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Eunice Kuhn

O PAPEL DO CIMENTO DE IONÔMERO DE VIDRO EM DENTINA INFECTADA DE MOLARES PERMANENTES COM LESÕES PROFUNDAS DE CÁRIE TRATADOS PELA MÍNIMA INTERVENÇÃO

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Tese apresentada ao Programa de Pós-Graduação em Odontologia, nível de Doutorado, da Universidade Estadual de Ponta Grossa, como pré-requisito para obtenção do título de Doutor em Odontologia, área de concentração Clínica Integrada.

Orientadora: Profa. Dra. Denise Stadler Wambier

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Eunice Kuhn

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Profa. Dra. Denise Stadler Wambier- Orientadora
Universidade Estadual de Ponta Grossa

Profa. Dra. Daniela Prócida Raggio
Faculdade de Odontologia/ Universidade de São Paulo

Profa. Dra. Estela Maris Losso
Universidade Positivo

Profa. Dra Alessandra Reis
Universidade Estadual de Ponta Grossa

Profa. Dra. Márcia Helena Baldani Pinto
Universidade Estadual de Ponta Grossa

DADOS CURRICULARES

Eunice Kuhn

Nascimento: 16.11.53	Ponta Grossa, Paraná - Brasil
Filiação:	João Kuhn Sobrinho Catarina Mayer Kuhn
1972-1975	Curso de Graduação em Odontologia. Universidade Estadual de Ponta Grossa (UEPG), Paraná, Brasil.
1975-1975	Especialização em Odontopediatria. Universidade Federal de Santa Catarina (UFSC), Santa Catarina, Brasil.
2000-2002	Mestrado em Saúde Pública. Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brasil.
2010-2013	Curso de Pós-Graduação em Odontologia. Área de Concentração: Clínica Integrada. Linha de Pesquisa Prevenção em Odontologia. Nível de Doutorado. Universidade Estadual de Ponta Grossa (UEPG). Ponta Grossa, Paraná, Brasil.
1986-atual	Professora Assistente do Departamento de Odontologia da Universidade Estadual de Ponta Grossa (UEPG). Ponta Grossa, Paraná, Brasil.

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A verdadeira dificuldade não está em aceitar idéias novas, mas em fugir das idéias antigas.

John Maynard Keynes

RESUMO

Kuhn, E**. O papel do cimento de ionômero de vidro em dentina infectada de molares permanentes com lesões profundas de cárie tratados pela mínima intervenção.** [Tese Doutorado em Clínica Integrada - Faculdade de Odontologia]. Ponta Grossa: Universidade Estadual de Ponta Grossa, 2013.

Este estudo descreveu as alterações em dentina infectada de dentes permanentes antes e após selamento cavitário, bem como a influência do material restaurador nesse processo. Foram selecionados 45 molares com lesões de cárie profunda e ativa de 35 indivíduos com idades entre 7 a 15 anos. Critérios clínicos, radiográficos e laboratoriais foram utilizados para comparar as condições iniciais da dentina infectada, 60 dias e 10-15 meses após procedimento restaurador. O grupo experimental (G1) recebeu proteção pulpar com cimento de ionômero de vidro (CIV) e o grupo controle (G2), um material inerte (cera). As restaurações foram realizadas em resina composta. A cavidade foi dividida em duas porções equivalentes (mesial e distal) e fragmentos de dentina infectada foram removidos em dois momentos: inicial (porção mesial) e após 60 dias de selamento (porção distal). Os critérios clínicos utilizados foram a fluorescência da dentina infectada nos G1 e G2 (inicial e 60 dias) e a qualidade das restaurações (critérios USPHS) (inicial, 60 dias e 10-15 meses). Nos critérios laboratoriais, foram realizadas a análise ultraestrutural (MEV), mineral (MEV/EDS – cálcio, fósforo e flúor) e radiográfica (subtração radiográfica - 60 dias e 10 a 15 meses de selamento) para os dois grupos. A análise imunohistoquímica da dentina (expressões de MMP-2, MMP-8 e MMP-9) foi realizada para G1 (inicial e 60 dias). Os exames radiográficos consistiram de: radiografias periapicais (diagnóstico) e radiografias interproximais padronizadas (pós-restauração; aos 60 dias e 10-15 meses). Os dados referentes à fluorescência da dentina em G1 e G2 foram comparados com o teste Wilcoxon Signed Rank e em cada período com o teste de Mann-Whitney; o teste t pareado foi usado para verificar as diferenças em relação aos valores iniciais da porcentagem em peso do cálcio, fósforo e flúor entre os dois grupos após 60 dias e o teste de ANOVA e Tukey entre os dois grupos e tempos, bem como as variações radiográficas nos níveis de cinza ao longo do tempo. A intensidade e distribuição da imunomarcação nos dois momentos foi analisada pelo teste qui-quadrado. Os resultados mostraram que, após 60 dias de selamento cavitário, foram detectadas reduções significativas na fluorescência dentinária entre os períodos para ambos os grupos ($p<0,05$), melhor organização tecidual, com um arranjo mais compacto das fibrilas de colágeno e redução no número de bactérias, bem como um aumento significativo na porcentagem em peso de cálcio ($p<0,001$) e fósforo ($p=0,003$) para ambos os grupos. Para o flúor, nenhuma diferença significativa foi observada entre os grupos ($p>0,05$). Houve redução significativa para a expressão de MMP-8 após 60 dias de selamento cavitário ($p=0,02$). As MMPs localizaram-se de maneira generalizada na região intertubular em ambos os períodos (MMP-2 - $p=0,172$; MMP-8 - $p=0,35$; MMP-9 - $p=0,28$) e intratubular ausente ou localizada ($p>0,28$). Após 10-15 meses de acompanhamento, as restaurações mostraram-se clinicamente adequadas e a subtração radiográfica demonstrou que as médias dos tons de cinza em dentina sadia e cariada aumentaram ($p<0,001$), independente do grupo. Concluiu-se que o material de forramento colocado sobre dentina infectada não é fundamental para a paralisação do processo carioso; quando a cavidade é devidamente selada o tecido tende a se reorganizar num período curto de tempo (60 dias) sendo o processo de remineralização contínuo durante períodos mais longos.

Palavras-chave: Cimento de ionômero de vidro. Lesões profundas de cárie. Dentes permanentes.

ABSTRACT

Kuhn, E. **The role of glass ionomer cement on infected dentin from permanent molars with deep caries lesions treated by minimal intervention.** [Thesis, Doctorate in Integrated Dental Clinics - School of Dentistry]. Ponta Grossa: Ponta Grossa State University, 2013.

This study described the changes in infected dentin in permanent teeth before and after cavity sealing, as well as the influence of lining material in this process. Forty five molars with deep and active caries lesions were selected, from patients aging 7 to 15 years old. Clinical, radiographic and laboratorial criteria were used to compare dentin conditions before and after 60-days and 10-15 months after dental restoration. The experimental group (G1) was lined with glass ionomer cement (GIC) and the control group (G2) with an inert material (wax). Composite resin was the selected restorative material for both groups. The caries cavities were divided into two equivalent portions (mesial and distal) and the dentin fragments were removed in two moments: baseline (mesial portion) and 60-days after restoration (distal portion). The clinical criteria for G1 and G2 were infected dentin fluorescence (baseline and 60-days) and the quality of the resin restorations (USPHS criteria) (baseline, 60-days and 10-15 months). For both groups, the laboratorial criteria were ultra structural (SEM), mineral (SEM/EDS - calcium, phosphorus and fluoride) and radiographic analysis (digital subtraction - 60-days and 10-15 months). The data from dentin fluorescence in G1 and G2 were analyzed by Wilcoxon Signed Rank test and in the different moments of evaluation with Mann-Whitney test. The paired Student's t test was used to verify differences in the percentage of weight of calcium, phosphorus and fluoride between groups after 60-days and the ANOVA and Tukey test between both groups and the moments of evaluation, as well as the radiographic changes in the gray levels along time. The intensity and distribution of immunostaining in both moments was analyzed with chi-square test. The results showed that, 60 days after restoration, there were significant reduction in dentin fluorescence in both groups ($p<0.05$), better tissue organization with a more compact fibril arrangement and a reduction in the number of bacteria, as well as a significant increase in the percentage in weight of calcium ($p<0.001$) and phosphorus ($p=0.003$) for both groups. No difference was observed in percentage of weight of fluorine ($p>0.05$). There was a significant reduction in the expression of MMP-8 60 days after restoration ($p=0.02$). The MMPs were generalized located in intertubular dentin in both evaluation moments ((MMP-2 - $p=0.172$; MMP-8 - $p=0.35$; MMP-9 - $p=0.28$) and in the intratubular dentin they were absence or localized ($p>0.28$). After 10-15 months, the resin restorations were judged clinically suitable and the digital subtraction of radiographic showed that the mean levels of gray tones incresead in sound and infected dentin ($p<0.001$) for both groups. It's possible to conclude that the lining material placed over infected dentin is not fundamental for caries arrestment; when the cavity is properly sealed, the tissue tends to reorganize in a short period of time (60 days) and the remineralization process continues for longer periods.

Keywords: Glass ionomer cement. Deep caries lesions. Permanent teeth

LISTA DE ABREVIATURAS E SIGLAS

ANOVA	Análise de variância
Bit	*Binary digit*
BSP	*Bone sialoprotein*
C	Cera utilidade
CIV	Cimento de ionômero de vidro
CIVs	Cimentos de ionômero de vidro
Cl	*Confidence interval*
DAB	3,3' – diaminobenzidina
DMP1	*Dentin matrix acidic phosphoprotein 1*
Dpi	*Dots per inch*
DPP	Fosfoforina
DSP	Sialoproteína da dentina
DSPP	Sialofosforoproteínas
DSR	*Digital subtraction radiography*
EDS	*Dispersive X-ray spectrometry energy*
EDS	Espectrometria dispersiva de raio X
EDTA	Ácido etileno diamino tetra-acético
G1	Grupo 1
G2	Grupo 2
GIC	*Glass ionomer cement*
H	Horas
H2O2	Peróxido de hidrogênio
i.e	Isto é
ICDAS	*International Caries Detection and Assesment System*
Kv	Quilovoltagem
kVp	Quilovoltagem pico
LF	*Laser fluorescence*
M	Molar
mA	Miliamperagem
MEV	Microscópio Eletrônico de Varredura
Min	Minutos
Ml	Mililitro
Mm	Milimetro

MMP	Metaloproteinase
MMPs	Metaloproteinases
mW/cm^2	Megawatt/ centímetros quadrados
NaOH	Hidróxido de sódio
Nm	Nanômetro
PBS	Solução tampão fosfato
PBS/BSA	Solução de tampão fosfato e soro-albumina bovina
pH	Potencial hidrogeniônico
s	Segundos
SEM	*Scanning electron microscope*
TGF	*Transforming growth factor*
TGF-ß1	*Transforming growth factor beta 1*
TIFF	*Tagged image file format.*
UEPG	Universidade Estadual de Ponta Grossa
USPHS	*United States Public Health Service*
Wt%	*Weight percent*

LISTA DE SIMBOLOS

#	Número
%	Porcentagem
<	Menor
=	Igual
>	Maior
±	Mais ou menos
°C	Graus Celsius
µm	Micrômetro
p	Probabilidade da hipótese nula ser verdadeira
x	Aumento de lente óptico
α	Alfa (nível de significância)

SUMÁRIO

1. INTRODUÇÃO

A remoção completa de tecido cariado, independente de sua extensão, foi por muito tempo considerada a estratégia ideal de tratamento das lesões de cárie[1-2]. Mas, esta prática, especialmente em lesões profundas, pode resultar em exposição pulpar que requer intervenções mais invasivas, entre elas endodontia e até mesmo exodontia do elemento dentário. Essa última opção de tratamento pode ser a única em pacientes jovens que vivem em áreas rurais, em geral, desprovidas de serviços especializados.

O clássico tratamento expectante de lesões profundas de cárie em dentes permanentes ou capeamento pulpar indireto passou a ser novamente estudado sob o título “stepwise excavation”, em pesquisas lideradas por Bjorndal[3]. A remoção passo a passo ou em etapas seguida de restauração provisória, visa evitar a exposição pulpar pela remoção de todo tecido desmineralizado na fase ativa da lesão. Essa conduta, ao isolar o biofilme da lesão, permite "desacelerar" o processo carioso, criando condições para uma ação fisiológica de defesa do complexo dentino-pulpar [4].

Na realidade, o tratamento em etapas assemelha-se ao capeamento pulpar indireto, pois nesse a remoção de tecido cariado é parcial, o material de proteção é o hidróxido de cálcio e o dente é reaberto em uma segunda sessão. A tendência atual é a de realizar uma única intervenção, dispensando a reabertura do dente[5] na expectativa de reparo do residual cariado.

Felizmente, o melhor conhecimento sobre a doença cárie tem direcionado para o manejo mais conservador das lesões em dentina[4-9]. Nesse contexto, a mínima intervenção em odontologia trouxe grande contribuição, motivando para uma série de pesquisas, discussões e revisões sistemáticas sobre esse tema[10]. A remoção completa passou a ser gradativamente substituída pela remoção parcial e seletiva, com manutenção da dentina afetada no assoalho pulpar[11].

Essas mudanças de conduta verificadas nos diferentes estudos, seja em cavidades médias[12] ou profundas[5,9], em dentes decíduos[7,13]e permanentes[4-6] estão baseadas na interrupção do processo carioso.

Na remoção seletiva[3,11] recomenda-se a retirada da dentina altamente infectada (camada externa), mantendo-se a porção afetada que com sua

rede de fibrilas colágenas possibilitaria a ligação de cristais de apatita e conseqüente remineralização do tecido dentinário. No entanto, a quantidade de tecido cariado deixado na cavidade depende de critérios subjetivos. Não há um limite seguro para orientar a remoção somente da parte infectada sem risco de exposição pulpar.

Outro fato a ser destacado é que os diferentes materiais de proteção em contato direto com a dentina profunda[9] determinaram percentuais semelhantes de sucesso clínico e radiográfico[14]. Existem ainda dados sugestivos de reorganização do tecido dentinário mesmo sem a remoção da dentina infectada[12,14]; e que o processo de remineralização não é material dependente[9,15-17].

A maior parte das pesquisas em cárie profunda empregou hidróxido de cálcio[6,18], considerado "padrão ouro" para o capeamento pulpar indireto, com o mais longo histórico de sucesso clínico[19]. Quanto aos cimentos de ionômero de vidro o maior argumento para sua indicação têm sido a liberação de fluoretos que poderia potencializar a remineralização de tecidos adjacentes à restauração[2]. Além disso, as propriedades de adesividade à estrutura dentária e coeficiente de expansão térmica-linear semelhante ao substrato dentário[20-21], proporcionam vedamento cavitário adequado.

Assim, algumas questões precisam ser melhor esclarecidas, como a real influência do material colocado diretamente sobre o tecido infectado para potencializar a remineralização, mas sem qualquer risco de alterações pulpares irreversíveis.

Ressalta-se que a dentina não deve ser analisada apenas sob a ótica de seu conteúdo mineral. O complexo dentino-pulpar compreende dentina mineralizada e tecidos moles vitais no seu interior, ou seja, odontoblastos e tecido pulpar[22]. Durante a progressão da cárie, os minerais dentinários são dissolvidos e a matriz orgânica de colágeno é degradada pela ativação de proteases endógenas (metaloproteinases – MMPs), supostamente ligadas a esta matriz ou provenientes da saliva[22].

Estas proteínas são secretadas pelos odontoblastos, têm papel fundamental durante o desenvolvimento dentário e permanecem inativas até que enzimas com potencial proteolítico as ative, como as proteinases bacterianas ou a queda de pH gerada pela atividade de cárie[23].

As enzimas MMP-8, secretadas pelos odontoblastos[24] têm como principal substrato o colágeno tipo I, que envolve 90% do componente orgânico da

dentina. As MMP-2, 8 e 9 foram identificados nas dentinas coronária e radicular, mas a ampla distribuição de MMP-2 e 8 em ambas as dentinas sugere que elas são as principais MMPs neste tecido[24-25].

As MMPs, são uma família de endopeptidases capazes de degradar praticamente todos os componentes da matriz extracelular, são expressas durante a formação normal do complexo dentino-pulpar e para sua manutenção. Sugere-se que a atividade de MMPs contribue para a degradação da matriz orgânica durante a progressão da cárie de dentina bem como para o seu reparo, promovendo reações de defesa nas células do complexo dentino-pulpar[22].

O acúmulo de MMP-2 e sialoproteína óssea (BSP) nos túbulos dentinários afetados por cárie podem indicar seu potencial envolvimento no mecanismo de defesa do hospedeiro que resulta em calcificação das regiões afetadas pelo processo de cárie[26].

A dentina, diferente do osso que não possui proteínas específicas, possui duas proteínas não colágenas, as quais são exclusivamente encontradas na matriz extracelular. Essas proteínas são as sialofosforoproteínas (DSPP) proteoliticamente divididas em fosfoproteína (DPP também chamada fosfoforina) e a sialoproteína da dentina (DSP). A DPP é o segundo mais abundante constituinte da matriz orgânica da dentina e provavelmente possui papel determinante na mineralização da dentina[27].

Na dentinogenese reacionária, sugere-se que a MMP-2 seja facilitadora da liberação de DSP da DSPP[28]. Como a DSP participa da mineralização[28] a MMP-2 deve, provavelmente, modular a formação da dentina reacional no processo de cárie.

A fosfoforina inicia e controla a deposição dos cristais de apatita e a sialoproteína dentinária, responsável por até 8% da matriz orgânica da dentina sinaliza a atividade odontoblástica na mineralização[29]. Desta forma, o presente estudo trabalhou com a hipótese de que há participação ativa de MMPs na dentina cariada de dentes permanentes e que a melhor organização desse substrato após selamento cavitário pode ter influência do processo remodelador destas proteinases.

Do exposto, objetiva-se elucidar os eventos em nível clínico e molecular na dentina infectada de dentes permanentes com cárie profunda selados com cimento de ionômero de vidro, utilizando-se dados clínicos, radiográficos e laboratoriais.

2. PROPOSIÇÃO

2.1 – PROPOSIÇÃO GERAL

Descrever o aspecto da dentina infectada profunda de molares permanentes antes e após selamento cavitário, bem como analisar a influência do CIV nesse processo.

2.2 – PROPOSIÇÕES ESPECÍFICAS

Considerando-se o forramento cavitário com cimento de ionômero de vidro e material inerte, este trabalho se propôs a:

-verificar os valores de laser fluorescência da dentina infectada, antes e após 60 dias de selamento cavitário;

-descrever micromorfologicamente as estruturas da dentina infectada antes e após 60 dias de selamento cavitário;

-quantificar o conteúdo mineral da dentina infectada antes e após 60 dias de selamento cavitário;

-observar a expressão de MMPs na dentina infectada antes e após 60 dias de selamento cavitário com cimento de ionômero de vidro e restauração;

-avaliar as condições clínicas e radiográficas dos dentes restaurados após 10 a 15 meses.

3. MATERIAL E MÉTODOS

Este estudo possibilitou a elaboração de três artigos descritos no capítulo **4** (**ARTIGOS**).

No 1º artigo: **The role of dental material on the repair of infected dentin of permanent molars after cavity sealing** foram avaliados os critérios clínicos laser fluorescência (LF), United States Public Health Service (USPHS), radiográficos (RX 60 dias após selamento cavitário), morfológicos (MEV) e de conteúdo mineral (EDS).

No 2º artigo: **The role of glass ionomer cement on infected caries sealing: A 10- to 15-month follow-up** foram analisados os critérios clínicos (USPHS), morfológicos (MEV), mineral (EDS) e radiográfico (período entre 60 dias e 10 a 15 meses de controle - subtração radiográfica).

No 3º artigo: **Effect of sealing infected dentin with glass ionomer cement on the abundance of MMP-2, MMP-8 and MMP-9** foram avaliados os critérios clínicos (USPHS), radiográficos (RX 60 dias após selamento cavitário) e de imunomarcação (imunohistoquímica).

3.1- Aprovação do estudo

Esta pesquisa foi aprovada pela Comissão de Ética em Pesquisa da UEPG (parecer nº 78/2011) (Anexo A) e autorizada pela Secretaria de Educação do Município de Ponta Grossa, pois envolveu o exame bucal de escolares de duas localidades rurais: Guaragi e Itaiacoca, no Estado do Paraná, Brasil (Anexos B e C).

3.2- Seleção da amostra e critérios de inclusão

Após uma triagem inicial de 772 indivíduos de escolas da área rural, 35 estudantes de ambos os sexos com idades entre 7 a 15 anos foram convidados para o estudo. Crianças com patologias sistêmicas não foram incluídas. Seus pais/responsáveis foram informados sobre os objetivos do estudo e assinaram um termo de consentimento livre e esclarecido permitindo a sua participação (Anexo D).

Os participantes foram submetidos a exames clínicos e radiográficos para a seleção dos molares permanentes. Estes deveriam apresentar lesão de cárie aberta e ativa na superfície oclusal, classificada com o código 06 do ICDAS (*International Caries Detection and Assesment System*)[30]. Quanto a profundidade, as lesões deveriam incluir a porção interna da dentina, com 2/3 ou mais de sua

espessura, mas sem exposição pulpar. Dentes com sinais ou sintomas de patologia irreversível, tais como fístula, mobilidade, alterações periapicais e dor espontânea não foram incluídos.

3.3- Desenho do estudo

Esta pesquisa consistiu num estudo clínico controlado e avaliou a reação da dentina infectada de dentes permanentes frente à proteção pulpar com material bioativo (cimento de ionômero de vidro) e um material inerte (cera utilidade) antes e após 60 dias de selamento cavitário e ao longo do tempo (acompanhamento de 10 a 15 meses), utilizando critérios clínicos, laboratoriais e radiográficos.

A avaliação inicial e após 60 dias consistiu na análise radiográfica (RX) de fluorescência dentinária (LF), critérios *United States Public Health Service* (USPHS), ultraestrutural (MEV), conteúdo mineral (EDS) e imunomarcação (imunohistoquímica). O acompanhamento ao longo do tempo foi realizado por meio dos critérios USPHS e de técnicas de subtração radiográfica digital, conforme fluxograma abaixo (figura 1).

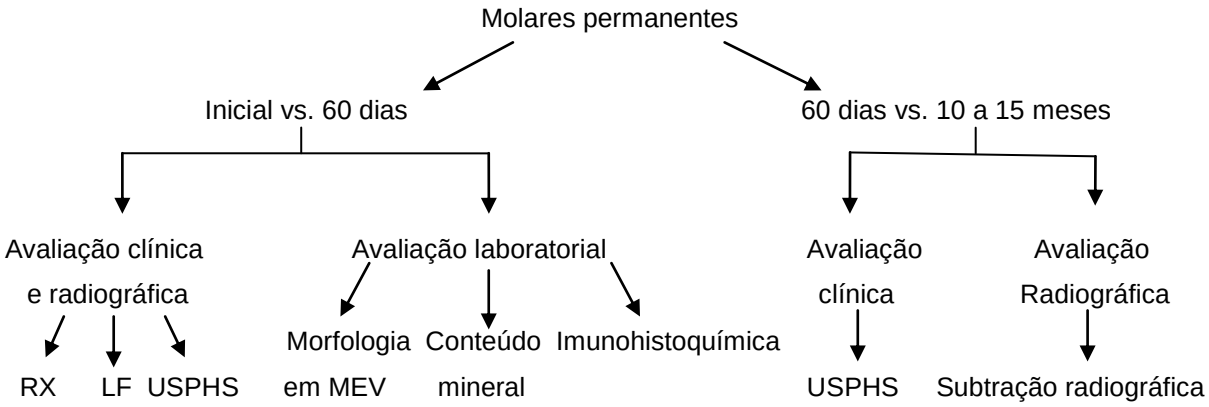

Figura 1 – Fluxograma da metodologia empregada neste estudo

3.4- Etapa clínica

3.4.1- Procedimentos clínicos:

Após cuidadosa seleção da amostra e diagnóstico da condição pulpar, a etapa clínica da pesquisa foi iniciada.

Os dentes selecionados foram aleatoriamente divididos em 2 grupos. No Grupo 1 (G1), a proteção pulpar foi realizada com cimento de ionômero de vidro

de alta viscosidade (Ketac Molar Easy Mix®, 3M ESPE, St. Paul, MN, EUA). Para o Grupo 2 (G2), a dentina cariada foi coberta por uma camada de 1 mm de material inerte (cera utilidade, Epoxiglass Ind. Com. de Produtos Químicos LTDA, Diadema, SP, Brasil). Em ambos os grupos, a restauração final foi realizada com adesivo dentinário (Adper Single Bond, 3M ESPE, St. Paul, MN, EUA), resina composta microhíbrida (Llis, FGM, Joinville, SC, Brasil) e selante para proteção superficial (FluroShield® Dentsply, Petrópolis, RJ, Brasil).

O protocolo de atendimento para G1 e G2 incluiu os seguintes procedimentos: anestesia local, isolamento absoluto, remoção do biofilme da coroa dentária com escova de dente e água, lavagem com água e secagem com ar, preparo cavitário minimamente invasivo, avaliação e coleta de amostra da dentina, forramento cavitário e restauração em resina composta. O preparo cavitário foi mínimamente invasivo e realizado com instrumentação manual. Consistiu na remoção do tecido cariado da margem cavosuperficial da cavidade, exclusivamente para permitir um selamento marginal adequado. Quando necessária a remoção do esmalte sem suporte, pontas diamantadas em alta rotação nº 1092 (KG SORENSEN São Paulo, SP, Brasil) foram utilizadas.

Objetivando-se comparar dados obtidos antes e após selamento cavitário, a cavidade foi dividida em duas porções equivalentes (mesial e distal), usando-se um esculpidor Hollemback em direção buco-lingual. Padronizou-se que a porção mesial seria o sítio para as aferições e coleta das amostras iniciais (controle) e a porção distal seria o sítio para aferição e coleta das amostras seladas por 60 dias (experimental) (figura 2 A).

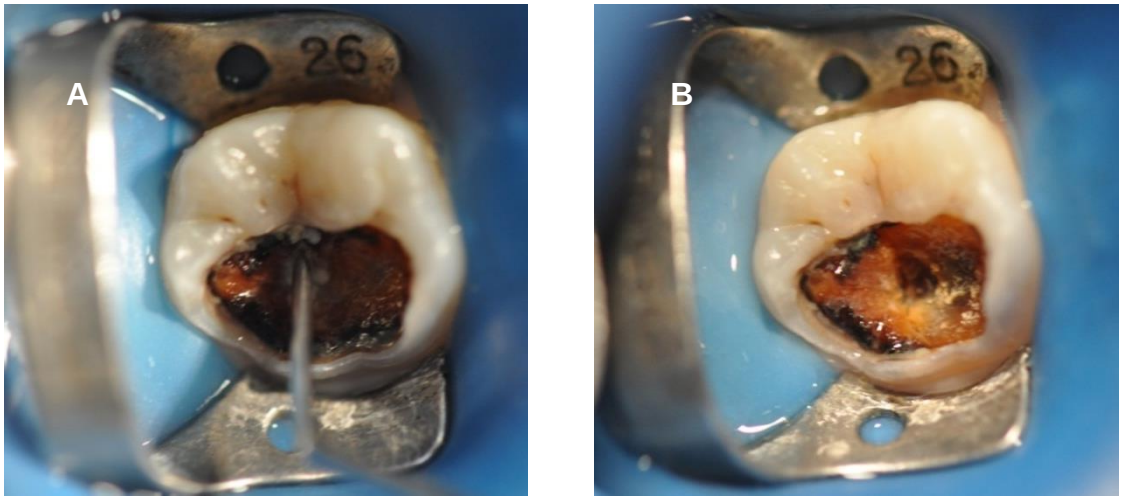

Figura 2 – (A) Divisão da dentina cariada em duas porções (mesial e distal). (B) Aspecto da cavidade após remoção da dentina cariada da porção mesial.

Após quantificar a fluorescência dentinária (descrita a seguir), dois fragmentos de dentina infectada foram removidos da porção mesial da cavidade com colher de dentina (avaliação inicial) e imediatamente processadas para análise ultraestrutural e imunohistoquímica. O tecido cariado da porção distal foi mantido na cavidade a fim de ser removido 60 dias após restauração (figura 2 B).

Sobre a dentina cariada infectada residual foram colocados cimento de ionômero de vidro (CIV - G1) ou cera utilidade (C - G2) de acordo com o grupo de estudo (figura 3).

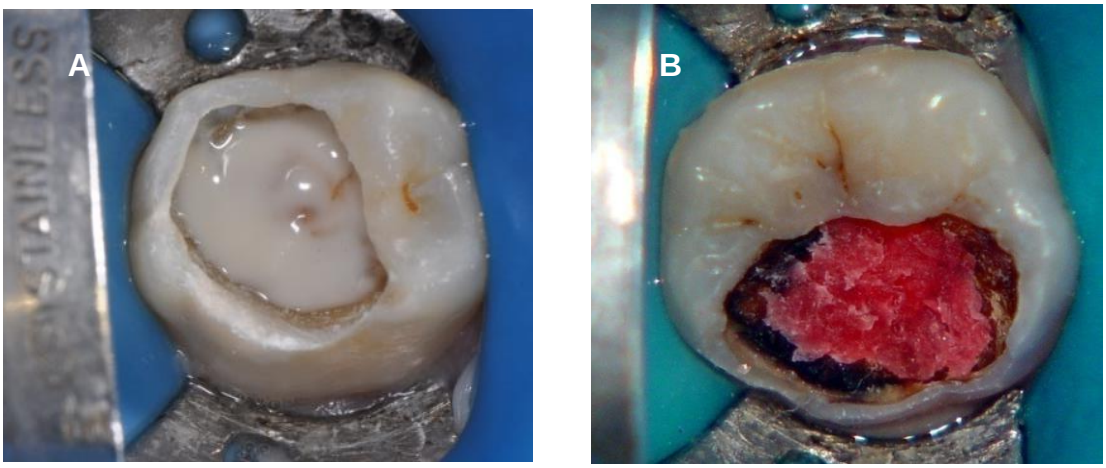

Figura 3 – Aspecto da cavidade após inserção do CIV (A) e cera (B).

As instruções do fabricante foram rigorosamente seguidas para o preparo e inserção do CIV na cavidade. Nenhum condicionamento da dentina foi feito neste momento para facilitar a remoção do CIV após 60 dias e coleta das amostras experimentais.

Os dentes foram então restaurados com resina composta de acordo com os seguintes passos: condicionamento de esmalte com ácido fosfórico por 20 segundos[31], sucção do ácido com cânula endodôntica e lavagem com água por 20 segundos. Resíduos de ácido foram removidos com um cotonete sem esfregar na superfície do esmalte. Todo cuidado foi tomado para evitar contato do ácido com os materiais de forramento. A secagem da cavidade foi feita com jatos de ar sem desidratação. O adesivo foi esfregado ao esmalte por 10 segundos, seguido de leves jatos de ar a uma distância de 20 centímetros para permitir a evaporação do solvente e água residual[32]. Após fotoativação do adesivo (10 segundos), a resina

composta foi aplicada pela técnica incremental,[33] fotoativada por 40 segundos cada incremento e a proteção superficial da restauração foi realizada com uma camada de selante de fossas e fissuras[34].

Sessenta dias depois, os pacientes foram chamados para uma segunda sessão clínica. Neste momento, em ambos os grupos os dentes foram avaliados radiograficamente e a qualidade das restaurações analisadas utilizando-se para isso os critérios descritos pelo *United States Public Health Service* (USPHS)[35]: (1) retenção / fratura, (2) forma anatômica, (3) adaptação marginal; (4) descoloração marginal; (5) cáries recorrentes; (6) sensibilidade pós-operatória; (7) textura da superfície; (8) alteração de cor. Em seguida o dente foi reaberto, foi feita a leitura da fluorescência dentinária na cavidade e obtidas amostras experimentais da porção distal, como realizado no momento inicial (figura 4 A).

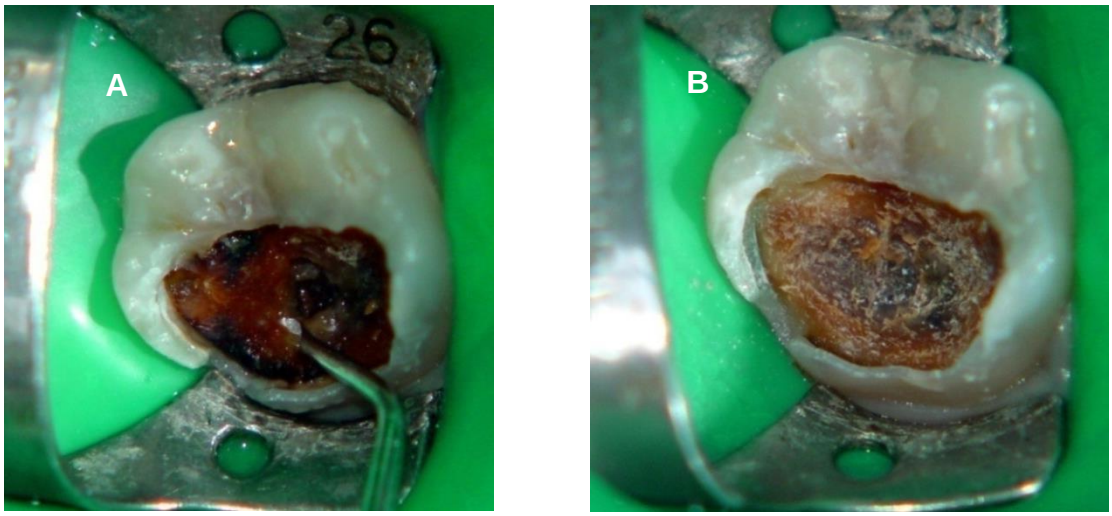

Figura 4 –(A) Aspecto da cavidade durante a remoção da dentina cariada da porção distal. (B) Aspecto da dentina após 60 dias de selamento no grupo G2.

A remoção da resina composta foi feita com alta rotação e a retirada da cera com colher de dentina. O CIV foi removido de forma cuidadosa com baixa rotação e curetas de dentina, secando-se com ar para permitir a visualização do material que se apresenta esbranquiçado e opaco à secagem. Após a remoção de fragmentos de dentina cariada da porção distal, os dentes foram forrados com CIV (G1) e restaurados em resina composta nos dois grupos (G1 e G2) (figuras 5 A e B).

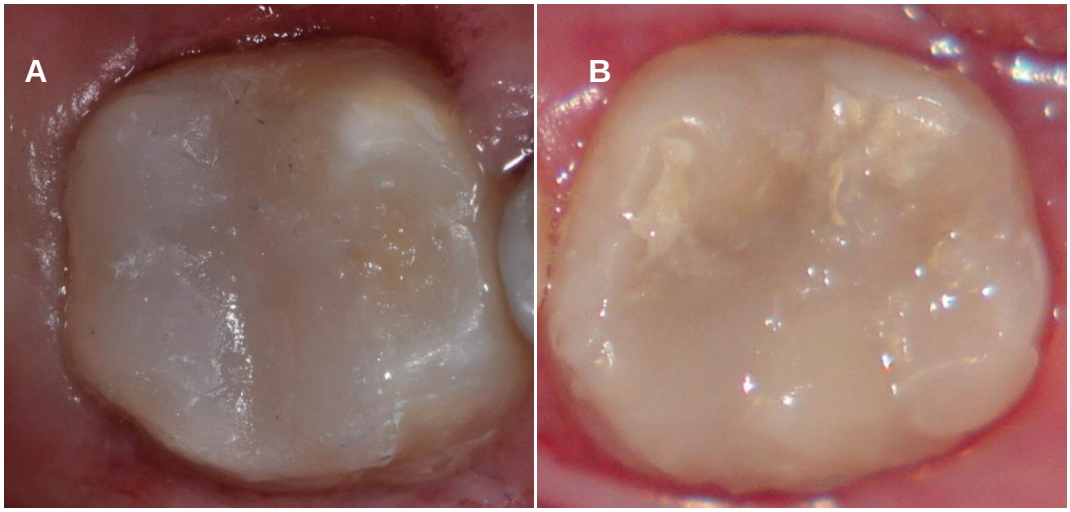

Figura 5 –(A) Restauração em resina composta (G1). (B) Restauração em resina composta (G2).

Todos os procedimentos clínicos foram realizados por um profissional experiente e treinado para realização do protocolo proposto.

3.4.2- Aferição da fluorescência dentinária:

A fluorescência da dentina infectada nos G1 e G2 em ambos os períodos de avaliação (inicial e 60 dias), foram realizadas com o aparelho DIAGNOdent 2095 (KaVo, Biberach, Alemanha), utilizando-se a ponta A (espefícica para superfícies oclusais). [36-38]

Antes de cada aferição, o aparelho de laser fluorescência foi calibrado em um objeto de referência (base cerâmica fornecida pelo fabricante). Em seguida o dente a ser analisado recebeu jatos de ar comprimido por 5 segundos[39] e a ponta A foi levada a uma das cúspides, seu sensor ativado e calibrado novamente em tecido hígido[40] antes do exame da lesão de cárie.

Na sequência, três medidas foram tomadas da dentina cariada infectada das porções mesial (avaliação inicial) e distal (avaliação 60 dias). A média dos valores obtidos foi calculada e considerada o valor de fluorescência dentinária naquele dado momento de avaliação.

3.4.3 Procedimentos radiográficos:

Todos os dentes pertencentes à amostra foram submetidos a quatro exames radiográficos: radiografia periapical (diagnóstico inicial); radiografias interproximais (pós-restauração no momento inicial; aos 60 dias e acompanhamento decorridos 10-15 meses).

Com o propósito de comparar as radiografias de controle pós-tratamento ao longo do tempo, posicionadores de filmes para radiografias interproximais (Indusbello, Londrina, PR, Brasil) foram padronizados para cada paciente, conforme descrito por Oliveira et al.[6] Uma resina acrílica quimicamente ativada (Jon, São Paulo, SP, Brasil) foi aplicada ao posicionador para obtenção da mordida do paciente (figura 6 A). Desta forma, o posicionador poderia ser recolocado na mesma posição em tomadas radiográficas subsequentes. Os ângulos horizontal e vertical, assim como a distância filme-foco das tomadas radiográficas, foram padronizados por meio da haste presente no posicionador e marcações no cilíndro do aparelho de raios-X (figuras 6 A e B).

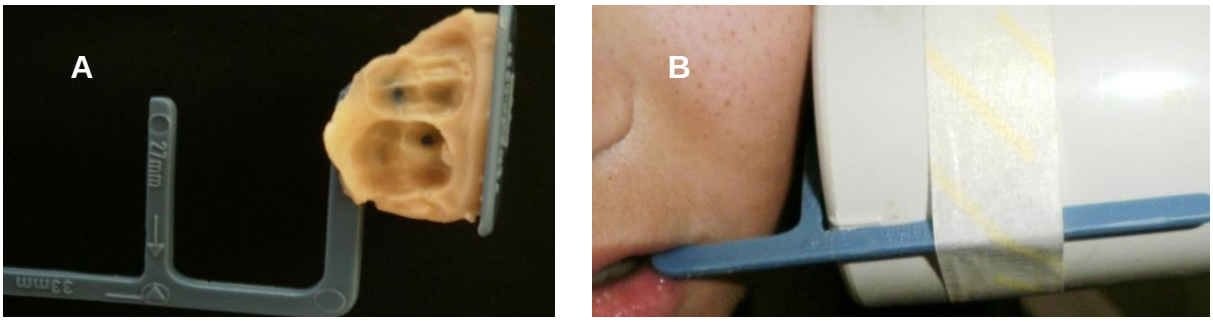

Figura 6 - (A) Imagem de um posicionador após individualização com resina acrílica. (B) Posicionamento do filme radiográfico com o posicionador individualizado.

Padronizou-se também o filme utilizado (Insight nº 0, Eastman Kodak, NY, EUA), o aparelho de raios X (Timex 70E, Gnatus, Ribeirão Preto, SP, Brasil) e os parâmetros de exposição (70 kVp, 7 mA e 0,4 s). Os procedimentos de revelação e fixação foram realizados por meio de um processador automático de radiografias (Revell, São Paulo, SP, Brasil) com soluções novas (Eastman Kodak, Worchester, EUA).

Radiografias obtidas aos 60 dias e após o período de acompanhamento foram digitalizadas utilizando-se um escaner com adaptador de transparência (HP Scanjet G4050, Hewlett-Packard, CA, EUA) com resolução de 600 dpi e escala de cinza de 8 bit. As imagens foram armazenadas no formato tagged image file format (TIFF).

3.4.4. - Acompanhamento clínico - Avaliação da qualidade das restaurações:

Os dentes restaurados foram acompanhados por um período médio de 12,5 meses (10-15 meses) e submetidos à nova avaliação clínica e radiográfica. Utilizando-se os critérios do USPHS[35], todas as restaurações sem sinais ou

sintomas de alterações pulpares foram reavaliadas de acordo com os escores (1) alfa; (2) bravo; (3) charlie por um único operador treinado. A seguir, foram realizadas novas tomadas radiográficas, conforme descrito nos procedimentos radiográficos.

4. Etapa laboratorial

4.1- Microscopia Eletrônica de Varredura (MEV) e Espectrometria dispersiva de raio X (EDS):

Os fragmentos de dentina cariada obtidos da porção mesial (amostra inicial) e distal (amostra 60-dias), foram imediatamente armazenados em solução de glutaraldeido a 2% com tampão fosfato de sódio de 0,1 M (pH 7,4) durante 7 dias. Passado este período, 3 lavagens de 30 minutos foram realizadas com solução tampão fosfato de sódio 50% (0.3M) em água destilada, seguidas por desidratação em acetona a 30%, 50%, 70%, durante 10 minutos, e 90%, durante 20 minutos, 100% por 10 minutos e 100% por 20 minutos. As amostras foram mantidas a 37°C durante pelo menos três dias para remover qualquer água residual. Após fixação em *stubs*, as amostras foram recobertas por uma camada de 10 micrômetros de espessura em ouro para análise em microscopia eletrônica de varredura (MEV) usando o modo de elétrons secundários com uma tensão de 12 kV. A mesma amostra foi analisada por meio de espectrometria de energia dispersiva raios X (EDS) e as percentagens em peso de fósforo, cálcio e flúor, antes (amostra controle) e depois do selamento cavitário (amostra 60-dias) foram avaliadas.

4.2- Análise imunohistoquímica – preparo e análise das lâminas:

As amostras coletadas para análise imunohistoquímica foram mantidas em formol a 10% por 24 a 48 horas no máximo, e em seguida foram descalcificadas. Para tanto, preparou-se solução de EDTA (41,5 gramas. de EDTA, 4,4 gramas de NaOH e 1 litro de água destilada). A solução foi trocada diariamente até que as lascas de dentina permitissem a passagem de um alfinete com facilidade. Este processo demorou até 5 meses.

Na sequência foi realizado o processamento histológico, onde o material foi desidratado (álcool 70%, 80%, 90% e três vezes de 100%, por 1 hora em cada solução), diafanizado (dois banhos de 30 minutos em xilol) e parafinizado (três banhos de 30 minutos em parafina diluída a 60ºC). Depois de todas essas etapas o

material foi emblocado em parafina fundida a 60ºC e mantido à temperatura ambiente.

Decorridas 24 horas as amostras foram cortadas em micrótomo (micrótomo Leica RM2125, Leica Biosystems, Wetzlar, Alemanha) numa espessura de 4 micrometros e colocadas em lâminas silanizadas StarFrost (Knittel, Alemanha) próprias para análise imunohistoquímica.

Cinco lâminas (4 cortes) das amostras controle e experimental de cada dente foram selecionados e tratados três vezes com peróxido de hidrogênio 20 volumes por 10 minutos cada, a fim de inativar a peroxidase endógena. Após uma rápida passagem em solução tampão fosfato (PBS), os cortes foram incubados com os anticorpos primários monoclonais para identificação da MMP-2, MMP-8, MMP-9, sialoproteina óssea e colágeno tipo I (Merck Millipore, Billerica, Massachusetts, EUA). Os anticorpos primários utilizados foram preparados em PBS/BSA (tampão fosfato/Albumina de Soro Bovino) 1%, na diluição de 1:500 e a incubação foi feita por 16 horas à 4ºC, em câmara úmida. Em seguida, os cortes foram lavados três vezes em PBS, por 5 minutos cada, e submetidos ao Kit de amplificação LSAB Dako LSAB+System-HRP (Dako, Glostrup, Denmark)**.** De acordo com o kit os cortes foram incubados com anticorpo secundário conjugado com biotina e posteriormente com a estreptoavidina conjugada com a peroxidase a 37^{o}C, por 30 minutos cada. Em seguida, foram lavados em PBS e a imunomarcação foi revelada incubando os cortes com substrato da peroxidase, 3,3 diaminobenzidina (DAB) (Saint Louis, MO, EUA) a 37ºC, por 5 minutos. Posteriormente os cortes foram lavados em água destilada e contra corados com Hematoxilina de Harris por 60 segundos. Depois, foram desidratados em séries crescentes alcoólicas (70% a 100%), por 2 minutos cada, e mergulhados duas vezes em xilol, por 2 minutos cada, à temperatura ambiente. Lamínulas foram limpas em diferenciador (99% de álcool e 1% ácido clorídrico) e montadas com resina sintética Permount (Fisher Chemical, Nova Jersey, EUA). Em um dos cortes de cada lâmina, para cada etapa do desenvolvimento analisada, o anticorpo primário foi omitido (controle negativo). A análise imunohistoquímica foi realizada no Grupo 1 em microscopia de luz, identificando a presença ou ausência de imunomarcação, bem como, sua localização intertubular ou intratubular.

As imagens foram capturadas em ampliação de 400 vezes (objetiva de 40), por meio de câmera de vídeo acoplada ao microscópio e analisadas em programa específico software Image J 1.45s (Wayne Rashband, National Institutes of Health, Bethesda, MD, EUA), por dois examinadores calibrados.

Descreveu- se a intensidade de imunomarcação, em cada imagem obtida do G1 no momento inicial e após 60 dias, para todas as MMPs analisadas de acordo com os seguintes critérios: imunomarcação reduzida; imunomarcação semelhante; imunomarcação aumentada, bem como, sua localização intertubular e intratubular de acordo com os critérios: ausente; localizada e generalizada.

4.3 - Análise radiográfica (Análise pela subtração radiográfica):

Pequenas diferenças em ângulos de projeção ou de contraste durante o processamento das radiografias ainda podem ocorrer mesmo utilizando-se técnicas padronizadas. A fim de se reduzir estas diferenças, as imagens foram importadas para o *Reggemy Image Registration and Mosaicking* (versão 0.2.43-RCB, DPI-INPE, São José dos Campos, SP, Brasil). Este *software* é capaz de fornecer algoritmos de registros de imagens e subtração de imagem, ou seja, corrige as discrepâncias geométricas e equaliza o contraste entre duas radiografias sequênciais para torná-las comparáveis e subtrai pixels análogos de duas imagens obtidas em sequência.

A radiografia obtida no período de acompanhamento de 60 dias foi denominada imagem de referência (Imagem A) e foi colocada lado a lado com a radiografia de controle do tratamento obtida após 10-15 meses (Imagem B). Selecionou-se manualmente pontos de referência (pontos imutáveis nas duas radiografias) nas duas imagens. Os pontos de referência servem de coordenadas para o *software* alinhar e corrigir a geometria da imagem B de acordo com a imagem de referência. Estruturas anatômicas claramente distinguíveis em ambas as imagens, como junção amelocementária e ponta de cúspides, atuaram como pontos de referência (figuras 7 A e B).

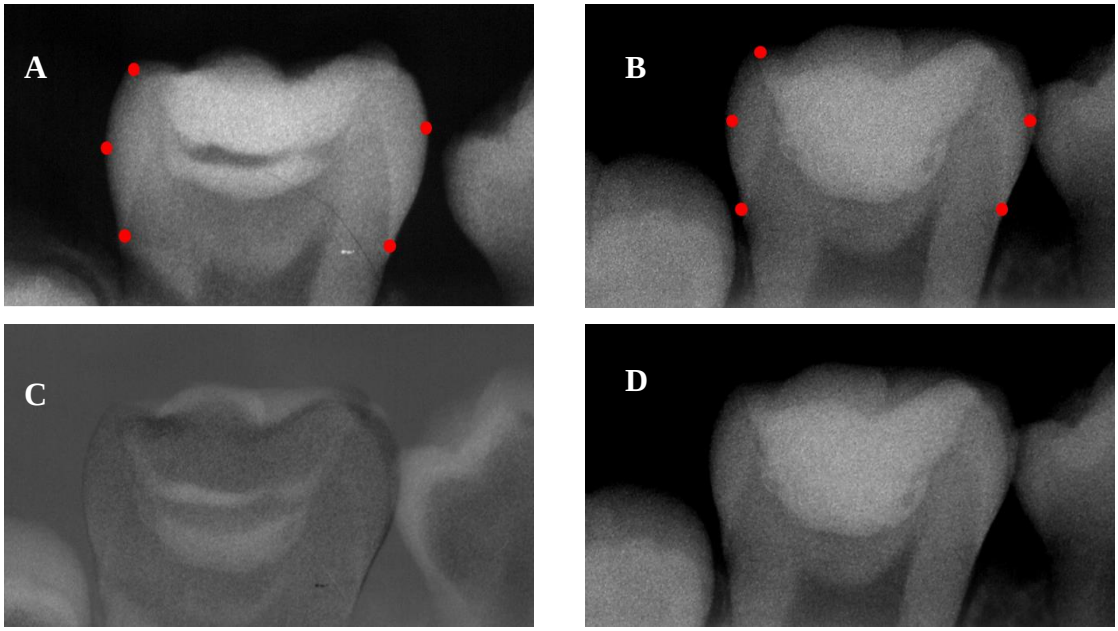

Figura 7– (A) Pontos de referência na radiografia de acompanhamento de 60 dias. (B) Pontos de referência na radiografia de acompanhamento 10 a 15 meses. (C) Subtração radiográfica A-B (D) Nova radiografia de acompanhamento.

A qualidade do registro de imagem foi determinada visualmente por meio da subtração radiográfica. O registro foi considerado adequado quando o ruído estrutural no dente de interesse estivesse reduzido ao menor nível possível e então gerou-se a imagem registrada (Imagem C). A imagem D passou a ser considerada a nova radiografia de acompanhamento de 10-15 meses, já que pequenas diferenças nos ângulos de projeção durante tomada radiográfica ou de contraste durante o processamento foram eliminadas (figura 7 C e D).

A imagem subtraída representa as alterações entre Imagem A e Imagem B. Todas as estruturas anatômicas comuns são mostradas como uma imagem de subtração perfeita em tons de cinza neutro (nível de cinza neutro=128 de 256 possíveis tonalidades de cinza)[4]. Se forem detectadas alterações entre os dois momentos de avaliação, as mudanças são mostradas em níveis de cinza mais escuros (<128) ou mais claros (>128), o que representa perda ou ganho mineral, respectivamente.

A imagem subtraída foi avaliada visualmente. Quando mudanças eram detectadas, uma segunda análise permitia quantificá-las por meio das médias dos níveis de cinza. Imagens A (60 dias) e D (10-15 meses de acompanhamento; registrada *a posteriori*) foram salvas em formato TIFF e importadas para o *software* Adobe Photoshop CS6 (versão 12.0x32, Adobe Systems Incorporated, San Jose, CA, EUA) para quantificar as alterações na dentina remanescente durante o período

de acompanhamento. A área de dentina onde alterações foram detectadas foi delimitada na radiografia subtraída com a ferramenta *polygonal lasso tool* e levada para a Imagem A. A média dos níveis de cinza na área selecionada foi obtida por meio da função histograma expandido. A mesma área foi novamente transportada para a Imagem D e a média dos níveis de cinza foi medida do mesmo modo. Níveis de cinza mais elevados indicam ganho mineral. Esta análise foi repetida 3 vezes e o valor médio das três medições foi calculado para cada dente (figura 8).

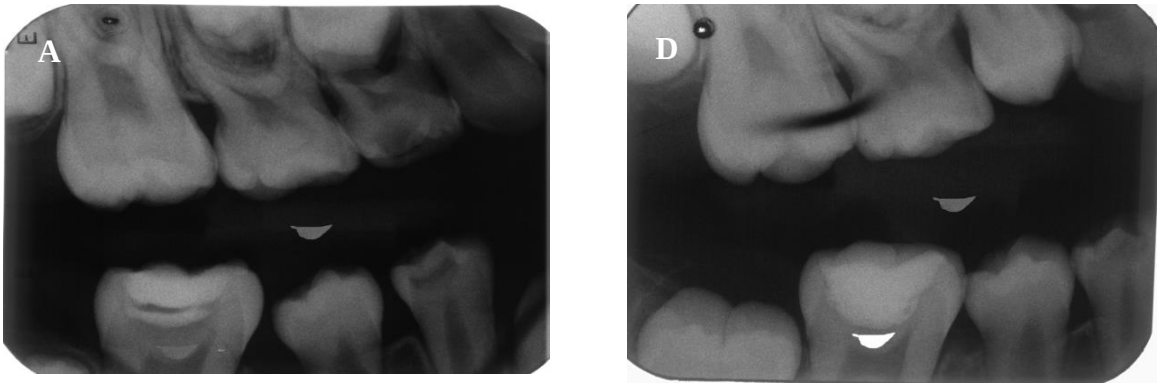

Figura 8 – (A) Radiografia de 60 dias. (D) radiografia de acompanhamento.

Realizou-se o mesmo procedimento em dentina considerada hígida nas porções mesial e distal de cada dente. Áreas padronizadas foram delimitadas com a ferramenta *rectangular marquee tool*. As medições seguiram a mesma rotina acima descrita. Todas as análises foram realizadas por um único operador.

5- Análise estatística

A análise dos dados foi realizada utilizando-se o dente como unidade experimental. Empregando-se os softwares Statistical Package for Social Sciences (SPSS® 17.0, Chicago, Illinois, EUA) e Sigma Plot (versão 11, Systat Software Inc., GmbH, Alemanha) com nível de significância de 0.05. Em cada grupo a laser fluorescência antes e após selamento cavitário foi comparada pelo teste Wilcoxon Signed Rank e em cada período, para os grupos G1 e G2 foi empregado o teste de Mann-Whitney.

O teste t pareado comparou as diferenças em porcentagem de peso do cálcio, fósforo e flúor entre os dois grupos após 60 dias em relação aos valores iniciais.

Os dados de cálcio, fósforo e flúor foram submetidos individualmente a duas medidas repetidas ANOVA e teste de Tukey para comparações pareadas ($\alpha = 0,05$).

Os dados de níveis médios de cinza apresentaram distribuição normal, de acordo com o teste de Kolmogorov-Smirnov ($p=0,493$), e, por conseguinte, foram submetidos ao teste ANOVA três critérios para medidas repetidas nos grupos (G1 e G2), condições da dentina (cariada ou sadia) e tempo de avaliação (60 dias e 10-15 meses) como os principais fatores. O teste de Tukey foi utilizado para comparações pareadas.

A alteração na intensidade da imunomarcação para as MMP-2, 8 e 9 após 60 dias foi analisada estatisticamente pelo teste qui-quadrado, bem como, a distribuição de todas as MMPs citadas acima, na amostra inicial e 60 dias. Em todos os testes, a significância estatística foi estabelecida em 5 %.

4. ARTIGOS

4.1 ARTIGO 1

The role of dental material on the repair of infected dentin of permanent molars after cavity sealing

Eunice Kuhn[1], Alessandra Reis[2], Ana Claudia Rodrigues Chibinski [1], Denise Stadler Wambier [1]

[1] DDS, MS, PhD, Department of Pediatric Dentistry, School of Dentistry, Ponta Grossa State University, Paraná, Brazil.

[2] DDS, PhD, Department of Restorative Dentistry, School of Dentistry, Ponta Grossa State University, Paraná, Brazil.

Running Title: Infected dentin sealing: material effect

Key Words: Dental pulp capping. Glass ionomer cement. Deep carious lesions.

Corresponding author: Eunice Kuhn. UEPG/Departamento de Odontologia, Bloco M, Campus de Uvaranas. Av. General Carlos Cavalcanti, 4748, CEP 84.030-900, Ponta Grossa, Paraná, Brazil. nice.kuhn@ig.com.br

contact adresses:

Eunice Kuhn - Universidade Estadual de Ponta Grossa, Departamento de Odontologia, Bloco M, Campus de Uvaranas. Av. General Carlos Cavalcanti, 4748, CEP 84.030-900, Ponta Grossa, Paraná, Brazil. nice.kuhn@ig.com.br

Alessandra Reis - Universidade Estadual de Ponta Grossa, Departamento de Odontologia, Bloco M, Campus de Uvaranas. Av. General Carlos Cavalcanti, 4748, CEP 84.030-900, Ponta Grossa, Paraná, Brazil , reis_ale@hotmail.com

Ana Claudia Rodrigues Chibinski - Universidade Estadual de Ponta Grossa, Departamento de Odontologia, Bloco M, Campus de Uvaranas. Av. General Carlos Cavalcanti, 4748, CEP 84.030-900, Ponta Grossa, Paraná, Brazil, anachibinski@hotmail.com

Denise Stadler Wambier - Universidade Estadual de Ponta Grossa, Departamento de Odontologia, Bloco M, Campus de Uvaranas. Av. General Carlos Cavalcanti, 4748, CEP 84.030-900, Ponta Grossa, Paraná, Brazil dswambier@yahoo.com.br

Clinical relevance statement: The paper showed that a restoration in young permanent teeth with deep caries lesions enables dentin reorganization when infected dentin remains in the cavity, regardless the lining material used. Therefore, the possibility of pulp exposures and the need of endodontic treatments will decreased in dental practice.

ABSTRACT

Purpose: This study evaluated the impact of the liner material on fluorescence, morphological and mineral characteristics of permanent carious dentin after cavity sealing. *Methods*: Thirty-five children (11.0 ± 2.7 years old) presenting at least one active deep caries lesion in 45 permanent molars, without signs or symptoms of pulpal pathology, were selected. The final sample consisted of 40 teeth from 30 children. Fragments of carious dentin were removed from teeth prior to lining the cavity (baseline samples) with high viscosity glass ionomer cement (G1) or an inert material (wax - G2). Cavities were restored with composite resin, re-opened 60 days later and other fragments were removed (60 days-sample). The laser fluorescence readings, morphological and mineral changes of both groups were compared. *Results:* After 60 days, lower laser fluorescence means were found (Wilcoxon Signed-Rank; $p < 0.05$) and a gain in calcium and phosphorus was detected for both groups (t-test, $p < 0.05$). An uptake of fluorine was only observed in G1 (t-test; $p < 0.05$). Regardless the group, baseline samples presented bacterial invasion and exposed collagen fibers; 60-day samples exhibited a better organized tissue with a more compact intertubular dentin. *Conclusions*: Caries arrestment with dentin reorganization occurs regardless of the dental material placed in contact with the infected dentin.

INTRODUCTION

Deep carious lesions in permanent molars challenge the dental practice in public and private dental clinics since complete caries removal can expose the pulp tissue and require endodontic treatment. Besides that, the clinical evaluation of sound dentin by visual and tactile methods is subjective and depends on the clinician's experience[1].

In the last years, the treatment of open carious lesions has changed significantly. Complete removal of carious tissue is no longer recommended, since partial caries removal [2-6] and even the sealing of infected dentin tissue [7, 8] allows dentin repair, prevents unnecessary tissue loss and permits the conservative treatment of deep carious lesions [2, 4].

In contrast with the stepwise excavation technique[9], a single clinical session (without tooth reopening) is the current trend in caries lesion management [3, 10, 11]. Previous studies reported tissue remineralization [2, 5, 12-14] reduction on the bacterial counts[1, 4, 6, 13, 15], and histological dentin reorganization with intertubular dentin thickening and formation of a dense collagen network, after cavity sealing.

The fact that authors observed exchange of calcium, phosphate and fluorine between demineralized dentin and glass ionomer cements (GIC) [12, 16] led to the discussion about the role of the provisional material on the reorganization of carious tissue. Some authors report that GIC can supply bioactive molecules promoting dentin regeneration [17], others argue that caries arrestment is not dependent on the materials' choice[1, 6, 10, 14].

This controversy highlights the need of further investigation as mentioned in a recent systematic review of the literature [18]. Therefore, this study aimed to evaluate the impact of the liner material on the morphological and mineral changes of carious lesions of permanent teeth after cavity sealing.

METHODS

This research was approved by the Ethics Committee of the State University of Ponta Grossa (Ponta Grossa, Paraná, Brazil) under protocol # 78/2011.

Sample Selection and Inclusion criteria

After an initial screening of 772 subjects from rural area schools, 35 students of both genders with ages ranging from 7 to 15 years (average = 11.0 ± 2.7) were invited for the study. Children with systemic pathologies were not included. Their parents/caregivers were informed about the objectives of this study and signed a consent form permitting their participation.

The participants were submitted to clinical and radiographic examinations for permanent molars selection. These teeth should present deep active caries, code 06 of ICDAS (International Caries Detection and Assessment System)[19], in order to get enough samples for all the tests by manual instrumentation. Lesions of caries should include the inner portion of dentin (2/3 or more of the dentin thickness), but teeth with signs or symptoms of pulp pathology (fistula, tooth mobility, periapical alterations, spontaneous pain) were excluded.

Study design

This clinical study evaluated infected permanent dentin at baseline and 60 days after cavity sealing (60-day), using laser fluorescence (LF) readings, morphological and mineral analysis under scanning electron microscopy (SEM).

Clinical procedures

Each patient attended two clinical sessions. After local anesthesia and rubber dam isolation, teeth were cleaned with a new toothbrush and water, washed thoroughly with air/water spray and air/dried without desiccation.

In order to facilitate the identification of the mesial and distal portions of the cavity, the caries lesion was divided into two equal portions, using a Hollembeck carver in a buccal-lingual direction [4]. The mesial portion was the baseline sample while the distal portion was the 60-day sample. This procedure was used for all the investigated criteria.

LF readings were performed in the mesial portion as described later in this session. A baseline dentin sample was removed using a sterile dentin excavator from the mesial portion while the distal portion was kept at the cavity in order to be removed 60 days after cavity sealing. Immediately after removal, the dentin fragments were processed for morphological and mineral analysis. A single operator removed the carious tissue from cavosurface margin in order to promote appropriate cavity sealing. However, no effort was done to remove the most superficial necrotic dentin layer in the pulp floor.

The teeth were designated to G1 or G2 randomly. In the glass ionomer cement (GIC) group (G1), the pulpal floor of the cavity covered with high viscosity

GIC (Ketac Molar Easy Mix®, 3M ESPE, St. Paul, MN, USA). Polyacrylic acid was not used to aid cavity reopening 60 days after the procedure. In the wax group (G2), a 1-mm thick piece of utility wax (Epoxiglass, Diadema, SP, Brazil) was placed in contact with the pulpal floor.

All cavities were acid etched with 37% phosphoric acid gel (Villevie, Joinville, SC, Brazil), rinsed out with water and slightly air dried. The 2-step etch-and-rinse adhesive (Adper Single Bond 2, 3M ESPE) were applied according to the manufacturer's instructions, and the composite resin (Llis, FGM, Joinville, SC, Brazil) were incrementally placed. After composite placement, a sealant (Fluroshield, Dentsply, Petrópolis, RJ, Brazil) was applied on the restoration margins for extra protection [20]. The light-curing steps were performed with a quartz-tungsten-halogen-light under ramped curing (DX Turbo Led 1200, D-X, Ribeirão Preto, São Paulo, Brazil). The initial light intensity was 450 mW/cm^2 with an automatic increase to 1200 mW/cm^2 after 10 s.

Sixty days after the procedure, the marginal integrity of the restorations was evaluated, using United States Public Health Service (USPHS)[21], criteria: (1) retention/fracture; (2) anatomic form; (3) marginal adaptation; (4) marginal discoloration; (5) recurrent caries; (6) post-operative sensitivity; (7) surface texture; (8) color match. Those variables were ranked in the following scores: alfa (no defect clinically detectable, needing just a polish), bravo (clinically acceptable, but repair is needed) and charlie (clinically unacceptable, needs restoration replacement). If there were signs or symptoms of pulp pathology, as well as restorations defects that compromised cavity sealing, the tooth was excluded.

Then, the restorative material was removed with a sterile diamond bur (#1092-KG Sorensen, São Paulo, Brazil) under water cooling until reaching the GIC and the wax. Manual instrumentation was used for complete removal of the material. LF readings were taken from the distal portion of the cavity. Then, a dentin sample, from the distal portion, was removed for morphological and mineral analysis in the same manner as described for the mesial portion.

Laser fluorescence (LF) readings

Dentin LF was measured with DIAGNOdent 2095 (KaVo, Biberach, Germany) following the manufacturer's instructions. The device was calibrated against a provided porcelain reference object prior the exam and then it was re-calibrated on a sound surface of every tooth. The values of LF may range from 0 to 99. Optimal cutt-off limits of the LF device to detect in vivo occlusal caries lesion depth are 0-14 - sound teeth; 15-21 - enamel lesions; 22-37 - caries lesion in the outer half of the dentin; >38 - caries lesion in the inner half of the dentin[22] . Three measurements on dentin in the mesial (baseline measurement) or distal portion (60 days after sealing) of the cavity were taken, and the mean value in each period was calculated to represent the individual tooth.

Morphological and mineral analysis

Dentin fragments from the mesial (baseline) and distal (60-day sample) portions were stored into a 2% glutaraldehyde solution with a sodium phosphate buffer of 0.1M (pH 7.4) for 7 days, rinsed thoroughly with 50% sodium phosphate buffer (0.3 M) and distilled water solution (three 30-min rinses) and dehydrated in acetone 30%, 50%, 70% for 10 min; 90% for 20 min, 100% for 10 min and 100% for 20 min. Samples were kept at 37° C for at least 3 days to remove any residual water.

Specimens were mounted on stubs and sputter-coated with a 10 nm gold layer. They were analyzed in the SEM using secondary electron mode with a voltage of 12 KV. The same sample was analyzed using dispersive X-ray spectrometry energy (EDS) to measure the relative percentage of calcium, phosphorus and fluorine among the twelve most common chemical elements in the dentin substrate. The weight percentages of calcium, phosphorus and fluorine, before and after cavity sealing were assessed at a magnification of x 200 for 100 s.

All the pictures were analyzed by the same examiner, who was blinded to the study groups. The characteristics evaluated were the presence of bacteria, the collagen network, the mineralization of inter e peritubular dentin. After this analysis, one representative image of each condition was selected for publication.

Statistical analysis

The tooth was employed as an experimental unit for data analysis. The Statistical Package for Social Sciences (SPSS® 17.0, Chicago, Illinois, USA) program was used for statistical analyses with the significance level set at 0.05. Before submitting the data to statistical analysis, the Kolmogorov–Smirnov test was performed to assess whether the data followed a normal distribution and the Barlett´s test was used to test if the assumption of equal variances was valid. As the data from the LF reading failed in both ANOVA assumptions ($p < 0.05$) we used non-parametric statistics for data analysis. In each group, the LF readings before and after cavity sealing were compared by Wilcoxon Signed Rank test. In each period, the LF readings of the GIC and wax groups were statistically analyzed with the Mann-Whitney test.

For the mineral data, the ANOVA assumptions were valid ($p > 0.05$) and therefore the more robust parametric statistics were employed. For each participant, the percentage value of each mineral obtained at 60-day was subtracted from the baseline value. The paired t-test was used to compare the differences in wt% of calcium, phosphorus and fluorine between the two study groups.

RESULTS

Of the 45 permanent molars at baseline, four teeth were not evaluated in the 60-day assessment: one had pulp necrosis (from GIC group) and three students did not attend the 60-day recall.

Clinical and radiographic evaluation

At baseline, all restorations were classified as alpha according to the USPHS criteria. After 60 days, only one restoration was classified as charlie, and it was not included in the sample. Thus, a total of 40 teeth, from 30 patients, were available for analysis. No radiographic changes or symptoms was seen 60 days after cavity sealing.

Laser fluorescence readings

No significant difference in LF readings was detected for GIC and wax groups in both periods (Mann-Whitney test, $p > 0.05$). Significant reductions in LF means were detected between periods for both groups (Wilcoxon Signed-Rank test, $p < 0.05$) (Table 1).

Morphological and mineral analysis

Baseline samples from both groups showed a highly infected tissue and a clearly exposed collagen matrix (Figures 1 and 2). After cavity sealing, fewer bacteria

with a more compact arrangement of collagen fibrils were seen in both groups (Figures 1 and 2). A similar gain in calcium and phosphorus was detected for both groups (t-test, $p > 0.05$; Table 2). An uptake of fluorine was significantly detected only for the GIC group (t-test, $p = 0.032$, Table 2).

DISCUSSION

The clinical success of conservative procedures (stepwise excavation/ indirect pulp capping) is achieved when the vitality of pulp is maintained, and no adverse symptoms are reported after treatment. In the present study, only one tooth out of 41 available for analysis was excluded for pulp necrosis. This results in an overall success rate of 98% (95% confidence interval 88 – 100%) which is in agreement with other published papers [13, 23].

Although this study was not a pioneering research in demonstrating caries arrestment after dentin carious lesions sealing [2, 4, 13] few papers compared a bioactive vs. an inert material as liner [6, 14, 24]. This article provided body of evidence that dentin reorganization and mineral changes were not dependent on the material placed in contact with the carious tissue, suggesting that the carious arrestment is a host-driven process rather than a material-induced process [6, 14, 24].

The concept that dentin remineralization is a material-driven process was based on the benefits of fluoride in the enamel remineralization [25, 26]. Some authors believe that fluoride release from GIC could favor the remineralization of carious dentin[5, 12, 18, 28]. However, if fluoride were necessary for dentin remineralization, we should have detected remineralization (by increased percentage of calcium and phosphorus) only in the GIC group, and this was not observed.

It is the cessation of caries process that allows biological response of the teeth. The sealing of the cavity isolates bacteria from the oral environment and active biofilm[5, 29, 30], arresting the carious process and providing time for host pulp-dentin complex defense. In general, repair and regeneration in the dentin-pulp complex are similar to the natural wound healing responses seen in many of the body's systems[31]. To respond to the inflammatory process induced by the caries lesion, odontoblasts produce a reactionary tertiary dentin matrix[31, 32] along with high growth factors secretion[33], in an effort of remodelling and repairing the extracellular matrix, damaged by the disease process[34]. Thus, the dense collagen matrix seen 60 days after sealing is the result of tissue remodeling. Considering that the used method is a destructive one, the obtained images are not from the same area, but the dentin sample collected was from the same tooth (which was re-opened after 60 days) and the same depth. This was possible because the clinically visible residue of carious tissue on the cavity floor was divided into 2 parts (bucco-lingual direction)[4].

The reduction of LF mean after cavity sealing is consistent with the bacterial reduction detected in the SEM images. The lower LF readings correlates with the reduction on bacterial aggregates and their metabolic by-products like protoporphyrin IX, meso-porphyrin and copro-porphyrin[35, 36], but not the mineral content of the dentin[37]. However, the LF mean in the 60-day sample is still far from the LF mean reported for sound dentin (which ranges between 3 and 6), and dentin caries (which ranges between 35 and 40)[23, 38]. A possible explanation is that the residual metabolic products can still be readable by LF device even after cavity sealing. Thus, we doubt if this device is an appropriate tool for diagnosis of dentin caries arrestment.

By no means has the present study suggested the placement of an inert material (such as wax) inside the cavity. The selection of the liner/provisional or

definitive restorative material should consider its biocompatibility, longevity, as well as its bonding ability to the dental structures. Lining the cavity with a GIC, in the so-called sandwich technique[39-41], may solve the problems related to deficient bonding of adhesive systems to caries-affected and caries-infected dentin[39-41].

CONCLUSION

Caries arrestment with dentin reorganization occurs regardless of the dental material placed in contact with the infected dentin.

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FIGURE LEGENDS

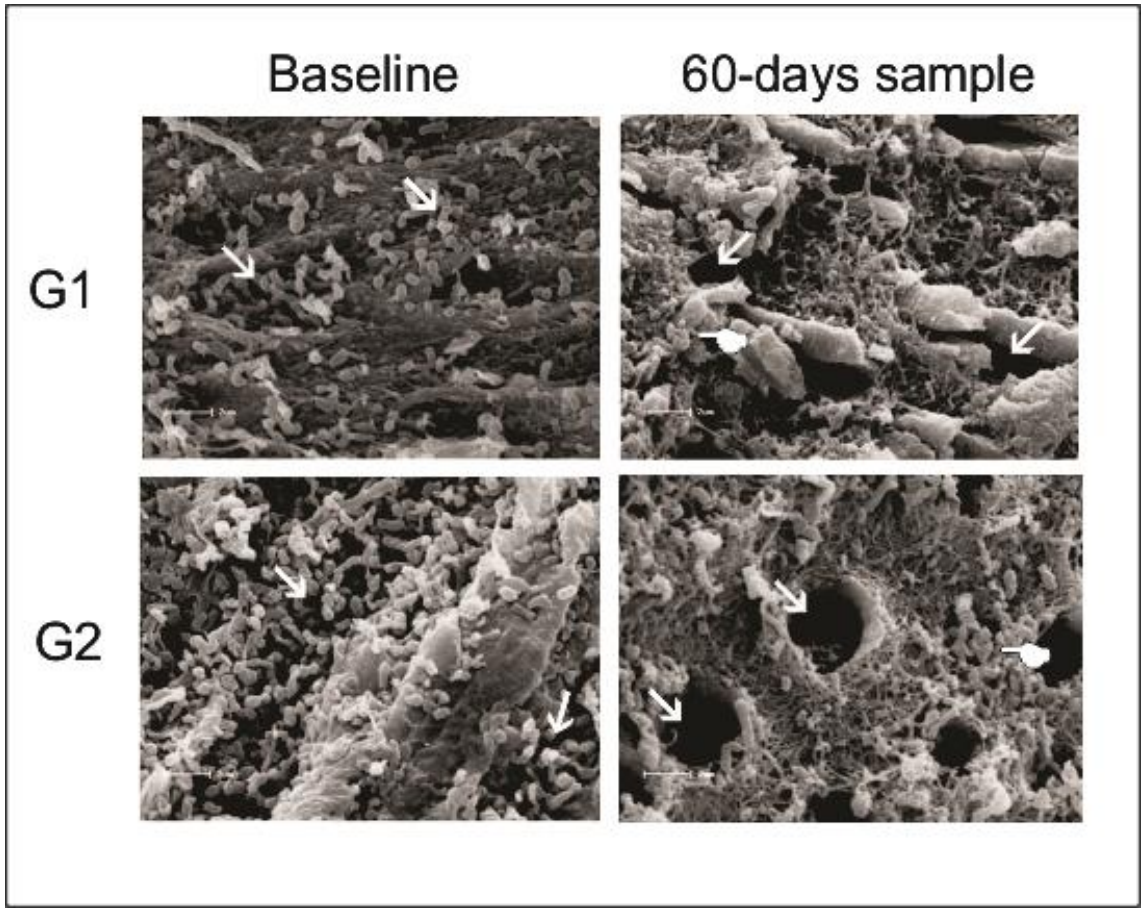
Figure 1 - Bacterial aggregates were seen at baseline for both groups (arrows). After restoration, we observed some dentinal tubules without microorganisms (arrows) for G1 and G2, and a decrease in the bacterial contamination is evident, although some microorganisms can still be seen in the intertubular dentin (pointer).

Figure 2 - The demineralized dentin tissue with exposed collagen fibers were observed at baseline for both groups (arrows). The 60-day samples exhibited a more compact tissue, particularly on G1. But the remineralization process also occurred on G2 in peritubular and intertubular dentin (arrows).

TABLE LEGENDS

Table 1 - Means and standard deviations of laser fluorescence for both groups at baseline and in the 60-day follow-up.

Table 2 – Means and standard deviations of the 60-day vs. baseline difference in the weight percentage of calcium, fluorine and phosphorus for both groups.


Baseline
60-days sample
G1
G2

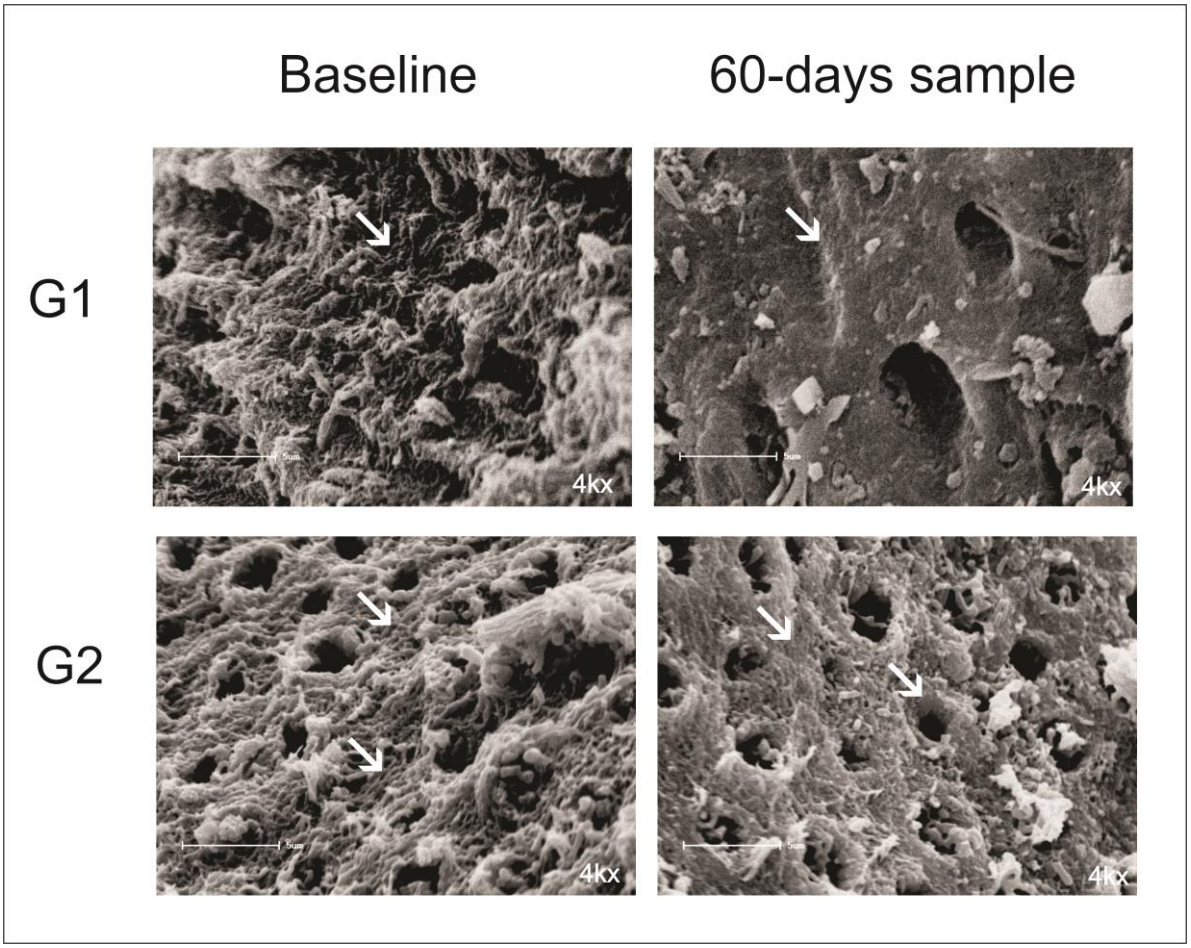

Baseline
60-days sample
G1
G2
4kx
4kx
4kx
4kx

Table 1 - Means and standard deviations of laser fluorescence for both groups at baseline and in the 60-day follow-up.

Groups(n.teeth)	Baseline	60-days	p-value*
G1(21)	86.5 ± 19.3	62.9 ± 28.2	< 0.001
G2(19)	83.5 ± 22.4	69.3 ± 29.4	0.002
p-value**	0.495	0.495	

(*)Wilcoxon Signed-Rank test (α = 0.05); (**)Mann-Whitney test (α = 0.05)

Table 2 – Means and standard deviations of the 60-day vs. baseline difference in the weight percentage of calcium, fluorine and phosphorus for both groups.

Groups(n.teeth)	Calcium	Fluorine	Phosphorus
G1 (21)	6.0 ± 6.5	0.1 ± 1.2	1.1 ± 3.3
G2 (19)	5.2 ± 6.4	-0.5 ± 0.6	1.6 ± 3.1
p-value*	0.715	0.032	0.66

** Student's t test (α = 0.05)*

4.2 ARTIGO 2

The role of glass ionomer cement on remineralization of infected caries sealing: An in vivo study

Eunice Kuhn[1], DDS, MS, PhD; Ana Claudia Rodrigues Chibinski[2], DDS, MS, PhD; Alessandra Reis[3], DDS, PhD; Denise Stadler Wambier[4], DDS, MS, PhD.

[1] Dr. Kuhn is an adjunct professor, Public Health Dentistry; [2] *Dr. Chibinski is an adjunct professor, Pediatric Dentistry;* [3] *Dr. Reis is an adjunct professor, Restorative Dentistry;* [4] *Dr.Wambier is an associate professor, Pediatric Dentistry,*
all in the School of Dentistry, Ponta Grossa State University, Ponta Grossa, Paraná, Brazil
Correspond with Dr. Kuhn at nice.kuhn@ig.com.br

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Corresponding author:

Eunice Kuhn

Universidade Estadual de Ponta Grossa

Departamento de Odontologia – Campus Uvaranas

Av. General Carlos Cavalcanti, 4748 - CEP 84030-900

e-mail: nice.kuhn@ig.com.br

Disclosure Statement

The authors state that there is no personal or financial conflict of interest for the study development that might introduce bias or affect their judgment.

Abstract

Objectives: This study investigated the role of the lining material on infected dentin. Methods: Forty-five permanent molars with deep caries lesions were selected from 35 children. Fragments of carious dentin were removed prior to lining the cavity (baseline sample) with high viscosity glass ionomer cement (G1) or an inert wax material (G2). Cavities were restored with composite resin, re-opened 60 days later and other fragments removed (60-day sample). The dentin morphology and mineral content (calcium, phosphorus, fluorine) of both samples were assessed under SEM. At the follow-up periods (60 days; 10-15 months), restorations were evaluated and standardized radiographs taken. A post-processing routine was used to identify changes in radiographic density between periods. Results: After 60 days, the dentin exhibited a better tissue organization, fewer bacteria and signs of remineralization. The wt% of calcium ($p<0.001$) and phosphorus ($p=0.003$) was higher 60 days after cavity sealing regardless of the group. Higher gray levels of carious and sound dentin were seen on the 10-15 month radiographs, irrespective of the group. The success rate of G1 and G2 were 89% and 88%, respectively. Conclusions: The lining material is not fundamental for caries arrestment. Early (60-day) and late (10-15 months) dentin changes occurred, indicating remineralization of dentin carious tissue.

Introduction

For a long time, the complete removal of carious tissue was considered the best strategy for the treatment of caries lesions [1, 2]. However, there is a high risk of pulp exposure when managing deep caries lesions [3], and the endodontic treatment is inaccessible to a significant part of the population from poor communities, especially those in rural areas. Other potential disadvantages are that the removal of carious tissue is based on subjective criteria and operator's tactile and visual perceptions [2, 4-6]. This fact reduces the predictability of the procedure and makes it very technique-sensitive.

Therefore, the management of deep caries lesions in young patients represents a challenge for clinicians. Similarly to the indirect pulp capping, the step-wise excavation [7, 8] advocates the partial carious tissue removal and a calcium hydroxide liner over the remaining carious tissue [4, 9]. The cavity should be re-opened 30 to 45 days later to remove the residual carious tissue. Although step-wise excavation has shown high rates of clinical success [7], it requires a second clinical session which increases the costs.

More recently, studies have reached clinical success without this second step of caries excavation [9-11]. In this new protocol, after partial caries removal, the infected and affected dentin is left at the bottom of the cavity floor [9, 12] followed by proper cavity sealing. A marked reduction on the number of bacteria over time [6, 13-16] suggests that the isolation of the caries lesion from the cariogenic biofilm is a reasonable approach for the management of deep carious lesions [17, 18].

The use of glass ionomer cements (GIC) has potential advantages over the other restorative materials for cavity sealing after partial caries removal. For instance, GIC bonds well to the moist tissue [19] and releases fluoride, which may aid in the remineralization of the carious lesions [1, 19, 20]. So far, only a few clinical studies have evaluated the role of GIC in sealing infected dentin [9, 21] and they showed that carious dentin tissue reorganizes in a short 60-day period [21].

However, the real role of fluoride from GIC in the caries lesion remineralization has not been determined yet. The positive changes in demineralized carious tissue in

contact with an inert material [4, 6, 10, 16] challenge the concept that the use of a bioactive GIC is essential to induce remineralization of the dentin carious lesions.

Therefore, the purpose of this study was to investigate the effect of a glass ionomer cement as a liner over infected unexcavated dentin after 60 days and 10-15 months.

Materials and methods

This research was approved by the Ethics Committee of the State University of Ponta Grossa (Ponta Grossa, Paraná, Brazil) under protocol # 78/2011.

Sample Selection and Inclusion criteria

After an initial screening of 772 subjects from schools in the rural area of Ponta Grossa (Paraná, Brazil), 35 students of both genders with ages ranging from 7 to 15 years (average = 11.0 ± 2.7) were invited for the study (Figure 2). Children with systemic conditions were not included. Their parents/caregivers were informed about the objectives of this study and signed a consent form permitting their participation.

There were excluded 338 patients with caries free molars, 349 patients with incipient molar caries, 41 molars with deep caries , and 9 patients for other reasons. The reasons for exclusion were the presence of fistula, tooth mobility, spontaneous pain, destroyed tooth crown that make restoration not possible or the treatment was not authorized.

The selected participants were submitted to clinical and radiographic examinations for tooth selection. Teeth should present active and open deep caries lesion in the occlusal surface of permanent molars. Lesions should include the inner portion of dentin (2/3 or more of the dentin thickness) and should be classified with an ICDAS code 06 (International Caries Detection and Assessment System) [22].Therefore, this study sample consisted of 45 permanent molars.

Study design

The dentin morphology and mineral content carious dentin of permanent molars were evaluated at baseline and 60 days after cavity sealing. The further long-

term assessment of remineralization could not be assessed using laboratory analysis since this would require sample removal. Thus, changes in the remineralization from the 60-day sample up to approximately 10 to 15 months were evaluated by radiographic assessment.

Clinical procedures

Each patient attended three clinical sessions. After local anesthesia and rubber dam isolation, teeth were cleaned with a new toothbrush and water, washed thoroughly with air/water spray and air/dried without desiccation.

In order to facilitate the identification of the mesial and distal portions of the cavity, the caries lesion was divided into two equal portions, using a Hollenbeck carver in a buccal-lingual direction [14, 21]. The mesial portion was the baseline sample while the distal portion was the 60-day sample.

The baseline sample was removed from the mesial portion, using a sterile dentin excavator. The distal portion was kept at the cavity in order to be removed 60 days after cavity sealing. Immediately after removal, the dentin fragments were processed for morphological and mineral analysis. A single operator removed the carious tissue from the cavo-surface margin in order to promote appropriate cavity sealing. However, no effort was done to remove the most superficial necrotic dentin layer from the pulp floor.

The teeth were then randomly assigned to two groups. A sortition was carried out to define the first material to be used. The second tooth was restored with the other material and so on until the last selected tooth.

In group 1, a high viscosity GIC (Ketac Molar Easy Mix®, 3M ESPE, St. Paul, MN, USA) was placed on the pulp floor of the cavity. Polyacrylic acid was not used to aid cavity reopening 60 days after the procedure. In group 2, a 1-mm thick piece of an inert material (Utility wax, Epoxiglass, Diadema, SP, Brazil) was placed in contact with the pulp floor. After lining, all cavities were acid etched with 37% phosphoric acid gel (Villevie, Joinville, SC, Brazil), rinsed out with water and slightly air-dried.

The 2-step etch-and-rinse adhesive (Adper Single Bond 2, 3M ESPE, St. Paul, MN, USA) were applied according to the manufacturer’s instructions and light-cured

for 10s. Then, the composite resin (Llis, FGM, Joinville, SC, Brazil) was incrementally placed and light-cured for 40s. After composite resin placement, a sealant (Fluroshield, Dentsply, Petrópolis, RJ, Brazil) was applied on the restoration margins for additional protection against marginal infiltration [23]. The light-curing steps were performed with a quartz-tungsten-halogen-light under ramped curing (DX Turbo Led 1200, D-X, RibeirãoPreto, São Paulo, Brazil). The initial light intensity was 450 mW/cm^2 with an automatic light intensity increase to 1200 mW/cm^2 after 10 s.

Sixty days after the procedure, the marginal integrity of the restorations was evaluated, using United States Public Health Service (USPHS) [24]: (1) retention/fracture; (2) anatomic form; (3) marginal adaptation; (4) marginal discoloration; (5) recurrent caries; (6) post-operative sensitivity; (7) surface texture; (8) color match. Those variables were ranked in the following scores: alfa (no defect clinically detectable, needing just a polish), bravo (clinically acceptable, but repair is needed) and charlie (clinically unacceptable, needs restoration replacement). If signs or symptoms of pulp pathology, described earlier in this section were observed, as well as restorations defects that compromised cavity sealing (score charlie in any of the USPHS items 1, 3, 4, 5, 6), the tooth was submitted to appropriate restorative and/or endodontic treatment. In this case, samples could not be collected in the 60-day recall as well as the radiographic density could not be evaluated 10-15 months later.

After clinical assessment, under local anesthesia and rubber dam, the restorative material was removed with a sterile diamond bur (#1092- KG Sorensen, São Paulo, Brazil) under water cooling until reaching the GIC and the wax. Manual instrumentation with spoon excavator was used to complete the removal of the material. Then, a dentin sample, from the distal portion, was collected for morphological and mineral analysis in the same manner as described for the mesial portion. No dentin was removed besides the samples collected at the 60-day session leaving carious infected and affected dentin on the pulpal floor. After that, all the teeth were restored with composite resin. For G1, the glass ionomer lining was kept. For G2, wax was removed and tooth restored with composite resin".

Morphological and mineral analysis

Dentin fragments from the mesial (baseline) and distal (60-day) portions were stored into a 2% glutaraldehyde solution with a sodium phosphate buffer of 0.1 M (pH 7.4). After 7 days, the specimens were rinsed thoroughly with 50% sodium phosphate buffer (0.3 M) and distilled water solution (three 30-min rinses). Next, they were dehydrated in acetone 30%, 50%, 70% for 10 min; 90% for 20 min, 100% for 10 min and 100% for 20 min. Samples were kept at 37°C for at least 3 days to remove any residual water. Specimens were mounted on stubs and sputter-coated with a 10 µm gold layer.

They were analyzed in the SEM using secondary electron mode with a voltage of 12 kV. The same sample was analyzed using dispersive X-ray spectrometry energy (EDS). The relative weight percentages of calcium, phosphorus and fluorine over the total content of the most common twelve chemical elements in dentin were assessed before and after cavity sealing at a magnification of x 200 for 100 s.

Radiographic examination

Periapical radiographs were used for initial diagnosis and sample selection. From 60 days on, changes in the dentin tissue could not be assessed by SEM evaluation as this would require the reopen of the teeth and increase the possibility of pulp exposure. Thus, radiographs taken at the 60-day and 10 to 15-month recalls were compared one another.

For this purpose, a standardized film holder was made for each patient. The film holder (Indusbello, Londrina, PR, Brazil) was individualized [25]. Self-cure acrylic resin (Jon, São Paulo, SP, Brazil) was applied to the film holder, and an impression of the treated tooth and its antagonist was obtained. This bite registration allowed the device to be placed in the same position after the follow-up period. Using the shaft present on the film holder and the markers on the X-ray cylinder, all radiographs were standardized in vertical and horizontal directions, as well as film-focus distances.

All the radiographs were taken with Insight #0 film (Eastman Kodak, NY, USA) and a Timex 70E equipment (Gnatus, RibeirãoPreto, SP, Brazil). Exposure parameters were set at 70 kVp, 7 mA and 0.4 s. Development and fixation procedures were carried out using an automatic processor (Revell, São Paulo, SP,

Brazil) with fresh solutions (Eastman Kodak, Worchester, USA). Radiographs taken at 60 days and after the follow-up period were digitized using a scanner with transparency adapter (HP Scanjet G4050, Hewlett-Packard, CA, USA) with a resolution of 600 dpi and 8-bit gray scale. The images were stored as a maximum-quality tagged image file format (TIFF).

Radiographic Analysis

Small differences in projection angles during exposure or processing still occur even using standardized techniques. In order to reduce these differences, the images were imported to Regeemy Image Registration and Mosaicking (version 0.2.43-RCB, DPI-INPE, São José dos Campos, SP, Brazil). This software provides image registration and image subtraction algorithms – *i.e.*, corrects geometric discrepancies and equalizes the contrast of 2 sequential radiographs in order to make them comparable and subtracts the analog pixels values from two sequential images.

The radiograph taken right after the placement of the restoration in the 60-day recall was the reference image (Figure 1A) and were put side-by-side with radiographs taken 10-15 months later (Figure 1B). Reference points (immutable points in both radiographs) were selected on both images manually. These reference points served as coordinates for the software to align and correct the geometry of the second image according to the reference one. Clearly distinguishable structures on tooth were selected as reference points, like cementum-enamel junction and cusps (Figure 1 A and B). The quality of image registration was visually determined with image subtraction routine.

The image registration was considered adequate when the structural noise was reduced to the lowest-possible level (Figure 1C). Structural noise can be defined as a discrepancy in the geometrical position between images, which appears as brighter or darker shades of gray in the subtracted radiograph. Figure 1C exhibited the least structural noise possible on the tooth of interest, therefore the registered image was generated (Figure 1D). Figure 1D was then considered the new follow-up radiographic without small differences in projection angles during exposure or processing.

The subtracted image represents the changes between Figure 1A and Figure 1D. All unchanged anatomical background structures are shown as a perfect subtraction image in neutral gray shade (gray level = 128 from 255 shades of gray) [17]. If alterations between the two moments of evaluation were detected, the changed areas were displayed in a darker (< 128) or lighter gray level (> 128), which represents mineral loss or mineral gain, respectively. Visual examination of subtracted image was accomplished. If changes were identified, a second analysis was done to quantify them by measuring means of gray pixels levels.

Figure 1A (60-day) and Figure 1D (10-15-month evaluation; registered *a posteriori*) were saved in uncompressed TIFF file and imported into Adobe Photoshop CS6 (version 12.0x32, Adobe Systems Incorporated, San Jose, CA, USA) to quantify the changes in the remaining dentin during the follow-up period. The changed dentin area under restoration marked on subtracted image was selected using the polygonal lasso tool and transported to Figure 1A. The mean gray level of the selected area was obtained using the expanded histogram function. The same area was transported into Figure 1D, and its mean gray level was measured in the same way. A high mean gray level indicates mineral gain. This analysis was repeated 3 times for each tooth, and the average value of the three measurements of the area under investigation was taken.

The same measurement was done on three areas judged to be sound in each tooth, and these values were averaged to provide a value for the tooth. Standardized areas were delimited with the rectangular marquee tool. Measurements followed the same routine described earlier. All the analyses were performed by a single and calibrated operator, blinded to the study objectives.

Statistical analysis

The tooth was employed as an experimental unit for data analysis. The Sigma Plot for Windows version 11 (Systat Software Inc., GmbH, Germany) program was used for statistical analyses with the significance level set at 0.05. Data from calcium, fluoride and phosphorus were individually subjected to a two-way repeated measures ANOVA and Tukey's test for pairwise comparisons ($\alpha = 0.05$).

The data from the mean levels of gray presented normal distribution, according to the Kolmogorov-Smirnov test (p = 0.493), therefore, the data was submitted to a three-way repeated measures ANOVA testing the Groups (Group 1 and 2), Condition of dentin (carious or sound) and Assessment time (60-day and 10-15 month) as the main factors. Tukey´s test was used for pairwise comparisons.

The per-protocol success rates and the 95% confidence interval for both groups were calculated. Teeth with pulp vitality and clinically acceptable restorations were considered cases of success. Failure was defined as the presence of either a clinical symptom (pain, swelling, or fistula) or radiologic abnormality at any of the study recalls.

Results

From the total of 45 permanent molars at baseline, five teeth were not evaluated in the 60-day assessment: one had pulp necrosis (Group 1 GIC), one showed dental fracture (Group 2 wax) and three students did not attend the 60-day recall (Figure 2). Thus, a total of 40 teeth, from 30 patients, were available for the 60-day evaluation.

After 10-15 months, four patients moved to other cities and two teeth presented pulp necrosis (Figure 2). Thus, only 31 teeth, from 26 patients were available for the radiographic analysis.

Clinical evaluation

At baseline, all restorations were classified as alpha according to the USPHS criteria. After 60 days, only one restoration from group 2 was classified as Charlie in the item retention/fracture. After 10-15 months, all restorations were classified as alpha in the USPHS items; however two of them showed pulp necrosis (Figure 2).

Morphological and mineral analysis

The morphological and mineral analysis was evaluated in 40 samples of infected dentin, being 21 and 19 from group 1 and 2, respectively. Baseline samples

from both groups showed a highly infected tissue and a clearly exposed collagen matrix (Figures 3 and 4). After cavity sealing, reduced number of bacteria with a more compact arrangement of collagen fibers were seen in both groups (Figures 3 and 4).

The means and standard deviations of the weight percentage of calcium, phosphorus and fluorine are shown in Table 1. A significant increase in the weight percentage of calcium ($p < 0.001$) and phosphorus ($p = 0.003$) were observed after 60 days for both groups. For fluoride, no significant difference was observed among groups ($p > 0.05$).

Radiographic Analysis

The radiographic analysis was evaluated in 31 teeth, being 16 from group 1 and 15 from group 2. The mean gray levels measurements of the baseline and 10-15 month radiographs are depicted in Table 2. Three-way ANOVA detected a significant effect only for the main factor Time ($p < 0.001$). Significant higher gray levels of carious and sound dentin were seen on the 10-15 month radiographs ($p < 0.001$), irrespective of the group.

Treatment success rate

After 10-15 months, 16 and 15 restorations from the groups 1 and 2 were available for evaluation. During this period, a total of 4 restorations failed clinically due to pulp necrosis (n = 2 teeth from group 1; and n = 1 tooth from group 2) and dental fracture (n = 1 tooth from group 2). Therefore, the success rate of groups 1 and 2 were 89% (95% CI 67 – 97%) and 88% (95% CI 66 – 97%), respectively. As no significant difference was observed between the groups, we calculated the overall success rate of study as being 89% (95% CI 74 – 95%).

Discussion

As reported in other clinical studies, carious dentin exhibits poor organization, with loose exposed collagen fibrils and evident microbiological contamination [14, 26, 27]. After 60 days of cavity sealing, signs of remineralization, *i.e.* histological dentin

reorganization with intertubular dentin thickening and formation of a dense collagen network [4, 13, 17, 26] and reduction of the viable bacteria are usually seen [14, 26, 27].

Other study on partial caries removal in permanent teeth showed similar results [16]. The authors found dentin hardening and decreased bacterial infection after lining the cavity with glass ionomer cement, calcium hydroxide cement or wax. Our paper goes further, since we didn't remove infected dentin and we show additional data about mineral content and radiographic follow-up.

Some authors believe that the repair of the carious lesion is the result of the possible role of fluorine, from the GIC, on the dentin remineralization [20, 28] However, the results of this study challenge this concept. The uptake of fluorine over the 60-day period was not statistically different between the study groups perhaps because we used different methodology [1] or even, different type of glass ionomer[19], however, this did not seem to alter the increase of calcium and phosphorus content, the reduction of bacteria, or the better dentin reorganization we observed. All of these findings occurred irrespective of the presence of a fluorine-releasing liner.

This suggests that dentin healing is not associated with the liner placed in contact with the infected dentin. Instead, it is likely that the isolation of the carious tissue from the dental biofilm [29] plays the most influential role on the repair process. After cavity sealing, only bacteria able of producing the enzymes to cleave the terminal sugars from the glycoproteins were recovered from the dentin[30]. An effective cavity sealing changes the environment of the lesion, stimulates tubular sclerosis, reduces permeability of the dentine and the amount of viable bacteria under restorations [30], which is a decisive factor in the dynamics of lesion progression. Microorganisms deprived of sugar become less metabolically active and allows caries arrestment.

Interference with the cariogenic environment via cavity sealing promotes favorable conditions and enhances defensive responses of the pulp-dentin complex by hard tissue formation. Although dentin formation is an age-related physiologic process, dental caries leads to inflammatory events that stimulate this defense mechanism. It has been suggested that bioactive molecules, especially transforming growth factor (TGF)-s, in the dentin matrix signal the differentiation of odontoblast-like

cells in order to replace the lost odontoblast cells and regulate reparative dentinogenesis [17, 31].

Pulp-dentin reactions of teeth with deep caries lesions may have promoted the increased mineral content observed in our study, allowing maintenance of pulp vitality. The pH neutralization is also vital as matrix metalloproteinases (MMPs) remain active [32]. It was already reported that MMPs may play important roles in tissue morphogenesis, development and remodeling [33]. Altogether these factors allow host-defense reactions of the dentin-pulp complex [17, 18] with resulting deposition of calcium (Figures 3 and 4).

This concept, *i.e.,* that dentin repair is not material dependent, is shared by other authors [4, 10, 16] and, thus cannot be considered a novel finding. The novelty of the present investigation is that we have not performed partial caries removal as most of the earlier studies. Instead, we have intentionally left infected dentin at the bottom of the cavity, and this did not seem to jeopardize dentin repair, as a high success rate was obtained. The literature shows success rates of 92% after a 3.5-4.5 year follow-up [7], 95% after a 6-year follow-up [34] and 96% after a 3-year follow-up [9]. These studies, as well as systematic reviews [35-37] indicated success rates up to 90%, which are similar to the success rate of 89% obtained in the present research. From a clinical standpoint, the results of this study facilitate the operative management of deep caries lesions. Clinicians no longer need to be concerned about the differentiation between infected vs. affected dentin during caries excavation.

To assess late changes on the dentin structure, we could not re-open the cavity again. To manage this problem we employed a non-destructive method to measure the mineral changes in a longer term. In this way, digital subtraction radiography (DSR) compared the grey levels of carious dentin in both follow-up periods (60-day and 10-15 month). This technique compares pairs of radiographs taken in different moments and identifies minimal variations in mineral content [17]. In this study, the DSR technique showed increased radiographic density on 10-15 months follow-up period, indicating mineral gain and dentin remineralization, regardless of the liner used. This finding is consistent with other studies in permanent [13, 16, 17, 25] and deciduous teeth [21].

It is noteworthy mentioning that not only caries-affected dentin showed increased gray levels after 10-15 months of clinical service, but also dentin areas judged to be sound. A possible explanation for this fact is that mineralization occurs as a continuous process in the whole dentin-pulp complex. Cavity sealing may entirely change the environment and the pathogenicity of the biofilm in the carious cavity [29]. Increased gray levels on radiographs suggest mineral deposition for the formation of hard tissue, which could be deposition of tertiary dentin, sclerosis or both [25].

The protocol described in this paper is interesting for a vital tooth with a deep caries lesion, especially in young patients. As the carious tissue is not removed, the risk of pulp exposure is reduced compared to complete caries excavation [3, 38]. There is no need to reopen the cavity and perform a second excavation for pulp vitality to be preserved [39]. Thus, keeping infected dentin in the cavity increases the probability to maintain the pulp vitality[39] and results in similar outcomes as incomplete excavation techniques[8] reduces the procedure costs since pulpotomies will be rarely required.

Based on that, the clinician's main concern should not be the material to be placed in contact with the carious lesion but the correct diagnosis of the pulp condition. In the present study, we only included teeth with no signs of pulp pathology. We believe that the three cases that showed pulp necrosis was due to inaccurate diagnosis of the pulp vitality at baseline.

Although similar dentin healing was observed in both study groups, this study does not aim to suggest the placement of a wax inside the dental cavity. This study was just designed to prove the concept that dentin remineralization is not material- but a host-driven process. The selection of the restorative material should consider several aspects such as its biocompatibility, longevity, as well as its bonding ability to the dental structures.

For instance, the material most employed for indirect pulp capping is calcium hydroxide [13, 16, 17, 25, 40-42]. Calcium hydroxide is capable of solubilizing bioactive proteins (such as TGF-β1) previously sequestered in the dentin matrix during development. The release of these molecules may stimulate the natural healing process of the tooth by two different mechanisms. Existing cells can be stimulated to

produce extracellular matrix for the deposition of new mineral, through the process of reactionary dentinogenesis or new cells can be recruited and after differentiation into odontoblast-like cells produce new mineral through reparative dentinogenesis [20].

GIC also offers additional advantages. Lining the cavity with a GIC, in the so-called sandwich technique [43, 44] solve the problems related to deficient bonding of adhesive systems to caries-affected and caries-infected dentin [45, 46].

Taken all together, we conclude that: 1.this study provides evidence that the lining material placed on infected dentin is not fundamental for caries arrestment; 2. when the cavity is properly sealed, carious dentin tissue tends to reorganize in a short period (60 days), and the remineralization process still continues for longer periods.

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Legends

Table 1 – Means and standard deviations of the percentage of calcium, fluoride and phosphorus for G1 (glass ionomer cement liner) and G2 (wax) at baseline and 60 days after sealing (N=40 teeth).

Table 2 - Means ± standard deviations of gray levels values of caries-affected and sound dentin from permanent teeth at baseline and 10-15 month radiographs G1 (glass ionomer cement liner) (n=16) and G2 (wax) (n=15).

Figure 1 - Illustrative images of post-processing procedures of dental radiographs. Radiographs of 60 days and 10-15 month follow-up are seen in A and B, respectivelly. Red dots in A and in B indicate the reference points points that aligned 1B to 1A before the image subtraction radiograph was obtained (C) (Image A - Image B). Success was achieved in subtraction, as structural noise was not observed for tooth #30. Structural noise was seen in the adjacent tooth (*). A brighter area (arrow) can be seen above the pulp chamber, indicating mineral gain. In D there is a recorded image obtained after computer alignment of the scanned images. Gray levels were measured by comparing the image A (60 days) with the image D (10-15 months of follow-adjusted geometric projection).

Figure 2 – Flowchart of the study design, showing the number of restorations evaluated in each recall. Np = number of patients; Nt = number of teeth.

Figure 3 - Bacterial aggregates can be seen at baseline for both groups (arrows). After restoration, it is possible to observe dentinal tubules free from microorganisms (pointer) in G1 (GIC) and G2 (wax). A decrease in the bacterial contamination is also evident, although some microorganisms can still be seen in the intertubular dentin (arrows).

Figure 4 - The demineralized dentin tissue with exposed collagen fibers was observed at baseline for both groups. The 60-day samples exhibited a more compact tissue, particularly in G1 (GIC). Neverteless, the remineralization process also occurred in G2 (wax), both in peritubular and intertubular dentin.

Table 1 – Means and standard deviations of the percentage of calcium, fluoride and phosphorus for G1 (glass ionomer cement liner) and G2 (wax) at baseline and 60 days after sealing (N=40 teeth).

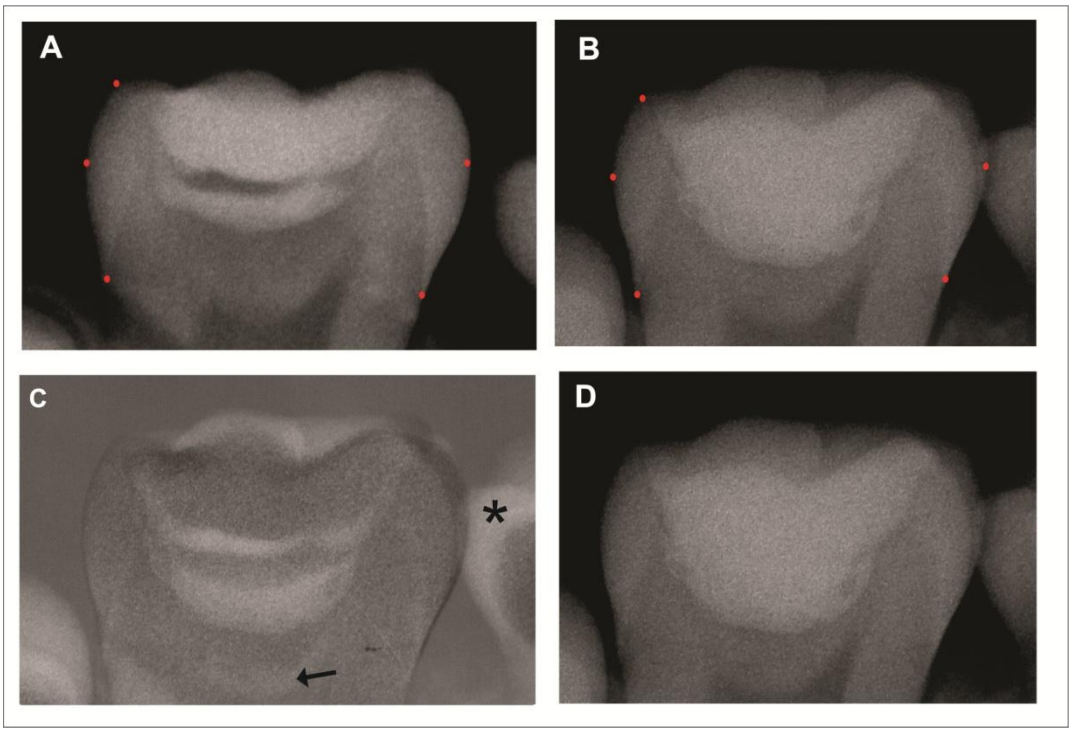
	Calcium		
	Baseline	**60-day**	**Main factor group***
G1	11.1 ± 4.7	17.2 ± 5.7	13.9 ± 5.9 a
G2	10.7 ± 4.9	16.1 ± 5.8	13.3 ± 5.9 a
Main factor Time*	10.9 ± 4.7 A	16.6 ± 5.7 B	
	Phosphorus		
	Baseline	**60-day**	**Main factor group***
G1	8.1 ± 2.4	9.8 ± 1.9	8.9 ± 2.3 a
G2	7.4 ± 2.5	8.9 ± 2.6	8.2 ± 3.5 a
Main factor Time*	7.8 ± 2.5 A	9.4 ± 2.1 B	
	Fluorine		
	Baseline	**60-day**	**Main factor group***
G1	0.7 ± 0.6	0.8 ± 1.0	0.7 ± 0.8 a
G2	0.8 ± 0.4	0.3 ± 0.5	0.6 ± 0.5 a
Main factor Time*	0.8 ± 0.5 A	0.6 ± 0.8 A	

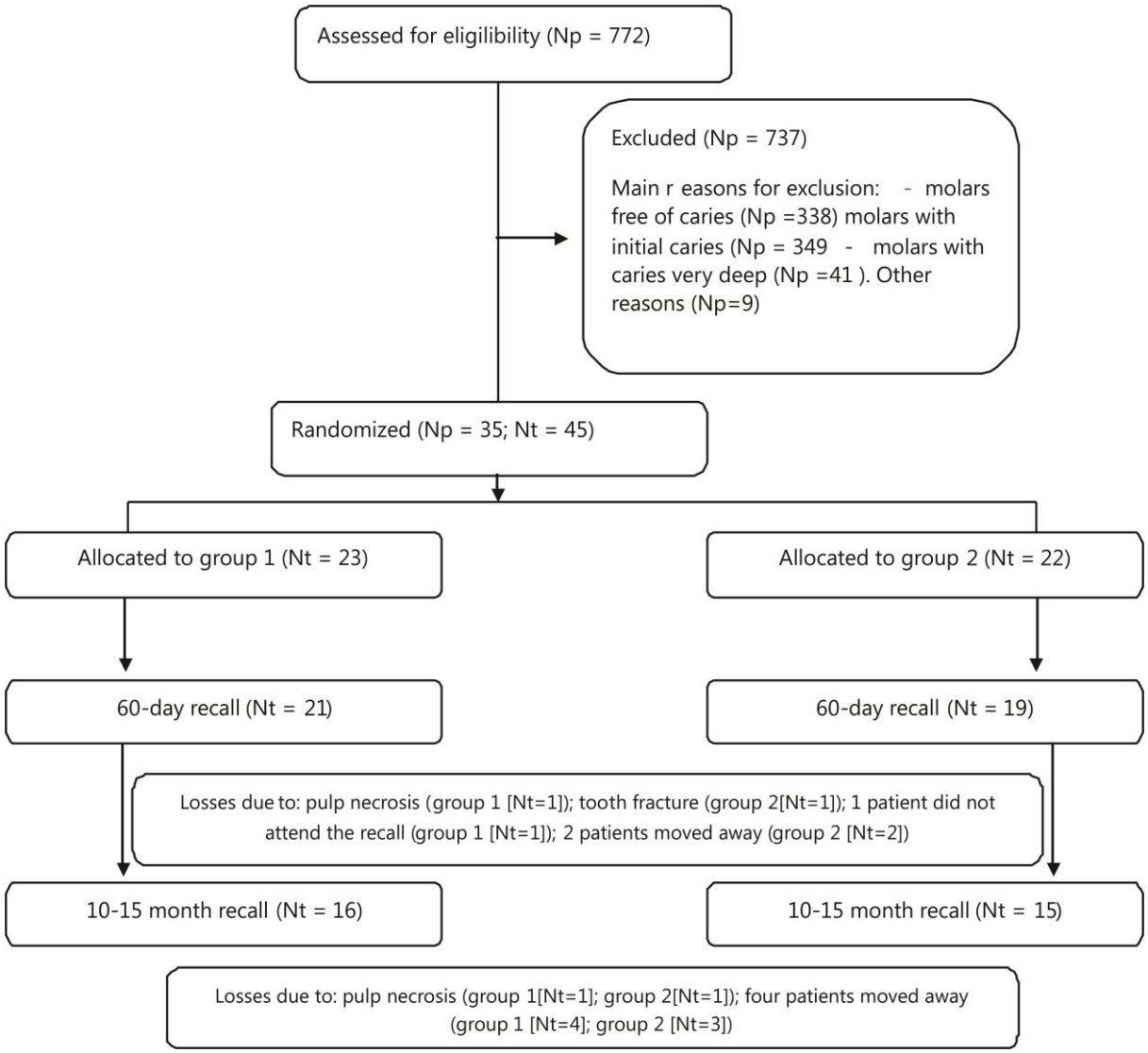
** Identical lowercase and uppercase letters indicate means statistically similar.*

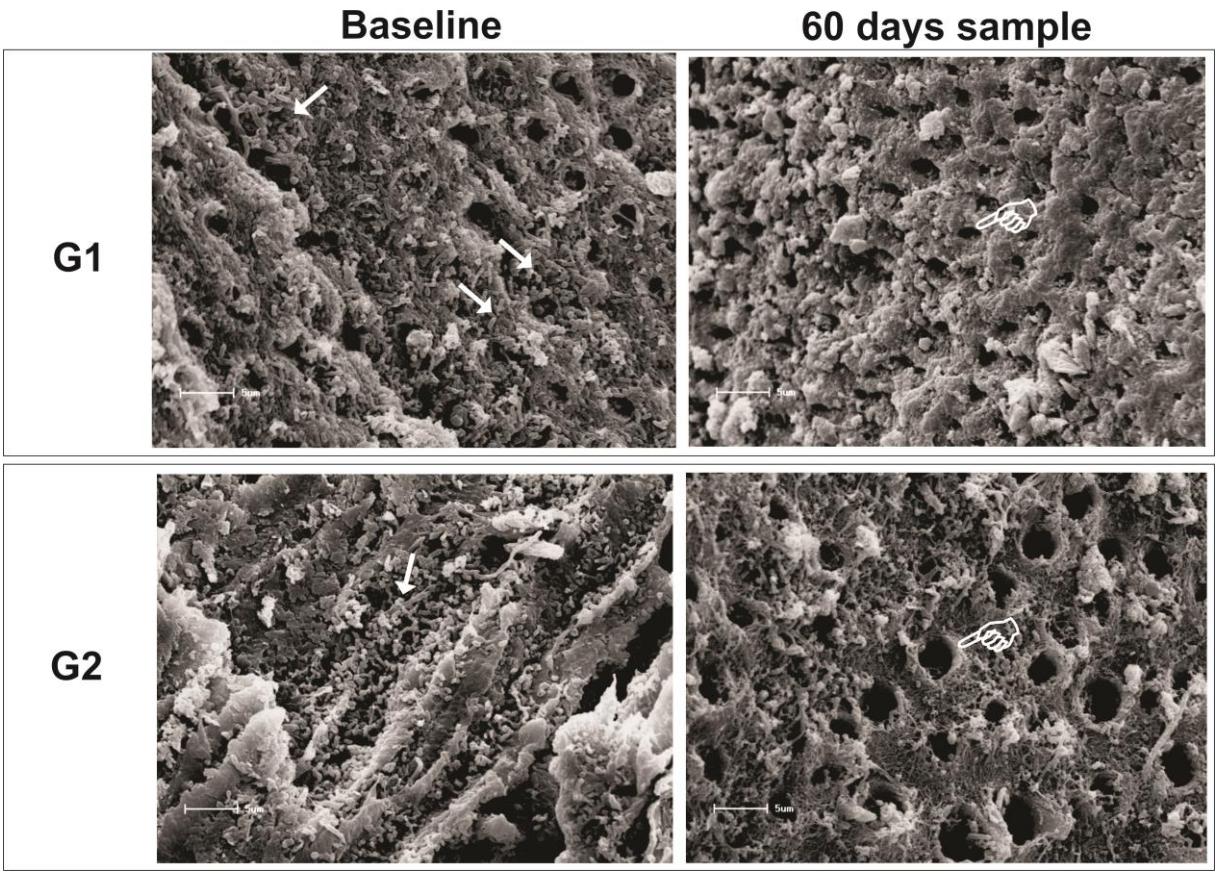
Table 2 - Means ± standard deviations of gray levels values of caries-affected and sound dentin from permanent teeth at baseline and 10-15 month radiographs G1 (glass ionomer cement liner) (n=16) and G2 (wax) (n=15).

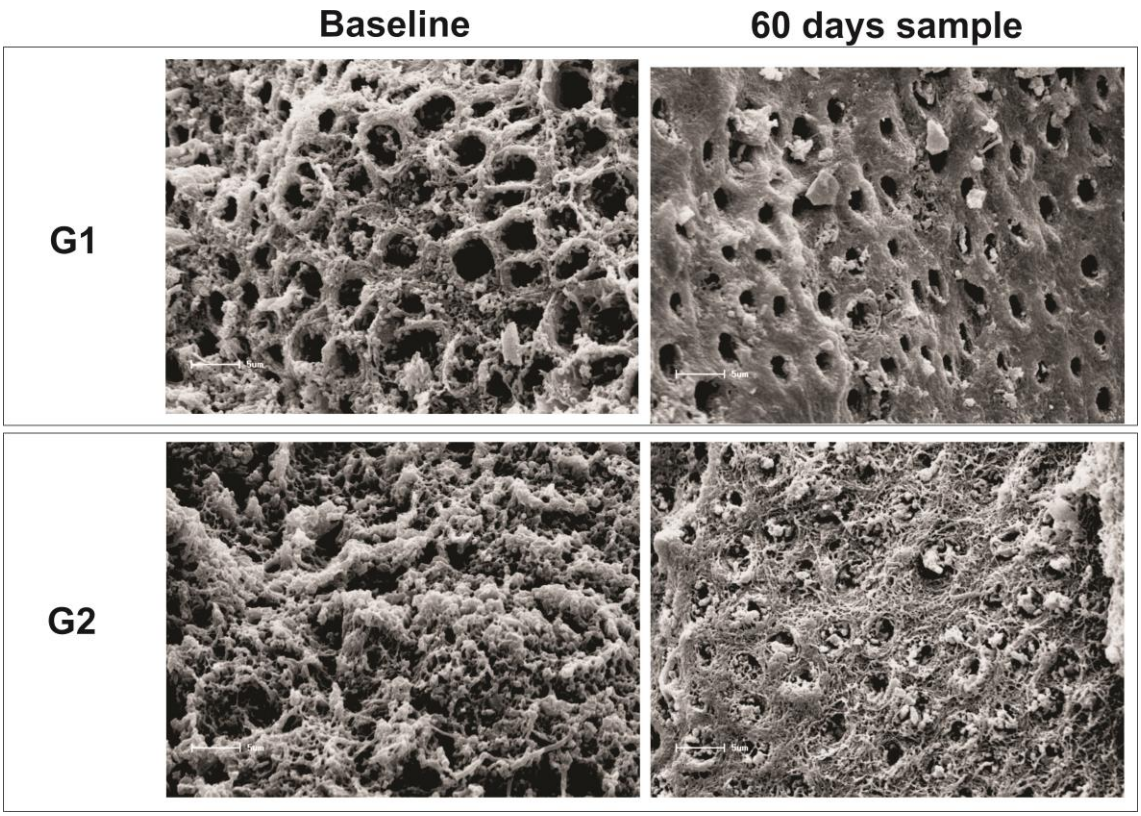
	Carious dentin		Sound dentin	
	G1	G2	G1	G2
Baseline	109.4 ± 23.0 A	109.1 ± 23.5 A	111.3 ± 21.6 A	108.7 ± 22.9 A
10-15 month	136.1 ± 20.0 B	130.0 ± 22.6 B	135.1 ± 19.3 B	129.0 ± 21.4 B

Three-way repeated measures ANOVA and Tukey´s test. Means identified with the same letters are statistically similar (p > 0.05).


A
B
C
*
D


Assessed for eligilibility (Np = 772)
Excluded (Np = 737)
Main r easons for exclusion: - molars free of caries (Np =338) molars with initial caries (Np = 349 - molars with caries very deep (Np =41). Other reasons (Np=9)
Randomized (Np = 35; Nt = 45)
Allocated to group 1 (Nt = 23)
Allocated to group 2 (Nt = 22)
60-day recall (Nt = 21)
60-day recall (Nt = 19)
Losses due to: pulp necrosis (group 1 [Nt=1]); tooth fracture (group 2[Nt=1]); 1 patient did not attend the recall (group 1 [Nt=1]); 2 patients moved away (group 2 [Nt=2])
10-15 month recall (Nt = 16)
10-15 month recall (Nt = 15)
Losses due to: pulp necrosis (group 1[Nt=1]; group 2[Nt=1]); four patients moved away (group 1 [Nt=4]; group 2 [Nt=3])


Baseline
60 days sample
G1
G2
5um
5um
5um
5um


Baseline
60 days sample
G1
G2

4.3 ARTIGO 3

EFFECT OF SEALING INFECTED DENTIN WITH GLASS IONOMER CEMENT ON THE ABUNDANCE OF MMP-2, MMP-8 AND MMP-9.

Short title: Expression of MMPs in active and sealed caries

Kuhn E[a]; Reis, A [a]; Campagnoli EB[a]; Chibinski, ACR [a]; Carrilho MRO[b]; Wambier, DS[a]

[a] *Department of Dentistry, Ponta Grossa State University, School of Dentistry, Ponta Grossa, Paraná, Brazil*
[b]*Departament of Dentistry, Bandeirante Anhanguera University, São Paulo, São Paulo, Brazil*

Abstract

This study compared the abundance and localization of MMP-2, MMP-8 and MMP-9 in infected dentin of permanent molars before and after sealing cavity. From a sample of 390 children (7-15 years old), 23 molars with deep caries lesions were selected. Infected carious dentin was removed from the the cavity at baseline (mesial portion) and 60 days after cavity sealing with GIC and resin composite (distal portion). The samples were fixed, demineralized and processed for immunohistochemistry assays using monoclonal antibodies for the localization of the MMPs with an avidin-biotin method. Digital images of the sections were obtained. Two calibrated operators analysed the baseline to 60-days samples according to the immunostaining intensity (reduced, remained unaltered or increased) and the localization of the MMPs (intertubular or intratubular and absent, localized or generalized). Statisical analyses used chi-square tests (alpha=0.05) for both conditions. The intensity of immunostaining for MMP-8 was significantly reduced after 60 days ($p = 0.02$). There was no statistical difference in immunostaining for MMP-2 ($p = 0.32$) and MMP-9 ($p = 0.14$). In general, the MMP distribution was generalized in the intertubular dentin with absent or located expression in the intertubular dentin, regardless of the period. It is possible to conclude that the sealing of infected carious dentin from permanent teeth modified the presence of MMP-8 suggesting a relationship with the initial remodeling process of the dentin matrix. The expression of MMP-2 and MMP-9 were not affected by cavity sealing.

Keywords: Glass ionomer cement. Carious dentin. Matrix Metalloproteinases

Introduction

Microbial invasion of carious lesions may continuously induce demineralization of the dentin surface [Chaussain-Miller et al., 2006; Hannas et al., 2007] and, in consequence, an adaptive response of the odontoblasts. Therefore, reactionary dentin matrix can be produced to limit bacterial invasion in dentinal tubules in attempt to control caries progression. Clinical studies that removed the most upper infected dentin layer showed dentin reorganization and tissue hardening [Alves et al., 2010; Corralo and Maltz, 2013; Maltz and Alves, 2013; Massara et al., 2002; Wambier et al., 2007]. The same phenomena were also observed in permanent [Gruythuysen et al., 2010; Mertz-Fairhurst et al., 1998] and deciduous teeth [Chibinski et al., 2013] even when the infected layer of carious tissue has not been removed.

While bacterial acids are directly implicated with dentin demineralization in caries progression, they are shown not to be able to degrade collagen in vitro [Van Strijp et al., 2003]. Degradation of the triple-helical type I collagen of dentin in caries seems to be more likely due to the activity of host-derived enzymes, such as those belonging to the family of matrix metalloproteinases (MMPs) [Sulkala et al., 2007; Tjaderhane et al., 1998] and the cysteine cathepsins[Nascimento et al., 2011].

Among the proteases identified so far in dentin, members of the MMPs family have been the most studied and they are known to degrade many components of the extracellular matrices, especially highly cross-linked triple-helical collagen. Thus, the identification of MMP-2 and sialoprotein in dentin tubules of carious dentin for instance may sign that they are part of the host defense mechanism with the aim to increase the mineralization of the caries-affected areas [Boushell et al., 2011].

Other MMPs may also have an effective participation on the processes of the dentin regeneration. The collagenase MMP-8 [Santos et al., 2009; Tjäderhane et al.,

2012] and the gelatinase MMP-9 [Niu et al., 2011; Tjäderhane et al., 2012] were also identified in sound and carious dentin [Charadram et al., 2012; Shimada et al., 2009; Tjaderhane et al., 1998; Toledano et al., 2010] and are probably the main proteases involved in the regenerative process of the dentin substrate [Santos et al., 2009; Sulkala et al., 2007].

Despite the significant advancement made with regard to the potential relationship between host-derived MMPs and caries progression, some of the fundamental issues such as the location of MMPs, expression in active caries lesions under in vivo conditions and their role in caries arrestment still remain unclear. Therefore, the objective of this study was to compare the expression and localization of MMP-8, MMP-2 and MMP-9 in caries lesions before and 60 days after sealing caries infected dentin of permanent teeth.

Materials and methods

This research was approved by the Ethics Committee of the State University of Ponta Grossa (Ponta Grossa, Paraná, Brazil) under protocol # 78/2011.

Sample Selection and Inclusion criteria

After an initial screening of 390 subjects from schools in the rural area of Ponta Grossa (Paraná, Brazil), 23 students of both genders with ages ranging from 7 to 15 years (11.0 ± 2.7 years) were invited for the participation in this study. Children with systemic pathologies were not included. Their parents/caregivers were informed about the objectives of this study and signed a consent form permitting their participation.

The participants were submitted to clinical and radiographic examinations for teeth selection. Permanent molars should present active and open deep caries lesion in the occlusal surface. Lesions should include the inner portion of dentin (2/3 or more of the dentin thickness) and should be classified with an ICDAS code 06 (International Caries Detection and Assessment System) [Ismail et al., 2007]. Teeth with signs or symptoms of pulp pathology (fistula, tooth mobility, periapical alterations, and spontaneous pain) were excluded.

Clinical procedures

Each patient attended two clinical sessions. After local anesthesia and rubber dam isolation, teeth were cleaned with a new toothbrush and water, washed thoroughly with air/water spray and air/dried without desiccation. A single operator divided the caries lesion into two equal portions in a buccal to lingual direction with a Hollenbeck carver [Chibinski et al., 2013; Wambier et al., 2007]. The mesial portion was considered the baseline sample while the distal portion was considered the 60-day sample.

The baseline sample was removed with a dental carver and the caries tissue from the cavo-surface margin was removed to promote appropriate cavity sealing. However, no effort was done to remove the most superficial necrotic dentin layer from the pulp floor. The infected dentin from the distal portion was left intact to be removed 60 days after cavity sealing. The whole cavity was lined with high viscosity glass ionomer cement (GIC, Ketac Molar Easy Mix®, 3M ESPE, St. Paul, MN, USA). Polyacrylic acid was not used to facilitate cavity re-opening 60 days after the procedure. After lining, all cavities were acid-etched with 37% phosphoric acid gel (Villevie, Joinville, SC, Brazil), rinsed out with water and slightly air-dried. The 2-step etch-and-rinse adhesive (Adper Single Bond 2, 3M ESPE, St. Paul, MN, USA) was

applied according to the manufacturer's instructions and light-cured for 10s. Then, the composite resin (Llis, FGM, Joinville, SC, Brazil) were incrementally placed and light-cured for 40s.

After composite resin placement, a sealant (Fluroshield, Dentsply, Petrópolis, RJ, Brazil) was applied on the restoration margins for additional protection against marginal infiltration [Houpt et al., 1994]. The light-curing steps were performed with a quartz-tungsten-halogen-light under ramped curing (DX Turbo Led 1200, D-X, Ribeirão Preto, São Paulo, Brazil). The initial light intensity was 450 mW/cm^2 with an automatic intensity increase to 1200 mW/cm^2 after 10 s.

Sixty days after the procedure, the marginal integrity of the restorations was evaluated, using United States Public Health Service (USPHS) [Barnes et al., 1995]: (1) retention/fracture; (2) anatomic form; (3) marginal adaptation; (4) marginal discoloration; (5) recurrent caries; (6) post-operative sensitivity; (7) surface texture; (8) color match. Those variables were ranked in the following scores: alfa (no defect clinically detectable, needing just a polish), bravo (clinically acceptable, but repair is needed) and charlie (clinically unacceptable, needs restoration replacement). If signs or symptoms of pulp pathology, described earlier in this section were observed, as well as restorations defects that compromised cavity sealing (score charlie in any of the USPHS items 1, 3, 4, 5, 6), the tooth were submitted to appropriate restorative and/or endodontic treatment. In this case, samples could not be collected in the 60-day recall.

After clinical assessment, the restorative material was removed with a sterile diamond bur (#1092, KG Sorensen, São Paulo, Brazil) under water-cooling until reaching the GIC. Manual instrumentation was used for complete removal of the

material. Then, a dentin sample, from the distal portion, was removed in the same manner as described for the mesial portion for immunohistochemical processing. Teeth were then lined with glass ionomer cement and restored with a composite resin as previously reported.

Immunohistochemical processing

The samples were processed for immunohistochemical analysis immediately after sample collection. After fixation in 10% formaldehyde until 48 h, the samples were demineralized using solution of EDTA (41.5 g EDTA, 4.4 g of NaOH and 1 L of distilled water). The solution was changed daily until the dentin samples could be easily penetrated with a thin needle. This process took up to 5 months.

Following histological processing, the material was dehydrated (three 1-hour immersions in each one of ascending ethanol concentrations (70%, 80%, 90% and 100%), diaphanized (two 30-min baths in xylene) and parafinized (three 30-min baths at 60 °C in paraffin). After all these steps the material was immersed in melted paraffin at 60 °C and kept at ambient temperature.

After 24 h, from each dentin sample, twelve 4-µm thick sections were obtained using a microtome (Leica RM2125, Leica Biosystems, Wetzlar, Germany). These sections were mounted on silane-coated slides (StarFrost, Knittel, Germany), in a way that each slide contained four sections, dewaxed in xylene, rehydrated from graded ethanol and washed thoroughly with distilled water. Sections were then incubated for 30 min in 0.3% H_2O_2 to quench endogenous peroxidase activity, prior to rinsing for 20 min with a PBS and submitted to monoclonal mouse anti-human antibodies for the identification of MMP-2, MMP-8 and MMP-9 (Merck Millipore-Billerica, Massachusetts, USA).

For the localization of MMP-2, MMP-8 and MMP-9, the antibodies were diluted 1:100 in a solution of 1% PBS/bovine serum albumin (PBS/BSA). Three sections of each slide received the monoclonal antibodies; the fourth section received only PBS/BSA solution and was considered the negative control of the reaction.

The slides were incubated overnight at 4°C in a moist chamber. Immune complexes were subsequently revealed using labeled streptavidin biotin (LSAB) reagents LSAB Dako LSAB+System-HRP (Dako, Glostrup, Denmark), which were used according to the manufacturer's instructions. All sections, including negative controls, were incubated with biotiny lated secondary antibodies and then with streptavidin peroxidase, both for 30 min at 37°C. After each reagent, slides were gently rinsed with PBS and the excess buffer was wiped out around sections before applying another solution. A substrate-chromogen (3,3’ diaminobenzidine chromogen solution - DAB) (Sigma Aldrich, Saint Louis, MO, USA) was used to develop the reaction (5 min, at 37°C), which resulted in a brown precipitate at the antigen site. The slides were rinsed with distilled water and, after dehydration in graded ethanol, a cover slip was placed over slide with synthetic resin permount mounting medium (Fisher Chemical, New Jersey, USA).

Analysis of the slides

Images of the sections were obtained using a biological microscope Olympus CX21 (Olympus, Shinjuku, Tokyo, Japan) and Cellsens digital imaging software (Olympus, Shinjuku, Tokyo, Japan) under 400x magnification. Due to the great variability in the immunostaining of the baseline samples, sections of the same tooth at both periods (baseline and 60-day) were observed side-by-side by two calibrated operators in order to detect whether the immunostaining intensity were reduced, remained unaltered or increased from baseline to 60-days. For each MMP, a chi-

square test (alpha = 0.05) was applied to identify changes in these three patterns. Similarly, the localization of the enzymes (intertubular or intratubular) was identified by the same operators and classified as absent, localized or generalized in the baseline and 60-day sections and evaluated by a chi-square test (alpha = 0.05).

Changes in the overall immunostaining intensity after 60 days were statistically analyzed by a chi-square test. The distribution of the immunostaining in the baseline and 60-day sample was statistically analyzed by the Fisher's exact test. In all tests, the statistical significance was set at 5%.

Results

Of the 23 permanent molars at baseline, one tooth had pulp necrosis and one patient did not attend the 60-day recall, resulting in 21 specimens that were processed for immunohistochemical analysis. At the end, a total of 17 (MMP-2 and MMP-8) and 16 (MMP-9) teeth were available for analysis. In two cases, the 60-day sample was not enough to be processed; in the remaining, we failed during immnohistochemical processing.

Clinical and radiographic evaluation

At baseline, all restorations were classified as alpha according to the USPHS criteria. No radiographic changes or symptoms were seen 60 days after cavity sealing, signalizing arrestment of the caries lesion.

Immunohistochemical analysis

The negative control specimens revealed no staining (Figure 1a). A high variation in the intensity of immunostaining was observed in the baseline samples for all MMPs.

Immunohistochemical processing for MMP-2 revealed the expression of this protease in the baseline and 60-day sections (Figure 1). For most of dentin sections, the intensity of immunostaining was similar or slightly increased, although this was not shown to be statistically significant (Table 1, $p = 0.32$). MMP-2 was predominantly located in the intertubular dentin with a generalized distribution in both periods of analysis (Figure 2a, $p = 0.172$).

Similarly, MMP-8 was seen in all specimens (Figure 1) at baseline and after 60-days after cavity sealing and it was predominantly located in the intertubular dentin with a generalized distribution in both periods (Figure 2b, $p = 0.35$). However, in most of the dentin sections, the intensity of immunostaining reduced significantly compared with baseline (Table 1; $p = 0.02$).

In regard to MMP-9, its immunostaining was observed in all baseline and 60-day specimens (Figure 1), with a generalized distribution in the intertubular dentin for both periods (Figure 2c; $p = 0.28$). Although most of the sections showed reduced or similar immunostaining, this was not statistically significant (Table 1; $p = 0.14$).

For most of cases, in general the MMPs distribution within dentin tubules was not detected or when present the staining was quite localized regardless the period of analysis (Figure 2a-c; chi-square test; $p > 0.28$).

Discussion

In the present study, we employed monoclonal antibodies, since they are very specific and allow for the detection of the selected MMPs only [Mazzoni et al., 2009]. As the negative control specimens revealed no staining, it is possible to state that no cross-reactions occurred between secondary antibodies and the dentin organic matrix. Therefore, the adequacy for labeling was established [Shimada et al., 2009].

Within the oral environment, extensive interest has been given to the detection, distribution and function of host-derived MMPs [Hannas et al., 2007]. MMPs seem to play a pivotal role in regulating and controlling physiological processes [Goldberg and Smith, 2004]. However, in addition to physiological roles, they have also been suggested to be involved in several pathological processes. Degradation of the dentin collagenous matrix, a prerequisite for cavity formation in caries development, has been for instance attributed to MMP activity [Tjaderhane et al., 1998; Tjäderhane et al., 1999]. Although, the identification of MMPs in human dentin is not a new finding, previous studies using immunostaining techniques have mostly concentrated their analysis *in vitro*, there by having extracted-teeth as the major source for these proteases identification [Boushell et al., 2011; Charadram et al., 2012; Shimada et al., 2009; Tjaderhane et al., 1998; Toledano et al., 2010]. More realistically, then, the present protocol has successfully identified the abundance of some members of MMPs family in an actual clinical scenario performing the analysis in non-extracted carious teeth.

Additionally, those previous studies discussed the possible involvement of MMPs into the progression of the caries lesions [Sulkala et al., 2007; Tjaderhane et al., 1998] and their role in the regeneration of the dentin-pulp complex [Sulkala et al., 2007; Tjäderhane et al., 2012], but they did not investigate whether or not these proteases are involved in the metabolism of the caries-affected dentin matrix that

occurs after cavity sealing. This was therefore one of the novelties of the present study, that is, we performed the identification of MMPs 2, 8 and 9 in infected-caries dentin samples, collected *in vivo*, in an active (baseline) and after the lesions have been sealed with GIC and composite resin in attempt to arrest caries (60 days after sealing). Besides that, two samples were collected from the same tooth and patient in two different stages, allowing us to control for individual variations.

We have chosen to investigate the expression of MMPs that had already been identified in dentin [Boushell et al., 2011; Charadram et al., 2012; Niu et al., 2011; Shimada et al., 2009; Sulkala et al., 2007; Toledano et al., 2010]. Yet it is not possible to describe the exact contribution of salivary, dentin-matrix or pulp-derived (via dentinal fluid) enzymes for dentinal caries lesions. Several MMPs were shown to be abundant in saliva and experimentally able to degrade exposed dentinal collagen matrix [Tjaderhane et al., 1998]. High relative detection of MMP-8 and -9 in outer caries layer compared to inner (caries-affected) layer [Shimada et al., 2009] indicates saliva as a patent source of these enzymes. Conversely, decreasing in MMP-2 staining from pulp towards the enamel [Niu et al., 2011] and absence of a significant difference in the level of MMP-2 between outer and inner carious dentin [Shimada et al., 2009] suggest this enzyme may not come from a prevalent source. These observations offer a rationale to interpret the present results, where in the sealing of dentin with GIC and composite resin for 60 days and privation of contact with saliva have, in general, reduced the immunostaining level for MMP-8 (i.e. 64% of the samples) and -9 (i.e. 56% of the samples) while keeping that for MMP-2 (total of 84% of the samples) similarly or slightly increased in comparison to the baseline results (Table 1).

The positive gene expression of several MMPs in mature odontoblast or pulp-cells [Palosaari et al., 2000; Tersariol et al., 2010] strongly indicated that, along with saliva, dentinal fluid may be a continuous source of proteolytic enzymes, hence assuming an important role in respect to caries and other remodeling process occurring in dentin matrix. It has been speculated that, in principal, when dentin is demineralized and collagen is exposed, odontoblasts and other pulp cells secrete enzymes, dentinal growth factors and other macromolecules that are involved in the hydrolysis of triple-helical, structured collagen. Thus, for instance, secreted molecules of the collagenase MMP-8 likely travel within dentin tubules and are distributed in the intertubular organic matrix in order to be in close contact with its target substrate, that is, with collagen type I fibrils [Chaussain-Miller et al., 2006]. This could explain why in the present study MMP-8 was predominantly located at this region (Figure 1).

After 60 days, however, the immuno-detection of MMP-8 was reduced, which seems to confirm the hypothesis that this protease is more related with the initial process of dentin matrix modeling (degradation and/or regeneration). As the time goes by, the cleaved, denatured collagen becomes substrate for other enzymes, such as the gelatinases MMP-2 and MMP-9 [Chaussain-Miller et al., 2006; Shimada et al., 2009]. At this stage, the presence of collagenases (i.e. MMP-8) seems not to be that important and gelatinases assume the major function of hydrolyzing denatured fragments of collagen and other small components, which may be the reason of why we did not detect reduction of MMP-2 over time.

In mineralized dentin, MMP-2 is probably the most abundant MMP. Unlike other MMP family members, MMP-2 production is constitutively expressed by numerous cell types and does not respond significantly to stimulation by most

biologic agents. This means that the activity of MMP-2 seems to be strictly regulated, predominantly on the posttranslational level [Mazzoni et al., 2012]. Active MMP-2, as most MMPs, is unstable in the absence of substrate, indicating that autolytic inactivation is an important mechanism for posttranslational control of MMP-2 activity [Sternlicht and Werb, 2001]. The complex interaction of MMP-2 with type I collagen may be crucially significant in dentin preservation and function. MMP-2 has been reported to split dentin matrix acidic phosphoprotein 1 (DMP1), a mineralization inhibitor molecule into two major peptides. After splitting, the C-terminal peptide portion can promote mineralization and has been shown as an important signaling molecule for transforming pulpal stem/progenitor cells and committing them to odontoblast-like lineage. These data suggest that a peptide generated through the proteolytic processing of DMP1 can regulate the differentiation of mesenchymal cells during dentinogenesis and thus sustain reparative dentin formation in pathological situations such as carious decay [Chaussain et al., 2009].

It should be mentioned there was a great variation in enzymes immunostaining among baseline samples (i.e with strong and slight immunostaining) extracted from different patients. This could be related with the differential degree of collagen degradation and enzymes activity. Although all caries lesions were classified into an ICDAS code 06, some variations in enzymes activity are expected between subjects [Nascimento et al., 2011] which is related not only with the individual proteolytic profile, but it can be also dependent on the degree of collagen degradation in different caries lesions depths (Niu et al. 2011). This variation was the reason why we have employed a scoring system to classify the comparative changes in the immunostaining of enzymes between the two periods of analysis, instead of using softwares to measure it.

The fact that caries lesion can be arrested with a sealing material is very well documented in the literature [Corralo and Maltz, 2013; Kidd, 2010; Paddick et al., 2005]. In an active condition, carious dentin exhibits poor structural organization, loose exposed collagen fibrils and evident microbiological contamination [Maltz et al., 2002; Massara et al., 2002; Wambier et al., 2007]. Caries lesion sealing for up to 60 days can changes this pattern. Signs of remineralization, *i.e.* histological dentin reorganization with intertubular dentin thickening, formation of a dense collagen network [Alves et al., 2010; Maltz et al., 2007; Marchi et al., 2008; Massara et al., 2002] and reduction of the viable bacteria is usually seen [Maltz et al., 2002; Massara et al., 2002; Wambier et al., 2007]. Therefore, the 60-day sample was representative of a more organized dentin tissue from an early arrested caries lesion.

Finally, it is worth to mentioning the limitations of the present study in an aim to guide future studies in this issue. We did not identify the most front line region of the carious dentin sample. This would have probably allowed us to identify the relative contribution of the different MMPs in the different dentin regions, which are in different degenerative conditions. Additionally, we did not collect sound dentin due to ethical concerns. This prevented us from comparing the expression of these MMPs in the caries lesion with a sound teeth: we do not know if the expression herein reported is in fact much more intense than in a sound condition. Lastly, a short period of time was employed, which only allows us to make inferences about the initial changes of the dentin tissue. From literature findings we know that dentin continues to change for months and years after cavity sealing [Alves et al., 2010; Maltz et al., 2007; Massara et al., 2002; Oliveira et al., 2006].

Natural caries lesions developed over extended time with numerous pH changes, de- and remineralization cycles and slowly progressing loss of minerals.

The changes in the dentin organic matrix may be slow and gradual. There is an urgent need for experimental in models that access the natural progression of dentinal lesions, including the time factor, to study the effect of collagenolytic enzymes and their inhibition in caries. We believe the present experiment is a reliable protocol and can constitute a handful tool to help one better understanding the mechanism and interplay of proteases in the pathology and regeneration of dentin matrix after a carious challenge.

Legends

Figure 1 – Immunostaining of the negative control and MMP-2, MMP-8 and MMP-9 at both study periods. The negative control reavealed no immunostaining. The immunostaining of the MMP-2 and MMP-9 were similar at both study periods (beige color in all figures). A reduction of the immunostaining was observed for MMP-8. The arrows in the images indicate the immunostaining in the dentin tubules, which was predominantely absent (▶) and localized (↙) at both periods. All MMPs studied was predominantely located in the intertubular dentin with a generalized distribution in the tissue a both periods.

Figure 2 – Expression and localization of the MMP-2 (a), MMP-8 (b), MMP-9 (c) in inter- and intratubular dentin at both study periods.

Table 1–Number of specimens (%) with a reduced, similar and increased immunostaining expression after 60 days compared to the baseline along with the statistical significance*.

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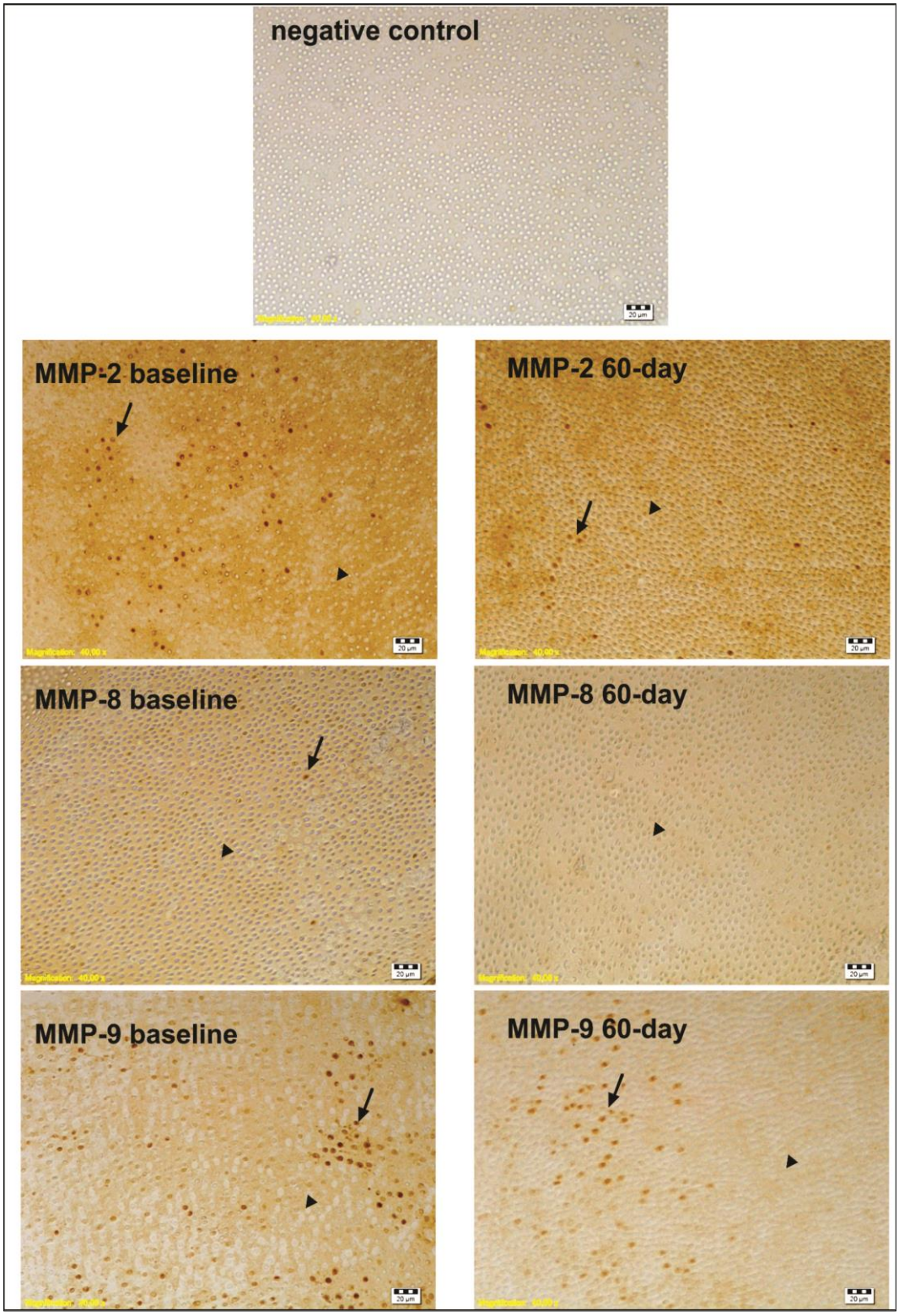
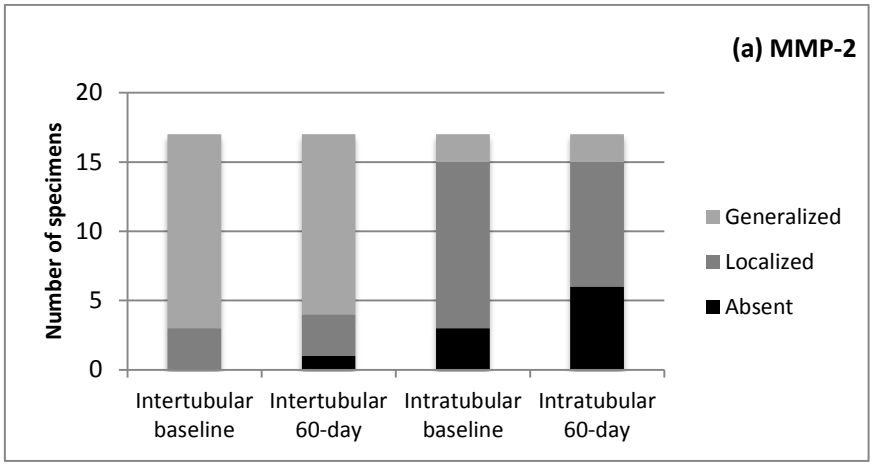
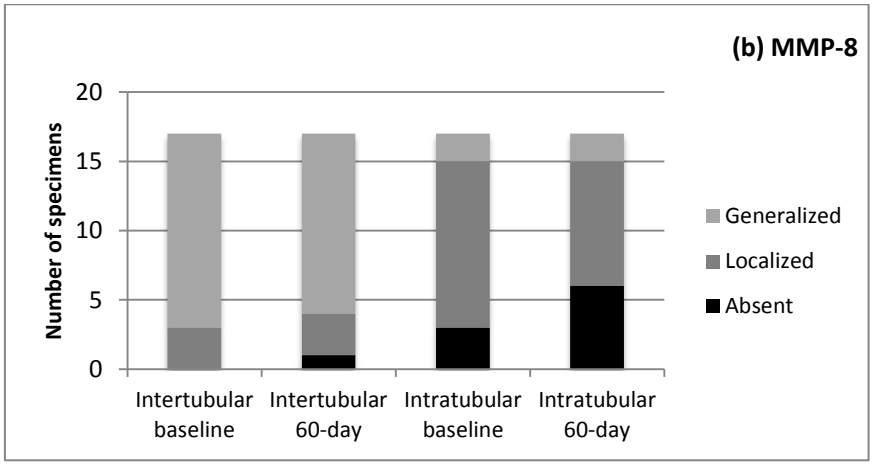

negative control
MMP-2 baseline
MMP-2 60-day
MMP-8 baseline
MMP-8 60-day
MMP-9 baseline
MMP-9 60-day


(a) MMP-2
Number of specimens
20
15
10
5
0
Intertubular baseline
Intertubular 60-day
Intratubular baseline
Intratubular 60-day
Generalized
Localized
Absent


(b) MMP-8
Number of specimens
20
15
10
5
0
Intertubular baseline
Intertubular 60-day
Intratubular baseline
Intratubular 60-day
Generalized
Localized
Absent

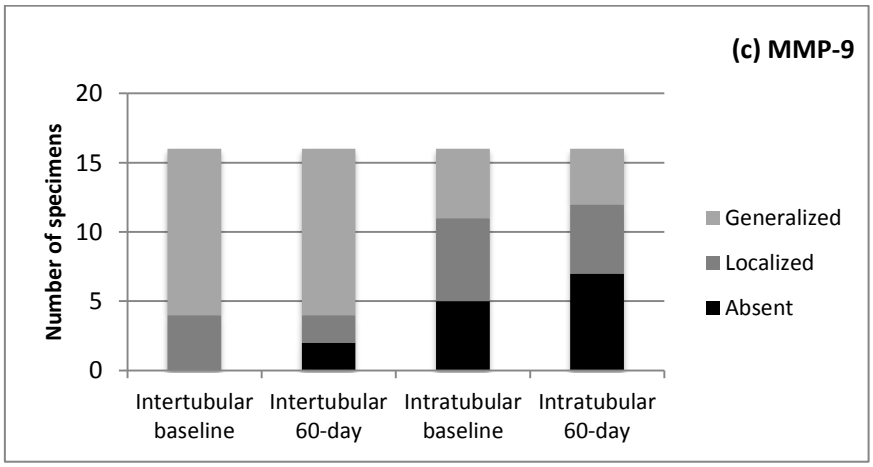

(c) MMP-9
Number of specimens
20
15
10
5
0
Intertubular baseline
Intertubular 60-day
Intratubular baseline
Intratubular 60-day
Generalized
Localized
Absent

Table 1–Number of specimens (%) with a reduced, similar and increased immunostaining expression after 60 days compared to the baseline along with the statistical significance*.

	Reduced	**Similar**	**Increased**	**p-value***
MMP-2 (n = 17)	3 (18)	8 (47)	6 (35)	0.32
MMP-8 (n = 17)	11 (64)	3 (18)	3 (18)	0.02
MMP-9 (n = 16)	9 (56)	3 (19)	4 (24)	0.14

** Chi-square test.*

5. DISCUSSÃO

A remoção parcial da dentina cariada, seja em dentes decíduos[7,13] ou em permanentes [4,6], sob uma restauração adequadamente selada[10,41], leva a uma reorganização do tecido dentinário[2,4,13]. Essa conduta está fundamentada na remoção seletiva de tecido cariado, proposta por Fusayama[11], e tem como meta eliminar a maior parte de dentina infectada, alcançando a camada afetada (desmineralizada) com potencial de recuperação devido a presença da trama colágena[11].

Porém, como na prática clínica, a diferenciação entre essas camadas de dentina (infectada e afetada)[11], está baseada somente em critérios subjetivos[42], alguns profissionais deixam de realizar técnicas de mínima intervenção, por não se sentirem seguros para fazê-las. E, além disso, a tentativa de eliminar todo tecido infectado, nas lesões profundas de cárie, amplia o risco de exposição pulpar[1].

Em contraste ao preparo cavitário tradicional, a remoção parcial ou seletiva já foi um avanço na Odontologia Restauradora. De forma mais ousada, por deixar tecido infectado no preparo cavitário, destacaram-se no cenário bibliográfico, as restaurações ultraconservadoras[12], realizadas com resina composta, contudo em preparos cavitários de média profundidade. O longo tempo de acompanhamento dessas restaurações (dez anos) com interrupção do processo carioso, na maior parte dos casos, sinalizou para a possibilidade de deixar dentina infectada, desde que haja adequado selamento marginal.

Essa série de pesquisas[2,4,6,7,11,13], e as revisões sistemáticas[8,43], elucidaram alguns fatos, derrubando mitos, entre esses, o da necessidade de uma segunda sessão para completar a remoção de tecido cariado [44], bem como, a super valorização do material de proteção, seja para proteger o tecido pulpar de algum dano, seja para fornecer minerais para o processo de remineralização dentinária[2,13] .

A capacidade de defesa do complexo dentino-pulpar é inquestionável, mas como isto ocorre, e qual a real participação das metaloproteinases nesse processo, é campo amplo de pesquisa, devido a carência de estudos nessa área. Visando preencher essa lacuna, na tentativa de compreender melhor o mecanismo biológico que conduz ao processo de cura, o presente estudo incluiu um conjunto de parâmetros (clínico, radiográfico e

laboratorial) para descrever as alterações no tecido dentinário infectado.

O protocolo de pesquisa foi elaborado para obter amostras de dentina infectada do mesmo dente e da mesma profundidade, antes e após proteção cavitária com material bioativo (CIV) e inerte (cera). Para tanto, o tecido cariado foi removido somente das margens da cavidade, que foi dividida em duas partes para a mensuração da laser fluorescência (DIAGNOdent®) e retirada de fragmentos de dentina infectada da porção mesial. Esse foi o local controle do estudo. Decorridos 60 dias, logo após a re-abertura do dente, procedeu-se a segunda mensuração da laser fluorescência e coleta de novos fragmentos de dentina infectada, agora da porção distal.

Assim, foi possível associar, numa mesma condição clínica, dados não subjetivos de dentina infectada (antes e após selamento cavitário), registrando-se valores numéricos da laser fluorescência e de minerais (cálcio, fosfato e flúor), bem como descrever as alterações micromorfológicas no tecido dentinário e a expressão das metaloproteinases (MMPs 2, 8 e 9).

Uma particularidade deste estudo, foi a utilização do DIAGNOdent em lesão ativa e aberta de cárie, ou seja de modo distinto à sua indicação principal, que é para o diagnóstico de lesões de cárie (em esmalte ou dentina). Foram notadas diferenças significativas nos valores de fluorescência dentinária pelo selamento cavitário, indicando modificações favoráveis em ambos os grupos. Embora, as médias de fluorescência após 60 dias de selamento tenham sido menores, elas ainda se mantiveram superiores aos limites de corte para cárie de dentina propostos na literatura (>38)[45]. A possível explicação para tais valores é que porfirinas, produtos metabólicos residuais bacterianos, podem ainda ser detectados pelo aparelho, mesmo após a reorganização do tecido dentinário.

Estas mudanças foram corroboradas pela análise ultraestrutural em microscopia eletrônica de varredura[2,7,9,13] mostrando nos dois grupos, no momento inicial um tecido dentinário altamente infectado e desorganizado com suas fibrilas colágenas expostas. Após o período de selamento ocorreu uma mudança positiva no aspecto desse tecido, com sinais de reorganização da dentina pela densa rede de colágeno[4,13,16,18] e redução de bactérias[7,13,46].

Alguns autores consideram que o processo de reparo da lesão de cárie é influenciado pelo flúor liberado dos CIVs[2,47], o qual participa na remineralização da dentina[47-48]. No entanto, os resultados do presente estudo,

quantificado pela espectometria dispersiva de raio X (EDS), mostraram que a absorção de flúor durante o período de 60 dias não foi estatisticamente diferente entre os grupos de estudo. No entanto, este fato não parece comprometer o aumento de cálcio e fósforo, a redução de bactérias e melhor reorganização da dentina nos dois grupos.

Entre os três minerais avaliados, o aumento foi significativo para o cálcio e fósforo nos dois grupos, sugerindo uma ação fisiológica de defesa, sem participação expressiva do material de forramento. Esse resultado é semelhante ao estudo de Massara et al.,[13]que usou CIV e detectou aumento significativo somente de cálcio.

A alteração mineral na dentina também foi observada ao longo do tempo, pelo acompanhamento radiográfico, por meio do método quantitativo de subtração que permite determinar em termos numéricos, qualquer mudança que possa ter ocorrido nas áreas em observação[6]. Verificou-se que esta alteração é um processo contínuo, envolvendo não apenas a dentina infectada, mas toda a estrutura dentinária após selamento cavitário, comprovado por um período médio de acompanhamento de um ano, nos dois grupos estudados e consistente com os resultados de outros estudos em dentes permanentes[4,6,18,] e dentes decíduos[49].

Isto sugere que a reestruturação da dentina não está associada com o forramento colocado em contacto com a dentina infectada e sim, ocorre, devido ao selamento cavitário que isola a lesão do biofilme dental[10,17,]associado a um processo fisiológico de reparo tecidual[7,13]. Um selamento efetivo da cavidade proporciona mudanças no ambiente da lesão, estimula a esclerose tubular, reduz a permeabilidade da dentina e a quantidade de bactérias viáveis sob restaurações[41], fator decisivo na dinâmica da progressão da lesão. Microorganismos privados de açúcar tornam-se menos ativos metabolicamente e permitem a paralisação do processo carioso.

A degradação da rede de colágeno exposta por ácidos bacterianos, é provavelmente realizada por enzimas derivadas de hospedeiro chamadas metaloproteinases (MMP)[22,50], uma vez que o colágeno tipo I é o substrato principal destas MMPs, bem como, os processos de reparação também podem envolver MMPs que provavelmente estimulam reações de defesa nas células do complexo dentino-pulpar[22,51].

Em geral, a reparação e regeneração do complexo dentino-pulpar, ocorre de forma semelhante às respostas naturais de cura de feridas observados em muitos sistemas do corpo[52]. Para responder ao processo inflamatório induzido pela lesão de cárie, odontoblastos produzem uma matriz de dentina terciária reacionária[51-52], juntamente com elevada secreção de fatores de crescimento[53], em um esforço de remodelação e reparação da matriz extracelular, danificada pela doença[54] .

Portanto, a densa matriz de colágeno observada nesta pesquisa, 60 dias após o selamento, é provavelmente o resultado da reestruturação do tecido dentinário, proporcionada pela neutralização do pH, essencial para que as metaloproteinases da matriz (MMP) permaneçam ativas[55] e juntamente com outros fatores possibilitem reações de defesa do hospedeiro, resultando na remodelação do tecido pelo complexo dentino-pulpar[4,55].

Neste estudo, optamos por analisar as MMPs 2, 8 e 9 no G1, por se tratar de um estudo preliminar com o uso de um material bioativo (CIV). Os resultados mostraram a presença destas metaloproteinases tanto intertubular quanto intratubular na dentina cariada infectada, no momento inicial e após 60 dias de selamento, porém, uma menor intensidade de imunomarcação da MMP-8 pode ser vista após o selamento, o que sugere que esta MMP pode estar mais relacionada com o processo inicial de regeneração do tecido.

Produzidas pelos odontoblastos e outras células da polpa[55], elas provavelmente viajam dentro dos túbulos dentinários e distribuem-se na matriz orgânica intertubular para clivar fibrilas de colágeno tipo I [56], que é o seu alvo principal. Com o passar do tempo, o colágeno clivado se torna substrato para gelatinases MMP- 2 e MMP- 9 [56-57] reduzindo a necessidade da MMP-8 no tecido dentinário. Nesta fase, a presença das gelatinases parece ser mais importante para degradar as fibrilas de colágeno anteriormente clivadas. Portanto, não foi detectada nenhuma redução de suas expressões 60 dias após o selamento da cavidade.

Assim, baseados nos resultados encontrados em uma amostra inicial de 45 molares permanentes com lesões de cárie profunda em dentina, após controle (média de 12,5 meses), somente 3 molares apresentaram necrose pulpar, portanto, a preocupação de muitos pesquisadores[58-60] em diferenciar a camada de dentina infectada da afetada, na remoção da dentina cariada, parece não ter grande relevância clínica, uma vez que o reparo tecidual tende a se processar em dentes

vitais devidamente selados mesmo na presença de dentina infectada na cavidade[12,14].

Finalmente, é importante relatar as limitações deste estudo, a fim de orientar novas pesquisas. Um dos grandes entraves foi convencer o Comitê de Ética em Pesquisa, por se tratar de uma pesquisa in vivo, com crianças, cuja dentina infectada não seria removida, e ainda explicar a razão da re-abertura do dente. Outra limitação foi a seleção da amostra, encontrando-se os dois extremos: dentes sem lesão ou muito destruídos não correspondendo aos critérios de inclusão estabelecidos, pois, as lesões deveriam ser profundas, mas sem comprometimento pulpar e de tamanho adequado para a obtenção das amostras nos dois tempos.

A obtenção de fundamentos científicos consistentes permite ampliar o emprego de abordagens alternativas de controle e tratamento da doença cárie, beneficiando um maior número de indivíduos e ainda, com chances reais de readequar a proporção entre o grau de necessidade de procedimentos odontológicos e a oferta de serviços na rede pública. Assim, este protocolo de trabalho pode ser inserido na Atenção Básica em Saúde Pública, reduzindo a necessidade de tratamento especializado, em geral, de alta demanda e alto custo, o que determina elevada frequência de exodontias, principalmente em pacientes jovens de áreas rurais.

6. CONCLUSÕES

O selamento de lesões profundas de cárie em molares permanentes vitais, mantendo-se dentina infectada no assoalho cavitário, independente do material de proteção aplicado antes da restauração, determinou:

- paralisação do processo carioso;
- reorganização da dentina, diminuição da fluorescência dentinária, ganho mineral e aumento de densidade radiográfica, sugestivos de remineralização a longo prazo;
- alteração na expressão da MMP-8 o que sugere participação dessa metaloproteína na remodelação da matriz dentinária.

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ANEXO A - Documento de aprovação da Comissão de Ética em Pesquisa da Universidade Estadual de Ponta Grossa

UEPG
UNIVERSIDADE ESTADUAL DE PONTA GROSSA

PROPESP
Pró-Reitoria de Pesquisa e Pós-Graduação

COEP
COMISSÃO DE ÉTICA EM PESQUISA

PARECER Nº 78/2011
Protocolo: 18647/10

No dia 28 de Julho de 2011, a Comissão de Ética em Pesquisa, **APROVOU** o protocolo de pesquisa intitulado **"O papel do material restaurador nas alterações em dentina cariada de dentes permanentes tratados pela mínima intervenção"** de responsabilidade da pesquisadora Eunice Kuhn.

Conforme Resolução CNS 196/96, solicitamos que sejam apresentados a esta Comissão, relatórios sobre andamento da pesquisa, conforme modelo (http://www.uepg.br/coep/).

Data para entrega do relatório Parcial: 01 de Julho de 2012.
Data para entrega do relatório Final: 01 de Julho de 2013.

Ponta Grossa, 28 de Julho de 2011.

UNIVERSIDADE ESTADUAL DE PONTA GROSSA
COMISSÃO DE ÉTICA EM PESQUISA - COEP

Prof. Dr. Ulisses Coelho
Coordenador

Av. Carlos Cavalcanti, 4748 - CEP: 84030-900 – Ponta Grossa - PR – BRASIL
Bloco M Sala 12 - Campus Universitário em Uvaranas
Fone (42) 3220-3108 – Fax: (42) 3220-3102
e-mail: seccoep@uepg.br Home page: www.uepg.br

ANEXO B - Autorização da Secretaria Municipal de Educação

PREFEITURA MUNICIPAL DA PONTA GROSSA
Secretaria Municipal de Educação
Av. Visconde de Taunay, 950 - Tel.: (042) 3220-1127 –Fax:3220-1221 -e-mail: assessoria-sme@pontagrossa.pr.gov.br - CEP: 84.051-900 -P.Grossa - PR

ASSESSORIA DE GESTÃO EDUCACIONAL
Em 13 de abril de 2011.
Of. nº 11/2011

Sra. Diretora

Informamos que foi autorizada por esta Secretaria a realização dos projetos intitulados "Avaliação in vivo das alterações em dentina cariada de dentes decíduos após selamento cavitário em crianças de diferentes faixas etárias" (protocolado nº. 0880432/2011) e "O papel do material retaurador nas alterações em dentina cariada de dentes permanentes tratados pela mínima intervenção" (protocolado nº. 0880436/2011) sob a responsabilidade das professoras Ana Cláudia Rodrigues e Eunice Kuhn, respectivamente, do Departamento de Odontologia, da Universidade Estadual de Ponta Grossa.

Portanto, solicitamos os seus préstimos no sentido de acolher as referidas professoras, colaborando com as ações previstas para a realização do projeto.

Cordialmente,

Teresa Jussara Luporini
Assessora de Gestão Educacional

Ilma. Sra
Bernadeth Malechi
Escola Municipal Professora Maria Elvira Justus Schmidit
Nesta

ANEXO C - Autorização da Secretaria Municipal de Educação

PREFEITURA MUNICIPAL DE PONTA GROSSA
Secretaria Municipal de Educação
Av. Visconde de Taunay, 950 - Tel.: (042) 3220-1127 -Fax.:2201221 -e-mail:assessoria-sme@pontagrossa.pr.gov.br - CEP:84.051-900 P.Grossa - PR

ASSESSORIA DE GESTÃO EDUCACIONAL
Em 05 de setembro de 2011.
Of. nº 052/2011

Senhora diretora:

Informamos que a Universidade Estadual de Ponta Grossa através do Departamento de Odontologia, tem a autorização desta Secretaria, para realizar a pesquisa intitulada: **O PAPEL DO MATERIAL RESTAURADOR NAS ALTERAÇÕES EM DENTINA DE DENTES PERMANENTES TRATADOS PELA MÍNIMA INTERVENÇÃO.**

Mediante o exposto, solicitamos que V.Sra. receba a pesquisadora e acompanhe a realização dos trabalho. Caso necessário, outros esclarecimentos poderão ser obtidos na Assessoria de Gestão Educacional – AGE/SME ou com a professora Eunice Kuhn, coordenadora da pesquisa, pelo telefone 3220 3111.

Atenciosamente,

Teresa Jussara Luporini
Assessora de Gestão Educacional

Ilma. Sra.
SUELI MIKA ANTUNES
Diretora da Escola Municipal Professor Eloy Avrechack
NESTA

ANEXO D - Termo de consentimento livre e esclarecido

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

5- **1.Título da pesquisa:** O papel do material restaurador nas alterações em dentina cariada de dentes permanentes tratados pela mínima intervenção

6- **2. Pesquisadores:** Denise Stadler Wambier; Eunice Kuhn; Eduardo Baulm Campagnoli

7- **3. Proposição:** estudar a reação dos dentes permanentes depois da realização do fechamento da cavidade (buraco do dente).

8- **4. Procedimentos:** Os dentes permanentes que tem cavidades de cárie serão radiografados e tratados por meio da remoção da cárie mais superficial com colheres para remover dentina (cárie amolecida) e em seguida o buraco do dente será fechado com um material odontológico apropriado para este tipo de obturação e a cárie que foi tirada do dente será estudada em laboratório. Para ver como o dente vai se comportar com o passar do tempo, uma nova radiografia será tirada e uma nova amostra de dentina será removida do dente 60 dias depois para novos estudos. Isto se faz necessário, pois se toda a dentina for removida no momento inicial, o risco de exposição pulpar (expor o nervo do dente) é bem maior e suas conseqüências também; havendo, muitas vezes, a necessidade de tratamento endodôntico (tratamento de canal).

9- **5. Local da Pesquisa**: O exame clínico e tratamento serão realizados nas Clínicas de Odontopediatria e Saúde Coletiva da UEPG ou na clínica da UEPG em Guaragi.

10- **6. Resultados Esperados**: espera-se, com este estudo, verificar a reação de dentes permanentes de crianças e adolescentes de 7 a 15 anos de idade, após fechamento da cavidade.

11- **7. Análise Crítica dos Riscos e Benefícios:** Como benefícios, os participantes desta pesquisa terão os seus dentes obturados. Também receberão orientações sobre saúde bucal, tratamento preventivo (aplicação de selantes e flúor) e acompanhamento clínico por pelo menos 12 meses. Desta forma, as crianças terão condições de manter sua saúde bucal. Em relação aos riscos, há a possibilidade de dor pós-operatória. Esta dor é bastante rara após a realização do fechamento da cavidade, no entanto, caso ocorra, os pesquisadores se comprometem a prestar o atendimento necessário para sua eliminação.

12- **8. Forma de Acompanhamento e Garantia de Esclarecimentos:** Os pais/pacientes terão a garantia de que receberão esclarecimento a qualquer dúvida, acerca dos procedimentos, riscos, benefícios e outros assuntos relacionados com a pesquisa. Os pesquisadores responsáveis assumem o compromisso de proporcionar informação atualizada obtida durante o estudo, ainda que esta possa afetar a vontade do indivíduo em continuar participando dele.

13- **9. Retirada do Consentimento:** Os pais/pacientes terão a liberdade de se recusar a participar da pesquisa ou de retirar seu consentimento a qualquer momento, sem sofrer qualquer tipo de prejuízo, ou represálias de qualquer natureza. O tratamento odontológico do paciente será completado mesmo que este deixe a pesquisa.

14- **10. Garantia de Sigilo:** os pesquisadores se comprometem a resguardar todas as informações individuais, tratando-as com impessoalidade e não revelando a identidade do sujeito que as originou.

15- **11. Forma de Ressarcimento de Despesas e Indenização:** Os indivíduos não deverão ter qualquer despesa extra, pois receberão o mesmo tratamento a que teriam direito caso não participassem da presente pesquisa. Caso ocorra algum dano ao paciente comprovadamente decorrente do procedimento experimental, será assegurado ao mesmo o tratamento e o ressarcimento das eventuais despesas.

5- Eu _____, certifico que, tendo lido as informações acima e suficientemente esclarecido de todos os itens, pelos pesquisadores e clínicos responsáveis: Denise Stadler Wambier, Eunice Kuhn; Eduardo Bauml Campagnoli concordo plenamente em participar nesta pesquisa. Assim, eu concordo com os termos do trabalho de pesquisa expostos acima. Certifico também ter recebido uma cópia deste Termo de Consentimento Livre e Esclarecido

6- Ponta Grossa, _____ de _____ de _____

7- Nome: _____ Idade: _____

8- RG:_____ Assinatura:

9- Eu, _____ _____, responsável pelo menor _____, certifico que, tendo lido as informações acima, e suficientemente esclarecido de todos os itens, pelos pesquisadores e clínicos responsáveis: Denise Stadler Wambier, Eunice Kuhn; Eduardo Bauml Campagnoli estou plenamente de acordo com a participação de meu filho nesta pesquisa. Assim, eu concordo com os termos do trabalho de pesquisa expostos acima.

10- Certifico também ter recebido uma cópia deste Termo de Consentimento Livre e Esclarecido.Ponta Grossa, _____ de _____ de 2_____.

11- Nome: _____ Idade: _____ RG: _____ Grau de parentesco: _____ Assinatura: _____

12- Para entrar em contato com os pesquisadores: (42) 3220-3104 : (42)9978-6540 e (42) 9911-9365

13- Denise Stadler Wambier, Eunice Kuhn, Eduardo Bauml Campagnoli

14- COMISSÃO DE ÉTICA EM PESQUISA EM SERES HUMANOS

15- Av.: Gen. Carlos Cavalcanti, 4748 CEP: 84030-900 Bloco M, Sala 12

16- Campus Uvaranas Ponta Grossa Fone: (42) 3220.3108 e-mail: seccoep@uegp.br