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Antimicrobial and anti-caries effects of a novel cystatin from sugarcane on saliva-derived multispecies biofilms

SUMMARY

This study evaluated the antimicrobial (anti-biofilm) and anti-caries (enamel demineralization prevention) effects of a new cystatin derived from sugarcane (CaneCPI-5). Microcosm biofilm was produced on bovine enamel specimens (4 × 4 mm; n = 48) from a mixture of human saliva and McBain saliva at the first 8 h. From this moment until the end of the experiment, the enamel specimens were exposed to McBain saliva containing 0.2% sucrose and, once a day, they were treated with the test solutions for 1 min. This treatment was performed for 5 days. The solutions evaluated were: PBS (negative control), 0.12% chlorhexidine (positive control), 0.1 mg/ml CaneCPI-5 and 1.0 mg/ml CaneCPI-5. The biofilm viability was determined by fluorescence using confocal microscopy and the enamel demineralization was quantified using transverse microradiography (TMR). The data were analyzed by ANOVA/Tukey or Kruskal-Wallis/Dunn tests for biofilm and enamel, respectively (p < 0.05). With respect to the antimicrobial effect, all treatment solutions significantly reduced the biofilm viability compared with PBS. The best antimicrobial effect was found for 1.0 mg/ml CaneCPI-5 (82.37 ± 10.01% dead bacteria) that significantly differed from 0.12% chlorhexidine (73.13 ± 15.07% dead bacteria). For the anti-caries effect, only 0.12% chlorhexidine (ΔZ: 2,610, 1,683–4,343) performed significantly better than PBS (ΔZ: 8,030, 7,213–9,115), but 0.12% chlorhexidine did not significantly differ from 0.1 mg/ml CaneCPI-5. Under this experimental model, CaneCPI-5 significantly reduced the biofilm viability, but this effect was not reflected on its anti-caries potential.

Introduction

The oral biofilm is formed by a community of microorganisms that are grouped three-dimensionally on the surface of the tooth structure. Within the oral biofilm, *Streptococcus mutans, Lactobacillus* spp, *Actinomyces, Fusobacterium, Porphyromonas, Selenomonas, Bifidobacteria, Scardovia,* and *Haemophilus* are involved in the initiation and development of caries (Tanner et al. 2011; Solbiati & Frias-Lopez 2018). Most of them are known to be acidogenic and acid tolerant. Thus, in the presence of fermentable carbohydrates, they produce acids that reduce the biofilm pH, which can lead, in the long term, to the development of a caries lesion (Pitts et al. 2017). In addition, some of these microorganisms produce insoluble extracellular polysaccharides from the sucrose in the functional biofilm matrix. Moreover, they protect the biofilm layer against the host defense, such as pH neutralization by salivary buffers and action of antimicrobial proteins (Koo et al. 2013).

It is important to emphasize that the biofilm is not harmful to the host as long as there is symbiosis. For this, the hypothesis of the ecological plaque was stipulated, which proposes that microorganisms related to diseases can be found in healthy sites, but at low levels. However, homeostasis can be broken, leading to shifts in the balance of the microflora, thereby predisposing sites to disease (Marsh 1994).

Despite being prevented through the reduction in sugar consume and mechanical disorganization of the oral biofilm by toothbrushing (Çolak et al. 2013; Rugg-Gunn 2013), dental caries is still highly prevalent worldwide (Peres et al. 2019). Fluorides are broadly used to prevent dental caries (Ten Cate & Buzalaf 2019), but patients with high risk may need other approaches, such as antimicrobial agents (Plemons et al. 2014). The search for new compounds and methods for prevention of dental caries is object of several studies.

Given this need, a study using proteomic tools showed that cystatin-B was increased around 13-fold in the acquired enamel pellicle (AEP) after exposure to lactic acid. It was suggested that this acid-resistant protein might protect against dental caries (Delecrode et al. 2015). However, when considering the clinical application of this protein, we must emphasize that an important factor is the cost for its production. In this sense, the use of human cystatin-B has not been feasible. For this reason, our group recently cloned and characterized a new cystatin derived from sugarcane, named CaneCPI-5 (Santiago et al. 2017). The protein has been shown to be soluble when produced in a bacterial expression system (in *Escherichia coli*), which facilitates its production and purification with a low cost. Furthermore, the treatment with CaneCPI-5 on bovine enamel surface protected against initial dental erosion and demonstrated strongly a binding force to enamel (Santiago et al. 2017).

Moreover, phytocystatins exhibit antimicrobial properties, since they inhibit cysteine peptidases (van Wyk et al. 2016). CaneCPI-5 was shown to strongly inhibit cathepsins B, K, and L (Ki = 6.87, 0.49, and 0.34 nM, respectively) (Santiago et al. 2017), which makes its use to control the oral biofilm especially attractive. In the present study, we evaluated the antimicrobial (anti-biofilm) and anti-caries (enamel demineralization prevention) effects of CaneCPI-5, using a microcosm biofilm model. The null hypotheses tested were that CaneCPI-5 does not possess neither 1) antimicrobial nor 2) anti-caries effect in the model employed.

Material and methods

Saliva collection

This study was approved by the local Ethics Committee (86780918.5.0000.5417) and the participants signed an informed consent. Stimulated saliva was collected from ten healthy donors who refrained from ingesting foods and drinks and from brushing their teeth for two hours prior to collection. The donors fulfilled the following criteria: 1) normal salivary flow (stimulated and non-stimulated salivary flows > 1 ml/min and > 0.3 ml/min, respectively), 2) previous history of caries, but no caries activity (no active white spot and/or cavitated lesions), 3) no gingivitis/periodontitis (gum bleeding or tooth mobility), 4) no ingestion of antibiotics three months prior to the experiment, and 5) no use of antimicrobial agents three months prior to the experiment. The saliva was collected under stimulation (> 1 ml/min) by chewing a rubber material for ten minutes in the morning. After collection, the pool of saliva (132 ml) was diluted in glycerol (70% saliva and 30% glycerol). Aliquots of 1 ml were stored at –80 °C (Pratten et al. 2003).

Tooth specimens preparation

Bovine incisors underwent a process of screening and cleaning (removal of soft tissue) before specimens' preparation. Forty-eight enamel specimens (4 mm × 4 mm) were prepared, using a semi-precision cutting machine ISOMET Low Speed Saw (Buehler Ltd., Lake Bluff, IL, USA). The specimens were polished in a metallographic polishing machine (Arotec, Cotia, Brazil) using water-cooled silicon-carbide discs (600-grade papers ANSI grit, Buehler, Enfield, USA) to remove grooves. The average surface roughness (Ra) was verified by contact profilometer coupled to Mahr Surf XCR 20 software (Mahr, Göttingen, Germany). Then, two thirds of the specimens surfaces were protected with nail polish to obtain control areas for the transverse microradiography (TMR) analysis and the specimens were sterilized using ethylene oxide (gas exposure time [30% ETO/70%CO$_2$] for 4 h under a pressure of 0.5 ± 0.1 kgF/cm^2).

Heterologous expression of CaneCPI-5

The sugarcane cystatin (CaneCPI-5) was recombinantly produced in bacterial strain (*E. coli Rosetta* DE3), transformed with the plasmid pET28aCaneCPI-5. The expressed protein was purified from the soluble fraction of bacterial cultures induced by isopropyl-beta-D-thiogalactosidium (IPTG) and submitted to centrifugation and sonication. Then, the purification was done by affinity chromatography, using columns containing nickel resin Ni-NTA Superflow (Qiagen) (Santiago et al. 2017).

Microcosm biofilm formation and treatment

The experiment was performed as previously described (Braga et al. 2018). Firstly, the human saliva was defrosted and mixed with McBain artificial saliva (McBain 2009) in a proportion of 1:50. The McBain saliva was composed by 2.5 g/l mucin from porcine stomach (type II), 2.0 g/l bacteriological peptone, 2.0 g/l tryptone, 1.0 g/l yeast extract, 0.35 g/l NaCl, 0.2 g/l KCl, 0.2 g/l CaCl$_2$, 0.1 g/l cysteine hydrochloride, 0.001 g/l hemin, 0.0002 g/l vitamin K1, at pH 7.0. Then, the specimens were randomly divided among the groups according to the Ra values (specimens with 0.100 μm until 0.300 μm of enamel surface roughness were accepted), allowing similar enamel surface roughness means among the groups (0.146 ± 0.033 μm). In 24-well plates, the solution of human and McBain saliva was

added to each well (1.5 ml/well) containing an enamel specimen and the microplates were incubated at 5% CO_2 and 37 °C. After eight hours, the enamel specimens were washed with phosphate-buffered saline (PBS, 5 s) and fresh McBain saliva (containing 0.2% sucrose) was added into the wells (1.5 ml/ well). Furthermore, the microplates were incubated at 5% CO_2 at 37 °C for further 16 hours, finishing the first day.

From the second to the fifth day, the specimens were treated with the respective solutions (1 ml/well) once a day for 60 s, according to the following groups (n = 12/group): 1) PBS (negative control); 2) alcohol-free 0.12% chlorhexidine digluconate solution (PerioGard®, São Bernardo do Campo, Brazil); 3) 0.1 mg/ml CaneCPI-5; 4) 1.0 mg/ml CaneCPI-5. After the treatment, each day, the solutions were removed, the specimens were washed with PBS (5 s) and fresh McBain saliva (containing 0.2% sucrose) was added (1.5 ml/well). The microplates were incubated at the same conditions previously described. The experiment was done in triplicate. Each experiment consisted of four specimens (total n = 12).

Bacterial viability analysis

After the experimental phase, the biofilm formed on enamel was stained with nucleic acid markers diluted in PBS (1 ml PBS + 1 μl SYTO9 + 1 μl propidium iodide, 10 μl/well) (Live & Dead® cells viability assay, Thermo Fisher Scientific, Waltham, USA) for 15 min, without the presence of light. The biofilm was stained with SYTO 9 and propidium iodide. Both live and dead bacteria are permeable to SYTO 9, which binds DNA and RNA, emitting green fluorescence. Propidium iodide only permeates dead or damaged cells, emitting a red fluorescence (Hannig et al. 2013). Then, the confocal laser scanning microscope (CLSM) (Leica TCS SPE, Mannheim, Germany) and Leica Application Suite-Advanced Fluorescence software (LAS AF, Mannheim, Germany) were used to analyze the biofilm. The specimens were analyzed using an objective of 40× magnification with oil. The laser 488 nm was used to detect the green color and emission wavelengths were adjusted to 500–590 nm; the laser to propidium iodide was 532 nm with 590–655 nm. The biofilm was analyzed in 3D images with step size of 1 μm. Three images (275 μm^2) were obtained from each specimen surface and analyzed using BioImage L 2.0 software, to quantify the presence of live and dead bacteria (%) (Chaves de Paz 2009)

Transverse microradiography (TMR)

The samples were transversally sectioned into three parts. The middle part, containing the demineralized and protected area of the enamel and the subjacent dentin, was polished (laterally) to obtain slices with 80–100 μm of thickness. The enamel slices were fixed in a specimen holder together with an aluminum calibration step wedge with 14 steps. A microradiograph was taken using an X-ray generator (Softex, Tokyo, Japan) on the glass plate at 20 kV and 20 mA (at a distance of 42 cm) for 13 min (Pires et al. 2018). The glass plates were developed for 5 min, rinsed in deionized water, fixed for 8 min in a dark environment, and then rinsed in running water for 10 min and air-dried (all procedures were done at 20 °C). The developed plate was analyzed using a transmitted light microscope fitted with a 20× magnification objective (Zeiss, Oberkochen, Germany), a CCD camera (Canon, Tokyo, Japan), and a computer. Two images per specimen were taken using data-acquisition (version 2012) and interpreted using calculation (version 2006) softwares from Inspektor Research System bv (Amsterdam, The Netherlands).

The mineral content was calculated, assuming the density of the mineral to be 3.15 kg l-1 and 87 vol% of mineral content for the sound enamel (Angmar et al. 1963). The lesion depth (LD, μm) and the integrated mineral loss (ΔZ, %vol. μm) were calculated (Arends & Ten Bosch 1992).

Statistical analysis

All experiments were performed in triplicate with three data points for each replicate. Data from biofilm viability and TMR were statistically analyzed using Graph Pad Instat for Windows software (GraphPad Software Inc., San Diego, USA). The normal distribution and homogeneity were checked using Kolmogorov & Smirnov and Bartlett's tests, respectively. The % live and dead microorganisms from biofilms treated with the different solutions were compared using ANOVA followed by Tukey's test, while TMR data were analyzed by Kruskal-Wallis followed by Dunn's test. The level of significance was set at 5%.

Results

Biofilm viability

All the treatment solutions, except 0.1 mg/ml CaneCPI-5, performed equally and were able to significantly reduce the number of live bacteria and increase the number of dead bacteria compared to the negative control (PBS) (Fig. 1). Moreover, 0.1 mg/ml CaneCPI-5 did not significantly differ from 1.0 mg/ml CaneCPI-5 and 0.12% chlorhexidine (Fig. 1). The best antimicrobial effect was found for 1.0 mg/ml CaneCPI-5 (82.37 ± 10.01% dead bacteria) that significantly differed from 0.12% chlorhexidine (73.13 ± 15.07% dead bacteria). The corresponding percentages for 0.1 mg/ml CaneCPI-5 and PBS were 65.62 ± 19.64 and 46.90 ± 21.64%, respectively. Figure 2 shows the representative images obtained for each group using a CLSM.

## Transverse microradiography (TMR)

The median (95% CI) mineral losses (ΔZ) found for PBS, 0.12% chlorhexidine, 0.1 mg/ml CaneCPI-5 and 1.0 mg/ml CaneCPI-5 were 8,030 (7,213–9,115), 2,610 (1,683–4,343), 7,850 (5,718–8,668) and 8,100 (7,435–9,108) %vol.μm, respectively. Only 0.12% chlorhexidine performed significantly better than PBS, but did not significantly differ from 0.1 mg/ml Cane-CPI-5. In addition, no significant differences were detected between the two tested concentrations of CaneCPI-5 that did not significantly differ from PBS (Tab. I; Fig. 3).

The median (95% CI) lesion depths for PBS, 0.12% chlorhexidine, 0.1 mg/ml CaneCPI-5 and 1.0 mg/ml CaneCPI-5 were 166 (135–240), 78 (64–101), 158 (124–185) and 149 (124–198) μm, respectively. Only 0.12% chlorhexidine presented the lowest lesion depth, significantly differed from all groups (Tab. I; Fig. 3).

Discussion

Due to the high prevalence of dental caries, several studies have been carried out to control the progression of this disease. To the best of our knowledge, this is the first study that evaluated the effect of CaneCPI-5 on the prevention of dental caries. We employed a microcosm biofilm model that represents the natural microbiota in its entirety (Braga et al. 2020; Hope & Wilson 2003) and is more appropriate than single-species (Ccahuana-Vasquez & Cury 2010) or abiotic models (Buzalaf et al. 2010). The microcosm biofilm is the closest in vitro model to the clinical situation (Ledder et al. 2009; Sim et al. 2016) since it mimics the oral environment and reproduces the conditions of a supragin-

Fig. 1 Mean (±SD) percentage of live and dead bacteria after five days of microcosm biofilm formed on enamel specimens treated with different solutions. Distinct letters denote significant differences among the treatments.
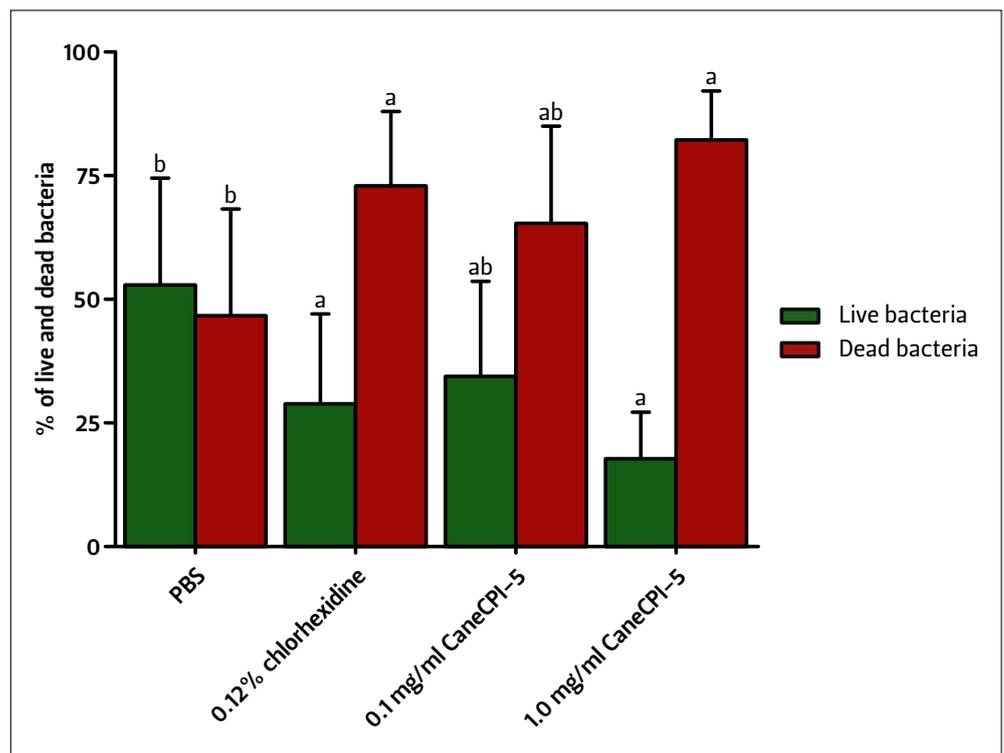
gival biofilm. Moreover, this model may become cariogenic upon exposure to sucrose (Tang et al. 2003; McBain 2009).

In the present study, as a positive control we chose 0.12% chlorhexidine which is a well-established antimicrobial agent for oral biofilm control (McGrath et al. 2019) but has side effects such as tooth staining and dysgeusia (Tartaglia et al. 2019), which limits its use in the long term. The mouth rinse Perio-

Tab. I Median (95% CI) of mineral loss (ΔZ) and lesion depth of bovine enamel specimens submitted to microcosm biofilm for five days and treated with different solutions. Distinct letters denote significant differences among the treatments.

Treatments	ΔZ (%vol.µm)	Lesion depth (µm)
PBS	8,030 (7,213–9,115)[b]	166 (135–240)[b]
0.12% chlorhexidine	2,610 (1,683–4,343)[a]	78 (64–101)[a]
0.1mg/ml CaneCPI–5	7,850 (5,718–8,668)[ab]	158 (124–185)[b]
1.0 mg/ml CaneCPI–5	8,100 (7,435–9,108)[b]	149 (124–198)[b]

For each variable, means followed by distinct letters are significantly different (Kruskal-Wallis and Dunn's test, p < 0.05). n = 12
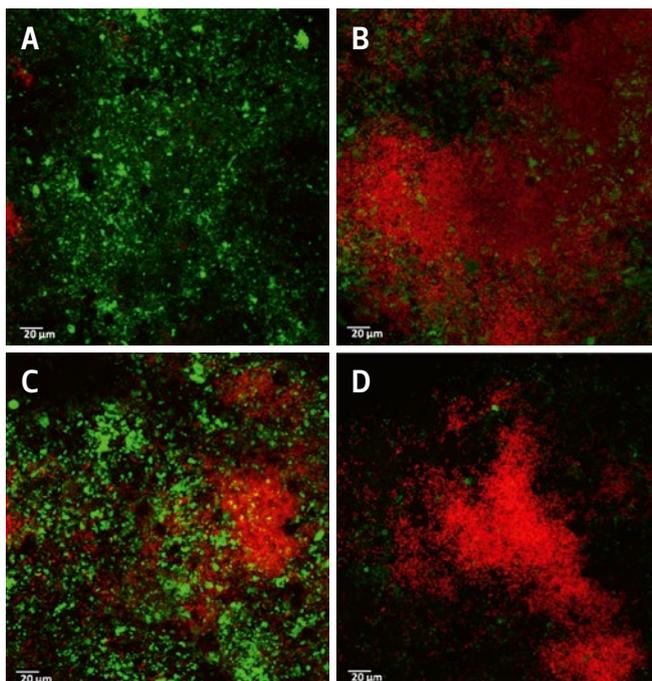

Fig. 2 Representative CLSM images from microcosm biofilms formed on enamel surface submitted to different treatments: A) PBS (negative control); B) alcohol-free 0.12% chlorhexidine digluconate solution; C) 0.1 mg/ml CPI–5; D) 1.0 mg/ml CaneCPI–5. Scale bar 20 µm.
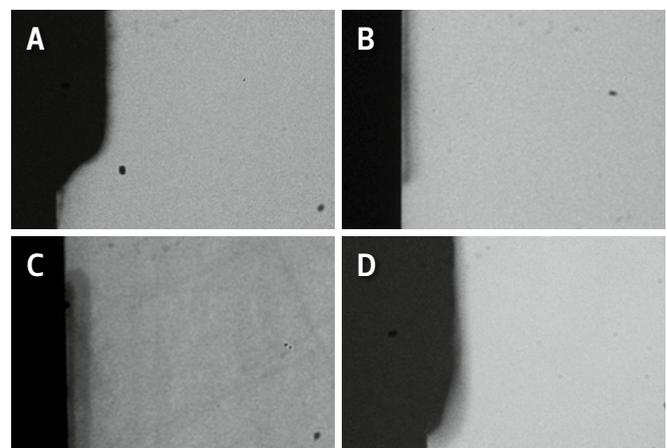

Fig. 3 Representative TMR images of enamel specimens submitted to microcosm biofilm for five days and treated with different solution: A) PBS (negative control); B) alcohol-free 0.12% chlorhexidine digluconate solution; C) 0.1 mg/ml CPI–5; D) 1.0 mg/ml CaneCPI–5.

Gard®· containing 0.12% chlorhexidine, has shown similar antimicrobial and anti-caries effects in previous studies (Coelho et al. 2017; Braga et al. 2018). The literature has shown that the effect of chlorhexidine compared with other antimicrobial agents is more pronounced in vitro than in vivo (Haerian-Ardakani et al. 2015). Chlorhexidine has already been shown to interfere with lactate production and during transport of sugar into the bacteria (Davies 1973; Walsh et al. 2015). However, chlorhexidine is also known to induce side effects such as tooth discoloration and astringent taste under uninterrupted use (James et al. 2017).

In addition, we tested two concentrations of CaneCPI-5. The concentration of 0.1 mg/ml was chosen based on the protective effect against initial dental erosion in vitro (Santiago et al. 2017). Since this is the first study employing this protein for protection against caries, we included a 10-fold higher concentration of CaneCPI-5 to see if the antimicrobial and anti-caries effect could be greater. Our results revealed that, except for 0.1 mg/ml CaneCPI-5, all treatment solutions had a similar performance and significantly reduced the biofilm viability compared to the negative control (Fig.1 and 2). These results led to the rejection of the first null hypothesis, since CaneCPI-5 at the higher concentration had antimicrobial effect, which might be due to its action of inhibitor of cysteine cathepsins (Santiago et al. 2017). However, the antimicrobial effect was not directly translated into anti-caries effect, since only 0.12% chlorhexidine significantly reduced the mineral loss and lesion depth in comparison to the negative control (Tab.I and Fig.3). Despite 0.1 mg/ml CaneCPI-5 did not significantly differ from 0.12% chlorhexidine regarding the mineral loss, it also did not significantly differ from placebo or 1.0 mg/ml CaneCPI-5. Thus, according with the present protocol, the second null hypothesis was accepted.

Many possibilities may help to explain the lack of correspondence between the antimicrobial and anti-caries effects found in the present study: 1) CaneCPI-5 might have reduced the viability of microorganisms that are not related to dental caries. Colony-forming unit experiments, evaluating different types of bacteria such as streptococci and lactobacilli, could shed light into the interpretation of these data; 2) the cariogenic challenge was very severe, considering the high degree of mineral loss and depth of the lesions formed (Tab.I). In this sense, experiments employing milder cariogenic challenges, such as those involving less days under microcosm biofilm could be elucidative; 3) in the present protocol, the treatment with the test solutions started after the formation of the AEP. Considering that CaneCPI-5 has strong ability to bind to hydroxyapatite (Santiago et al. 2017) and that cystatins in the AEP are resistant to removal by lactic acid (Delecrode et al. 2015), it would be interesting to perform experiments in which CaneCPI-5 is applied before the first incubation with saliva, in order to increase the amount of this acid-resistant protein in the AEP. This could potentialize its anti-caries effect that would then rely not only on its antimicrobial activity but also on its ability to reinforce the AEP. In addition, when we think about the possible clinical use of CaneCPI-5 to control caries, it is important to mention that both concentrations tested in the present study did not present a cytotoxic effect on human gingival fibroblasts, as evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay (unpublished data).

In conclusion, this is the first study evaluating the use of CaneCPI-5 to prevent dental caries. According to the protocol employed, the protein had antimicrobial activity that was not translated into anti-caries effect. These apparently not favorable results can be attributed to the protocol employed and should not discourage further studies using different designs. Future studies evaluating the types of bacteria affected by CaneCPI-5 should be conducted, as well as studies with reduced duration, since cavitation was observed in many specimens using the present model. Moreover, in future experiments the enamel specimens should be treated with CaneCPI-5 before incubation with saliva, in order to take advantage of its ability to bind to hydroxyapatite and act as an acid-resistant protein in the AEP. Our results open a new avenue for the development of dental products containing CaneCPI-5, such as a mouthwash. However, additional studies with other vehicles of application (such as gels) and on other surfaces (dentin) need to be conducted.

Acknowledgements

Conflict of interest statement
University of São Paulo and Federal University of São Carlos hold a patent pending at INPI (BR 10 2017 008691 7) on "Recombinant sugarcane cystatin to protect against erosive tooth wear and dental caries".

Zusammenfassung
Einleitung
Oraler Biofilm ist eine komplexe Schicht aus verschiedenen in Matrix eingebetteter Mikroorganismen, der die äusseren Zahnoberflächen bedeckt. Unsere Gruppe klonte und charakterisierte kürzlich ein neues Cystatin aus Zuckerrohr namens CaneCPI-5. Dieses Protein schützte gegen In-vitro-Schmelzerosion. Hier untersuchten wir die antimikrobielle und die antikariöse (Verhinderung von Schmelzläsionen) Wirkung von CaneCPI-5 in einem Mikrokosmos-Biofilm-Modell.

Material und Methoden
Mikrokosmos-Biofilm wurde auf 48 bovinen Schmelzproben (4 mm × 4 mm) angezüchtet, und zwar aus einer Mischung von menschlichem Speichel (10 gesunde Spender) und McBain-Speichel während der ersten acht Stunden. Danach und bis zum Ende des Experiments wurden die Schmelzproben in McBain-Speichel mit 0,2% Saccharose aufbewahrt und einmal täglich mit den Testlösungen für eine Minute behandelt. Diese Behandlung erfolgte über fünf Tage. Vom zweiten bis zum fünften Tag wurden die Proben mit folgenden Lösungen behandelt (1 ml/ Probe): 1) Ringerlösung (Negativkontrolle); 2) 0,12% Chlorhexidindiglutonat (PerioGard®); 3) 0,1 mg/ml CaneCPI-5; 4) 1,0 mg/ml CaneCPI-5. Die Biofilmvitalität wurde mittels einer Fluoreszenzmethode konfokal-mikroskopisch gemessen. Die lebenden Bakterien wurden mit SYTO9 gefärbt (grüne Fluoreszenz), die toten Keime mittels Propidiumiodid/SYTO9 (rote

Fluoreszenz). Der Mineralgehalt der Schmelzproben wurde mittels Transversaler Mikroradiographie (TMR) bestimmt unter der Annahme, dass gesunder Schmelz $3,15\,kg/dm^3$ und 87 Vol% Mineral enthält. Läsionstiefe (LT, µm) und der integrierte Mineralverlust (ΔZ, %Vol µm) wurden so berechnet. Die Daten wurden mittels ANOVA/Tukey- oder Kruskal-Wallis/Dunn-Tests zwischen den Gruppen verglichen ($p < 0,05$).

Resultate

Alle Behandlungen ausser 0,1 mg/ml CaneCPI-5 reduzierten die Anzahl der toten und erhöhten die Menge der lebendigen Bakterien signifikant gegenüber der Ringerlösung. Jedoch unterschied sich 0,1 mg/ml CaneCPI-5 nicht signifikant ($p > 0,05$) von 1,0 mg/ml CaneCPI-5 und 0,12% Chlorhexidin. Bezüglich Mineralverlust unterschied sich nur 0,12% Chlorhexidin von der Ringerlösung, aber unterschied sich wiederum nicht signifikant von 0,1 mg/ml Cane-CPI-5.

Diskussion

Wir wählten 0,12% Chlorhexidin als Kontrollbehandlung, weil diese Lösung sich klinisch zur dentalen Biofilmkontrolle etabliert hat. Es wurden zwei Konzentrationen von CaneCPI-5 untersucht. Der diesbezüglich beobachtete Antibiofilm-Effekt übertrug sich allerdings nicht messbar auf den Mineralverlust. Dieses Resultat könnte sich durch den starken kariösen Effekt im verwendeten Modell erklären und/oder durch die Zugabe des untersuchten Proteins CaneCPI-5 nach einer ersten Inkubation im Speichel. Unter den Bedingungen dieses Experiments hatte also CaneCPI-5 eine antimikrobielle Wirkung, die sich aber nicht in einen antikariösen Effekt übertrug.

Résumé

Introduction

Le biofilm oral est une couche complexe composée de différents microorganismes inclus dans une matrice, qui recouvre les surfaces externes des dents. Notre groupe a cloné et caractérisé récemment une nouvelle cystatine de la canne à sucre, appelée CaneCPI-5. Cette protéine a protégé *in vitro* contre l'érosion de l'émail. Nous avons investigué ici l'effet antimicrobien et anticariogène (prévention des lésions de l'émail) de la CaneCPI-5 dans un modèle de biofilm microcosmique (n.d.t. : *CPI: cysteine protease inhibitor*).

Matériel et méthodes

Un biofilm microcosmique a été cultivé sur 48 échantillons d'émail bovin (4 mm × 4 mm), dans un mélange de salive humaine (10 donneurs sains) et de salive artificielle (McBain) pendant les huit premières heures. Ensuite et jusqu'à la fin de l'expérience, les échantillons d'émail ont été conservés dans de la salive McBain avec 0,2% de saccharose, et traités une fois par jour pendant une minute avec les solutions à tester. Ce traitement a été réalisé pendant une période de cinq jours. Du deuxième au cinquième jour, les échantillons ont été traités avec les solutions suivantes (échantillon de 1 ml) : 1) solution de Ringer (contrôle négatif) ; 2) diglutonate de chlorhexidine à 0,12% (PerioGard®) ; 3) CaneCPI-5 à 0,1 mg/ml ; 4) CaneCPI-5 à 1,0 mg/ml. La vitalité du biofilm a été mesurée par microscopie confocale en utilisant une méthode de fluorescence. Les bactéries vivantes ont été colorées avec du SYTO9 (fluorescence verte), les germes morts avec de l'iodure de propidium/SYTO9 (fluorescence rouge). La teneur en minéraux des prélèvements d'émail a été déterminée par microradiographie transverse (TMR) en supposant que l'émail sain contient $3,15\,kg/m^3$ et 87% en volume de minéraux. La profondeur des lésions (LT, µm) et la perte minérale intégrée (ΔZ, % vol µm) ont été calculées de cette manière. Les données des différents groupes ont été comparées à l'aide des tests ANOVA/Tukey ou Kruskal-Wallis/Dunn ($p < 0,05$).

Résultats

Tous les traitements – sauf la solution de CaneCPI-5 à 0,1 mg/ml – ont réduit le nombre de bactéries mortes et augmenté la quantité de bactéries vivantes de manière significative comparativement à la solution de Ringer. Cependant, 0,1 mg/ml de CaneCPI-5 ne différait pas significativement ($p > 0,05$) de 1,0 mg/ml de CaneCPI-5 et de 0,12% de chlorhexidine. En ce qui concerne la perte de minéraux, seule la solution à 0,12% de chlorhexidine différait de la solution de Ringer, mais là encore, elle ne différait pas de manière significative de la solution à 0,1 mg/ml de CaneCPI-5.

Discussion

Nous avons choisi la chlorhexidine à 0,12% comme traitement de contrôle car cette solution s'est imposée cliniquement pour le contrôle du biofilm dentaire. Deux concentrations de CaneCPI-5 ont été testées. Cependant, l'effet antibiofilm observé alors n'a pas eu de répercussion mesurable sur la perte de minéraux. Ce résultat pourrait s'expliquer par l'effet carieux important du modèle utilisé, et/ou par l'ajout de la protéine étudiée CaneCPI-5 après une première incubation dans la salive. Dans les conditions de cette expérience, la CaneCPI-5 a donc eu un effet antimicrobien, mais cela ne s'est pas traduit par un effet anticariogène.

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