UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de São José dos Campos
Instituto de Ciência e Tecnologia

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AVALIAÇÃO DO EFEITO PROTETOR, REMINERALIZANTE E ANTIMICROBIANO DE DENTIFRÍCIOS CONTENDO PARTÍCULAS BIOATIVAS

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Tese apresentada ao Instituto de Ciência e Tecnologia, Universidade Estadual Paulista (Unesp), Campus de São José dos Campos, como parte dos requisitos para obtenção do título de DOUTOR, pelo Programa de Pós-Graduação em ODONTOLOGIA RESTAURADORA.

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"A persistência é o caminho do êxito". Charles Chaplin

SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS

Al^{3+}	Alumínio
BO^{3-}_{3}	Borato
CFU	*Colony forming units*
CSMH	*Cross-sectional microhardness*
F^{-}	Fluoreto ou íon flúor
Na^{+}	Sódio
SiO^{2-}_{3}	Silicato
SMH	*Surface microhardness*
SML	*Surface microhardness loss*
SMR	*Surface microhardness recovery*
S. mutans	*Streptococcus mutans*
S-PRG	*Surface pre reacted glass ionomer*
Sr^{2+}	Estrôncio
WSL	*White spot lesions*

Spinola MS. Avaliação do efeito protetor, remineralizante e antimicrobriano de dentifrícios contendo partículas bioativas [tese]. São José dos Campos (SP): Universidade Estadual Paulista (Unesp), Instituto de Ciência e Tecnologia; 2021.

RESUMO

O objetivo deste trabalho foi avaliar o potencial protetor contra a desmineralização e o efeito remineralizante de dentifrícios experimentais contendo diferentes concentrações de partículas de vidro ionomérico pré-reagido (S-PRG - *surface prereacted glass ionomer cement*). Adicionalmente, o potencial antimicrobiano foi avaliado. Foram preparados 168 espécimes cilíndricos (4mm - diâmetro; 2mm - altura) de esmalte bovino hígido e polido para avaliação do potencial protetor (n=84) e remineralizante (n=84). Estes foram estratificados nos seguintes grupos de tratamento (n=12), de acordo com a concentração das partículas bioativas (S-PGR) incorporadas nos dentifrícios: 0%; 1%; 5%; 20% e 30%. Um dentifrício contendo NaF (1450 µg F/mL) foi utilizado como controle positivo e a água ultrapurificada foi utilizada como controle negativo. Os tratamentos com as suspensões de dentifrícios (1:3 com saliva artificial) foram realizados 2x/dia – 5 min/8 dias, intercalados com a ciclagem des/remineralizante. Para avaliação do potencial protetor dos tratamentos contra a desmineralização, os espécimes foram imersos em solução desmineralizante por 4 h e em solução remineralizante por 20 h. Para a avaliação do potencial remineralizante dos dentifrícios, os espécimes foram submetidos à formação de lesão de mancha branca artificial em solução desmineralizante por 20 h e então foram submetidos aos mesmos tratamentos e ciclagem des/re (2 h em solução des e 22 h em solução re). Após a ciclagem, os espécimes foram analisados quanto a dureza superficial, subsuperficial. Adicionalmente, o pH da suspensão de dentifrício preparada em água destilada foi determinado. A avaliação do efeito dos dentifrícios sobre a adesão bacteriana e crescimento do biofilme foi realizada por meio de testes em uma cepa padrão de *S. mutans* (UA159) e em uma cepa clínica de *S mutans*. Para cada cepa, 35 espécimes de esmalte bovino polido (6mm - diâmetro; 2mm - altura) foram distribuídos aleatoriamente nos mesmos grupos de tratamento (n=5), porém para a avaliação do efeito antimicrobiano, um dentifrício contendo 1450 µg F/mL + triclosan foi utilizado como controle positivo. Os espécimes foram tratados com as suspensões (5 min) e então inseridos em uma placa contendo sacarose, saliva artificial e uma suspensão *S. mutans* (padrão e clínica) para permitir a formação do biofilme. Então, foi realizada a contagem de unidades formadoras de colônias por mL (UFC/mL) após 48 h. O efeito antimicrobiano sobre um biofilme recém-formado e maduro também foi avaliado. Para isso, 35 blocos de esmalte bovino foram distribuídos aleatoriamente nos sete grupos citados anteriormente (n=5). Os espécimes foram inseridos em uma placa contendo sacarose, saliva artificial e uma suspensão *S. mutans* para permitir a adesão bacteriana. Após 4 h e 24 h da formação inicial do biofilme, os espécimes foram tratados com um dos dentifrícios contendo diferentes concentrações de S-PRG e controles e retornaram ao meio de cultura. Após 48 h, a contagem de UFC/mL foi realizada. Análises estatísticas independentes foram realizadas entre os grupos para cada estudo. Os dados foram analisados com ANOVA e teste de Tukey (5%). Os dentifrícios contendo S-PRG apresentaram potencial protetor contra a

desmineralização e o dentifrício com 30% S-PRG foi o mais eficaz, diferindo do controle positivo ($p<0,05$). Para a remineralização, dentifrícios contendo S-PRG diferiram do controle negativo ($p<0,05$), mas não diferiram entre si e não foram superiores ao dentifrício contendo NaF. Uma diminuição significativa na adesão de microrganismos foi observada para todos os grupos tratados com os dentifrícios contendo S-PRG e para a cepa UA159 os dentifrícios com 20 e 30%S-PRG apresentaram efeito superior ao dentifrício contendo NaF+Triclosan ($p<0,05$). Efeito antimicrobiano sobre o biofilme recém-formado (4 h) também foi observado para os grupos tratados com dentifrícios contendo S-PRG, mas não foi observado efeito superior ao dentifrício contendo NaF+Triclosan ($p>0,05$). Para o biofilme maduro, efeito antimicrobiano dos dentifrícios contendo S-PRG foi observado apenas para a cepa clínica ($p<0.05$), sendo inferior ao exercido pelo controle positivo. Concluiu-se que os dentifrícios contendo S-PRG apresentam capacidade de proteger o esmalte contra a desmineralização, bem como capacidade remineralizante, além de serem capazes de impedir a adesão bacteriana e atuar sobre o crescimento do biofilme cariogênico.

Palavras-chave: Cárie dental. Desmineralização. Remineralização dentária. Partícula bioativa. S-PRG. Biofilme dental.

Spinola MS. Evaluation of the protective, remineralizing and antimicrobial efficay of toothpastes containing bioactive particles [doctorate thesis]. São José dos Campos (SP): São Paulo State University (Unesp), Institute of Science and Technology; 2021.

ABSTRACT

The aim of the present study was to evaluate the protective, remineralizing, and antimicrobial potential of experimental toothpastes containing different concentrations of pre-reacted glass ionomer particles (S-PRG). Cylindrical specimens (n=84, 4mm-diameter, 2mm-height) of sound and polished bovine enamel were prepared to evaluate the protective and remineralizing potential of the toothpastes. These were stratified into the following treatment groups (n=12), according to the concentration of bioactive particles (S-PGR) incorporated to the toothpastes: 0%; 1%; 5%; 20%; and 30%. A toothpaste containing 1450 µg F/mL was used as a positive control and distilled water as a negative control. Treatments with toothpastes' slurries (1:3 with artificial saliva) were performed 2x/day - 5 min / 8 days, interposed with de/remineralization cycling. To evaluate the protective potential of the toothpastes, specimens were immersed in demineralizing solution for 4 h and in a remineralizing solution for 20 h. To evaluate the remineralizing potential of toothpastes, the specimens were submitted to the formation of white spot lesion in demineralizing solution for 20 h and then submitted to the same treatments de- and remineralizing pH-cycling (2 h in de- and 22 h in remineralizing solution). Specimens were analysed for surface and cross-sectional hardness. Additionally, the pH of the slurries prepared in deionized water was assessed. The effect of toothpastes over microorganisms adhesion and their antimicrobial potential over a newly formed and mature biofilms were also evaluated. To evaluate the effect of toothpastes on microorganisms adhesion and biofilm development, two different studies were performed using a S mutans strain (UA159) and a S mutans clinical strain. 35 specimens of polished bovine enamel (6mm-diameter, 2mm-height) were randomly distributed in the same treatment groups (n=5). The specimens were treated with the suspensions and then inserted into a plate containing sucrose, artificial saliva and a standard suspension of S. mutans to allow microorganisms adhesion and then colony forming units per ml (CFU/mL) counting was performed after 48 h. The antimicrobial effect on a newly formed and mature biofilms was also evaluated. For this, 35 blocks of bovine enamel were randomly distributed into the seven previously mentioned groups (n=5). The specimens were inserted into a plate containing sucrose, artificial saliva and a standard suspension of S. mutans to allow biofilm formation. After 4 h and 24 h of the initial formation of the biofilm, the specimens were treated with one of the toothpastes containing different concentrations of S-PRG and were return to the culture medium. After 48 h the CFU/mL counting were performed. Independent statistical analyses were performed for each study. Data were analysed with ANOVA and Tukey test (5%). The S-PRG containing toothpastes presented protective potential and the 30% S-PRG was the most effective, differing from the positive control ($p<0.05$). For remineralization, toothpastes containing S-PRG differed from the negative control and 0% S-PRG ($p<0.05$), but did not differ from each other and were not superior to toothpaste

containing NaF ($p>0.05$). A significant decrease in the adhesion of microorganisms was observed for all groups treated with the S-PRG containing toothpastes and for the UA159 strain the 20 and 30% S-PRG toothpastes had a superior effect than the NaF+Triclosan ($p<0.05$). Antimicrobial effect on the newly formed biofilm (4 h) was also observed for the groups treated with S-PRG, but no greater effect was observed than that of NaF+Triclosan ($p>0.05$). For mature biofilm, antimicrobial effect of S-PRG toothpastes was observed only for the clinical strain ($p<0.05$), and were inferior than NaF+Triclosan toothpaste. It could be concluded that, toothpastes containing S-PRG presented higher efficacy in protecting enamel against demineralization and in promoting remineralization, as well as inhibiting the cariogenic biofilm development.

Keywords: Dental caries. Demineralization. Remineralization. Bioactive particles. S-PRG. Dental biofilm.

1 INTRODUÇÃO

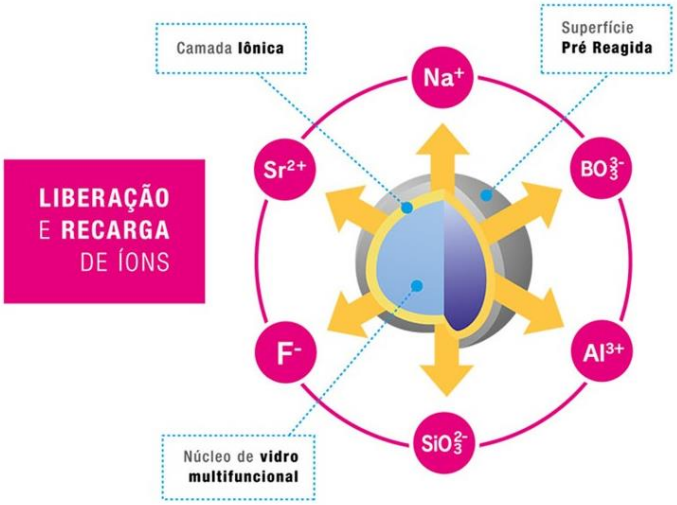
A cárie dental é uma doença multifatorial, que resulta no desequilíbrio entre os minerais do dente e os minerais presentes nos fluidos orais (fluido do biofilme e saliva). Esse desequilíbrio ocorre pela diminuição do pH no interior do biofilme dental quando este é exposto a açúcares provenientes da dieta (Dawes, 2003). Nos últimos anos, o declínio da prevalência de cárie dental vem sendo associado a diferentes fatores, como mudanças nos hábitos alimentares, melhores condições de higiene bucal e, adicionalmente, devido ao desenvolvimento de produtos que visam inibir a desmineralização inicial do esmalte, promover sua remineralização e atuar como agente antimicrobiano diminuindo a quantidade de microrganismos cariogênicos (Bratthall et al., 1996).

Além dos produtos fluoretados, comumente presentes no mercado com potencial anticárie, produtos contendo diferentes agentes bioativos têm se mostrado altamente promissores no controle da cárie dentária. São considerados compostos bioativos aqueles capazes de produzir uma resposta biológica específica resultando na interação entre os tecidos circunjacentes e o material (Vallittu et al., 2018). Diferentes materiais bioativos têm sido utilizados na odontologia, como os vidros bioativos, que são capazes de induzir a formação de hidroxiapatita para reparo ósseo, bem como do esmalte e dentina comprometidos; materiais biocerâmicos, que estimulam a formação de dentina terciária e fosfopeptídeo de caseína/fosfato de cálcio amorfo (CPP-ACP), com potencial de promover a proteção contra a desmineralização do esmalte e remineralização de lesões de mancha branca (Ramadoss et al., 2021; Attiguppeet al., 2019; Thierens et al., 2019).

Dentre os compostos bioativos, destacam-se ainda as partículas de vidro pré-reagido denominado S-PRG *(surface pre-reacted glass ionomer)*. Seu processo de obtenção envolve inicialmente a fabricação de um vidro à base de fluorboroaluminosilicato, que é triturado para obtenção de partículas micro e nanométricas. Estas são então tratadas com uma solução contendo polisiloxano e álcool, sob alta temperatura, que resulta na formação de uma camada superficial modificada de sílica porosa. Posteriormente, o material é pulverizado com solução

aquosa de ácido poliacrílico para se obter as partículas com superfície pré-reagida. Este tratamento gera uma reação ácido-base na subsuperfície da partícula, promovendo a formação de uma camada intermediária de hidrogel ionomérico (Nakatsuka et al., 2003). A partícula de S-PRG então possui uma estrutura trilaminar, composta por um núcleo de vidro funcional, circundado por uma fase de ionômero de vidro e por fim, apresentando a camada superficial modificada (Figura 1).

Figura 1 – Ilustração da partícula de *S-PRG* formada a partir da tecnologia *GIOMER*



Fonte: Esquema apresentado pelo fabricante (Shofu Inc., 2021).

As partículas de fluorboroaluminosilicato são formadas, dentre outros compostos, por fluoreto de estrôncio e carbonato de estrôncio (Fujimoto et al., 2010). Por apresentarem fluoreto na sua composição, essas partículas são capazes de liberar flúor (F^-), bem como se recarregar desse íon quando expostos a produtos fluoretados, com potencial de elevação dos níveis de F^- nos fluidos orais para interferir com os processos de des e remineralização do esmalte (Kamijo et al., 2009). Dentre seus mecanismos de ação, os fluoretos podem estar relacionados à dificuldade de crescimento e adesão das bactérias cariogênicas, como os estreptococos do grupo

mutans (*S. mutans*) (Buzalaf et al., 2011), além de inibir a metabolização de açúcar por essas bactérias (Kitagawa et al., 2018).

O diferencial desta partícula consiste em liberar também os demais íons que fazem parte da sua composição como estrôncio (Sr^{2+}), alumínio (Al^{3+}), silicato (SiO^{2-}_3), sódio (Na^+) e borato (BO^{3-}_3) (Fujimoto et al., 2010). A liberação do SiO^{2-}_3 e Sr^{2+} está associada à capacidade de remineralização, induzindo a formação de hidroxiapatita e estrôncio-apatita. Além disso, estudos demonstraram que o Sr^{2+}, Na^+ e Al^{3+} liberados pelas partículas de S-PRG são capazes de aumentar o pH do meio (Kaga et al., 2014; Shimizubata et al., 2020), atuando na neutralização de ácidos provenientes da metabolização de carboidratos por bactérias da cavidade oral, por exemplo (Ma et al., 2021). O Na^+ também é responsável por catalisar as reações dos demais íons liberados pelas partículas de S-PRG (Fujimoto et al., 2010). Já a liberação de BO^{3-}_3 está relacionada ao efeito antimicrobiano dos produtos contendo S-PRG, pois dificulta a adesão da placa bacteriana já que inibe o mecanismo de comunicação entre as células microbianas (Dembitsky et al., 2011), enquanto o Al^{3+} liberado tende a formar complexos com o F^-, o que parece interessante para a obliteração de túbulos dentinários (Etsuo et al., 2013).

O fabricante denomina *Giomer* o grupo de materiais odontológicos que possuem a tecnologia S-PRG em sua composição, devido à fase ionomérica (Glass IOnoMER). Estudos com resinas compostas, sistemas adesivos, selantes e vernizes têm mostrado resultados favoráveis quanto à prevenção da cárie, por atuarem como agente inibidor da formação do biofilme (Tadayuki et al., 2004; Saku et al., 2010; Yoneda et al., 2015; Nomura et al., 2018) e da desmineralização de esmalte e dentina (Mukai et al., 2009; Kawasaki, Kambara, 2014; Spinola et al., 2020). Este fato encorajou a adição de partículas de S-PRG a agentes de uso tópico contínuo, como dentifrícios e enxaguatórios bucais. Estudos prévios com produtos experimentais demonstraram que dentifrícios contendo S-PRG apresentaram efeito contra a desmineralização do esmalte frente a desafios ácidos a que os dentes são expostos diariamente e na presença de biofilme (Iijima et al., 2017; Amaechi et al., 2017). Entretanto, apesar da partícula de S-PRG apresentar resultados promissores na prevenção de cárie, pouco ainda se sabe sobre quais concentrações dessas partículas são as mais favoráveis quando incorporadas aos dentifrícios para atuar

frente à prevenção da desmineralização do esmalte. Além disso, parece não haver nenhum estudo que demonstrou qual a melhor concentração de S-PRG adicionada a dentifrícios que pode favorecer o processo de remineralização do esmalte e apresentar maior potencial antimicrobiano sobre a formação do biofilme dental cariogênico.

Assim, a investigação de dentifrícios contendo diferentes concentrações de S-PRG torna-se relevante como alternativa para a futura disponibilização de agentes anticárie que possam ser eficazes na proteção contra a desmineralização do esmalte, bem como na ativação da remineralização, além de exibirem potencial efeito sobre o biofilme de *S. mutans*.

2 ARTIGO(S)

2.1 Artigo – Spinola MS, Moreira LS, Torres CRG, Borges AB. Eficácia de dentifrícios contendo S-PRG na proteção contra a desmineralização do esmalte e remineralização de lesões de mancha branca / *Efficacy of S-PRG containing toothpastes on enamel protection against demineralization and remineralization of white spot lesions******

RESUMO

Objetivos: Avaliar o potencial protetor e remineralizador de dentifrícios experimentais contendo diferentes concentrações de partículas pré-reagidas de vidro ionomérico (S-PRG). Design: Foram preparados 84 espécimes cilíndricos de esmalte bovino hígido e polido para avaliar o potencial protetor e 84 espécimes para avaliar o potencial remineralizador dos dentifrícios. Os espécimes de cada estudo foram estratificados nos seguintes grupos de tratamento (n=12): 0%; 1%; 5%; 20% e 30% S-PRG, 1450 µg F/mL (controle positivo) e água destilada (controle negativo). Foram realizados tratamentos com suspensões de pasta de dente 2x/dia/5min/8dias, intercalados com ciclagens de des/remineralização. Para avaliar o potencial protetor dos tratamentos, as amostras foram imersas em solução desmineralizadora por 4h e em solução remineralizante por 20h e para avaliar o potencial remineralizante, as amostras foram submetidas à formação de lesão de manchas brancas artificiais em solução desmineralizante por 20h e submetidas aos mesmos tratamentos e ciclagem des/re (2h em solução des e 22h em solução re). Após a realização das ciclagens, análises de microdureza superficial e transversal foram realizadas. Análises estatísticas independentes para ambos os estudos foram realizadas utilizando ANOVA e teste de Tukey (5%). Resultados: Para proteção contra a desmineralização, os dentifrícios contendo S-PRG diferiram significativamente do controle negativo e 0% S-PRG ($p<0,05$) apresentando efeito protetor e o dentifrício com 30% S-PRG foi o mais eficaz diferindo do controle positivo ($p<0,05$). Para a remineralização, dentifrícios contendo S-PRG diferiram do controle negativo ($p<0,05$), mas não diferiram entre si ($p>0,05$). Os dentifrícios contendo S-PRG não foram superiores ao NaF. Conclusão: Dentifrícios contendo S-PRG apresentaram efeito protetor e remineralizante, embora para remineralização, estes não foram superiores ao dentifrício contendo NaF.

Palavras-chave: Cárie dental. Desmineralização. Remineralização. Dentifrícios. *S-PRG*.

ABSTRACT

Objectives: To evaluate the anticaries and remineralizing potential of experimental toothpastes containing different concentrations of surface pre-reacted glass ionomer (S-PRG). Design: Cylindrical specimens of sound and polished bovine enamel were used to evaluate the protective (n=84) and the remineralizing (n=84) potential of the toothpastes. Specimens of each study were stratified into the treatment groups (n=12): 0%, 1%, 5%, 20%, and 30% S-PRG, 1450µg F/mL (positive control), and distilled water (negative control). Treatments with toothpastes slurries were performed 2x/day-5min-8days, interspersed with de/remineralizing cycling. To assess the protective potential of treatments, specimens were immersed in demineralizing solution for 4h and in remineralizing solution for 20h and to evaluate the remineralizing potential, specimens were submitted to artificial white spot lesion formation in demineralizing solution for 20h, submitted to the treatments and de/re cycling (2h in de and 22h in re solution). Surface and cross-sectional microhardness analyses were performed. Independent statistical analyses were applied for both studies using ANOVA and Tukey test (5%). Results: For protection, toothpastes containing S-PRG differed significantly from the negative control and 0% S-PRG ($p<0.05$) presenting protective effect and 30% S-PRG was the most effective, differing from the positive control ($p<0.05$). For remineralization, toothpastes containing S-PRG differed from negative control group ($p<0.05$), but did not differ from each other ($p>0.05$). Toothpastes containing S-PRG were not superior to the NaF. Conclusion: Toothpastes containing S-PRG were effective as anticaries and remineralizing agents. The 30%S-PRG showed the superior efficacy against enamel demineralization, being the most promising concentration to be added to toothpastes.

Keywords: Dental caries; Demineralization; Remineralization; Toothpastes; *S-PRG*.

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Introduction

Although fluoridated products are consistent for preventing caries development and the initial remineralization of early caries lesions, caries can still develop in high-risk individuals and, because of that, additional preventive measures are worthy of investigation (Amaechi et al., 2021). Considering this, the addition of bioactive particles to oral health products has shown to be highly promising in the control of dental caries (Amaechi et al., 2017; Spinola et al., 2020). Among the bioactive particles added to dental products, pre-reacted glass ionomer particles, named S-PRG, have called attention. These particles represent a proprietary active ingredient initially incorporated into commercial products containing the nominated GIOMER technology by the manufacturer, because they present a "Glass IOnoMER" phase. The process for obtaining these particles involves the grinding of a fluoroboroaluminosilicate glass in micro or nanoparticles, which are treated with a solution containing polysiloxane and ethanol, followed by drying. Subsequently, the material is disintegrated in a mixer under the addition of polyacrylic acid in the presence of water to obtain particles with a pre-reacted surface. This treatment creates an acid-base reaction on the particle surface, promoting the formation of an ionomeric hydrogel layer (Nakatsuka et al., 2003). The S-PRG particle then has a trilaminar structure, composed of a functional glass core, surrounded by a glass ionomer phase and finally, presenting a modified surface layer. From the pre-reacted ionomeric phase, multiple ions (fluoride, strontium, borate, sodium, aluminum, and silicate) are released in the presence of moisture (Fujimoto et al., 2010).

The addition of these particles to different dental materials such as composite resins and varnishes has led to positive results in terms of caries prevention, as they act as an inhibitor of enamel (Spinola et al., 2020) and dentin (Mukai et al., 2009) demineralization. This fact encouraged the addition of S-PRG particles to daily use products, such as toothpastes and mouthwashes. Previous studies with experimental products have shown that dentifrices containing S-PRG had an effect against enamel demineralization in the face of acid challenges to which teeth are exposed daily and in the presence of biofilm (Iijima et al., 2017; Amaechi et al., 2017; Amaechi et al., 2018).

However, although the S-PRG particles show promising results in preventing caries, little is known about which concentrations of these particles are the most favourable when incorporated into toothpastes to act in the prevention of enamel demineralization. Furthermore, to the best of our knowledge, no study has shown the proper concentration of S-PRG added to toothpastes that could favour enamel remineralization. In this context, the objectives of the present study were to investigate the potential of toothpastes containing different concentrations of S-PRG in protecting enamel against demineralization as well as in promoting remineralization. The null hypotheses tested were that the experimental S-PRG toothpastes do not protect the tooth enamel against demineralization or promote white spot lesions remineralization. Additionally, pH and concentration of available fluoride in the slurries were also assessed.

Material and Methods

Experimental design

Experimental toothpastes containing different concentrations of S-PRG (0, 1, 5, 20, and 30%) and a dentifrice with 1450 µg F/mL were evaluated for their potential to protect enamel against demineralization, in a cycling model favouring demineralization, as well as to provide remineralization, in a cycling model favouring remineralization. For this, 84 bovine enamel blocks were obtained for each experiment and stratified to one of the following treatment groups according to the amount of S-PRG fillers added to the toothpaste (n = 12): 0% wt S-PRG; 1% wt S-PRG; 5% wt S-PRG; 20% wt S-PRG; 30% wt S-PRG; 1450 µg F/mL (positive control) and distilled water (DW) (negative control). The specimens were then be subjected to 8-day cycles favouring demineralization (to assess protection against demineralization) or favouring remineralization (to assess the ability to remineralize early carious lesions previously induced in the specimens). During cycling, the specimens were treated twice with dentifrice suspension in artificial saliva (1:3) with one of the treatments above. The pH and fluoride concentration in the slurries were assessed. After cycling, surface microhardness and cross-sectional microhardness were performed.

Assessment of pH in the slurries

The pH of the toothpaste slurries prepared in artificial saliva was analysed using a pH meter (Seven Multi, Mettler Toledo, Switzerland) coupled with specific electrode (micro pH electrode, Hanna Instruments, USA), calibrated with standard solutions (pH 4.00 and 6.86). Slurries pH were read in triplicate and the mean of the three readouts was considered as the final pH value.

Specimens' preparation

The crowns of fresh, non-damaged bovine incisors were separated from the roots and stored into 0.1% thymol solution at 4 ºC until required. A hundred and sixty-eight cylindrical enamel specimens (4 mm in diameter) were obtained from the labial surface of the teeth, using a diamond-coated trephine mill adapted to a circular cutting machine. The enamel specimens were embedded in acrylic resin and polished using SiC sandpapers in sequential grits of 1200, 2400, and 4000 (FEPA-P, Struers, Copehnagen, Denmark), under constant water irrigation for 10, 30 and 60 s, respectively, using an automatic polishing machine (Tegramin 25, Struers). After each paper grit change, specimens were kept in an ultrasonic bath for 5 min, to remove debris and abrasive grains. The specimens were examined under a stereomicroscope (Carl Zeiss – Stemi 2000 - 20X) to ensure the absence of cracks or other surface defects (Ávila et al., 2017). Then, 84 specimens were used for the protective effect against demineralization test and 84 specimens for the remineralization potential test.

Microhardness analysis for enamel blocks selection

For specimens' selection, surface microhardness (SMH) analysis of enamel with Knoop diamond were performed. Three indentations were performed on the surface of each specimen with a load of 25 g for 5 s and with a lateral spacing of 100 µm between each one. The mean of the 3 values of each specimen were calculated and specimens presenting a microhardness value (KHN) above 300 were selected.

Groups division

Enamel specimens were stratified into 7 treatment groups, according to the microhardness values. Each group (n = 12) was treated with a toothpaste containing

0, 1, 5, 20, 30% S-PRG, 1450 µg F/mL (positive control) and distilled water (DW) (negative control). Toothpastes were prepared and provided by the manufacturer (Shofu Inc., Kyoto, Japan) containing: silicic anhydride, sodium carboxymethylcellulose, glycerol, sorbitol solution, perfume, and 1µm of S-PRG particles (1 – 30 wt%). The fluoride toothpaste presented similar composition, with no S-PRG.

Demineralizing pH cycling

For the protective effect against demineralization test, a pH cycling was used to simulate a cariogenic challenge, where the demineralization process was greater than the remineralization process (Queiroz et al., 2008). For this, the sound specimens were immersed in a demineralizing solution for 4 h (6.25 mL/mm^2), pH 5.0, containing 0.05 M acetate buffer, 1.28 mM Ca, 0.74 mM Pi and 0.03 µg F/mL and for 20 h in a remineralizing solution (3.12 mL/mm^2), pH 7.0, containing 0.1 M Tris buffer, 1.5 mM Ca, 150 mM KCl, 0.9 mM Pi and 0.05 µg F/mL at 37°C for 8 days. Specimens were washed with distilled water and immersed in one of the treatments (toothpaste/artificial saliva (1:3)) for 5 minutes at room temperature before and after immersion in the demineralizing solution. The solutions were changed after the fourth day of cycling and after the eighth day of cycling. Specimens were maintained in remineralizing solution for additional 24 h before the surface microhardness analysis (Queiroz et al., 2008).

White spot lesion formation

For the remineralization potential test, after the initial microhardness analysis, enamel specimens were submitted to white spot lesion formation by immersion in a demineralizing solution, pH 5.0, 37°C, containing: 0.05 M acetate buffer, 1.28 mM Ca, 0.74 mM Pi and 0.03µg F/mL for 20 h (Queiroz et al., 2008). New microhardness analysis was performed after white spot lesions.

Remineralizing pH cycling

To evaluate the activation of enamel remineralization, a pH cycling regimen was performed, in which the remineralization condition was greater than the demineralization. Specimens with white spot lesions were kept in demineralizing solution for 2 h and in remineralizing solution for 22 h at 37°C for 8 days. Specimens

were washed with distilled water and submitted to one of the treatments for 5 minutes before and after immersion of the specimens in the demineralizing solution. The solutions were changed on the fourth day of the pH cycling.

Surface microhardness measurement after cycling

Surface microhardness analyses were performed again on specimens' surface after pH cycling regimens. A new set of three indentations was performed at 150 µm from the initial indentations. The percentage of loss of surface microhardness loss (%SML) and surface microhardness recovery (%SMR) after demineralization and remineralization cycles were determined using the following equations:

$$\%SML = ((SMH_{sound\ enamel} - SMH_{after\ pH\ cycling}) / SMH_{sound\ enamel}) * 100$$

$$\%SMR = ((SMH_{treated\ enamel} - SMH_{carious\ enamel}) / SMH_{sound\ enamel} - SMH_{carious\ enamel}) * 100$$

Cross-sectional microhardness measurement

After surface microhardness analyses, specimens had their halves embedded in acrylic resin. The non-recessed part was ground and polished with carbide sandpapers #1200, 2400 and 4000 until the internal parts of the samples submitted to demineralization and remineralization cycling were exposed for cross-sectional microhardness analysis (CSMH). In each specimen, three rows with 10 indentations at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µm depths were performed.

Statistical analysis

Brown-Forsythe test was used to analyze homogeneity of variances and Shapiro-Wilk test to analyze the normality assumptions of the data after pH-cyclings. Surface alteration (%SMR) data obtained after the remineralizing pH-cycle were transformed to log to satisfy the premises of normality. Differences for surface alteration (%SML and %SMR) were analyzed using one-way ANOVA and Tukey's tests. The absolute microhardness values for each depth of CSMH were also analyzed with ANOVA and Tukey's tests. The analyses were performed with Statistica for Windows (StatSoft, Hamburg, Germany) and the significance level of 5% was adopted.

Results

pH in toothpastes slurries

Table I shows the results of pH for each experimental group.

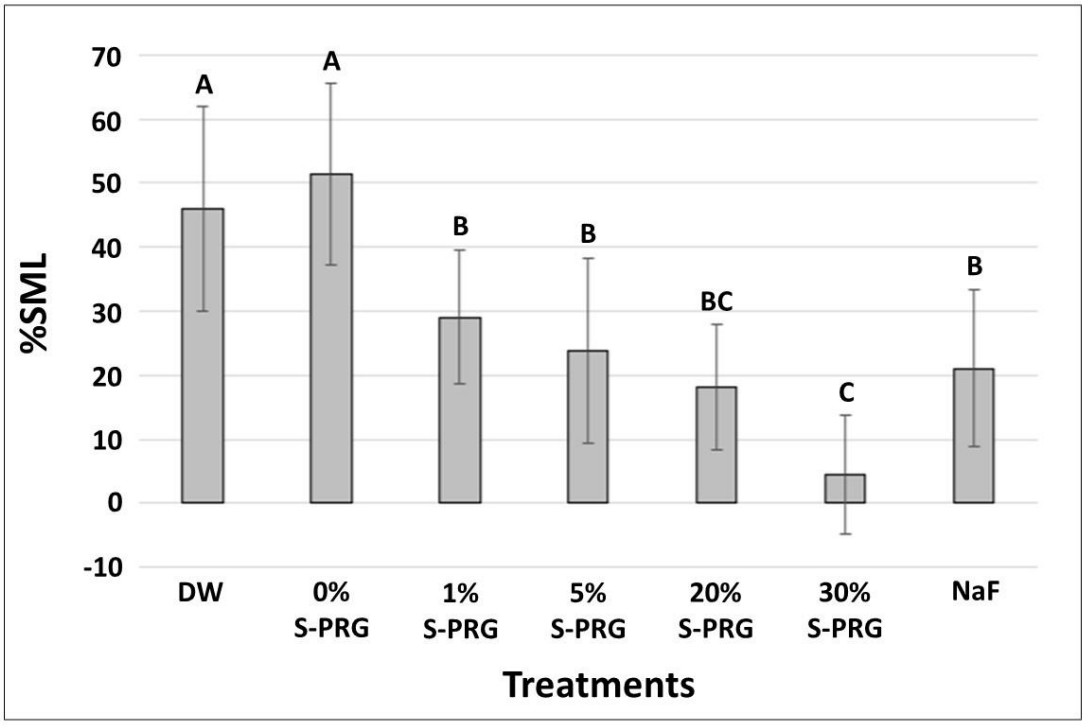
Table I: Mean and standard deviation values of the slurries' pH for each group.

Toothpastes	*pH*
0% S-PRG	*5.59 ± 0.02*
1% S-PRG	*5.58 ± 0.03*
5% S-PRG	*5.96 ± 0.02*
20% S-PRG	*6.08 ± 0.02*
30% S-PRG	*6.19 ± 0.00*
NaF	*5.66 ± 0.01*

Surface microhardness

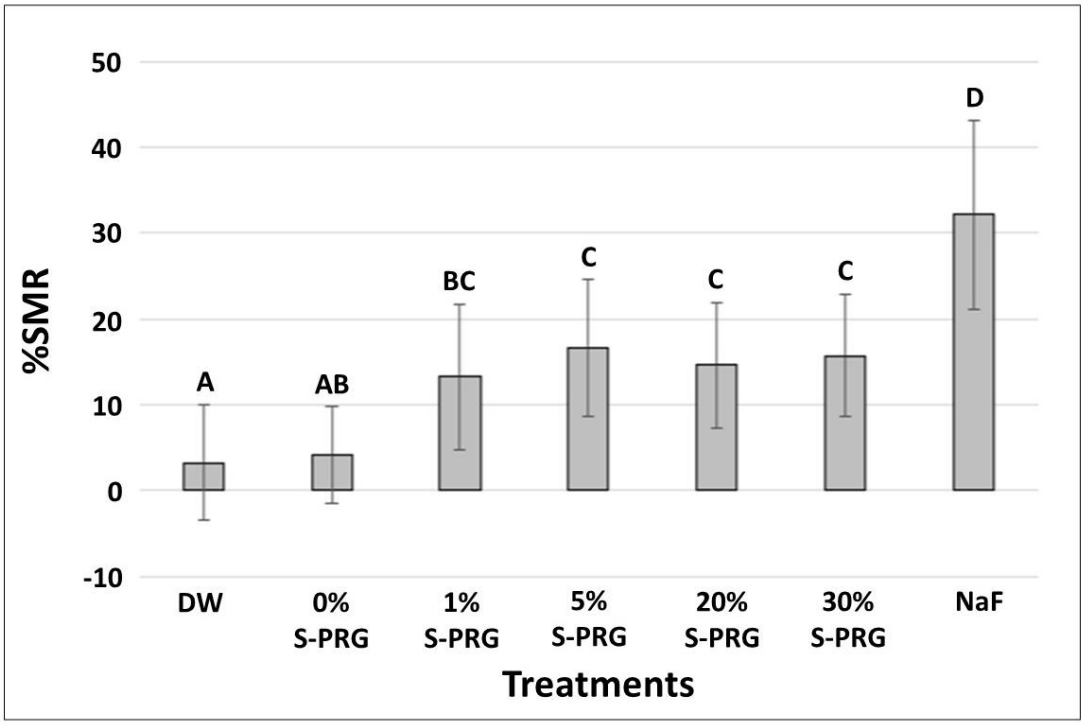
S-PRG containing toothpastes were able to protect enamel against demineralization and to remineralize early caries lesions. For demineralizing pH-cycling, S-PRG (1 – 30%) and NaF toothpastes differed significantly from the DW and the 0% S-PRG ($p < 0.05$). Toothpastes containing 1, 5 and 20% S-PRG presented similar effect to NaF, while the 30% S-PRG toothpaste presented the highest efficacy in protecting enamel against demineralization (lowest %SML) (figure 1).

Figure 1: Values (means ± SD) of %SML after the demineralizing pH-cycling. Higher values mean higher demineralization. Different letters mean significant differences according to Tukey's test (5%).



For the remineralizing potential analysis, S-PRG (5 – 30%) and NaF toothpastes differed significantly from the DW and the 0% S-PRG ($p < 0.05$), but no significant differences could be observed for the %SMR among the groups treated with 1 – 30% S-PRG toothpastes ($p > 0.05$). NaF toothpaste differed from the groups treated with S-PRG ($p < 0.05$) and presented the highest values for %SMR (figure 2).

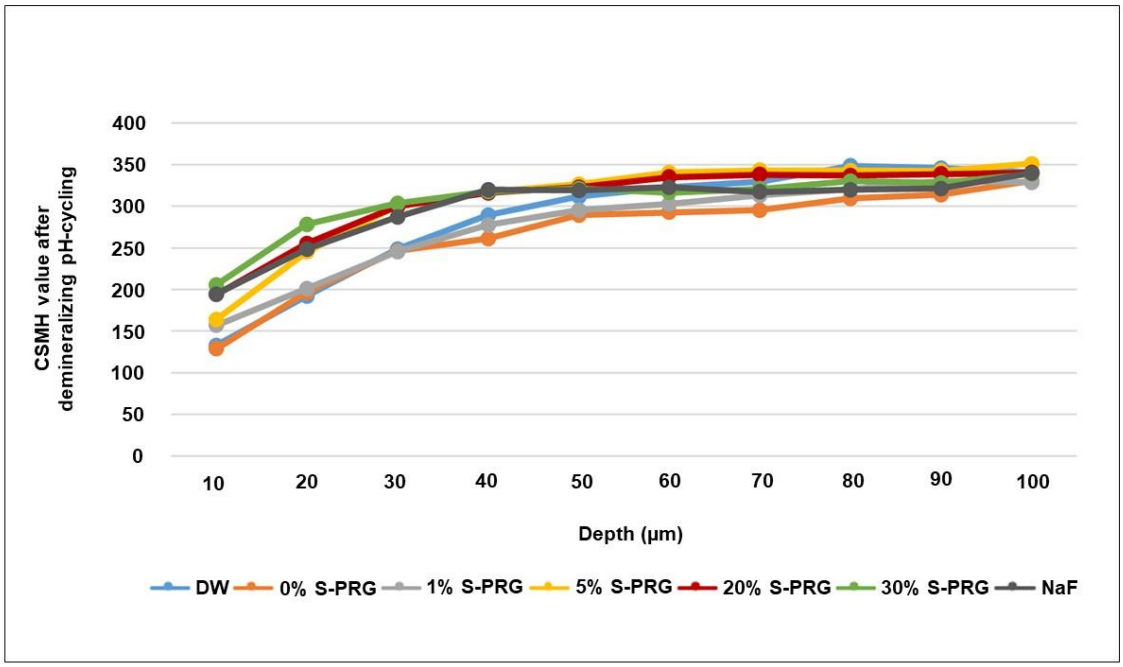
Figure 2: Values (means ± SD) of surface microhardness recovery (%SMR) after the remineralizing pH-cycling. Higher values mean higher remineralization potential. Different letters mean significant differences according to Tukey's test (5%).



Cross-sectional microhardness

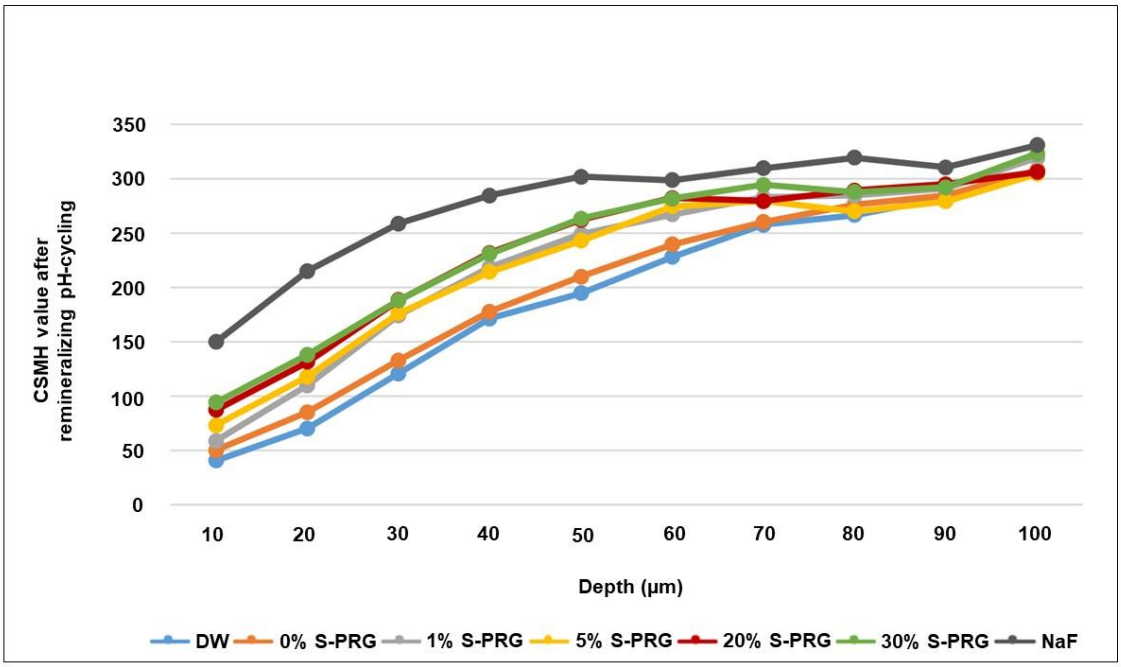
Figures 3 and 4 show the results of the cross-sectional microhardness (CSMH), from 10 µm to 100 µm, for both experiments. For the demineralizing pH-cycling, it was observed at 10 and 20 µm depths that 20, 30% S-PRG and NaF toothpastes were able to protect enamel against demineralization and differed significantly from DW and the 0% S-PRG groups ($p < 0.05$), with 20 and 30% S-PRG presenting a similar effect to NaF at 10 µm and 20 and 30% S-PRG presenting a higher efficacy than NaF at 20 µm. At 30 um, 5, 20 and 30% S-PRG presented a higher efficacy than NaF. From 40 µm to 100 µm, no significant differences were observed among the groups ($p > 0.05$).

Figure 3: Values of CSMH at different depths after demineralizing pH-cycling. For the mean and SD values details, refer to Table I of the supplemental material.



For the remineralizing pH-cycling, it was observed that 20 and 30 % S-PRG and NaF toothpastes differed from DW and placebo groups ($p < 0.05$) until 30 µm. Toothpaste containing NaF presented higher values of CSMH at depths 10 to 50 µm at 60 µm, 20, 30 and NaF differed from the negative control ($p < 0.05$) after 70 µm, no differences were observed among the groups tested ($p > 0.05$).

Figure 4: Values of CSMH at different depths after remineralizing pH-cycling. For the average and SD values details, refer to table II of the supplemental material.



Discussion

In order to investigate if the addition of S-PRG particles to toothpastes could exhibit anticaries effect and remineralization potential, defining the most appropriate concentration of these particles to be added to toothpastes, two different experiments were conducted. The pH-cycling models used here are well stablished and were proved effective to evaluate fluoridated products dose-response in de- and remineralizing processes (Queiroz et al., 2008; Moi et al., 2008; Spinola et al., 2020). The surface and cross-sectional microhardness analysis were performed since it is able to provide relevant information about the mechanical properties of the lesion through a hardness value that can be related to the relative mineral content of the analyzed area (Featherstone, 1992).

According to our results, S-PRG containing toothpastes were effective in protecting enamel against demineralization and in remineralizing white spot lesions, thus rejecting the null hypotheses tested. Since for the present study the composition

of S-PRG toothpastes were the same, changing only the concentration of the active ingredient, the results observed can be directly related to the action of the ions released on enamel and/or surrounding media. For demineralization protection, toothpastes containing 5 and 20% S-PRG were as effective as toothpaste containing NaF, while 30% S-PRG was the most effective toothpaste presenting a superior effect than NaF toothpaste. This result is in accordance with previous studies, which also showed that the addition of high concentration of S-PRG particles to toothpastes and varnishes, such as 30 and 40%, promoted higher protective effect than fluoride only products. This may be a consequence of more ions released, which are available to interact with hydroxyapatite and surrounding media (Amaechi et al., 2017; Spinola et al., 2020; Suge and Matsuo, 2020).

The use of a NaF toothpaste as a positive control is imperative, since its efficacy against enamel demineralization is well stablished, even in low concentrations in biofilm or oral fluids. This occurs because it induces mineral precipitation on the tooth structure in the form of fluorapatite when the demineralization process is occurring, in an effect called reduction of demineralization (Tenuta and Cury, 2010).

The concentration of F^- released from S-PRG toothpastes is probably much lower than the amount released by the NaF toothpaste containing 1450 µg F/mL. The similar effect found for 5% S-PRG, 20% S-PRG and NaF toothpastes might be then associated with the amount of Sr^{2+} concomitantly released by S-PRG. Although the amount of Sr^{2+} was not assessed in the present study, unpublished data provided by the manufacturer showed the amount of Sr^{2+} released from S-PRG to be much higher than F^- and to increase considerably with the increase in the concentration of S-PRG. It is known that Sr^{2+} plays an important role in caries prevention as it can substitute calcium in apatite lattice reducing its solubility, which contributes to the increased resistance to further acid challenges (Ashrafi, 1980; Wang et al., 2019). Besides, Sr^{2+} may also contribute to an acid buffer capacity. Previous studies reported that S-PRG particles are associated with the pH increase of the surrounding media (Fujimoto et al., 2010; Kaga et al., 2014; Hirayama et al., 2018). An increase in the pH occurs because Sr^{2+}, Na^+ and Al^{3+} released from S-PRG act as strong bases that exert a buffering capacity by neutralizing the acids, thus protecting enamel against demineralization (Ma

et al., 2012). A similar effect could be observed in the present study and is shown in table I as S-PRG toothpastes were able to increase the pH of the demineralizing solution used in the pH cycling immediately after the preparation of the slurries.

All these factors together might explain why S-PRG exhibited enamel protection and the 30% S-PRG was more effective than NaF toothpaste. The potential of S-PRG containing materials to protect enamel against demineralization seems to be even higher when a microbiological model is used (Amaechi et al., 2018). This occurs because they present antimicrobial capacity, mainly promoted by BO_3^{3-}, also released from S-PRG particles, which difficult bacteria adhesion and growth (Dembitsky et al., 2011).

In the present study, we were able to extend the knowledge on the mechanisms of S-PRG containing toothpastes, as we also investigated their remineralizing potential. It was observed that all concentrations of S-PRG added to toothpastes were able to remineralize the white spot lesions, although no significant differences could be observed between the groups treated with the different concentrations of S-PRG. The remineralizing action of S-PRG containing products has been demonstrated on dentin (Ito et al., 2011) and etched enamel (Miyauchi, 2009; Iijima et al., 2014). It has been shown that the ions released can enhance the mineral deposition and this may be mainly associated with F^-, Sr^{2+} and Si_3^{2-}.

Toothpastes containing F^- exhibited a higher remineralizing efficacy, showed by the surface microhardness test. This superior effect was also evident in the cross-sectional microhardness analysis, up to 30 µm. The higher efficacy observed for NaF toothpaste may be related to fluoride mechanism of action itself. Fluoride is able to partially remineralize enamel by forming fluorapatite when pH inside biofilm increases, by the buffering action of saliva, after a cariogenic challenge in a process named activation of remineralization (Tenuta and Cury, 2010). The higher efficacy observed for NaF toothpaste may be related to the fact that the concentration of F^- available to remineralize white spot lesions might be considerably higher for NaF toothpaste than those found in 30% S-PRG toothpaste, the highest concentration of S-PRG containing toothpastes tested. The lack of difference for remineralization observed among the groups treated with different concentrations of S-PRG might indicate a similar amount

of F^- released by the S-PRG groups. Previous studies have reported that besides F^- alone, the association between Sr^{2+} and F^- seems to enhance F^- remineralizing potential of enamel and dentin (Thuy et al., 2008; Yassen et al., 2012; Dai et al., 2021), but the mechanism of action of this association needs to be further investigated. Although the remineralizing effect of NaF toothpaste was higher, all the S-PRG toothpastes were also effective as remineralizing agents.

It is also important to highlight that silicate ions (Si_3^{2-}) play a role on the remineralization potential. It is reported that Si_3^{2-} seems to promote hydroxyapatite formation as silica gel induces apatite nucleation because of its silanol groups that interacts with calcium and phosphorous from the surrounding media, creating a biologically active apatite on the silica gel surface (Li et al., 1993). Bioactive glasses can release Si_3^{2-} that are adsorbed on the substrate surface, providing sites for apatite nucleation. Once nucleated, it will spontaneously grow to form a bone-like apatite layer (Tanahashi et al., 1994). However, apatite formation seems to be more effective in dentin than in enamel due to presence of phosphophoryn, a major noncollagenous protein present in dentin organic matrix, which is believed to have a primary role in dentin mineralization (Saito et al., 2003). Nevertheless, much lower amount of Si_3^{2-} is released when compared to the other ions (Fujimoto et al., 2010). Thus, the action of this ion on enamel remineralization promoted by S-PRG toothpastes should be also further investigated.

Toothpastes are usually considered excellent vehicles for the availability of active ingredients on caries prevention, due to its association to hygiene habits and biofilm control, used in high frequency. Nevertheless, it must be considered that they present some limitations as S-PRG particle delivery vehicle compared to other products, as varnishes and sealants, such as the short period of time in contact with teeth, immediately dilution by saliva, and rinsing after use, which might interfere with the ions availability. On the other hand, in toothpastes, the active ingredient is readily accessible and in direct contact with the tooth surface. The association of toothpastes with other daily used or professional products, might represent an interesting alternative for individuals with high risk of caries, but this strategy needs further investigation. Additionally, studies simulating more relevant clinical conditions, as the

presence of biofilm, salivary flow and clearance, must be conducted to strengthen S-PRG toothpastes anticaries and remineralizing potential evidence.

Conclusion

The S-PRG containing toothpastes were able to protect enamel against demineralization and to promote remineralization. The 30% S-PRG toothpaste presented higher efficacy than NaF toothpaste in protecting enamel against demineralization, being the most promising concentration to be added to toothpastes.

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Authors contributions statement

Manuela da Silva Spinola: Conceptualization; Methodology; Data analysis; Writing-Original draft preparation; Writing-Reviewing. **Letícia Silva Moreira**: Methodology. **Carlos Rocha Gomes Torres**: Conceptualization; Writing-Reviewing. **Alessandra Bühler Borges**: Conceptualization; Data analysis; Writing-Original draft preparation; Writing-Reviewing.

Highlights

- *S-PRG toothpastes were effective on the protection against demineralization;*
- *S-PRG toothpastes were effective in remineralizing white spot lesions;*
- *30% S-PRG seems to be the most appropriate concentration in toothpastes;*
- *S-PRG are promising agents to prevent dental caries.*

Supplemental material

Table 1: Mean cross-sectional microhardness values (SD) for each depth after demineralizing pH-cycling (n = 12). Different capital letters mean differences among the treatments for each depth (one-way ANOVA followed by Tukey's test).

Treatments	***Depth (µm)***									
	10	***20***	***30***	***40***	***50***	***60***	***70***	***80***	***90***	***100***
DW	*132.73 (53.74) A*	*191.90 (42.73) A*	*248.87 (44.67) A*	*289.18 (58.94) AB*	*311.73 (63.24) A*	*323.10 (45.86) A*	*329.66 (48.18) A*	*348.61 (30.92) A*	*345.74 (35.83) A*	*340.82 (34.27) A*
0% S-PRG	*128.71 (36.96) A*	*196.66 (52.34) AB*	*246.45 (48.37) A*	*261.20 (46.89) A*	*289.37 (58.97) A*	*292.81 (43.33) A*	*295.68 (45.78) A*	*309.62 (46.87) A*	*313.77 (32.20) A*	*330.53 (20.45) A*
1% S-PRG	*157.09 (36.06) AB*	*200.93 (33.61) ABC*	*245.60 (51.76) A*	*277.77 (53.13) AB*	*295.26 (53.87) A*	*302.87 (45.40) A*	*313.05 (48.49) A*	*320.47 (51.12) A*	*330.92 (46.00) A*	*328.72 (44.20) A*
5% S-PRG	*164.00 (40.71) AB*	*245.33 (37.11) BCD*	*300.77 (29.28) B*	*317.33 (32.72) B*	*326.84 (37.67) A*	*340.59 (39.14) A*	*343.30 (40.68) A*	*343.14 (42.68) A*	*342.57 (36.51) A*	*351.34 (32.08) A*
20% S-PRG	*193.97 (30.78) B*	*255.42 (49.80) D*	*299.95 (42.35) B*	*315.98 (35.53) AB*	*322.81 (34.54) A*	*335.16 (36.74) A*	*338.20 (28.67) A*	*337.00 (26.14) A*	*338.94 (30.87) A*	*340.47 (27.96) A*
30% S-PRG	*205.56 (32.98) B*	*278.38 (26.74) D*	*303.57 (23.87) B*	*317.61 (28.32) B*	*321.72 (32.62) A*	*316.56 (31.25) A*	*321.07 (26.74) A*	*330.43 (35.00) A*	*327.86 (48.09) A*	*338.87 (31.86) A*
NaF	*194.57 (48.38) B*	*248.38 ± 42.31 CD*	*287.19 ± 40.84 AB*	*320.05 (48.34) B*	*319.34 (51.38) A*	*322.70 (54.24) A*	*317.34 (56.21) A*	*319.88 (53.77) A*	*321.32 (44.46) A*	*340.28 (38.84) A*

Table 2: Mean cross-sectional microhardness values (SD) for each depth after remineralizing pH-cycling (n = 12). Different capital letters mean differences among the treatments for each depth (one-way ANOVA followed by Tukey's test).

Treatments	***Depth (µm)***									
	10	***20***	***30***	***40***	***50***	***60***	***70***	***80***	***90***	***100***
DW	*40.87 (9.85) A*	*70.35 (25.04) A*	*120.98 (40.65) A*	*171.66 (52.00) A*	*194.80 (46.85) A*	*228.13 (54.70) A*	*257.86 (45.06) A*	*266.51 (38.12) A*	*283.00 (31.55) A*	*307.73 (21.23) A*
0% S-PRG	*50.28 (14.74) AB*	*85.48 (27.34) AB*	*133.03 (29.63) AB*	*178.00 (44.77) AB*	*210.12 (38.94) AB*	*239.73 (54.72) AB*	*260.44 (62.73) A*	*276.42 (64.61) A*	*284.74 (50.03) A*	*308.13 (27.57) A*
1% S-PRG	*58.82 (13.12) ABC*	*109.91 (36.67) ABC*	*174.39 (43.16) BC*	*218.69 (48.60) AB*	*249.14 (54.51) ABC*	*267.18 (62.18) AB*	*283.13 (61.82) A*	*284.70 (54.61) A*	*291.39 (45.63) A*	*319.39 (21.24) A*
5% S-PRG	*73.26 (19.60) BCD*	*118.19 (21.71) BC*	*176.43 (40.34) BC*	*214.51 (45.79) AB*	*243.19 (57.82) ABC*	*274.88 (58.91) AB*	*279.75 (58.85) A*	*270.04 (61.14) A*	*279.46 (50.44) A*	*304.67 (37.90) A*
20% S-PRG	*87.29 (30.04) CD*	*131.54 (42.73) C*	*188.50 (33.03) C*	*231.95 (39.89) BC*	*262.31 (46.96) BC*	*282.38 (40.83) AB*	*279.65 (28.54) A*	*289.30 (23.22) A*	*295.12 (31.33) A*	*306.35 (37.40) A*
30% S-PRG	*94.18 (36.81) D*	*138.35 (37.51) C*	*188.10 (34.51) C*	*231.12 (52.24) BC*	*263.67 (48.98) BC*	*282.00 (55.78) AB*	*294.46 (67.05) A*	*288.07 (55.15) A*	*292.44 (47.51) A*	*323.37 (49.62) A*
NaF	*150.03 (25.79) E*	*215.35 (46.95) D*	*258.96 (49.78) D*	*284.82 (43.62) C*	*302.19 (43.94) C*	*298.84 (45.41) B*	*309.86 (46.70) A*	*319.63 (46.34) A*	*310.65 (40.21) A*	*331.09 (26.85) A*

2.2 Artigo – Spinola MS, Mendonça JL, Garcia MT, Junqueira JC, Torres CRG, Borges AB. Eficácia antimicrobiano de dentifrícios contendo S-PRG sobre o desenvolvimento de biofilmes de *S. mutans* / Antimicrobial e*fficacy of S-PRG containing toothpastes on S. mutans biofilm development******

RESUMO

Objetivos: o objetivo do presente estudo foi investigar o potencial antimicrobiano de dentifrícios contendo partículas bioativas de vidro pré reagido (S-PRG) sobre a colonização inicial e maturação de biofilmes de *Estreptococos mutans* (*S. mutans*). Design: Uma cepa de referência (UA 159) e uma cepa clínica de *S. mutans* (SM6) foram utilizadas. Espécimes de esmalte bovino foram aleatoriamente alocadas em um dos seguintes grupos (n=5): dentifrícios contendo 0%; 1%; 5%; 20% e 30% de S-PRG; controle positivo (NaF+triclosan); e controle negativo (água destilada). Para formação do biofilme, os espécimes foram inseridos em uma placa de 24 poços contendo saliva artificial (4h), seguido por adição de 1mL de saliva artificial e meio de cultura BHI e 225µL de suspensão de *S. mutans*. Os tratamentos com as supensões de dentifrícios foram aplicados antes ou depois de 4 e 24h da formação do biofilme. As amostras foram incubadas por 48h a 37°C em 5%CO_2 e o biofilme foi então removido dos espécimes e semeado em placas de Petri para contagem de UFC/mL. Os dados foram analisados por ANOVA teste de Tukey (5%). Resultados: uma diminuição significativa na adesão de microrganismos ($p<0.05$) foi observada para todos os grupos tratados com os dentifrícios contendo S-PRG. Para a cepa SM6, os dentifrícios contendo S-PRG apresentaram resultados semelhantes ao dentifrício contendo NaF+triclosan e para a cepa UA159 o dentifrício com 30%S-PRG apresentou efeito superior ao dentifrício contendo NaF+Triclosan. Efeito antimicrobiano sobre o biofilme recém-formado (4h) também foi observada para os grupos tratados com dentifrícios contendo S-PRG groups, mas não foi observado efeito superior ao dentifrício contendo NaF+Triclosan. Para o biofilme maduro, efeito antimicrobiano dos dentifrícios contendo S-PRG foi observado apenas para a cepa clínica, mas não foi maior que o efeito antimicrobiano exercido pelo dentifrício contendo NaF+Triclosan. Conclusão: Dentifrícios experimentais contendo S-PRG foram eficazes na inibição do desenvolvimento de biofilmes de *S. mutans,* sendo promissores agentes para prevenir a o desenvolvimento de biofilme cariogênico.

Palavras-chave: *Estreptococos mutans*. Biofilme. Esmalte dental. Giomer.

ABSTRACT

Objectives: The antimicrobial effects of toothpastes containing bioactive surface pre-reacted glass particles (S-PRG) on Streptococcus mutans (S. mutans) biofilms in adherence, initial colonization and maturation stages were investigated. Design: Reference UA 159 and a clinical S. mutans (SM6) strains were used. Bovine enamel specimens were randomly allocated into the groups (n=5): toothpastes containing 0%; 1%; 5%; 20%; 30% S-PRG; positive control dentifrice (NaF+triclosan); and negative control (distilled water). For biofilm formation, samples were placed in a 24-well plate containing artificial saliva (4h), followed by adding 1mL of artificial saliva, BHI broth and 225µL of S. mutans suspension. The treatments with dentifrices were applied previously or after 4h and 24h of biofilm formation. Samples were incubated for 48h at 37°C in 5%CO_2 and biofilm was detached and seeded in Petri dishes for determining the number of viable cells. Data were analyzed by ANOVA and Tukey test (5%). Results: Significantly lower microorganisms adherence ($p<0.05$) was obtained for all S-PRG toothpastes, with similar results to NaF+triclosan for SM6 and 20 and 30%S-PRG groups exhibiting higher inhibition effect than the NaF+Triclosan for UA159. Antibacterial effect on the early-stage biofilm was also observed for the S-PRG groups, but was not superior to the NaF+Triclosan toothpaste. For the mature biofilm, effective antimicrobial potential of S-PRG toothpastes was observed only for the SM6 clinical strain, but was not higher than the positive control. Conclusions: Experimental S-PRG toothpastes were effective to inhibit S. mutans biofilm growth by exhibiting antimicrobial activity, being promising agents to prevent cariogenic biofilm development.

Keywords: Streptococcus mutans; Biofilm; Dental enamel; Giomer.

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Introduction

Dental caries is a biofilm and sugar-dependent disease that results in mineral loss of tooth surface. The control of the disease is directly related to the biofilm monitoring, which can be achieved by means of a controlled diet, with less intakes of carbohydrates, and with efficient oral hygiene, to disaggregate the cariogenic biofilm formed over the tooth surface (Machiulskiene et al., 2020). Although these methods are well established, and the prevalence and severity of dental caries have declined in recent years, the cariogenic biofilm tends to remain in regions that are difficult to access by brushing, such as the occlusal pit and fissures, and interproximal regions, making it still one of the most prevalent diseases at a world level (Frencken, 2018).

Strategies to improve the control of the acidogenic microorganisms that are prevalent in the cariogenic biofilm using products that have multiple biological effects such as antibacterial properties and biofilm formation inhibition have been tested (Hickl et al., 2018; Phillip et al. 2019).

The surface pre-reacted glass ionomer (S-PRG) filler is an innovative bioactive material composed by a 3-layer structure derived from a proprietary technology. A fluoroboroaluminosilicate multifuncional glass is covered with polysiloxane, creating a porous superficial silica-glassy layer. Then, a polyacrylic acid solution is sprayed on the particle and penetrates the surface porosities, reacting with the inner glass core. This creates a thin intermediate stable pre-reacted glass ionomer phase (Nakatsuka et al., 2003). The dental products containing S-PRG fillers are named GIOMER, due to their "Glass IOnoMER" phase. The bioactive effect of these materials is exerted by the multi-ions release (fluoride, strontium, borate, sodium, aluminium, and silicate) derived from the multifunctional glass in the presence of moisture (Fujimoto et al., 2010).

These fillers can be incorporated into preventive and restorative materials. The antimicrobial/anticaries/remineralizing effects have been shown with surface barrier materials, varnishes, and sealants (Okuyama et al., 2016; Spinola et al., 2020; Shimazu et al., 2012). Favorable results have been observed regarding the antimicrobial activity of restorative materials using Giomer technology (Saku et al., 2010; Miki et al., 2016), besides the ability to reduce the demineralization of dentin

adjacent to restorations (Shiiya et al., 2016), and exert acid-buffering properties, which might reduce the susceptibility to secondary caries (Kaga et al., 2014).

This motivated the development of toothpastes containing the S-PRG particles, providing the benefit of releasing the multi-ions into the oral environment, as an alternative for caries prevention. Studies have shown that experimental S-PRG based toothpastes were effective on enamel demineralization prevention (Amaechi et al., 2018; Iijima et al., 2017; Nakamura et al., 2017). However, despite previous studies present promising results regarding the addition of S-PRG to dental products, little is still known about the ideal concentration of these particles in dentifrices concerning their antimicrobial efficacy on dental cariogenic biofilms, aiming to promote strong scientific evidence so that commercial products can be developed and be further available in the market. Thus, the present study reports the findings on the inhibitory effect of experimental dentifrices containing different concentrations of S-PRG on adherence, initial colonization and maturation phases of S. mutans biofilms. The null hypotheses tested was that there is no difference in the S. mutans biofilm inhibition among the toothpastes in the different phases tested.

Material and Methods

Ethical aspects

The study protocol was approved by the local Ethics Committee in Research (CAAE:005611/2017).

Microorganisms

In this study, one reference S. mutans strain (UA159) and one clinical strain were used. The clinical strain was previously isolated from the active cavities of a subject according to de Carvalho et al., 2006, was identified by PCR (Oho et al., 2000) and confirmed by automatic sequencing (Dereeper et al., 2008), named SM6.

Study design

Six independent experiments were conducted (three for the S. mutans UA159 strain, and three for the SM6 strain). Each experiment presented one factor under investigation (treatment toothpastes) at 7 levels (distilled water as negative control (DW); commercial NaF+Triclosan-based toothpaste as positive control; and toothpastes containing S-PRG: 0%; 1%, 5%, 20%, and 30%). For both strains, the following effects were investigated: the inhibitory effect on biofilm adherence, initial colonization and maturation (n = 5/group). UFC/mL was the response variable.

Specimens' preparation

Fresh, non-damaged bovine incisors were collected for this study. The crowns were separated from the roots and stored into 0.1% thymol solution at 4ºC until required. Seventy cylindrical enamel specimens (6 mm in diameter) were obtained from the labial surface of the teeth, using a custom-made diamond-coated trephine mill adapted to a circular cutting machine. The enamel specimens were polished using SiC sandpapers in sequential grits of 1200, 2400, and 4000 (FEPA-P, Struers, Copenhagen, Denmark), under constant water irrigation for 10, 30 and 60 s, respectively, using an automatic polishing machine (Tegramin 25, Struers). After each paper grit change, specimens were kept in an ultrasonic bath for 5 min, to remove debris and abrasive grains. The specimens were examined under a stereomicroscope (Carl Zeiss – Stemi 2000 - 20X) to ensure the absence of cracks or other surface defects (Borges et al., 2016). The specimens were autoclaved before the beginning of the experiments.

Preparation of the standardized suspension of S. mutans

The microorganisms were cultured in brain-heart infusion broth (BHI Himedia, Mumbai, India) at 37ºC for 48 h (5% CO_2). The microbial cells in culture were centrifuged (1300 rpm for 10 min), and the pellet was rinsed twice with 0.85% NaCl (Labimpex, Sao Paulo, Brazil). The cells suspensions were adjusted to 10^8 cells/mL using a spectrophotometer at a wavelength of 398 nm and optical density of 0.620 (B5B2, Micronal, São Paulo, Brazil) (Terra Garcia et al., 2018).

Groups division

For each experiment, testing both the reference and the clinical strain, the specimens were divided in seven groups (n = 5 each). The experimental toothpastes were prepared by Shofu Inc. (Kyoto, Japan), and contained silicic anhydride, sodium carboxymethylcellulose, glycerol, sorbitol solution, perfume, and 1 μm of S-PRG particles: 0% (placebo), 1%, 5%, 20%, and 30% (in weight). The distilled water was the negative control and a commercial toothpaste containing NaF (1450 ppm F^-) and Triclosan (Colgate Total 12, Colgate-Palmolive, São Paulo, Brazil) was the positive control.

S. mutans biofilm formation

Specimens were placed in 24-well plates containing 2 mL of mucin containing artificial saliva (Klimek et al., 1982) and incubated for 4 h at 37°C to allow the formation of a surface pellicle. After 4h, specimens were rinsed twice with 2 mL of PBS solution, placed in a 24-well plate with 1 mL of BHI broth supplemented with 5% sucrose and 1 mL artificial saliva. Then, 225 μL of the standardized S. mutans suspension was added in each well, and the plates were incubated for 24 h at 37°C (5% CO_2). The wells were washed three times with 2 mL PBS for the removal of weakly adhered cells, and 1 mL of BHI broth supplemented with 5% sucrose and 1 mL artificial saliva was added. The plates were incubated for an additional 24 h at 37°C.

Antibacterial potential of the toothpastes on S. mutans biofilm development

Specimens were treated with dentifrices slurries prepared in artificial saliva (1:3) for 5 min in 3 different phases: before the immersion in artificial saliva to evaluate the effect of the treatments on the microorganisms adherence phase, after 4 h of biofilm growth to evaluate the antibacterial potential over the early-stage biofilm, and after 24 h of biofilm growth to evaluate the antibacterial potential over biofilm maturation.

Cell count for biofilm analysis

After final incubation, the specimens were transferred to Falcon tubes containing 4 mL PBS. The adhered biofilm was detached using an ultrasonic homogenizer (Sonoplus KD2200, Bandelin Eletronic, Berlin, Germany) at 7 W for 30 s. Serial dilutions were prepared from the obtained solution and plated on Petri dishes

containing BHI agar. The plates were incubated for 48 h at 37ºC (5% CO_2) to determine the number of colony-forming units (CFU/mL).

Statistical analysis

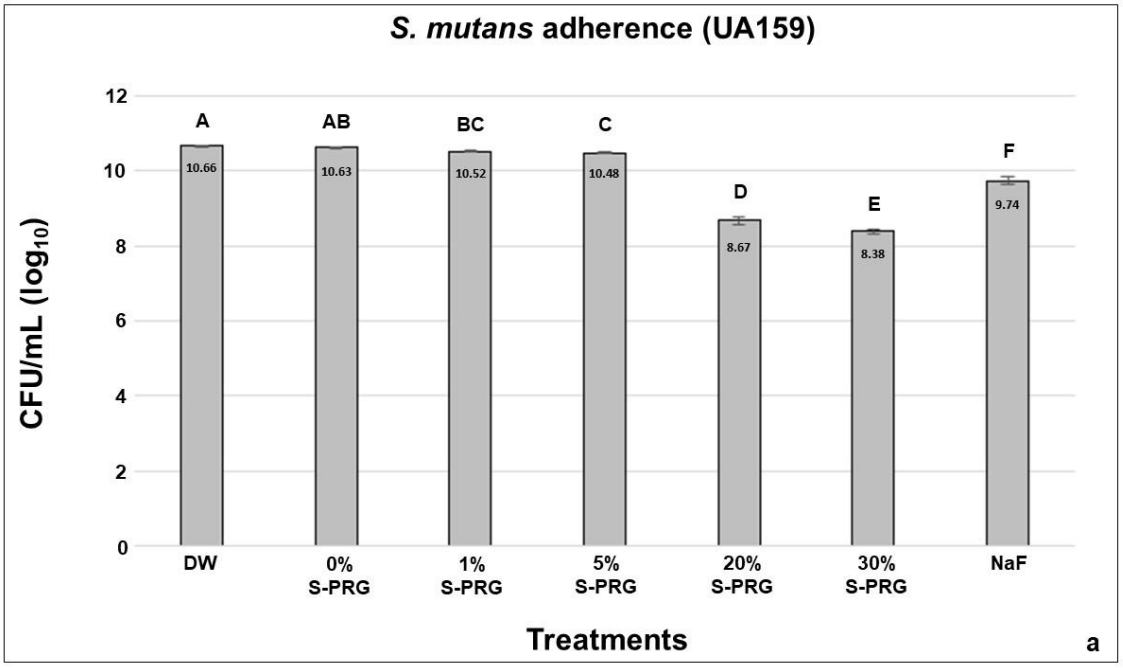
Data of CFU/mL were transformed in log10 and means ± standard deviation were calculated for each treatment and analysed by one-way ANOVA followed by Tukey's test with a p value set at 5%. Data was analysed using Statistica for Windows (StatSoft, Hamburg, Germany).

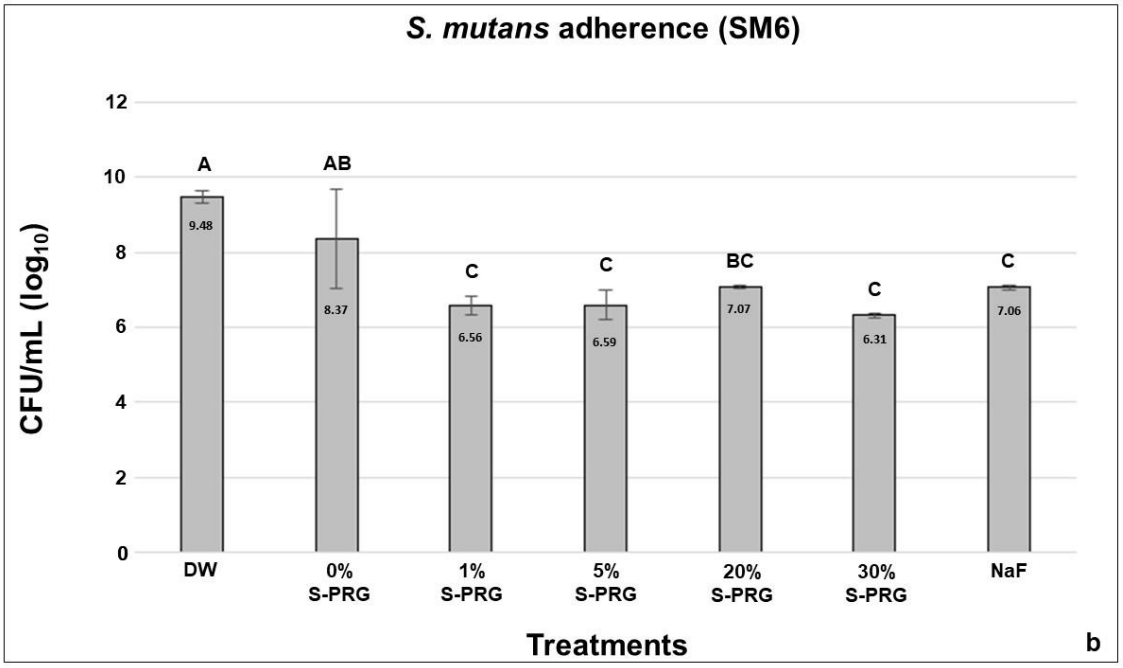
Results

Potential of the toothpastes on S. mutans adherence

Figures 1 a and b show mean and standard deviation data for the tested groups, using the reference UA159 and the SM6 clinical strains, respectively. The results of ANOVA one-way indicated significant difference for the groups ($p<0.05$). The inhibiting effect was higher when more concentrated toothpastes were used, indicating a dose-dependent effect. According to Tukey's test, the toothpastes containing S-PRG were capable to inhibit S. mutans adherence when compared to the negative control ($p<0.05$) for both strains. For UA159 strain, both 20 and 30% S-PRG toothpastes showed significantly lower S. mutans adherence than the positive commercial toothpaste containing NaF and Triclosan. For SM6 strain, 1 to 30% were similar to the positive control. The 0% S-PRG toothpaste presented no significant S. mutans adherence inhibition potential compared to negative control ($p>0.05$).

Figure 1 – Mean CFU/mL (log_{10}) and standard deviation data when toothpastes were applied previously to S. mutans adherence, from UA159 reference (a) and SM6 clinical (b) strains.

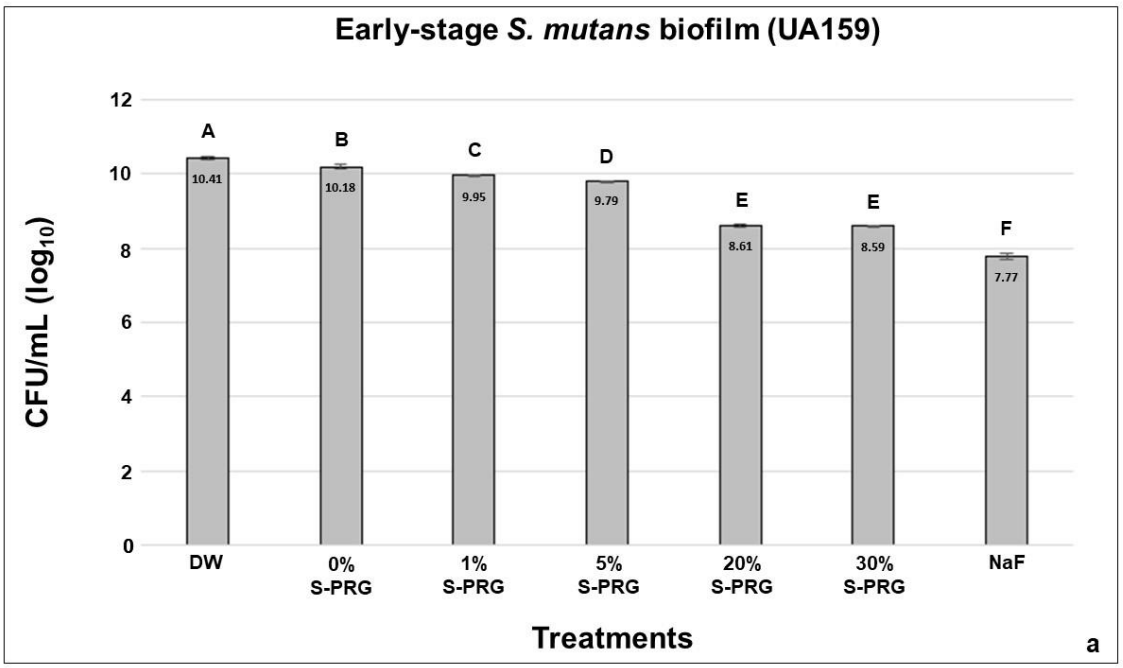


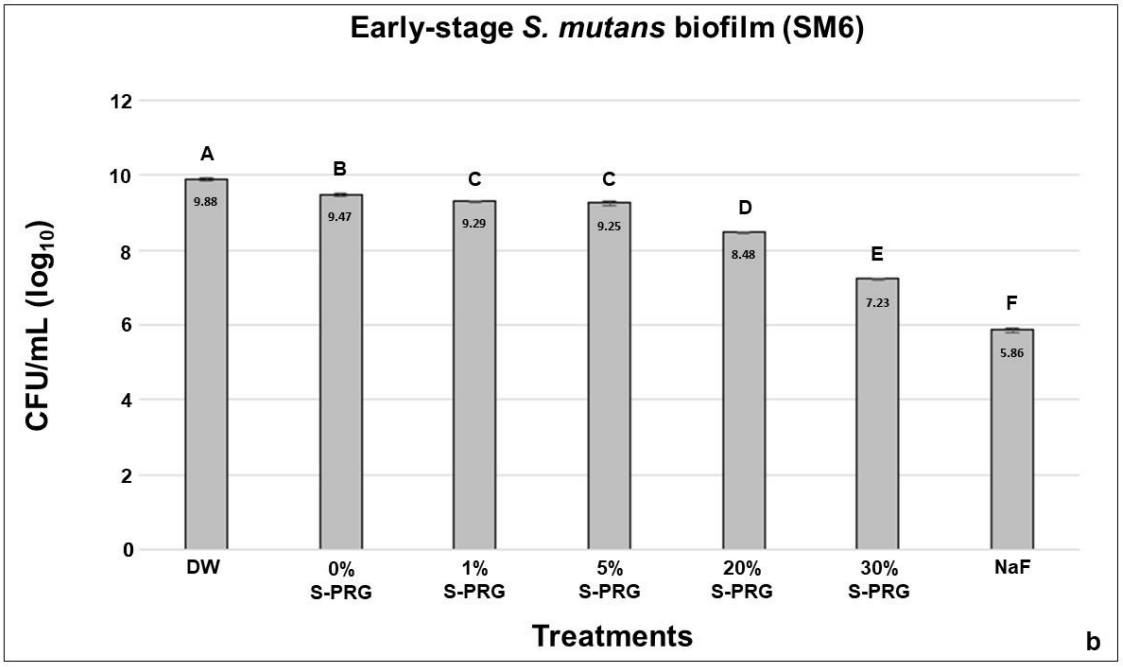


Antibacterial potential of the toothpastes on early-stage S. mutans biofilm

The mean and standard deviation data for the treatments applied after 4 h of reference and clinical S. mutans strains biofilm formation are shown in figure 2 a and b, respectively. ANOVA one-way indicated significant difference for the tested groups ($p<0.05$). According to Tukey's test results, all S-PRG containing toothpastes were significantly different from the negative control for both strains ($p<0.0001$). The most effective products were the 20% and 30% S-PRG toothpastes. Nevertheless, all were significantly different from the positive commercial product (NaF + Triclosan) that showed the higher antimicrobial effect against early-stage biofilms ($p<0.0001$).

Figure 2 – Mean CFU/mL (log_{10}) and standard deviation data for antibacterial potential of treatments on early-stage S. mutans biofilms (after 4 h) cultivated from UA159 reference (a) and SM6 clinical (b) strains.

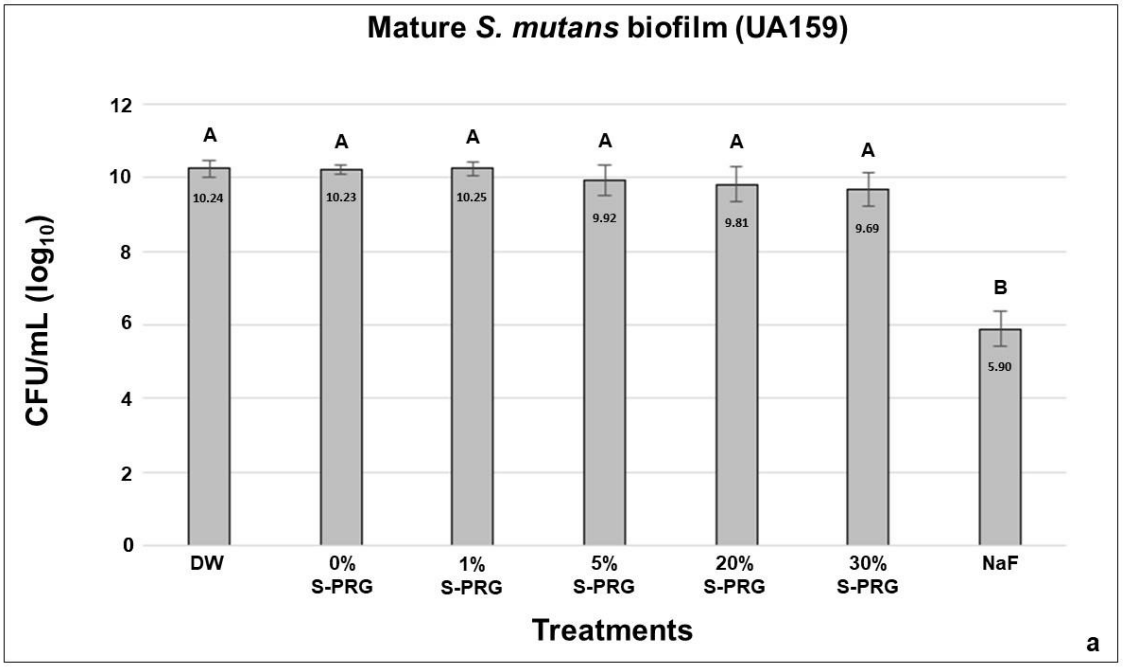


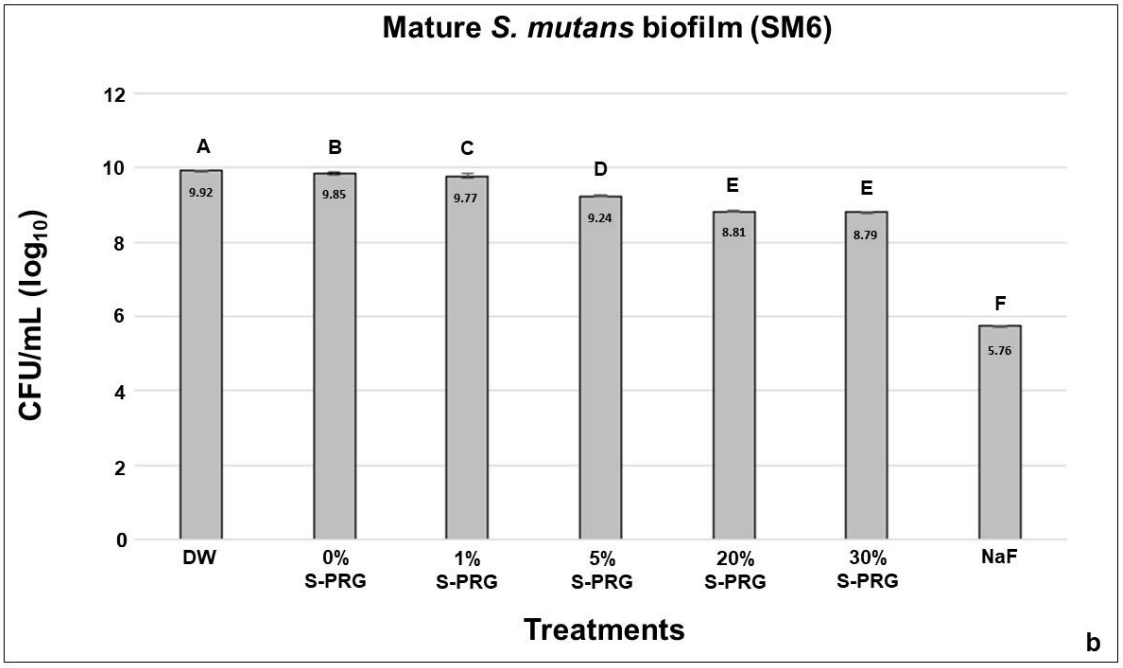


Antibacterial potential of the toothpastes on mature S. mutans biofilm

The mean and standard deviation data of cell counts for the treatments applied after 24 h of reference and clinical S. mutans biofilm formation are shown in figure 3 a and b, respectively. ANOVA one-way showed significant difference for the groups ($p<0.05$). According to the Tukey's test, there were no significant differences for the S-PRG containing toothpastes compared to the negative control, when reference UA159 strain was tested ($p>0.05$). For the SM6 clinical strain, the experimental toothpastes exhibited significant antimicrobial effect compared to the negative control, with higher efficacy when 20 and 30% S-PRG were used, but they were not superior than the commercial NaF toothpaste used as positive control ($p<0.05$).

Figure 3 – Mean CFU/mL (log_{10}) and standard deviation data for antibacterial potential of treatments on S. mutans biofilms maturation (after 24 h) cultivated from UA159 reference (a) and SM6 clinical (b) strains.





Discussion

The effect of toothpastes containing different concentrations of S-PRG was evaluated at three distinct moments: before, after 4 h, and after 24 h of biofilm development, to represent the different clinical situations found in the oral cavity. A standard strain of S. mutans (UA 159) was used because this microorganism represents one of the most important microorganisms in the etiology of caries disease (Takahashi and Nyvad, 2011). Additionally, a clinical strain, previously isolated from dental caries (SM6), was used in order to extend the knowledge of the antimicrobial properties of the S-PRG toothpastes to a more real condition (Terra Garcia et al., 2021). The model used here for biofilm growth has been widely used in the microbiology and cariology areas (Pereira et al., 2013; Terra Garcia et al., 2018) to simulate the conditions of a cariogenic biofilm due to the presence of 5% sucrose added to the culture medium. The biofilm was cultivated over bovine tooth specimens.

The use of bovine specimens in substitution of human in caries lesion studies is a viable alternative due to their similar mineral content (Edmunds et al., 1988) and biofilm cariogenicity pattern with both substrates (Ayoub et al., 2020). Besides, they present great advantages, such as easier acquisition and the presence of a large and flat surface to obtain the specimens.

The null hypothesis was rejected, as the toothpastes containing S-PRG were able to inhibit the adherence of the microorganisms and all the concentrations tested reduced the development of the early-stage biofilm. The toothpastes containing 20% and 30% were superior to the positive control in inhibiting biofilm development for the standard strain, while toothpastes from 1 – 30% were had similar effect to the positive control in inhibiting biofilm development for the clinical strain. These results agree with previous studies, in which the presence of S-PRG in composite materials (Hotta et al., 2014; Miki et al., 2016) and eluate (Suzuki et al., 2014; Nomura et al., 2018) inhibited the growth of S. mutans biofilm. Inhibition of initial microorganisms is an important factor in prevention of the biofilm development because coaggregation influences the properties of plaque (Huang et al., 2011). The observed inhibition of biofilm growth and effect on the newly formed biofilm are probably associated with the release of BO_3^{3-},

F^- and Sr^{2+} as it has been suggested that these ions are able to diffuse into the biofilm (Kato et al., 2020).

It is known that BO_3^{3-} presents great ability to prevent bacterial growth, because it acts by inhibiting quorum sensing inside bacteria (Dembitsky et al., 2011). Quorum sensing is characterized as a highly specific system that controls different bacterial activities such as the production of signalling molecules, transport, and perception of the surrounding environment. This system is responsible for the process of bacterial adhesion to surfaces, the production of the extracellular polysaccharide matrix, and the virulence of the biofilm (Nadell et al., 2008; Sifri, 2008).

The F^- ions are inhibitors of carbohydrate metabolism in oral Streptococci (Bibby and van Kesteren, 1940), because they can penetrate the cell and bind to enolase and ATPase, inhibiting these enzymes and leading to the reduction of carbohydrate metabolism (Marquis, 1990; Marquis et al., 2003; van Loveren et al., 2008). The inhibition of enolase causes a reduction in phosphoenolpyruvate levels and, consequently, a reduction in glucose uptake by phosphoenolpyruvate phosphotransferase system (Kanapka and Hamilton, 1971; Hamilton, 1977), while the inhibition of ATPase causes ineffective extrusions of protons (Quivey et al., 2000), leading to an acidification of cell cytoplasm and reduced metabolism and acid tolerance by the cells (Sheng and Marquis, 2006; van Loveren et al., 2008).

Regarding the Sr^{2+} released from the S-PRG fillers, the cariostatic activity caused by the presence of such ions in oral products is not completely understood, since it seems that its concentration in saliva and plaque is usually not able to exert an important antimicrobial effect (Lippert and Hara, 2013). Nevertheless, the release of Sr^{2+} might represent an important ally in products containing S-PRG, since its cariostatic properties may act synergistically to F^- to inhibit bacterial activity (Dabsie et al., 2009).

In mature biofilms (24h), S-PRG toothpastes had no effect on the standard strain, but all concentrations of S-PRG were able to decrease the number of bacteria in the biofilm cultivated from the clinical strain SM6. Nevertheless, this effect was lower than the NaF + triclosan toothpaste. Triclosan, in the concentration of 0.3% in the dentifrice used as positive control, has well-established antimicrobial properties when

added to toothpastes to act against dental plaque (Valkenburg et al., 2019), although it has been replaced in the recently launched toothpastes due to its potential hormonal disfunction properties (Zorrilla et al., 2009). This active agent may be released in a higher concentration than the ions present in the S-PRG particle, to exert its antimicrobial effect on the mature biofilm, but this was not tested in the present study.

It was previously shown that the inhibitory effect of a S-PRG eluate was less pronounced when the eluate was applied in the post-logarithmic phase of biofilm growth, suggesting that the main action of these bioactive particles is related to the inhibition of S. mutans virulence and growth (Nomura et al., 2018). The fact that S-PRG could decrease the number of bacteria in the clinical strain, but not in the standard strain may be related to the fact that S. mutans standard strain UA 159 is known to be a highly cariogenic strain, besides being adapted to the culture medium since it is the strain of choice for many studies in cariology. The difference between the cariogenicity and adaptation potential of the standard strain UA159 and the clinical strain SM6 is clear when we observed that for the three types of biofilms tested, the products presented better results with the clinical strain.

These less favourable results observed with the mature biofilms can be explained by the fact that the dental products containing S-PRG particles, in general, are characterized as agents that constantly release the ions present in their composition. However, the amount of ions released from the experimental toothpastes might not be high enough to act in the rupture of a mature biofilm. In addition, the short time of exposure to the treatments and the dilution of the products in artificial saliva prior to the treatment of enamel blocks, to simulate the dilution caused by saliva in the oral cavity, may also have influenced the efficacy of the products. Previous studies have shown that low concentrations of S-PRG in eluates, for example, were not effective on mature biofilm and that higher concentrations of these products were needed to cause a rupture in the mature biofilm when compared to the concentration to inhibit the biofilm development (Suzuki et al., 2014). This may be due to the fact that the properties of a mature biofilm are completely different when compared to a newly formed biofilm. In a mature cariogenic biofilm, formed by the interaction between bacteria and sucrose, we can observe the presence of an extracellular polysaccharide

matrix that is responsible for its virulence and that interfere with the physical and chemical properties of the biofilm (Flemming and Wingender, 2010; Koo et al., 2013).

The presence of the extracellular matrix promotes a support for the development of the biofilm because it encourages bacterial adhesion, besides hindering the diffusion of substrates from the medium to the interior of the biofilm (Flemming and Wingender, 2010), thus impairing the action of the ions inside the biofilm.

Although the results with the experimental toothpastes containing S-PRG are promising, especially those with higher concentrations of particles in their composition, more studies are needed to confirm the action of these particles on the dental biofilm in more relevant clinical conditions.

Conclusion

Experimental dentifrices containing S-PRG showed antimicrobial activity mainly on microorganisms adherence and on early-stage S. mutans biofilm, being promising agents to prevent the cariogenic biofilm growth and development.

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Manuela da Silva Spinola: Conceptualization; Methodology; Data analysis; Writing-Original draft preparation; Writing-Reviewing. **Maira Terra Garcia**: Methodology; Data analysis. **Jacqueline Landi Mendonça**: Methodology. **Juliana Campos Junqueira**: Methodology; Writing-Reviewing. **Carlos Rocha Gomes Torres**: Conceptualization; Writing-Reviewing. **Alessandra Bühler Borges**: Conceptualization; Data analysis; Writing-Original draft preparation; Writing-Reviewing.

Highlights

- *S-PRG toothpastes were effective against S. mutans biofilm adherence;*
- *S-PRG toothpastes showed antimicrobial properties against S.mutans biofilms;*
- *S-PRG are promising agents to prevent development of cariogenic biofilms.*

3 CONSIDERAÇÕES GERAIS

Com base nos artigos apresentados, observou-se que os dentifrícios contendo S-PRG são agentes promissores no controle da cárie, pois foram capazes de apresentar efeito protetor, remineralizante e antimicrobiano.

Dentre todas as concentrações de S-PRG testadas (1, 5, 20, 30% S-PRG), podemos considerar que o dentifrício contendo 30% S-PRG foi o mais promissor, pois apesar de não ter diferido dos outros dentifrícios com diferentes concentrações de S-PRG na capacidade de promover a remineralização ou não ter sido superior ao controle positivo (dentifrício contendo NaF+triclosan) no efeito antimicrobiano sobre biofilmes recém formado e maduro, foi o que apresentou melhor resultado para inibir a perda mineral inicial do esmalte e inibir a adesão dos microrganismos para formação do biofilme.

Assim, podemos sugerir que a adição de partículas bioativas de S-PRG em dentifrícios é uma abordagem promissora como alternativa capaz de mostrar efeito protetor contra a cárie dental. Porém ainda devem ser testados em investigações futuras, utilizando modelos *in situ* capazes de simular condições mais próximas às condições clínicas e também em ensaios clínicos, para confirmar a efetividade desses dentifrícios. Ainda, análises adicionais envolvendo o potencial abrasivo de tais dentifrícios, efeitos sobre os tecidos moles e custo devem ser considerados para que estes produtos possam efetivamente ser disponibilizados no mercado.

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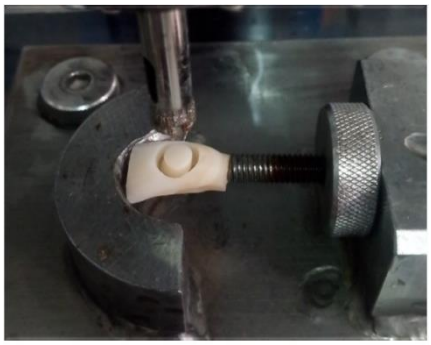
APÊNDICE A – Detalhamento da metodologia de confecção dos espécimes

1. Espécimes submetidos às ciclagens de pH.

Para a realização das ciclagens de pH para avaliação da proteção contra a desmineralização e da remineralização de lesões de mancha branca, espécimes provenientes de dentes bovinos foram cortados em cortadeira de amostras circulares com uma broca trefina de 4 mm de diâmetro interno, sob refrigeração em água (Figura 1).

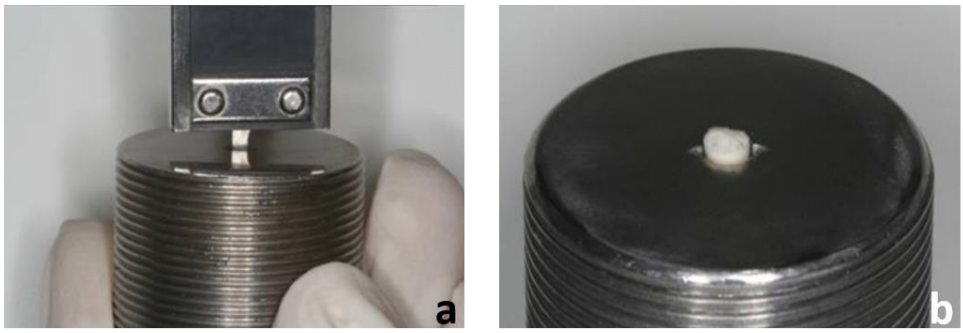
Figura 1 – Corte do espécime de dente bovino em cortadeira circular



Fonte: Elaborado pelo autor.

Após o corte, os espécimes foram inseridos em um dispositivo metálico, com orifício central de 4 mm de diâmetro e regulado em 2 mm de altura (Figura 2a), com o esmalte voltado para a parte interior do dispositivo para realizar a regularização da base em dentina dos mesmos (Figura 2b).

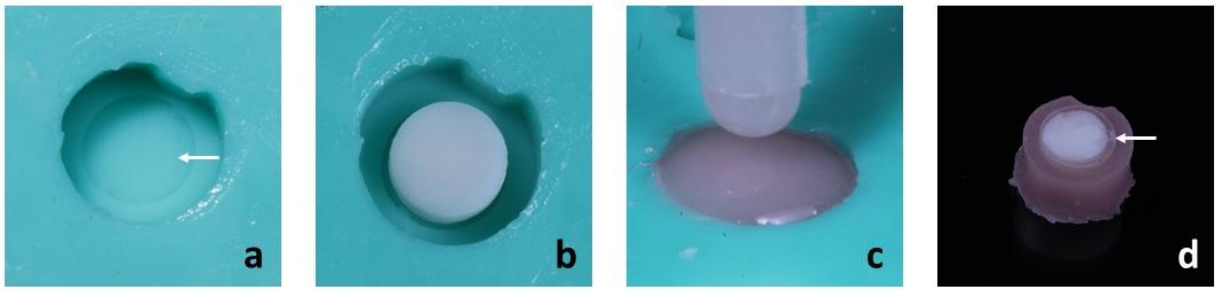
Figura 2 – Planificação da base dos espécimes



Legenda: a) regulação do dispositivo metálico em 2 mm de profundidade; b) inserção do espécime com o esmalte voltado para o interior do dispositivo e a porcao de dentina voltada para fora para planificação da base.
Fonte: Elaborado pelo autor.

Os espécimes foram então embutidos em resina acrílica com o auxílio de uma matriz de silicone (6mm x 3 mm) contendo um recuo de 0,1 mm em seu interior (Figura 3a). Os espécimes foram inseridos com o esmalte voltado para a parte interior da matriz (Figura 3b). Resina acrílica (JET - Clássico, São Paulo, Brasil) foi aplicada sobre os espécimes (Figura 3c) e, então o conjunto espécime + matriz + resina acrílica foi inserido em uma polimerizadora de pressão por 10 minutos para evitar a formação de bolhas durante a polimerização da resina acrílica. Após a polimerização, o espécime ficou embutido em resina acrílica (Figura 3d), deixando apenas 0,1 mm da superfície de esmalte exposto acima da resina para, posteriormente, ser desgastado em uma politriz automática (Tegramin 25, Struers).

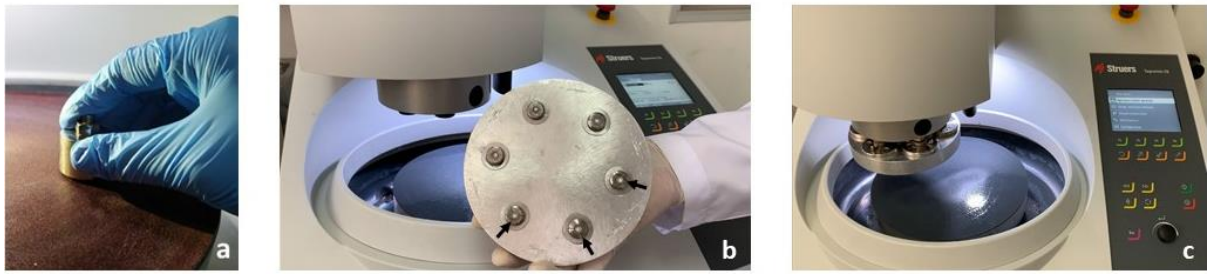
Figura 3 – Embutimento dos espécimes em resina acrílica



Legenda: a) matriz de silicone para o embutimento dos espécimes com 0,1 mm de recuo no interior (seta branca); b) espécime inserido na matriz de silicone com o esmalte voltado para o interior da matriz; c) inserção da resina acrílica sobre o espécime; d) aspecto final do espécime embutido com 0,1 mm de esmalte acima da resina acrílica (seta branca).
Fonte: Elaborado pelo autor.

Os espécimes foram inseridos em um dispositivo metálico com o esmalte voltado para dentro e a base dos espécimes embutidos com resina acrílica voltada para fora, a fim de ser individualmente polidos (Figura 4a). Após a regularização da base dos espécimes, estes foram individualmente inseridos com o esmalte voltado para fora em dispositivos metálicos customizados (Figura 4b) e acoplados ao braço vertical da politriz automática (Figura 4c), permitindo o aplainamento e manutenção do paralelismo entre a base e a superfície do espécime. Os espécimes foram então polidos com uma sequência de lixas de carbeto de silício nas seguintes granulações: #1200, #2400 e #4000 por 10, 30 e 60 segundos, respectivamente.

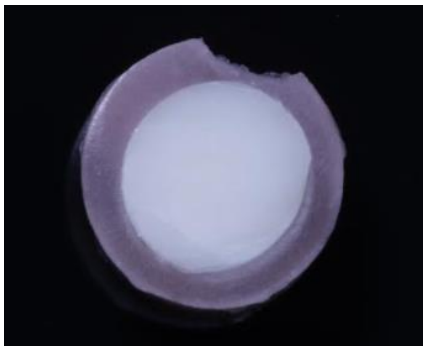
Figura 4 – Polimento dos espécimes



Legenda: a) base do espécime sendo individualmente polida; b) espécimes inseridos no dispositivo metálico customizado para a politriz automática (setas pretas); c) dispositivo metálico acoplado ao braço vertical da politriz automática.
Fonte: Elaborado pelo autor.

Após o polimento, obtivemos o aspecto final dos espécimes embutidos em resina acrílica com apenas a superfície de esmalte exposta (Figura 5).

Figura 5 – Aspecto final do espécime utilizado nas ciclagens de des e remineralização



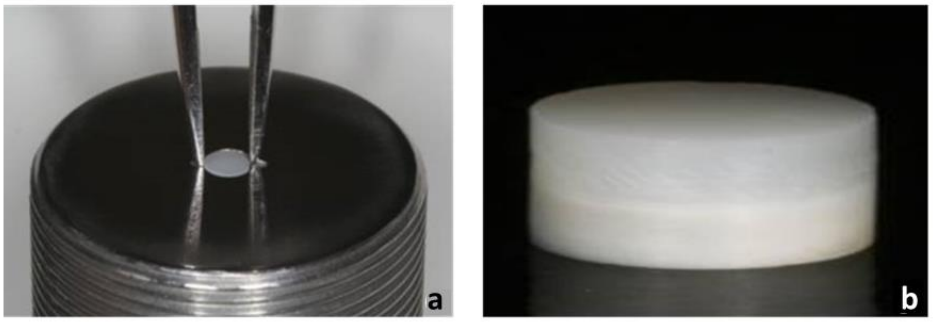
Fonte: Elaborado pelo autor.

1.1. Espécimes submetidos à formação de biofilme.

Para a obtenção dos espécimes do estudo que avaliou o efeito dos dentifrícios sobre a formação de biofilme, espécimes de 6 mm de diâmetro foram cortados em uma cortadeira de amostras circulares e tiveram sua base regulada como demonstrado anteriormente (Figuras 1 e 2).

Após a regularização da base dos espécimes, os mesmos foram então inseridos no dispositivo metálico com o esmalte voltado para fora (Figura 6a) para planificação e polimento da superfície do esmalte em uma politriz automática (Tegramin 25, Struers) com lixas de carbeto de silício nas seguintes granulações: #1200, #2400 e #4000 por 10, 30 e 60 segundos, respectivamente, até a obtenção do aspecto final (Figura 6b).

Figura 6 – Planificação da superfície de esmalte dos espécimes utilizados para avaliação antimicrobiana



Legenda: a) inserção do espécime com o esmalte voltado para fora para planificação da superfície de esmalte; b) aspecto final do espécime de 6 mm.

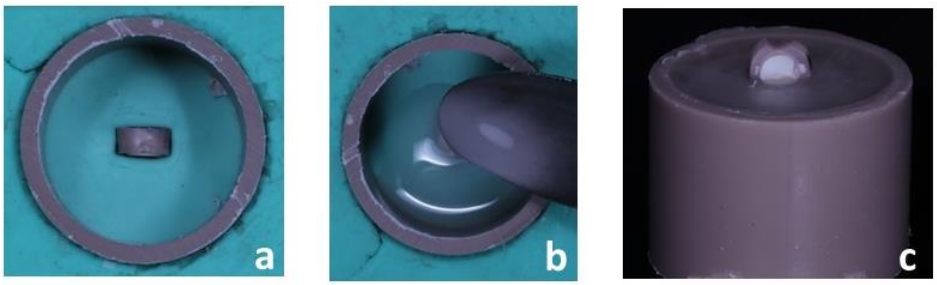
Fonte: Elaborado pelo autor.

2. Embutimento das amostras para análise de dureza subsuperficial

As amostras submetidas às ciclagens des e remineralizante foram embutidas em resina acrílica após a análise de microdureza superficial, para que tivessem as metades desgastadas e pudessem expor a parte interna do esmalte para análise de microdureza subsuperficial nas profundidades: 10, 20, 30, 40, 50, 60, 70, 80, 90 e 100 µm.

Inicialmente, os espécimes foram inseridos em uma matriz de silicone com uma projeção interna nas dimensões laterais do espécime (6 mm largura x 2 mm altura) junto a um tubo de PVC com 2 cm de altura para reter a resina acrílica (Figura 7a). A resina acrílica foi então inserida na matriz sobre o espécime (Figura 7b) para embutimento. Após a polimerização da resina acrílica, os espécimes ficaram com metade da amostra submersa em resina acrílica e metade exposta para ser posteriormente desgastada e polida para permitir que a dureza subsuperficial fosse realizada (Figura 7c).

Figura 7 – Embutimento dos espécimes em resina acrílica para análise subsuperficial



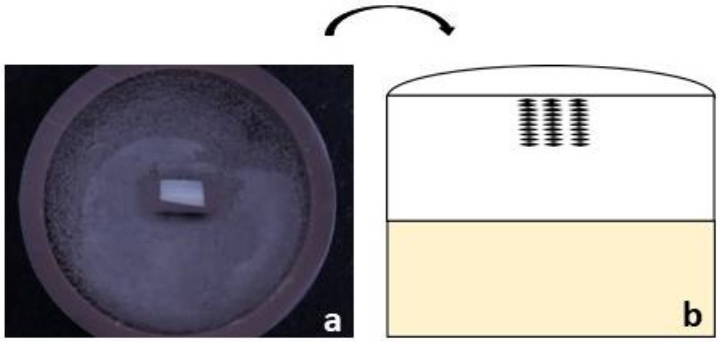
Legenda: a) espécime inserido no interior da matriz de silicone junto com um tubo de PVC; b) inserção da resina acrílica sobre o espécime; c) espécime com metade não inserida para ser posteriormente desgastada e polida.

Fonte: Elaborado pelo autor.

Os espécimes tiveram então a metade exposta desgastada em politriz automática (Tegramin 25, Struers) e foram polidos com uma sequência de lixas de

carbeto de silício nas seguintes granulações: #1200, #2400 e #4000 por 10, 30 e 60 segundos, respectivamente, até a obtenção do aspecto final das amostras (Figura 8).

Figura 8 – Aspecto final da amostra para análise da dureza subsuperficial



Legenda: a) espécime inserido em resina acrílica com a porção interna do esmalte exposta para análise de dureza subsuperficial; b) desenho esquemático demonstrando as endentações da análise de dureza subsuperficial no esmalte.
Fonte: Elaborado pelo autor.

ANEXO A – Comprovante de artigo submetido.

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