UNIVERSIDADE FEDERAL DO AMAPÁ
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

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Atividade antibiofilme de nanopartículas poliméricas carregadas com ácido anacárdico e seu efeito desmineralizante em esmalte bovino

Macapá
2018

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal do Amapá para obtenção do Título de Mestre em Ciências Farmacêuticas.

Orientador: **Prof. Dr. Francisco Fábio Oliveira de Sousa**

Macapá
2018

Dados Internacionais de Catalogação na Publicação (CIP)
Biblioteca Central da Universidade Federal do Amapá
Bibliotecária Orinete Costa Souza CRB-11/920

612.311
P436a Pereira, Yane dos Santos.
1.1.1. Atividade antibiofilme de nanopartículas poliméricas carregadas com ácido anacárdio e seu efeito desmineralizante em esmalte bovino / Yane dos Santos Pereira ; orientador, Francisco Fábio Oliveira de Sousa. – Macapá, 2018.
41 f.

Dissertação (Mestrado) – Fundação Universidade Federal do Amapá, Programa de Pós-Graduação em Ciências Farmacêuticas.

1. Cáries dentarias. 2. Anacárdico. 3. Nanopartícula. 4. *Streptococcus mutans*. I. Sousa, Francisco Fábio Oliveira de, orientador. II. Fundação Universidade Federal do Amapá. III. Título.

Programa de Pós-Graduação em Ciências Farmacêuticas
da Universidade Federal do Amapá

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Data: 21/05/2018

Dedico esta tese a minha família, em especial a Pedro Macena (avô) e Ivaneide Pereira (Tia) que hoje descansam ao lado do Pai e minha mãe Irene Pereira, por todo apoio e incentivo, por acreditarem no meu potencial.

AGRADECIMENTOS

Primeiramente a **DEUS,** por estar sempre me guiando no caminho certo, me protegendo e dando forças para continuar quando tudo parece não fazer sentido. Principalmente nos momentos mais difíceis, onde pensei que não conseguiria.

A toda a minha família, pois ressalto que Deus não poderia ter me dado a melhor, que de perto ou de longe estão sempre torcendo pelo meu sucesso, seja através de palavras de incentivo e de carinho ou simplesmente pelo fato que existirem em minha vida, especialmente aos meus pais amados, **IRENE PEREIRA** e **MANOEL CARLOS,** meu pai avô **PEDRO MACENA**, que hoje está descansando ao lado do Pai, minha mãe avó **IONE LIMA,** vocês me ensinaram os primeiros passos da vida e são exemplo de força e determinação. Meus irmãos, **DAIARA PEREIRA, DAYANE PEREIRA** e **DANIEL SANTOS,** vejo o quanto torcem pela minha felicidade pessoal e profissional, meu filho do coração **PABLO GUSTAVO** que nas ausências, principalmente as noites torcia para eu terminar a dissertação, pois já contava os dias e ao meu sobrinho e afilhado **PEDRO LORENZZO** meu encanto**,** amo muito vocês. Vocês são a minha base, sem vocês tudo se tonaria muito mais difícil.

Ao meu noivo, **JORGE MONTEIRO,** pois sempre embarca comigo nos sonhos e objetivos, apoiando e incentivando em todos os momentos, entendendo minha ausência e sempre torcendo pela minha realização profissional. Obrigada amor pela dedicação, cuidado, companheirismo e paciência. Sou grata a você pelas palavras de incentivo e pelos abraços nos momentos em que eu me desesperava achando que não iria conseguir. Te amo!

Ao meu orientador **PROF DR FRANCISCO FÁBIO OLIVEIRA DE SOUSA**, obrigada primeiramente por ceder à oportunidade de ser sua orientanda, sou grata por todo conhecimento transmitido, apoio, carinho, disponibilidade e principalmente pelas cobranças na orientação, pois você me ensinou que tudo depende de planejamento e dedicação ao máximo, pois bons frutos serão colhidos, e através destes nos tornaremos os protagonistas de nossos projetos acadêmicos. Isso é muito típico de um bom orientador.

As professoras **DRª. LÍLIAN GRACE DA SILVA SOLON** e **DRª RAMILLE DE ARAÚJO LIMA** pela disponibilidade em participar da banca avaliadora desta defesa.

Agradeço, ao Laboratório de Controle de Qualidade, Bromatologia e Microbiologia (LCBM) do Curso de Ciências Farmacêuticas, em especial a **JENNIFER ARAÚJO** por ceder as nanopartículas desenvolvidas durante seu projeto de monografia.

Ao Laboratório de Pesquisa do Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará, por aceitaram essa parceria. A todos que direta ou indiretamente contribuíram para o desenvolvimento desta pesquisa, em especial a doutoranda **ERNANDA SALES** que desde o inicio, sem me conhecer, me recebeu com toda gentileza, obrigada por todo ensinamento e contribuição, grata pela sua disponibilidade em ajudar, pela forma em que compartilhou seus conhecimentos, com todo cuidado, atenção e preocupação em ensinar passo-a-passo.

As minhas amigas de fortaleza, **MARIA CELIA** e **LAYDIANE MATOS** por me receberem em suas casas, me acolhendo e estimulando principalmente no momento mais dolorido de minha vida, minha perda familiar. Vocês foram muito importantes nesse momento, pois me consolaram e não me deixaram desistir. O estimulo de vocês foi primordial, pois me mostram que Deus é maior e Ele nos dá apenas o peso o qual podemos carregar.

Ao meu amigo **CLEBER NUNES,** por ter segurado as pontas quando eu precisava me ausentar do trabalho para assistir as aulas. Você que sempre muito cavalheiro dizia, vai lá, dou conta aqui, não se preocupa, resolvo aqui. És muito querido e foi muito importante no inicio da realização deste sonho.

A **IALLE GURGEL,** uma pessoa muito simples e querida que sempre se mostrou atenciosa e preocupada. Desde o inicio se dispôs a ajudar no desenvolvimento desta pesquisa. Obrigada pela ajudar na coleta e preparo dos espécimes, por ceder seu consultório sempre que eu precisava. Agradeço por ter te conhecido e termos construído uma bela amizade.

Aos meus colegas do laboratório, **ANANDA COUTINHO,** amiga de longa data, que retornou a casa agora como futura mestranda, obrigada pela ajuda nos experimentos e por todo incentivo, meio a dificuldade estava lá comigo contribuindo sempre, acreditando no meu potencial. E não menos importante aos meus **ALBERTO GOMES** e **WALTER TAVARES** pela ajuda em um dos experimentos, vocês me ensinaram e ajudaram quando eu mais precisei e senti dificuldades.

Ao amigo querido **RAFAEL MATOS** que Deus me possibilitou conhecer na graduação, agradeço pelo apoio, ajuda e por me socorrer em um dos momentos de desespero e preocupação, você veio como aquela luz no fim do túnel, estimulando e falando vamos fazer, você é capaz e vai conseguir. Sua amizade é um presente! Sua ajuda foi essencial na conclusão deste projeto.

A você querido **JACKSON LEITÃO,** por ter aparecido em minha vida quando me encontrava desestimulada e sem vontade de continuar. Você com seu jeito meigo, carinhoso, atencioso e disposto a contribuir sempre, me estimulou a continuar e ensinou muito da vida. És muito importante!

Aos meus colegas do **HOSPITAL SÃO CAMILO**, empresa que trabalho, obrigada pelo incentivo e contagem para que chegasse o dia da defesa para comemoramos.

A todos que contribuíram direta ou indiretamente para a conclusão deste trabalho, os meus sinceros agradecimentos.

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ΔS	Grau de desmineralização
ζ	Potencial Zeta
AaNp-L-GIV	Nanopartículas de Ácido Anacárdico
ANOVA	Análise de Variância
ART	Tratamento Restaurador Traumático
BHI	Brain Heart Infusion
BNp-C-giv	Nanopartículas Brancas
CFU	Unidades Formadoras de Colônia
CIV/GIC	Cimento Ionômero de Vidro
CHX	Gluconato de Clorexidina
ENA	Dente
KDa	Kilodalton
LCC	Líquido da Castanha de Cajú

Atividade antibiofilme de nanopartículas poliméricas carregadas com ácido anacárdico e seu efeito desmineralizante em esmalte bovino

RESUMO

Introdução: A cárie dentária representa um problema de saúde pública, que apesar dos métodos profiláticos, ainda /é muito prevalente. Pode ser desencadeada por diversos fatores, mas seu inicio se dá pela formação do biofilme bacteriano. **Objetivo:** Avaliar a atividade profilática anticárie e a desmineralização resultante de nanopartículas poliméricas de zeína carregadas com ácido anacárdico em blocos de esmalte bovino. **Metodologia:** Foram confeccionadas cavidades de dimensões padronizadas em 36 blocos, restaurados com cimento ionômero de vidro convencional submetido a diferentes tratamentos. Os blocos restantes (12 blocos), mantiveram-se hígidos foram utilizados como grupos controle, 6 lavados com as nanopartículas e os 6 últimos com solução salina estéril. Estes foram levados a ensaio de formação de biofilme de *Streptococcus mutans* em caldo BHI suplementado com glicose a 10%, cultivadas em microanaerobiose. A analise do efeito antimicrobiano foi feita através da contagem de UFC/mg do peso seco de biofilme. A perda de dureza (ΔS) foi mensurada nas distâncias de 50µm e 150 µm em diferentes profundidades. Os resultados foram submetidos à análise de variância (ANOVA) com pos-test de Tukey, assumindo um nível de confiança de 5%. **Resultados e discussão:** Apesar dos grupos CHX e AaNp-L-GIC serem proeminentemente superiores aos seus pares, não foram encontradas diferenças significativas na inibição do biofilme. Porém, os grupos tratados com as nanoparticulas de ácido anacárdico apresentaram notoriamente uma redução na média de crescimento quando comparados com os demais grupos, demonstrando o seu efeito antimicrobiano. Em relação à perda de dureza, o grupo BNp-C-GIC diferiu estatisticamente de alguns grupos, apresentando a maior desmineralização, o que poderia estar relacionado a retirada de nutrientes necessários a manutenção do biofilme. **Conclusões:** Os resultados indicaram que o uso de nanopartículas poliméricas de ácido anacárdico polimérico, incorporadas ao CIV ou na forma de enxague, poderia ser uma opção profilática contra o biofilme de *S. mutans* tanto no esmalte restaurado como não restaurado e, como tal, poderia ser testado como um potencial agente anticárie.

Palavras-Chave: Cárie. Ácido Anacárdico. Cimento Ionômero de Vidro. Nanopartícula. Zeína. *Streptococcus mutans*.

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Agradecimentos: PPGCF, UNIFAP, UFC, CNPq.

ANTI-BIOFILM ACTIVITY OF ANACARDIC ACID LOADED POLYMERIC NANOPARTICLES IN BOVINE ENAMEL AND ITS DEMINERALIZATION EFFECT

Introduction: Caries represents a public health problem, which despite the prophylactic methods, is still very prevalent. It can be triggered by several factors, but its formation is dependente on the bacterial biofilm. **Objective:** To evaluate the anticaries prophilatic activity and the demineralization associated with anacardic acid loaded zein nanoparticles in bovine enamel slabs. **Methodology:** Standard cavities were made in 36 slabs, restored with conventional glass ionomer cement subjected to different treatments, while 12 sound slabs, without cavities were used as controls, 6 cleansed with the test nanoparticles and 6 with sterile saline solution. The slabs were submitted to *Streptococcus mutans* biofilm formation assay in BHI sterile broth supplemented with 10% glucose, cultured in microanaerobiosis. The antimicrobial effect was assessed by counting the CFU per mg of biofilm dried weight. The microhardness loss (ΔS) was measured at distances of 50 μm and 150 μm at different depths. The results were submitted to analysis of variance (ANOVA) with Tukey post-test, assuming a confidence level of 5%.**Results and discussion:** Although the CHX and AaNp-L-GIC groups were prominently superior to their pairs, no significant difference was found in the biofilm inhibition within the groups. However, the groups treated with anacardic acid nanoparticles presented a notorious reduction in the mean growth in comparasion to the other groups, demonstrating its antimicrobial effect. Regarding the loss of hardness, BNp-C-GIC group differed statistically from some groups, presenting the highest demineralization, what could be related to the nutrients removal from the biofilm. **Conclusions:** Our findings indicate that the use of polymeric anacardic acid nanoparticles, incorporated into the GIC or as cleanser, could be a prophylactic option against the *S. mutans* biofilm in both restored and unrestored enamel and as such could be tested as a potential anticaries agent.

Keywords: Caries. Anacardic acid. Glass Ionomer Cement. Nanoparticles. Zein. *Streptococcus mutans.*

Acknowledgements: PPGCF, UNIFAP, UFC, CNPq.

1 INTRODUÇÃO

A cárie dentária está entre os problemas de saúde pública de maior relevância, é considerada mundialmente como uma doença de grande impacto social, por provocar limitações no cotidiano do individuo, não somente devido a sua prevalência, mas também em virtude dos danos passíveis, podendo desencadear dores e até mesmo perdas dentárias. Isso contribui na criação de mudanças biopsicossociais que irão interferir na qualidade de vida das pessoas. É considerada uma doença biofilme-açúcar-dependente, que progride de forma lenta quando não tratada, porém, bastante invasiva, destruindo tecidos mineralizados, formando cavidades. Portanto, existe a necessidade de ser buscar/aperfeiçoar as medidas de prevenção e combate aos processos infecciosos e danosos as estruturas dentais. (FERRACANE, 2012; COSTA et al. 2013).

Vários são os fatores promotores do processo cariogênico, dentre eles estão envolvidas condições biológicas, alimentares, comportamentais e socioeconômicas, bem como fatores que influenciam o acesso a bens de consumo e aos serviços de saúde (FRIAS et al. 2007).

O processo cariogênico tem início a partir da formação do biofilme, uma camada fina, amorfa, acelular, constituída por proteínas salivares adsorvidas e outras moléculas, que constitui uma barreira entre a saliva e o dente (SIMÓN-SORO & MIRA, 2015).

Para que a cárie dental se instale, ocorre inicialmente a adesão de bactérias sob a superfície dentária que em contato com os componentes da saliva são adsorvidos pelo esmalte. Na primeira linha de bactérias colonizadoras do biofilme encontra-se o S*treptococcus mutans*, espécie Gram-positiva, anaeróbia facultativa, maior representante do início do processo cariogênico (KLEIN et al. 2015).

Há muito tempo, diversos esforços tem sido feitos no intuito de se buscar a prevenção e combate a formação do biofilme bacteriano, além de ser a principal estratégia de prevenção é um fator determinante no inicio de agravos de maior impacto. A formação de biofilme sobre os materiais dentários ocasiona cáries ao redor da restauração, o que frequentemente leva a perda de peças dentais (SIMÓN-SORO & MIRA, 2015).

As cáries ao redor da restauração, também chamadas de cáries recorrentes ou recidivantes, são detectadas nas margens das restaurações e coroas existentes. Essas áreas são de fácil limpeza, mas tendem a acumular placa bacteriana, a qual dá inicio ao

desenvolvimento da deterioração. As fraturas das bordas das restaurações e do esmalte das margens cavitárias, contração dos materiais restauradores, restaurações com margens rugosas ou com excessos e má adaptação, estão entre as principais causas de cárie secundária (MJÖRE TOFFENETTI, 2000; FIGUEROA, 2009).

Os materiais restauradores desempenham um importante papel na função mastigatória, sendo que grande parte deles interage e entra em contato com os tecidos e fluidos orais, sendo a escolha do material extremamente relevante, devendo-se levar em consideração as suas propriedades físicas, mecânicas e biocompatibilidade destes com o entorno biológico (SILVA et al. (2010).

O Cimento Ionômero de Vidro (CIV) é o nome genérico utilizado para classificar um grupo de materiais produzidos originalmente a partir do pó de vidro de silicato e uma solução aquosa de ácidos alquenóicos, empregado em restaurações, proteção da dentina (material de base) e cimentação (BONIFACIO et al., 2009, ANUSAVICE et al. 2013).

No presente estudo, foi utilizado o Maxxion R®, um CIV convencional que não exige fotopolimerização, utilizado na dentística para restaurações de dentes decíduos, restaurações tipo classe III e V, reparos de erosões em regiões cervicais não cariosas em tratamento restaurador atraumático (ART) e cimentações provisórias de coroas. Além de ser um cimento de presa rápida, possui uma boa adesão ao esmalte e a dentina sendo desnecessária a criação de retenções, pois é biocompatível e apresenta maior capacidade de liberação de flúor com finalidade anticariogênica, tendo ainda CE (Certificação Europeia) (PEDRO et al., 2014).

Com o intuito de melhorar os cuidados a saúde bucal, diversas intervenções inovadoras tem sido ensaiadas. A incorporação de nanopartículas poliméricas em materiais restauradores, por exemplo, é uma das linhas de pesquisa inovadoras mais contemporâneas conduzidas no âmbito da Odontologia (PITTS, 2011).

Por volta dos anos 90 a nanotecnologia começou a conquistar seu espaço e ganhar importância através do conhecimento de que a propriedades dos materiais (óticas, mecânicas, magnéticas, catalíticas, etc) dependem fortemente do tamanho das partículas do material. E essa característica, tem o poder de potencializar propriedades físicas e químicas, delegando características que antes não eram apresentadas, devido ao fato de estruturas possuírem dimensões que resultam em uma elevada superfície, com maior grau de dispersão e exibição de fenômenos químicos, físicos e biológicos novos e modificados (DEMARCO, 2012; HAMOUDA, 2012).

A nanotecnologia evidencia-se na ciência farmacêutica por permitir a obtenção de sistemas de carreamento de moléculas de interesse terapêutico através de nanopartículas

constituídas por polímeros ou materiais lipídicos capazes de controlar liberação ou atuar *in loco*. Esses colóides sólidos estáveis possuem tamanho que variam geralmente de 10 a 1000 nm no qual a molécula pode estar dissolvida, recoberta, encapsulada ou dispersa em sua matriz. (USKOKOVIC, 2007; ZHANG & WEBSTER, 2009).

Como exemplo, pôde-se observar que na odontologia restauradora materiais resinosos tem recebido a introdução de nanopartículas e outros nano-objetos que podem ser dissolvidos em um meio macroscópico (matriz) e polimerizados no sistema resinoso, alterando as propriedades deste meio macroscópico no qual estão inseridos (SCHLEYER, 2000; MELO, 2012). Como resultado observa-se a melhora no comportamento óptico das restaurações, capacidade de manutenção do polimento e de propriedades físicas como resistência às forças mastigatórias e ao desgaste (KANAPARTHY e KANAPARTHY, 2011; LAINOVIĆ et al. 2012; SUBRAMANI e AHMED, 2012;).

Apesar de todo avanço e melhoria nas características mecânicas e ópticas dos materiais resinosos nanoparticulados, a cárie dental provocada por bactérias, continua sendo ainda o principal desafio a ser superado no aprimoramento desses materiais (FERRACANE, 2012).

Diversos estudos têm sido realizados para avaliar a atividade antibiofilme de nanopartículas poliméricas associadas a produtos, materiais e equipamentos, sendo recente o uso destas tecnológicas no campo odontológico (SOUSA et al 2014). Como é o caso do estudo de Montanaro et al. (2004) que avaliou a adesão de *Streptococcus mutans* sobre diferentes materiais restauradores dentários, Fúcio et al. (2009) que analisaram a formação de biofilme sobre materiais restauradores dentários, cerâmica, resina composta, ionômero de vidro convencional, e ionômero de vidro modificado com resina e o estudo de Burgers et al. (2009), no qual os autores avaliaram a atividade antibacteriana de uma resina composta contendo nanopartículas de prata.

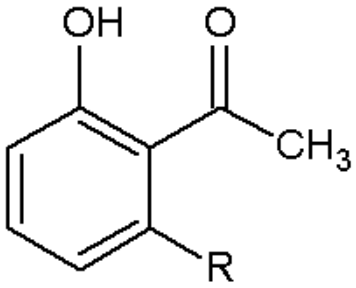
Deste modo, levando em consideração a participação do biofilme oral na formação e evolução da cárie, a incorporação de componentes com propriedades antimicrobianas em cimento de ionômero de vidro pode ser uma estratégia eficaz e bastante promissora para modificar a cariogenicidade do biofilme, contribuir para a eliminação de infecção residual, minimizar o risco de cárie e inibir o desenvolvimento de lesões de cárie ao redor das restaurações (DE CASTILHO et al. 2013; SALES, 2016).

A zeína é uma proteína proveniente do milho, com massa molecular variando de 19 – 22 kDa. Tem caráter hidrofóbico, o que a torna insolúvel em água, sendo solúvel em álcool (65 – 95%), éter etílico, solução alcalina (pH 11 ou superior) e glicóis (KIM & XU, 2008; TAVARES, 2010). Partículas nanométricas a base desta proteína tem sido

desenvolvidas para liberação controlada de fármacos, com aplicabilidade médica. Estudos mostram que nanopartículas de zeína expressam grande potencial de liberação tanto *in vivo* quanto *in vitro (*PATEL, 2010; LAI 2011; REDDY & YANG 2011).

O ácido anacárdico (**Figura 1A**) representa cerca de 70% do Líquido da Castanha do Caju (LCC), líquido escuro, cáustico, extraído da casca do caju (MAZZETTO; LOMONACO; MELE, 2009), sendo os demais constituintes majoritários os fenóis cardol (**Figura 1B**) e cardanol (**Figura 1C**) (AGOSTINI-COSTA et al. 2005). Os ácidos anacárdicos são divididos em quatro subtipos, e apresentam um anel fenólico com 15 carbonos em uma cadeia lateral, podendo conter uma, duas ou três ligações ou até mesmo não contê-las (TREVISAN et al. 2006).

Figura 1 - Estrutura química genérica dos Ácidos Anacárdicos.



Fonte: Próprio autor.

Por apresentarem diversas atividades, dentre elas, antioxidante (KUBO et al. 2006; TREVISAN et al. 2006) e antitumoral (KUBO et al. 1993; HEMSHEKHAR et al. 2011), as pesquisas estes bioativos são intensas. Na área odontológica, grande interesse está relacionado às suas propriedades antimicrobianas (SOUSA, 2014), sobretudo frente a bactérias Gram-positivas como o *Streptococcus mutans* (HIMEJIMA & KUBO 1991; MUROI & KUBO 1993; LIMA, PASTORE & LIMA 2000; GREEN et al. 2008; SOUSA 2014). Para Muroi e Kubo et al. (1993), essa ação esta diretamente relacionada com o número de duplas ligações existentes da cadeia lateral. Para ele quanto maior o numero de duplas ligações, maior atividade antimicrobiana.

Os achados na literatura científica apontam que a adição de nanopartículas poliméricas em materiais restauradores apresentaria resultados significantes, o que implica no interesse em estudar a adesão bacteriana sobre os materiais que se tornam parte da cavidade bucal. O uso do ácido anacárdico associado a esta tecnologia

representaria ainda um grande avanço, haja vista a sua notória ação frente ao principal agente iniciador da cárie dentária. Diante do exposto, o presente trabalho buscou avaliar a sua utilização na forma nanoparticulada incorporada ou como enxague na profilaxia do biofilme de *Streptococcus mutans* em esmalte bovino.

2 OBJETIVOS

OBJETIVO GERAL

Avaliar a atividade antibiofilme e o efeito desmineralizante de nanopartículas de zeína carregadas com ácido anacárdico em esmalte bovino.

OBJETIVOS ESPECÍFICOS

a) Avaliar o efeito profilático de nanopartículas de zeína carregadas com ácido anacárdico na formação do biofilme de *Streptococcus mutans* em esmalte bovino;
b) Avaliar o efeito desmineralizante de nanopartículas de zeína carregadas com ácido anacárdico em esmalte bovino submetido à ação de biofilme de *Streptococcus mutans in vitro*;
c) Comparar o efeito antibiofilme e desmineralizador das nanopartículas de zeína carregadas com ácido anacárdico, nanopartículas brancas e digluconato de clorexidina em esmalte bovino.

Anti-biofilm and demineralization effects of anacardic acid loaded polymeric nanoparticles in bovine enamel

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Abstract

Objectives: This paper aimed to evaluate the antibiofilm and demineralization effects of anacardic acid nanoparticles (AAN) on restored and unrestored bovine enamel. **Materials and Methods:** Thirty-six bovine enamel slabs with cavities, restored with the following treatments: glass ionomer cement (GIC), GIC washed with and containing AAN (AaNp-L-GIC), GIC washed with (BNp-C-GIC) and containing BN (blank nanoparticles) (BNp-L-GIC) and GIC washed with chlorhexidine digluconate (CHX) (CHX-C-GIC) together with blocks without cavities: 6 untreated (negative control) and 6 washed with AAN (ENA-C-AaNp) were submitted to an *ex-vivo Streptococcus mutans* biofilm formation assay. The response variables colonies forming units (CFU)/mg of biofilm and loss of hardness (ΔS) at distances of 50μm and 150 μm have been determined. The significant level used was 5%. **Results:** Despite the groups CHX and AaNp-L-GIC being prominently superior to their pairs, no significant differences were found in the biofilm inhibition. Regarding the loss of hardness, the group BNp-C-GIC statistically differed from BNp-L-GIC, CHX-C-GIC and ENA-C-AaNp groups, showing the highest desmineralization. **Conclusions:** The data obtained indicate that the use of polymeric anacardic acid nanoparticles used as a cleanser or incorporated in GIC could be a prophylactic option against *S. mutans* biofilm in both restored and unrestored enamel and as such, could be assayed as a potential anti-caries agent. **Clinical Relevance:** The AAN when used as a cleanser agent or incorporated into GIC could be an option for caries prevention.

Keywords: Anacardic acid. Caries. Glass Ionomer Cement. Nanoparticles. Biofilm. *Streptococcus mutans*.

Introduction

Dental caries is a multifactorial disease that affects humans worldwide [1,2], causing limitations in the daily life, triggering pain and eventually the tooth loss [3]. It is the result of a chronic process of demineralization, caused by organic acids originated from the bacterial carbohydrates fermentation. Its rising and severity is a result of three factors: host, diet and microbiota [4,5].

The major challenge in developing restorative materials is to find one that restores the tooth function, exhibits adequate abrasion resistance, marginal adaptation, biocompatibility, and reproduces the natural color of teeth [6]. Most defects related to the use of restorative materials are associated with polymeric contraction or occlusal and proximal wear due to marginal infiltration, spotting, caries around the restoration lesions and fractures [7].

In order to overcome these issues, nanotechnology was introduced as a possible alternative. Resins containing nanoparticulate materials could provide greater mechanical resistance, reducing wear, chew and keeping the surface smooth and bright for longer periods. In addition, they could be designed to prevent recurrent caries and caries around restorations [8].

The corn protein zein has been used as a nanocarrier to control the release of drugs, with medical applicability [9, 10]. Anacardic acid has recently received great attention in dentistry due to its antimicrobial properties, especially against cariogenic bacteria [11].

Therefore, the aim of this work was to evaluate the antibiofilm and demineralization effects of anacardic acid loaded zein nanoparticles using bovine enamel unrestored and restored with conventional glass ionomer cement to simulate caries and caries around the restoration *ex-vivo*.

Material and methods

Nanoparticles preparation

Zein (Sigma-aldrich®, Saint Louis, Missouri) nanoparticles containing anacardic acid (9.337mcg/ ml) (AaNp) were prepared by the nanoprecipitation of the protein. Blank nanoparticles (BNp) were prepared in the same manner, except for the absence of the drug. Size, polydispersity index (pdI), ζ potential and pH for the nanoparticles were: AaNp (381.6nm ± 2.122, 0.067, -15.9mV and 4.4) and BNp (376.5nm ± 3.951, 0.137, +6.56mV and 4.71).

Enamel slabs preparation

The bovine teeth were kindly donated by Braga Empreendimentos LTDA. After collection, they were cleaned and stored at 4°C in 0.01% w/v thymol solution. Forty-eight slabs (8.0mm x 8.0mm x 4.0mm) were obtained from the lower incisors, free of apparent enamel defects, macroscopic cracks, abrasions and staining (assessed by visual examination). The surfaces to be treated were polished for 30s using a 5 mm alumina/water suspension in order to expose fresh enamel. The slabs were randomly distributed in eight experimental groups (Table 1).

Table 1 Experimental groups

Groups	Description
ENA	Sound enamel slabs (negative control)
CHX-C-GIC	GIC restored enamel slabs cleansed with chlorhexidine digluconate at 0.12% (positive control)
ENA+GIC	GIC restored enamel slabs (negative control)

ENA-C-AaNp	Enamel slabs cleansed with anacardic acid nanoparticles
BNp-C-GIC	GIC restored enamel slabs cleansed with blank nanoparticles
BNp-L-GIC	GIC restored enamel slabs containing blank nanoparticles
AaNp-C-GIC	GIC restored enamel slabs cleansed with anacardic acid nanoparticles
AaNp-L-GIC	GIC restored enamel slabs containing anacardic acid nanoparticles

Enamel cavities preparation and restoration procedure

Standard circular cavities (diameter 4.0 ± 1, and depth of 1.5mm) were centrally made in the enamel on buccal surfaces, using cylindrical diamond burs # 2294 (KG Sorensen, Barueri, São Paulo) and a diamond bur # 3053 (KG Sorensen, Barueri, São Paulo) that had a stop to limit the depth, used in a high-speed turbine with air–water spray (Kavo do Brasil, Joinville, Brazil), with the exception of the ENA and ENA-C-AaNp unrestored groups. The diamond burs were changed after 5 treatments.

After preparation, all the slabs were sterilized by autoclaving in saline solution at 121°C for 15 min and randomly divided into eight groups of 6 specimens according to Table 1. The cavities present in the enamel slabs were restored according to their respective material (Table 1) under aseptic conditions.

The GIC MaxxionR® (FGM, Joinville, Brazil) was handled according to the manufacturer's specifications, and the AaNp and BNp, when indicated, were incorporated at ca. 3.3µl per cavity during the material preparation. After restoration, the specimens were stored for 24 h in a 100% humid environment prior to the antibiofilm assay.

Inoculum preparation

Streptococcus mutans ATCC 25175 was the bacteria used. The inoculum was prepared by dispersing 3-4 previously grown colonies in Tryptic Soy sterile broth (Kasvi®, Liofilchem, China) supplemented with glucose 10% w/v (Dynamics, Diadema, Brazil), incubated for 18 hours in a bacteriological oven with 5% CO_2 at 37°C. Prior to use, it was diluted in sterile broth to reach the equivalent to 0.5 in the Mac-Farland scale, approximately 10^8 colony forming units (CFU)/ml [12].

Acquired pellicle formation

Natural saliva was collected from a healthy adult donor with natural dentition, without active caries or periodontal disease and use of antibiotics in the previous 3 months. Saliva was collected in the morning, with the donor stimulated. Initially, 25ml of saliva was collected and kept in an ice bath. The saliva was centrifuged for 10 minutes at 3,600 rpm (Centrífuga, Edutec®, Curitiba, Brazil) and the supernatant was subjected to sterile vacuum filtration (Kasvi®, China). A 25ml of PBS (pH 7.0) was added to 25ml of filtered saliva, resulting in 50ml. From that, an aliquot of 500µl was replaced for 500 µl of PMSF adsorption buffer (0.1 M α-phenylmethylsufonyl fluoride, Sigma-Aldrich®, Darmstadt, Germany) and vigorous homogenized. An aliquot of 2 ml of the final product was added to each 24-plate well (Costar®, USA) containing the specimens, submitted to orbital shaking for one hour to form the pellicle [13].

Biofilm model

A 5 days biofilm assay was used to induce cariogenic formation in bovine enamel prophylactically treated, according to the experimental groups (Table 1). The enamel slabs were immersed for 2 minutes in the 24-well plates (Costar®, USA) containing the treatments (except for ENA group), cleansed twice with sterile saline solution (NaCl

0.85% w/v) and transferred to wells containing the inoculum in order to allow the *S. mutans* biofilm formation. The inoculation was performed solely only in the first day of the experiment to evaluate the prophylactic effect. The broth was substituted every 24 hours along the experimental period [14]. The incubation was carried at 37ºC in a bacteriological oven with 5% CO_2.

After 5 days, the formed biofilms were collected and transferred to pre-weighted sterile microtubes containing 1ml sterile saline solution. To determine the CFU, a serial dilution (1:10, 1:100, 1:1,000, 1: 10,000, 1: 100,000, 1: 1,000,000 and 1: 10,000,000) was made. Aliquots (10 µl) from each dilution were seeded in triplicate over sterile BHI agar plates for 24 hours at 37°C with 5% CO_2.

An aliquot of 200 µl from the biofilm suspension was collected in clean and pre-weighed microtubes mixed with 600 µl ethanol (99.9%) at -20°C and stored under this condition for at least 24 h. The suspensions were homogenized using a vortex stirrer (Biomixer QL-901) and centrifuged (Kasvi®, China) for 10 min at -4°C. After 24 h, the supernatant was discarded and 500 µl of ethanol 70% was added again. The suspension was again homogenized and centrifuged for 5 min at -4° C, discharging the supernatant. Finally, the microtubes containing the precipitates were stored in a desiccator until the complete removal of the internal moisture and then weighed on a precision scale (Shimadzu, Tokyo, Japan) to determine the dry biofilm weight [15].

Cross-section microhardness testing

Enamel slabs were longitudinally sectioned though the center. Each half section was embedded in acrylic resin and serially polished with 320, 600, 1,200 water droplets and felt disks using polyurethane (Buehler, Lake Buff, USA). Cross-sectional microhardness measurements were made with a microhardness tester using a Knoop indentation (Future-Tech FM, Kanagawa, Japan) with 5 grams of charge per 5 seconds. Eighteen indentations were performed in two lanes with a distance of 50 µm and 150 µm from the preparation margin. The indentations were made at the following depths: 10, 30, 50, 70, 90, 110, 150, 200 and 250 µm from the outer enamel. Indentations, for restored teeth, were made from the margin of the restoration, and for the healthy ones, from the center of the specimen. The ΔS (integrated demineralization) calculation was performed in the following way [13]: first the values of the Knoop hardness numbers (KHN) at distances 10, 30, 50, 70, 90, 110, 150, 200 and 250 µm from the outer enamel were obtained. Second, KHN was plotted against depth for each slab and the integrated hardness profile of the treated enamel was calculated. For depths higher than 100 µm, the mean KHN was used as a measure of the integrated hardness profile of inner sound enamel. Finally, to compute ΔS parameters, the hardness profile of the treated enamel was subtracted from that obtained for sound enamel and compared among the groups.

Statistical analysis

The obtained data were checked for normality and homogeneity. The microbiological results were submitted to one-way ANOVA and microhardness to a two-way ANOVA analysis followed by Tukey´s HSD post-hoc test, assuming a significance level of 5%. The software GraphPad Prism® 5.0 3 was used for the analysis.

Results

Regarding the microbiological composition of the biofilm formed on the tested slabs, despite the groups CHX (completely inhibited the biofilm formation) and AaNp-L-GIC being prominently superior to their pairs, no statistically significant differences has been found in the biofilm inhibition between the treatments in terms of CFU or biofilm dry weight (Table 2).

Table 2 Microbiological analysis of dental biofilm according to treatment (mean values with their standard deviation and p-value for each analysis).

	Treatments								
	ENA	CHX-C-CIG	ENA + GIC	ENA-C-AaNp	BNp-C-GIC	BNp-L-GIC	AaNp-C-GIC	AaNp-L-GIC	*p*-value
Streptococcus mutans ($x10^6$ CFU/mg)	1.33 ± 2.00	0.0 ± 0.0	0.81 ± 1.30	1.19 ± 1.13	0.02 ± 0.02	0.13 ± 0.10	0.03 ± 0.42	0.01 ± 0.01	0.3857
Dry weigth (mg)	0.02±0.02	0.0±0.0	0.08±0.08	0.01±0.01	0.07±0.069	0.01±0.00	0.02±0.02	0.07±0.08	0.1091

CFU: colony-forming units ; Data were expressed as mean ± standard deviation. C- means cleansed and L – loaded

In the analysis of microhardness loss (ΔS), there was no significant difference when comparing the distances 50 and 150 µm (Fig. 1 and Table 3) among the treated specimens. In contrast, when comparing the differences within the treatments, only BNp-L-GIC differed from BNp-C-GIC, CHX-C-CIG and ENA-C-AaNp in both distances evaluated, showing the highest demineralization (ΔS) (Table 3) amongst the treatments. Comparing the interaction between groups and distances, no difference has either been observed (Fig. 1).

Table 3 Demineralization (ΔS) according to the studied distances for each treatment

Treatment/groups	Distance from the restoration margin (µm)	
	50	150
ENA (negative control)	5868.18 ± 3666.80[a,b]	5613.19 ± 3406.69[a,b]
CHX-C-GIC (positive control)	1703.96 ± 110.00[a]	1612.91 ± 116.70[a]
ENA + GIC (negative control)	7628.94 ± 5903.38[a,b]	6679.68 ± 4980.92[a,b]
ENA-C-AaNp	2300.05 ± 520.16[a]	2266.61 ± 480.87[a]
BNp-C-GIC	10318.11 ± 5660.61[a]	10519.22 ± 5880.26[a]
BNp-L-GIC	1735.22 ± 147.61[b]	1868.01 ± 279.71[b]
AaNp-C-GIC	7893.06 ± 5659.70[a,b]	6753.70 ± 4788.91[a,b]
AaNp-L-GIC	7668.21 ± 6014.79[a,b]	7665.05 ± 5683.81[a,b]

Data were expressed as mean ± standard deviation

Discussion

Nanotechnology has contributed significantly to the development of new direct restorative products, commonly used in the clinical practice. Recent studies have incorporated silver nanoparticles, zinc oxide, calcium phosphate, calcium fluoride, hydroxyapatite and fluorohydroxyapatite, among other substances [15] to restorative materials.

Caries around the restoration are lesions that occur along the margins of an existing restoration over time [16, 17]. It is still considered the main cause for replacement of restorations; therefore, their control is an important clinical issue.

This study proposed to explore the effects of anacardic acid zein nanoparticles on the initiation caries and caries around the restoration in a *S. mutans* biofilm assay [18]. The bovine enamel slabs were restored with glass ionomer cement, which is a common restorative material to the clinical practice. The incorporation of the nanoparticles (AaNp and BNp) was successfully performed as the material remained unaltered over the assay.

Regarding the antimicrobial effect, despite no significant differences were found, which might be attributed to the biofilm processing and biological variations, the anacardic acid nanoparticles when applied as cleanser (AaNp-C-GIC) or incorporated (AaNp-L-GIC) to glass ionomer cement presented an upright performance comparatively to the control restored enamel slabs (ENA + GIC) and could be subject to further investigations. Additionally, the unrestored

enamel slabs treated with those nanoparticles (ENA-C-AaNp) presented an evident decreasing in the average CFU/mg ratio compared to the untreated sound enamel (ENA). Anacardic acids have already aroused interest in dentistry due to its antimicrobial characteristics against *Streptococcus mutans* [19, 20], what confirms its potentiality.

Unrestored enamel slabs treated with anacardic acid nanoparticles (AaNp) improved slightly the antibacterial activity, though increased the demineralization. On the other hand, the restored enamel slabs treated with AaNp did not presented the same behavior regarding the demineralization associated, as it remained similar to the restored control group (ENA + GIC), while greatly contributed to the biofilm inhibition observed. As such, the caries around the restoration prevention could be improved by the anacardic acid nanoparticles treatment, nearly on the same level as chlorhexidine digluconate, which controversially presents many adverse effects in the chronic usage [21].

The significant reduction associated with the BNp-C-GIC treatment could be associated to the ability of zein to attrap solutes, such as drugs, aminoacids and other substances. In this case, as it has been used as a cleansing agent, it could have removed organic and inorganic residues from the pellicle and enamel, including essential nutrients the *S. mutans* nutrition, as such, it impacted positively in the biofilm inhibition and negatively in the demineralization, as it made the enamel surface more susceptible to acid attack from a more virulent bacteria. This effect was not noticeable in the loaded nanoparticles treatments, where the nanocarriers remained onto the glass ionomer surface and as such could impede directly the *S. mutans* adherence. Further investigations are needed to evaluate such controversial aspects.

Conclusions

The use of anacardic acid nanoparticles as a preventive agent to caries and caries around the restoration has shown to be prominent. The glass ionomer incorporated nanoparticles presented nearly the same biofilm inhibition as chlorhexidine digluconate, while no relevant demineralization has been noticed. Blank nanoparticles were more hazardous while cleansing restored enamel, as it has increased the demineralization, though it was very effective in terms of biofilm inhibiton. Therefore, further studies are needed to improve the understanding and evaluate other conditions, such as the daily application of the nanoparticles as a cleanser or the effect on pre-stablished mature biofilm, where the antimicrobial effect of the anacardic acid nanoparticles can be also valuable.

Compliance with Ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

This study was funded by PAPESQ Program of the Federal University of Amapa, Macapa, Brazil. Authors do not have a financial relationship with none of the companies listed in this paper.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

For this type of study, formal consent is not required.

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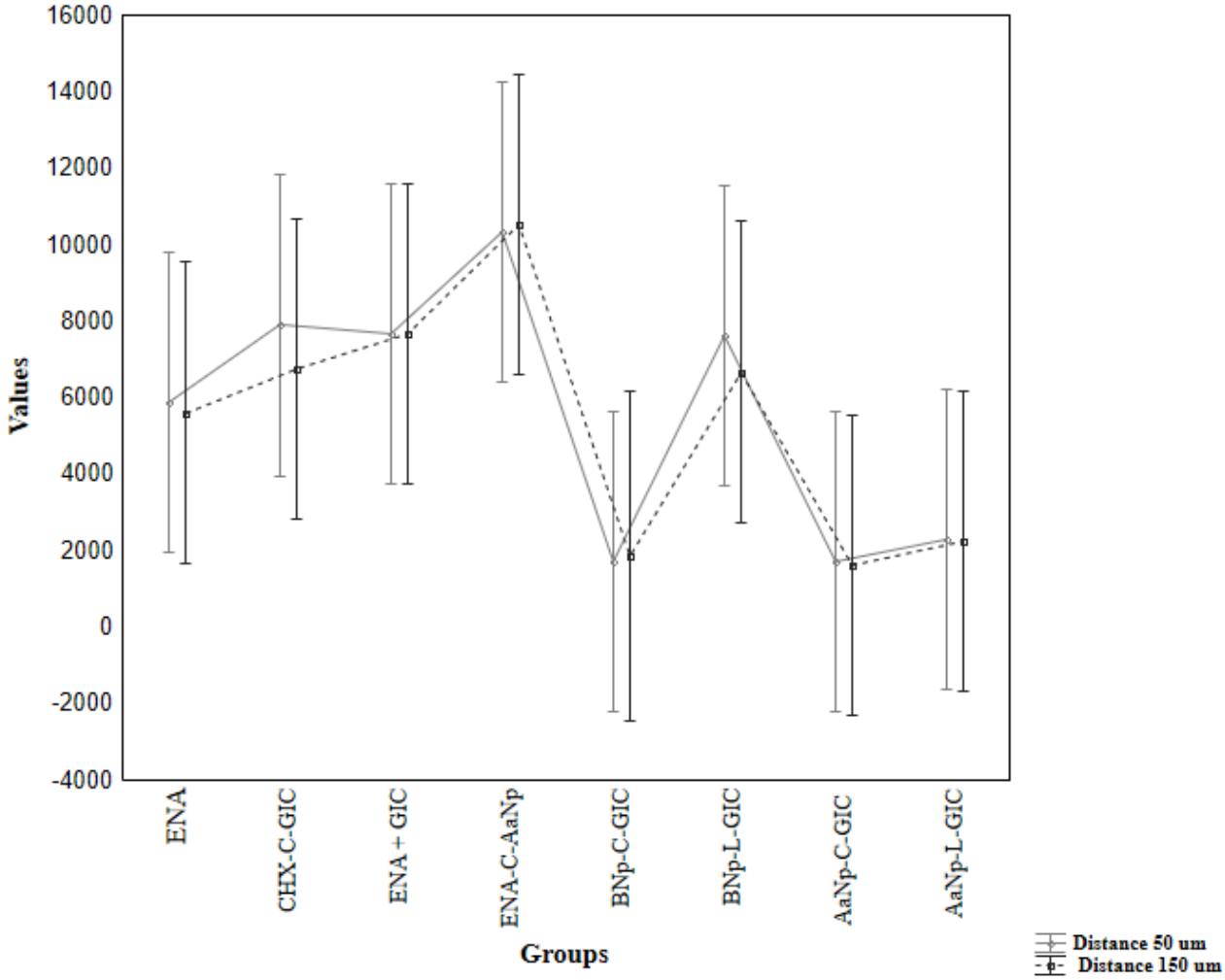
Figure captions:

Fig. 1 Enamel demineralization (ΔS) observed between the assayed treatments in the *Streptococcus mutans* biofilm model.



4 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

O uso de nanopartículas de ácido anacárdico demonstrou ser uma estratégia eficiente como agente profilático da formação do biofilme cariogênico de *Streptococcus mutans* em esmalte bovino, servindo como uma estratégica promissora na prevenção de cáries e cáries ao redor da restauração. As nanopartículas incorporadas ao cimento ionômero de vidro apresentaram quase a mesma inibição na formação do biofilme que o digluconato de clorexidina (padrão ouro na Odontologia), enquanto nenhuma desmineralização relevante foi notada. A aplicação das nanopartículas brancas para a lavagem do esmalte restaurado foi mais incerta, pois se pode observar um aumento na desmineralização, embora tenha sido muito eficaz em termos de inibição do biofilme. Portanto, mais estudos são necessários para melhorar a compreensão e avaliar outras condições, como a aplicação diária ou o efeito sobre o biofilme maduro pré-formado, onde o efeito antimicrobiano das nanopartículas de ácido anacárdico poderiam ser de grande valia.

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