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CENTRO DE CIÊNCIAS DA SAÚDE
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CARACTERIZAÇÃO DAS INTERAÇÕES QUÍMICAS E ANÁLISE DA INTERFACE ENTRE OS MONÔMEROS FUNCIONAIS 10-METACRILOILOXI-DECIL-DI-HIDROGENO-FOSFATO E N-METACRILOIL GLICINA DE ADESIVOS SELF-ETCH E A DENTINA EM LESÕES CERVICAIS NÃO CARIOSAS

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Tese apresentada ao Programa de Pós Graduação em Odontologia Integrada da Universidade Estadual de Maringá para obtenção do título de Doutora.

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Orientadora: Profª. Drª. Renata Corrêa Pascotto

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Oliveira BMB. Caracterização das interações químicas e análise da interface entre os monômeros funcionais 10-metacriloiloxi-decil-di-hidrogeno-fosfato e n-metacriloil glicina de adesivos self-etch e a dentina em lesões cervicais não cariosas [tese]. Maringá: Universidade Estadual de Maringá; 2018

RESUMO

Objetivo: Caracterizar as interações químicas e analisar a interface entre os monômeros funcionais 10-Methacryloyloxydecyl dihydrogen phosphate (10-MDP) e N-methacryloyl glycine (Methacrylamide) de adesivos *self-etch* e a dentina em lesões cervicais não cariosas (LCNCs) em comparação à dentina esclerótica em defeitos artificiais preparados (DAs). **Métodos:** Foram utilizados 20 dentes humanos com LCNCs. Para o controle, foram confeccionados DAs (classe V) na face lingual hígida dos mesmos dentes similares às LCNCs. Os espécimes foram divididos em dois grupos (n=10) de acordo com o monômero funcional do sistema adesivo utilizado: 10-MDP (G1) e Metacrilamida (G2). Os DAs e LCNCs foram caracterizados quanto à composição mineral (CM) e grau de desmineralização (GD) usando a espectroscopia micro-Raman; as interações químicas (IQ) foram observadas através da Espectroscopia Fotoacústica no infravermelho; e a morfologia da interface foi avaliada por microscopia eletrônica de varredura (MEV) e microscopia de luz (ML). Os valores obtidos da CM, GD e IQ foram submetidos aos testes Shapiro Wilk e T de Student ($p<0,05$). **Resultados:** As LCNCs apresentaram-se hipermineralizadas em comparação aos DAs ($p<0,05$). No G1, as IQ, o GD nos primeiros 2 µm e as projeções do adesivo nas LCNC e DA foram semelhantes. Uma fina camada de colágeno exposto foi observada nos DAs, enquanto que nas LCNCs o colágeno foi pouco evidenciado. Para o G2, as IQ não puderam ser identificadas, porém observou-se alterações nos componentes minerais. O GD em DAs e LCNCs foi estatisticamente semelhante, enquanto que as imagens MEV evidenciaram falha da adesão na interface das LCNCs. O GD e exposição do colágeno nos DAs e LCNCs foram mais pronunciados que no G1. **Conclusão:** Os resultados sugerem que o G1 pode ser aplicado diretamente na superfície esclerótica das LCNCs. Em contraste, para o G2, deve ser realizado o preparo prévio da cavidade nas LCNCs para melhorar a retenção micromecânica com a dentina.

Palavras-chave: Adesão, Dentina, Análise Espectral Raman, Espectroscopia Infravermelho Transformada de Fourier, Microscopia Eletrônica de Varredura.

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ABSTRACT

Objective: To characterize the chemical interactions and analyze the interface of adhesive systems containing 10-Methacryloyloxydecyl dihydrogen phosphate (10-MDP) and N-methacryloyl glycine (methacrylamide) functional monomers with the dentin in non-carious cervical lesions (NCCLs) in comparison with artificial defects (ADs). **Methods:** Twenty human teeth with natural NCCLs on the buccal surface were used. Class V cavities, similar to NCCLs, were created on the lingual surface to serve as controls. Teeth were randomly allocated to two groups according to the functional monomer in the adhesive (N=10): G1, 10-MDP; and G2, methacrylamide. NCCLs and ADs were characterized for their mineral composition (MC) and degree of demineralization (DD) using micro-Raman spectroscopy; adhesive/dentin chemical interactions (CI) were assessed with infrared photoacoustic spectroscopy; and interface morphology was evaluated with scanning electron and light microscopy. MC, CI and DD data were submitted to Shapiro Wilk and Student's t tests ($p<0.05$). **Results:** In comparison to ADs, dentin in NCCLs was hypermineralized ($p<0.05$). In G1, CI and DD in the first 2 µm, and adhesive projections in NCCLs and ADs interface were similar. Additionally, a thin layer of dentin collagen was observed in ADs, while it was hardly present in NCCLs. In G2, although CI could not be identified, changes in the mineral components were observed. DD in ADs and NCCLs were statistically similar, while SEM showed a lack of adhesion at NCCLs interface. DD and collagen exposure in ADs and NCCLs were more pronounced than in G1. **Conclusions:** Results suggest that the G1 adhesive could be applied directly on the superficial sclerotic layer in NCCLs. In contrast, previous cavity preparation should be conducted to improve the micromechanical interaction of G2 with the dentin.

Key words: Adhesion; Dentin; Raman Spectral Analysis; Fourier Transform Infrared Spectroscopy; Scanning Electron Microscopy.

SUMÁRIO

1 CAPÍTULO DE CONTEXTUALIZAÇÃO

1.1 LESÕES CERVICAIS NÃO CARIOSAS

As lesões cervicais não cariosas (LCNCs) foram primeiramente descritas na literatura como "defeitos angulares" por Zsigmondy (1894). Posteriormente, Miller (1907) descreveu as LCNCs como "perda de tecido dentário", caracterizada pela perda lenta e progressiva de estruturas dentárias resultando em um defeito com superfície lisa e em forma de cunha na região da junção cemento-esmalte.

O sucesso do diagnóstico e planejamento do tratamento das LCNCs requer uma observação minuciosa, completa e cuidadosa avaliação do histórico do paciente. (GRIPPO; SIMRING; SCHEREINER, 2004). Por constituírem um grupo de lesões de complexidade diagnóstica na prática clínica odontológica devido a sua etiologia multifatorial (LEVITCH et al., 1994; PEREZ et al., 2012), Grippo, Simring e Schereiner (2004), propuseram uma classificação atualizada e revisada para as lesões de superfície dos dentes, com o objetivo de padronizar e esclarecer essas lesões para os clínicos.

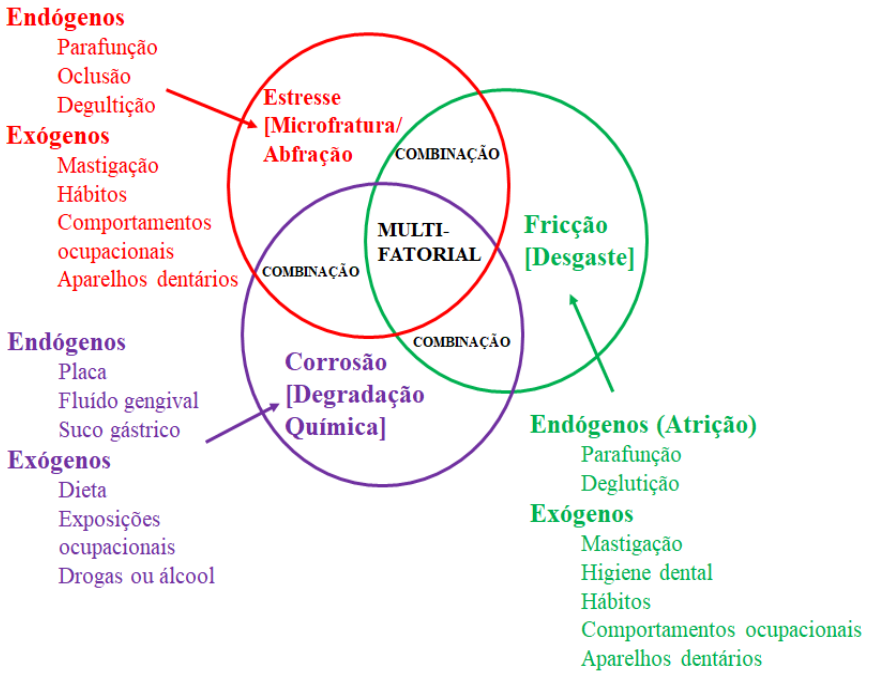
Os fenômenos atrição, abrasão, corrosão e abfração estão relacionados à formação das LCNCs. A atrição é o movimento de fricção das superfícies oclusais e incisais durante a deglutição e apertamento dos dentes (LEHMAN; MEYERS, 1966; SHORE, 1976; STRAUB 1960; KYDD, 1957). O ato de friccionar agentes externos contra os dentes é chamado de "abrasão". Esse tipo de lesão pode ocorrer como resultado de uma escovação exagerada, uso inapropriado do fio dental, ou em função de hábitos como mascar tabaco, morder objetos duros como canetas ou cachimbos, ou roer unhas (GRIPPO; SIMRING; SCHEREINER, 2004).

Os autores também propõem que o termo "erosão" deveria ser substituído por "corrosão" para denominar a dissolução química do dente. Essa corrosão pode ser ocasionada por fatores endógenos como por exemplo a bulimia, ou exógenos como a ingestão de alimentos e bebidas ácidas. Por fim, a abfração é descrita como uma perda microestrutural do dente em áreas de concentração de estresse.

Mais comumente, ocorre na região cervical do dente, onde a força de flexão pode levar a ruptura da camada extremamente fina de esmalte, assim como as microfraturas do cemento e dentina (MCCOY, 1893; LEE; EAKLE, 1984).

No entanto, a perda da estrutura dentária está frequentemente relacionada à ocorrência associada, alternada ou sequenciada de mais de dois desses mecanismos, ilustrados na figura 1.

Figura 1. Esquema dos mecanismos patógenos, mostrando alguns dos fatores etiológicos das LCNCs.



GRIPPO, J. O.; SIMRING, M.; SCHREINER, S. Attrition, abrasion, corrosion and abfraction revisited: a new perspective on tooth surface lesions. J Am Dent Assoc, v. 135, n. 8, p. 1109-18, 2004.

1.2 DENTINA ESCLERÓTICA

As características químicas e estruturais do substrato dental têm forte influência nos mecanismos de adesão e consequente resultado na resistência adesiva dos sistemas adesivos (PERDIGÃO, 2010). Assim, adesão à dentina esclerótica é clinicamente relevante, uma vez que se trata de um substrato com alterações patológicas, resultando em uma obliteração parcial ou completa dos

túbulos dentinários (GWINNETT; JENDRESEN, 1978; HARNIRATTISAI et al., 1993; VAN MEERBEEK et al., 1994; YOSHIYAMA et al., 1996).

A composição da dentina na superfície das LCNCs pode ser bem diferente da composição da dentina saudável (ROBERSON et al., 2006). A camada hipermineralizada da dentina esclerótica de LCNCs naturais tem aproximadamente 10-20 µm de espessura (TAY; PASHLEY, 2004), com teor de fosfato cerca de 2 vezes maior do que a dentina normal (XU et al., 2009). Um estudo utilizando a microscopia Raman (XU et al., 2009) demonstrou que a dentina esclerótica das LCNCs naturais apresenta-se hipermineralizada e com maior cristalinidade, quando comparada à dentina saudável. Além disso, a composição mineral das LCNCs apresenta-se com um teor muito baixo de carbonato em cerca de 2%, enquanto que na dentina saudável esse teor chega ser próximo de 8%. Em relação à composição orgânica, existe uma notável redução nas taxas de colágeno na dentina esclerótica das LCNCs. Assim, a proporção mineral/orgânica das LCNCs é 2 à 3 vezes maior que a dentina saudável (XU et al., 2009; MIXSON et al., 1995).

1.3 SISTEMAS ADESIVOS AUTOCONDICIONANTES

A penetração do sistema adesivo na dentina desmineralizada foi observada e denominada como "camada híbrida" por Nakabayashi (1982). O mecanismo fundamental da adesão ao esmalte e dentina é baseado no processo de troca, onde os minerais são removidos dos tecidos dentários duros e substituídos pelos monômeros resinosos, que se tornam micromecanicamente interligados. Esse processo é conhecido como "hibridização".

A principal característica dos sistemas adesivos autocondicionantes é a incorporação de monômeros ácidos em sua formulação (TAY; PASHLEY, 2001). O mecanismo de ação ocorre de forma que a dentina e o esmalte sofram desmineralização, devido ao baixo pH do sistema, e ao mesmo tempo infiltrem no tecido dentário, atuando simultaneamente como condicionador e primer. Assim, a

técnica permite uma redução do tempo de trabalho clínico, além de tornar baixa ou inexistente a possibilidade de discrepância entre a profundidade de condicionamento e de infiltração dos monômeros (VAN MEERBEEK et al., 2003).

Os monômeros ácidos em adesivos/primers autocondicionantes são ésteres originados da reação de um álcool bivalente com ácido metacrílico e derivados do ácido fosfórico/carboxílico. Cada adesivo autocondicionante contém um monômero específico, que na grande maioria, determina a sua performance adesiva através de uma interação química com o substrato dentinário (YOSHIDA et al., 2000, 2004), suplementando a retenção micromecânica. Diversos monômeros funcionais estão disponíveis no mercado com diferentes formas de adesão química ao substrato dentário, como por exemplo o 10-metacriloiloxi-decil-di-hidrogeno-fosfato (10-MDP) capaz de se ligar aos componentes inorgânicos, e n-metacriloil glicina (metacrilamida) que interage quimicamente com os compostos orgânicos.

O mecanismo de adesão do 10-MDP é baseado na interação química primária do monômero com a hidroxiapatita que permanece ao redor de colágeno parcialmente exposto (YOSHIDA et al., 2004; FU et al., 2005), mantendo a hidroxiapatita como um abrigo natural ao redor do colágeno. Essa interação forma sais estáveis de Cálcio-MDP, dando origem à chamada "nanocamada" composta por duas moléculas de 10-MDP com os grupos metacrilatos voltados para si e o grupo hidrogênio fosfato afastados um do outro, onde o metacrilato se liga aos íons de cálcio e o grupo hidrogênio fosfato à dentina (YOSHIHARA et al., 2010). A literatura tem demonstrado que a adesão química promovida pelo 10-MDP é mais efetiva e estável à degradação hidrolítica quando comparada a outros monômeros funcionais, tais como 4-metacriloiloxietil ácido trimelítico (4-META) e o 2-metacriloxietil fenilhidrogeno-fosfato (phenyl-P) (YOSHIDA et al., 2004; YOSHIHARA et al., 2010). Essas ligações podem explicar a alta estabilidade de adesão dos adesivos à base de MDP, que vem sendo provado

tanto em estudos laboratoriais quando em estudos clínicos (YOSHIDA et al., 2012; YOSHIHARA et al., 2010, 2011; YOSHIDA, INOUE, 2012; PEUMANS et al., 2015; PENA et al., 2016; HASS et al., 2017; OLIVEIRA et al., 2017)

O monômero funcional metacrilamida foi desenvolvido com o objetivo de obter um primer autocondicionante mais efetivo, com estabilidade hidrolítica mais duradoura (NISHIYAMA et al., 2004). A hidrólise de monômeros em adesivos dentários autocondicionantes pode ser evitada usando monômeros sem porções de ésteres, como por exemplo, os derivados de acrilamida (SALZ, BOCK, 2010). Nishiyama et al. (2000), determinaram uma forte interação de uma série de primers à base de metacrilamida com o colágeno da dentina. A amida ou o ácido carboxílico do monômero são os responsáveis por interagir com a macromolécula do colágeno da dentina, demonstrando uma forte correlação entre a força de adesão com a dentina condicionada com o primer contendo metacrilamida, através de ligações do tipo pontes de hidrogênio (NISHIYAMA et al., 1998). Possivelmente devido à essas ligações, o monômero funcional metacrilamida vem apresentando bons resultados à longo prazo em dentina saudável quando comparada à sistemas adesivos à base de metacrilato (SALZ; BOCK, 2010, PENA et al, 2016).

1.4 ADESÃO À DENTINA ESCLERÓTICA

A necessidade de restauração das LCNCs naturais ocorre devido fatores como hipersensibilidade, comprometimento estético, prevenção do acúmulo de alimentos, necessidade de retenção de próteses ou simplesmente para impedir a progressão da lesão (LEVITCH et al., 1994, SPRANGER, 1995). No entanto, a manutenção das restaurações adesivas em LCNCs ainda é um desafio. A longevidade da restauração é altamente dependente da qualidade da camada híbrida formada (YOSHIDA; INOUE, 2012).

Tay et al. (2000), listaram quatro fatores que podem influenciar a diminuição nas resistência de união à dentina esclerótica das LCNCs naturais: 1) a presença de uma matriz microbiana hibridizada com bactérias aderidas; 2) incapacidade do adesivo/primer autocondicionante em dissolver e penetrar a espessa superfície hipermineralizada; 3) presença de uma camada de colágeno desnaturado na base da zona hipermineralizada; 4) presença de detritos escleróticos residuais que obliteram os túbulos e impedem a formação de tags. Como estratégia para melhorar esses fatores, alguns autores defendem a necessidade de uma asperização e/ou um condicionamento ácido previamente aos procedimentos restauradores em LCNCs naturais (LOPES et al., 2003, 2004; VAN DIJKEN, 2000, 2004).

Kwong et al. (2002), avaliaram *in vitro* as forças de adesão de um sistema adesivo autocondicionante em dentina esclerótica, com e sem a presença do condicionamento com ácido fosfórico. Apesar de ter sido observado que a remoção de detritos foi incompleta em ambos os grupos, os autores sugerem que a remoção da camada esclerótica da dentina e/ou condicionamento com ácidos mais fortes pode ser benéfica para obter uma adesão mais efetiva à dentina esclerótica. Camargo et al. (2008), avaliaram a influência do tratamento da superfície e o tempo do condicionamento ácido na força de microtração em dentina esclerótica de dentes bovinos, sugerindo que o tratamento da superfície da dentina esclerótica influencia significativamente na força de adesão de um sistema adesivo *etch and rinse.*

Camargo et al. (2008), defendem que o tratamento da superfície com uma ponta diamantada ou pasta é capaz de melhorar a adesão à dentina esclerótica bovina, alcançando valores próximos à dentina saudável. No entanto, a infiltração do adesivo dentro dos túbulos dentinários pode ser prejudicada com a remoção da camada hipermineralizada antes do procedimento adesivo em LCNCs devido a presença da *smear layer* causada pelo preparo com pontas diamantadas (TAY; PASHLEY, 2004). Luque-Martinez et al. (2013) testaram a performance adesiva de sistemas autocondicionantes na dentina

esclerótica de dentes bovinos e demonstraram que os resultados da superfície não preparada foram superiores quando comparada à superfície preparada com pontas diamantadas. Além disso, Oliveira et al. (2017) sugerem que a remoção da camada hipermineralizada não é necessária, uma vez que pode diminuir o potencial de adesão química de um sistema adesivo autocondicionante contendo o monômero funcional 10-MDP.

Apesar dos baixos resultados na força de adesão obtida nas LCNCs naturais, os estudos clínicos não têm corroborado com os resultados *in vitro*. Um estudo clínico (VAN DIJKEN, 2005) encontrou que as diferenças entre as dentinas escleróticas e não escleróticas com ou sem a asperização da superfície não são significantes. Recentemente, Loguercio et al. (2018), testando a influência da asperização da superfície dentária no comportamento de um sistema adesivo aplicado no modo autocondicionante e *etch-and-rinse*, demonstrou que o desgaste prévio à aplicação do adesivo não afetou o comportamento clínico das restaurações em LCNCs naturais.

O número reduzido de estudos clínicos que comparam a influência da asperização da dentina no grau de retenção de restaurações em LCNC submetidas a diferentes tratamentos de superfície impede que revisões sistemáticas sejam realizadas (SANTOS et al., 2014) e os estudos clínicos apresentam resultados controversos (VAN DIJKEN, 2000, 2004, 2005, 2010)

Essa controvérsia da literatura pode ser explicada pela grande variedade de sistemas adesivos utilizados e disponíveis no mercado, que apresentam diferentes formas de interação com o substrato dentinário. Assim, destaca-se a necessidade de trabalhos que avaliem o desempenho de diferentes sistemas adesivos com o objetivo de indicar a melhor estratégia clínica para o procedimento restaurador em LCNCs naturais especificamente de acordo com o adesivo de escolha do cirurgião dentista.

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2 ARTIGO

2.1 INTRODUCTION

Non-carious cervical lesions (NCCLs) form a group of lesions of difficult characterization in the dental practice because of their multifactorial etiology[1,2]. NCCLs result from the slow and progressive loss of mineralized dental structure caused by the association of different phenomena such as erosion, abrasion and abfraction[3].

Laboratory studies have demonstrated that adhesion to dentin affected by NCCLs may lead to adhesive failures and compromise the longevity of restorations[4-6]. The mean reason for this phenomenon is the molecular/chemical structural changes that occurs at the interface, which result in less favorable adhesion to the substrate[7]. Because the dentin in NCCLs is sclerotic, the formation of a hybrid layer in the dentin/adhesive interface is compromised by irregular primer diffusion and reduced adhesive infiltration[8].

Although the main adhesive mechanism to the dental substrate is based on the micromechanical retention resulting from the formation of a hybrid layer and resin tags, attention has recently been focused on the benefit of additional chemical interactions between the functional monomers present in adhesive systems and the components of the dental substrate[9-12]. Chemical interactions can occur through ionic bonds established by acid monomers such as 10-Methacryloyloxydecyl dihydrogen phosphate (10-MDP) that react with the hydroxyapatite, forming monomer-Ca salts that are stable to degradation[9,10]. On the other hand, the interaction of adhesive systems containing methacrylamide can result in bonds with dentin collagen fibrils through the additional reactive groups present in monomeric acids. Since dentin collagen contains reactive groups such as amino or hydroxyl, the aldehyde or anhydride groups of the adhesive system can establish covalent bonds with collagen fibrils[13].

The aim of this study was to analyze the chemical interactions of two self-etching adhesive systems, one containing the 10-MDP and the other methacrylamide functional monomers with the

dentin in NCCLs and artificial defects (ADs), in order to evaluate the requirement for additional substrate preparation prior to adhesive procedures.

2.2 MATERIALS AND METHODS

This *in vitro* study was approved by the Local Ethics Committee (CAAE: 47305015.7.0000.0104). Teeth with natural non-carious cervical lesions extracted for periodontal and/or orthodontic reasons were used. All teeth presented grade 4 of dentin sclerosis, according to the scale modified by Ritter et al [14]. Grade 4 is attributed to NCCLs with significant presence of sclerosis, in which the dentin is dark-yellow or brownish with a petrified appearance, significant translucency or evident transparency.

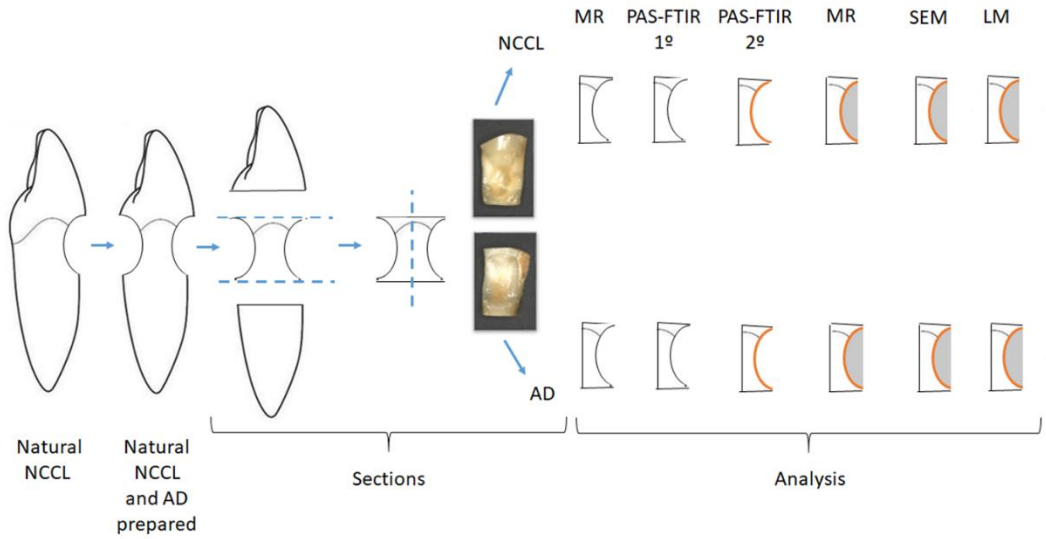
2.2.1 Specimen preparation

A total of 20 teeth with natural NCCLs located in the cervical region of the buccal surface were used in the experiment. They were randomly divided into two groups (N=10); G1, to be restored with an adhesive system containing 10-MDP; and G2, to be restored with an adhesive system containing methacrylamide. After extraction, the teeth were cleaned with sterile gauze and saline solution. Any remaining periodontal tissues were removed with the aid of periodontal curettes. After cleaning, the teeth were stored in saline at 4°C.

Artificial defects (ADs) in the shape of Class V cavities were created in the lingual surface of the same tooth with a CVDentus (C1 1.0 x 4.0 mm) cylindrical diamond tip coupled to an ultrasound device (CVDent 1000, CVDVale, São Carlos, Brazil) under continuous water cooling. ADs were prepared in sound dentin with dimensions and shape approximately the same as those of the corresponding NCCL, and served as control[15].

Then, dental specimens containing the NCCLs and DAs were obtained from each tooth. The teeth were sectioned with a diamond disk at low speed, under water cooling in the following sequence: first, just above the lesion to remove the crown, and then, just below the lesion to remove the remaining 2/3 apical root. Finally, a section was made along the long axis of the tooth to separate specimens containing NCCLs from ADs. Once the dental specimens were obtained, they were ready to be employed in the sequence of analyzes described below (Figure 1).

Figure 1. Diagram illustrating the preparation of the specimens and the analysis sequence.



Abbreviations: NCCL: Non carious cervical lesions; AD: Artificial defect.; MR: Micro-Raman spectroscopy; PAS-FTIR: Fourier transform infrared photoacoustic spectroscopy; SEM: Scanning electron microscopy; LM: Light microscopy.

2.2.2 Adhesive system application

The composition of the adhesive systems used in this study and their recommended mode of application are described in Table 1. Light-curing unit used was Translux Power Blue (Heraeus Kulzer,

Hanau, Germany) at 1000 mW/cm² of irradiance. The light-curing time for each adhesive systems was according to the manufacturer's instructions.

Table 1. Composition and mode of application of the adhesive systems used in the experimente.

Group	System	Composition	Application mode
G1	Clearfill SE Bond 2 (Kuraray Noritake Dental Inc., Tokyo, Japan)	*Primer:* MDP HEMA Dimethacrylate Canphorquinone Water *Adhesive:* MDP Bis-GMA HEMA Dimethacrylate Canphorquinone Initiators Accelerators Silanizaded coloidal silica (pH 2)	Apply primer for 20 seconds Gently air dry for 5 seconds Apply the adhesive Photopolymerize for 10 seconds
G2	Xeno V+ (Dentsply Sirona, York, USA)	Bifunctional acrylate Acrylate Acid Esters of phosphoric acid Water Tertiary butanol Initiators Stabilizing (pH 1.38)	Actively apply the adhesive for 20 seconds Gently air dry for 5 seconds Photopolymerize for 10 seconds

Abbreviations: MDP: 10-methacryloxydecyl dihydrogen phosphate; Bis-GMA: Bisphenol-A Bismethacrylate; HEMA: 2-hydroxyethylmethacrylate.

2.2.3 Dentin mineral composition

Mineral composition analysis of the dentin in ADs (control) and NCCLs was performed with micro-Raman (MR) spectroscopy. The analysis was conducted with a micro-Raman spectrometer (Bruker Optik GmbH, Ettlingen, Germany), equipped with a SENTERRA confocal microscope. The equipment works based on infrared light scattering, i.e., the source of light irradiation excites the studied matter. In this interaction, the Raman effect is obtained, which allows to study vibrations at a molecular level.

Specimens had their spectra measured at 3 different points on the dentin surface. All measurements were collected at a resolution of 4 cm^{-1} in the spectral region between 3500-450 cm^{-1}. Each spectrum was obtained from an average of 60 readings in order to decrease the signal-to-noise ratio, with a laser wavelength of 785 nm, power of 100 mW and objective gain of 100 x. In addition to the high number of readings, signal pattern was improved by decreasing detector temperature to -84°C. In all readings, the surface area selected for measurement, the support mirror used and manual focusing followed by autofocusing were performed following the same standardized procedures.

All spectra were placed on the same baseline and normalized with the aid of an Opus spectroscopy software (Bruker Optics, Ettlingen, Germany). Additionally, the Origin Software (OriginPro 8 Corporation, Northampton, MA, USA) was used to obtain numerical quantifications of MR spectra by integrating each curve of the band at 961 cm^{-1} (phosphate) to calculate the respective area and mean of the 3 distinct points measured in the sound dentin of AD specimens and in the sclerotic dentin of natural NCCLs.

2.2.4 Adhesive/dentin chemical interactions

Specimens had their spectra measured with Fourier transform infrared photoacoustic

spectroscopy (FTIR-PAS) before and after being submitted to the adhesive treatment. The technique provides the optical absorption bands of the sample, which are considered as the fingerprint of specific molecules. The information on the chemical modifications within the specimen are expressed by means of changes and/or emergence of new peaks.

The experiments were performed with a nicolet Spectrometer (MTEC Photoacoustics, Ames, USA) equipped with a MTEC 200 photoacoustic cell model. This equipment allows monitoring the absorption of the substance of interest at specific specimen depths, providing the distribution profile of the substances along the thickness studied. All spectra were collected at a resolution of 8 cm^{-1}, with scanning speed of 0.5 cm/sec. The spectral region of the measurements lies in the energy range between 4000 and 400 cm^{-1}. After inserting the specimen, the photoacoustic cell was filled with helium to minimize interference on the optical absorption spectra of oxygen and water molecules present in the air and on the surface of the specimen.

In order to determine the depth of the analysis in the present experimental condition, FTIR-PAS test measurement depth was defined by the thermal diffusion length. The thermal diffusivity of the adhesive was measured by the thermal lens technique[16] as described by Oliveira et al[15]. The inspection depth of the technique for the readouts taken in this study was about 6 μm for G1 and 4 μm for G2. Since the adhesive film depth can range from 2-3 μm[17,18], it may be stated that the technique was able of reading not only the hybrid region, but also the dentin under the adhesive.

To evaluate the spectrum of the adhesive system, a disc of pure adhesive was prepared applying 1 mL of the material on a histological glass slide. After photoactivation for 20 seconds, the disc was inserted into the measuring equipment. This is an important step to differentiate the composition of the adhesive from that of the dental structure and to verify the differences between photoacoustic absorption peaks of the adhesive and the dentin.

Data collected were transferred to the Origin Software. Graphs were generated for each tooth individually, from which the average spectrum of ADs and natural NCCLs dentin specimens were calculated. Bands identified as chemical interactions between the dentin and the adhesive system were selected, and the respective intensities in the ADs and natural NCCLs before and after the application of the adhesive were compared.

2.2.5 Degree of Demineralization (DD)

After FTIR-PAS analysis, all cavities were filled with composite resin (Filtek Z250-3M ESPE, St Paul, MN, USA) and subsequently sectioned for MR scanning analysis. Dental specimens were cut longitudinally with a sectioning machine (Buehler Isomet 1000 Precision Saw, Lake Bluff, USA) using a diamond disc (Buehler, Diamond Wafering Blade, Series 15LC, Arbon Size ½, 12.7mm, Lake Bluff, USA) under water cooling.

MR spectra were obtained by scanning the inner region of the composite resin toward the deeper layers of the dentin in NCCLs and ADs. All spectra were obtained under the same conditions as previously described for mineral composition analysis. Raman spectra were acquired at 1 μm intervals in 20 μm long lines at a distance of 10 μm between lines. Scans were individually analyzed, and spectra reading presenting any flaws, possibly due to the presence of bubbles, were excluded from the analysis. All spectra were placed on the same baseline and normalized with the aid of the Opus spectroscopy software, and analyses were conducted with the Origin Software. DD, as a function of location, was determined at the bands at 961 cm^{-1} (PO_4 of the dentin) in relation to the band at 1458 cm^{-1} (CH_2), according to the equation:

$$DD = \left(1 - \frac{961cm^{-1}\ intensity\ interface / 1458cm^{-1}\ intensity\ interface}{961cm^{-1} intensity\ dentin / 1458cm^{-1} intensity\ dentin}\right) \text{ x } 100\%$$

2.2.6 Interface morphology

The analysis of the adhesive system/dentin substrate interface morphology was performed with scanning electron microscopy (SEM). Sample treatment was performed according to the protocol suggested by Monticelli et al[19]. After MR analyzes, samples were embedded in acrylic resin and polished with an increasing wet sandpaper grit sequence (220, 400, 600, 1200, 1800, 2000). Then, specimens were submitted to demineralization with phosphoric acid 37% for 10 sec, deproteinization with sodium hypochlorite 2% for 1 min, dehydration with 100% alcohol for 2 min in an ultrasonic vat (Bio-Free-Gnatus Ribeirão Preto, Brazil), and drying with air jets. Specimens were sputter-coating with gold and evaluated with SEM (Shimadzu, model SS-550 Superscan, Kyoto, Japan) with a magnification of 1000 ×.

2.2.7 Collagen fiber exposure

To examine collagen fiber exposure at the adhesive system/dentin interface, four additional teeth were prepared according to the protocol described above, up to the application of the adhesive system, and histologically analyzed under light optical microscopy.

Immediately after the application of the adhesive system, samples were fixed in Karnovsky's solution (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.3) for 48 hours and washed in running water for 4 hours. The samples were placed in a decalcifying solution (20% sodium citrate and 50% formic acid) for 25 days. After that, specimens were washed in running water for 4 hours, dehydrated in an increasing alcohol sequence, and embedded in paraffin. Histological sections with 6 μm thick were serially made using a tungsten carbide blade coupled to a microtome (Leica RM2265, Leica Microsystems, Wetzlar, Germany).

Goldner-modified Masson trichrome[19-21] was used to stain specimens. Before staining, samples

were prepared according to the protocol proposed by Wang and Spencer[20]. Sections were first stained with Weigert's iron hematoxylin solution for 5 min, immersed in Masson solution for 10 min and rinsed twice in 0.2% acetic acid solution. Afterwards, they were kept in mordant solution for 5 min and washed with 1% acetic acid again. Finally, they were stained with a light green solution for 5 min and rinsed twice with 0.2% acetic acid solution. For sample dehydration, sections were immersed in 96% and 100% alcohol, twice for 1 minute. Specimens were immersed twice in xylol for 3 and 5 min and mounted within histological slides. Sections were examined and photographed at a magnification of 100x under a light optical microscope (Olympus BX41, Tokyo, Japan).

2.2.8 Statistical analysis

Statistical analysis was performed using the R i386 3.0.2 software (R statistical software, R Foundation for Statistical Computing, Vienna, Austria). The areas of the band at 961 cm^{-1} (PO_4) for the phosphate present in the dentin, the intensities related to the chemical interactions found with FTIR-PAS, and DD were submitted to Shapiro Wilk and Student's t test ($p<0.05$).

2.3 RESULTS

2.3.1 Adhesive/dentin chemical interactions

Figure 2A shows the spectrum of the pure adhesive system containing the 10-MDP monomer (G1), with its organic and inorganic functional groups: OH (3445 cm^{-1}), CH_2 (2940 cm^{-1}), methacrylate monomer [carbonyl C=O (1720 cm^{-1}), CH_2CH_3 (1457 cm^{-1})], BIS-GMA [C=C (1638 cm^{-1}), C-O-C (1140 cm^{-1}), $(CH_3)_2$-C (1300 cm^{-1}), C_6H_4 (840 cm^{-1})], and load [SiO_2 (1105 cm^{-1})]. Figure 2B shows the dentin spectra of the natural NCCLs and ADs. The bands associated with mineral and organic

composition were observed at the following wavenumber: phosphate (1179 cm^{-1}), amide I (1650 cm^{-1}), amide II (1550 cm^{-1}), amide III (1240 cm^{-1}).

Figure 2. Photoacoustic absorption spectra: **(A)** pure adhesive system contaning 10-MDP (G1); and **(B)** dentin in ADs and natural NCCLs.

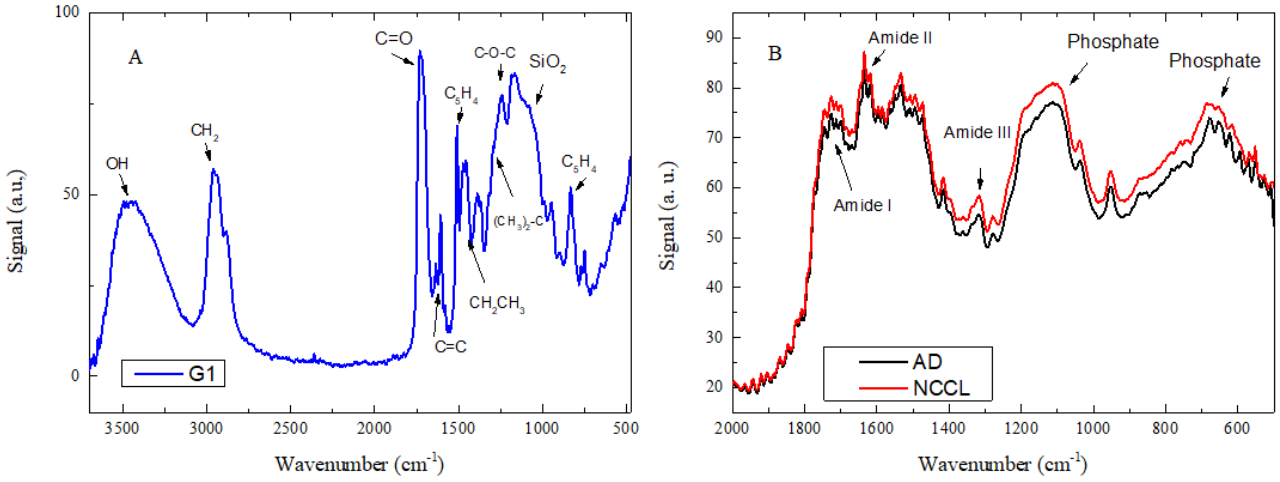


Figure 3A and 3B illustrate NCCL spectra before and after application of the adhesive system containing the 10-MDP monomer (G1). Circles and bars indicate changes in spectra, suggesting chemical interactions between the dentin and the adhesive system. Table 2 shows the bands and functional groups identified as possible chemical interactions between the dentin and the adhesive system containing 10-MDP monomer and the means and standard deviations of the intensities obtained from ADs and natural NCCLs characterizing chemical interactions. Although no statistically significant differences were observed between the two groups, mean values for natural NCCLs in all band intensities were numerically higher than those of ADs.

Figure 3. Photoacoustic absorption spectra obtained from the adhesive and the NCCL before and after application of the adhesive system containing the 10-MDP monomer (G1). **(A)** Spectral region 2100 to 550 cm^{-1}, and **(B)** Spectral region between 1800 and 1200 cm^{-1}

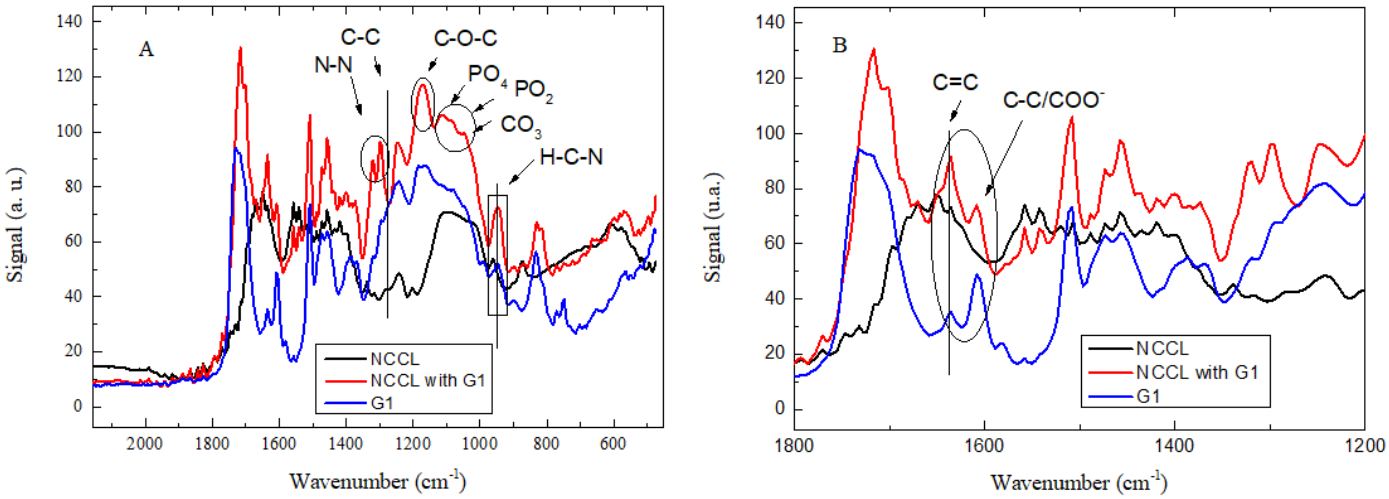


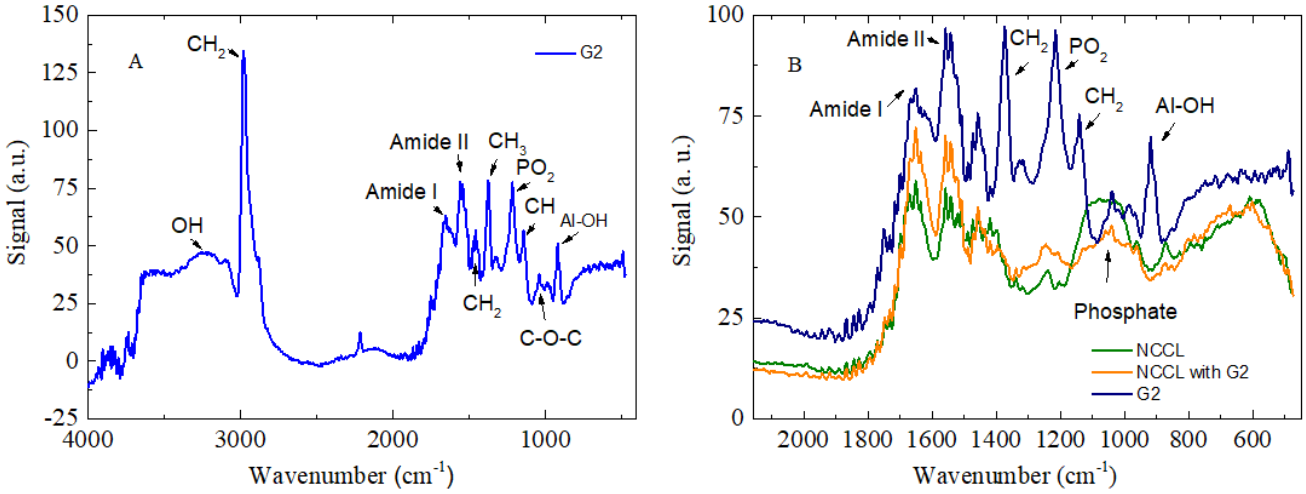
Table 2. Means and standard deviations (SD) of the intensities of the bands that characterize chemical interactions in ADs and natural LCNCs with the adhesive containing the 10-MDP monomer (G1).

Assignment	Band	Group	Mean	SD	p
C=C	1635 cm^{-1}	AD	60.82	3.83	0.25
		NCCL	63.24	3.8	
C-C/COO^-	1608 cm^{-1}	AD	49.66	3.67	0.19
		NCCL	52.47	3.92	
N-N stretching	1323 cm^{-1}	AD	59.86	6.22	0.52
		NCCL	62.45	8.26	
C-C stretching	1295 cm^{-1}	AD	64.28	7.23	0.58
		NCCL	66.82	9.55	
C-O-C	1169 cm^{-1}	AD	76.7	8.67	0.58
		NCCL	79.93	12.34	
PO_4	1112 cm^{-1}	AD	68.94	7.86	0.41
		NCCL	72.77	9.23	
PO_2 symmetric stretching	1081 cm^{-1}	AD	67.4	7.55	0.36
		NCCL	71.56	8.77	
CO_3	1045 cm^{-1}	AD	64	6.73	0.25
		NCCL	68.52	7.4	
H-C-N bending	946 cm^{-1}	AD	49.26	5.07	0.36
		NCCL	52	5.67	

*Student's t test ($p<0.05$)

Figure 4A shows the photoacoustic absorption spectra of the pure adhesive containing the methacrylamide monomer (G2), with the following functional groups: OH (3445 cm^{-1}), CH_2 (2940 cm^{-1}), CH_2 (1456 cm^{-1}), CH_3 (1373 cm^{-1}), PO_2 (1265 cm^{-1}), CH (1139 cm^{-1}), C-O-C (1040 cm^{-1}), Al-OH (920 cm^{-1}). Figure 4B shows the dentin in the natural NCCLs before and after the application of the adhesive system, suggesting a change in the inorganic components like phosphate and calcium. However, no indication of chemical interactions can be observed.

Figure 4. (A) Photoacoustic absorption spectra obtained from the pure adhesive containing the methacrylamide monomer (G2); and **(B)** NCCL before and after the application of the adhesive system. The arrow indicates structural modifications in the region of the inorganic component of dentin.



2.3.2 Dentin mineral composition

Means and standard deviations of the measured spectra obtained from the integrated areas at the band at 961 cm^{-1} (PO_4) on the surface of ADs and natural NCCLs are shown in Table 3. Student's t test showed that the mineral content in dentin in natural NCCLs was significantly higher than that found in the ADs ($p<0.05$).

Table 3. Means and standard deviations the integrated areas of the band at 961cm^{-1} (PO_4) of ADs and natural NCCLs.

Group	**Mean**	**Standard deviation**	***p***
ADs	297364.6	43571.92	0.002*
NCCLs	387726.7	80574.17	

*Student's T Test ($p<0.05$)

2.3.3 Degree of Demineralization (DD)

MR scanning analysis of the natural NCCLs and ADs with the adhesive systems used are represented by Figures 5 and 6. It can be observed that, in G1, the behavior in natural NCCLs (Figure 5 A) and ADs (Figure 5B) was similar. However, in G2, the dentin in the ADs (Figure 6A) underwent deeper demineralization when compared to natural NCCLs (Figure 6B).

Figure 5. MicroRaman spectra of the dentin/adhesive system interface in G1: **(A)** artificial defects (ADs), and **(B)** natural non-carious cervical lesions (NCCLs).

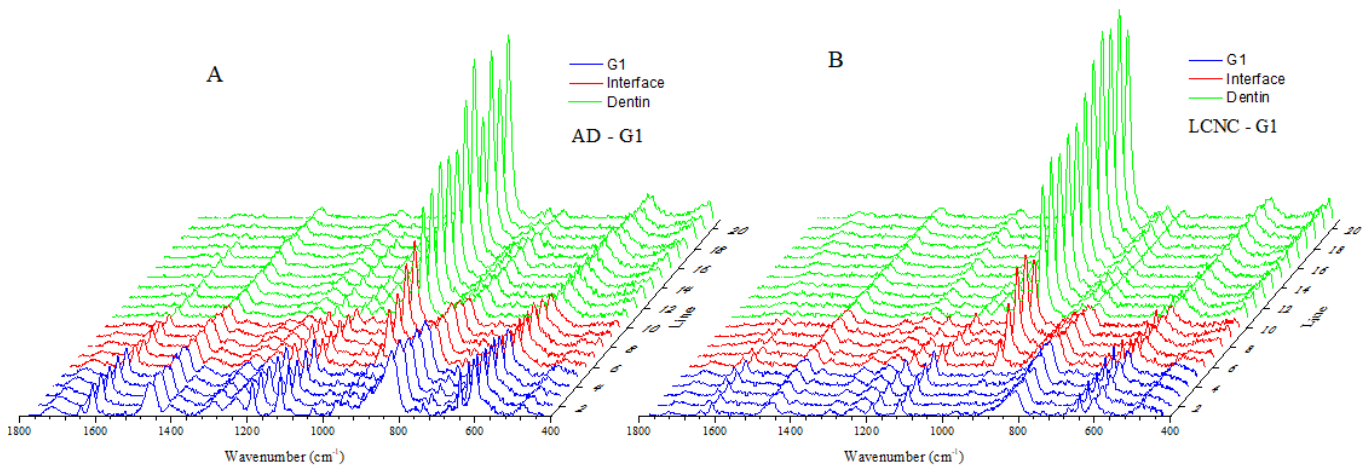
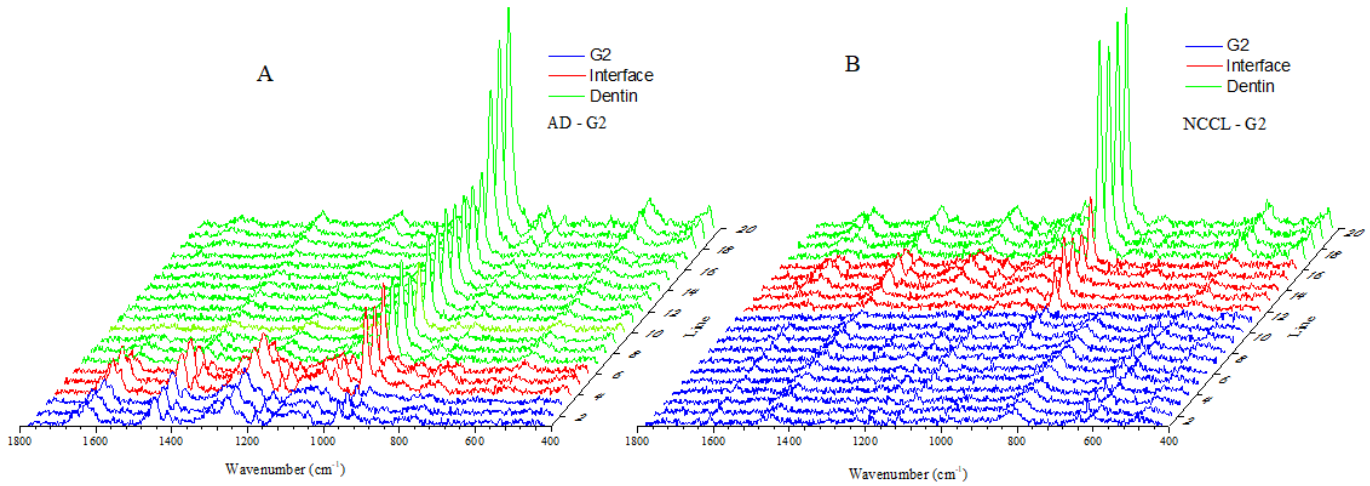


Figure 6. MicroRaman spectra of the dentin/adhesive system interface in G2: **(A)** artificial defects (ADs), and **(B)** natural non-carious cervical lesions (NCCLs).



Mean of the DD (%) of the dentin using the systems G1 and G2 are presented in Table 4. DD in G1 had similar behavior in the first 2 µm of the hybrid layer, whereas from 3 µm, a statistically

significant difference ($p<0.05$) between ADs and natural NCCLs was observed. In G2, DD presented no statistically significant differences between ADs and natural NCCLs.

Table 4. Degree of demineralization (%) in ADs and NCCLs in groups G1 and G2.

Group	Dentin	Degree of demineralization							
		1 µm	***p***	**2 µm**	***p***	**3 µm**	***p***	**4 µm**	***p***
G1	**AD**	93%	0.557	91%	0.242	87%	*0.005	81%	*0.016
	NCCL	94%		87%		75%		70%	
G2	**AD**	91%	0.514	91%	0.515	89%	0.202	83%	0.076
	NCCL	89%		87%		80%		67%	

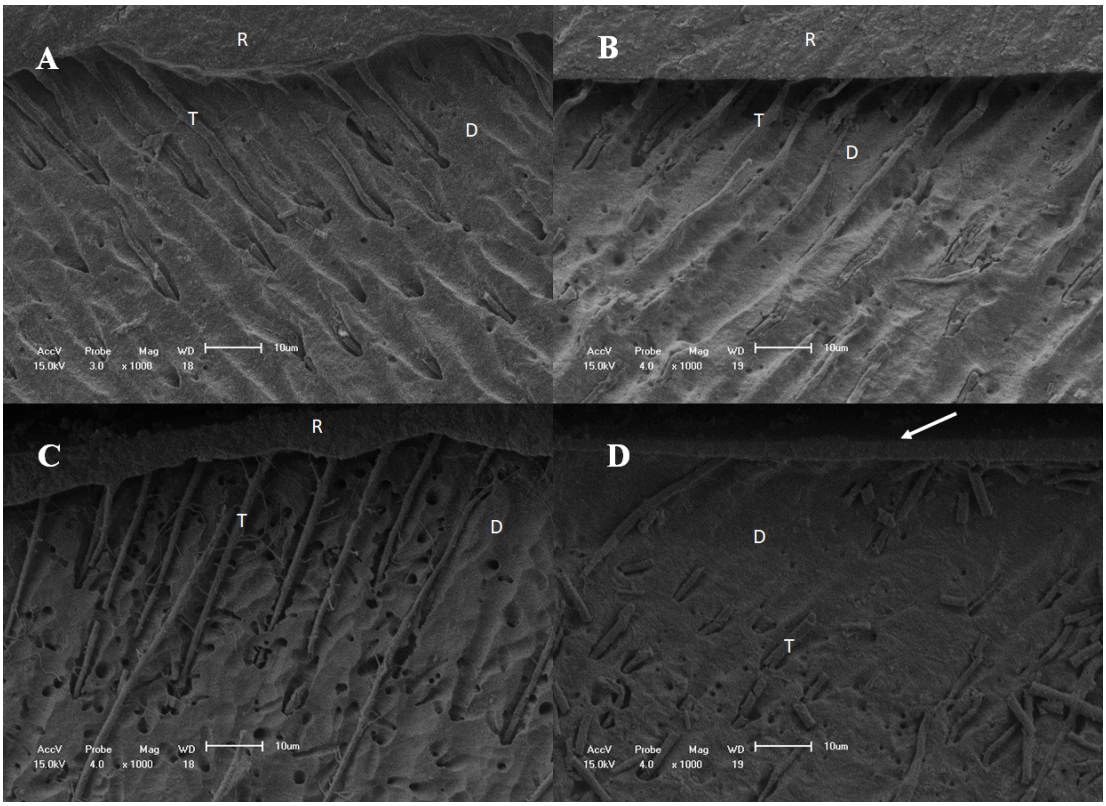
*Student t test ($p<0.05$).

2.3.4 Interface morphology

Scanning electron microscopy analysis showed differences in the adhesive system/dentin substrate interface between G1 and G2 (Figure 7). In G1, images demonstrated similar projections of the adhesive within the demineralized dentin in ADs (Figure 7A) and natural NCCLs (Figure 7B), although the sclerotic aspect of the dentin with greater mineralization can be identified in natural NCCLs. In G2, when applied to ADs (Figure 7C), the methacrylamide-containing adhesive provided greater inter- and peritubular demineralization, with well-defined projections within the dentin. On the other hand, the interface in the natural NCCLs (Figure 7D) was indefinite and more obliterated due to the higher degree of mineralization, compromising the formation of a hybrid layer due to irregular

primer diffusion and reduced adhesive infiltration, leading to the failure of the restoration (indicated by the arrow in Figure 7D).

Figure 7. The dentin/adhesive interface observed with scanning electron microscopy: **(A)** G1 in AD; **(B)** G1 in natural NCCL; **(C)** G2 in AD; **(D)** G2 in natural NCCL. Arrow in Figure 7D indicates restoration failure. R: restoration; T: tags; D: dentin.



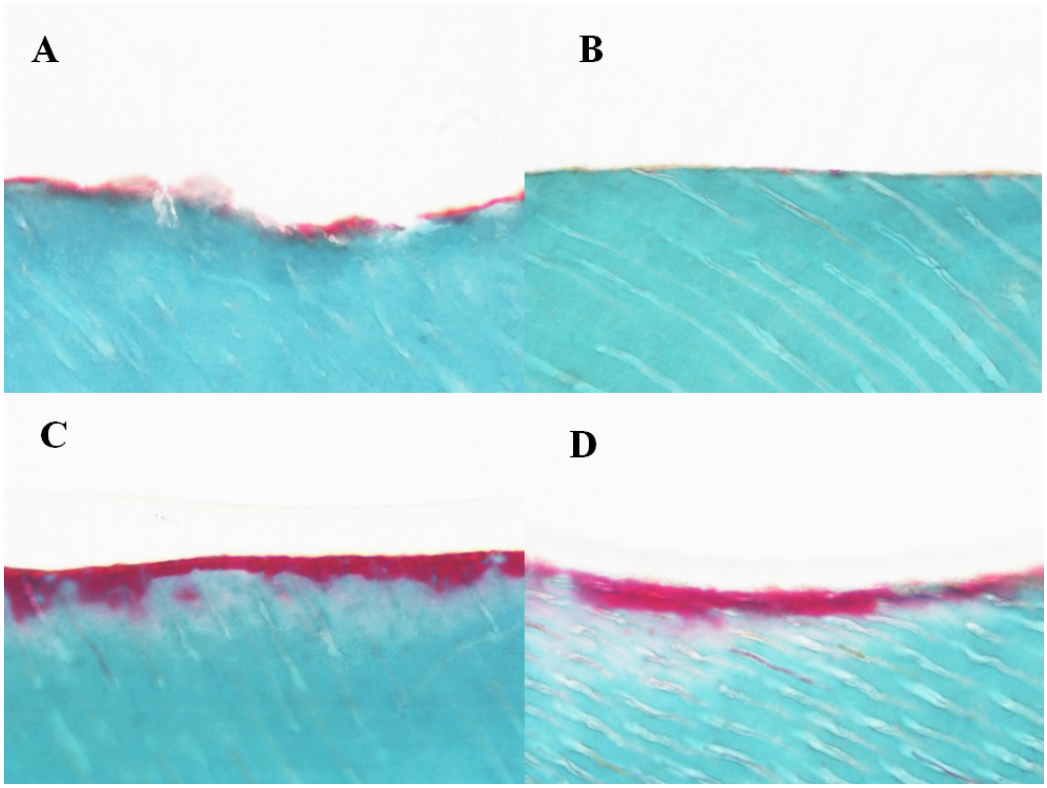
2.3.5 Collagen fiber exposure

The photomicrographs of the samples stained with Goldner's Modified Masson's Trichrome are shown in Figure 8. The dentin appears stained in green, the exposed unprotected collagen is evidenced in red/pink, while pure adhesive is unstained[22,23].

In G1, histomorphological analysis demonstrated a thin layer (pink) of exposed collagen in the

dentin in ADs (Figure 8A). In natural NCCLs (Figure 8B), collagen was hardly evident. In G2, the photomicrographs illustrate deeper demineralization of the dentin and more pronounced collagen exposure (dark red) in ADs (Figure 8C) as well as natural NCCLs (Figure 8D).

Figure 8. Photomicrographs representative of natural NCCLs and ADs stained with Goldner's modified Masson's Trichrome, showing the dentin (green) and exposed collagen (red/pink): **(A)** G1 – AD; **(B)** G1 **–** natural NCCL; **(C)** G2 – AD; and **(D)** G2 – natural NCCL.



2.4 DISCUSSION

Self-etching adhesive systems with different functional monomers were used in this study in an attempt to identify strategies that may increase the longevity of esthetic restorations in natural NCCLs.

Controversy on the best strategy to restore NCCLs still exists. A previous systematic review of the literature failed to find sufficient evidence to support a particular adhesive system or adhesive strategy for the restoration of natural NCCLs[24], highlighting the necessity to investigate the adhesive interface in these situations.

In the present study, MR analyzes showed that the areas corresponding to the phosphate band (961 cm^{-1}) in the dentin in ADs and NCCLs were statistically different, clearly demonstrating that dentin in NCCLs was more mineralized than in ADs. This analysis was performed in this study with the objective to confirm the presence of sclerotic dentin and ensure that specimens with natural LCNCs were in similar conditions. These findings corroborate previous studies that evaluated the molecular and structural differences in the mineral/organic components in the dentin of natural NCCLs and ADs using MR[7,15] and FTIR-PAS[25].

It has already been demonstrated that the 10-MDP functional monomer present in G1 is capable of forming chemical bonds with the hydroxyapatite (calcium salts-MDP), improving adhesion and the longevity of restorations[10,15,26,27]. In the present study, FTIR-PAS analysis demonstrated that the peak intensities related to the chemical interactions (Table 2) in ADs were similar to those in natural NCCLs. The DD (Table 4) of natural NCCLs and ADs was also similar for the first 2 µm of the hybrid layer, becoming statistically different from the depth of 3 µm. However, SEM images (Figure 7a and 7b) demonstrated a similar behavior in terms of adhesive infiltration, despite the higher degree of dentin mineralization in natural NCCLs.

Histomorphological photomicrographs of the adhesive systems tested in this study revealed the

presence of exposed and unprotected collagen (Figure 8). For the formation of an ideal hybrid layer, collagen should be fully protected by the monomer in the adhesive system, preventing any collagen labeling[23]. Studies show that fully exposed collagen is marked by a strong red color, and when partially coated by the adhesive, is marked by lighter colors[22]. In the present study, it is possible to observe a thin layer of light red/pink collagen in G1, indicating that the adhesive partially enveloped the demineralized dentin in the ADs (Figure 8A). In the natural NCCLs, the system was able to encapsulate collagen more closely to the ideal, and was very little evidenced by the dye (Figure 8B).

The molecular/chemical alterations of the hypermineralized sclerotic dentin in NCCLs may result in a less favorable substrate for adhesion[7]. Some studies advocate the removal of the superficial sclerotic layer in order to increase intra-tubular retention[5,28-30]. However, according to Tay and Pashley[8] the removal of the hypermineralized layer in NCCLs may not increase adhesive strength, since the sclerotic dentin may still contain crystals capable of preventing the infiltration of the adhesive into dentinal tubules. Additionally, another systematic review was also unable to determine any differences in survival rates, because of the small number of studies comparing the influence of dentin roughness on the retention of restorations in NCCLs[31].

However, Luque-Martinez et al[32] demonstrated that the adhesive strength of self-etching systems containing 10-MDP in unprepared sclerotic bovine dentin was superior when compared to the same surface prepared with diamond burs. Corroborating these findings, Oliveira et al[15], using human teeth with natural NCCLs also observed that adhesion of a self-etching system containing 10-MDP was stronger in natural NCCLs when compared to ADs, probably due to the chemical affinity of the monomer with the hydroxyapatite.

The results of the present study suggest that the adhesive system containing 10-MDP functional monomer (G1) can be applied directly on the superficial sclerotic layer in NCCLs, since the intensity

of chemical interactions and the degree of demineralization of natural NCCL and ADs were similar. This perception is reinforced by the fact that G1 was able to involve collagen in natural NCCLs more completely. As a result, apart from promoting unnecessary dental structure wear, surface preparation could actually hinder adhesion due to the presence of debris.

The methacrylamide monomer present in the G2 system interacts with the collagen present in the dentin[33], showing high hydrolytic stability of the amide portion[34], as well as the ability to demineralize the dentin[35], providing good long-term adhesive strength in healthy dentin when compared to methacrylate-based adhesive systems[36].

In G2, MR analyzes demonstrated that the DD in ADs (Figure 6A) was probably caused by the high demineralizing power of the system (pH 1.38), and the absence of a smear layer. In contrast, in natural NCCLs the demineralization was less intense due to the hypermineralized characteristic of the surface (Table 4). SEM images (Figures 7C and 7D) confirm these findings and demonstrated adhesive failure in the NCCL restoration. A clinical study using the Xeno Select adhesive system, which also has the same functional monomer in G2, demonstrated that the adhesive was not able to fulfil the ADA criteria for restoration failure rate of less than 5% after 6 months of clinical performance in natural NCCLs[37].

In natural NCCLs with G2, DD in the first 4 microns of the hybrid zone was similar to the ADs (Table 4). Furthermore, FTIR-PAS analysis demonstrated changes in dentin spectra after application of the adhesive. These modifications suggest a change in the inorganic components, such as phosphate and calcium, but do not indicate the occurrence of chemical interactions (Figure 4). Zhou et al[38] when testing adhesives with 10-MDP (Clearfil S3 Bond), 4-META (GBond) and methacrylamide (Xeno V) in sound and deproteinized dentin, also observed that -C=C-COO- chemical bonds could not be identified in the spectrum of the methacrylamide-based adhesive. The absence of a signal in the infrared

spectrum of the Xeno V adhesive group indicated low affinity with the dentin. The band at 1718 cm^{-1} of Xeno V was much weaker than the other adhesives, which may explain the absence of a signal after being applied to the dentin surface[38].

In the present study, a thick band of dark red collagen could be observed in the histomorphometric analysis of specimens in G2, indicating that the adhesive system demineralized dentin at a greater depth than in G1, both in ADs and natural NCCLs. Additionally, they revealed that the functional monomer did not involve the collagen in neither dentin substrates tested with G2. This result confirms FTIR-PAS and SEM findings, which also demonstrated that no hybrid layer was optimally formed to provide the expected chemical interaction between the collagen in natural NCCLs and ADs with G2.

Thus, the results of the present study demonstrated that the infiltration of the methacrylamide-based adhesive in NCCLs was limited due to the presence of a more obliterated dentin, compromising hybrid layer formation at the interface. Considering that in the present study, G2 demineralized the dentin more deeply in ADs when compared to natural NCCLs, the superficial sclerotic layer should be removed by means of cavity preparation to provide micromechanical interaction and effectively bond to the dentin in NCCLs.

In vitro studies, which attempt to simulate actual clinical conditions present some important limitations. Despite that, cavities were tested in a paired way, i.e, the same tooth received the control cavity on the surface opposite to the natural NCCLs, to allow direct comparison between groups and avoid possible differences in the permeability between teeth, which could interfere in the results obtained from dentin in NCCLs and ADs. Further studies testing adhesive systems with different chemical compositions and dentin surface preparation mode are required to help dentists to use strategies that may increase the longevity of esthetic restorations in natural NCCLs.

2.5 CONCLUSIONS

The use of self-etch adhesive system in NCCLs requires different substrate preparation strategies according to the functional monomer present in its composition. Because the G1 adhesive containing the 10-MDP monomer was shown to react chemically with the mineral component present in the sclerotic dentin, it may be applied directly into the surface of NCCLs. On the other hand, the G2 adhesive containing methacrylamide demonstrated that the superficial sclerotic layer on NCCLs needs to be removed prior to adhesive application in order to obtain improved micromechanical interaction.

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