, the samples were exposed to 1.5 ml of the treatment solution. After 60 s, the solution was removed, and fresh medium was added to the well (Araujo et al. 2021; Zhang et al. 2013).

Evaluation of metabolic activity by the Resazurin assay

After the treatments, the specimens were washed with 1.0 ml PBS to remove dead/non-adherent bacteria. Subsequently, the specimens were allocated to other 48-well sterile plates, and 1.0 ml of 0.0016% resazurin dye diluted in PBS was added. The plates were protected with aluminum foil and then incubated for 2 hours at 37ºC and 5% CO_2 (Ahmed et al. 1994; Alonso et al. 2017; Jiang et al. 2011). The microplates containing the resazurin dye were taken for the absorbance reading to be performed. The Synergy H1 microplate reader (Biotek®) was used to read absorbance (570 nm and 600 nm), coupled with the Gen5 software. Thus, the metabolic activity of the cariogenic biofilm after exposure to the different treatments was determined based on the percentage of viability (Jayme et al. 2017). The dentin

specimens removed from the solution were stored in plastic tubes for TMR analysis.

Colony Forming Units (CFU) Count

The biofilms were dispersed by sonication on ice at 40 mW, 1 pulse s^{-1} for 1 min (Single UltraSonic Cell Disruptor, Merse, Campinas, Brazil) and homogenized in vortex. Then, 100 µL of the bacterial suspension were diluted 1:100.000 and spread on the Petri dishes (25 µL/dish) containing different types of agar: (1) brain heart infusion agar (BHI, Difco, Detroit, MI, USA) for total microorganisms; (2) Mitis Salivarius agar (MSA, Neogen, Indaiatuba, Brazil) containing 20% sucrose and 1% potassium tellurite for total streptococci; (3) SB-20 M containing 15 g of bacto-casitone (Difco), 5 g of yeast extract (Kasvi, Curitiba, Brazil), 0.2 g of L-cysteine hydrochloride (Sigma, Steinheim, Germany), 0.1 g of sodium sulfite (Sigma), 20 g of sodium acetate (Synth), 200 g of coarse granular cane sugar, 15 g of agar (Kasvi) and 0.2 U ml^{-1} of bacitracin (Sigma, Steinheim, Germany) in 1 liter distilled water (autoclaved) for the determination of mutans group streptococci (*S. mutans* and *S. sobrinus*) and (4) Rogosa (Kasvi) supplemented with 0.13% glacial acetic acid to assess the number of *Lactobacillus* sp. The plates were incubated at 5% CO_2 and 37 °C for 48 h and the CFU numbers were counted and transformed to $\log_{10}$ CFU ml^{-1}.

Analysis of demineralization by transverse micro-radiography (TMR)

The dentin samples were prepared for the transverse micro-radiography assay. For this, half of the specimen was additionally sectioned longitudinally in the center of the surface, using a precision cutting device (Buehler, USA). Several fragments were obtained, including protected and demineralized areas. The fragments were manually polished with 600 grit sandpaper (Extec, USA), making it possible to reach appropriate thicknesses for the analysis (approximately 100-120 µm).

Subsequently, the samples were glued with adhesive tape to the sample holder, containing the aluminum pattern with different thicknesses, which generates different step wedges (gray gradations). The sample holder was inserted into the cassette, together with the glass plate in a dark environment, which was wrapped in a black bag and inserted into the X-ray generator (Softex, Japan). Thus, the glass

plate was sensitized by X-ray (20 kv and 20 mA) for 13 min. After each exposure, the glass plate was developed, fixed in a dark environment at 20ºC and washed.

The image analysis was performed using a microscope (Zeiss, Germany) with CCD camera (Canon, Japan) and accessories coupled to a computer with 2012 software for capture and 2006 for analysis (Inspektor Research System, Netherlands). Mineral loss (ΔZ) and depth of the lesion (μm) were calculated from gray values of standard micro-radiographs using the formula of Angmar et al. 1963. ΔZ (% vol.μm) is defined as integrated mineral loss, calculated from the difference between the percentage of mineral volume in healthy dentin (50%) and the percentage of mineral volume in demineralized dentin multiplied by the lesion depth (μm). The depth of the lesion is defined by the distance from the surface (LD, 0% vol min) to the depth at which the dentin has a mineral content equal to or greater than 95% of the mineral content of the healthy dentine (μm), equivalent to 47.5% (Arends and ten Bosch, 1992).

Statistical analysis

GraphPad InStat software version 3.0 for Windows (GraphPad Software Inc., La Jolla, Ca, USA) was used. The Kolmogorov-Smirnov test was applied to verify whether the data showed normal distribution and the Bartlett test to assess their homogeneity. After this initial check, the data were evaluated by the Kruskal-Wallis non-parametric test, followed by the Dunn´s test for individual comparisons. The level of significance adopted was 5%.

Results

Metabolic activity by the Resazurin test

The Kruskal-Wallis test revealed a significant difference between the groups (KW = 21.236, p = 0.0003). Dunn's test revealed that only 0.12% chlorhexidine significantly reduced the metabolic activity of the microcosm biofilm in relation to the negative control and the other treated groups ($p < 0.01$), which did not differ significantly from each other ($p > 0.05$) (Table 1).

Colony Forming Units (CFU) Count

The results of CFU counts for different groups are presented in Figure 1. There was no significant difference between the tested CaneCPI-5 concentrations and the control group with respect to CFU count. Fluoride showed a significant effect in reducing the CFU count for the Total microorganisms and Mutans Group Streptococci. The best efficacy against all analyzed microorganisms was found for chlorhexidine (positive control) that significantly differ from PBS.

Demineralization by transverse micro-radiography (TMR)

The Kruskal-Wallis test revealed a significant difference between the groups (KW = 17.213, p = 0.0018) in relation to the integrated mineral loss (ΔZ) of the dentin after different experimental conditions. Dunn's test revealed that CaneCPI-5 group at a concentration of 0.05 mg/ml induced higher demineralization compared to the other groups ($p<0.001$) (Table 2). Regarding the lesion depth, there was no statistically significant difference among the groups (p = 0.2044).

Discussion

Several studies have investigated alternatives that can be as efficient as brushing and fluoride in order to prevent or reverse carious lesions, and that can be used safely by the population. Among these alternatives is CaneCPI-5, a cystatin cloned from sugarcane, capable of inhibiting cysteine-cathepsins, with high binding strength to hydroxyapatite, which inhibits initial erosion *in vitro* (Santiago et al. 2017) and *in vivo* (Carvalho et al. 2020). Due to the ability of CaneCPI-5 to bind to hydroxyapatite, there was a pre-exposure of the specimens to the treatment solutions before incubation in saliva. In addition, CaneCPI-5 has high thermal stability and low production cost. Due to these characteristics, we hypothesized that the protein could have a protective effect against dental caries, acting on several fronts. Because it binds to hydroxyapatite, it could strengthen the acquired pellicle, reducing demineralization by bacterial acids (Santiago et al., 2017). By inhibiting bacterial cysteine-cathepsins (Santiago et al. 2017; Klein et al. 2016; Kolenbrander 2000), it could have antimicrobial action and by inhibiting dentin cysteine-cathepsins, it could delay the degradation of the demineralized organic matrix (Nascimento et al. 2011; Buzalaf et al. 2015).

In this work, the microcosm biofilm model was used, as it presents desirable

characteristics for mimicking the oral environment, reproduces the conditions of a supragingival biofilm and presents complex microbial communities. It can be produced in the laboratory (controlled environment), reducing the possibility of bias (Arthur et al. 2013; Tang et al. 2003). Using this model, it was evaluated the in vitro protective effect of different concentrations of CaneCPI-5 in solution form, before and during the formation of the bacterial biofilm for 5 days, on the bacteria metabolism and caries development.

Resazurin method is an effective alternative for viewing the reading of tests on microplates. This compound works as a chromogenic substrate for dehydrogenase enzymes, acts as an oxy-reduction indicator, and is reduced (by hydrogen gain) by flavins linked to enzymes related to the transport system during cellular metabolism (Vaz Crippa et al. 2020). However, in the evaluated concentrations, CaneCPI-5 was not able to reduce the metabolic activity of the microcosm biofilm in relation to the negative control, having a lower performance than that of the positive control (0.12% chlorhexidine).

These results indicate that its action front does not seem to be related to the inhibition of bacterial cysteine-cathepsins, at least not in the concentrations used in the present study. In addition, no antimicrobial effect was seen for CaneCPI-5 on the CFU count. In a recent study conducted by our group using a similar protocol but employing enamel specimens, CaneCPI-5 at 0.05, 0.1 and 0.5 mg/ml significantly reduced the activity of microcosm biofilms compared to PBS (Araujo et al. 2021; Pelá et al. 2021). Moreover, all concentrations of CaneCPI-5 significantly reduced the lactobacilli and total streptococci, while for the mutans group streptococci, 0.05 mg/ml CaneCPI-5 performed better than 0.12% CHX (Araujo et al. 2021). As reported in relation to the results of viability, the absence of a protective effect of CaneCPI-5 (and CHX 0.12%) on dentin demineralization was also identified.

Understanding the difference in the results obtained for enamel and dentin specimens, using the same experimental design, requires a discussion about the differences between these two types of substrates, as well as the biofilms that are established on them. Regarding the types of bacteria present in biofilms installed over enamel and dentin, in the latter, in addition to acidogenic/aciduric bacteria commonly found in enamel, such as *Streptococcus mutans*, lactobacilli and bifidobacteria, there are also proteolytic species such as Prevotella and Propionebacterium (Takahashi and Nyvad 2016). In this way, it is possible that

CaneCPI-5 served as a substrate for these bacteria, and for this reason there was an increase in the biofilm viability by the Resazurin test (Figure 1), which may be further confirmed using other approaches. This might justify the lack of anticaries action of CaneCPI-5.

The possible lack of effect of CHX is possibly associated with low concentration. Despite the reduction in the number of mutans group streptococci in the present study, other acidogenic bacteria could be present in the biofilm, producing enough acids to induce demineralization. Further studies are needed to elucidate this finding. However, if a higher concentration were used, there could have been an effective reduction in the production of acids and consequently in the formation of the lesion, as reported in the findings of Dos Santos et al. 2019.

Enamel is the tissue with the highest mineral content in our body, being made up of 95% apatite, while dentin has about 70% mineral and about 30% organic matter. Thus, the formation of enamel caries is an essentially chemical process of dissolving hydroxyapatite by bacterial acids, produced by the consumption of sugars. In the case of dentin, the dissolution of the mineral content, given by the production of bacterial acids, not only promotes the exposure of the dentin organic matrix, but also triggers a mechanism of activation of host enzymes (supposedly linked to the organic matrix or from saliva), which would then lead to the degradation of collagen and other components of the organic dentinal matrix (Tjäderhane et al. 2015). These enzymes are mainly from two families: matrix metalloproteinases (MMPs) and cysteine cathepsins (CTs) (Femiano et al. 2016; Hannas et al. 2007; Mazzoni et al. 2015). These enzymes would then degrade the demineralized organic matrix, accelerating the progression of the carious lesion.

Thus, it was hypothesized that CaneCPI-5 would have an even greater effect in preventing caries in dentin compared to enamel, because this enzyme is a cysteine-cathepsin inhibitor, being able to efficiently inhibit a large number of cysteine-peptidases, including cathepsin B (Santiago et al. 2017), which is resistant to cystatin inhibition. Inhibition of cathepsin B could be important for the preservation of the demineralized organic matrix of dentin, as it has been observed that the expression of cathepsin B, as well as the nonspecific activity of CCs in decayed dentin are significantly higher when compared to those of healthy dentin (Nascimento et al. 2011). However, this was not the case in the present study. In our previous study, the Kis of CaneCPI-5 on cathepsins B, K and L were 6.87, 0.49 and 0.34 nM,

respectively (Santiago et al. 2017). In the present study, the concentrations of CaneCPI-5 used were 1.64 and 3.28 µM, therefore about two orders of magnitude higher than those necessary for the inhibition of cathepsins. It must be considered that the study by Santiago et al. 2017 was a simple assay of enzymatic kinetics without interfering, whereas in the present study the cathepsins were embedded in the dentinal matrix, which might have impaired the effect of the tested solutions.

Conclusions

According to the protocol of the present study, CaneCPI-5, at the tested concentrations, did not have antimicrobial effect on the biofilm and did not protect dentin against demineralization. Further mechanistic studies should be conducted to understand the lack of anticaries efficacy of CaneCPI-5 in dentin, considering that promising results were recently reported for enamel caries using similar protocols.

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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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Table 1. Median (95% CI) of the metabolic activity (resazurin test) of the microcosm biofilm formed on bovine dentin samples for 5 days, submitted to different treatments.

Treatment groups	Median (CI 95%)
PBS	100.0 (100.0-100.0)[a]
0.12% Chlorhexidine	12.5 (6.0 -18.5)[b]
500 ppm F (NaF)	118.1 (64.8-135.4)[a]
0.025 mg/ml CaneCPI-5	111.9 (76.6-131.3)[a]
0.05 mg/ml CaneCPI-5	121.8 (65.9-135.1)[a]

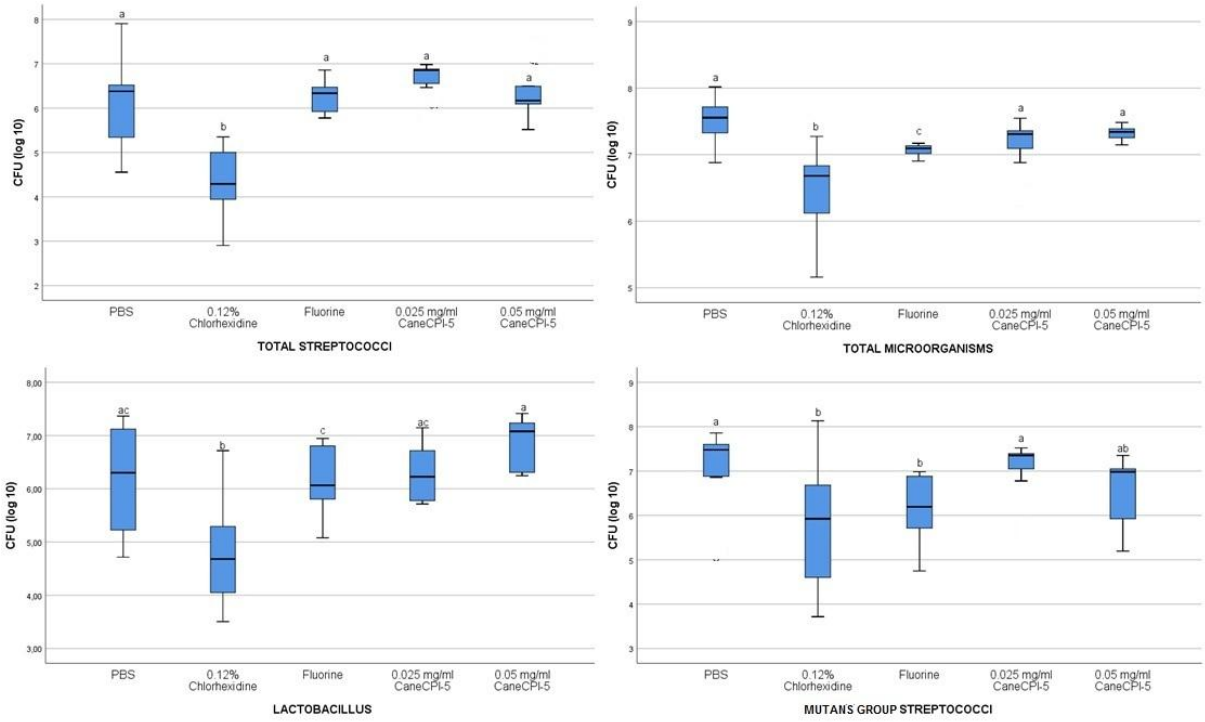
Values higher than 100% show high metabolic activity, while lower values point out reduction of metabolic activity. Distinctt letters indicate significant differences between the groups (Kruskal-Wallis and Dunn´s tests, $p < 0.05$). n = 9.

Table 2. Median (95% CI) of integrated mineral loss (% vol x µm) and lesion depth (µm) after treatments and formation of microcosm biofilm on dentin for 5 days.

Treatment groups	Integrated Mineral Loss (%vol x µm)	Lesion depth (µm)
PBS	875.0 (675.0-1300.0)[a]	63.4 (28.5-102.7)[a]
0.12% Chlorhexidine	650.4 (127.5-990.0)[a]	52.5 (13.8-86.6)[a]
500 ppm F (NaF)	960.0 (175.0-1093.3)[a]	71.5 (8.2-117.0)[a]
0.025 mg/ml CaneCPI-5	937.5 (475.0-1924.0)[a]	70.0 (17.2-125.4)[a]
0.05 mg/ml CaneCPI-5	1300.0 (914.0-1982.0)[b]	93.7 (78.3-116.8)[a]

Distinct letters indicate significant differences between the groups (Kruskal-Wallis and Dunn´s tests, $p < 0.05$). $n = 9$.

Figure 1. Viability of total microorganisms, total streptococci, mutans group streptococci and total lactobacilli in different groups.



3 DISCUSSION

3 DISCUSSION

Dental caries is a chronic oral disease, considered one of the most prevalent worldwide. It is a dysbiosis that arises from an imbalance in the biofilm caused by excessive intake of sugars, which leads to a predominance of acidogenic and aciduric microorganisms that promote dental demineralization (Mathur et al., 2018).

The initial phase of dental caries is characterized by the appearance of white spot lesions, which, if treated early, prevent cavitation on the tooth surface. Since the 1960´s it is known that the removal of the biofilm from the white spot lesion is capable of leading to lesion reversal, without any additional treatment (Backer Dirks et al., 1961). However, it is very difficult to remove all the biofilm with brushing and flossing. Thus, the use of fluorides has emerged as a great ally of toothbrushing and has led to a decline in the prevalence of caries worldwide, as it favorably intervenes in the de and remineralization reactions, shifting the balance in favor of remineralization (ten Cate & Buzalaf, 2019).

Thus, thinking of viable alternatives for the control of dental caries, a new cystatin derived from sugarcane was produced (CaneCPI-5). This protein presents a strong binding force to hydroaxypatite, inhibiting dental erosion in vitro and in vivo (Santiago et al., 2017; Carvalho et al., 2020). More recently, it was demonstrated, for the first time, that CaneCPI-5 in concentrations ranging between 0.05 and 0.5 mg/ml reduces the metabolic activity of the microcosm biofilm, as well as enamel demineralization. It was also observed a significant reduction in the counts of total streptococci and lactobacilli (Araújo et al., 2021). The microcosm biofilm model was used in order to reproduce the complexity of the oral environment, being a microbiological model that seeks to mimic dental demineralization through the addition of sucrose. The biofilm is formed from salivary inocula and/or dental plaque, which are natural resources of the oral microflora, whose primary characteristic is ecological complexity, with the advantage to control in vitro variables (Santos et al., 2019).

It was hypothesized that CaneCPI-5 could have an additional protective effect against dentinal caries due to its antimicrobial action by inhibiting bacterial

cysteine cathepsins (Santiago et al., 2017; Klein et al., 2016; Kolenbrander, 2000). Moreover, slow down the degradation of the demineralized organic matrix by inhibiting dentinal cysteine cathepsins (Nascimento et al., 2011; Buzalaf et al., 2015).

This was the first study to investigate the effect of a sugarcane-derived cystatin (CaneCPI-5) on biofilm viability and demineralization of bovine dentin in a microcosm biofilm model. Based on our results, the null hypothesis was confirmed, since CaneCPI-5 neither prevent dentin demineralization nor reduce the amount of microorganisms or their metabolic activity. Similar findings were reported in the study carried out by Pelá et al. (2021), i.e., CaneCPI-5 did not show anti-caries effect at concentrations of 0.1 mg/ml and 1.0 mg/ml using enamel samples in microcosm biofilm. However, in the study by Pelá et al. (2021), CaneCPI-5 was applied after the acquired pellicle had been formed, thus not favoring its main mechanism of action that is based on its binding to hydroxyapatite. When CaneCPI-5 was applied on the enamel surface, at concentrations ranging between 0.05 and 0.5 mg/ml, before the formation of the acquired enamel pellicle, it was able to protect enamel against demineralization under a similar microcosm biofilm model (Araújo et al., 2021).

In the present study, the concentrations of CaneCPI-5 tested were established based on a pilot study, in which concentrations of 0.025, 0.05 and 0.1 mg/ml were evaluated. Considering the cost/benefit ratio, we chose to evaluate lower concentrations in the present study. However, at the concentrations evaluated, CaneCPI-5 was not able to reduce the metabolic activity of the microcosm biofilm in relation to the negative control, having a lower performance than the positive control (chlorhexidine 0.12%). This indicates that the mechanism of action of CaneCPI-5 is not related to the inhibition of bacterial cysteine cathepsins, at least not in the concentrations evaluated. Moreover, we found no antimicrobial effect on the CFU counts, differently from which was observed for enamel at concentrations of 0.05, 0,1 and 0.5 mg/ml, using a similar model (Araújo et al., 2021; Pelá et al., 2021).

In order to understand these differences, it is important to consider the intrinsic differences between enamel and dentin, as well as between the biofilms that are established on them. Enamel contains typically acidogenic/aciduric bacteria, such as S. mutans, lactobacilli and bifidobacterial, while dentin also contains species that degrade proteins, such as Propionebacterium and Prevotella (Takehashi and Nyvad, 2016). Thus, one cannot exclude the possibility that, due to its nature, CaneCPI-5 served as substrate for these proteolytic species, which led to an increase in the

viability of the biofilm, as evaluated by the resazurin test. This might help to explain the lack of anticaries effect of CaneCPI-5 in dentin.

The lack of effect of CHX might be associated with the low concentration employed. In the present study, we observed reduction in the number of mutans streptococci in the group treated with CHX. However, other acidogenic bacteria might have produced acids, leading to demineralization. It is possible that higher CHX concentrations could have led to protection against demineralization, as previously reported (Dos Santos et al., 2019).

In conclusion, CaneCPI-5, at the concentrations evaluated, did not have antimicrobial effect on the biofilm and did not protect dentin against demineralization. Future mechanistic studies are required to shed light into the reasons leading to the lack of anticaries effect of CaneCPI-5 in dentin, since encouraging results have been reported for enamel employing similar protocols.

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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 **009/2019**
Recebido em: 03/05/2019
Maristela
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:	Abr/2019 a Mar/2021
Título da pesquisa:	Avaliação do efeito de uma nova cistatina derivada da cana-de-açúcar (CaneCPI-5) sobre o perfil e viabilidade de biofilme microcosmo, bem como na prevenção da desmineralização da dentina
Pesquisador Responsável:	Profa. Dra. Marília Afonso Rabelo Buzalaf
Pesquisador Executor:	João Victor Frazão Câmara
Colaboradores:	Vinícius Taioqui Pelá
Nota Fiscal/Termo de Doação adquirido: 90	Termo de Doação - Natara Dias Gomes da Silva (009/2018) **Total**
Nº Lote / Data do Abate	- / -
Nº utilizados / Nº de grupos:	15/5

ANNEX 2

USP - FACULDADE DE ODONTOLOGIA DE BAURU DA USP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação do efeito de uma nova cistatina derivada da cana-de-açúcar (CaneCPI-5) sobre o perfil e viabilidade de biofilme microcosmo, bem como na prevenção da desmineralização da dentina

Pesquisador: João Victor Frazão Câmara

Área Temática:

Versão: 3

CAAE: 14953719.1.0000.5417

Instituição Proponente: Faculdade de Odontologia de Bauru

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.720.167

Apresentação do Projeto:

idem ao parecer Consubstanciado 3.456.956, de 16 de Julho de 2019.

Objetivo da Pesquisa:

idem ao parecer Consubstanciado 3.456.956, de 16 de Julho de 2019.

Avaliação dos Riscos e Benefícios:

idem ao parecer Consubstanciado 3.456.956, de 16 de Julho de 2019.

Comentários e Considerações sobre a Pesquisa:

idem ao parecer Consubstanciado 3.456.956, de 16 de Julho de 2019.

Considerações sobre os Termos de apresentação obrigatória:

idem ao parecer Consubstanciado 3.456.956, de 16 de Julho de 2019.