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Dissertação de Mestrado

Potencial dos agentes *cross-linkers* na biomodificação da dentina radicular

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Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade de Ciências da Saúde da Universidade de Brasília, como requisito parcial à obtenção do título de Mestre em Odontologia.

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RESUMO

Há uma série de limitações atreladas aos tratamentos atuais disponíveis para a cárie radicular, que variam desde dificuldades técnicas até custo-efetividade. Assim, explorar o uso de fontes renováveis para o desenvolvimento de técnicas alternativas que visem preservar a dentina e seu arcabouço orgânico podem ser futuramente aplicáveis na prática clínica, uma vez que são alternativas potencialmente biológica e economicamente viáveis. Uma opção seria o uso de agentes *cross-linkers*, que por interagirem com o arcabouço colágeno formando ligações inter e intramoleculares, são capazes de melhorar bioquímica e mecanicamente o colágeno presente no tecido dentinário *in vitro*, favorecendo não só o controle de lesões cariosas, como também a remineralização. A biomodificação dentinária por agentes *cross-linkers* já é objeto de destaque em estudos de adesão, considerando a necessidade clínica de promover uma maior longevidade da camada híbrida e, consequentemente, dos tratamentos restauradores. Dessa forma, a presente dissertação de mestrado teve como objetivo estudar a capacidade de agentes que atuam como *cross-linkers* no controle da degradação proteolítica e formação de lesões cariosas dentinárias (artigo I –cap. 2), além de investigar a interação do ácido anacárdico saturado (LDT11), um potencial *cross-linker* natural, com a dentina radicular bovina (artigo II – cap. 3). Metodologia: Por meio de uma revisão sistemática de estudos *in vitro,* comparou-se o tamanho de lesão e a alteração na liberação de hidroxiprolina (biodegradação) em amostras de dentina submetidas a desafios cariogênicos após tratamento com diferentes agentes *cross-linkers* ou sem tratamento prévio (cap. 1). Ainda, no cap. 2, utilizou-se o método de espectroscopia de infravermelho com transformada de Fourier no modo de reflexão total atenuada (FTIR-ATR) para verificar o efeito do potencial agente cross-linker (LDT11) na dentina bovina pré-tratada em uma concentração de 100µg/mL durante 72h. Resultados: Foram incluídos 50 artigos e identificados 31 agentes *cross-linkers*. Dos 50, 39 foram de baixo risco de viés, 10 médio risco e apenas 1 apresentou alto risco de viés. Aponta-se um efeito positivo do tratamento com *cross-linkers* naturais e sintéticos sobre os desfechos de tamanho de lesão e biodegradação em amostras de substrato dentinário expostas a desafio cariogênico. O ácido anacárdico LDT11, por sua vez, promoveu alterações na matriz orgânica dentinária sugestivas de biomodificação no experimento de FTIR-ATR. Conclusão: os agentes *cross-linkers* parecem atuar positivamente no controle de lesões cariosas dentinárias *in vitro*, com destaque para agentes naturais como naringin, quercetina e proantocianidinas e agentes sintéticos como o glutaraldeído. São necessários estudos clínicos para avaliar a real eficácia e implicação desses compostos sobre a dentina radicular humana, visto que o ambiente bucal é complexo por sua composição salivar, microbiana e de pH. Nesse cenário, o LDT11 pode ser um promissor agente no controle das lesões e longevidade de tratamentos se comprovada sua efetividade na melhora das propriedades mecânicas da dentina e no aumento da resistência a biodegradação.

Palavras-Chave: Colágeno; Dentina; Cárie radicular; Hidroxiprolina; Técnicas *in vitro*

ABSTRACT

There are several limitations associated with current treatments available for root caries, from technical difficulties to cost-effectiveness. Therefore, exploring the use of renewable sources for developing alternative techniques aimed at preserving dentin and its organic structure may potentially be applicable in clinical practice, as they represent biologically and economically feasible alternatives. An alternative approach involves employing cross-linking agents. Through interaction with the collagen framework, these agents form both inter and intramolecular bonds, enhancing the biochemical and mechanical characteristics of collagen within dentin tissue *in vitro*. This could not only favor the management of carious lesions but also could facilitate remineralization. Dentin biomodification through cross-linking agents is already a highlighted subject in adhesion studies, considering the clinical need to promote greater longevity of the hybrid layer and consequently, of restorative treatments. Thus, this dissertation aimed to study the capacity of agents acting as cross-linkers in controlling proteolytic degradation and the formation of dentin carious lesions (Article I – Chapter 2), as well as investigating the interaction of saturated anacardic acid (LDT11), a potential natural cross-linker, with bovine root dentin (Article II – Chapter 3). Methodology: A systematic review of *in vitro* studies compared lesion size and changes in hydroxyproline release (biodegradation) in dentin samples subjected to cariogenic challenges after treatment with different cross-linking agents or without prior treatment (chapter 1). Additionally, in chapter 2, Fourier-transform infrared spectroscopy in attenuated total reflection mode (FTIR-ATR) was used to verify the effect of the potential cross-linking agent (LDT11) with bovine dentin pretreated at a concentration of 100µg/mL for 72 hours. Results: 50 articles were included, identifying 31 cross-linking agents. Out of these, 39 presented a low risk of bias, 10 a medium risk, and only 1 showed a high risk of bias. A positive effect of treatment with natural and synthetic cross-linking agents on lesion size and biodegradation outcomes in dentin substrate samples exposed to cariogenic challenge was observed. In the FTIR-ATR experiment, the anacardic acid LDT11 induced changes in the root dentin organic matrix suggestive of biomodification. Conclusion: Cross-linking agents seem to positively impact the control of in vitro dentin carious lesions, especially natural agents like Naringin, quercetin, and proanthocyanidins, as well as synthetic agents like glutaraldehyde. Clinical studies are necessary to assess the effectiveness and implications of these compounds on human root dentin, considering the oral environment's complexity due to its salivary, microbial, and pH composition. In this scenario, LDT11 might be a promising agent in controlling lesions and treatment longevity if its effectiveness in improving dentin's mechanical properties and increasing resistance to biodegradation is confirmed.

Keywords: Collagen; Dentin; Root caries; Hidroxyproline; In Vitro Techniques.

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1.1 INTRODUÇÃO

Apesar dos inúmeros avanços na odontologia, cárie ainda está entre as doenças de maior prevalência na população mundial (1). A doença cárie ainda afeta cerca de 44% da população mundial. A prevalência de cárie não tratada chegou a afetar mais de 2 bilhões de pessoas em 2010 (1), número que não se mostrou diferente no *Global Burden of Disease* de 2017 (2). Nesse contexto, com o aumento da expectativa de vida decorrente das alterações demográficas, algumas doenças têm se tornado cada vez mais presentes em determinados grupos etários, como é o caso da cárie radicular na população adulta-idosa (3). A exposição dos tecidos radiculares ao meio bucal ocorre através da recessão gengival, decorrente de muitos fatores, dentre eles por meio de métodos inadequados de controle do biofilme, e a região exposta é mais susceptível às quedas de pH no biofilme, ocasionadas pelos produtos finais do metabolismo microbiano de carboidratos fermentáveis (4). Isso se dá pelo alto conteúdo orgânico, cerca de 30% de seu volume, e o menor conteúdo mineral presente no cemento e na dentina (5), tecidos que compõe a porção radicular, quando comparados com o esmalte. Assim, sugere-se que a degradação proteica esteja envolvida em um segundo estágio de desenvolvimento da cárie em tecidos duros da raiz (6).

O papel de proteases endógenas no processo de degradação orgânica da matriz dentinária tem sido muito estudado (7), enquanto a participação microbiana, apesar de sugerida (8,9) ainda precisa ser melhor elucidada. Nesse contexto, alguns micro-organismos como o *S. mutans* têm sido associados a cárie radicular por sua alta frequência em lesões desse tipo (4,10–13) e pela superexpressão de suas colagenases em lesões de cárie radicular (9).

Existem muitas limitações nos tratamentos disponíveis para a cárie radicular. O alto conteúdo orgânico da dentina radicular, rica em colágeno do tipo I, limita a realização de procedimentos adesivos pelo difícil controle de umidade e de um campo operatório limpo e seco, necessário para a qualidade do procedimento adesivo (14). Além disso, o padrão de lesão nessa região também difere do observado na região coronária, sendo caracterizado por uma progressão lateral, resultando em lesões

largas, porém rasas (6), o que também contribui para uma menor retentividade de materiais restauradores, quando indicados. Nesse sentido, tratamentos não invasivos, como o uso de dentifrícios fluoretados com 5000ppm de flúor têm se mostrado como melhor alternativa no controle da cárie radicular (15). Entretanto, esse ainda pode ser considerado um tratamento relativamente custoso ao paciente e é dependente da colaboração e disciplina do mesmo, além de ter eficiência limitada quando se trata de lesões em superfícies proximais cavitadas, aonde o controle de biofilme cariogênico pela higienização é menos eficiente (16), e no manejo de áreas estéticas.

Paralelamente a isso, com o surgimento da hipótese do envolvimento da degradação colagenolítica em cárie de raiz (6), métodos voltados para a inibição da degradação de matriz orgânica passaram a ser mais explorados. Nesse contexto, alguns compostos naturais começaram a ser estudados na cariologia por suas características antimicrobianas e anticolagenolíticas como possíveis terapêuticas. Esse é o caso, por exemplo, do extrato do suco de *cranberry* (17,18) e dos ácidos anacárdicos (AA), cardanol e cardol (19–26), que são óleos derivados do líquido da casca da castanha de caju (CNSL, em inglês *cashew nut shell liquid*) (27). Assim como eles, diversos compostos de origem natural apresentam atividade inibitória de proteases endógenas relacionadas ao desenvolvimento de cárie, tendo potencial para a prevenção e controle da doença em dentina e cemento.

No que tange ao tecido dentinário, alguns desses agentes naturais vem sendo explorados também como *cross-linkers* (17,28–32). A capacidade de reticulação com as moléculas de colágeno presentes na dentina são a base para a estabilidade da mesma. Esses compostos exógenos em contato com as fibrilas colágenas expostas resultam na formação de ligações cruzadas covalentes intra e intermoleculares (33), tendo como resultado a promoção de uma melhora de suas propriedades mecânicas, como resistência à tração final, resistência à desmineralização/biodegradação, módulo de elasticidade, resistência flexural, além de uma influência positiva na resistência de união. Essas características se traduzem em um tecido mais resistente aos desafios cariogênicos (30,34–38). Ademais, esse "*cross-link*" ou reticulação com o colágeno dentinário é de suma importância para permitir que haja uma remineralização de camadas mais profundas nas lesões cariosas. Foi relatado que a segunda camada de dentina cariada em lesões dentinárias, anterior à dentina hígida, possui precursores de *cross-links* que, em ambiente de pH neutro, seriam capazes de voltar a condição de *cross-link* (39). Assim, em condições adequadas, promoveriam

um retorno às fibrilas colágenas normais da dentina. Isso sugere o papel desses agentes na facilitação da remineralização.

Apesar de ter sido relatado efeito de *cross-linker* para alguns compostos derivados do CNSL, como é o caso do cardol e do cardanol (32), ainda não se sabe quanto ao ácido anacárdico, que representa quase 90% da composição do óleo obtido do processamento da castanha de caju (22). Aliado ao seu potencial antimicrobiano contra bactérias cariogênicas como o *S. mutans* e atividade inibitória de colagenases endógenas, o AA pode ser um promissor agente natural de menor custo para a prevenção e controle de cárie radicular. O AA utilizado no presente estudo é o LDT11, que é um ácido anacárdico saturado e apresenta bom perfil inibitório para colagenases do hospedeiro (24,26) e do *S. mutans* (40). A substância foi sintetizada a partir da mistura de ácidos anacárdicos extraída da casca da castanha de caju, no Laboratório de Desenvolvimento de Inovações Terapêuticas (LDT) do Núcleo de Medicina Tropical da Universidade de Brasília pela equipe do professor Luiz Romeiro (FS/FAR).

Somado a isso, existe uma necessidade de ampliar o entendimento acerca dos agentes *cross-linkers* já conhecidos e sobre sua capacidade no controle da biodegradação dentinária. Apesar de algumas revisões de literatura terem caracterizado esses agentes previamente (41–43), ainda não há, na literatura, nenhum estudo que revisou sistematicamente a capacidade dessas substâncias em prevenir ou controlar a progressão da degradação colagenolítica *in vitro*, ponto chave para o desenvolvimento de lesões dentinárias.

Assim, a presente dissertação contará com três capítulos que abordarão os temas supracitados. No capítulo I será apresentada uma revisão da literatura sobre contexto dos agentes reticuladores em cárie radicular/dentinária. Em seguida, serão apresentados dois artigos, sendo o primeiro (capítulo II), uma revisão sistemática sobre os efeitos de agentes *cross-linkers* em lesões cariosas dentinárias *in vitro,* com foco para desfechos colagenolíticos. Já o segundo artigo (capítulo III) trará uma análise inicial da interação de um agente *cross-linker*, o LDT11 (ácido anacárdico saturado), com espécimes de dentina bovina, utilizando como metodologia a Espectroscopia Infravermelho com Transformada de Fourier e Reflectância Total Atenuada (FTIR/ATR).

1.2 REVISÃO DE LITERATURA

1.2.1 A Dentina

A dentina é a estrutura mineralizada que compõe a maior parte do dente, sendo revestida em sua porção coronária pelo esmalte, estrutura altamente mineralizada, e em sua porção radicular pelo cemento, tecido que participa da ancoragem dentária ao osso alveolar. Sua composição consiste em cerca de 70% de conteúdo mineral, 18% de material orgânico e 12% de água (44). Segundo Linde (45), em termos de volume, o que traria uma visão mais relevante, a parte mineral corresponderia a 50% enquanto a fase orgânica a 30%. A matriz orgânica dentinária apresenta como principal componente proteico o colágeno, que constitui cerca de 90% desta, sendo quase exclusivamente do tipo I. Além disso, em sua composição remanescente apresenta proteínas não colágenas, como os proteoglicanos, e lipídeos (44,46).

1.2.2 O Colágeno

Para entender como processos fisiológicos e patológicos ocorrem no tecido dentinário, se faz necessário conhecer o principal componente do arcabouço de sua matriz orgânica, o colágeno. Os membros da família do colágeno possuem como característica a presença de um tri peptídeo rico em prolina de estrutura ”Gly-X-Y”, que forma uma tripla hélice de cadeias polipeptídicas na qual o resíduo de glicina é posicionado no centro e é flanqueada por pequenos segmentos não helicoidais denominados telopeptídeos (47–49). Frequentemente, “X” e “Y” correspondem as proteínas prolina e hidroxiprolina, respectivamente. Essa última sendo essencial para a formação de ligações de hidrogênio intermoleculares (*cross-links*) (Fig.1) de moléculas de colágeno em fibrilas, que são a base para a estabilidade da tripla hélice.

O colágeno do tipo I, principal componente da matriz extracelular como comentado anteriormente, apresenta uma estrutura fibrilar e uma tripla hélice de composição heterotrimétrica, possuindo duas cadeias α1 idênticas e uma cadeia α2. Essas cadeias são sintetizadas em meio intra-celular para formar um precursor, o pró-colágeno. Na dentina, os odontoblastos são os responsáveis pela síntese da proteína. Após passar por modificações pós-translacionais (processos de hidroxilação, por exemplo), esse precursor é excretado ao meio extra-celular, clivado e associado em

fibrilas (46,48). Durante o processamento e modificações extracelulares, as fibrilas são adicionalmente estabilizadas por ligações covalentes (*cross-links*) que contribuem para a sua resiliência mecânica (47,48).

1.2.2.1 *Cross-Links*

O processo de reticulação (*cross-link*) endógeno pode ocorrer de forma enzimática e não enzimática. A forma não enzimática pode estar relacionada a ligação de regiões susceptíveis da proteína com açúcares redutores por meio da reação conhecida como reação de Maillard. Nesse caso, então, a grande maioria dos *cross-links* se baseia em processos de oxidação e glicação (50). Já a maior parte dos *cross-links* enzimáticos, durante a estabilização das fibrilas, são mediados pela enzima lisil oxidase (LOX), que é uma enzima dependente de cobre e tem sua atividade influenciada por fatores hormornais, ambientais e nutricionais (51–53). Esse mecanismo enzimático é baseado na formação de aldeídos a partir de resíduos específicos de telopeptídeo lisina ou hidroxilisina que são espontaneamente condensados com outros aldeídos ou lisinas e hidroxilisinas não reagidas para formar uma variedade de ligações cruzadas covalentes intra e intermoleculares que podem, ainda, passar por um rearranjo para formar *cross-links* mais estáveis (48,54). Foi relatado na literatura que a inclinação da curva tensão-deformação elástica do colágeno aumenta proporcionalmente ao aumento do grau de *cross-links* (55), o que sugere uma correlação positiva entre os *cross-links* mediados por enzimas e as propriedades mecânicas desse tecido (41).

Não só a reticulação, como também os processos pós-translacionais anteriores são de suma importância para a melhora das propriedades mecânicas da matriz colagena, como por exemplo as reações de hidroxilação. Essas irão definir qual o tipo de *cross-link* será formado e consequentemente sua resposta a alterações no meio como a exposição a um pH ácido ou elevações de temperatura, por exemplo (54). Reticulações intermoleculares são a modificação pós-translacional final do colágeno e são a base para a estabilidade das fibrilas.

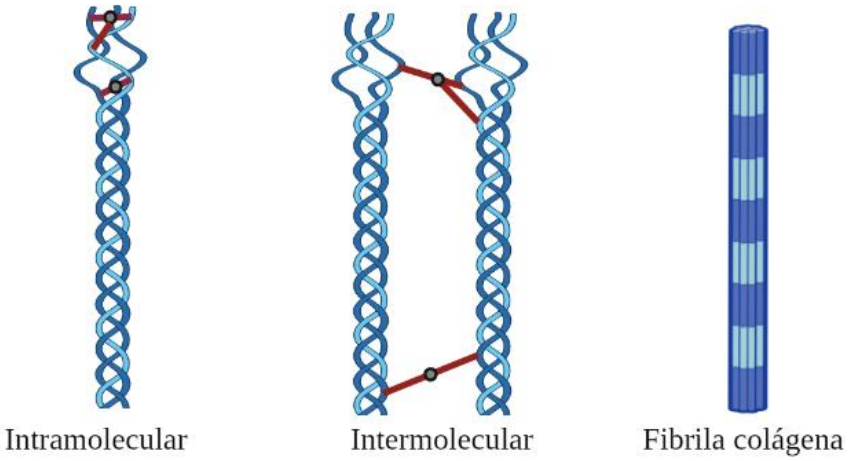

Figura 1. Representação esquemática de *cross-links* em colágeno do tipo I. Agentes promotores de ligações cruzadas covalentes estão representados pelos círculos pretos. (Imagem adaptada de Bedran-Russo e Zamperini, 2017 (41)).

Os *cross-links* nas fibrilas colágenas também podem ocorrer de forma exógena. Nesse caso podem ser induzidos por fontes de reações não enzimáticas como agentes físicos ou químicos (41) que serão discutidos em uma das próximas sessões. A atual odontologia biomimética tem focado no estudo de agentes biomodificadores que atuam como agentes reticuladores exógenos estabilizando o colágeno dentinário.

Nesse contexto, tendo em vista que na odontologia as doenças bucais mais prevalentes envolvem degradação de colágeno, como é o caso da doença cárie quando envolve dentina e da doença periodontal, o entendimento da constituição e da biomecânica dessa matriz orgânica rica em colágeno é determinante para se pensar em estratégias de biomodificação para a prevenção e controle da progressão da doença.

1.2.3 A Doença Cárie: Uma Breve Atualização

A hipótese ecológica da placa para a cárie definida por Marsh (56) sugeriu que uma alteração do meio para um ambiente de pH ácido, causada pelo aumento da frequência de consumo de carboidratos fermentáveis, levaria a uma mudança ecológica da microbiota. Essa é caracterizada pelo aumento de micro-organismos acidogênicos e acidúricos, favorecendo o processo de desmineralização. Quando o balanço de desmineralização é favorecido em decorrência da remineralização, ocorre

o início da formação de lesões cariosas, que está relacionado com a interação entre a superfície dentária, o biofilme e o açúcar, podendo também sofrer a influência de outros fatores que podem atuar como protetivos ou de risco. São fatores protetivos uma dieta balanceada, uma frequência de escovação adequada com uso de dentifrício fluoretado, uso de selantes preventivos ou terapêuticos em populações de risco, função salivar normal, dentre outros (57).

A doença que anteriormente era entendida como infecciosa, transmissível e multifatorial, hoje tem sua compreensão consolidada como uma disbiose da microbiota residente, assim, não transmissível, e as abordagens de prevenção passaram a ser focadas no controle de fatores de risco, que são, inclusive, considerados também para o desenvolvimento de outras doenças crônicas, como é o caso do perfil de consumo de açúcar (58).

1.2.4 Cárie Radicular

Apesar de muito se falar em cárie na infância, têm se observado uma mudança de curso de prevalência de lesões não tratadas da infância para a vida adulta. Isso sugere que, apesar do aumento das ações preventivas na idade escolar, os cuidados passam a ser negligenciados em seguida na vida adulta, contribuindo para essa alteração de cenário (1). Hoje, com o aumento da expectativa de vida e a construção de uma abordagem de saúde mais preventiva do que curativa, observa-se no contexto odontológico uma realidade que se traduz em adultos e idosos com cada vez mais dentes em boca. Havendo, consequentemente, uma necessidade de conhecer, prevenir e tratar os problemas bucais dessa faixa da população, como é o caso da cárie radicular, que é vista frequentemente nesses pacientes.

As lesões radiculares têm início a partir da exposição dos tecidos duros da raiz ao meio bucal. Esses tecidos são caracterizados por uma maior solubilidade em relação ao esmalte dentário, tecido altamente mineralizado que reveste a coroa dentária. Isso faz com que os tecidos radiculares, principalmente a dentina que é rapidamente exposta ao meio bucal após desgaste da fina camada de cemento, exijam uma menor queda de pH para o início de sua desmineralização (4,59). Diferentemente da cárie coronária, o processo de desenvolvimento de lesões cariosas radiculares é constituído por duas fases. Assim, após uma fase de dissolução mineral e exposição da matriz orgânica colágena dentinária por ácidos microbianos, há uma

fase de degradação proteolítica do conteúdo orgânico (6). A literatura relata que essa degradação enzimática ocorreria a partir da ativação de proteases endógenas como as metaloproteases da matriz (MMPs) e catepsinas de cisteína B e K (60).

Em uma revisão sistemática prévia, demonstramos que as MMPs 2, 9, 13 e catepsinas de cisteína B e K parecem estar aumentadas em dentina cariada com relação a dentina hígida, podendo sugerir seu maior envolvimento com a cárie. Dos estudos incluídos, apenas dois observaram lesões radiculares, sugerindo uma maior presença da MMP-13. Entretanto, é necessário salientar que as evidências da revisão foram consideradas muito incertas, reforçando a necessidade de mais estudos primários com maior número amostral. A revisão em questão reforça que a presença de MMPs é indiscutível e deve estar ligada à desnaturação do colágeno (61).

Alguns estudos já relataram a possibilidade de participação de colagenases microbianas na cárie dentinária (8,9,62–65). Na mesma revisão sistemática que avaliou a presença de proteases endógenas e bacterianas em dentina cariada em estudos *ex vivo* (61), mostramos que ainda existe uma quantidade significativamente menor de trabalhos abarcando a proteólise microbiana nesse contexto quando comparada a pesquisas voltadas para colagenases do hospedeiro. Entretanto, a presença de genes codificadores de proteases colagenolíticas e bactérias capazes de degradar proteínas foram detectados em lesões cariosas e poderiam sinalizar um possível alvo para o desenvolvimento de tratamentos em cárie dentinária (61).

1.2.4.1 Manejo da Cárie Radicular

As particularidades das lesões radiculares também tornam seu tratamento mais complexo. O manejo das lesões tem como base inicial o diagnóstico diferencial entre lesões ativas e inativas. Lesões com características de atividade podem exigir diferentes tipos de intervenções restauradoras ou não, enquanto lesões inativas muitas vezes requerem apenas um acompanhamento periódico do paciente e de seus hábitos e reforço das instruções de higiene oral (66). As abordagens de prevenção são indicadas não somente para os pacientes que apresentem alto risco de desenvolver a doença, mas também devem ser aplicadas a pacientes que já a apresentem para evitar o surgimento de novas lesões. Os fatores moduladores para o aparecimento dos sinais da doença são muito similares para a cárie coronária e radicular, assim, podem ser aplicados os mesmos manejos de prevenção (66). Eles

incluem o controle da dieta, controle do biofilme e favorecimento da remineralização (67). A literatura relata um pobre prognóstico de sobrevivência de restaurações em raiz, que pode ser atribuído às próprias características dessas lesões. A própria composição altamente orgânica da dentina e sua consequente umidade podem se mostrar um desafio para a adesão nesse tecido (68,69). Isso sugere uma prioridade aos manejos mais conservadores em detrimento do tratamento restaurador nesse tecido, quando possíveis (66).

1.2.4.1.1 Tratamentos Não-Invasivos

A abordagem não-invasiva tem por objetivo paralisar lesões em atividade, impedindo sua progressão e evitando o surgimento de novas lesões. Para isso, deve-se considerar não somente os tratamentos disponíveis como as peculiaridades de cada paciente e sua adesão ao tratamento. Muito se tem estudado sobre o uso de fluoretos na prevenção e no controle das lesões cariosas coronárias e radiculares. A literatura mostrou que a concentração de flúor nos dentifrícios pode afetar sua efetividade no controle e paralização dos sinais da cárie radicular. A exemplo disso, um estudo que avaliou a incidência e paralisação de lesões radiculares em grupos que utilizaram o dentifrício de NaF 1.450ppm ou 5.000ppm/F mostrou um desempenho muito superior para o de alta concentração de flúor (70). Além dos dentifrícios, os fluoretos têm sido utilizados no manejo não restaurador em forma de géis e vernizes de aplicação tópica e bochechos. Eles atuam na redução da desmineralização e no favorecimento da remineralização quando presentes no meio bucal, podendo ainda influenciar no metabolismo microbiano (71,72).

Nesse sentido, uma tradução e adaptação cultural do consenso internacional/ *European Organization for Caries Research (ORCA) e European Federation of Conservative Dentistry (EFCD)* para dentistas brasileiros (73) apresentou as recomendações atuais para prevenção e tratamento. A nível de paciente, pensando em prevenção, o foco foi a redução da frequência de açúcar, higiene bucal diária, escovação com dentifrício fluoretado (≥1.500ppm/F) ao menos duas vezes ao dia e utilização de dispositivos orais de suporte, no caso de pacientes idosos. Quanto ao manejo não invasivo de lesões radiculares, recomenda-se inicialmente a diferenciação entre lesões ativas e inativas. Lesões inativas devem ser apenas monitoradas, enquanto as ativas devem ser tratadas considerando sua localização e profundidade.

Nesse último caso, sugere-se que as lesões acessíveis devem ser escovadas diariamente, dando preferência ao dentifrício de alta concentração de flúor (5.000ppm/F). A aplicação de verniz fluoretado (>20.000ppm/F) e o tratamento com diamino fluoreto de prata (concentração >30%). Também podem ser consideradas alternativas para as lesões em raiz (73,74).

Recentemente, uma revisão sistemática de 11 estudos que avaliou o prognóstico de tratamentos não-invasivos para cárie radicular e o custo-efetividade dos respectivos manejos observou que o tratamento de maior custo efetividade, considerando o mercado brasileiro e as taxas de prognóstico, foi o dentifrício de alta concentração de flúor (5.000ppm/F), apesar de seu alto custo no país (15). O estudo mostrou que a taxa de progressão de lesões para pacientes que utilizaram placebos ou estavam em grupos controle foi 0.68, o que indicou que a cada dois meses haveria o surgimento de uma nova lesão. Já para os tratamentos caseiros como o uso de dentifrícios e colutórios bucais, a taxa foi de – 0.08 (-3.68 a 2.3), enquanto para os procedimentos realizados em consultório, como é o caso dos vernizes e soluções tópicas, esse número foi de 0.03 (-0.01 a 0.51). A menor taxa de progressão mensal, atribuída ao dentifrício de 5.000ppm/F, foi de -3.68. Para o uso de gel de flúor-fosfato acidulado 1.2% e o verniz de clorexidina a taxa de progressão correspondeu ao surgimento de uma nova lesão a cada cerca de 4 meses. O dentifrício convencional teve um resultado ainda pior, indicando um prognóstico de surgimento de cerca de 2 lesões radiculares mensalmente. Os achados reforçam o uso de estratégias não invasivas como o verniz fluoretado, 5000ppm/F, SnF2 e diamino fluoreto de prata com iodeto de potássio, corroborando com as recomendações atuais anteriormente citadas (73). Nesses casos um prognóstico favorável acima de 90% foi observado nos tratamentos (15). Em outro estudo anterior, quatro metanálises de rede sugeriram que o uso de selantes + verniz de fluoreto de sódio (NaF) a 5%, a infiltração de resina + verniz de NaF a 5% e o creme dental ou gel de 5.000ppm/F (NaF a 1,1%) foram os mais eficazes para interromper ou reverter lesões oclusais e proximais não cavitadas e lesões radiculares cavitadas e não cavitadas de dentes decíduos e/ou dentes permanentes, respectivamente com evidência de certeza baixa a moderada (75).

Apesar do bom desempenho e comprovada efetividade das estratégias citadas, ainda existem limitações e desvantagens relacionadas ao seu uso. A desvantagem do do diamino fluoreto de prata (SDF) se encontra na pigmentação escurecida. Nesse caso, a lesão adquire coloração preta após a aplicação, sendo uma limitação para as

áreas estéticas, por exemplo (76). Já nas estratégias caseiras, como é o caso do dentifrício de alta concentração de flúor, além do alto custo do produto quando comparado ao dentifrício convencional, a efetividade está diretamente relacionada a colaboração do paciente e sua adesão ao tratamento.

1.2.4.1.2 Tratamentos Invasivos

Quando há uma impossibilidade de higienização adequada, seja por dificuldade de acesso à região dentária ou por limitações motoras do paciente, além de situações em que existe uma demanda estética, pode se lançar mão de uma grande variedade de materiais restauradores. Contudo, o tratamento restaurador nessa região apresenta suas complexidades como um controle de umidade adequado, devido à proximidade com a margem gengival. Além do isolamento, se faz necessário um selamento de margens eficiente dentre outros aspectos que também são sensíveis à técnica e operadores dependentes. Não menos importante, o tratamento restaurador muitas vezes ocorre às custas da estrutura dentária, em que pode haver remoção de tecido sadio durante os preparos cavitários. Esse fator também é operador dependente e é responsável por uma possível posterior evolução para tratamentos mais invasivos, acelerando a diminuição da vida útil dentária.

Dentre a diversa variedade de materiais disponíveis para a restauração de lesões cariosas, o que parece ter melhor desempenho na região radicular é o cimento de ionômero de vidro (CIV). Estudos prévios relataram a capacidade desse material de se ligar quimicamente a dentina, mesmo em condições de umidade e prevenir o aparecimento de lesões adjacentes à restauração pela liberação de fluoretos, se mostrando vantajoso em comparação a outros materiais (77). Apesar da ação antimicrobiana contra alguns patógenos como o *S. mutans* e *Lactobacillus,* o mesmo não apresenta ação contra *C. albicans* (78), importante colonizador da região radicular e que pode estar relacionado ao desenvolvimento da cárie de raíz (79) . Além disso, o CIV apresenta limitações quanto às suas propriedades mecânicas inferiores (80) e em relação a estética no que diz respeito a cor e polimento da superfície (81,82), quando comparado a resina composta, por exemplo.

Uma alternativa, o CIV modificado por resina apresenta uma melhora na resistência e na cor, entretanto ainda se mostra inferior às resinas compostas em ambos os aspectos (83). O uso das resinas compostas, entretanto, também inclui

algumas desvantagens no tratamento restaurador das superfícies radiculares. Além da maior susceptibilidade da adesão à hidrólise, causada pela adsorção de água na dentina, a ativação de proteases endógenas durante o condicionamento ácido pode também comprometer a longevidade das restaurações (84). A fratura do material, irritação pulpar e lesões de cárie adjacentes também foram relatadas (85). Uma revisão sistemática e meta-análise sobre manejo de cárie radicular mostrou que, apesar da superioridade da resina diante dos outros materiais (CIV convencional e modificado por resina), todos mostraram taxas de falha anuais bastante altas na maioria dos estudos e as evidências foram baseadas em um baixo número de estudos prospectivos com um risco alto de viés (86).

Considerando a complexa microbiota e ambiente bucal relacionados à cárie radicular em adultos e idosos, se faz necessário que haja uma melhora das características dos materiais restauradores disponíveis, além do desenvolvimento de novas tecnologias para prevenção e tratamento. Os estudos acerca do desempenho de novos materiais para a restauração das superfícies radiculares ainda são pouco numerosos e existe uma demanda de pesquisas sobre o uso de materiais restauradores na população idosa, para que, então, isso seja levado à prática clínica (87). Nesse contexto, a remineralização a longo prazo, a atividade antimicrobiana, a inibição de proteases endógenas/microbianas e a melhora das propriedades mecânicas do substrato podem ser promissores, sendo o estudo de materiais bioativos e de agentes biomodificadores do colágeno dentinário a melhor opção atual para o desenvolvimento das novas estratégias de prevenção e tratamento.

1.2.5 Agentes Biomodificadores

Como comentado anteriormente, a estratégia de biomodificação da dentina tem sido estudada para desenvolvimento de tratamentos futuros e tem como base a formação de *cross-links* inter e intramoleculares que resultam na estabilização do colágeno constituinte da matriz orgânica dentinária. Esses *cross-links* exógenos têm sido amplamente estudados e podem ser de origem natural ou sintética (41,42).

1.2.5.1 Agentes Sintéticos

Os agentes sintéticos podem ser classificados de acordo com o método de ação. Agentes físicos podem atuar por meio da foto-oxidação como é o caso da riboflavina ao ser exposta a radiação ultra-violeta (UV). A riboflavina, também conhecida como vitamina B2, atua através da formação de radicais reativos de oxigênio. Assim, ao ser exposta a radiação UV promove a formação de ligações entre grupos amina (N-H) e grupos carbonil (C=O) (41,42), contribuindo para a estabilização do colágeno. Em estudos prévios sua aplicação foi capaz de melhorar as propriedades da dentina desmineralizada e não-desmineralizada (88,89). Contudo, a utilização de radiação UV ainda levanta questões sobre a segurança desse método e, ademais, uma outra limitação relacionada é o manchamento amarelado da dentina associado ao uso da riboflavina em altas concentrações (88).

Existem também agentes sintéticos que atuarão quimicamente. Dentre eles podemos citar os aldeídos, que tem como representante mais estudado o glutaraldeído (GA), e o cloridrato de carbodiimida (EDC). O glutaraldeído possui dois grupos aldeídos que são capazes de reagir com grupos amina de resíduos de lisil e hidroxilisil do colágeno para, então, formar ligações cruzadas. Estudos prévios relataram um aumento no módulo de elasticidade, na resistência à biodegradação e um favorecimento da remineralização da dentina após o tratamento com o GA (28,90,91). Apesar disso, a alta citotoxicidade proveniente desse agente precisa ser considerada (92). Nesse sentido, o EDC pode ser uma alternativa ao glutaraldeído, visto que é menos citotóxico. Ele atua reticulando o colágeno sem a ligação de grupos adicionais. Nesse processo, a ativação de grupos carboxílicos forma um intermediário capaz de reagir com grupos amina, liberando ureia, que posteriormente é eliminada pelos tecidos (42). Além de seu papel na melhora das propriedades mecânicas e biológicas da dentina (30), o composto foi capaz de inibir MMP´s (30,93,94).

1.2.5.2 Agentes Naturais

Os agentes naturais são caracterizados por uma menor toxicidade em relação aos sintéticos e podem ser considerados renováveis. A grande maioria desses agentes são derivados de plantas e são seus metabólitos secundários. Os polifenóis,

como são classificados, podem se subdividir em ácidos fenólicos, flavonóides, estilbenos e lignanas (41). Os flavonoides polifenólicos contêm um anel fenil contendo um grupo hidroxila com propriedades anfifílicas que os fazem capaz e interagir com o colágeno (42). Na odontologia, os mais estudados são os flavonoides proantocianidinas, também chamados de PACs. Eles possuem uma composição diversa, podendo ser encontrados em muitas fontes de nutrientes como sementes, frutas, vegetais, entre outros. Esses agentes são capazes de formar *cross-links* a partir de ligações covalentes e ligações de hidrogênio (92).

As proantocianidinas são divididas em duas classes de acordo com o padrão de hidroxilação nos anéis A ou B de seu esqueleto flavonoide que é composto por unidades de flavan-3-ol. Os PACs mais abundantes são os que possuem como unidade de constituição a epicatequina ou a catequina e denominados procianidinas. Os PACs podem ser encontrados em formas oligoméricas, poliméricas (taninos) ou monoméricas (epicatequina, epigalocatequina, catequina e galocatequina) (43). Seus monômeros podem, através de reações de condensação com outros monômeros ou com outros polifenóis como o ácido gálico (formando galatos) e reações de conjugação com açúcares, dar origem as formas oligoméricas ou poliméricas (43). O chá-verde (*Camellia sinensis*), por exemplo, possui componentes como o galato de epigalocatequina e foi relacionado em estudo prévio a uma redução da biodegradação de colágeno dentinário (95). Vidal e colaboradores (96) também estudaram o efeito do tratamento dentinário com PACs e derivados do ácido gálico, mostrando efeito positivo e significativo para a melhoras das propriedades mecânicas e resistência a biodegradação *in vitro* em comparação ao grupo controle (96). Proantocianidinas derivadas do extrato de semente de uva demonstraram favorecer a remineralização de lesões radiculares *in vitro* (29), além de aumentar a resistência dentinária a biodegradação do colágeno (17,29). Essa estabilização colagenolítica também foi observada para PACs presentes no extrato do suco de cranberry (17).

Além dos PACs, outros tipos de polifenóis também têm sido estudados como biomodificadores, sendo capazes de melhorar as propriedades mecânicas da dentina como é o caso do ácido tânico, que é capaz de formar ligações de hidrogênio com os grupos amina do colágeno (97). A hesperidina, um flavonoide glicosídico extraído de frutas cítricas, possui um mecanismo semelhante de *cross-link* com relação aos demais flavonoides e foi associado a uma inibição da degradação proteolítica (98). Já a genipina é um composto extraído do fruto da Gardênia *Jasminoides Ellis* que

também foi descrito por sua capacidade de formar ligações cruzadas com o colágeno do tipo I, aumentando a resistência da dentina bovina a degradação enzimática *in vitro* (28). Entretanto, esse composto apresentou a desvantagem clínica de uma pigmentação de tom azulado dos substratos (41).

Nesse contexto, é possível observar que esses agentes podem ser multifuncionais, estando associados a uma melhora das propriedades mecânicas (99,100), da remineralização (29) e da inibição de proteases endógenas (101,102). Assim como a estabilização da matriz colágena pela formação de *cross-links*, essas características compõem aspectos importantes que podem ser alvos de estratégias futuras de controle da cárie radicular visando a preservação tecidual.

Nesse cenário, um agente natural que vem sendo bastante estudado por suas inúmeras atividades antimicrobianas (20,26,103), anti-colagenases (23,24) e dentre outras relacionadas à cárie é o ácido anacárdico, que será explorado a seguir.

1.2.6 Ácido Anacárdico

O cajueiro, de nome científico *Anacardium occidentale*, é uma planta da família *Anacardiaceae* cujo fruto é a castanha de caju. O produto do processamento industrial da casca desse fruto é um líquido oleoso de característica viscosa (*Cashew nutshell liquid* – CNSL) que representa uma das fontes principais e mais baratas de lipídios fenólicos não isoprenóides de ocorrência natural, como ácidos anacárdicos, cardóis, cardanóis, metilcardóis e materiais poliméricos (104). Enquanto os ácidos anacárdicos compreendem cerca de 80% ou mais da composição do CNSL, em uma fração menor estão presentes o cardol (cerca de 15%) e uma pequena quantidade de Cardanol (105). Dentro disso, os ácidos anacárdicos (AA) são derivados com núcleo salicílico que apresentam cadeia lateral alquílica com 15 carbonos. Os AA têm sido amplamente estudados por suas diversas atividades biológicas e terapêuticas como efeito antioxidante, anticancerígeno, antiinflamatório, antimicrobiano, antiobesidade e inseticida (22). Esse agente também tem sido caracterizado como inibidor da atividade de metaloproteinases da matriz (MMP's), especialmente MMP’s 2, 9 e 14 (23,25,106,107). A inibição dessa atividade catalítica relacionada com a degradação da matriz orgânica dentinária foi demonstrada previamente após desafios erosivos *in vitro* (24). Além disso, observou-se que os AA apresentam forte atividade antibacteriana contra organismos gram-positivos, como o *Streptococcus mutans*

(19,103,108) e outros presentes em biofilmes orais (40,109), o que o torna substância de interesse clínico para a odontologia.

1.2.6.1 Composição Química

Os AA constituem cerca de 90% do CNSL (27). Quimicamente, são constituídos por uma mistura de vários compostos orgânicos intimamente relacionados consistindo em um núcleo de ácido salicílico com uma cadeia lateral alquílica não isoprenoide de 15-17 carbonos que pode se apresentar saturada ou insaturada nas formas: monoeno, dieno e trieno, em que as ligações duplas estão localizadas respectivamente nas posições 8, 8, 11 e 8, 11, 14 (104) (Figura 2).

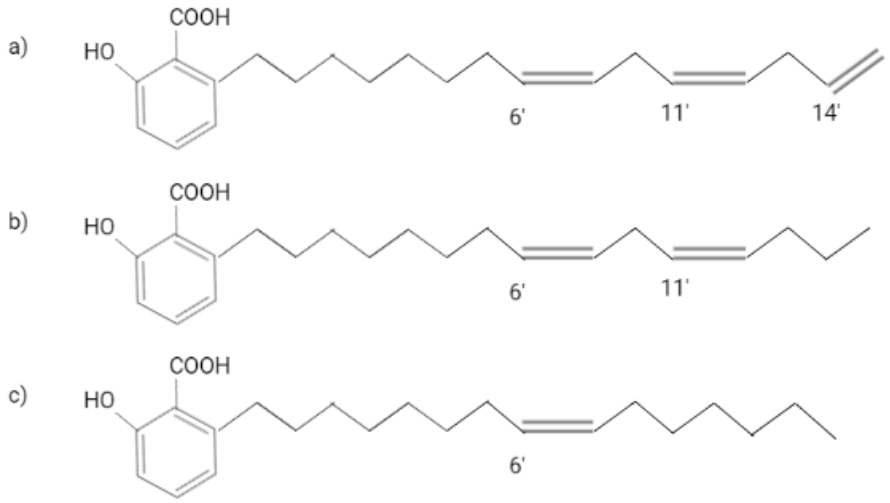

Figura 2. Estrutura química da molécula de ácido anacárdico em suas formas a) Trieno; b) Dieno e c) Monoeno.

A cadeia lateral alquilica é a responsável pelo aumento drástico na atividade antimicrobiana desses ácidos ao comparar-se com apenas o ácido salicílico. O tamanho da cadeia lateral e suas insaturações também parecem influenciar diretamente a atividade antimicrobiana. A exemplo disso, Gellerman et al. (110) obseravaram uma diminuição na atividade bactericida contra o *S. mutans* em AA com cadeias laterais com menos insaturações. Em suma, as características conformacionais de cadeias laterais insaturadas com suas curvas e comprimentos

mais curtos, criam uma desordem maior na membrana de bicamada de fluido do micro-organismo, resultando em uma atividade antimicrobiana mais potente (111).

1.2.6.2 Ação Antimicrobiana – O AA e a microbiota bucal

Como mencionado anteriormente, o mecanismo de ação antibacteriano dos AA está relacionado a sua atuação como agente tensoativo, alterando fisicamente a membrana lipídica microbiana (20,21), sem, contudo, descartar mecanismos bioquímicos (21). Em estudo prévio, que investigou a susceptibilidade de micro-organismos orais a extratos de plantas e enxaguatórios bucais, tendo como controle a clorexidina e povidona, observou-se que o *S. mutans* foi o micro-organismo mais susceptível a *Anacardium occidentale* (112). Essa atividade antimicrobiana já foi relatada previamente na literatura, porém com diversas variações de concentração. Green et al. (108) e Kubo et al. (21) observaram uma Mínima Concentração Bactericida (MBC) de 6,25µg/ml e uma mínima concentração inibitória (MIC) de 1,56µg/mL para o *S. mutans*. Outros estudos relataram uma MIC variando entre 3,13 – 7µg/mL (113,114). Apesar dos valores citados terem sido descritos para AAs insaturados, mais comumente encontrados na natureza e caracterizados como mais potentes antimicrobianos, discute-se sobre a possibilidade de um efeito antibacteriano insuficiente dos AAs insaturados contra o *S. mutans* na prática clínica. Sugere-se, então, uma abordagem sintética como solução, de forma a reduzir a resistência antimicrobiana e a instabilidade das cadeias insaturadas (108). Nesse contexto, estudos *in vitro* com AAs saturados, como o LDT11, por exemplo, têm apresentado comportamento semelhante para a cepa testada (103).

Recentemente, com o aumento do interesse clínico em pesquisas envolvendo a microbiota cariogênica e esse agente natural, Lima et al. (26) demonstraram um efeito preventivo na formação de biofilme de *S. mutans* (UA159) *in vitro* com a utilização de nanopartículas contendo AA a uma concentração de 9,375 µg/mL, com resultados indicando um forte efeito antiplaca. Entretanto, não apresentou efeito em biofilmes maduros. Em contrapartida, outro estudo mais recente constatou atividade bactericida em concentrações a partir de 5µg/mL em células planctônicas (103), sugerindo que o efeito antiplaca observado por Lima et al. (26) possa estar relacionado a uma redução do número de células, e não há um efeito antiplaca em si. Em contrapartida, de Souza et al. (109) testaram o efeito do composto em biofilmes

uniespécie com 24 horas de formação, mostrando que houve uma redução na biomassa de até 70% na concentração de 1,56µg/mL. A despeito de uma alta atividade inibitória e bactericida em células planctônicas de bactérias orais ter sido demonstrada previamente (26,103), a literatura sobre o efeito do AA em biofilmes multiespécie ainda é escassa (109) e mais estudos são necessários antes de sugerir seu potencial para uso clínico.

Nesse contexto pode-se citar ainda, outro estudo recente (40) utilizando um ácido anacárdico saturado (LDT11). Demonstramos a inibição de bactérias bucais gram-positivas e gram-negativas por concentrações de LDT11 acima de 5µg/mL. A exemplo disso, foi observada potente ação bacteriostática contra *V. parvula*, um micro-organismo anaeróbio, essencial no microbioma bucal. Além disso, nosso resultado em biofilmes pré-formados mostrou uma dose-resposta na viabilidade bacteriana, indicando um bom efeito antimicrobiano do LDT11 contra biofilmes maduros em altas concentrações. Ainda, observou-se que os derivados do CNLS testados (LDT11 e LDT409 – cardanol saturado) apresentaram baixa citotoxicidade em células-tronco pulpares (máximo de 30% e chegando a 0 mortes após 1 semana), sendo ainda capazes de estimular a proliferação e diferenciação celular (40). Com isso, estudos futuros com o AA e seus derivados devem ter como um dos enfoques o efeito desses agentes sobre modelos de biofilmes complexos.

1.2.6.3 Ação Anticolagenolítica

Além de suas diversas atividades antimicrobianas, biológicas e terapêuticas (22), o AA também tem sido caracterizado como inibidor da atividade de metaloproteinases da matriz (MMP's) (23,25,106,107). Omanakuttan et al. (23) sugeriu que a inibição da atividade proteolítica ocorreria pela ligação do AA ao sítio ativo da MMP, quelando o íon zinco catalítico. Além de uma regulação a nível de pró-enzima e atividade enzimática, esse composto de ocorrência natural também atua a nível transcripcional, regulando a expressão das gelatinases e inibindo ativadores endógenos das mesmas (MMP-14 e EMMPRIN), enquanto ativam seus inibidores (RECK e Spry2) (25).

O potencial inibitório do AA para as diversas MMP’s foi relatado com muitas variações na literatura, sem um consenso. Contudo, sabe-se que essa regulação ocorre de forma dose-dependente (25). Omanakuttan et al. (23) mostraram inibição

significativa da MMP-2, uma das principais gelatinases relacionadas a degradação de colágeno dentinário, a partir de 1µg/mL (3µM) de AA, podendo chegar a uma inibição de 98% na concentração de 200µM. A mesma capacidade inibitória foi observada também para a atividade de gelatinase da MMP-9. Enquanto isso, Nambiar et al.,(25) constataram inibição das MMP's 2 e 9 a partir de 5µM. Já Silveira et al. (24) analisaram o controle de perda de tecido dentinário após desafio erosivo in vitro comparando a inibição da atividade da MMP-2 e 9 pelo AA saturado com compostos fenólicos naturais e o NaF. O ácido se mostrou superior em relação aos demais, sendo capaz de reduzir a erosão em concentrações de 100 µmol/L. A possibilidade do uso desse promissor agente natural em colutórios bucais para o controle de erosão dentinária e lesões cariosas, foi avaliada por Araújo et al. (115). O composto não apresentou genotoxicidade na concentração testada (9,375µ/mL), sugerindo-se que seja seguro para uso a curto prazo. Para tal, são necessárias mais pesquisas *in vivo*. Ainda com relação ao potencial de uso clínico, a incorporação do ácido anacárdico em sistema adesivo universal aparenta não reduzir as propriedades do mesmo, além de ter efeito antimicrobiano significativo em biofilmes multiespécie (109).

Além de seu efeito em proteases endógenas, existem evidências recentes sobre a inibição de colagenases microbianas. Em estudo previamente descrito (40), a atividade de colagenase de biofilmes de *S. mutans* foi significativamente inibida por 34µg/mL de LDT11 quando comparada ao controle. Somado a isso, em concentração de 100µg/mL, tanto o LDT11 quanto o LDT409 (compostos derivados do CNSL) foram capazes de inibir significativamente a forte ação colagenolítica de *Porphyromonas gingivalis*, chegando a níveis de 96.8% de inibição. Esse resultado pode ser de extrema importância, visto que essa *P. gingivalis* é considerada a bactéria bucal proteolítica de maior relevância (40).

Essa atividade colagenolítica do biofilme sobre a matriz orgânica têm se tornado de grande interesse, visto que micro-organismos de lesões radiculares apresentaram superexpressão de colagenases (9). Dessa forma, caso comprovada, a inibição de proteases microbianas pelo AA poderia levar a novos caminhos terapêuticos em doenças orais.

1.3 OBJETIVO GERAL

Estudar a capacidade de agentes que atuam como *cross-linkers* no controle da degradação proteolítica e formação de lesões cariosas dentinária, investigando principalmente o efeito de *cross-link* em dentina radicular bovina após tratamento com o ácido anacárdico saturado (LDT11).

1.3.1 Objetivos Específicos

- Comparar a alteração na liberação de hidroxiprolina em amostras de dentina submetidas a desafios cariogênicos *in vitro* após tratamento com diferentes agentes *cross-linkers* ou sem tratamento prévio (CAPÍTULO 2);
- Comparar o tamanho da lesão em amostras de dentina tratadas e não tratadas com agentes *cross-linkers* submetidas a desafio cariogênico (CAPÍTULO 2);
- Verificar se o tratamento da dentina radicular bovina com ácido anacárdico saturado (LDT11) em uma concentração de 100µg/mL promove efeito de *cross-link* com o colágeno dentinário por meio de metodologias de espectroscopia infravermelho com transformada de Fourier no modo de reflexão total atenuada - (ATR-FTIR) (CAPÍTULO 3).

1.3.2 Perguntas de Pesquisa

PERGUNTA CAPÍTULO 2 (REVISÃO SISTEMÁTICA):

Baseada no acrônimo PICOs:

P= Amostras de dentina (placas, discos, blocos), humanas ou bovinas, submetidas a qualquer desafio cariogênico/desmineralização

I = agentes *cross-linkers*

C = controles negativos ou outros *cross-linkers*

O = efeito na liberação de hidroxiprolina e tamanho da lesão

S = estudos *in vitro*

A pergunta de pesquisa é: agentes *cross-linkers* alteram a liberação de hidroxiprolina e/ou o tamanho da lesão em amostras de dentina submetidas a desafio cariogênico?

PERGUNTA CAPÍTULO 3 (ESTUDO *IN VITRO*)

Baseada no acrônimo PICOs, onde:

P= colágeno da dentina radicular bovina

I = tratamento com composto derivativo de AA saturado

C = tratamento com outros *cross-linkers* ou controles negativos

O = *cross-link* entre fibras colágenas

S = estudos *in vitro*

A pergunta dessa pesquisa é: o tratamento da dentina radicular com ácido anacárdico saturado (LDT11) resulta na reticulação (*cross-link*) deste com o colágeno dentinário?

1.4 JUSTIFICATIVA

Como já mencionado, ainda existe uma série de limitações atreladas aos tratamentos atuais disponíveis para a cárie radicular, que variam desde custo a questões biológicas e estéticas. Assim, explorar o uso de fontes renováveis para o desenvolvimento de estratégias que visem preservar a dentina e seu arcabouço orgânico devem ser consideradas relevantes para a prática clínica do futuro, uma vez que são alternativas potencialmente biológica e economicamente viáveis. Além disso, a biomodificação dentinária por agentes que promovem *cross-links* têm se tornado objeto de destaque em estudos de adesão, considerando a necessidade clínica de promover uma maior longevidade da camada híbrida e, consequentemente, dos tratamentos restauradores. Para isso, são necessários mais estudos que tenham como alvo agentes estabilizadores da matriz colágena dentinária e seus efeitos na biodegradação.

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CAPÍTULO 2. EFFECTS OF CROSS-LINKING AGENTS ON HYDROXYPROLINE RELEASE AND ROOT CARIES LESION SIZE *IN VITRO*: A SYSTEMATIC REVIEW WITH NETWORK META-ANALYSIS

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2.1 ABSTRACT

Introduction/Aim: Treatments targeting the control of root caries lesions have inherent limitations and require significant enhancement. One potential strategy involves the use of cross-linking agents, which may have the ability to increase the quantity of inter- and intra-molecular links within collagen, thereby reducing the rates of dentin matrix biodegradation and enhancing the biomechanical characteristics. Various natural and synthetic agents have been tested *in vitro*, yet there is a lack of consensus on their efficacy and in determining the most suitable products for translational research. This review seeks to address the following research question: “Do cross-linking agents change the hydroxyproline release and/or lesion depht in dentin samples submitted to cariogenic challenge?” **Methods:** The inclusion criteria comprised *in vitro* study designs assessing the impact of any cross-linking agent on dentin, specifically in terms of reducing lesion depth or measuring collagen degradation through hydroxyproline release. A search strategy was adapted to eight databases, and the methodological quality of the included studies was assessed using QUINN Tool for *in vitro* studies in Dentistry. A Bayesian network meta-analysis (NMA) was performed to compare different agents considering the lesion depth

outcome, and the heterogeneity between studies was estimated using Chi^2 and I^2 statistics.

Results: A total of 2280 records were retrieved after duplicates removal, and 50 studies were included for qualitative synthesis. Thirty one cross-linking agents were tested across the literature, from which grape seed extract and glutaraldehyde were the most tested. Regarding the outcome of hydroxyproline release, the performance of the agents varied depending on the concentration used, emphasizing the positive effect of agents like proanthocyanidins derived from grape seeds, among others, even at lower concentrations. Most studies had a low risk of bias. The NMA for lesion depth included 284 samples analyzed in 36 paired comparisons. The ranks of cross-linking agents for reducing lesion depth were as follows: Naringin > Quercetin > Riboflavin UVA-activated > Proanthocyanidins > Hesperidin > Glutaraldehyde > Cranberry > Grape seed extract > Control groups without treatment. However, only Naringin, Quercetin, Proanthocyanidins and Glutaraldehyde presented evidence of efficacy when compared to control without treatment. **Conclusion:** Most cross-linking agents showed evidence of efficacy compared to the untreated group for both analyzed outcomes. Naringin was the best agent in reducing lesion depth; however, these results should be interpreted cautiously. Clinical studies are needed considering the complexities of the oral environment to determine the actual effectiveness of pretreatment with these agents, and the most recommended for further research are Naringin, Proanthocyanidins, Quercetin and Glutaraldehyde due to their efficacy demonstrated *in vitro*.

Keywords: dentinal caries, root caries, dentin biomodification, systematic reviews, network metanalysis

2.2 INTRODUCTION

Untreated dental caries in permanent teeth is still the most prevalent oral disease in the world's population, affecting 2.5 billion people (1). This underscores the importance of exploring treatment options designed to manage lesion development and slow down its progression, particularly on the dentin and root surfaces (1–3). Dental root surfaces are especially susceptible to caries due to its high organic content, primarily composed of type I collagen (4). The current strategies for treatment still have limitations and need significant improvement, as a lack of evidence regarding their longevity has been discussed (3).

One potential strategy involves the use of cross-linking agents, though investigations are currently limited to *in vitro* studies. These substances may have the ability to augment the quantity of inter- and intra-molecular links within collagen, thereby diminishing the rates of dentin matrix biodegradation substantially and enhancing the biomechanical characteristics of healthy tissue *in vitro* (5–7). Several agents have previously been documented as having the capability to create cross-links in dentin. These include synthetic agents like glutaraldehyde and carbodiimide hydrochloride (EDC), as well as a variety of natural agents derived from plants, including polyphenolic flavonoids derived from green tea (*Camellia sinensis*), grape seed extract, cranberry juice extract, among others (8,9). Previous studies have demonstrated their ability to reduce the size of artificial carious lesions *in vitro* (10–12). Furthermore, prior exposure to the agents has also been associated with a reduction in organic degradation by collagenases, measured by the release of hydroxyproline (12–15). The chemical or physical biomodification of the affected dentin using these agents appears to be a potential means of slowing the progression of carious lesions, offering a way to reduce the speed of progression of root carious lesions.

Given the potential role of cross-linking agents in dentin and root caries, it is crucial to investigate the existing evidence to enable the development of new clinical research focused on the most effective agents *in vitro* rather than investing time in those with lower efficacy. Although some literature reviews have already been published on agents currently studied for cross-linking with collagen (8,9), none of them systematically evaluated the ability of these agents to control collagenolytic degradation and formation of dentin carious lesions. As the studies on the topic have

employed *in vitro* methodologies and considering the absence of clinical data, this review included only *in vitro* designs to filter the most effective agents studied so far, maybe defining a gold-standard product for further analysis. Therefore, this study seeks to address the following research question: “Do cross-linking agents change the hydroxyproline release and/or lesion size in dentin samples submitted to cariogenic challenge?”

2.3 MATERIALS AND METHODS

2.3.1 Protocol and Registration

This systematic review was reported according to the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) checklist (78). A study protocol was designed and registered at the International Prospective Register of Systematic Review (PROSPERO) database, under the identification number CRD42023404911.

2.3.2 Eligibility Criteria

The acronym PICOs (Population/participants; Intervention; Comparator; Outcomes; type of Studies included) was used to design the research question: P= Dentin samples (slabs, discs, blocks), either human or bovine, submitted to any cariogenic/demineralization challenge; I= Cross-linking agents; C= Negative controls or other cross-linking agents; O= Effect on hydroxyproline release and lesion size; S= *in vitro* studies.

2.3.2.1 Inclusion Criteria: Studies eligible for this review were *in vitro* designs that assessed the effect of using cross-linking agents on dentin samples (slabs/discs/blocks, being human or bovine) that were submitted to any type of cariogenic challenge.There was no limitations regarding the language and year of publication in the search.

2.3.2.2 Exclusion Criteria: The exclusion criteria were as follows: (1) Studies performed on enamel surface/cell cultures; (2) Studies evaluating collagenase inhibitors that do not act as a cross-linking agent, but through enzymatic inhibition; (3) Studies that used remineralizing agents as controls; (4) Different outcomes of hydroxyproline release and lesion size, including those that analyzed lesion size qualitatively through microscopy, microbial or gene expression outcomes, and (5) Reviews, letters, conference abstracts, personal opinions, book chapter, protocols and animal studies. (6) While there was no limitation regarding language during the search, studies in non-Latin alphabets were excluded due to the high risk of imprecision in online translations.

2.3.3 Data Sources and Search Strategy

The search was performed in March 2023. The terms “Dentin”, “Dental caries” and “Cross-linker”, together with their synonyms and variations were used as main terms for develop the PubMed search strategy, which was adapted for each electronic database: MEDLINE via PubMed, LILACS, Web of Science, Scopus, and EMBASE. The search was also performed in gray literature on Livivo, Google Scholar, and ProQuest (dissertations and theses). Reference lists of included studies were reviewed to identify other studies that were potentially eligible. The complete search strategy is described in Appendix 1. Duplicates were detected using EndNoteWeb (Clarivate Analytics, Mumbai) and then manually identified in Rayyan QCRI® (Qatar Computer Research Institute, Qatar).

2.3.4 Study Selection and Data Extraction

The selection of included articles was carried out by two independent reviewers (IMS and CBB). The reviewers individually examined all titles and abstracts retrieved in the search using a web tool for systematic reviews (Rayyan QCRI®, Qatar Computing Research Institute). The same reviewers (IMS and CBB) conducted a full text reading for a final decision. Discrepancies were checked by a third reviewer (ER).

Data were collected independently by the same reviewers (IMS and CBB) using Excel spreadsheet exclusively designed to this study. A third reviewer (ER) judged any conflict between first and second reviewers. The following data were collected from

each selected study: Author and year; Country; Number of samples (test group); Number of samples (control group); Intervention; Control; Product application method; Sample type (human/bovine); Sample location (coronal or radicular dentin); Pre-treatment with acids; Method of product application; Storage of samples (before and after treatment); Demineralization method (cycles with acids, bacteria, etc.); Lesion size (mean ± SD and p value); Type of microscopy; and the Release of hydroxyproline (mean ± SD and p value).

2.3.5 Methodological Quality Assessment and Certainty of Evidence

The assessment of methodological quality of all included studies was independently conducted in duplicate (IMS and CBB) using the QUINN tool (16), specifically designed for *in vitro* studies in Dentistry. Twelve criteria were evaluated, scored as 0 to 2 or NA (not applicable). A score of 2 points was assigned when the answer for the criterium was “adequately specified”; 1 point for “inadequately specified”, and 0 points for “not specified”. In cases where NA was used, the criteria were excluded from the total score. The total score was determined by the following formula, as recommended by the instrument (16): **Final score** = (Total score x100)/(2x n° applicable criteria).

Then, the final score was used to classify the study as having low risk of bias (> 70%), moderate risk (50-70%) or high risk (< 50%). The certainty of evidence was not evaluated, since currently there is no GRADE approach adaptation for *in vitro* studies.

2.3.6 Data Synthesis and Statistical Analysis

A rigorous narrative synthesis of the results was carried out for both outcomes (lesion depth and hydroxyproline release) according to the tested agents. It was not possible to conduct a quantitative analysis for the outcome of hydroxyproline release due to the large variability in concentrations and the lack of standardization of units of measurement across primary studies. Despite that, a quantitative analysis was performed for the most direct and clinically relevant outcome: Lesion Depth (LD). Data on LD in mm mean scores (and standard deviations) after treatment with each agent were extracted for every included study. For studies that did not provide numerical

data, the WebPlotDigitizer tool (4.6 version) was used to extract the data from the graphs presented. When necessary, an online calculator was used to convert data into mean and standard deviation (available in https://smcgrath.shinyapps.io/estmeansd/, accessed on December 2023). Using the MetaInsight software v5.1.0 (17) for continuous variables (available on https://crsu.shinyapps.io/MetaInsight/, accessed on December 2023), a Bayesian network meta-analysis (NMA) was performed to compare different agents. In such cases, when different concentrations of the same agent were present within the same study, the lowest concentration capable of promoting an effect on the analyzed outcomes compared to the untreated group was chosen. Inverse variance statistics were used to calculate mean differences (MD) with a 95% confidence interval. The statistic model (fixed or random effects) was chosen according to the deviance information criterion (DIC) for consistency and inconsistency models. The model with the lowest DIC was chosen. Since an inconsistency analysis through node-split in Bayesian NMA was not feasible due to absence of closed loops arising from independent studies, a global inconsistency was performed.

We calculated the relative ranking of cross-linking agents using surface under the cumulative ranking (SUCRA), where a higher SUCRA value indicates a better treatment.

For agents that were tested in more than one study, a conventional pair-wise meta-analyses were conducted to investigate heterogeneity. The meta-analysis utilized Review Manager software, Version 5.4. Mean difference (MD) with 95% confidence intervals (CI) was employed for continuous variables. Statistical heterogeneity was assessed through the I^2 test. The random effect method was selected as the suitable approach for conducting this meta-analysis.

2.4 RESULTS

2.4.1 Characteristics of The Included Studies

A total of 2284 records were found by searching the databases. After removing duplicates, 2280 (databases + websites) remained for titles and abstracts reading. From 80 records selected for full-text reading, 50 studies were included for qualitative

synthesis (Figure 1) as they met the eligibility criteria. Out of 50 articles, the majority were conducted in the USA (40%) and China (30%) (Supplementary Figure 1). The reasons for exclusion are available in Figure 1, and the complete list of excluded studies with reasons can be found in Supplementary Table 6.

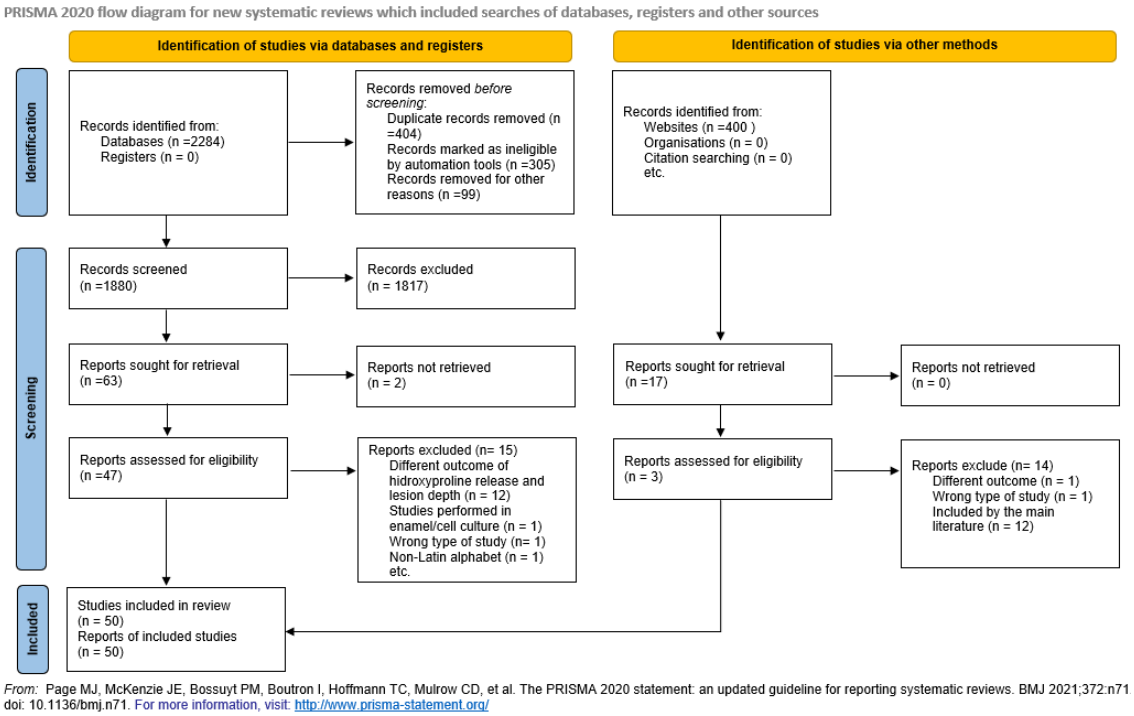


Figure 1. Flow diagram of the identification, screening and inclusion criteria, adapted from PRISMA 2020.

Among the included studies, a total of 31 cross-linking agents were identified (Table 1). The most studied was grape seed extract (GSE) (13–15,18–29,58), followed by glutaraldehyde (GA) (10–12,15,31–38) (Table 1). While 16 studies have been documented for examining the impact of GSE as a cross-linking agent, this count might be underestimated if we consider the studies categorizing the cross-linking agent as proanthocyanidin (PAC) that can originate from grape seeds.

Table 1. Cross-linking agents evaluated across the literature and corresponding number of studies.

Cross-linker	Studies
Black tea	1 (Liu et al., 2021)
Cardanol	1 (de Paula et al., 2022)
Cranberry juice	3 (Islam et al., 2021; Wang et al;. 2021 ; Boteon et al., 2017)
N-(3,4-dihydroxyphenethyl)methacrylamide (DMA)	1 (Li et al., 2021)
EDC	3 (Daood et al., 2019 ; Haught et al., 2016 ; Tezvergil-Mutluay et al., 2012)
EDC/NHS	2 (Hass et al., 2022 ; Wang et al., 2021)
EDC/TMC	1 (Wang et al., 2022c)
Epigallocatechin	1 (Hass et al., 2022)
Epigallocatechin gallate	4 (Chen and Huang, 2014; Hass et al., 2022; Nagpal et al., 2020; Wang et al., 2022)
Galla Chinesis extract	1 (Deng et al., 2013)
Genipin	4 (Hiraishi et al., 2013 ; Walter et al., 2008 ; Scheffel et al., 2014 ; Uemura et al., 2019)
Glutaraldehyde	12 (Arends et al.,1989 ; Bedran-Russo et al., 2011 ; Chen et al. 2016 ;Deng et al., 2013 ; Gong et al., 2022 ; Hiraishi et al., 2013 ; Macedo et al. 2009 ; Haught et al., 2016 ; Islam et al., 2021 ; Scheffel et al., 2014 ; Walter et al., 2008 ; Lee, and Sabatini, 2017)
Grape seed extract	16 (Boteon et al., 2017 ; Benjamin et al., 2012 ; Bedran-Russo et al., 2011 ; de Paula et al., 2022; Epasighe et al., 2013; Fawzy et al., 2017, Hass et al., 2021; Islam et al., 2012; Ismai et al., 2017; Leme-Kraus et al., 2017 ; Leme-Kraus et al., 2020 ; Liu et al., 2021 ; Macedo et al. 2009 ; Pavan et al., 2011 ; Wang et al., 2021; Xie et al., 2008)

Green tea extract	1 (Wang et al., 2021)
Hesperetin	1 (Liu et al., 2017)
Hesperidin	5 (Hiraishi et al., 2011; Hiraishi et al., 2013; Islam et al., 2012; Liu et al., 2017; Nagpal et al., 2020)
Lemon essential oil	1 (Ma et al., 2020)
Limonene	1 (Ma et al., 2020)
Lignin	1 (de Paula et al., 2022)
Luteolin	1 (Omar et al., 2022)
Miswak extract	1 (Khunkar et al., 2021)
Narigenin	1 (Liu et al., 2017)
Naringin	1 (Epasinghe et al., 2016)
Nordihydroguaiaretic acid - NDGA	2 (Gong et al., 2018; Gong et al., 2022)
Proanthocyanidin	4 (Gong et al., 2018; Walter et al., 2008; Wang et al., 2022a; Wang et al., 2022b)
Quercetin	2 (Epasinghe et al., 2016; Hass et al., 2021)
Riboflavin	3 (Fawzy et al., 2012a; Fawzy et al., 2012b; Fawzy et al., 2013)
Riboflavin + Chitosan	1 (Fawzy et al., 2013)
Sodium ascorbate	1 (Ismai et al., 2017)
Tannic acid	1 (Hass et al., 2022)
Urushiol	1 (Zhao et al., 2022)

Supplementary Table 1 (Supplementary File) provides a summary of the data for all included studies, along with their results for each outcome. Only 5 studies used bovine dentin samples (12,24,30,39,40), and all others used human dentin as the substrate for testing the efficacy of cross-linking agents. A broad range of sample sizes was observed, ranging from 4 to 600 samples per study and totaling 3,366 samples across all included studies. Among these, coronal dentin samples (15,19,21,23,24,27,29,31–38,41–56) and root samples (10–12,20,22,25,30,39,40,57–60) were used. As for the application of cross-linking agents, most studies immersed the substrates in solutions, with immersion times ranging from 30s to 30 days. Few studies mentioned that the samples were “treated” with the agents, suggesting either application or rubbing on the surface. For most agents, small variations in treatment time (e.g., 30s > 1min. > 5min.), did not seem to significantly affect their performance. On the other hand, when comparing wider variations of treatment time for the same cross-linking agent, increased treatment duration seemed to improve the agents' effects on the tested outcomes (18,41,52), even though it is important to only consider timeframes closer to clinical reality.

Most studies pretreated the substrate with acid demineralization to initiate the lesion formation, as well as to expose the collagen to the cross-linking agent. The phosphoric acid was usually applied in concentrations ranging from 5 to 37% and varying in application time. The most commonly used acid protocol was 10% phosphoric acid (13,14,19,21,27,29,31–33,35–38,42–44,46–49,51–55). Samples were then exposed to demineralizing and remineralizing cycles, with or without the addition of collagenases during remineralization. Some studies degraded the collagenolytic matrix of the substrates using bacterial collagenase, usually from *Clostridium histolyticum* (12–15,19–21,24,28,29,31–36,38,42–56,59,61).

Outcomes evaluated included lesion sizes, mostly measured using microscopy images, although they represented a small number of studies. When this information was included, some methodologies were used, such as Confocal Laser Scanning Microscopy (CLSM) (12,22,25,60); Transverse microradiography (10,20,30,57,58); Light microscopy (11,18,39) and scanning electron microscope (59).

As for the outcome “release of hydroxyproline”, the results of collagen degradation after treatment with the respective cross-linking agents were presented in the form of graphs or numerical tables. In cases where studies only provided results in

graphical form, the WebPlotDigitizer tool (4.6 version) was used to extract numerical values (11,21,24,36–38,40,43,47,51–53,55,56,58,61).

2.4.2 Qualitative Synthesis of the Results

2.4.2.1 Glutaraldehyde

All studies evaluating GA presented a significant increase in the biostability of dentin collagen when subjected to cariogenic challenges. One study used the GA-based desensitizer Gluma (Heraeus Kulzer, Hanau, Germany), showing no differences in the release of hydroxyproline when compared to the negative control after 1 week of treatment, and a significant superiority of the Gluma after 4 weeks (32). On the other hand, there was a better result of Proanthocyanidins (PACs) in relation to GA in reducing LD in root caries (but not for hydroxyproline release), although it demonstrated good performance in relation to the untreated group and similar performance compared to Genipin, a compound derived from gardenia fruits (12).

Regarding the hydroxyproline related outcome, GA was better than GSE (15), but similar to Galla Chinesis extract 4% (35). GA was also similar to Nordihydroguaiaretic acid (NDGA) (36) and EDC (31,38) for the release of hydroxyproline. At a concentration of 0.02%, GA was inferior to other agents such as hesperidin, PAC and EGCG at a concentration of 0.5% (37).

2.4.2.2 Grape Seed Extract

Six studies evaluated grape seed extracts for the LD (18,20,22,24,25,58), while 11 analyzed its effect on hydroxyproline release (13,14,19,21,23,24,27–29,33,58). One study evaluated the proportion of degraded collagen between treated and untreated groups (15).

Overall, GSE showed positive results in reducing the release of hydroxyproline. GSE (10%) was similar to Cranberry (10%), chlorhexidine (0.012%) and NaF gel (1.23%) (24). Samples treated with GSE 0.65% were more resistant to degradation compared to the groups treated with green tea and EDC/NHS (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride/NHS: N-hydroxysuccinimide (21).

In contrast, in other studies, treatment with GSE at different concentrations was significantly better than chlorhexidine (27,58). When used in nanoparticles or considering the time factor in the hydroxyproline release, this agent also performed better than the studied controls (13,14,28). Also, it presented a similar result to GA (5 and 25%), when applied at a concentration of 6.5% (33), lignin and cardanol (concentrations of 2 and 4%) (23) with no statistical difference in terms of resistance to biodegradation but performed worse at a lower concentration (0.65%) (33) and 0.4% when compared to Black Tea (19). Meanwhile, for the 1% concentration, lignin treatment was statistically more favorable than the other groups. In relation to sodium ascorbate, the effect of the GSE appears to be affected by the exposure time (18).

2.4.2.3 Other Cross-linking Agents

Less frequently discussed agents also exhibited favorable effects on the outcomes when compared to untreated groups, further emphasizing the "protective" action facilitated by the stabilization of dentin collagen. The exception was Quercetin, which demonstrated negligible collagen interactions and low collagen biostability (47) and EGCG (Epigallocatechin gallate) (46). For instance, EGCG was able to promote cross-links with collagen, but these were highly metabolized by salivary esterases, thus compromising biostability (46).

2.4.3 Risk of Bias

Out of the 50 studies included, 38 had a low risk of bias, 11 had a moderate risk, and only 1 presented a high risk of bias (Table 2). Among the main reasons for an increased risk of bias, one can mention the lack of description regarding the randomization and allocation process of samples, sampling technique, and sample size calculation. Criteria for blinding, operator details, and calibration were considered not applicable to the studies in question. The list of each article with its respective score for each question and final score is presented in Supplementary File (Supplementary Table 2.). Considering that this systematic review only included *in vitro* studies, the GRADE approach for evaluating the certainty of evidence was not applied.

Table 2. Risk of Bias of the included studies based on scores for the 12 criteria of the QUINN tool (16) for *in vitro* studies in Dentistry.

Article	Final Score (%)	Risk of Bias
Arends et al.,1989	66,66%	-
Bedran-Russo et al., 2011	66,66%	-
Benjamin et al., 2012	66,66%	-
Boteon et al., 2017	72,22%	+
Chen et al. 2016	72,22%	+
Chen and Huang, 2014	72,22%	+
Daood et al. 2019	77,77%	+
de Paula et al., 2019	61,11%	-
Deng et al., 2013	77,77%	+
Epasinghe et al., 2017	77,77%	+
Epasinghe et al., 2013	72,22%	+
Epasinghe et al., 2015	77,77%	+
Epasinghe et al., 2016	77,77%	+
Fawzy et al., 2013	72,22%	+
Fawzy et al., 2012a	72,22%	+
Fawzy et al., 2017	83,33	+
Fawzy et al., 2012b	72,22%	+
Gong et al., 2022	72,22%	+
Gong et al., 2018	66,66%	-
Hass et al., 2022	83,33	+
Hass et al., 2021a	72,22%	+
Hass et al., 2021b	72,22%	+
Haught et al., 2016	77,77%	+
Hiraishi et al., 2011	72,22%	+
Hiraishi et al., 2013	77,77%	+
Islam et al., 2012	66,66%	-
Islam et al., 2021	66,66%	-
Ismai et al., 2017	50%	-
Khunkar et al., 2021	66,66%	-
Lee, and Sabatini, 2017	77,77%	+
Leme-Kraus et al., 2017	44,44%	X
Leme-Kraus et al., 2020	61,11%	-
Li et al., 2021	77,77%	+
Liu et al., 2021	77,77%	+
Liu et al., 2017	77,77%	+
Ma et al., 2020	77,77%	+
Macedo et al. 2009	77,77%	+
Nagpal et al., 2020	72,22%	+
Omar et al., 2022	77,77%	+
Pavan et al., 2011	77,77%	+
Scheffel et al., 2014	77,77%	+
Tezvergil-Mutluay et al., 2012	72,22%	+

Uemura et al., 2019	61,11%	-
Walter et al., 2008	77,77%	+
Wang et al., 2022a	72,22%	+
Wang et al., 2022b	77,77%	+
Wang et al., 2022c	77,77%	+
Wang et al., 2021	77,77%	+
Xie et al., 2008	77,77%	+
Zhao et al., 2022	77,77%	+

* + Low; - Moderate; X High.

2.4.4 Lesion Depth NMA

A NMA for the LD outcome was performed, including eight studies and nine interventions. The network comprised a total of 284 samples, with 36 paired comparisons. Among these, 13 involved direct data resulting in a connected network with five two-arm studies and three multi-arm studies.

The comparisons of all agents with the control are shown in Figure 3-B, presenting the mean differences and the credibility interval (95%). The cross-linking agents hesperidin, GSE, Cranberry and riboflavin UVA-activated (RF-UVA) showed no effect when compared to the untreated control group for the outcome lesion depth ($p>0.05$). Meanwhile, the efficacy of the cross-linking agents GA, Naringin, PAC and Quercetin was confirmed as they presented better performance in the analyzed outcome when compared to the control.

The average LD was 123.6µm (Supplementary Table 3, supplementary file), and the components of the NMA are shown in Figure 3. The SUCRA (Surface Under the Cumulative Ranking curve) (Figure 3-A) shows the proportion of efficacy of each agent compared to a hypothetical ideal agent (62). Rank values are described in Table 3. Narigin was the cross-linking agent presenting the highest rank, followed by Quercetin, Riboflavin UVA-activated, and PAC. The worse rank was attributed to the negative controls.

Table 3. SUCRA (Surface Under the Cumulative Ranking curve) rank of cross-linking agents evaluated across studies included in the network metanalysis (n=9) for reducing lesion depth in cariogenic challenges *in vitro.*

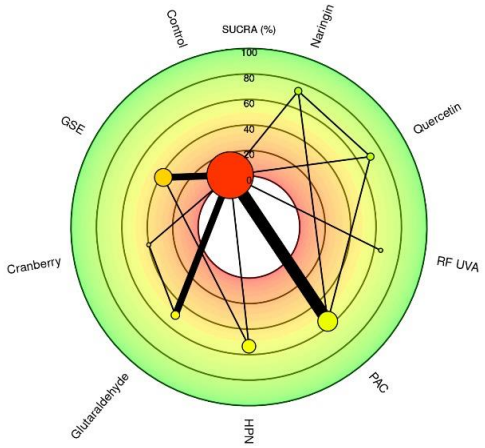
Treatment	SUCRA (%)
Naringin	73.49
Quercetin	70.14
Riboflavin UVA-activated	64.94
Proanthocyanidins	56.41

Hesperidin	53.62
Glutaraldehyde	50.03
Cranberry	40.25
Grape seed extract	37.90
Control (untreated)	3.21

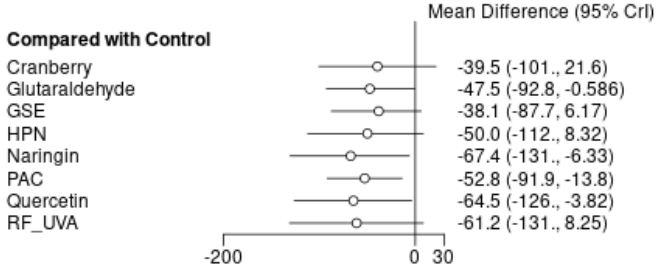
A global inconsistency was analyzed through deviance from NMA model graph (Figure 3-C), confirming the absence of inconsistency. Additionally, a frequentist NMA with random effects was carried out only to evaluate the inconsistency, showing no p-values <0.05 for any available direct/indirect comparisons (Supplementary table 4, supplementary file).

Cross-linking agents tested in multiple studies were tested for heterogeneity (Figure 3-D). PAC and GA were favorable over untreated control groups. PAC had low heterogeneity, while GA and GSE had considerable heterogeneity but no inconsistency since all effect estimates showed the same direction. The high heterogeneity may be explained by the precision of included studies, which resulted in a poor 95% CI overlapping. Strategies to reduce the heterogeneity were excluding studies with bovine samples from the NMA to avoid any type of bias related to the different origin type of sample (12,30,39). Also, studies that used a method that was not comparable to others to produce (24) or to measure the lesion (22,25) were not included in the NMA.

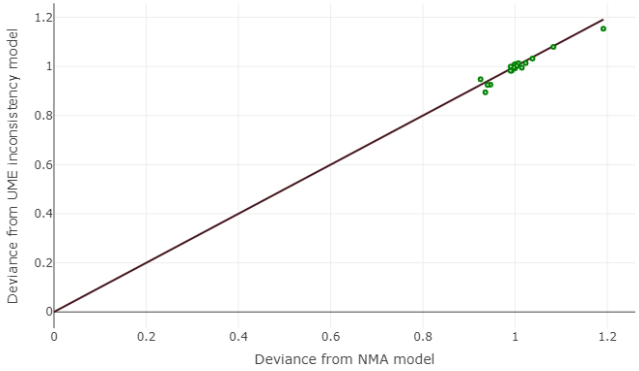
A


SUCRA (%)
100
80
60
40
20
Control
Naringin
Quercetin
RF UVA
PAC
HPN
Glutaraldehyde
Cranberry
GSE

B


Mean Difference (95% CrI)
Compared with Control
Cranberry -39.5 (-101., 21.6)
Glutaraldehyde -47.5 (-92.8, -0.586)
GSE -38.1 (-87.7, 6.17)
HPN -50.0 (-112., 8.32)
Naringin -67.4 (-131., -6.33)
PAC -52.8 (-91.9, -13.8)
Quercetin -64.5 (-126., -3.82)
RF_UVA -61.2 (-131., 8.25)
-200
0
30

C


Deviance from UME inconsistency model
Deviance from NMA model
0
0.2
0.4
0.6
0.8
1
1.2

D

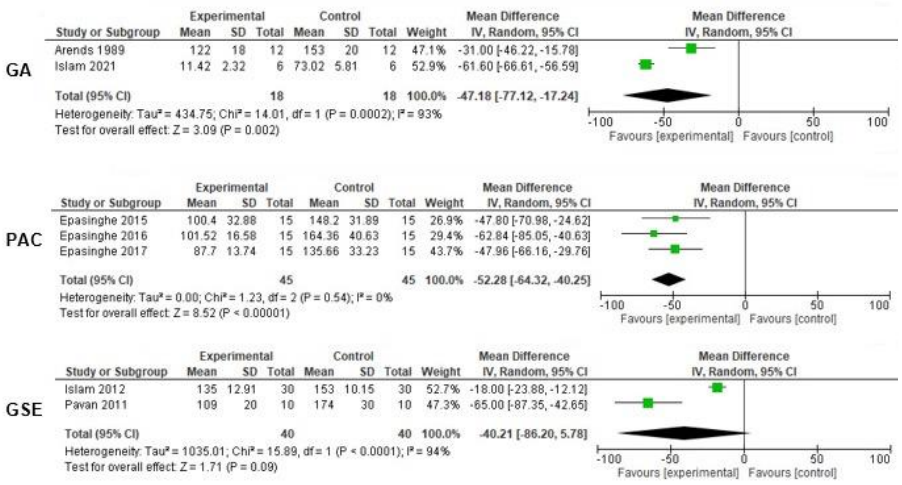

GA
Experimental
Control
Mean Difference
Mean Difference
Study or Subgroup Mean SD Total Mean SD Total Weight IV, Random, 95% CI
IV, Random, 95% CI
Arends 1989 122 18 12 153 20 12 47.1% -31.00 [-46.22, -15.78]
Islam 2021 11.42 2.32 6 73.02 5.81 6 52.9% -61.60 [-66.61, -56.59]
Total (95% CI) 18 18 100.0% -47.18 [-77.12, -17.24]
Heterogeneity: Tau² = 434.75; Chi² = 14.01, df = 1 (P = 0.0002); I² = 93%
Test for overall effect: Z = 3.09 (P = 0.002)
-100 -50 0 50 100
Favours [experimental] Favours [control]
PAC
Experimental
Control
Mean Difference
Mean Difference
Study or Subgroup Mean SD Total Mean SD Total Weight IV, Random, 95% CI
IV, Random, 95% CI
Epasinghe 2015 100.4 32.88 15 148.2 31.89 15 26.9% -47.80 [-70.98, -24.62]
Epasinghe 2016 101.52 16.58 15 164.36 40.63 15 29.4% -62.84 [-85.05, -40.63]
Epasinghe 2017 87.7 13.74 15 135.66 33.23 15 43.7% -47.96 [-66.16, -29.76]
Total (95% CI) 45 45 100.0% -52.28 [-64.32, -40.25]
Heterogeneity: Tau² = 0.00; Chi² = 1.23, df = 2 (P = 0.54); I² = 0%
Test for overall effect: Z = 8.52 (P < 0.00001)
-100 -50 0 50 100
Favours [experimental] Favours [control]
GSE
Experimental
Control
Mean Difference
Mean Difference
Study or Subgroup Mean SD Total Mean SD Total Weight IV, Random, 95% CI
IV, Random, 95% CI
Islam 2012 135 12.91 30 153 10.15 30 52.7% -18.00 [-23.88, -12.12]
Pavan 2011 109 20 10 174 30 10 47.3% -65.00 [-87.35, -42.65]
Total (95% CI) 40 40 100.0% -40.21 [-86.20, 5.78]
Heterogeneity: Tau² = 1035.01; Chi² = 15.89, df = 1 (P < 0.0001); I² = 94%
Test for overall effect: Z = 1.71 (P = 0.09)
-100 -50 0 50 100
Favours [experimental] Favours [control]

Figure 3. Network meta-analysis (NMA) components for the lesion depth outcome. A) **Radial SUCRA plots.** The figure shows SUCRA considering the network meta-analysis. Higher SUCRA values indicate better treatments; Size of nodes represent number of participants and thickness of lines indicate number of trials conducted (RF UVA: Riboflavin UVA activated; PAC: proanthocyanidins; HPN: hesperidin; GSE: grape seed extract and control: untreated). B) **Forest plot from NMA using Bayesian method**. Numerical values for the mean difference and 95% credibility interval (95% CrI). C) **Residual deviance from NMA model and UME inconsistency model for all studies.** This plot represents each data points' contribution to the residual deviance for the NMA with consistency (horizontal axis) and the unrelated mean effect (ume) inconsistency models (vertical axis) along with the line of equality. The points on the equality line means there is no improvement in model fit when using the inconsistency model, suggesting that there is no evidence of inconsistency. Points above the equality line means they have a smaller residual deviance for the consistency model indicating a better fit in the NMA consistency model and points below the equality line means they have a better fit in the ume inconsistency model. D) **Heterogeneity approach for meta-analysis** of lesion depth reduction for treatments with glutaraldehyde - GA, proanthocyanidins - PAC and grape seed extract - GSE.

2.5 DISCUSSION

The discovery of new treatments that minimize the progression of caries lesions is desirable (1,63). Therefore, the aim of this study was to systematically review the efficacy of cross-linking agents in reducing caries lesion depth and collagen degradation *in vitro*. These agents may be promising treatments by biomodifying dentin collagen through the promotion of a better collagen matrix stability (8,20,22). The results obtained suggest the possibility of using Naringin, Quercetin, PACs, and GA in further research as they presented a higher probability of reducing lesion depth *in vitro*. These products also seemed promising for the outcome “hydroxyproline release” corresponding to the surrogate measurement for collagen breakdown in the qualitative analysis.

Positive outcomes have been demonstrated for agents as non-invasive therapies for root caries (64–66). Most studies, however, are focused on the antimicrobial capacity of these therapeutic agents and limited information is available regarding substances with physical-chemical potential to modify the affected dentinal tissue (67–69). Some of these agents might promote collagen cross-linking, significantly reducing the biodegradation rates of the dentin matrix and increasing the biomechanical properties of dentin (12). GA, formaldehyde, carbodiimide, and epoxy

compounds are among the most studied cross-linking agents (12,21). However, some adverse effects of their use, such as toxicity and instability, limit their application *in vivo*. Therefore, the possibility arises of using natural compounds considered stable and biocompatible, such as PAC, genipin, and cranberry extracts (21). pH cycling models show that both PAC and GSE (grape seed extract containing proanthocyanidins) could stabilize affected collagen and remineralize root carious lesions *in vitro,* respectively (12,22). Likewise, significant results were observed when using hesperidin for remineralization in areas of affected dentinal collagen. Here, we confirmed the efficacy of a few alternative natural compounds to cross-link dentin substrates, mostly from the flavonoid family.

In our findings, naringin performed the best in the rank for the outcome of lesion depth. Naringin is a flavone glicosyde found mostly in onions and apples and despite its similarity to PAC's molecular structure, it has a smaller molecular size. This may facilitate its penetration into demineralized dentine and enhance the remineralization effect (57). This compound, along with PAC, hesperidin, cranberry, among others, belongs to the flavonoid family, possessing antioxidant, antibacterial, and anti-inflammatory properties (70). They are easily found in fruits and vegetables and are known to interact with proteins like collagen (22,57). They have a common basic structure but differ in the number and type of groups attached to the benzene ring. This difference may account for alterations in their biological effects (71).

Despite naringin ranking first in the SUCRA, these results need to be interpreted with caution (Table 3) as it was tested in only one study with a relatively small sample size (n=15/per group). The study had a moderate risk of bias due to limitations in sample size calculation description, sampling technique, and randomization. Additionally, it should be considered that the evidence upon which the ranks are based is of low confidence/certainty. This occurs because in various ranks generated, an intervention might be the best for a particular outcome (e.g., cost; adverse effects) but the worst for another (72). In other words, the interpretation of these results also depends on relevant clinical aspects (73). For instance, it is known that GA has previously been associated with higher toxicity than natural compounds (74). These aspects should be considered when choosing the best agents for testing in translational research. Another limitation of SUCRA would be its failure to consider the magnitude of differences in effects between treatments and its inability to account for the possibility of randomness (72). Taking this into consideration, to reduce the

possibility of overinterpreting the results described here, a comparison of the cross-linkers with a constant group (untreated control) as a common comparator across the groups was used. Also, it is important to mention that the Bayesian method was chosen for the NMA in this study for its conservatism, and the and the forest plot summarizing the results of comparisons with the common comparator was based on it (Figure 3-B). It was observed that some agents did not show a statistically significant differences from the control, including well-ranked agents, such as riboflavin (Figure 3-B). Studies that showed significance between the tested agents versus control for the lesion depth outcome in the study level did not perform similarly in the NMA. This likely occurred because NMA considers not only direct comparison evidence but also indirect evidence. Thus, when evaluated in the network, agents like grape seed extract, riboflavin, hesperidin, and cranberry, despite resulting in smaller lesion depths, did not statistically differ from untreated groups. Naringin, on the other hand, along with PAC and quercetin, showed a difference compared to the control, and these three agents had similar values in SUCRA. In addition to this, if considered factors such as lower imprecision and the risk of bias in studies included in the NMA, it could be suggested that other agents might be tested in future research even before Naringin. This is the case with PAC, for instance, which has a larger sample size and has been tested in more studies, all of them with low risk of bias. As for the hydroxyproline release results, PAC demonstrated good performance, showing higher cross-linking and biostability efficacy compared to quercetin (47). However, when compared to GA, its performance varied according to the concentrations tested for both agents (12, 15, 33, 35, 37).

As we aimed to evaluate the collagen cross-linking capacity of the tested agents, we did not evaluate the groups including remineralizing substances or collagenase inhibitors. For instance, studies testing sodium monofluorophosphate (25), Chlorhexidine (24,30,39,58), sodium fluoride (24,57,60,75), TCP (Tri-calcium phosphate) (60), CPP-ACFP and CPP-ACP (75), Galardin and Benzalkonium chloride (50) and Galangin (51) were not included in the NMA to avoid heterogeneity. However, it is known that some cross-linkers can also hold the ability to inhibit endogenous collagenases, which are directly related to collagenolytic degradation during the carious process (76), a very desirable outcome. It was also noted that adding a cross-linking agent to treatments with remineralizing compounds reduced collagen degradation and lesion size, inhibiting demineralization and enhancing

remineralization (20,60,75). These findings reinforce the potential of these substances for use in combined treatments involving different types of agents.

There was a variety of methodologies using pH cycling, acid demineralization, or bacterial collagenases to degrade collagen from the dentin substrates treated with the cross-linking agents in test. It was observed that studies assessing the lesion depth used pH cycling or exposure to acidic solutions, while hydroxyproline release was measured after exposure to collagenases with or without involvement of pH cycles. The first method consists of applying demineralizing and remineralizing substances to the sample surface in order to constantly alternate the demineralization and remineralization processes (pH cycle). This is only interrupted during the short period of application of the products under investigation. Each article used its own methodology regarding application time and protocol, however, the cycling substances were very similar. Benjamin et al. (25), for example, used the following order of pH cycle: treatment solutions (10 minutes), acidic buffer at pH 5.0 (30 minutes) and neutral buffer at pH 7.0 (10 minutes) for 8 days consisting of 6 cycles per day. Epasinghe et al. (60), on the other hand, immersed the substrates in demineralizing solution (2.2 mM CaCl2.2H2O, 2.2 mM KH2PO2, 50 mM acetate, pH 4.6) for 96 hours at 37 °C to create lesions. However, these differences seemed not to affect the result as in the NMA. In the pairwise meta-analysis for PAC (Figure 3-D), for instance, despite studies using different acids and cycle times, low heterogeneity was found. This may suggest that the cycle methodology might not significantly affect the outcome. Therefore, researchers can opt for what is most convenient for their research.

The presence of bacterial collagenases in the solutions causes the degradation of the collagen and simulates the protein loss occurring during the progression of dentin carious lesions. Determining collagen content in a sample can be done by quantifying the amino acid hydroxyproline. N=31 studies assessed hydroxyproline release after treatment with cross-linking agents. However, the high number of studies lacked standardization in the units of measurement for the results (Supplementary Table 5, supplementary file), making comparisons difficult. Additionally, several concentrations of each compound were tested for the pretreatment of samples, with a trend/higher repetition observed for concentrations of 6.5% for GSE and 5% for glutaraldehyde. The aim here was not to evaluate a dose-response relationship for the agents but rather their efficacy in the studied outcomes. Nevertheless, this is an important factor to consider for conducting future studies. Another variable to be

considered is the treatment duration, which also varied across different articles and should be as short as possible to be clinically translatable.

Another collected data from the included studies was the storage conditions of the dentin specimens before and after treatments (Supplementary Table 1.1), although not all articles described it. We strongly believe that this information is important when considering replicating methodologies for future research. Among the most commonly used substances, thymol solutions, chloramine, and phosphate-buffered saline or NaCl containing sodium azide were mentioned and seem to be appropriate to keep the quality of the samples and to prevent bacterial growth.

2.5.1 Limitations of the Study and Implications for Future Research

One of the limitations of this systematic review is the lack of a meta-analysis for the hydroxyproline outcome. However, we believe that the lesion depth outcome is closer to clinical reality and therefore can be highly valuable for exploring potential treatments and preventive measures. One of the reasons for the complexity of conducting NMA for the mentioned outcome was the vast number of different concentrations tested for various agents, significantly increasing the number of groups to be compared in the network and impairing it analysis. Also, a limitation is recognized due to the use of manual extraction aided by software (WebPlotDigitizer version 4.6) in a few articles that did not directly provide numerical data. This strategy is sensitive to a margin of human error, considering there was no prior calibration, although this step was performed by only one researcher (IMS). Moreover, direct comparisons for NMA of lesion depth were limited in some cases, and these comparisons usually provide greater certainty of evidence for network estimates (77). Lastly, it's essential to acknowledge the inherent limitations of *in vitro* methodologies, as *in vivo* conditions are more complex, and the studied agents may perform differently in an environment like the oral cavity, which presents other factors such as salivary composition that could contribute to a reduction in the clinical bio-stability of these treatments (46).

Despite the limitations, this study managed to gather mostly low bias risk evidence (39 studies), aiding in narrowing down potential treatment choices for future research. Furthermore, no inconsistencies were found in the findings (Figure 3-C.). Managing carious lesions in dentin substrate is challenging, and the emergence of new

control and treatment options may assist clinicians in tissue preservation, reducing tooth wear and improving the quality and longevity of restorative treatments.

2.6 CONCLUSION

- The study encompassed research on 31 cross-linking agents, with the most studied being GSE and GA;
- The majority of cross-linking agents showed efficacy compared to the untreated group for both analyzed outcomes, except for GSE, RF_UVA, Cranberry and Hesperidin that presented no statistical differences with the controls without treatments in the NMA for lesion depth;
- Naringin was the best-performing cross-linking agent in reducing lesion depth; however, these results should be interpreted cautiously as it was only present in one study, which was considered to have a moderate risk of bias;
- Clinical studies are needed considering the complexities of the oral environment to determine the actual effectiveness of pretreatment with these agents, and the most recommended for further research are Naringin, PAC, Quercetin and GA.

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2.8 SUPPLEMENTARY MATERIAL

The supplementary material is available through the link: https://drive.google.com/file/d/1eVhkgIQfuMcIlV6GvbChmxfomw3aX_fs/view?usp=drive_link

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CAPÍTULO 3. ANACARDIC ACID LDT11 IS A POTENTIAL CROSS-LINKING AGENT FOR DENTIN BIOMODIFICATION: AN *IN VITRO* STUDY

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3.1 ABSTRACT:

The use of cross-linking agents can be adjunctive strategies for controlling root caries due to their capacity to promote intra- and intermolecular bonds with dentin collagen, enhancing its mechanical and chemical properties. The objective of this study was to evaluate the interaction of a potential cross-linking agent with bovine dentin. **Methodology**: Two hemi-synthetic saturated cashew-nut shell liquid derivative compounds were selected (LDT11-anacardic acids-derivative and LDT409-cardanol-derivative). N=9 samples of bovine dentin were prepared and randomly allocated into 3 groups in triplicates: negative control (untreated), positive control (100µg/mL of cardanol - LDT409), and treatment with 100µg/mL of LDT11. Samples were demineralized with 10% phosphoric acid for 30min. and treated by immersion with the respective solutions for 72h. The Fourier Transform Infrared Spectroscopy associated with Attenuated Total Reflectance technique (FTIR/ATR) was used to measure the spectra in triplicates. **Results**: The resulting spectra suggest alterations in the samples treated with LDT11 and a similar behavior to that observed for the cardanol-derivative positive control. Changes related to characteristic peaks of collagen were observed, such as reduced intensity in the peak around ~1400cm-1, reduction in Amide II (~1550cm-1) with the appearance of new peaks, and increased intensity with new peaks in Amide I (~1630cm-1). Principal component analysis revealed differentiation among the groups, indicating high similarity between the cardanol-positive control and the anacardic acid-test group (LDT11), and both presented low similarity when compared to the negative control without treatment. **Conclusion**: The saturated anacardic acid LDT11 may exhibit chemical interaction with dentin collagen similar to cardanol, suggesting its potential as a biomodifier cross-linking. Its capacity to controlling and treating carious lesions involving the dentin substrate could be explored in new experimental models.

Keywords: root caries, dentin biomodification, collagen cross-links, Spectroscopy, Fourier Transform Infrared

3.2 INTRODUCTION

Root caries lesions affecting aged groups are preceded by gingival recession and exposure of the root structure to the oral environment. Considering the theory that the development of these lesions would involve the degradation of the dentin organic matrix after demineralization (1), and the limitations of currently available approaches for treating root caries, biomodifying agents may be alternatives as adjunctive treatments for controlling these lesions. These agents act through exogenous cross-links and can interact with the predominantly present type I collagen framework in the organic matrix of root dentin tissue, promoting stability and improving mechanical and biochemical properties. Apart from this potential role in root caries lesion control, these agents have shown potential in contributing to the hybrid layer durability in dentin bonding (2–5).

Cross linkers can have synthetic origin, like glutaraldehyde, also recognized for its ability to enhance dentin bio-stability (6), and EDC (1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide) that presented promising results in dentin adhesion studies (7,8). Natural substances can also be collagen cross-linkers, as seen in some polyphenols such as green tea and proanthocyanidins derived from cranberry or grape seeds (9,10). In this context, natural agents have gained greater attention due to their renewable, sustainable nature and lower cytotoxic potential compared to synthetic agents. An example of this is the grape seed extract, extensively researched in vitro and previously described as a potential adjunctive agent for preventing root caries (9).

Recently, two natural agents have been reported for their ability to cross-link with dentin collagen: cardanol and cardol, both derived from the liquid obtained through the processing of cashew nut shell liquid (CNSL) (5,11). An increase in the modulus of elasticity and resistance to biodegradation after treatment were observed. Another derivative from the CNSL is the anacardic acid (AA), which represents about 90% of its composition and is more clinically stable. This substance has been described for its inhibitory activity on matrix metalloproteinases (12,13), enzymes associated with collagenolytic degradation in the process of root and dentin caries (14). In addition, we previously characterized its antimicrobial activity against gram-positive and gram-negative bacteria, its anti-collagenase effect, and its biocompatibility with dental pulp stromal cells (15). However, there are still no studies characterizing the chemical interaction of AA with dentin for biomodification purposes.

Considering the importance of studying cross-linking agents for the development of future strategies for dentin caries prevention and control, this study aimed to test whether saturated AA (LDT11) is capable of interacting with dentin's organic matrix collagen forming cross-links. The null hypothesis is that AA, unlike cardanol, is not a cross-linking agent for dentin.

3.3 MATERIALS AND METHODS

3.3.1 Sample Preparation

For dentin sample preparation, roots from bovine incisors were used. Teeth were stored in freshly prepared 0.5% chloramine to prevent cross-contamination for at least five days (16). For sectioning, the teeth were initially embedded in acrylic resin within circular plastic devices, immersed up to the cementoenamel junction. Subsequently, the apical third was removed using diamond discs and a handpiece. Dental sample sections then were obtained using a high-precision cutting machine (Accutom, Struers, Spain), resulting in two approximately 500 µm-thick and 8mm wide samples from each incisor. Longitudinal sections were made along the long axis of the tooth, obtained from mesial and distal portions, excluding regions of the root canal and root cementum (Figure 1). The samples were then stored in distilled water until further use.

3.3.2 Experimental Design and Groups

The compounds examined in this study were saturated anacardic acid LDT11 and cardanol LDT409 (15). Both were extracted from the CNSL and modified at the Laboratory of Therapeutic Innovations Development (LDT) at the Tropical Medicine Center of the University of Brasilia (FS/FAR). For use, solutions were prepared from isolated and dehydrated compounds added to 1mL of 95% ethanol and diluted until de final concentration in the same substance with a final volume of 1mL.

The resulting specimens were randomly allocated into 3 treatment groups (n = 3/group):

Group A. Cardanol (LDT409 - positive control) 100µg/mL
Group B. Anacardic Acid (LDT411) 100µg/mL
Group C. Negative control (distilled water)

3.3.3 Demineralization and Treatments

Prior to treatments, samples were randomly placed in a 24-well plate and demineralized with 10% phosphoric acid (ACS Científica, SP, Brazil) for 30min to expose the dentin organic matrix following the protocol established by Wang et al. (10) Subsequently, they were immersed in 1mL of the respective treatment solution for 72 hours (17). The specimens were vigorously rinsed with distilled water for 30s to remove non-adhered cross-linkers before the outcome analysis. Additionally, samples were dried in vacuum to avoid interferences of the reading in OH region.

3.3.4 Outcome: Fourier Transform Infrared Spectrometer with Module Attenuated Total Reflectance (FTIR/ATR)

The interactions between the agents (LDT11 and LDT409) and demineralized bovine dentin were analyzed by FTIR using a Bruker spectrometer (Vertex 70 model, Massachusetts, U.S.). The analysis was performed using the module attenuated total reflectance (ATR). The measurements were averaged over 96 scans, which were taken at a resolution of 4cm-1 from 400 to 4000cm-1. The background signal was averaged over 96 scans before each measurement. Each experiment was performed in triplicate.

3.3.5 Statistical Analysis

To assess the correlation between different groups and the multiple spectra, as well as respective peaks obtained from each sample, a Principal Component Analysis (PCA) analysis was performed using Minitab® 17.1.0. The calculation of the areas of peaks in Amide I was performed using Fitik software version 0.9.8 with the Voigt function. Analysis of Variance (ANOVA) was used to compare groups. The power of the study was performed by using the mean difference of the Amide I peak and

considering a 95% two-tailed confidence interval using Openepi tool (https://www.openepi.com/Power/PowerMean.htm).

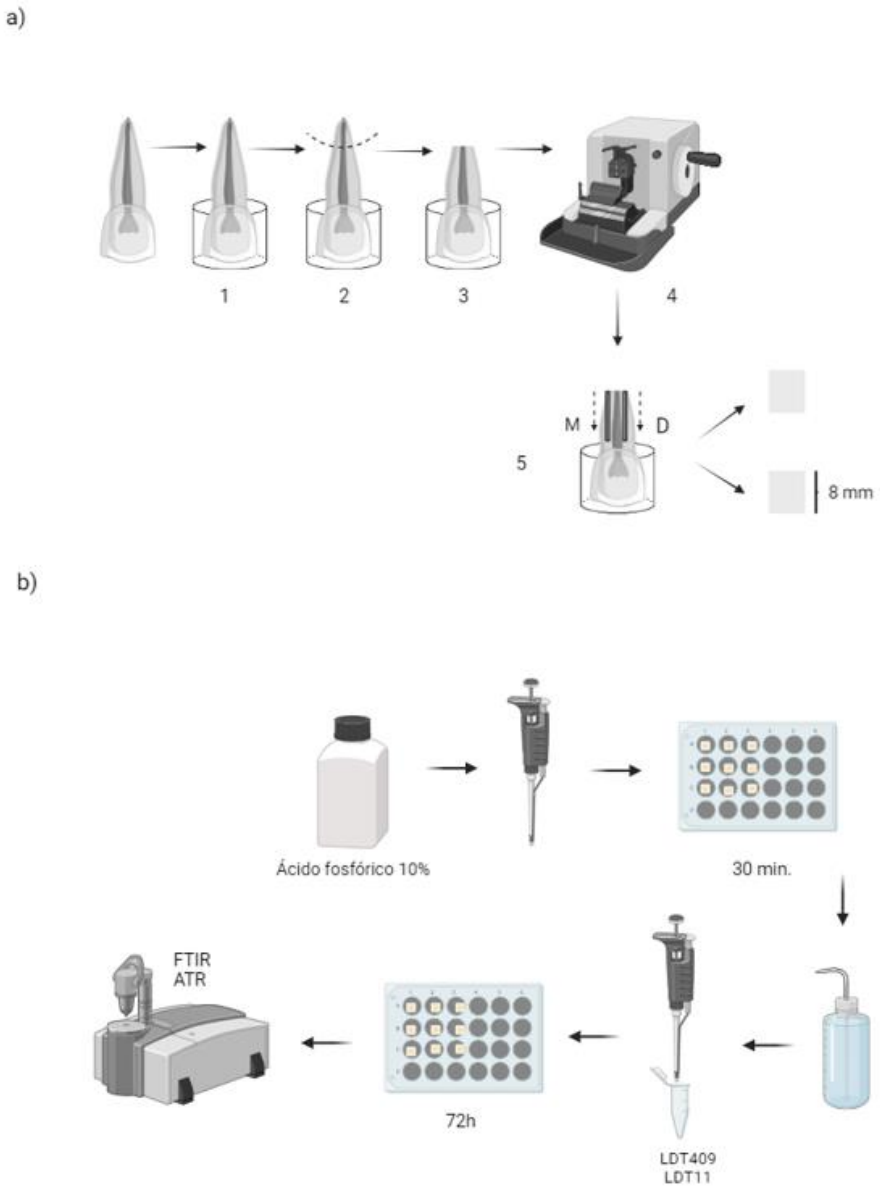

Figure 1. Methodology. a) Sample preparation; b) Demineralization and preliminary treatments.

3.4 RESULTS

The results of the FTIR/ATR spectral analysis are summarized in Figure 2. Each spectrum is the average of 9 spectrum from each group (triplicate measurements from each of 3 samples/group). Two altered spectra from a control group and two additional spectra from the remaining groups were excluded from the analysis to match

the sample number, totaling n = 7 spectra/group. The characteristic bands of dentin collagen were identified in all samples as follows: amide I at ~1630cm^{-1}, amide II at ~1550cm^{-1}, CH2 bending at ~1450cm^{-1}, and amide III at ~1239cm^{-1}.

It was observed that the interaction of substances LDT11 and positive control (LDT409) with the collagen framework of bovine dentin led to a reduction in the peak intensity at ~1400cm^{-1}, as well as the emergence of new peaks in amide I and amide II. In the former, an increase in intensity at 1630cm-1 was also observed. Regarding the peak at Amide III (~1239cm^{-1}), an intensity increase was observed for the LDT409 treatment (Fig. 2). Furthermore, alterations in peaks in the region between 1000-1200 cm^{-1} such as broadening and increased intensity were observed for both treated groups, with the emergence of a shoulder at ~1120 cm^{-1} for the group treated with AA.

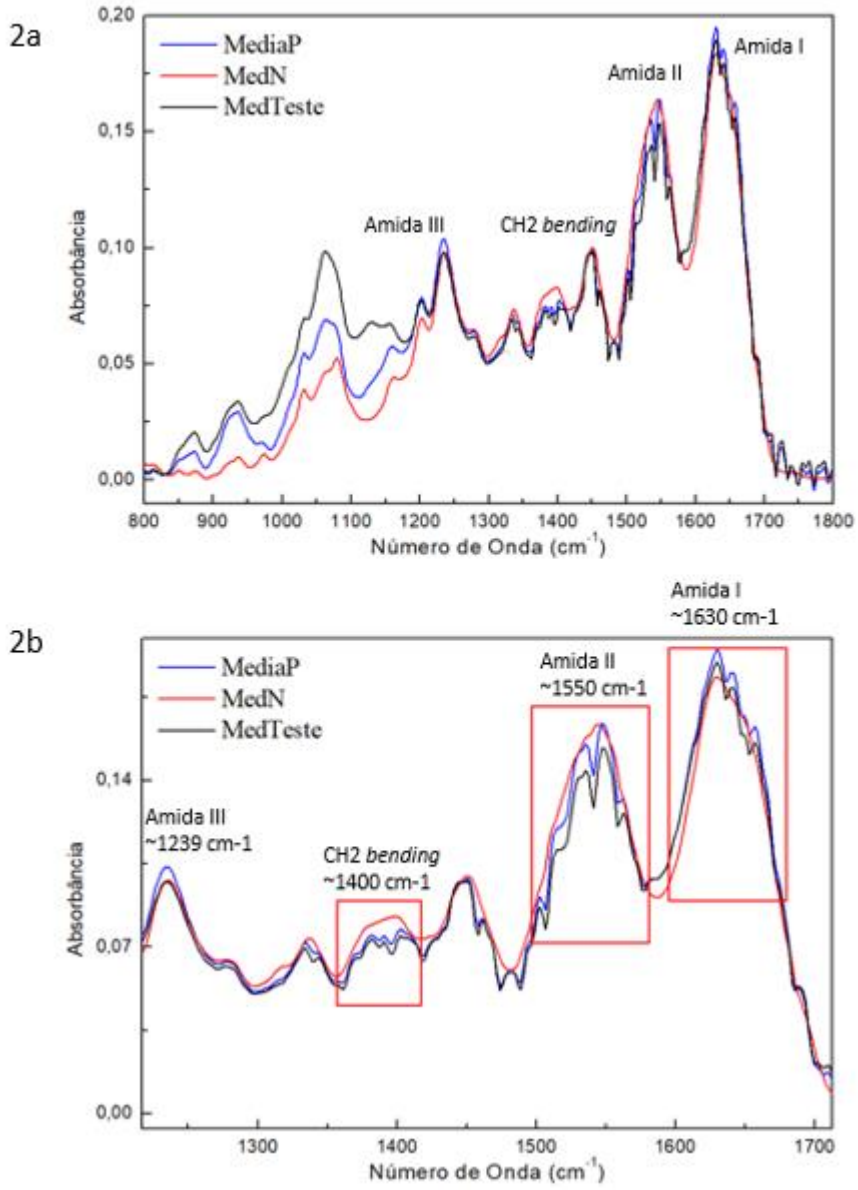

Figure 2. Average of the obtained spectra (“MediaP” = LDT409; “MedN” = negative control, and “MedTeste” = LDT409) (2a) and augmentation of the spectral region highlighting the observed changes in characteristic collagen peaks (amide I; amide II; Amide III e CH2 bending) (2b)

The result of Principal Component Analysis (PCA) PC2xPC3 is presented in Figure 3. A clear separation among the groups is observed and indicating a positive correlation between the positive control group (LDT409/cardanol) and the test group (LDT11/anacardic acid). This may suggest a similar outcome between their spectra. Conversely, a negative correlation was observed with the negative control group (untreated), in an opposite direction. In summary, concerning the studied chemical interactions with dentin samples, LDT11 and LDT409 groups exhibit similar behavior and yield different results compared to the untreated group.

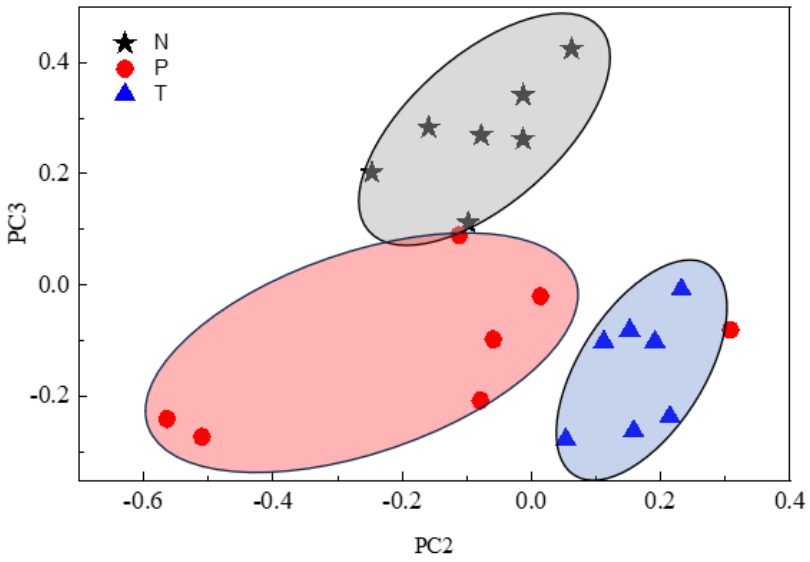

Figure 3. Principal Component Analysis (PCA) using PC2xPC3 for 7 spectra obtained from each group (P = positive control; N = negative control; T = test (LDT11)).

A statistically significant difference was found between the groups (ANOVA, p = 0.011). The study's power was >80% considering a 95% two-tailed confidence interval.

3.5 DISCUSSION

We have been exploring potential substances as adjunctive treatments to manage root caries lesions, aiming to either inhibit collagenolytic activity or protect the dentin matrix. Notably, promising outcomes have emerged from our investigations into a bio-based compound developed at the University of Brasilia, known as LDT11 (18) The multifunctionality and biocompatibility of LDT11, fostering the proliferation and differentiation of dental pulp stem cells while inhibiting bacterial collagenases, suggest its potential for protecting collagen in root caries (15). Nevertheless, further characterization is crucial to understand the specific impact of LDT11 on dentin. The results of the present study indicate a biomodification of the dental structure, possibly due to the cross-linking effect of anacardic acid LDT11 with dentin collagen, similar to the one caused by cardanol treatment. Cardanol success in cross-linking the dentin collagen was previously demonstrated (5,11), therefore this substance can be considered a positive control.

Some natural agents can perform dentin biomodification through their capacity to form intra- and intermolecular bonds with the collagen present in the organic matrix. Thus, one way to analyze the chemical interaction of bonds between molecules of these agents and the dentin substrate is through vibrational spectroscopy. This method provides molecular information about the structure and composition of chemical and biological samples by measuring vibrational energy levels associated with molecular/chemical bonds in a sample. In this study, the choice was made to utilize the FTIR/ATR technique, which represents a valuable tool for protein studies (19). When a sample is exposed to infrared light, the molecules within it absorb radiation at specific energies or frequencies, transitioning from a lower-energy ground state to a higher-energy excited state. This absorption process of infrared energy, during the transition of certain molecules or functional groups, leads to the formation of characteristic bands in their FTIR spectrum. These bands are determined by the intrinsic physicochemical properties of the corresponding molecule. There are two widely used FTIR sampling techniques, one of which is attenuated total reflection (ATR). In FTIR/ATR, the infrared beam traverses a crystal possessing a high refractive index, referred to as an internal reflection element placed beneath the sample. This wave extends past the crystal's surface, penetrating the sample in the form of an

evanescent wave, reaching a shallow depth (<2µm) before returning to the crystal. The infrared absorption by the sample attenuates the evanescent wave, resulting in the generation of an FTIR spectrum (20).

Considering the characteristics of this method, the choice of technique was based not only on its advantages in quickly obtaining spectra but also on the characteristics of the samples. The prepared specimens had an average thickness of 500µm, which would be limiting for the use of Raman spectroscopy or FTIR transmission techniques, for instance. In such cases, samples need to be around 10µm thick to avoid absorption saturation (20). In the case of FTIR/ATR, as the penetration occurs only in the first few micrometers of the surface, it was possible to conduct the analyses even with a thicker sample. A pilot study was carried out with Raman, however the high fluorescence of the tissue also compromised the analyses, favoring the choice of FTIR.

We opted for using a demineralization protocol before treating dentin samples with the LDT11, as previously described by Wang et al. (10). For the treatment time, we opted for a longer treatment as the diffusion of the cross-link in a surface of 500µm thickness uniformly would be difficult to achieve in a short treatment time (10). Therefore, considering the existing literature, a treatment time of 72h was chosen to ensure agent penetration throughout the surface (17). One can argue that this is not clinically relevant, however, pre-clinical *in vitro* studies are used to test the effect of the substance, and a translation to the clinics should be later tested in other relevant time points.

The concentration used for treatments was previously tested by Damé-Teixeira et al. (15), as 100µg/mL of LDT11 and LDT409 significantly inhibited the activity of *P. gingivalis* collagenase, one of the most relevant oral bacteria in proteolysis, achieving up to 96.8% inhibition, while still biocompatible. This data is crucial when considering dentin caries, i.e., the same concentration that is capable to inhibit a relevant collagenase can also biomodify the dentin.

Regarding the origin of the specimens, bovine samples were chosen due to their ease of acquisition and standardization. Despite some differences in the organic and inorganic composition between bovine and human dentin (21), similar biological functions have been reported among the constituent molecules of the two organic matrices (22). Furthermore, bovine dentin has proven to be a reliable substrate for *in vitro* studies on the progression and inhibition of root caries (23,24). A previous meta-

analysis of *in vitro* studies found no significant difference between different human and bovine substrates for adhesion outcomes, whether in enamel or dentin (25), suggesting its suitability for this study.

Regarding the resulting spectra, alterations in the peaks corresponding to the regions of Amide and CH2 bending were analyzed. The amide I band is mostly attributed to stretching vibrations of the peptide groups C=O. Meanwhile, amide II absorbance arises from NH bending vibrations coupled with C–N stretching vibrations. Lastly, Amide III is attributed to C–N stretching vibrations and N–H bending of amide bonds, as well as swing vibrations of CH2 groups in the glycine structure and in the side chains of proline (19). A previous study that evaluated the interaction of other types of plant-derived polyphenols with dentin observed a reduction in intensity at the peak around ~1400cm^{-1}, suggesting a dehydration effect induced by cross-linking with collagen caused by a hydrophobic effect of polyphenols (10,26–28). This finding would indicate cross-link formation and increased stability, supporting what was observed for the treatment with AA and cardanol tested here. The authors also reported that a reduction in intensity for the amide II peak (~1550cm^{-1}) and the formation of shoulders would be indicative of hydrogen bonding, also suggesting cross-link formation. In Figure 2b, similar findings can be observed, focusing on the formation of new peaks in this region, suggesting a change in the treated samples. Likewise, an increase in the amide I peak (~1630cm^{-1}) was also associated with this biomodification (Fig. 2b) (10). This latter peak was used as the basis for calculating the areas to determine the study's power as it accounts for a significant part of protein secondary structure analysis, which is the most common application of FTIR in these studies (19). In contrast, another study comparing cardanol with other polyphenol compounds like proanthocyanidins and lignins observed an increase in all peaks in Amide I, II, and III, along with the appearance of a shoulder at 1400cm^{-1} for all tested compounds (29). However, the treatment time for the samples was only 15s, and their thickness was not described in the study. Additionally, the changes observed in the region between 1000-1200 cm^{-1} may correspond to an aliphatic amine. Peaks of moderate intensity in this region have previously been associated with collagen hydrolysis in the case of degradation (30). On the other hand, in the treated groups, hydrogen bonding may be occurring between the hydroxyl groups of AA and the amine group of collagen. Considering that the negative control was in the same condition of demineralization and exposed to the same chances to degradation as the test and positive controls, we can speculate that

the second hypothesis is more plausible. Cardanol has also been demonstrated as a cross-linker in two other studies using another type of vibrational spectroscopy (Raman). In these cases, the authors observed an increase in the peak corresponding to amide III and the appearance of a shoulder in one of the cases. Raman, however, operates differently and can be considered a complementary method to infrared spectroscopy. To our knowledge, this study is the first to chemically test the interaction of AA (LDT11) with dentin, acting as a biomodifier, and to test cardanol using the ATR technique.

Our study presents some limitations. Regarding the sample thickness, studies have employed samples with a thickness around 10 µm. This choice facilitates uniform infiltration of cross-linking agents across the surface within a clinically feasible timeframe. Additionally, it enables thorough removal of non-adhered cross-links from the organic matrix, ensuring a more precise comparison among various compounds (10,20). However, we believe that the thickness of the samples did not affect the result as significant differences between test and negative control groups were found. Additionally, it is necessary to test the treatment of LDT11 within a timeframe compatible with clinical routine to validate its subsequent use. Nonetheless, the results presented here suggest that LDT11 seems to be a potential biomodifying agent for dentin due to its ability to interact with the collagen of the organic matrix. The next steps of this research should include studying the effect of this compound on the mechanical and biochemical properties of dentin, as well as the microbial adhesion characteristics in the biomodified dentin.

3.6 CONCLUSION

Saturated anacardic acid LDT11 may exhibit chemical interaction with dentin collagen similar to cardanol, suggesting its potential as a biomodifier cross-linking agent. Its capacity to controlling and treating carious lesions involving the dentin substrate could be explored in new experimental models. Studies evaluating mechanical and biodegradation outcomes are also needed for a better understanding of the results of this interaction.

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CAPÍTULO 4. DISCUSSÃO GERAL E CONCLUSÃO

4.1 DISCUSSÃO GERAL

O tratamento da cárie radicular se tornou desafiador na odontologia devido as limitações presentes nas técnicas restauradores nessa região. Assim, estratégias que visem melhorar a qualidade do substrato dentinário presente na raiz, assim como o controle não invasivo das lesões, se tornam de suma importância para a manutenção de dentes vitais e funcionais na população idosa. No presente trabalho, agentes que apresentam capacidade de promover *cross-links* no colágeno dentinário se mostraram uma alternativa viável a ser explorada em pesquisas futuras para o manejo de lesões cariosas radiculares, com enfoque para os diversos agentes naturais testados. Alguns destes se mostraram eficazes em controlar a profundidade de lesões cariosas produzidas *in vitro,* a exemplo do Naringin, Quercetina e das Proantocianidinas. Além disso, outras evidências também apontam para o papel desses agentes no controle da biodegradação, aqui mensurado indiretamente pela liberação de hidroxiprolina. Esses resultados demonstram o uso promissor desses *cross-linkers* para lesões radiculares, além de fornecerem suporte para a escolha de tratamentos em novos estudos.

Dentro dos agentes naturais, testamos o ácido anacárdico LDT11, que também se mostrou um potencial biomodificador dentinário. No nosso estudo, esse agente foi capaz de interagir com o colágeno dentinário, modificando suas características químicas significativamente quando comparado com um controle sem tratamento. Com isso, os próximos passos dessa pesquisa visam estudar desfechos mecânicos e desafios colagenolíticos para melhor investigar o efeito do tratamento da dentina radicular com essa substância.

4.2 CONCLUSÃO

Os agentes *cross-linkers* atuam positivamente no controle de lesões cariosas dentinárias *in vitro*, com destaque para agentes naturais como naringin, quercetina e proantocianidinas e agentes sintéticos como o Glutaraldeído. São necessários estudos clínicos para avaliar a real eficácia e implicação desses compostos sobre a dentina radicular humana, visto que o ambiente bucal é complexo por sua composição

salivar, microbiana e de pH. Nesse cenário, o LDT11 também pode emergir como um promissor agente no controle das lesões e longevidade de tratamentos se comprovada sua efetividade na melhora das propriedades mecânicas da dentina e no aumento da resistência a biodegradação.

CAPÍTULO 5. PRESS RELEASE

O aumento significativo da população idosa trouxe desafios aos sistemas de cuidados para garantir uma qualidade de vida sustentável para essa parcela cada vez mais expressiva da população. Um exemplo disso é a diminuição da perda de dentes nas faixas etárias mais avançadas, aumentando a prevalência de alguns problemas como a cárie radicular. As lesões de cárie se apresentam na raiz do dente, em porções próximas à margem gengival. De forma geral, elas se desenvolvem quando há uma elevada frequência de consumo de carboidratos fermentáveis, somados a uma higiene bucal deficiente e ao fato dos tecidos que cobrem a raiz do dente terem um menor conteúdo mineral e maior conteúdo orgânico. Os tratamentos para cárie de raiz são relativamente diferentes da cárie da coroa dentária. Existem limitações atreladas a esses tratamentos, que variam desde custo até questões biológicas e estéticas. Assim, explorar o uso de alternativas renováveis para o desenvolvimento de estratégias que visem preservar os tecidos que cobrem a raiz devem ser consideradas de extrema importância, podendo representar novos tratamentos no futuro. Um exemplo disso são agentes capazes de interagir com um dos principais componentes dessa matriz orgânica (o colágeno) e melhorar as propriedades do tecido, chamados de *cross-linkers*. Alguns estudos sugerem que essa interação pode promover uma maior resistência dentária aos agravos como a cárie, além de possibilitar uma maior longevidade aos tratamentos restauradores disponíveis. Neste trabalho, estudos com o objetivo de analisar o efeito do tratamento com esses agentes em situações que simularam o desenvolvimento de lesões cariosas radiculares foram sistematicamente analisados. Nesse contexto, considerando a importância da exploração de fontes renováveis, este estudo também testou um agente natural derivado da castanha de caju, desenvolvido por pesquisadores da Universidade de Brasília, quanto ao seu possível efeito como um *cross-linker* e potencial adjuvante para tratamentos futuros de cárie radicular.