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Role of antimicrobial peptide on cariogenic biofilm and dental enamel demineralization

Papel de peptídeo antimicrobiano no biofilme cariogênico e na desmineralização do esmalte dentário

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ABSTRACT

Objective: To investigate the effect of antimicrobial peptide (AMP) D1-23 on antibiofilm activity and enamel demineralization by using a microcosm biofilm model. **Material and Methods:** Human saliva biofilm was grown on bovine enamel discs, which were divided into 4 groups according to the 24-hour growth treatment: 0.2 mM AMP D1-23; 1 mM AMP D1-23; Defined Medium with Mucin (DMM; negative control); and 2% chlorhexidine (CHX; positive control). The biomass and number of cultivable cells of a 4-day-old biofilm were evaluated for antibiofilm activity. For analysis of enamel mineral loss by the 7-day-old biofilm and investigated treatments, the Knoop surface microhardness method was performed. ANOVA and Tukey tests were used to analyze data from biomass and enamel surface microhardness. Culturable cells data were analyzed by a pairwise Kruskal-Wallis test. A significance level of 5% was considered for all tests. **Results**: Although the two concentrations of AMP D1-23 had similar effects on the amount of biofilm biomass ($p>0.05$), a reduction in biomass was observed when compared to the control group ($p < 0.05$). The antimicrobial activity of 1 mM AMP D1-23 was similar to that of 2% CHX against *Streptococcus mutans*, whereas against *Streptococci sp* and *Candida sp* it was lower ($p <0.05$). Also, the 1 mM AMP D1-23 significantly reduced enamel demineralization ($p < 0.05$). **Conclusion**: Under the limitations of this study, the 1 mM AMP D1-23 appears to have positive effect on controlling dental enamel demineralization and may be considered a potential treatment for non-cavitated carious lesions.

KEYWORDS

Antimicrobial cationic peptides; Dental caries, Dental plaque; Public health; Tooth remineralization.

RESUMO

Objetivo: Investigar o efeito do peptídeo antimicrobiano (AMP) D1-23 na atividade antibiofilme e na desmineralização do esmalte usando um modelo de biofilme microcosmo. **Material e Métodos:** Biofilme de saliva humana foi cultivado em discos de esmalte bovino, que foram divididos em 4 grupos: AMP D1-23 0,2 mM; AMP D1-23 1 mM; Meio Definido com Mucina (DMM; controle negativo); e clorexidina 2% (CHX; controle positivo). A biomassa e o número de células cultiváveis do biofilme com 4 dias foram avaliados quanto à atividade antibiofilme. Para análise da perda mineral do esmalte um biofilme com 7 dias foi cultivado e realizado pelo método de microdureza superficial Knoop. Os testes ANOVA e Tukey foram utilizados para analisar dados de biomassa e microdura superficial. Os dados de células cultiváveis foram analisados por um teste de Kruskal-Wallis pareado. Foi considerado nível de significância de 5% para todos os testes. **Resultados**: Embora as duas concentrações de AMP D1-23 tenham tido efeitos semelhantes na quantidade de biomassa do biofilme ($p>0,05$), foi observada uma redução na biomassa quando comparado ao grupo controle ($p <0,05$). A atividade antimicrobiana de AMP D1-23 1 mM foi semelhante à de CHX 2% contra *Streptococcus mutans*, enquanto contra *Streptococci* sp e *Candida* sp foi menor ($p<0,05$). Além disso, o AMP D1-23 1 mM reduziu significativamente a desmineralização do esmalte ($p < 0,05$). **Conclusão:**

O AMP D1-23 1 mM parece ter efeito positivo no controle da desmineralização do esmalte danificado e pode ser considerado uma alternativa de tratamento em potencial para lesões de cárie não cavitadas.

PALAVRAS-CHAVE

Cárie dentária; Peptídeos catiônicos antimicrobianos, Placa dentária; Saúde pública; Remineralização dentária.

INTRODUCTION

Although preventable, dental caries is a disease of major public health concern, that imposes an expensive burden on health systems. The importance of maintaining a healthy lifestyle and controlling oral biofilm through moderate refined sugar consumption is important for long-term disease control. However, behavioral changes in diet are difficult to achieve and to maintain, especially current eating habits [1].

There is no doubt that fluoride will continue to be the pillar of any caries prevention protocol, as it still remains the most effective and cost-effective protective agent against tooth decay [2,3]. However, fluoride alone does not offer complete protection against caries disease, and its efficacy can be improved when in combination with additional cariostatic agents [1,2]. Silver diamine fluoride (SDF) has been recommended in the treatment of early childhood caries (ECC) because of its interesting role in the control of enamel demineralization [3,4]. Nonetheless, SDF promotes dentin discoloration, which has negative aesthetic effects [3]. New preventive therapies involving salivary peptides have been proposed [5,6]. Antimicrobial peptides (AMPs) have been investigated since they are responsible for modulating the host's immune response, keeping the microbiota balanced in different niches (Wiesner and Vilcinskas, 2010). The main AMPs in saliva/crevicular fluid are α- and β-defensins (hBD) and cathelicidins [7,8]. The role of AMPs in dental caries control has raised interest, as these molecules are identified as a new class of antibiotics. The long length of their amino acid chains has hampered the production of peptides for therapeutic uses; resulting in the interest of investigating peptide fragments against cariogenic bacteria [9]. The peptide Defb14-1CV(1-23) or D1-23, derived from Defb14 (the mouse ortholog of human β-defensin-3), showed promising preliminary results against planktonic microbial strains and in monospecies biofilms [9,10].

No evidence has yet been found regarding the antimicrobial potential of AMP D1-23 in complex biofilms and its effect on tooth enamel demineralization. Based on the need for a more effective treatment to control the progression of dental caries disease, this study aimed to evaluate antibiofilm activity and the ability to inhibit dental enamel demineralization of AMP D1-23. The hypotheses tested were: the concentration of AMP D1-23 does not influence: (1) the antibiofilm activity and (2) enamel demineralization.

METHODS

Ethical aspects

This study was approved by the Research Ethics Committee (Protocol number [CAAE] 87670818 9 0000 5165), and written informed consent was obtained from the saliva donor.

Experimental design

The microcosm biofilm model was used in this *in vitro* study. To provide a multispecies biofilm, human stimulated saliva was used as the inoculum. Biofilms were grown on bovine enamel discs, which were divided into 4 groups according to the 24-hour treatment [11]: 1 mM AMP D1-23, 0.2 mM AMP D1-23, defined medium with mucine (artificial saliva/DMM; negative control), 2% chlorhexidine (positive control). The experiments were performed in triplicate.

Biofilm inoculation on enamel discs was performed with human donor saliva and DMM as the culture medium. The DMM was renewed twice a day, and enamel-biofilm discs were subjected to cariogenic challenge through cyclic exposure to sucrose, A 4-day-old biofilm and a 7-day-old biofilm were used for microbiological tests [11] and microhardness test [12], respectively. After that, the enamel discs were treated for 24 hours according to the different groups. The microhardness test was performed to assess mineral loss, which was

recorded as the percentage change in enamel surface microhardness (%SMH).

Preparation of antimicrobial agents

AMP D1-23 (FLPKTLRKFFARIRGGRAAVLNA), derived from Defb14 (the mouse ortholog of human β-defensin-3) (AminoTech Research and Development Ltda, Diadema, SP, Brazil) [10], was resuspended in sterilized deionized water at 20 mM and stored at -20°C. Two concentrations of AMP D1-23 were investigated: 0.2 mM and 1 mM. DMM (Interlab, São Paulo, SP, Brazil) and a 2% chlorhexidine solution (GeneralMed, São Paulo, SP, Brazil) were used as negative and positive control, respectively.

Preparation of bovine enamel discs

Thirty two enamel discs (5 mm in diameter x 2 mm in thickness) were prepared from the mid-height of the dental crown of bovine incisors. The enamel surfaces were sequentially polished with 100/600/1200 grit silicon carbide paper discs. Next, the bovine disc specimens were covered with nail polish, exposing only the tooth enamel surface.

Microcosmos biofilm growth

Thirty milliliters of saliva stimulated by paraffin film (Parafilm "M", American National CanTM, Chicago, IL, USA) were collected from a good general health human donor [11].

The enamel discs were inoculated with 400 μL of homogenized saliva and were incubated for 1 hour at 37°C. For the cariogenic challenge, 1.8 mL of artificial saliva (DMM)[13] was supplemented with 1% sucrose (Interlab, São Paulo, SP, Brazil) for 6 hours under anaerobic conditions (5-10% CO_2 < 1% O_2) at 37° C, without shaking.

The cariogenic challenge was repeated daily, for 4 or 7 days [13], depending on the experiments, with two changes: DMM enriched with 1% sucrose for 6 hours and DMM without sucrose for 18 hours. After 4 and 7 days of biofilm growth, for antibiofilm evaluation and enamel surface microhardness evaluation, respectively, the discs were divided into four groups, according to the investigated treatment.

In order to detach the biofilm from the enamel discs, the samples were sonicated (Ultrasonic Cell Tip-Disruptor Sonicator, Model: DES500, Unique, São Paulo, SP, Brazil).

Total biomass and microbial composition of the biofilm

The total biofilm biomass was evaluated by the dry weight method [11], determined by the weight difference between the microtube containing the biofilm and the empty microtube. Biofilms were dehydrated with ethanolic solutions (99% and 75%) and stored in a glass desiccator (LaborQuimi Vidrolabor - Poá, SP, Brazil) for 48 hours.

The quantification of viable cells was performed after disaggregation of the treated biofilm. Serial dilutions of the biofilm (from 0 to 10^{-6}) were inoculated in Petri dishes with CHROMagar culture medium (Difco Laboratories, Franklin Lakes, New Jersey, USA) for *Candida sp* cultures; Mitis Salivarius agar culture medium (Difco Laboratories) supplemented with 0.2 U/mL bacitracin (Sigma-Aldrich, St. Louis, MO, USA) for *Streptococcus mutans* cultures; and Mitis Salivarius agar culture medium for streptococci cultures. *S. mutans* and *Streptococci* cultures were incubated under anaerobic conditions for 72 hours, while *Candida* sp was grown under aerobic conditions. The number of colony forming units (CFU) in each culture was determined by a trained researcher and the results were expressed in CFU/mg of biofilm (dry weight).

Microhardness change analysis

The microhardness test was performed to assess mineral loss, which was recorded as the percentage change in enamel surface microhardness (% SMH).

Enamel demineralization was analyzed by the surface microhardness (SMH) Knoop test [12] at two different times, before biofilm formation (healthy enamel; initial microhardness) and after biofilm growth and treatment (final microhardness).

The initial SMH was determined based on the SMH measured with a Knoop diamond (Future-Tech Corp., Tokyo, Japan) under a 25 g load for 5 seconds, using three equidistant indentations in the center of the enamel discs (150 μm). The mean microhardness was calculated and analyzed by ANOVA test to select samples that were statistically equivalent at baseline ($p > 0.05$). The selected

specimens were subjected to cariogenic challenge by the biofilm model.

After 7 days of biofilm growth, the specimens were treated for 24 hours according to the investigated groups. Final microhardness was evaluated using three equidistant indentations (150 μm) from the initial readings. The enamel surface microhardness was calculated using the equation: % SMH (Δ) = 100 * (initial reading - final reading) / initial reading.

Statistical analysis

Data were analyzed using SigmaPlot 12.0 software (Systat Software Inc., San Jose, USA). The ANOVA and Tukey's multiple comparisons post hoc tests were used to assess total biofilm biomass and microhardness. The presence of culturable cells in the biofilms was compared among groups by ANOVA with Tukey's post-test post hoc paired comparisons. Data from microbial biofilm composition were transformed in log 10, and the significance level was set at 5%.

RESULTS

Total biofilm biomass

The results of quantification of total biomass by dry weight of biofilm AMP D1-23, in both studied concentrations, reduced biofilm biomass. However, these results were not statistically different from the positive control group ($p > 0.05$).

Following are the dry weight and standard deviation data: DMM (15.5+1.8), AMP D1-23 0,2mM (13.0+3.12), AMP D1-23 1mM (9.83+3.55), Chlorhexidine (20.8+3.01).

Quantification and identification of biofilm culturable cells

Figure 1 shows that AMP D1-23 at a concentration of 1 mM showed an antimicrobial activity similar to chlorhexidine against *S. mutans* ($p > 0.05$), while it did not present antimicrobial activity against *Streptococci* or *Candida sp* in the investigated concentrations ($p > 0.05$).

Different letters represent statistical difference. ($p < 0.05$, ANOVA with Tukey's post-test).

Effect of AMP D1-23 on enamel demineralization

The SMH results are shown in Table I. Loss of enamel surface hardness was showed in all groups after 7 days of biofilm growth and 24 hours of treatment. When the cariogenic biofilm was treated with 1 mM AMP D1-23, it was observerd the lowest enamel demineralization ($p < 0.05$).

DISCUSSION

AMP D1-23, at the investigated concentrations, did not play an important role in controlling the cariogenic biofilm. However, it positively affected the control of enamel demineralization following cariogenic biofilm growth.

The microcosm biofilm model, used in this study, was applied in preclinical evaluations to evaluate the potential of antimicrobials in the development of biologically active materials [11,14].

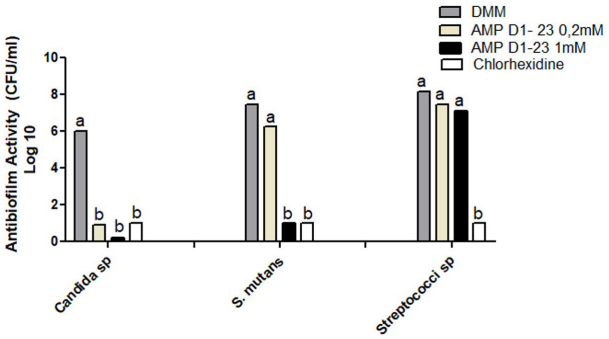

Figure 1 - Graphical representation of antibiofilm activity through quantitative analysis of Colony Forming Units (CFU) of complex biofilm of *Candida sp*, *S. mutas* and *Streotococci sp*, treated with AMP D1-23 0.2mM and AMP D1-23 1mM.

Table I - Analysis of Knoop surface microhardness of dental enamel according to the treatment performed

Treatment	Surface microhardness (baseline)* Mean ± SD	Surface microhardness After treatment Mean± SD**	Percentage Enamel demineralization after treatment
Defined Medium Mucine (DMM)	327.7±35.2	28.4±2.5	91.26$^{a\#}$
D1-23 0.2 mM	271.6±33.6	58.3±6.4	78.47^{a}
D1-23 1 mM	286.6±63.5	112.1±19.0	58.04^{b}
Chlorhexidine 2% (0.03mM)	284.7±81.1	72.9±12.8	73.55^{a}

* Surface microhardness of sound tooth enamel (baseline). ** Surface microhardness after 7 days of biofilm growth and 24h-treatment. # Different letters represent statistically different values ($p<0.05$. ANOVA with Tukey test).

It is important to mention that the mature biofilm, rich in extracellular polysaccharide matrix, is characterized by an organized structure that makes its mechanical removal [15] and the action of antimicrobials difficult [16]. Thus, approaches to control the formation of pathogenic biofilms can be a strategy for the prevention of dental caries [15]

Bovine tooth discs were used as substrate to develop the microcosm biofilm model, which has advantages such as its easy obtention and handling. Furthermore, it is known that the structure of bovine enamel and dentin is similar to that of human enamel and dentin in terms of quantity, size, shape, diameter, and density of dentin tubules [17].

Antibiofilm activity was evaluated by quantifying the total biomass and culturable cariogenic microorganisms present in the biofilm after antimicrobial treatment. Although no studies were found with AMP D1-23 in complex biofilms [9], it was showed the effect of AMP D1-23 on planktonic cells and simple *S. mutans* biofilm. Greater reduction in total biofilm biomass was observed by confocal microscopy after biofilm treatment with AMP D1-23 than with chlorhexidine [9]. The effect of antimicrobial agents on biomass depends on treatment depletion in the wet phase, antibiotic penetration, and biome physiology [16]. However, only antimicrobial penetration and biofilm formation and maturation can be associated with biomass tolerance in the *in vitro* microcosm biofilm model.

When the number of viable cells in the dental biofilm was evaluated, AMP D1-23, at both concentrations, showed effects similar to those of the negative control group, that is, low efficacy against the studied strains. Contrary to our results, Kreking et al. [9] observed a more significant effect of 0.2 mM AMP D1-23 compared to 0.03 mM (2%) chlorhexidine against *S. mutans*. However, it was shown that more mature and thicker biofilms are invariably less susceptible to antimicrobial agents than younger and less dense biofilms due to the more complex structural nature of the extracellular polysaccharide matrix [16], which could explain the difference between the two studies. In our study, 4-day complex biofilms, for the first time investigated under the effect of AMP D1-23, were obtained from human saliva, contrary to the previous investigation, where the effect of AMP D1-23 was evaluated on a 48-hour *S. mutans* biofilm [9]. Biofilm tolerance to antimicrobials is known to comprise the large number of challenges that can influence susceptibility in a specific biofilm, such as microbial composition, substrate material, cell density or biofilm thickness, and biofilm age [11].

S. mutans is considered one of the initial colonizers of the cariogenic biofilm, being a potent acid producer, which helps in modulating the presence of other microorganisms and makes the biofilm more pathogenic [17]. Therefore, a reduction in *S. mutans* levels may contribute to a lower acidogenicity of the biofilm by reducing sugar metabolism and biofilm complexity [15]. *Streptococci*, which were also evaluated in this study, are related to the constant maintenance of the acidic biofilm pH and the progression of caries lesions. In this study, AMP D1-23 had the same performance as the negative control against *Streptococci*, which differed from Kreling et al. [9], who observed higher bactericidal activity of AMP D1-23 against the same species in planktonic state. *Candida sp* contributes to the formation of multispecies biofilms, increasing the chances of adhesion of other microorganisms in the oral environment. In our study, the count of *Candida* species in the donor was low, corroborating previous studies that showed that *Candida* constitutes less than 1% of the total of microorganisms found in the non-biofilm composition of saliva [18]. However, the same donor, at different times of repeated collections, has a significantly different microflora composition, suggesting that the observed transition in dental biofilm composition between health and disease is driven by a microbiota response to environmental changes [18].

The antimicrobial effect of AMP D1-23 in complex biofilm is thought-provoking, since it is known that the effect of an antimicrobial is different and limited in complex biofilms when compared to microorganisms in planktonic state [19]. This considers the fact that microorganisms in biofilms are incorporated into the extracellular polysaccharide matrix, which offers physical protection against mechanical and chemical challenges [20].

There is no doubt that chlorhexidine has a potent antimicrobial effect. However, although the broad-spectrum antimicrobial action of chlorhexidine is well established in the literature [21], the suppression of oral microbiota by chlorhexidine represents a disadvantage [22],

since an effective antimicrobial must be associated with the re-establishment of oral microbiome balance and not its total elimination.

A reduction in enamel demineralization after treatment with 1 mM of AMP D1-23 was observed in our study. Although no previous studies on the effect of AMP D1-23 on tooth enamel have been found, the AMP TVH19 (TKRQQVVGLLWHLLHHLLH-NH_2), synthesized with bifunctional activity (antimicrobial and remineralizing action), showed a remineralizing effect on tooth enamel.

The control of dental enamel demineralization by AMP D1-23 at the two investigated concentrations observed in this study could be explained by the reduction of total biofilm biomass, since the initial demineralization process occurs by complex biofilm formation [12] and the interruption of this process could contribute to the reduction of biofilm acidogenicity. However, more studies are necessaries to explain AMP D1-23 activity.

Positive results regarding the action of AMP fragments on complex biofilms open up interesting possibilities for their use in the literature, taking into account that the cost of synthesizing this material decreases when studying its fragments [4]. Despite the promising results of the present study, some limitations prevent our findings from being extrapolated to clinical practice. Although a microcosm biofilm resembling the dynamics of the dental biofilm was used, it still represents an *in vitro* model, which does not fully portray the reality of biofilm formation. This process depends on the availability of nutrients, as well as the substrate made available by each individual and the biofilm pH, which changes according to the substrate being metabolized by the microorganisms [23].

The search for agents with anti-cariogenic properties such as antimicrobial capacity [24], antibiofilm properties [4], and the ability to inhibit demineralization and to stimulate remineralization [24] still represents a challenge in cariology.

Even with the limitations of our results, the possibilities of studies with peptide fragments become more viable every day [9]. Among the advantages of investigating these molecules, we described the fact that dentistry is no longer looking for materials that eliminate microorganisms of the oral cavity, but those that provide the rebalancing of the oral microbiome [20]. In this context, AMP D1-23 may represent a promising strategy against dental caries, as it has antibiofilm action and an ability to control demineralization. However, investigations on the effect of AMP D1-23 in the production of extracellular and intracellular polysaccharides are still needed for controlling biofilm acidity, as well as on the effectiveness of its incorporation into fluoride varnishes and restorative materials.

CONCLUSIONS

Based on the results of this study, the following conclusions can be made:

1. AMP D1-23 regardless of concentration reduced cariogenic biofilm biomass, which is an important factor in controlling demineralization.
2. 1mM AMP D1-23 decreased mineral loss, controlling the progression of caries lesions.
3. AMP D1-23 may be a potential treatment of non-cavitated initial carious lesions. The findings of cariogenic biofilm control and prevention of demineralization are relevant in the control and treatment of dental caries, making the results promising. However, further studies are needed to assess the mechanism of action and efficacy in clinical treatment.

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Author's Contributions

PVS: Data Curation, Writing- Original, Draft Preparation; TPC: Methodology, Resources, Writing- Review & Editing; JLSU: Data Curation; ARSJ: Formal Analysis, Writing- Review & Editing; MSC: Methodology, Validation, Writing- Review & Editing; AMFA: Conceptualization, Funding Acquisition, Project Administration, Supervision, Validation, Writing- Review & Editing.

Conflict of Interest

The authors have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

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Regulatory Statement

This study was conducted in accordance with all the provisions of the local human subjects oversight committee guidelines and policies of the Research.

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