

UNIVERSIDADE ESTADUAL DE PONTA GROSSA
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA – DOUTORADO
ÁREA DE CONCENTRAÇÃO: CLÍNICA INTEGRADA

ANA CLÁUDIA RODRIGUES CHIBINSKI

**AVALIAÇÃO *IN VIVO* DAS ALTERAÇÕES EM DENTINA INFECTADA DE
DENTES DECÍDUOS APÓS TRATAMENTO RESTAURADOR
MINIMAMENTE INVASIVO**

PONTA GROSSA
2012

UNIVERSIDADE ESTADUAL DE PONTA GROSSA
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA – DOUTORADO
ÁREA DE CONCENTRAÇÃO: CLÍNICA INTEGRADA

ANA CLÁUDIA RODRIGUES CHIBINSKI

**AVALIAÇÃO *IN VIVO* DAS ALTERAÇÕES EM DENTINA INFECTADA DE
DENTES DECÍDUOS APÓS TRATAMENTO RESTAURADOR
MINIMAMENTE INVASIVO**

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.

Orientador: Profa. Dra. Denise Stadler Wambier

PONTA GROSSA
2012

Catálogo na Fonte
Elaborada pelo Setor Tratamento da Informação BICEN/UEPG

- C532a Chibinski, Ana Cláudia Rodrigues
Avaliação *in vivo* das alterações em dentina infectada de dentes decíduos após tratamento restaurador minimamente invasivo / Ana Cláudia Rodrigues Chibinski. Ponta Grossa, 2012.
119 f.
- Tese (Doutorado em Odontologia – área de concentração : Clínica Integrada), Universidade Estadual de Ponta Grossa.
Orientadora: Profa. Dra. Denise Stadler Wambier
1. Dentina. 2. Cárie dentária. 3. Dente decíduo. I. Wambier, Denise Stadler. II. Universidade Estadual de Ponta Grossa. Doutorado em Odontologia. III.T.

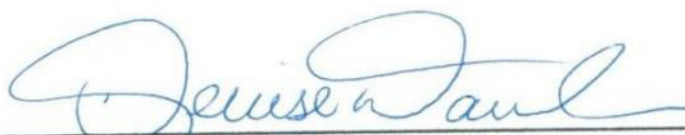
CDD: 617.67

ANA CLAUDIA RODRIGUES CHIBINSKI

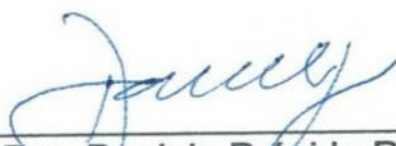
**AVALIAÇÃO *IN VIVO* DAS ALTERAÇÕES EM DENTINA INFECTADA DE
DENTES DECÍDUOS APÓS TRATAMENTO RESTAURADOR
MINIMAMENTE INVASIVO**

Tese apresentada como pré-requisito para obtenção do título de Doutor em
Odontologia na Universidade Estadual de Ponta Grossa, no Curso de Doutorado em
Odontologia – Área de concentração: Clínica Integrada.
Linha de Pesquisa: Prevenção.

Ponta Grossa, 07 de dezembro de 2012.



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



Profa. Dra. Daniela Procida Raggio
Universidade de São Paulo



Prof. Dr. Rafael Gomes Ditterich
Universidade Federal do Paraná



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



Profa. Dra. Stella Kossatz Pereira
Universidade Estadual de Ponta Grossa

DADOS CURRICULARES

Ana Cláudia Rodrigues Chibinski

NASCIMENTO 19.06.1971	Ponta Grossa, Paraná - Brasil
FILIAÇÃO	Cleide Aparecida Faria Rodrigues Jorge Rodrigues
1989 - 1992	Curso de Graduação em Odontologia. Universidade Estadual de Ponta Grossa (UEPG). Ponta Grossa, Paraná, Brasil.
1997 - 1998	Especialização em Odontopediatria. Universidade Federal do Paraná (UFPR). Curitiba, Paraná, Brasil.
2002 - 2004	Curso de Pós-Graduação em Odontologia. Área de Concentração: Clínica Integrada. Linha de Pesquisa: Prevenção em Odontologia. Nível de Mestrado. Universidade Estadual de Ponta Grossa (UEPG). Ponta Grossa, Paraná, Brasil.
2009 - 2012	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.
2009 - atual	Professora Assistente do Departamento de Odontologia da Universidade Estadual de Ponta Grossa (UEPG). Ponta Grossa, Paraná, Brasil.

Ao **Eduardo**

Espírito sábio e generoso,
que me escolheu e
vem me ensinando a ser MÃE.

O abraço que você veio me dar,
exatamente no momento em que eu
tentava traduzir meus sentimentos
em palavras, confirmou o que eu
sempre soube: nossa conexão é de
alma e coração.

Dedico este trabalho a você,
meu pequeno grande homem.

Agradecimentos especiais a minha família:

A minha mãe **Cleide**,
que me apontou o caminho e me deu os
instrumentos necessários para perseguir meus
sonhos. Hoje, tenho orgulho de ter seguido
seus passos na docência e esperança de me
tornar, no futuro, uma verdadeira
Mestre, como você é.

A minha avó **Josefina**
(*in memoriam*), que lá de cima deve estar cheia
de orgulho por mais esta etapa concluída...

Ao meu pai **Jorge** e irmã **Simone**,
pela torcida, palavras de incentivo e apoio
presente em todas as horas.

Aos queridos **Guilherme** e **Marina**,
pelos sorrisos sinceros e olhinhos brilhando
com que me brindam quando nos encontramos
e por me deixarem ser um pouco
sua MÃE também

Ao meu marido **Maurício**,
por ser um grande incentivador da minha
caminhada profissional,
há pelo menos 20 anos...

*"Podem acreditar em mim: é uma tranquilidade não desejar ser
querida por ser impecável e se saber querida inclusive nas falhas."*

Ana Paula Padrão

AGRADECIMENTOS

À **Universidade Estadual de Ponta Grossa (UEPG)**, que, capitaneada por seu reitor **Prof. Dr. João Carlos Gomes**, vem mostrando seu potencial e se firmando como um centro de destaque na pesquisa e pós-graduação. Obrigada pela oportunidade de fazer parte desta história de crescimento contínuo desde a minha graduação.

À **Profa. Dra. Osnara Maria Mongruel Gomes**, coordenadora do Programa de Pós-Graduação *Stricto Sensu* em Odontologia da UEPG, pela competência e firmeza com que desenvolve seu trabalho, sempre voltado para a busca da qualidade na pós-graduação.

A minha orientadora **Profa. Dra. Denise Stadler Wambier**, que no decorrer destes anos de convivência se tornou minha grande amiga. Obrigada pelo sorriso franco, pela discussão profícua e pela conversa "jogada fora", pelo estímulo constante e por acreditar que eu conseguiria quando eu mesma estava duvidando. Agradeço pelas oportunidades e pela parceria que se consolidou nestes 4 anos e que eu espero continue por muito tempo...

À **Profa. Dra. Alessandra Reis**, pesquisadora nata, de mente brilhante e raciocínio rápido, que tive o prazer de conhecer no início deste curso. Obrigada pela generosidade com que compartilhou comigo suas idéias e amplo conhecimento e, principalmente, pelas palavras "certeiras" de estímulo no momento em que mais precisei.

Ao **Prof. Dr. José Rosa Gomes**, um entusiasta da pesquisa e professor por vocação. Obrigado por me acolher em seu laboratório e me conduzir com clareza e precisão pelo mundo da imunohistoquímica.

A **profa. Ms. Eunice Kuhn**, minha colega de aventuras pelas estradas rurais de Ponta Grossa. Obrigada pela companhia e pela troca de experiências.

À **Escola Municipal Profa. Maria Elvira Justus Schmidt**, na pessoa da sua diretora **Profa. Bernardete Malechi**, por sua acolhida carinhosa e esforço conjunto para que este trabalho de pesquisa fosse realizado.

Ao **CAIC Reitor Alvaro Augusto Cunha Rocha/UEPG**, na pessoa da sua diretora **Profa. Audrey Pietrobelli de Souza**, uma instituição que valoriza a qualificação de seus colaboradores e estimula seu crescimento profissional.

Às profissionais da Seção de Saúde do CAIC Reitor Alvaro Augusto Cunha Rocha, **Cléa Berger Silva, Josélia Jacob e Jussara Lepinski**, pelo carinho e amizade que sempre me dedicaram, mas principalmente pelo apoio durante o curso e pela "torcida" para que tudo desse certo.

Ao técnico de laboratório **Dr. Milton Domingos Michél**, pelas análises em microscopia eletrônica de varredura, à técnica **Janaína Diniz**, pelo auxílio com os meios de cultura e ao técnico em radiologia **José Milano**, pela ajuda no processamento automático das radiografias.

À técnica de laboratório **Maria Inês Cordeiro** e à mestrande em Biologia **Kamila Camargo**, pela ajuda na confecção das lâminas de imunohistoquímica.

Aos **professores** e **colegas** do curso de Doutorado em Odontologia da UEPG, pela troca de conhecimento, pela amizade e companheirismo.

À **Fundação Araucária** de Apoio ao Desenvolvimento Científico e Tecnológico do Paraná, pelo financiamento do projeto de pesquisa que possibilitou o desenvolvimento desta tese.

E, acima de tudo, **aos pequenos pacientes**, razão maior da existência deste trabalho.

***A mente que se abre a uma nova idéia,
jamais retorna ao seu tamanho original***

Albert Einstein

RESUMO

Chibinski, ACR. **Avaliação *in vivo* das alterações em dentina infectada de dentes decíduos após tratamento restaurador minimamente invasivo.** [Tese Doutorado em Clínica Integrada - Faculdade de Odontologia]. Ponta Grossa: Universidade Estadual de Ponta Grossa, 2012.

Este estudo avaliou a dentina infectada de dentes decíduos antes e após selamento cavitário, com critérios clínicos (cor, consistência e fluorescência dentinária) e laboratoriais (avaliação microbiológica, ultraestrutural, mineral e imunohistoquímica). As condições clínicas e radiográficas também foram verificadas ao longo do tempo por um período médio de 11 meses. Foram selecionados 45 molares decíduos com lesões de cárie ativa e sem sinais ou sintomas de patologias pulpares. A dentina infectada foi dividida em duas partes para avaliação e remoção de fragmentos em momentos distintos: antes do selamento cavitário (porção mesial) e 60 dias após restauração (porção distal). Escores foram utilizados para registro da cor e da consistência dentinária e a fluorescência foi medida com equipamento DIAGNOdent 2095. Fragmentos de dentina cariada foram coletados para as análises microbiológica (total de viáveis), ultraestrutural (MEV); de conteúdo mineral (MEV/EDS - cálcio, fósforo e flúor) e imunohistoquímica (expressões de MMP-2, MMP-8, MMP-9, colágeno tipo I e sialoproteína óssea [BSP]). As cavidades foram restauradas com cimento de ionômero de vidro (CIV) de alta viscosidade e reabertas após 60 dias, repetindo-se as mesmas análises, porém na porção distal. A remoção de tecido cariado foi restrita à coleta dos fragmentos necessários para os estudos, os dentes foram novamente restaurados com CIV e acompanhados clínica e radiograficamente. As características das restaurações foram avaliadas pelos critérios do *United States Public Health System*. Técnicas de subtração radiográfica em radiografias padronizadas permitiram definir diferenças no conteúdo mineral da dentina cariada mantida sob a restauração ao longo do tempo por meio de variações nos tons de cinza. Os dados referentes à cor, consistência, fluorescência e total de bactérias viáveis foram submetidos ao teste de Wilcoxon; as porcentagens em peso de cálcio, fósforo e flúor, assim como as expressões de MMP-2, MMP-8, MMP-9, colágeno tipo I e BSP foram comparadas por meio do teste t pareado; as características das restaurações após 10-13 meses de acompanhamento foram verificadas com o teste de McNemar; as variações nos níveis médios de cinza ao longo do tempo foram avaliadas com o teste ANOVA dois critérios para medidas repetidas ($\alpha = 0.05$). Após 60 dias de selamento cavitário, as amostras de dentina infectada apresentaram valores mais baixos de fluorescência dentinária ($p < 0.0001$) e total de microorganismos viáveis ($p < 0.0001$), assim

como um aumento na consistência ($p<0.0001$), nos conteúdos de cálcio ($p=0.001$) e fósforo ($p=0.002$) e na expressão de MMP-8 ($p=0.018$), colágeno tipo I ($p=0.040$) e BSP ($p<0.001$). Não foram detectadas diferenças significativas na cor, conteúdo de flúor e expressões de MMP-2 e MMP-9. As alterações ultraestruturais foram evidentes, mostrando poucas bactérias e melhor organização tecidual com estreitamento de túbulos dentinários e dentina intertubular mais compacta e mineralizada. Após 10-13 meses de acompanhamento, as restaurações mostraram-se clinicamente adequadas, com alterações detectadas apenas para descoloração cavosuperficial ($p=0.008$), desgaste ($p<0.001$) e textura superficial ($p<0.001$). A subtração radiográfica demonstrou que as médias dos tons de cinza em dentina sadia e cariada aumentaram ($p<0.001$). Conclui-se que o selamento de dentina decídua infectada promove a reorganização tecidual e favorece a remineralização da dentina cariada remanescente, num processo que se mantém contínuo ao longo do tempo.

Palavras-chave: Dentina. Cárie Dentária. Dente Decíduo.

ABSTRACT

Chibinski, ACR. **In vivo evaluation of infected dentin changes from deciduous teeth after minimally invasive restorative treatment.** [Thesis Doctorate in Integrated Dental Clinics - School of Dentistry]. Ponta Grossa: Ponta Grossa State University, 2012.

This study evaluated the infected dentin from deciduous teeth before and after cavity sealing, using clinical criteria (dentin color, consistency and fluorescence) and laboratorial criteria (microbiological, ultra-structural, mineral and immunohistochemical evaluations). Clinical and radiographic aspects from the treated teeth were also assessed after a mean period of 11 months in oral function. Forty-five deciduous molars with active caries lesions and without signs or symptoms of pulp pathologies were selected. The infected dentin was divided in two portions for evaluation and removal in different moments: before cavity sealing (mesial portion) and 60 days after restoration (distal portion). Scores were used to register dentin color and consistency; dentin fluorescence was measured with DIAGNOdent 2095. Carious dentin samples were collected for microbiological analysis (total viable counts), ultra-structural (SEM) and mineral analysis (SEM/EDS - calcium, phosphorus and fluoride) and immunohistochemical assays (expressions of MMP-2, MMP-8, MMP-9, type I collagen and bone sialoprotein [BSP]). The cavities were restored with high viscosity glass ionomer cement (GIC) and reopened after 60 days, when the same tests were done in the distal portion. After removal of the caries samples needed for the different analysis, the teeth were restored with GIC again and were clinical and radiographic followed-up. The characteristics of the restorations were evaluated using United States Public Health System criteria. Radiographic subtraction techniques in standardized radiographics allowed the detection of differences in the mineral content from the carious dentin bellow restoration over time, using the evaluation of gray levels changes. Data from dentin color, consistency, fluorescence and total viable counts were analysed using Wilcoxon signed rank test; the weight percentage of calcium, phosphorus and fluoride, and the expressions of MMP-2, MMP-8, MMP-9, collagen type I and BSP were compared using paired t test; the restorations characteristics after 10-13 follow-up period were analysed using McNemar test; the mean gray levels over time were compared using two way ANOVA ($\alpha=0.05$). Sixty days after cavity sealing, the infected dentin samples exhibited lower values of fluorescence ($p<0.0001$) and total viable counts ($p<0.0001$), as well as higher values of consistency ($p<0.0001$), calcium ($p=0.001$) and phosphorus content ($p=0.002$) and expressions of MMP-8 ($p=0.018$), type I collagen ($p=0.004$) and BSP ($p<0.001$). There were no statistical differences in dentin color, fluoride

content and expressions of MMP-2 and MMP-9. Ultra-structural changes were clear: the infected dentin showed much less bacteria and better tissue organization, with narrower dentinal tubules and a more mineralized and compact intertubular dentin. After 10-13 months follow-up, the restorations were acceptable clinically, with alterations detected only for cavosurface discoloration ($p=0.008$), wear ($p < 0.001$) and surface texture ($p < 0.001$). The radiographic subtraction routine showed that the mean of gray levels in sound and carious dentin increased ($p<0.001$). It is possible to conclude that sealing infected dentin from deciduous teeth promotes tissue reorganization and favors the remineralization of remained carious dentin, in a constant process over the time.

Key words: Dentin. Dental Caries. Tooth, Deciduous.

LISTA DE ABREVIATURAS E SIGLAS

AAPD	American Academy of Pediatric Dentistry
ANOVA	Análise de variância
BHI	<i>Brain heart infusion</i>
bit	<i>Binary digit</i>
BSP	<i>Bone sialoprotein</i>
CFU	<i>Colony forming units</i>
CIV	Cimento de ionômero de vidro
CO	<i>Color</i>
COEP/UEPG	Comissão de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Ponta Grossa
CONEP/MS	Conselho Nacional de Saúde/ Ministério da Saúde
COS	<i>Consistency</i>
DAB	3,3' - diaminobenzidina
dpi	<i>Dots per inch</i>
DSR	<i>Digital subtraction radiography</i>
ECM	<i>Extra-celular matrix</i>
EDS	<i>Dispersive X-ray spectrometry energy</i>
EDS	Espectrometria dispersiva de raios X
EDTA	Ácido etilenodiamino tetra-acético
GIC	<i>Glass ionomer cement</i>
h	Horas
ICDAS	<i>International Caries Detection and Assessment System</i>
IPT	<i>Indirect pulp therapy</i>
Kv	Quilovoltagem
kVp	Quilovoltagem pico
LF	<i>Laser fluorescence</i>
M	Molar
mA	Miliamperagem
MC	<i>Mineral content</i>
MEV	Microscopia eletrônica de varredura
ml	Mililitro
MMP	Metaloproteinase
MMPs	Metaloproteinases

NCPs	<i>Non collagenous proteins</i>
nm	Nanômetro
PBS	Solução tampão fosfato
PBS/BSA	Solução de tampão fosfato e soro-albumina bovina
pH	Potencial hidrogeniônico
RGB	Red, green, blue
s	Segundos
SEM	<i>Scanning electron microscope</i>
SIBLINGs	<i>Small integrin-binding ligand n-linked glycoproteins</i>
TGF- β 1	<i>Transforming growth factor beta 1</i>
TIFF	<i>Tagged image file format</i>
TIMPs	<i>Tissue inhibitors of metalloproteinases</i>
TPI	Tratamento pulpar indireto
TVC	<i>Total viable counting</i>
USPHS	<i>United States Public Health Service</i>

LISTA DE SÍMBOLOS

#	Número
%	Porcentagem
<	Menor
=	Igual
>	Maior
±	Mais ou menos
°C	Graus Celsius
p	Significância estatística
x	Aumento de lente óptico
α	Alfa (nível de significância)

SUMÁRIO

1	INTRODUÇÃO	17
2	PROPOSIÇÃO	22
2.1	PROPOSIÇÃO GERAL	22
2.2	PROPOSIÇÕES ESPECÍFICAS	22
3	MATERIAL E MÉTODOS	23
4	ARTIGOS	34
4.1	ARTIGO 1	34
4.2	ARTIGO 2	55
4.3	ARTIGO 3	78
5	DISCUSSÃO	102
6	CONCLUSÕES.....	109
	REFERÊNCIAS	110
	ANEXO A - Documento de aprovação da Comissão de Ética em Pesquisa da Universidade Estadual de Ponta Grossa	118
	ANEXO B - Carta de aceite do artigo 3	119

1 INTRODUÇÃO

A remoção "completa" de tecido cariado já não é mais um requisito fundamental para a restauração de elementos dentários afetados por lesões de cárie. A estratégia atual preconiza a menor intervenção operatória possível, na expectativa da defesa biológica do dente, seguindo os princípios da Odontologia Minimamente Invasiva (ERICSON, 2004; SELWITZ; ISMAIL; PITTS, 2007). Nessa estratégia, há dois procedimentos que são os mais difundidos para serem empregados na prática clínica: o tratamento pulpar indireto e a restauração atraumática.

Em ambos os casos, o que se propõe é a remoção incompleta do tecido cariado, guiada pela distinção entre as duas camadas da dentina cariada, ou seja, remove-se a camada externa (dentina infectada), extremamente contaminada e desestruturada, e mantém-se a camada interna (dentina afetada), que conserva uma rede de fibras colágenas, possibilitando a ligação de cristais de apatita e a reorganização deste substrato (BERTASSONI et al., 2009).

O tratamento pulpar indireto (TPI) é especialmente interessante em lesões de cárie profundas, que conduziriam a uma exposição pulpar caso todo tecido cariado fosse removido (HAYASHI et al., 2011). Consiste na remoção parcial da dentina cariada e proteção do tecido remanescente com material biocompatível (THOMPSON et al., 2008).

Já as restaurações atraumáticas ampliaram as indicações da remoção incompleta de tecido cariado, associando instrumentação manual e selamento cavitário com cimento de ionômero de vidro, o que possibilitou seu uso tanto em situações de campo quanto em consultório odontológico (MASSARA; ALVES; BRANDÃO, 2002). Para os dois procedimentos, a condição fundamental para sucesso do tratamento é o bom vedamento marginal, que previne a infiltração do substrato para metabolismo dos microorganismos remanescentes (BUYUKGURAL; CEHRELI, 2008; THOMPSON et al., 2008).

O tecido afetado mantido na cavidade após selamento cavitário tende a se reestruturar, exibindo, com o passar do tempo, melhor organização tecidual e redução significativa na população bacteriana (WAMBIER et al.,

2007; DUQUE et al., 2009; LULA et al., 2009). Observações em microscopia eletrônica mostraram que a dentina intertubular torna-se mais densa e, a rede de fibras colágenas, mais compacta (MASSARA; ALVES; BRANDÃO, 2002; WAMBIER et al., 2007). Estudos clínicos longitudinais corroboram estes achados, já que apresentaram evidências radiográficas de formação de dentina reacional (MALTZ et al., 2007; ALVES et al., 2010).

Em todas as pesquisas citadas, o selamento cavitário efetivou-se após remoção parcial da dentina cariada e este procedimento foi o suficiente para paralisar o desenvolvimento do processo carioso e dar condições para a deposição de dentina terciária. Portanto, segundo revisões sistemáticas disponíveis na literatura, o selamento cavitário após remoção parcial de tecido cariado pode ser considerado procedimento de escolha no tratamento de lesões de cárie profundas (RICKETTS et al., 2006; THOMPSON et al., 2008; FERREIRA et al., 2012).

Na prática, o profissional tem o desafio de diferenciar as duas camadas dentinárias (IWAMI et al., 2004) e definir qual o limite seguro para remoção, considerando a possibilidade de reparo tecidual e o risco de exposição pulpar. Esta diferenciação é complexa e subjetiva do ponto de vista clínico, já que ainda não há uma técnica capaz de distinguir as duas camadas com precisão (NGO et al., 2006). Sabe-se que, mesmo após a remoção da camada infectada, são observados microorganismos viáveis na dentina afetada remanescente (MALTZ et al., 2002; TOI; BONECKER; CLEATON-JONES, 2003; ERSIN et al., 2006). Deve-se questionar, portanto, até que ponto a eliminação de toda dentina infectada da cavidade é importante para o sucesso do tratamento.

Um estudo longitudinal clássico na literatura odontológica mostra, após 10 anos de acompanhamento que, em dentes permanentes, a paralisação do processo carioso independe da remoção da dentina infectada, desde que o selamento da cavidade se mantenha (MERTZ-FAIRHURST et al., 1998). Em dentes decíduos, são raros os estudos sobre selamento mantendo-se dentina infectada na cavidade. Pode-se citar um estudo transversal utilizando a associação de cimento de ionômero de vidro e antibióticos (PINHEIRO et al., 2005) e uma pesquisa longitudinal, com 48 meses de acompanhamento, no qual os autores restauraram dentes decíduos cariados

com coras de aço inoxidável sem qualquer preparo prévio (INNES; EVANS; STIRRUPS, 2011).

Considerando-se, portanto, a permanência de dentina decídua infectada na cavidade, o presente estudo emprega um conjunto de métodos para descrever as alterações nesse tecido, na tentativa de esclarecer o mecanismo biológico que conduz ao processo de reparo.

O ponto de partida é comum a maioria dos estudos com dentina cariada, ou seja, os critérios visual (cor) e tátil (consistência), que são os parâmetros clínicos mais habitualmente usados para orientação dos profissionais no momento da remoção do tecido cariado (GURBUZ; YILMAZ; SENGUL, 2008; IWAMI et al., 2008; MARCHI et al., 2008; DUQUE et al., 2009) e a contaminação bacteriana da dentina, que costuma ser o parâmetro laboratorial mais frequente nas pesquisas (MALTZ et al., 2002; BONECKER et al., 2003; ERSIN et al., 2006; WAMBIER et al., 2007; DUQUE et al., 2009; LULA et al., 2009; LULA et al., 2011).

A estes critérios, foram acrescentados outros elementos. Na expectativa de oferecer ao clínico uma possibilidade de avaliação *in situ* da dentina cariada, a fluorescência dentinária foi mensurada. Sabe-se que o grau de fluorescência de tecidos dentais sadios e cariados é diferente quando estimulados com laser de comprimento de onda de 655nm. Avaliações da dentina cariada realizadas com o DIAGNOdent (Kavo, Biberach, Alemanha), que é um equipamento odontológico periférico, não alteram o tecido, são passíveis de realização na clínica e podem ser relacionadas com profundidade da lesão de cárie (MENDES et al., 2005) e/ou contaminação bacteriana (IWAMI et al., 2011).

O conteúdo mineral da dentina cariada selada é um dado indicativo de reorganização/remineralização tecidual e pode ser associado à consistência da dentina. Após selamento cavitário, a remineralização da dentina foi demonstrada em dentes permanentes, por meio de avaliação radiográfica (OLIVEIRA et al., 2006; MALTZ et al., 2007; ALVES et al., 2010). Em dentes decíduos, o aumento no conteúdo mineral da dentina foi constatado por meio de espectrometria dispersiva de raios X (EDS) (MASSARA; ALVES; BRANDÃO, 2002) e microdureza (SANTIAGO et al., 2005; MARCHI et al., 2008; FRANZON et al., 2009).

Ao longo do tempo, também é possível observar mudanças no conteúdo mineral da dentina por meio de acompanhamento radiográfico. Todavia, está demonstrado que a avaliação visual de pares de radiografias sequenciais resulta em falhas de concordância intra-examinador (CHRISTGAU et al., 1998; PETRIKOWSKI et al., 1998), além de não detectar mudanças sutis de conteúdo mineral (ONO et al., 2011). Desta forma, o acompanhamento radiográfico das restaurações que compõe a amostra deste estudo foi feito por meio da obtenção dos registros das imagens e subtração radiográfica.

Todavia, a dentina não deve ser analisada apenas sob o aspecto mineral. Este tecido é um complexo formado por túbulos dentinários, prolongamentos odontoblásticos e fluido dentinário, delimitados por um tecido altamente mineralizado (dentina peritubular) e cercados por uma matriz tecidual mineralizada (dentina intertubular) (ZAVGORODNIY; ROHANIZADEH; SWAIN, 2008). Uma das suas principais características é o elevado conteúdo orgânico (30 a 35% em peso), representado principalmente pelo colágeno tipo I (ZAVGORODNIY; ROHANIZADEH; SWAIN, 2008; MARANGOS et al., 2009; ZHENG et al., 2011), que constitui aproximadamente 98% da matriz orgânica secretada pelos odontoblastos (ORSINI et al., 2012). É a partir da estrutura oferecida pela rede de fibras colágenas que os cristais de apatita são depositados (LEE et al., 2006; ZAVGORODNIY; ROHANIZADEH; SWAIN, 2008), sob influência de outros elementos da matriz orgânica como as proteínas não colagênicas (MAZZONI et al., 2012), seja para formação da dentina primária, secundária ou terciária.

Com o avanço do processo carioso, as alterações na dentina intertubular são imediatamente sentidas uma vez que as toxinas microbianas se espalham e o pH do meio cai (FRANK, 1990). Desta forma, não haverá apenas perdas minerais com a dissolução da apatita pelos ácidos bacterianos, mas também a ativação de metaloproteinases endógenas (MMPs) que degradam a matriz orgânica da dentina e permitem a continuidade do processo carioso e a formação da cavidade (TJADERHANE et al., 1998; CHAUSSAIN-MILLER et al., 2006; HANNAS et al., 2007). Estas proteinases são secretadas pelos odontoblastos e tem papel fundamental durante o desenvolvimento dentário. Sua ativação pode ocorrer por diversos mecanismos, como a interação com outras proteinases, ou um pH mais ácido, o que é proporcionado

pela atividade de cárie. Neste processo, há a participação ativa de pelo menos duas gelatinases, que atuam em colágeno desnaturado (MMP-2 e MMP-9) e uma collagenase, que degrada fibras colágenas intactas (MMP-8) (TJADERHANE et al., 1998).

Ao se esperar uma reorganização tecidual da dentina cariada selada, as MMPs provavelmente assumiriam sua função precípua e presente na grande maioria dos tecidos do corpo humano, que é a de remodelação/degradação da matriz extracelular tanto em tecidos saudáveis quanto em condições patológicas (HANNAS et al., 2007), envolvendo alterações morfológicas e organização espacial de tecidos que requerem síntese e organização de matriz extracelular para posterior mineralização (BOURD-BOITTIN et al., 2005). Desta forma, o presente estudo trabalhou com a hipótese de que há participação ativa de MMPs na dentina cariada de dentes decíduos e que a dentina "melhorada" que se obtém após selamento cavitário teve influência do processo remodelador destas proteinases.

É necessário salientar que, na literatura odontológica, apesar de haver estudos sobre a ação das metaloproteinases e proteínas não colagênicas no processo carioso (TJADERHANE et al., 1998; SHIMADA et al., 2009; BOUSHELL; NAGAOKA; YAMAUCHI, 2011), não é do conhecimento desta autora relatos desta investigação em relação a dentes decíduos.

Portanto, considerando-se a importância de se caracterizar a capacidade de reação de dentes decíduos, esta pesquisa propõe a associação entre dados clínicos/laboratoriais, incluindo o estudo de eventos em nível molecular na dentina infectada, que podem ter efeito no processo de reparo e mineralização deste tecido após selamento cavitário.

2 PROPOSIÇÃO

2.1 PROPOSIÇÃO GERAL

Descrever as características da dentina infectada de dentes decíduos antes e após tratamento restaurador minimamente invasivo.

2.2 PROPOSIÇÕES ESPECÍFICAS

- Identificar parâmetros clinicamente detectáveis, como alterações visuais (cor), táteis (consistência) e de fluorescência da dentina infectada antes e após selamento cavitário;
- Verificar o total de microorganismos viáveis antes e após selamento cavitário;
- Descrever micromorfolologicamente as estruturas da dentina infectada nos momentos inicial e após selamento cavitário;
- Avaliar as alterações no conteúdo mineral da dentina infectada de dentes decíduos antes e após selamento cavitário;
- Verificar a presença de MMPs na dentina infectada antes e depois do tratamento restaurador minimamente invasivo;
- Descrever as condições clínicas e radiográficas dos elementos tratados pela mínima intervenção após acompanhamento mínimo de 10 meses.

3 MATERIAL E MÉTODOS

O presente estudo recebeu a aprovação da Comissão de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Ponta Grossa - COEP/UEPG (processo nº 15821/09) (Anexo A).

Um levantamento inicial, envolvendo aproximadamente 850 escolares, foi realizado, e 33 pacientes de ambos os sexos, com idades entre 3 e 10 anos (6.02 ± 2.12) foram selecionados. Os critérios de inclusão foram a presença de pelo menos um molar com lesão ativa e cavitada de cárie em dentina (código 6 de severidade da lesão cariosa do *International Caries Detection and Assessment System* - ICDAS) (ISMAIL et al., 2007), sem sinais ou sintomas de pulpopatias. A cavidade deveria estar limitada à superfície oclusal e com envolvimento de pelo menos 2/3 da espessura da dentina (verificado radiograficamente). Fluorescência dentinária entre 89 e 99, medida com o aparelho DIAGNOdent (KaVo, Biberach, Alemanha), também foi um critério de inclusão, já que leituras elevadas de fluorescência indicam lesão aguda na porção interna da dentina (BRAGA; MENDES; EKSTRAND, 2010). Crianças com patologias sistêmicas foram excluídas. Todos os pais/responsáveis pelas crianças selecionadas foram informados a respeito da natureza da pesquisa e assinaram um Termo de Consentimento Livre e Esclarecido. Após exame clínico e radiográfico dos pacientes selecionados, a amostra ficou constituída por 45 dentes.

Modelo do estudo

Um estudo clínico foi realizado para avaliação da dentina infectada de cada dente selecionado em dois momentos (antes e após selamento cavitário - amostra inicial e 60 dias, respectivamente). Para tanto, foram utilizados critérios clínicos e laboratoriais. Os critérios clínicos foram cor e consistência dentinária e aferição da fluorescência dentinária. Os critérios laboratoriais incluíram avaliação microbiológica, ultraestrutural, mineral e imunohistoquímica da dentina. Aos 60 dias e após um período médio de 11 meses (entre 10 e 13 meses), as restaurações foram novamente analisadas de acordo com os critérios de *United States Public Health System* (USPHS)

(BARNES et al., 1995), assim como a dentina remanescente também foi avaliada radiograficamente.

Procedimentos clínicos e experimentais

Foram necessárias duas sessões clínicas para cada dente para a obtenção das amostras de dentina cariada antes e após selamento cavitário. Em cada consulta, após anestesia local, a sequência de procedimentos foi a seguinte: (1) isolamento absoluto do campo operatório; (2) limpeza do dente com escova dental nova e água; (3) lavagem do dente com *spray* ar/água e (4) secagem sem desidratação da superfície.

A fim de padronizar o procedimento de obtenção das amostras, a lesão de cárie foi dividida em 2 partes equivalentes, usando-se um esculpador Hollembach em direção buco-lingual. A porção mesial foi considerada a amostra controle e a distal foi definida como amostra experimental (WAMBIER et al., 2007). Este procedimento foi usado para todos os critérios pesquisados.

Neste ponto, as características clínicas da dentina foram avaliadas, como descrito posteriormente. A seguir, amostras da porção mesial da dentina cariada foram removidas usando uma colher de dentina estéril, enquanto a porção distal foi mantida na cavidade a fim de ser removida 60 dias após selamento cavitário. Imediatamente após obtenção das amostras, os fragmentos dentinários foram processados para as análises microbiológica, ultraestrutural e imunohistoquímica. Todo tecido cariado do ângulo cavosuperficial foi removido a fim de garantir o selamento adequado da cavidade.

Os dentes foram selados com o cimento de ionômero de vidro de alta viscosidade Ketac Molar Easy Mix (3M ESPE, Saint Paul, Minnesota, EUA). O cimento foi protegido com uma camada de Adper Single Bond (3M ESPE, Saint Paul, Minnesota, EUA) para prevenir alterações na estrutura do ionômero até completa geleificação. Para facilitar a reabertura da cavidade após 60 dias sem remoção de tecido adicional, na primeira sessão clínica não foi utilizado condicionador de dentina.

Na segunda sessão, após 60 dias, a integridade marginal das restaurações foi avaliada, utilizando-se para isso os critérios descritos pelo *United States Public Health Service* (USPHS) (BARNES et al., 1995), no que diz respeito a descoloração cavosuperficial, lesões de cárie recorrentes, perda

de substância (desgaste); integridade marginal, textura superficial. Estas variáveis foram analisadas segundo os escores alfa (sem defeito clinicamente detectável, necessidade apenas de um polimento), bravo (aceitável clinicamente, mas é necessário reparo) e charlie (clinicamente inaceitável, é necessária a troca da restauração).

Então, o material restaurador foi removido com uma ponta diamantada estéril (nº 1016 - KG Sorensen, São Paulo, SP, BR) sob refrigeração e colheres de dentina. Após secagem sem desidratação, as características clínicas da dentina na porção distal foram avaliadas e novas amostras foram coletadas da porção distal para as análises microbiológica ultraestrutural e imunohistoquímica conforme descrito anteriormente para a porção mesial.

Avaliação clínica: cor e consistência dentinária

A cor e consistência da dentina cariada foram avaliadas no momento inicial e após 60 dias de selamento cavitário. A consistência da dentina cariada foi classificada em: (0) macia (a sonda exploradora penetra no tecido sem qualquer resistência à sua remoção); (1) média (há alguma resistência à remoção da sonda); (2) dura (resistência comparável à dentina hígida) (BJORNDAL; LARSEN; THYLSTRUP, 1997). A cor foi avaliada de acordo com os seguintes escores: (0) amarelo claro; (1) amarelo; (2) marrom claro; (3) marrom (adaptados de BONECKER et al., 2003). Ambas as análises foram feitas por um único examinador treinado, com índice Kappa de 0.8 para a cor e 0.9 para a consistência da dentina. O índice Kappa para a cor foi obtido com a análise de fotos clínicas; para a consistência, o índice foi obtido por meio do exame clínico de cavidades de cárie em pacientes. Em ambos os casos, o período entre as duas avaliações foi de aproximadamente 10 dias e as cavidades selecionadas para calibração do operador apresentavam as mesmas características dos dentes incluídos no estudo.

Medição da Fluorescência Dentinária

A fluorescência dentinária foi obtida usando o equipamento DIAGNOdent 2095 (KaVo, Biberach, Alemanha). Seguindo as instruções do fabricante, o equipamento foi calibrado em um objeto de referência (padrão de

porcelana fornecido pelo fabricante) previamente ao uso e recalibrado em uma superfície sadia do dente antes de cada avaliação. Os valores de fluorescência que podem ser obtidos variam de 0 a 99.

Foram realizadas três medições em dentina para cada cavidade, nas porções mesial (avaliação inicial) ou distal (avaliação de 60 dias). A média dos valores obtidos foi calculada para representar o valor de fluorescência do dente naquele determinado período de avaliação.

Avaliação microbiológica

Os fragmentos dentinários da porção mesial (amostra inicial) e distal (amostra de 60 dias) foram armazenados em 1ml de solução salina estéril e processados imediatamente. Após agitação por 1 minuto, diluições nas frações de 1:10, 1:100 e 1:1000 foram obtidas e, em triplicata, 100 µL de cada diluição foram semeados no meio de *Brain Heart Infusion* - BHI (Difco-BD, Sparks, Md., EUA). As placas foram incubadas a 37°C por 48 horas e o número de colônias contado para determinar o total de microorganismos viáveis. O total obtido em cada placa foi registrado, multiplicado pelo fator de diluição e o valor médio foi calculado para representar cada dente.

Análise ultraestrutural e de conteúdo mineral

Fragmentos de dentina das porções mesial (amostra inicial) e distal (amostra de 60-dias) foram armazenados em solução de glutaraldeído 2% com tampão fosfato de sódio de 0.1M (pH 7.4) por 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. Os espécimes foram fixados em *stubs* e receberam uma camada de ouro de 10 µm de espessura para análise em microscopia eletrônica de varredura (MEV) e espectrometria dispersiva de raios X (EDS). As porcentagens em peso de cálcio, fósforo e flúor, antes e após selamento cavitário, foram obtidas numa magnificação de 200x por 100s.

Análise Imunohistoquímica - preparo das lâminas

As expressões de MMP-2, MMP-8, MMP-9, colágeno tipo I e sialoproteína óssea, foram avaliadas das amostras de dentina decídua cariada antes e após 60 dias de selamento cavitário.

O processamento das amostras teve início imediatamente após coleta e consistiu no protocolo descrito a seguir.

Assim que foram removidas dos dentes, as amostras de dentina foram fixadas em formaldeído 10% por 48 h. O processo de desmineralização em ácido etilenodiamino tetra-acético 4,13% (EDTA) se deu em aproximadamente 2 meses, seguido pela desidratação das amostras em concentrações graduadas de etanol a fim de possibilitar sua inclusão em parafina.

As amostras foram então seccionadas em cortes de 5 µm de espessura (micrótomo Leica RM2125, Leica Biosystems, Wetzlar, Alemanha). Os cortes foram montados em lâminas silanizadas e passaram por um processo de desparafinização com o uso de xilol e re-hidratação com sequência de etanol. Após lavagem em água destilada, as lâminas foram incubadas em água oxigenada 0.3% (H₂O₂) (3 banhos de 10 minutos), para bloqueio da peroxidase endógena antes da lavagem com solução tampão fosfato (PBS).

Quatro secções de uma mesma amostra foram montadas em cada lâmina, sendo uma marcada como controle negativo da reação. Desta forma, para cada amostra dentinária coletada, foram confeccionadas 5 lâminas (com 4 cortes) para identificação das expressões de MMP-2, MMP-8, MMP-9, colágeno tipo I e sialoproteína óssea.

Anticorpos monoclonais foram utilizados para a identificação de MMP-2, MMP-8, MMP-9, colágeno tipo I e sialoproteína óssea (Merck Millipore, Billerica, Massachusetts, EUA). Os anticorpos foram diluídos em uma solução de tampão fosfato e soro-albumina bovina (PBS/BSA) nas proporções de 1:200 para o colágeno tipo I e sialoproteína dentinária e 1:100 para as MMPs.

Os anticorpos diluídos para os diferentes antígenos pesquisados foram aplicados diretamente em três secções de cada lâmina; na

quarta secção, aplicou-se apenas PBS/BSA (controle negativo da reação). As lâminas foram incubadas *overnight* em câmara úmida na temperatura de 4°C.

Na sequência, os complexos imunes foram marcados com o *kit* LSAB Dako LSAB+System-HRP (Dako, Glostrup, Denmark), de acordo com as instruções do fabricante. As secções, incluindo o controle negativo, foram incubadas com o anticorpo secundário biotinilado (30 minutos; 37 °C), seguido pelo conjugado peroxidase-estreptoavidina (30 minutos; 37 °C). Após cada etapa, as secções foram lavadas com PBS e o excesso de água removido cuidadosamente antes da aplicação do próximo reagente.

A reação foi revelada com a utilização de um cromógeno (3,3' - diaminobenzidina - DAB) por 5 minutos, a 37°C aplicado sobre as secções, o que resultou em um precipitado amarronzado nos sítios onde os antígenos pesquisados estavam presentes.

Para finalização da montagem das lâminas, os cortes foram lavados em água destilada, desidratados em séries de concentrações crescentes de etanol e montados com resina sintética Permount (Fisher Chemical, Nova Jersey, EUA).

Análise Imunohistoquímica - avaliação das lâminas

Imagens das amostras de dentina cariada foram obtidas com a utilização de um microscópio biológico Olympus CX21 (Olympus, Shinjuku, Tóquio, Japão) e o *software* específico Cellsens (Olympus, Shinjuku, Tóquio, Japão), em magnificação de 40x.

A intensidade da marcação de imunohistoquímica foi efetivada com o uso do *software* ImageJ 1.45s (Wayne Rashband, National Institutes of Health, Bethesda, MD, EUA - <http://rsb.info.nih.gov/ij>). A intensidade média dos canais RGB (Red, Green, Blue) em cada secção foi obtida, numa escala de 0 (imagens mais escuras) a 255 (imagens mais claras). Portanto, uma maior expressão dos antígenos estará relacionada a médias mais baixas de intensidade RGB (GOMES et al., 2011). Em cada fragmento de dentina, 3 secções foram analisadas para cada antígeno. O valor médio foi obtido e considerado como a intensidade de marcação imunohistoquímica do fragmento para aquele antígeno específico.

Acompanhamento de 10-13 meses

Os dentes restaurados foram acompanhados por um período médio de 11 meses (10-13 meses) e submetidos a nova avaliação clínica e radiográfica. Utilizando-se os critérios do USPHS (BARNES et al., 1995), 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, como descrito abaixo.

Avaliação radiográfica

Após os 60 dias iniciais de avaliação, as alterações no tecido dentinário não poderiam mais ser avaliadas por meio de microscopia eletrônica de varredura, já que este método exigiria remoção de novas amostras de dentina. Sendo assim, radiografias obtidas no período de avaliação de 60 dias foram comparadas com as de 10-13 meses de acompanhamento.

Para este propósito, posicionadores de filmes para radiografias interproximais (Indusbello, Londrina, Paraná, BR) foram padronizados para cada paciente, conforme descrito por Oliveira et al. (2006). Acrílico autopolimerizável (Jon, São Paulo, São Paulo, BR) foi aplicado ao posicionador para obtenção da mordida do paciente. Desta forma, o posicionador pode 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 cilindro do aparelho de raios-X.

Todas as radiografias foram realizadas com o filme *Insight* nº 0 (Eastman Kodak, Nova York, EUA) e o aparelho Timex 70E (Gnatus, Ribeirão Preto, São Paulo, BR) e parâmetros de exposição fixados em 70kVp, 7mA e 0.4s. Os procedimentos de revelação e fixação foram realizados por meio de um processador automático de radiografias (Revell, São Paulo, São Paulo, BR) com soluções novas (Eastman Kodak, Worchester, EUA).

Radiografias obtidas imediatamente depois de confeccionada a restauração final das cavidades de cárie 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).

Análise 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, São Paulo, BR). 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 sequenciais para torná-las comparáveis e subtrai pixels análogos de duas imagens obtidas em sequência.

A radiografia obtida imediatamente após a restauração no período de acompanhamento de 60-dias foi denominada imagem de referência (Imagem 1) e foi colocada lado a lado com a radiografia obtida 10-13 meses após (Imagem 2). 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 2 de acordo com a imagem de referência. Estruturas anatômicas claramente distinguíveis em ambas as imagens, como junção amelo-cementária e ponta de cúspides, atuaram como pontos de referência.

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 (Figura 1) e então gerou-se a imagem registrada (Imagem 3). A imagem 3 passou a ser considerada a nova radiografia de acompanhamento de 10-13 meses, já que pequenas diferenças nos ângulos de projeção durante tomada radiográfica ou de contraste durante o processamento foram eliminadas (Figura 2).

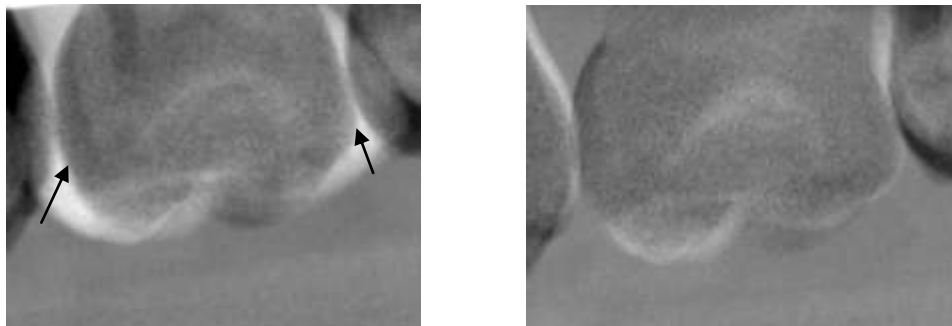


Figura 1 - Imagens ilustrativas obtidas após subtração radiográfica. Na esquerda, há muito ruído estrutural (setas). Na imagem da direita, o ruído estrutural foi reduzido e a imagem 3 pode ser registrada

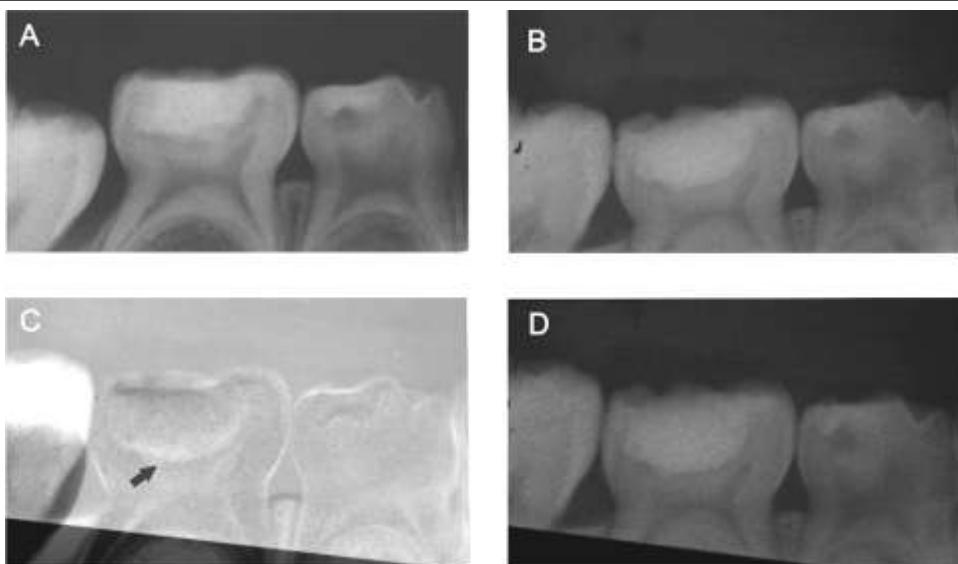


Figura 2 - Imagens ilustrativas dos procedimentos de pós-processamento de radiografias dentais. As radiografias de 60 dias e de 10-13 meses de acompanhamento são vistas em A e B. Em C, a imagem de subtração radiográfica é apresentada (imagem A - imagem B). Todo processo foi realizado para o dente 85. Obteve-se sucesso na subtração, já que ruído estrutural não é observado. No entanto, uma área mais clara (seta) pode ser vista sob a restauração, indicando ganho mineral. Em D, observa-se a imagem registrada obtida após alinhamento computadorizado das imagens digitalizadas. Os níveis de cinza serão medidos comparando-se a imagem A (60 dias) com a imagem D (10-13 meses de acompanhamento com projeção geométrica ajustada)

A imagem subtraída representa as alterações entre Imagem 1 e Imagem 3. Todas as estruturas anatômicas comuns são mostradas como uma imagem de subtração perfeita em tons de cinza neutro (nível de cinza neutro=128 de 255 possíveis tonalidades de cinza) (ALVES et al., 2010). 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. Se mudanças foram detectadas, uma segunda análise foi feita para quantificar esta alteração por meio das médias dos níveis de cinza.

Imagens 1 (60 dias) e 3 (10-13 meses de acompanhamento; registrada *a posteriori*) foram salvas em formato TIFF e importadas para o *software* Adobe Photoshop CS5 (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 1. 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 3 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.

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.

Análise estatística

A análise dos dados foi realizada utilizando-se o dente como unidade experimental. Para cor, consistência, fluorescência e número total de microorganismos viáveis, os dados não seguiram uma distribuição normal (testes de Shapiro-Wilk e Kolmogorov-Smirnov). Desta forma, as diferenças antes e depois do selamento cavitário (60 dias) foram verificadas por meio do teste de Wilcoxon. As porcentagens em peso de cálcio, fósforo e flúor, assim como as expressões médias de MMP-2, MMP-8, MMP-9, colágeno tipo I e sialoproteína óssea apresentaram distribuição normal e foram comparadas por meio do teste t pareado. Estatística descritiva foi utilizada para demonstrar as

distribuições de frequência dos critérios do USPHS após 60 dias e 10-13 meses de acompanhamento das radiografias e as diferenças entre os períodos de avaliação foram comparadas usando o teste de McNemar. Os níveis médios de cinza apresentaram distribuição normal (teste de Komogorov-Smirnov) e os dados foram submetidos ao teste ANOVA dois critérios para medidas repetidas, tendo como fatores principais a condição da dentina (cariada ou hígida) e o momento de avaliação (60 dias ou 10-13 meses). As análises estatísticas foram realizadas utilizando-se os *softwares Statistical Package for Social Sciences* (SPSS® 17.0, Chicago, Illinois, EUA) e SigmaPlot 12.0 (Systat Software Inc., San Jose, Califórnia, EUA) com nível de significância de 0.05.

4 ARTIGOS

4.1 ARTIGO 1

Clinical, mineral and ultrastructural changes on infected dentin left in sealed cavities from primary molars

Ana Claudia Rodrigues Chibinski ^a Fábio André dos Santos ^a Alessandra Reis ^a Denise Stadler Wambier ^a

^a *Department of Dentistry, Ponta Grossa State University, School of Dentistry, Ponta Grossa, Paraná, Brazil*

Running head: Changes on infected dentin after cavity sealing

Key Words: Dental caries. Tooth, deciduous. Dentin. Dental restoration.

SUMMARY

Aim: This in vivo study described the changes on infected dentin of primary teeth by means of clinical, mineral and ultrastructural analysis before and after cavity sealing with a glass ionomer cement.

Design: Dentin samples from 45 primary molars with deep and active carious lesions were evaluated before (baseline samples) and 60 days after cavity sealing (experimental samples). Clinical analysis consisted of dentin color (CO), consistency (COS) and laser fluorescence (LF). Laboratory procedures included: bacterial counting (CFU), mineral content (MC) and ultrastructural evaluations. Data from CO, COS, LF and CFU were analyzed by Wilcoxon Sign-Rank test while MC was evaluated by paired t-test.

Results: After 60 days, it was observed lower values of dentin fluorescence and bacterial counts, and a higher dentin consistency with an increase in calcium and phosphorus contents. No differences were detected on dentin color or fluorine weight percentage. Baseline samples showed enlarged dentinal tubules with bacterial invasion; 60-day samples exhibit a better organized tissue, with a more compact intertubular dentin and narrower dentinal tubules.

Conclusion: The complete removal of infected dentin before restoration is not an essential step to promote beneficial changes in the lesion environment of primary teeth with deep caries lesions.

INTRODUCTION

Minimal intervention dentistry associates the control of caries disease and lesion activity using preventive procedures and maximum preservation of the tooth structure, when a restoration is necessary¹. This philosophy is influencing the manner pediatric dentists conduct the treatment of children with active caries in a very positive way.

The changes in the treatment of deep carious lesions in primary teeth is an example of this concept. Contemporary AAPD guidelines state that indirect pulp therapy (IPT) is a choice for vital pulp therapy in primary teeth where caries approach the pulp². In the absence of pulpal diseases, IPT allows the preservation of dental structure and reduces the possibility of pulpal exposure^{3, 4}. The usual choice of treatment - pulpotomy - may be out of date, since IPT shows success rates of 90% or greater⁵.

The principle of minimal intervention also dictates the clinical steps for caries removal in Atraumatic Restorative Treatment, where the infected dentin layer is removed using manual excavators and the cavity is restored with high viscosity glass ionomer cement⁶.

Most of the studies about partial caries removal aimed to keep only affected dentin in the cavity and considered that point as the cut-off limit when the interruption of carious process is the goal⁶⁻⁸. Their results shows histological and clinical positive changes. It is seen a better tissue organization and a significant reduction on bacterial counts^{3, 6, 9}, leading to a thicker intertubular dentin and a more compact collagen network⁷, without caries progression. Clinical researches with longitudinal follow-ups confirm these findings, since radiographic evidence is compatible with tertiary dentin formation

¹⁰⁻¹². The dentin becomes darker, thicker and drier, which are arrested lesion characteristics ⁴. There are reports of dentin remineralization¹³ and enhanced microhardness ¹⁴⁻¹⁶.

However, it must be pointed out that the selective removal of decayed tissue, as preconized by the former cited papers, offers some difficulty to the clinicians, who have trouble in differentiating infected from affected dentin layers^{13, 17}. There isn't a reliable way to differentiate these two layers in the daily practice¹³, since the criteria used are based on operator's visual (color) and tactile (hardness) perceptions, and being so is highly subjective ¹.

One may question if all infected dentin is being removed during cavity preparation or, on the other hand, if practitioners are "afraid" of leaving this tissue behind and are overpreparing cavities, in a way that dental tissue is being displaced unnecessarily. If so, Minimal Intervention Dentistry principles are not being accomplished.

Since primary teeth have a defined biological cycle, it is interesting to keep as much dental tissue as possible in order to avoid pulp exposure and keep pulp vitality until exfoliation. Thus, an intriguing question is how important is the total removal of infected dentin to achieve a successful restoration?

Mertz-Fairhurst et al.¹⁸ got favorable results in permanent teeth treated with ultraconservative restorations and showed that, ten years after sealing infected dentin with composite restorations, there was no prejudice to the restoration or lesion progress as long as the cavity sealing was adequate. But, to the best of our knowledge, literature still lacks information on whether infected dentin can be maintained in primary teeth or not. Therefore, the aim of

this clinical study was to characterize the reactions on primary dentin by means of clinical, microbiological and dentin ultrastructure analysis after cavity sealing with a glass ionomer cement without removal of infected dentin.

METHODS

The present study received approval from the Ethics Committee of the State University of Ponta Grossa (Ponta Grossa, Paraná, Brazil) under protocol #15821/09.

Sample selection

After an initial screening of approximately 850 school children, 33 patients of both genders, with ages ranging from 3 to 10 years (average = 6.0 ± 2.1) were selected, to obtain a power of the test of 80% at a level of significance of 5%. The inclusion criteria were children having at least one second primary molar with cavitated and active carious lesion in dentin (ICDAS code 6), without signs or symptoms of pulpal pathology, checked by clinical and radiographic examination. The lesions were deep (2/3 or more of the dentin thickness) and limited to occlusal surface. Dentin laser fluorescence between 89 and 99 measured with DIAGNOdent device (KaVo, Biberach, Germany) was also an inclusion criterion because it indicates caries activity in the inner part of dentin ¹⁹. Children with systemic pathologies were excluded. All the parents or caregivers were informed about the nature of the study and signed a consent form, according to the Helsinki Declaration (version 2002) and the

Resolution #196/96 (National Health Council, Brazil). The final sample consisted of 45 teeth that met the inclusion criteria.

Study design

The carious dentin of each selected tooth was evaluated in two moments (before and after cavity sealing - baseline and 60-day, respectively), using clinical and laboratorial criteria. The clinical criteria were: dentin color and consistency; laser fluorescence readings. The laboratorial analysis included: microbiological evaluation and ultrastructural analysis.

Clinical and experimental procedures

Each patient attended two clinical sessions. At every session, after local anesthesia, teeth were: (1) isolated with a rubber dam; (2) cleaned using a new toothbrush and water; (3) washed thoroughly with air/water spray; and (4) air dried without desiccation.

At this point, the dentin clinical characteristics were evaluated as described later on in this section. Then, all carious tissue was removed from the cavosurface margins of the cavity. The infected layer over pulp floor was not removed.

Samples of infected dentin removed prior to restoration (baseline samples) were compared with samples taken 60 days after cavity sealing (experimental samples). In order to standardize the sample collection, the caries lesions were divided into two equivalent portions using a Hollembeck carver in a buccal-lingual direction. Fragments of infected dentin from the mesial portion were collected prior to the temporary restorations. The infected

dentin in the distal portion was left in the cavity in order to be removed 60 days after cavity sealing. Dentin samples were removed using sterile dentin excavators and immediately processed for microbiological and ultrastructural analysis. This routine was used for all the investigated criteria.

Teeth were sealed with Ketac Molar Easy Mix (3M ESPE, St. Paul, MN, USA), a high viscosity glass ionomer cement. The cement was covered with Adper Single Bond 2 (3M ESPE, St. Paul, MN, USA) to prevent changes in glass ionomer structure during the first moments after restoration placement. Dentin conditioner was not used when the cavity was sealed for the first time to facilitate the reopening of the cavity. A single operator (ACRC) performed all the clinical procedures for standardization purposes.

The integrity of each restoration was evaluated after sixty days according to the following criteria of United States Public Health Service (USPHS)²⁰: (1) cavosurface marginal discoloration; (2) recurrent caries; (3) contour or loss of substance (wear); (4) marginal integrity; (5) surface texture. Those variables were ranked in the following scores: alfa (no defect clinically detectable, needing just a polish), bravo (clinically acceptable, but repair is necessary) and charlie (clinically unacceptable, needs restoration substitution).

In order to reopen the cavity, the restorative material was carefully removed with sterile diamond burs (#1016 - KG Sorensen, São Paulo, SP, Brazil) under water cooling and dentin excavators. At this phase, air spray was constantly applied to make the GIC opaque, what helped to distinguish the restorative material from the dentin tissue.

The clinical characteristics of the dentin were re-evaluated and fragments of infected dentin from the distal portion (60-day samples) were

removed for microbiological and ultrastructural analysis. After this, the teeth were restored using Ketac Molar Easy Mix (3M ESPE, St. Paul, MN, USA), according to manufacturer's instructions.

Clinical evaluation: Color and consistency evaluation

Color and consistency were evaluated at baseline and sixty days after sealing. Carious dentin consistency was classified in: (0) soft (probe penetrates tissue with no resistance when removing probe), (1) medium (slight resistance when removing probe) or (2) hard (comparable to unaffected dentin)²¹. The color was evaluated using the following scores: (0) light yellow; (1) yellow; (2) light brown; (3) brown. Both analyses were made by a single trained examiner (ACRC) who achieved a Kappa index of 0.80 for the dentin color and 0.90 for the dentin consistency evaluation. Intra-examiner agreement for color was obtained using clinical photos; consistency index was obtained after in vivo examination of caries lesions. In both cases, the selected cavities presented the same characteristics as those in the study.

Laser fluorescence readings

Dentin laser fluorescence (LF) was obtained using DIAGNOdent 2095 (KaVo, Biberach, Germany) following manufacturer's instructions. The device was calibrated against a provided porcelain reference object prior the examination and then it was re-calibrated on a sound surface of every tooth. The possible values of LF range from 0 to 99. 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 single tooth.

Microbiological analysis

Dentin fragments from the mesial (baseline sample) and distal (60-day sample) portions were stored into sterile saline solution and processed immediately. In the laboratory, the samples were vortexed for 60s to break up aggregates of bacteria and aliquots of 100 μ L of decimal dilutions were plated in triplicate onto brain and heart infusion agar (BHI) (Difco-BD, Sparks, Md., USA). Plates were incubated at 37°C for 48 h and counted to determine total viable counting (TVC). The number of colonies obtained in each plate was registered and multiplied by the dilution factor, and the mean value was calculated to represent the single tooth.

Ultra structural and mineral analysis

Dentin fragments from the mesial (baseline sample) and distal (60-day sample) portions were stored into a 2% glutaraldehyde solution with a sodium phosphate buffer of 0.1 M (pH 7.4) for 7 days, rinsed thoroughly with 50% sodium phosphate buffer (0.3 M) and distilled water solution (three 30' rinses) and dehydrated in acetone. Specimens were then mounted on stubs and sputter-coated with a 10 nm gold layer to be analyzed in the scanning electron microscope (SEM) using secondary electron mode. The same sample was analyzed using dispersive X-ray spectrometry energy (EDS). The weight percentages of calcium, phosphorus and fluorine, before and after cavity sealing were obtained at a magnification of x200 for 100 s.

Statistical Analysis

The tooth was used as the experimental unit for data analysis. For dentin color, consistency, laser fluorescence and number of total viable counts, the Shapiro-Wilk and Kolmogorov-Smirnov tests showed non-normal distribution of data and therefore the non-parametric Wilcoxon Signed Rank test was used to compare the differences before and after cavity sealing. For the number of no growth samples, Fisher's exact test was employed. Finally, paired t-test was used to compare the weight percentage of calcium, phosphorus and fluorine. 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.

RESULTS

Two teeth were lost and the final sample consisted of 43 teeth from 31 patients. One tooth had pulp exposure on the second clinical session and the other was not available because the patient moved away from the town.

Clinical Evaluation

No tooth sensitivity was reported during the study period. The radiographic and clinical analysis did not reveal any sign of necrosis or pulpitis during the follow-up period. All provisional restorations after 60 days were ranked as alfa in the USPHS criteria, confirming that an appropriate sealing was obtained throughout the experimental period.

The results of dentin color, consistency and laser fluorescence readings are depicted in Table 1. No significant difference of dentin color was observed between the two periods of evaluation ($p = 0.366$). A significant change on dentin consistency was observed 60 days after cavity sealing ($p < 0.0001$). An increased resistance to manual removal of dentin tissue could be observed. Statistical differences on laser fluorescence readings before and after cavity sealing were also observed ($p < 0.0001$).

Microbiological, ultra structural and mineral analysis

Baseline samples showed higher bacterial counts compared to the 60-day ones and this difference was statistically significant. After cavity sealing, a remarkable decrease in CFU/sample was detected along with an expressive number of samples without bacterial growth (Table 2).

Mineral gain was also detected in the 60-day samples when compared to the baseline. Higher percentages of calcium and phosphorus were observed ($p < 0.003$) in the 60-day samples and no significant difference was noted in the percentage of fluorine (Table 2).

Ultra structural analysis by SEM photographs corroborate these findings. Baseline samples showed: (1) enlarged tubules diameter; (2) highly infected dentin, with bacteria aggregates present in both intertubular dentin and dentinal tubule openings; (3) clearly exposed collagen matrix, disposed in a flaccid arrange network (Fig. 1a, 1b). On the other hand the 60-day samples showed: (1) substantial bacterial reduction; (2) dentin tissue reorganization with a more compact arrangement of collagen fibers and (3) narrowing of dentinal tubules (Fig. 1c, 1d).

DISCUSSION

The main difference from the present and earlier studies that evaluate dentin features after partial caries removal and cavity sealing is that no attempt was made to remove the infected dentin, except from the cavosurface margin. Our results demonstrated that this procedure yielded similar results than those observed with partial caries removal. Thus, the concern about the differentiation between infected and affected dentin may be of little clinical significance. Restoring deep caries lesion in primary teeth enables dentin reorganization when infected dentin remains in the cavity. After 60 days of sealing, carious dentin showed a better tissue organization, substantial bacterial reduction and narrowing of dentinal tubules. Other researchers reported similar results^{6, 7} when all infected dentin has been removed.

The present study design allowed the effective comparison between baseline and 60-day conditions, since both samples were obtained from the same tooth and patient. Therefore, all individual variations were eliminated. The evaluation of cavity sealing after the follow-up period was considered appropriate and no signs of pain or pulp alterations were noted clinically or radiographically, except for one tooth that were excluded from the analysis.

Drastic reductions on bacterial counts is a common finding in the literature when caries cavity is sealed^{3, 6, 9}. This finding was also observed in the present investigation. An important change in bacterial environment is provided by cavity sealing, which eliminates nutrients and blocks the communication with the biofilm on the tooth surface. This process leads to a

decreased number of bacteria and a less cariogenic microbiota that makes caries activity not feasible⁶.

The interruption or deceleration of caries process allows biological response of the teeth, which can be seen clinically as changes in laser fluorescence readings and consistency. It was observed that 60-day samples showed reduced fluorescence when compared to the baseline samples. Although consistent with the bacterial reduction observed 60 days after cavity sealing, the laser fluorescence mean from the 60-day sample in the present study is far from the values reported for sound teeth or even from the cut-off limits suggested by the literature as dentin caries (> 38)²². The Diagnodent device is a quantitative method used for caries diagnosis, but evidence published so far suggests that this device does not detect variations in mineral changes, but the amount of metabolic by-products produced by bacteria¹⁷, mainly protoporphyrin IX, meso-porphyrin and copro-porphyrin²³. As porphoryns are very diffusible molecules²³ and active caries lesions present marked demineralization, it should be expected that these molecules penetrate deep into dentinal tissue and become incorporated in new reorganized tissue after cavity sealing. Therefore it may still be readable for the LF device.

It has been reported that this LF device has a good performance in detecting dentinal caries in primary teeth with a positive correlation with the lesion depth^{19, 24}. So, the subtle differences in the fluorescence readings between baseline and 60-day samples can only be interpreted as a reduction in bacterial contamination associated with deep caries lesion, but cannot be considered threshold for such diagnosis.

Dentin color did not change significantly in this study after cavity sealing. There was a trend of carious dentin to become darker, but this was not detected by the statistical analysis. Changes from a light brown to a darker brown are reported in the literature after cavity sealing^{3, 9}; however it seems that these changes take longer to occur. These reports were observed in studies with follow-up periods superior to 3 months^{3, 9} and in the present study a shorter follow-up period was employed.

Changes in mineral content of samples were also noted. Significant higher weight percentage of calcium and phosphorus were detected after cavity sealing. The mineral gain is in agreement with the harder dentin consistency observed in the present and in other studies by tactile means^{3, 9, 25}, microhardness measurements¹⁴⁻¹⁶ and EDS analysis⁷. The changes seen on SEM photographs after 60 days also corroborate this finding. The exposed collagen fibers and gap zones seen on baseline samples are not visible after 60 days, which is evidence of apatite mineralization. Other signs of dentin mineralization can be seen in Figures 1C and 1D. After 60 days, dentin tubules became narrower with the presence of a mineralized mass composed of apatite aggregates on their entrance.

Although there are data showing calcium and phosphate exchange between demineralized dentin and bioactive cements²⁶ or glass ionomer cements¹³, the mineral gain in the carious dentin are not only due to ionic changes between restorative material and teeth. There are many non-collagenous dentin proteins like dentin sialoprotein and dentin phosphoprotein and metalloproteinases²⁷ that are involved both in caries process and in the reorganization of carious tissue. Therefore, these changes in the carious dentin

seems to be a result from host-derived tissue regeneration mechanisms, activated when a friendlier environment was created by cavity sealing and not an effect of restorative material itself. The similar changes that occur when inert materials such as gutta-percha and wax are placed between the restorative material and the affected dentin tissue ^{15, 25} strengthen this hypothesis.

Another interesting finding of this research is about fluorine gain. The main fluorine source was from the glass ionomer cement (GIC) used for cavity sealing. The literature reports that GIC can release bioactive molecules to contribute to the process of dentin regeneration ²⁸. There are studies that show fluorine exchange between teeth and glass ionomer cement in a pattern compatible with dentin remineralization¹³. Although an increase in the weight percentage of fluorine was detected after cavity sealing, this increase was not statistically significant, in accordance to an earlier study⁷. Whether or not fluorine ion is important for the remineralization process of carious dentin is yet to be addressed.

Finally, the outcomes of the present study were positive in all evaluated criteria. Nevertheless, there are important aspects that must be mentioned: the correct diagnosis of pulp condition² and the adequate cavity sealing^{29, 30}. Taken all together, the complete removal of infected dentin before restoration is not an essential step to promote beneficial changes in the lesion environment and the protocol suggested may be an option for the treatment of primary teeth with deep caries lesions.

Acknowledgements: This work was supported by the brazilian research agency Fundação Araucária - Apoio ao Desenvolvimento Científico e

Tecnológico do Paraná (grant # 525/10). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

- 1 Iwami Y, Yamamoto H, Hayashi M, Ebisu S. Relationship between laser fluorescence and bacterial invasion in arrested dentinal carious lesions. *Lasers Med Sci.* 2011; **26**: 439-444.
- 2 American Academy of Pediatric Dentistry reference manual 2010-2011. *Pediatr Dent.* 2010; **32**: 1-334.
- 3 Duque C, Negrini Tde C, Sacono NT, Spolidorio DM, de Souza Costa CA, Hebling J. Clinical and microbiological performance of resin-modified glass-ionomer liners after incomplete dentine caries removal. *Clin Oral Investig.* 2009; **13**: 465-471.
- 4 Ribeiro CC, de Oliveira Lula EC, da Costa RC, Nunes AM. Rationale for the partial removal of carious tissue in primary teeth. *Pediatr Dent.* 2012; **34**: 39-41.
- 5 Coll JA. Indirect pulp capping and primary teeth: is the primary tooth pulpotomy out of date? *J Endod.* 2008; **34**: S34-39.
- 6 Wambier DS, dos Santos FA, Guedes-Pinto AC, Jaeger RG, Simionato MR. Ultrastructural and microbiological analysis of the dentin layers affected by caries lesions in primary molars treated by minimal intervention. *Pediatr Dent.* 2007; **29**: 228-234.
- 7 Massara ML, Alves JB, Brandao PR. Atraumatic restorative treatment: clinical, ultrastructural and chemical analysis. *Caries Res.* 2002; **36**: 430-436.
- 8 Maltz M, de Oliveira EF, Fontanella V, Bianchi R. A clinical, microbiologic, and radiographic study of deep caries lesions after incomplete caries removal. *Quintessence Int.* 2002; **33**: 151-159.
- 9 Lula EC, Almeida LJ, Jr., Alves CM, Monteiro-Neto V, Ribeiro CC. Partial caries removal in primary teeth: association of clinical parameters with microbiological status. *Caries Res.* 2011; **45**: 275-280.
- 10 Alves LS, Fontanella V, Damo AC, Ferreira de Oliveira E, Maltz M. Qualitative and quantitative radiographic assessment of sealed carious dentin: a 10-year prospective study. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2010; **109**: 135-141.
- 11 Maltz M, Oliveira EF, Fontanella V, Carminatti G. Deep caries lesions after incomplete dentine caries removal: 40-month follow-up study. *Caries Res.* 2007; **41**: 493-496.

- 12 Oliveira EF, Carminatti G, Fontanella V, Maltz M. The monitoring of deep caries lesions after incomplete dentine caries removal: results after 14-18 months. *Clin Oral Investig*. 2006; **10**: 134-139.
- 13 Ngo HC, Mount G, Mc Intyre J, Tuisuva J, Von Doussa RJ. Chemical exchange between glass-ionomer restorations and residual carious dentine in permanent molars: an in vivo study. *J Dent*. 2006; **34**: 608-613.
- 14 Santiago BM, Ventin DA, Primo LG, Barcelos R. Microhardness of dentine underlying ART restorations in primary molars: an in vivo pilot study. *Br Dent J*. 2005; **199**: 103-106.
- 15 Marchi JJ, Froner AM, Alves HL, Bergmann CP, Araujo FB. Analysis of primary tooth dentin after indirect pulp capping. *J Dent Child*. 2008; **75**: 295-300.
- 16 Franzon R, Gomes M, Pitoni CM, Bergmann CP, Araujo FB. Dentin rehardening after indirect pulp treatment in primary teeth. *J Dent Child*. 2009; **76**: 223-228.
- 17 Gurbuz T, Yilmaz Y, Sengul F. Performance of laser fluorescence for residual caries detection in primary teeth. *Eur J Dent*. 2008; **2**: 176-184.
- 18 Mertz-Fairhurst EJ, Curtis JW, Jr., Ergle JW, Rueggeberg FA, Adair SM. Ultraconservative and cariostatic sealed restorations: results at year 10. *J Am Dent Assoc*. 1998; **129**: 55-66.
- 19 Braga MM, Mendes FM, Ekstrand KR. Detection activity assessment and diagnosis of dental caries lesions. *Dent Clin North Am*. 2010; **54**: 479-493.
- 20 Barnes DM, Blank LW, Gingell JC, Gilner PP. A clinical evaluation of a resin-modified. Glass ionomer restorative material. *J Am Dent Assoc*. 1995; **126**: 1245-1253.
- 21 Bjorndal L, Larsen T, Thylstrup A. A clinical and microbiological study of deep carious lesions during stepwise excavation using long treatment intervals. *Caries Res*. 1997; **31**: 411-417.
- 22 Diniz MB, Rodrigues JA, de Paula AB, Cordeiro Rde C. In vivo evaluation of laser fluorescence performance using different cut-off limits for occlusal caries detection. *Lasers Med Sci*. 2009; **24**: 295-300.
- 23 Hibst R, Paulus R, Lussi A. Detection of Occlusal Caries by Laser Fluorescence: Basic and Clinical Investigations. *Medical Laser Application*. 2001; **16**: 205-213.
- 24 Mendes FM, Siqueira WL, Mazzitelli JF, Pinheiro SL, Bengtson AL. Performance of DIAGNOdent for detection and quantification of smooth-surface caries in primary teeth. *J Dent*. 2005; **33**: 79-84.
- 25 Pinto AS, de Araujo FB, Franzon R et al. Clinical and microbiological effect of calcium hydroxide protection in indirect pulp capping in primary teeth. *Am J Dent*. 2006; **19**: 382-386.
- 26 Peters MC, Bresciani E, Barata TJ et al. In vivo dentin remineralization by calcium-phosphate cement. *J Dent Res*. 2010; **89**: 286-291.
- 27 Chaussain-Miller C, Fioretti F, Goldberg M, Menashi S. The role of matrix metalloproteinases (MMPs) in human caries. *J Dent Res*. 2006; **85**: 22-32.

- 28 Ferracane JL, Cooper PR, Smith AJ. Can interaction of materials with the dentin-pulp complex contribute to dentin regeneration? *Odontology*. 2010; **98**: 2-14.
- 29 Lula EC, Monteiro-Neto V, Alves CM, Ribeiro CC. Microbiological analysis after complete or partial removal of carious dentin in primary teeth: a randomized clinical trial. *Caries Res*. 2009; **43**: 354-358.
- 30 Gruythuysen RJ, van Strijp AJ, Wu MK. Long-term survival of indirect pulp treatment performed in primary and permanent teeth with clinically diagnosed deep carious lesions. *J Endod*. 2010; **36**: 1490-1493.

FIGURE LEGENDS

Table 1 - Medians and interquartile range for dentin color, consistency and laser fluorescence readings (n=43).

Table 2 - Means (standard deviations) of bacterial colony-forming units (CFU/sample) from microbiological and mineral analysis (% weight) of primary caries dentin for baseline and 60-day samples (n=43).

Figure 1 - Representative secondary electron SEM images of primary carious dentin on baseline (A) and 60-day follow-up period (C). In baseline samples (A), there is intense dentin demineralization. A higher magnification (B) shows exposed collagen fibers (point) and a highly infected dentin (arrow). After cavity sealing (C), 60-day samples exhibit narrowing of dentinal tubules. The higher magnification (D) of the same image shows evident signs of mineralization and a better tissue organization. The bar represents 5 μm .

TABLES

Table 1 - Medians and interquartile range for dentin color, consistency and laser fluorescence readings (n=43).

	Baseline	60-day	p value*
Color	2 (1.5-2)	2(2-3)	0.366
Consistency	0 (0-1)	1 (1-2)	< 0.0001
Laser Fluorescence	99 (99-99)	71 (57-78)	< 0.0001

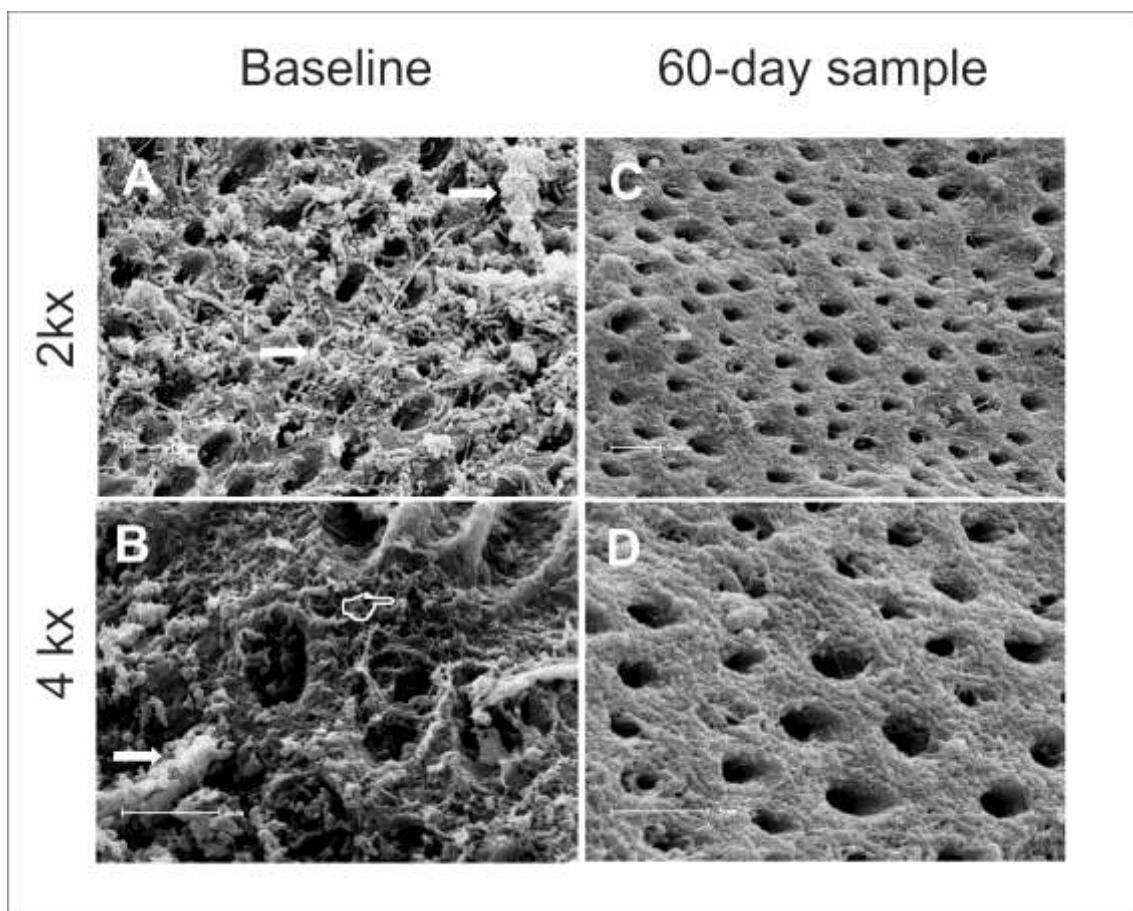
*Wilcoxon signed rank test

Table 2 - Means (standard deviations) of bacterial colony-forming units (CFU/sample) from microbiological and mineral analysis (% weight) of primary caries dentin for baseline and 60-day samples (n=43).

		Baseline	60-day	p value [‡]
Microbiological analysis	Total viable counts*	1905.2 (5.6)	31.0 (47.4)	< 0.0001
	No growth samples**	n=0	n=17	< 0.0001
Mineral analysis	Calcium	11.4 (5.3)	14.5 (4.2)	0.001
	Phosphorus	9.0 (3.0)	10.6 (2.3)	0.002
	Fluorine	1.3 (0.7)	1.6 (0.8)	0.067

[‡] Wilcoxon signed rank test; ** Fisher's exact test

Figure 1 - Representative secondary electron SEM images of primary carious dentin on baseline (A) and 60-day follow-up period (C). In baseline samples (A), there is intense dentin demineralization. A higher magnification (B) shows exposed collagen fibers (point) and a highly infected dentin (arrow). After cavity sealing (C), 60-day samples exhibit narrowing of dentinal tubules. The higher magnification (D) of the same image shows evident signs of mineralization and a better tissue organization. The bar represents 5 μm .



4.1 ARTIGO 2

Expression of MMPs, type I collagen and bone sialoprotein before and after sealing infected carious dentin in primary teeth

Ana Claudia Rodrigues Chibinski ^a José Rosa Gomes^b Alessandra Reis ^a
Denise Stadler Wambier ^a

^a *Department of Dentistry, Ponta Grossa State University, School of Dentistry, Ponta Grossa, Paraná, Brazil*

^b*Departament of Molecular Biology and Genetics, Ponta Grossa State University, School of Dentistry, Ponta Grossa, Paraná, Brazil*

Running head: Metalloproteinases, collagen and bone sialoprotein in infected dentin

Key Words: Dentin. Dental Caries. Tooth, Deciduous. Metalloproteases.

ABSTRACT

AIM: The objective of this in vivo study is to compare the expressions of MMP-2, MMP-8, MMP-9, type I collagen and bone sialoprotein (BSP) in infected dentin of primary teeth before and after cavity sealing with glass ionomer cement.

DESIGN: Dentin samples from 45 primary molars with deep and active carious lesions were collected before (baseline sample) and after cavity sealing (60-day samples). The samples were fixed, demineralized and processed for immunohistochemistry assays. Monoclonal antibodies were used for the localization of the cited antigens with an avidin-biotin method. Digital images of the sections were captured and analyzed with ImageJ software. The mean intensity of RGB channels in the images were obtained and compared using a Student's t test ($\alpha=0.05$).

RESULTS: The expressions of the MMPs, type I collagen and BSP increased after sealing, but statistical differences were observed only for MMP-8, type I collagen and BSP. MMP-2 and MMP-9 were more concentrated around dentin tubules; MMP-8 and collagen showed strong expressions throughout the organic matrix; BSP exhibit strong expression both in the matrix and around dentin tubules.

CONCLUSION: Sealing of infected carious dentin in primary teeth modified the expression of MMP-8, type I collagen and BSP in a pattern that suggests dentin matrix remodelling.

Key Words: Dentin. Dental Caries. Tooth, Deciduous. Metalloproteases.

INTRODUCTION

The caries process is the result of demineralization of the dental structure by acids produced by cariogenic bacteria, followed by the destruction of the collagenous organic matrix of dentin (Mazzoni et al., 2012). Although bacterial acids initiate the caries process with enamel and dentin demineralization (Chaussain-Miller et al., 2006), bacterial enzymes are not capable of degrading the organic matrix of dentin (Hannas et al., 2007), which is mediated mainly by host-derived matrix metalloproteinases (MMPs) (Hannas et al., 2007; Tjaderhane et al., 1998).

These MMPs are a family of zinc and calcium-dependent proteolytic enzymes (Mazzoni et al., 2012), which are fundamental to remodel the connective tissue in physiological and pathological conditions (Charadram et al., 2012). In human dentin, MMP-8 (human neutrophil collagenase) is the most effective in hydrolyzing type I collagen fibrils and MMP-9 appears to be the predominant gelatinolytic enzyme in dentin caries lesions (Tjaderhane et al., 1998). These MMPs, along with MMP-2, are the main proteases associated with dentin caries (Tjaderhane et al., 1998).

Although removal of demineralized dentin tissue and its replacement by restorative materials has been the most traditional approach for treatment of caries lesions, a less invasive restorative dentistry, with a minimal removal of dentin tissue, has shown long records of clinical success (Al-Zayer et al., 2003; Alves et al., 2010; Bjorndal et al., 1997; Gruythuysen et al., 2010), with the advantage of preserving tooth structure and allowing biological response of pulp (Ribeiro et al., 2012). The sealing of carious lesion allows

significant reductions of the number of microorganisms (Duque et al., 2009; Lula et al., 2011; Wambier et al., 2007), resulting in caries arrestment (Alves et al., 2010; Mertz-Fairhurst et al., 1998) and tissue remineralization (Alves et al., 2010; Maltz et al., 2007; Oliveira et al., 2006).

All the characteristics listed before signalize a "healing" process of the dentin, *i.e.*, they describe a more organized tissue, achieved after changing cavity environment by a dental restoration. 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 (Smith et al., 2012). To respond to the inflammatory process induced by the caries lesion, odontoblasts produce a reactionary tertiary dentin matrix (Smith et al., 2012; Tjäderhane et al., 2012), along with high MMPs secretion (Wang et al., 2012) in an effort of the pulp in remodelling and repair the extracellular matrix (ECM) that was damaged in the disease process (Corbel et al., 2000).

Besides that, we cannot rule out the role of other non-collagenous proteins (NCPs), grouped under the name of Small Integrin-Binding Ligand N-linked Glycoproteins (SIBLINGs). The SIBLINGs proteins can form specific complexes with different MMPs, being able to activate the latent form of MMPs and promote tissue remodeling even in the presence of tissue inhibitors of MMPs (TIMPs) (Chaussain-Miller et al., 2006). Bone sialoprotein (BSP) is part of the SIBLING group and it is able to bind to hydroxyapatite and collagen. Despite BSP has been localized in human dentin, its role in dentin mineralization is still unknown (Boushell et al., 2011).

Although the expression and role of MMPs on the progression of carious lesion has already been focus of many studies (Shimada et al., 2009; Sulkala et al., 2007; Tjaderhane et al., 1998), to our knowledge, the expression of MMPs and other non-collagenous proteins on primary carious and arrested carious dentin has not yet been well elucidated. Since information about the different expressions before and after carious dentin sealing is fundamental for the understanding of the success of minimally invasive approaches and the development of newer predictable regenerative therapies, this study attempted to identify and quantify the expression of these proteases as well as other proteins likely involved in dentin mineralization, on the caries arrestment produced by sealing carious dentin. Therefore, the aim of this study was to compare the expression of MMP-2, MMP-8, MMP-9, type I collagen and BSP in carious dentin of primary teeth before and 60 days after cavity sealing.

METHODS

The present study received approval from the Ethics Committee of the State University of Ponta Grossa (Ponta Grossa, Paraná, Brazil) under protocol #15821/09.

Sample selection

Thirty three patients of both genders, with ages ranging from 3 to 10 years (average = 6.0 ± 2.1) were selected, after an initial screening of approximately 850 school children. The inclusion criteria were children having at

least one second primary molar with acute open carious lesion in dentin (ICDAS code 6) (Ismail et al., 2007), without signs or symptoms of pulp pathology. Caries lesions included in the sample exhibited a soft or medium consistency on tactile analysis (Bjorndal et al., 1997) and were limited to occlusal surface, involving 2/3 or more of dentin thickness. Children with any systemic pathology were excluded.

All the parents or caregivers were informed about the nature of the study and signed a consent form, according to the Helsinki Declaration (version 2002) and the Resolution 196/96 (National Health Council, Brazil). After clinical and radiographic examination of these children, 45 teeth met the inclusion criteria.

Study design

This clinical study was designed to identify the expression of MMP-2, MMP-8 and MMP-9, type I collagen and BSP, in carious dentin of primary teeth before [baseline sample] and after [60-day sample] cavity sealing, using immunohistochemistry techniques.

Clinical and experimental procedures

Each patient attended two clinical sessions. In each clinical session, teeth were (1) anesthetized, (2) isolated with a rubber dam, (3) cleaned using a new toothbrush and water, (4) washed thoroughly with an air/water spray, and (5) air dried without desiccation.

In order to standardize the sample collection, the carious lesion was divided into mesial and distal portions using a dental carver in a buccal-lingual direction. The mesial portion was considered the baseline sample while the distal portion was the 60-day sample.

The baseline sample was removed using a sterile dentin excavator and immediately processed for immunohistochemical analysis; while the distal portion was kept in the cavity and only removed 60 days after cavity sealing. Soft dentin tissue from cavosurface margin was removed and the cavities were provisionally restored. Sixty days after restoration (60-day sample), all cavities were reopened, and dentin sample from the distal portion of the cavity were removed and processed again by immunohistochemistry analysis.

The restorative material used in both periods was high viscosity glass ionomer cement (Ketac Molar Easy Mix - 3M ESPE, St. Paul, MN, USA). The material was applied according to manufacturer's instructions. After restoration a coat of the adhesive Adper Single Bond (3M ESPE) was placed on the glass ionomer cement to prevent structure changes during the initial material setting. The dentin substrate was not conditioned with polyacrylic acid in order to facilitate cavity reopening without removal of dentin tissue.

In the 60-day evaluation, all teeth were submitted to clinical and radiographic examination and the restorations were evaluated using United States Public Health Service (USPHS) (Barnes et al., 1995). If signs or symptoms of pulp pathology as well as restorations defects that compromise cavity sealing were detected, the tooth was excluded from the study and

appropriately managed. All restorative procedures and sample collection were performed by a single operator (ACRC) to allow standardization of the procedures.

Immunohistochemical processing

The samples were processed for immunohistochemical analysis immediately after sample collection. After fixation in 10% formaldehyde for 48 h, the samples were demineralized using 4.13% EDTA for 2 months and placed in phosphate buffered saline (PBS, pH 7.4). After an overnight wash, each sample was dehydrated in graded ethanol and embedded in wax paraffin.

From each dentin sample, twenty 5- μ m thick sections were obtained using a microtome (Leica RM2125, Leica Biosystems, Wetzlar, Germany). These sections were mounted on silane-coated slides, 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% H₂O₂ 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, type I collagen and BSP (Merck Millipore-Billerica, Massachusetts, USA).

For the localization of type I collagen and BSP, the respective antibodies were diluted 1:200 in a solution of 1% PBS/bovine serum albumin (PBS/BSA); for the localization of MMP-2, MMP-8 and MMP-9, the antibodies were diluted 1:100 in the same solution.

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 humidified chamber. Immune complexes were subsequently revealed using labeled streptavidin biotin (LSAB) reagents (Dako LSAB+System-HRP, Glostrup, Denmark), which were used according to the manufacturer's instructions. All sections, including negative controls, were incubated with biotinylated 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) 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 permount mounting medium.

Images of the sections were obtained using a biological microscope Olympus CX21 (Olympus, Shinjuku, Tokyo, Japan) and Cellsens digital imaging software for research (Olympus, Shinjuku, Tokyo, Japan) under 400x magnification. The immunostaining intensity of these images was evaluated for the pixel intensity with ImageJ 1.45s software (Wayne Rashband, National Institutes of Health, Bethesda, USA; available at <http://rsb.info.nih.gov/ij>). The mean of the intensity of the RGB channels in the images were obtained, on a scale of 0 (darker images) to 256 (lighter images). Higher expressions of the antigens were reported as low RGB means.

From each dentin sample, 20 sections were evaluated (3 for each antibody; being one the negative control). The immunostaining intensity of the three sections from the same tooth at each assessment time was considered for statistical purposes.

Statistical analysis

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. After observing the normality of the data distribution and the equality of the variances, the baseline and 60-day sample were compared by a Student's t test. 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.

RESULTS

The final sample of this study consisted of 34 teeth. Nine teeth were excluded from the study for different reasons. In seven teeth, it was not possible to collect enough dentin structure after 60 days; in another tooth, pulp exposure was observed in the second clinical session and a pulpotomy and a composite resin restoration were made; the other one was excluded because the patient moved away from town and the contact was lost.

Clinical and radiographic evaluations showed no signs of pulp pathologies and all the restorations were considered clinically acceptable according to USPHS criteria after 60 days.

Immunohistochemical assays revealed the presence of the MMPs, type I collagen and BSP in primary carious dentin before and after cavity sealing, whereas the negative control specimens showed no labeling on the surface. The expressions of the MMPs, type I collagen and BSP increased after sealing, but statistical differences were observed only for MMP-8, type I collagen and BSP (Table 1).

MMP-2 and MMP-9 expression were detected before and after cavity sealing. These proteases were more concentrated around dentin tubules; the labeling was weak in the intertubular matrix. MMP-8 showed significantly stronger expression after cavity sealing and was distributed throughout the organic matrix. A strong expression of MMP-8 in the dentin tubules could be detected in all sections (Figure 1).

Type I collagen labeling showed a slight increase after sealing, with strong expression in the intertubular matrix of carious dentin. BSP expression was significantly stronger after sealing, both in the intertubular matrix and around the dentin tubules (Figure 2).

DISCUSSION

This study showed that the cavity sealing with remaining infected dentin modified the expression of MMP-8, type I collagen and BSP in

primary caries lesions. Most of the research about MMPs and dental caries focused on the localization and/or expression of MMPs in sound and carious dentin from extracted permanent teeth (Shimada et al., 2009; Tjaderhane et al., 1998; Toledano et al., 2010) or permanent demineralized dentin (Mazzoni et al., 2007; Sulkala et al., 2007; Van Strijp et al., 2003).

Although these studies produced important findings, the use of extracted teeth do not allow the demonstration of the role of ECM components in the progression of dentin caries as well as the host-defense response upon regenerative treatment for dentin caries. Therefore, to our knowledge, the present study is the first one that attempted to identify the expressions of different ECM components *in situ* before and after cavity sealing of dentin carious lesions in primary teeth.

This investigation employed only primary monoclonal antibodies, which are very specific and allow the detection of particular molecules in the ECM of carious dentin. The negative control specimens revealed no staining, confirming that cross-reactions did not occur between the secondary antibodies and the dentin organic matrix. Both dentin samples were collected from the same tooth in order to allow the comparison of infected dentin before and after sealing, as well as to minimize individual differences.

A strong expression of MMP-8 was observed in both evaluation periods. MMP-8 is expressed by odontoblasts and pulp tissue and it is the major collagenase in sound and demineralized human dentin (Sulkala et al., 2007). It specifically cleaves native triple helical type I collagen molecules, resulting in the release of 3/4 and 1/4 length peptides. The collagen fiber degraded is the

substrate for gelatinases MMP-2 and MMP-9 (Chaussain-Miller et al., 2006; Shimada et al., 2009).

In an active and open caries lesions, as the ones selected for this study, strong expression of MMP-8 was expected at baseline due to the close contact with gingival crevicular fluid and saliva, other potential sources of MMP-8 (Palosaari et al., 2000). However even after eradicating the external sources of MMP-8 by cavity sealing, the expression of this protease was higher at the 60-day samples. This suggests that the changes in the environment produced by cavity sealing originated suitable conditions for a dentin-pulp reaction resulting in an increased production of MMP-8 by the odontoblasts.

MMP-8 is thought to be involved in the organization of dentin matrix before mineralization, in association with odontoblast-derived gelatinases MMP-2 and MMP-9. Thus, cooperation between the interstitial collagenases and gelatinases is thought to be essential for remodeling and/or degrading interstitial collagens in connective tissues (Toledano et al., 2010).

Although the expression of MMP-2 and MMP-9 was not statistically different between baseline and 60-day samples, one cannot rule out the fact that their expressions were still high compared to that of sound teeth (Shimada et al., 2009). This hypothesis can be reinforced by the fact that immunoreactivity for these MMPs in sound dentin was only shown to be present in predentin and enamel dentin junction (Boushell et al., 2011), but not around dentin tubules as detected in this study.

The evident labeling of MMP-2 surrounding dentin tubules may be related to newly MMP-2 secreted by odontoblasts as a reparative reaction; a finding already demonstrated in early studies of dentin caries lesions (Boushell

et al., 2011; Toledano et al., 2010). Thus, the weak detection of MMP-2 in the intertubular dentin may be due to the short evaluation period after cavity sealing, which was probably not enough to allow diffusion of the newly secreted MMP-2 to the intertubular dentin (de Las Heras et al., 2000). In addition we also hypothesize that the MMP-2 detected in the intertubular dentin in this study is probably related to the diffused fossilized MMP-2 released from the mineralized dentin matrix during the caries attack.

Unfortunately, comparison with sound dentin is difficult. In this study we could not collect sample from sound teeth due to ethical concerns and further comparisons with literature findings is hampered by the fact that the previous studies that identified MMP-2 and MMP-9 in sound dentin worked with extracted permanent teeth (Mazzoni et al., 2008; Shimada et al., 2009; Toledano et al., 2010; Niu et al., 2011).

In regard to MMP-9, labeling were detected in dentin tubules as well as in the intertubular dentin (Mazzoni et al., 2008). The expression of MMP-9 in the present investigation can be considered moderate, but it was still higher than MMP-2. Although there are studies that report that MMP-2 is the major gelatinase in dentin (Niu et al., 2011), this was not corroborated by the present findings in deciduous carious dentin and therefore, deserves further investigations.

Type I collagen expression was higher in the 60-day sample, signaling that odontoblasts have not only produced and secreted MMPs, but also collagen fibrils, as shown in a previous study (Lee et al., 2006). An increase in organized collagen area has been reported after sealing infected dentin by glass ionomer cements (Pinheiro et al., 2010). On the other hand,

without cavity sealing, the degraded collagen level increased significantly in demineralized dentin specimens as compared to controls (Van Strijp et al., 2003), which again reinforces that cavity sealing per se was able to change the cavity environment and favor an odontoblastic response.

It is known that the type I collagen is the main component of the organic matrix of dentin providing the scaffold for the dentin mineralization. In association with NCPs and proteoglycans, it produces a three-dimensional organic network reinforced by apatite mineral crystallites (Bertassoni et al., 2012). In demineralized dentin, it has been suggested that remnant intrafibrillar mineral crystallites that are present within the collagen might act as sites for apatite nucleation and re-growth (Bertassoni et al., 2011). BSP provides the connection between collagen and the inorganic phase of dentin due to its high affinity to calcium and hydroxyapatite (Charadram et al., 2012) and has been identified around dentin tubules from caries dentin (Boushell et al., 2011). In fact, the higher expression of BSP after cavity sealing, particularly around dentin tubules, may be considered evidence of the remineralization process to which the carious dentin is susceptible after sealing.

Early studies advocate that MMP-2 and BSP are specific binding partners (Fedarko et al., 2004). Consequently, their detection in similar distribution in caries dentin, just like we observed in this study, is suggestive of a functional relationship of these proteins in the host response to the carious process (Boushell et al., 2011).

ECM modification/degradation requires the formation of specific complexes with MMPs (Boushell et al., 2011), which are activated in a tissue location and during a period of time that is appropriate and beneficial to the

tissue (Butler et al., 2002). In our study, this process was probably more connected to a remodeling process than to degrading activity and signals a repair mechanism. Controlling the ratio between deposition and degradation of ECM is the key of the remodeling process.

MMPs activities are regulated by the interaction with endogenous tissue inhibitors of metalloproteinases (TIMPs), that have already been observed in human dental tissues (Leonardi and Loreto, 2010). Another important molecule is transforming growth factor beta 1 (TGF- β 1), which has been detected in dentin and regulate tissue repair in teeth by activating odontoblasts to secrete MMPs and other ECM components for reparative dentin formation (Palosaari et al., 2000 ; Shimada et al., 2009). Therefore, further research on the interaction of MMPs with other ECM components is necessary to fully understand the reorganization of infected dentin in deciduous teeth.

Taking all together, the cavity sealing of infected dentin in deciduous teeth modified the expression of MMP-8, type I collagen and BSP in a pattern that appears to be suggestive of dentin matrix remodeling.

REFERENCES

Al-Zayer MA, Straffon LH, Feigal RJ, Welch KB (2003). Indirect pulp treatment of primary posterior teeth: a retrospective study. *Pediatr Dent* 25(1):29-36.

Alves LS, Fontanella V, Damo AC, Ferreira de Oliveira E, Maltz M (2010). Qualitative and quantitative radiographic assessment of sealed carious dentin: a 10-year prospective study. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 109(1):135-141.

Barnes DM, Blank LW, Gingell JC, Gilner PP (1995). A clinical evaluation of a resin-modified. Glass ionomer restorative material. *J Am Dent Assoc* 126(9):1245-1253.

Bertassoni LE, Habelitz S, Marshall SJ, Marshall GW (2011). Mechanical recovery of dentin following remineralization in vitro--an indentation study. *J Biomech* 44(1):176-181.

Bertassoni LE, Orgel JP, Antipova O, Swain MV (2012). The dentin organic matrix - limitations of restorative dentistry hidden on the nanometer scale. *Acta Biomater* 8(7):2419-2433.

Bjorndal L, Larsen T, Thylstrup A (1997). A clinical and microbiological study of deep carious lesions during stepwise excavation using long treatment intervals. *Caries Res* 31(6):411-417.

Boushell L, Nagaoka H, Yamauchi M (2011). Increased matrix metalloproteinase-2 and bone sialoprotein response to human coronal caries. *Caries Research* 45(5):453-459.

Butler WT, Brunn JC, Qin C, McKee MD (2002). Extracellular matrix proteins and the dynamics of dentin formation. *Connective tissue research* 43(2-3):301-307.

Charadram N, Farahani RM, Harty D, Rathsam C, Swain MV, Hunter N (2012). Regulation of reactionary dentin formation by odontoblasts in response to polymicrobial invasion of dentin matrix. *Bone* 50(1):265-275.

Chaussain-Miller C, Fioretti F, Goldberg M, Menashi S (2006). The role of matrix metalloproteinases (MMPs) in human caries. *J Dent Res* 85(1):22-32.

Corbel M, Boichot E, Lagente V (2000). Role of gelatinases MMP-2 and MMP-9 in tissue remodeling following acute lung injury. *Braz J Med Biol Res* 33(7):749-754.

Duque C, Negrini Tde C, Sacono NT, Spolidorio DM, de Souza Costa CA, Hebling J (2009). Clinical and microbiological performance of resin-modified glass-ionomer liners after incomplete dentine caries removal. *Clin Oral Investig* 13(4):465-471.

Fedarko NS, Jain A, Karadag A, Fisher LW (2004). Three small integrin binding ligand N-linked glycoproteins (SIBLINGs) bind and activate specific matrix metalloproteinases. *The FASEB journal* 18(6):734-736.

Gruythuysen RJ, van Strijp AJ, Wu MK (2010). Long-term survival of indirect pulp treatment performed in primary and permanent teeth with clinically diagnosed deep carious lesions. *J Endod* 36(9):1490-1493.

Hannas AR, Pereira JC, Granjeiro JM, Tjaderhane L (2007). The role of matrix metalloproteinases in the oral environment. *Acta Odontol Scand* 65(1):1-13.

Ismail AI, Sohn W, Tellez M, Amaya A, Sen A, Hasson H *et al.* (2007). The International Caries Detection and Assessment System (ICDAS): an integrated system for measuring dental caries. *Community Dent Oral Epidemiol* 35(3):170-178.

Leonardi R, Loreto C (2010). Immunohistochemical localization of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in human carious dentine. *Acta Histochemica* 112(3):298-302.

Lula EC, Almeida LJ, Jr., Alves CM, Monteiro-Neto V, Ribeiro CC (2011). Partial caries removal in primary teeth: association of clinical parameters with microbiological status. *Caries Res* 45(3):275-280.

Maltz M, Oliveira EF, Fontanella V, Carminatti G (2007). Deep caries lesions after incomplete dentine caries removal: 40-month follow-up study. *Caries Res* 41(6):493-496.

Mazzoni A, Mannello F, Tay F, Tonti G, Papa S, Mazzotti G *et al.* (2007). Zymographic analysis and characterization of MMP-2 and-9 forms in human sound dentin. *Journal of Dental Research* 86(5):436-440.

Mazzoni A, Pashley DH, Tay FR, Gobbi P, Orsini G, Ruggeri A *et al.* (2008). Immunohistochemical identification of MMP-2 and MMP-9 in human dentin: Correlative FEI-SEM/TEM analysis. *Journal of Biomedical Materials Research Part A* 88(3):697-703.

Mazzoni A, Breschi L, Carrilho M, Nascimento FD, Orsini G, Ruggeri A *et al.* (2012). A review of the nature, role, and function of dentin non-collagenous proteins. Part II: enzymes, serum proteins, and growth factors. *Endodontic Topics* 21(1):19-40.

Mertz-Fairhurst EJ, Curtis JW, Jr., Ergle JW, Rueggeberg FA, Adair SM (1998). Ultraconservative and cariostatic sealed restorations: results at year 10. *J Am Dent Assoc* 129(1):55-66.

Niu L, Zhang L, Jiao K, Li F, Ding Y, Wang D *et al.* (2011). Localization of MMP-2, MMP-9, TIMP-1, and TIMP-2 in human coronal dentine. *Journal of dentistry* 39(8):536-542.

Oliveira EF, Carminatti G, Fontanella V, Maltz M (2006). The monitoring of deep caries lesions after incomplete dentine caries removal: results after 14-18 months. *Clin Oral Investig* 10(2):134-139.

Palosaari H, Wahlgren J, Larmas M, Ronka H, Sorsa T, Salo T *et al.* (2000). The expression of MMP-8 in human odontoblasts and dental pulp cells is down-regulated by TGF-beta1. *J Dent Res* 79(1):77-84.

Pinheiro SL, Gallassi PC, Saldanha TC, Bincelli IN, Barros PP, Silva GH (2010). Repairing Collagen in Dentin Carious Lesions. Influence of Sealing the Material: A Morphometric Study. *Journal of Clinical Pediatric Dentistry* 34(3):223-228.

Ribeiro CC, de Oliveira Lula EC, da Costa RC, Nunes AM (2012). Rationale for the partial removal of carious tissue in primary teeth. *Pediatr Dent* 34(1):39-41.

Shimada Y, Ichinose S, Sadr A, Burrow MF, Tagami J (2009). Localization of matrix metalloproteinases (MMPs-2, 8, 9 and 20) in normal and carious dentine. *Aust Dent J* 54(4):347-354.

Smith A, Scheven B, Takahashi Y, Ferracane J, Shelton R, Cooper P (2012). Dentine as a bioactive extracellular matrix. *Archives of Oral Biology* 57(2):109-121.

Sulkala M, Tervahartiala T, Sorsa T, Larmas M, Salo T, Tjaderhane L (2007). Matrix metalloproteinase-8 (MMP-8) is the major collagenase in human dentin. *Arch Oral Biol* 52(2):121-127.

Tjaderhane L, Larjava H, Sorsa T, Uitto VJ, Larmas M, Salo T (1998). The activation and function of host matrix metalloproteinases in dentin matrix breakdown in caries lesions. *J Dent Res* 77(8):1622-1629.

Tjäderhane L, Carrilho MR, Breschi L, Tay FR, Pashley DH (2012). Dentin basic structure and composition—an overview. *Endodontic Topics* 20(1):3-29.

Toledano M, Nieto-Aguilar R, Osorio R, Campos A, Osorio E, Tay F *et al.* (2010). Differential expression of matrix metalloproteinase-2 in human coronal and radicular sound and carious dentine. *Journal of dentistry* 38(8):635-640.

Van Strijp A, Jansen D, DeGroot J, Ten Cate J, Everts V (2003). Host-derived proteinases and degradation of dentine collagen in situ. *Caries Research* 37(1):58-65.

Wambier DS, dos Santos FA, Guedes-Pinto AC, Jaeger RG, Simionato MR (2007). Ultrastructural and microbiological analysis of the dentin layers affected by caries lesions in primary molars treated by minimal intervention. *Pediatr Dent* 29(3):228-234.

Acknowledgements: This work was supported by the brazilian research agency Fundação Araucária - Apoio ao Desenvolvimento Científico e Tecnológico do Paraná (grant # 525/10). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

FIGURES

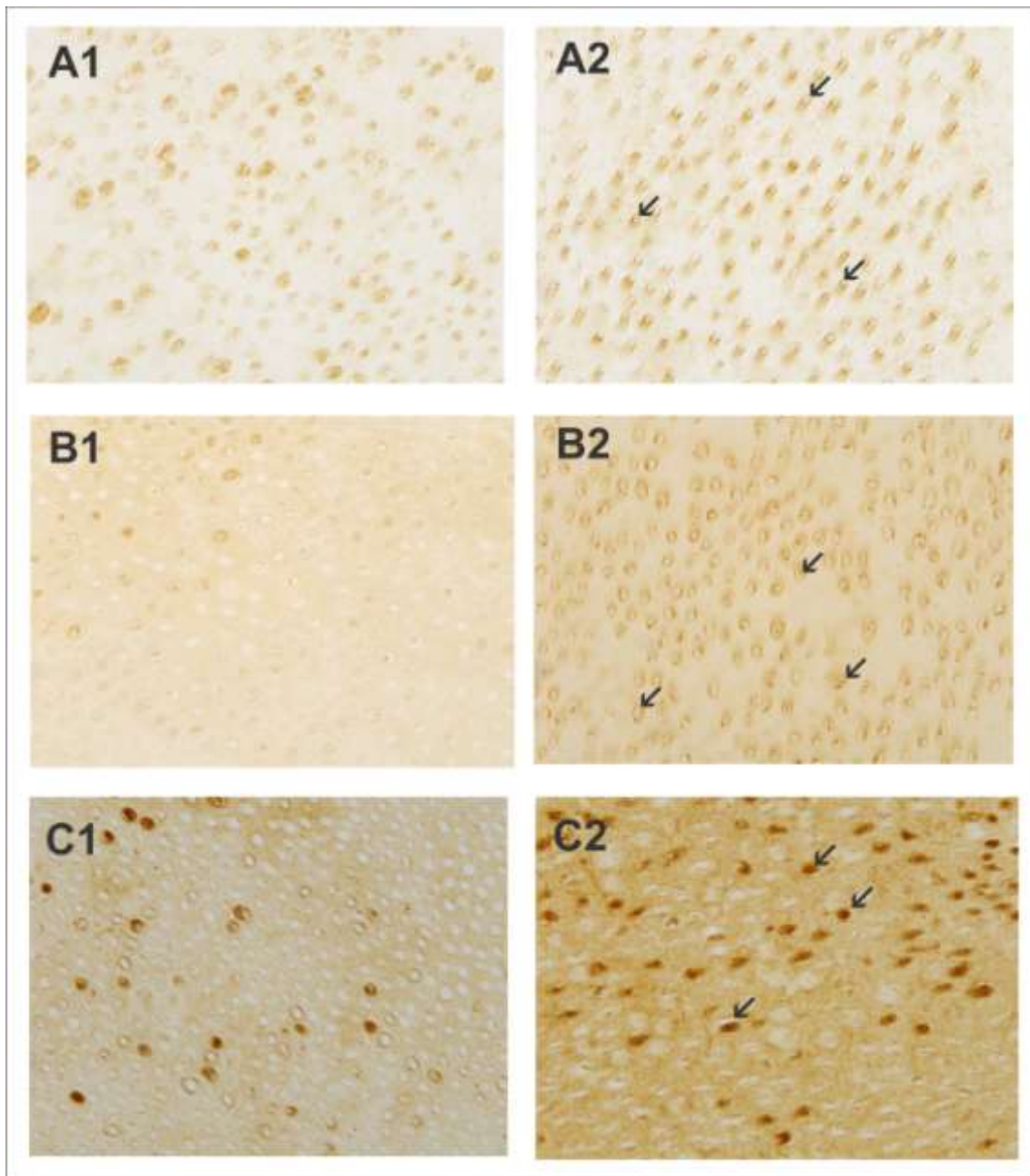


Figure 1 - Immunohistochemical labeling of MMP-2 (A), MMP-9 (B) and MMP-8 (C) in deciduous carious lesion before (1) and after (2) cavity sealing (x 1000). Visually, the expressions of the MMPs increased after sealing. MMP-2 (A1; A2) and MMP-9 (B1; B2) were distributed around the dentin tubules (arrows). MMP-8 expressions differed visually and statistically between the two assessment times (C1; C2); the expression of MMP-8 was stronger after cavity sealing (C2) in the intertubular matrix of carious dentin, as well as in the dentin tubules (arrow).

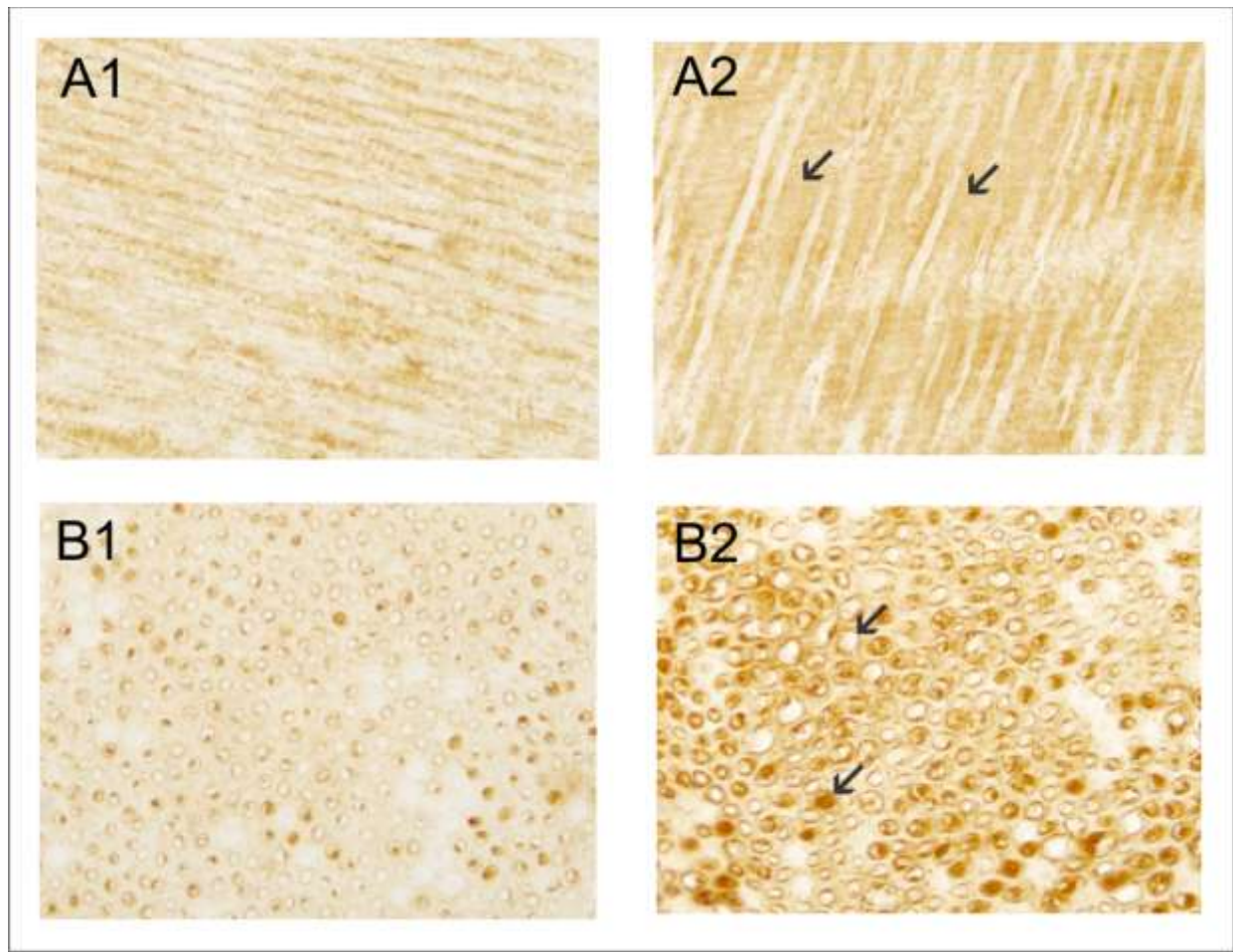


Figure 2 - Immunohistochemical labelling of type I collagen (A) and bone sialoprotein (B) before (1) and after (2) cavity sealing (x 1000). The collagen labelling showed a slight increase after sealing (A2), with strong expression in the ECM of carious dentin (arrows). Bone sialoprotein expression was stronger after sealing (B2), both in the ECM matrix and in the dentinal tubules (arrows).

TABLES

Table 1 – Expression of MMP-2, MMP-8 and MMP-9, type I collagen and BSP in primary carious dentin before (baseline sample) and after cavity sealing (60-day sample).

	Baseline	60-day	p-value*
MMP-2	170.3 ± 12.1	167.6 ± 12.1	0.228
MMP-8	152.0 ± 18.2	142.4 ± 17.3	0.018
MMP-9	175.8 ± 8.8	171.3 ± 12.1	0.092
type I collagen	185.9 ± 20.9	172.4 ± 38.6	0.040
BSP	174.8 ± 22.5	159.0 ± 24.8	<0.001

* Paired Student's *t*-test. Values are means ± standard deviations.

4.3 ARTIGO 3

Evaluation of primary carious dentin after cavity sealing in deep lesions: a 10-13 month follow-up*

Ana Claudia Rodrigues Chibinski ^a Eliane Kreich ^a Jefferson Tanaka^b
Alessandra Reis ^a Denise Stadler Wambier ^a

^a *Department of Dentistry, Ponta Grossa State University, School of Dentistry, Ponta Grossa, Paraná, Brazil*

^b *Department of Dentistry, Londrina State University, School of Dentistry, Londrina, Paraná, Brazil*

Running head: Primary carious dentin after restoration

Key Words: Tooth, deciduous. Dental caries. Dentin. Follow-Up Studies.

* accepted for publication - Pediatric Dentistry

ABSTRACT

Purpose: To describe the primary dentin reactions after restoration without complete removal of infected dentin. *Methods:* Fragments of carious dentin from 43 teeth with acute deep lesions were removed at baseline and compared to samples taken 60 days after (SEM photographs). At follow-up periods (60-day; 10-13-month), restorations were evaluated using USPHS criteria and standardized radiographs. Post-processing routine of radiographs were used to identify changes in radiographic density between periods. *Results:* At baseline, SEM photographs showed a disorganized tissue and bacterial invasion. After 60 days, dentin exhibited a better organization and signs of remineralization. Differences in restoration characteristics were detected for cavosurface discoloration ($p = 0.008$), wear ($p < 0.001$) and surface texture ($p < 0.001$) after 10-13 months. Digital subtraction radiographs showed that mean gray levels of carious and sound dentin increased after 10-13-month ($p < 0.001$). Differences in mean gray levels were detected between carious and sound dentin only for the 60-day images ($p = 0.03$). *Conclusions:* Removal of infected dentin wasn't fundamental for caries arrestment. Carious dentin tended to reorganize in a short period when properly sealed and the remineralization process continued for longer periods. Correct diagnosis of pulp vitality seems fundamental for the success of this protocol.

INTRODUCTION

The treatment of deep caries in primary teeth using minimally invasive techniques is supported by the literature¹⁻⁶. It involves removal of the infected dentin followed by sealing the cavity with a long-lasting material⁷. This technique exhibits good outcomes in terms of pulp tissue preservation and allows the activation of pulp defense mechanisms, such as the formation of reparative dentin and dentinal sclerosis¹. It promotes a drastic reduction in microbiological counts^{8, 9} with a less cariogenic flora¹⁰, which leads to a harder and drier tissue^{11, 12}. In spite of these findings, discussion continues about the clinical threshold for removal of carious tissue, because the determination of infected and affected dentin is based on subjective criteria (color, consistency) and operator's perceptions¹³. It is highly possible that clinicians are sealing teeth with infected dentin remaining below the restoration. However, a more aggressive removal of carious dentin could jeopardize tooth vitality, mainly in primary teeth with deep caries lesions.

It is recognized that remineralization/reorganization of affected dentin¹⁰ is possible after partial caries removal and proper sealing of the cavity^{2, 14-16}. However, it has yet to be confirmed whether these positive changes also occur when infected dentin is left in the cavity, as there is only one study in primary teeth that consider the conservation of infected dentin before cavity sealing¹⁷.

Long-term evaluation of minimally invasive restorative techniques are based on clinical and radiographic assessments. The absence of clinical or radiographic symptomatology related to pulp pathology is considered as a successful outcome^{4, 5}. In addition, remineralization of the

remaining dentin is a crucial indicator of the interruption of the caries process, as well as evidence of tooth vitality^{18, 19}, and constitutes a decisive criterion that needs to be investigated.

Remineralization can be evaluated by reopening the cavity and analyzing dentin consistency by tactile methods^{11, 20, 21} or through radiographic examinations. Visual evaluation of pairs of radiographic images is subjected to discrepancies in clinical practice¹⁸. Studies have demonstrated lack of inter-examiner agreement on consecutive interpretations of sequential radiographs^{22, 23}. Another difficulty is that visual examination of radiographic images does not have the capacity to detect subtle mineral changes²⁴, which occur during dentin remineralization.

There are techniques based on computational resources that can be used to overcome these difficulties. Digital subtraction radiography (**DSR**) is one of them. DSR distinguishes differences between sequential radiographs that are too small to be recognized by the human eye. The process subtracts the value of the corresponding pixels of two images and uses it to calculate a new one. Changes between them will appear as brighter or darker areas²⁵. Using this technique, changes in the mineral content of affected dentin can be detected.

Although there are studies in the literature documenting changes in mineralized tissues of permanent teeth^{18, 20, 26, 27}, to the authors' knowledge, the literature still lacks information about the use of DSR on primary teeth. Therefore, the aims of the present study were to: (1) evaluate the structure of dentin and (2) measure mineral uptake by carious dentin of primary teeth after cavity sealing without removal of infected dentin.

METHODS

This study was approved by Ethics Committee of State University of Ponta Grossa (Ponta Grossa, Paraná, Brazil) under protocol #15821/09.

Sample and inclusion criteria

The sample consisted of 43 primary molars from 33 patients (11 males; 22 females), with ages ranging from 3 to 10 years (average = 6.0 ± 2.1 years). All the parents or caregivers were informed about the nature of the study and signed a consent form, according to the Helsinki Declaration (version 2002) and the Resolution #196/96 (National Health Council, Brazil).

The selected teeth met the following inclusion criteria: (1) primary molars with active open caries lesions in dentin (ICDAS code 6)²⁸ limited to the occlusal surface; (2) absence of clinical or radiographic signs of pulpal pathology. Diagnosis of the caries lesion in dentin was made using dentin consistency, fluorescence readings, and radiographs. Caries lesions included in the sample exhibited a soft or medium consistency on tactile analysis²⁹, laser fluorescence readings higher than 89³⁰, and lesion depth on radiography extending to the inner half of dentin.

Study design

The ultrastructural analysis of carious dentin of each tooth was 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 mineralization from the 60-day sample up to approximately 11 months were evaluated by radiographic assessment.

Restorative procedures

After local anesthesia and rubber dam isolation, teeth were cleaned using a new toothbrush and water, washed thoroughly with air/water spray, and air-dried. The carious lesion was divided into mesial and distal portions by making a groove using a dental carver in a buccal-lingual direction. The mesial portion was considered the baseline sample while the distal portion was the 60-day sample.

The baseline sample was removed using a sterile dentin excavator and processed for qualitative ultrastructural analysis under scanning electron microscopy (**SEM**), while the distal portion was maintained in the cavity. Cavities were provisionally restored after removal of soft dentin tissue from the cavosurface margin to promote appropriate cavity sealing. Sixty days after restoration (60-day sample), all cavities were reopened, and the distal portions of carious dentin were removed and processed for SEM evaluation using the same protocol as the baseline samples.

The restorative material used in both periods was a high viscosity glass ionomer cement (Ketac Molar Easy Mix - 3M ESPE, St. Paul, MN, USA), according to the manufacturer's instructions. The cement was covered, with Adper Single Bond (3M ESPE), to prevent changes in glass ionomer structure immediately after restoration placement.

Ultra structural analysis

Dentin fragments from the mesial (baseline sample) and distal (60-day sample) portions were stored into a 2% glutaraldehyde solution with a sodium phosphate buffer of 0.1 M (pH 7.4) for 7 days. Then they were rinsed thoroughly with 50% sodium phosphate buffer (0.3 M) and distilled water solution (three 30 seconds rinses) and dehydrated in acetone. Specimens were mounted on stubs and sputter-coated with a 10 nm gold layer to be analyzed in SEM using secondary electron mode with a voltage of 12 Kv.

Clinical Evaluation

At the 60-day evaluation and after a mean period of 11 months (ranging from 10-13 months), restorations without clinic or radiographic signs of pulp pathologies were assessed according to United States Public Health Service (USPHS) criteria³¹ using scores (1) alfa; (2) bravo; (3) charlie (Table 1) by a single and trained operator.

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 extraction of the teeth. Thus, radiographs taken at the 60-day recall and 10-13 month-recall were compared one another.

For this purpose, a standardized film holder was made for each patient. The film holder (Indusbello, Londrina, PR, Brazil) was individualized²⁷. 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, Ribeirão Preto, 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 immediately after final restoration placement 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 contrast during 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 (image 1) and were put side-by-side with radiographs taken 10-13 months later (image 2). 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 cemento-enamel junction and cusps. The quality of image registration was visually determined with image subtraction routine. The image registration was considered adequate when the structural noise on the teeth of interest was reduced to the lowest-possible level (Figure 1), and then the registered image was generated (image 3) (Figure 2). Image 3 was then considered the new follow-up radiographic without small differences in projection angles during exposure or contrast during processing.

The subtracted image represents the changes between image 1 and image 3. All unchanged anatomical background structures are shown as a perfect subtraction image in neutral gray shade (gray level = 128 from 256 shades of gray)¹⁸. 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.

Images 1 (60-day) and 3 (10-13-month evaluation; registered *a posteriori*) were saved in uncompressed TIFF file and imported into Adobe

Photoshop CS5 (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 image 1. The mean gray level of the selected area was obtained using the expanded histogram function. The same area was transported into image 3, 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 mean value of the three measurements of the area under investigation was taken.

The same measurement was done on sound dentin from mesial and distal portions of each tooth. Standardized areas were delimited with the rectangular marquee tool. Measurement followed the same routine describe above. All the analyses were performed by a single and calibrated operator, blinded to the study objectives.

Statistical analysis

The experimental unit for data analysis was the tooth. Descriptive statistics were used to demonstrate the frequency distribution of the evaluated USPHS criteria. In each group, the differences between the two recall times (baseline vs. 10-13-month period) for each criterion was compared using the McNemar's test. The intra-examiner agreement was assessed by Cohen's Kappa statistics. The data from median levels of gray presented normal distribution, according to the Kolmogorov-Smirnov test, and, therefore, data were submitted to a two-way repeated measures ANOVA having Condition of dentin (caries or sound) and Assessment time (60-day and 10-13-month) as

the main factors. Tukey's test was used for pairwise comparisons. The statistical analysis was done using SigmaPlot 12.0 (Systat Software Inc., San Jose, CA, USA) software, with significance level set at 0.05.

RESULTS

From the sample of 43 teeth at baseline, 30 teeth were evaluated on 10-13-month follow-up period. Teeth were excluded for diverse reasons: physiological exfoliation (n=4); tooth fracture and restoration replacement (n=2); endodontic treatment (n=3); contact with the patient was lost (n=4).

Ultrastructural analysis

Ultrastructural analysis under SEM evaluation (Figure 3) showed a much disorganized tissue on baseline samples. It was possible to see enlarged dentin tubules and a high infected dentin tissue. Demineralization was evident, since the collagen network was exposed and gaps between fibers could be easily seen in all samples. Otherwise, 60-day samples exhibited a more organized tissue, with a substantial bacterial reduction, narrowing of dentinal tubules and mineralization over collagen network.

Clinical Evaluation

The results of the clinical evaluation using USPHS criteria are depicted in Table 2. At baseline, all restorations were ranked as alpha. After 10-13 months no significant differences were detected in the following items: recurrent caries ($p = 1$) and marginal integrity ($p = 0.125$). Significant

differences were detected for cavosurface marginal discoloration ($p=0.008$), wear ($p < 0.001$) and surface texture ($p < 0.001$). Restorations after 10-13 months of clinical service exhibited more surface wear without dentin exposure and a gritty surface texture. The intra-examiner agreement, assessed by Cohen's Kappa statistics was considered excellent (0.85).

Radiographic Analysis

The reproducibility of gray levels analysis in the present study can be considered excellent. The inter-class correlation coefficient for the mean gray levels was 0.99. All subtracted images showed changes seen as brighter areas. The mean gray levels measurements of baseline and 10-13-month radiographs are depicted in Table 3. Two-way ANOVA detected a significant effect for the cross-product interaction condition of dentin vs. assessment time. Significant higher mean gray levels of carious dentin were seen on the 10-13-month radiographs ($p < 0.001$). The same could be observed for sound dentin ($p < 0.001$). Significant differences were detected between carious and sound dentin on the 60-day images ($p = 0.03$), but these differences were not significant after the follow-up of 10-13 months ($p = 0.36$).

DISCUSSION

This study aimed to evaluate early and late changes in the carious dentin tissue after cavity sealing without removal of infected dentin. Early changes (from baseline to 60 days after cavity sealing) in the demineralized and infected dentin and its capacity of remineralization/reorganization were observed under SEM analysis. However, unfortunately, this

methodology needs tooth reopening and consecutive sample collection. This is the reason why we used a noninvasive diagnostic tool - the digital subtraction radiography (DSR) - to continue the evaluation of mineral gain below restorations for longer periods of time, without the need of new dentin samples or tooth extraction.

In 2002, the first paper of a series of four^{18-20, 27} suggested the use of DSR as an effective method to measure mineral changes in carious dentin after cavity sealing. It was demonstrated, through DSR, that there was a mineral gain in the radiolucent zone of sealed carious dentin^{18, 20, 26}. This method has also been used to investigate caries arrest in approximal surfaces^{26, 32, 33}.

The present investigation showed that this method is also feasible for primary teeth. The main challenge in DSR is to obtain comparable radiographs on two different assessment periods, mainly for the primary dentition, that undergoes many physiologic changes. There are several factors that influence the final image, such as angular variations, differences between image developments, position of the teeth in the alveolar bone²⁴, attrition of the crown and root resorption of primary teeth³⁴.

In order to minimize these interferences, film holders were individualized to standardize the position and direction of the exposure^{18-20, 27}, but small differences may still occur. Thus, computerized alignment of digital images were done to achieve the same geometry on the two sequential radiographs and make DSR procedures possible^{18, 25, 26}. Another source of variation between two sequential radiographs is the tightness of the patient's bite on the film holder, which is likely variable between exposures and may

produce artifacts³⁵. This bias was eliminated in the present study by subtracting upper and lower jaw separately and focusing in the treated tooth for reference points.

The high Cohen's kappa index obtained in this study for intra-examiner evaluation reflects the accuracy and reproducibility of DSR. The method attempts to eliminate operator's individual differences when interpreting radiographs, once the area to be analyzed has been delimited and quantified by the software, which allows numeric comparison^{27, 33}.

This study demonstrated by SEM analysis that reorganization and remineralization of carious dentin still occurs even when infected dentin remains in the cavity. The findings of this study were similar to those observed after partial caries removal^{8, 36} and thus concerns about the differentiation between infected vs. affected dentin may not be of clinical significance during caries excavation. The SEM photographs demonstrated a better tissue organization, an evident decrease in microorganisms and a more compact dentin 60 days after cavity sealing. The radiographic analysis indicates that these positive changes continued to occur over time, since an increased radiographic density from 60-day up to 10-13 months was observed, indicating mineral gain and apparent arrest of the carious process. Previous studies have already detected increased levels of calcium in the affected dentin some months after cavity sealing³⁶ as well as increased radiographic density^{18-20, 27}, which is in agreement with the results of this investigation.

It is noteworthy mentioning that not only caries-affected dentin showed increased gray levels after 10-13 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 cavity¹⁰. Increased gray levels on radiographs suggest mineral deposition for the formation of hard tissue, which could be deposition of tertiary dentin, sclerosis or both²⁷.

The continuing mineralization of dentin found in the present, and earlier studies is certainly associated with properly sealed restorations, since remineralization of dentin only occurs under a suitable restoration^{1, 2, 16}. Statistical differences between baseline and 10-13-month restorations were detected for cavosurface marginal discoloration, wear and surface texture. At the short evaluation time of 10-13-month evaluation, these differences only jeopardized the esthetics of the restoration as the discoloration was only superficial and could be easily removed by finishing and polishing procedures. Although it is widely known that marginal discoloration is a common problem in direct posterior restorations³⁷, this may be responsible for the failure of the restoration in longer evaluation periods. Thus, the clinical approach, herein presented, should be further evaluated in after longer periods of clinical service. In the other relevant items related to sealing ability, such as recurrent caries or marginal integrity, no significant differences from baseline were detected.

It should be mentioned that 3 teeth from our sample had to receive endodontic treatment. Although only teeth with no signs of pulpal pathology were included in the sample, this evaluation was performed by clinical and radiographic examinations, which may lead to false negative findings at baseline, because bacteria and toxins progressing ahead of caries can cause areas of undetectable pulp necrosis or irreversible pulpitis³⁸. This

might be the reason of why endodontic treatment was required in three teeth. The use of more objective methods to evaluate the pulp vitality, such as laser Doppler or pulse oximetry, may be a way to allow a more accurate diagnosis of the pulp vitality at the baseline and during follow-up periods, but even these quantitative methods have shortcomings ³⁹.

On the other hand, taking into account the sample at 10-13 months follow-up (30 teeth), the success rate would be 90%. Literature shows success rates of 95% after 6 years follow-up⁴⁰ and 96% after 3 years follow-up ¹⁶. These studies, as well as systematic reviews^{2, 41, 42}, related success rates up to 90%, similar to that obtained in the present research. It is essential to emphasize that we kept infected dentin in the cavity and achieved similar results as those presented by other studies with indirect pulp capping, which indicates that the procedure can be an option for restoration of deciduous teeth with deep caries lesions.

CONCLUSIONS

Taken all together the results of this study provide evidence that the removal of infected dentin seems not to be fundamental for caries arrestment in the short period of time evaluated. When 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. Nevertheless, the favorable outcome is dependent of a good clinical diagnosis to determine pulp vitality and its capacity to respond positively to the treatment.

REFERENCES

1. Ribeiro CC, de Oliveira Lula EC, da Costa RC, Nunes AM. Rationale for the partial removal of carious tissue in primary teeth. *Pediatr Dent* 2012;34:39-41.
2. Thompson V, Craig RG, Curro FA, Green WS, Ship JA. Treatment of deep carious lesions by complete excavation or partial removal: a critical review. *J Am Dent Assoc* 2008;139:705-12.
3. Ricketts DN, Kidd EA, Innes N, Clarkson J. Complete or ultraconservative removal of decayed tissue in unfilled teeth. *Cochrane Database Syst Rev* 2006:CD003808.
4. Marchi JJ, Froner AM, Alves HL, Bergmann CP, Araujo FB. Analysis of primary tooth dentin after indirect pulp capping. *J Dent Child* 2008;75:295-300.
5. Franzon R, Gomes M, Pitoni CM, Bergmann CP, Araujo FB. Dentin rehardening after indirect pulp treatment in primary teeth. *J Dent Child* 2009;76:223-8.
6. Orhan AI, Oz FT, Orhan K. Pulp exposure occurrence and outcomes after 1- or 2-visit indirect pulp therapy vs complete caries removal in primary and permanent molars. *Pediatr Dent* 2010;32:347-55.
7. Weber CM, Alves LS, Maltz M. Treatment decisions for deep carious lesions in the Public Health Service in Southern Brazil. *J Public Health Dent* 2011;71:265-70.
8. Wambier DS, dos Santos FA, Guedes-Pinto AC, Jaeger RG, Simionato MR. Ultrastructural and microbiological analysis of the dentin layers affected by caries lesions in primary molars treated by minimal intervention. *Pediatr Dent* 2007;29:228-34.
9. Lula EC, Monteiro-Neto V, Alves CM, Ribeiro CC. Microbiological analysis after complete or partial removal of carious dentin in primary teeth: a randomized clinical trial. *Caries Res* 2009;43:354-8.
10. Kidd EA. Clinical threshold for carious tissue removal. *Dent Clin North Am* 2010;54:541-9.
11. Duque C, Negrini Tde C, Sacono NT, Spolidorio DM, de Souza Costa CA, Hebling J. Clinical and microbiological performance of resin-modified glass-ionomer liners after incomplete dentine caries removal. *Clin Oral Investig* 2009;13:465-71.
12. Lula EC, Almeida LJ, Jr., Alves CM, Monteiro-Neto V, Ribeiro CC. Partial caries removal in primary teeth: association of clinical parameters with microbiological status. *Caries Res* 2011;45:275-80.
13. Iwami Y, Yamamoto H, Hayashi M, Ebisu S. Relationship between laser fluorescence and bacterial invasion in arrested dentinal carious lesions. *Lasers Med Sci* 2011;26:439-44.
14. Silva NR, Carvalho RM, Pegoraro LF, Tay FR, Thompson VP. Evaluation of a self-limiting concept in dentinal caries removal. *J Dent Res* 2006;85:282-6.

15. Buyukgural B, Cehreli ZC. Effect of different adhesive protocols vs calcium hydroxide on primary tooth pulp with different remaining dentin thicknesses:24-month results. *Clin Oral Investig* 2008;12:91-6.
16. Gruythuysen RJ, van Strijp AJ, Wu MK. Long-term survival of indirect pulp treatment performed in primary and permanent teeth with clinically diagnosed deep carious lesions. *J Endod* 2010;36:1490-3.
17. Innes NP, Evans DJ, Stirrups DR. Sealing caries in primary molars: randomized control trial, 5-year results. *J Dent Res* 2011;90:1405-10.
18. Alves LS, Fontanella V, Damo AC, Ferreira de Oliveira E, Maltz M. Qualitative and quantitative radiographic assessment of sealed carious dentin: a 10-year prospective study. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2010;109:135-41.
19. Maltz M, Oliveira EF, Fontanella V, Carminatti G. Deep caries lesions after incomplete dentine caries removal: 40-month follow-up study. *Caries Res* 2007;41:493-6.
20. Maltz M, de Oliveira EF, Fontanella V, Bianchi R. A clinical, microbiologic, and radiographic study of deep caries lesions after incomplete caries removal. *Quintessence Int* 2002;33:151-9.
21. Pinto AS, de Araujo FB, Franzon R, Figueiredo MC, Henz S, Garcia-Godoy F, et al. Clinical and microbiological effect of calcium hydroxide protection in indirect pulp capping in primary teeth. *Am J Dent* 2006;19:382-6.
22. Petrikowski CG, ElBadrawy HE, Boehlau EE, Grace MG. Interobserver variability in pediatric radiographic quality assessment. *J Can Dent Assoc* 1998;64:36-41.
23. Christgau M, Hiller KA, Schmalz G, Kolbeck C, Wenzel A. Accuracy of quantitative digital subtraction radiography for determining changes in calcium mass in mandibular bone: an in vitro study. *J Periodontal Res* 1998;33:138-49.
24. Ono E, Medici Filho E, Faig Leite H, Tanaka JL, De Moraes ME, De Melo Castilho JC. Evaluation of simulated external root resorptions with digital radiography and digital subtraction radiography. *Am J Orthod Dentofacial Orthop* 2011;139:324-33.
25. Gormez O, Yilmaz HH. Image post-processing in dental practice. *Eur J Dent* 2009;3:343-7.
26. Martignon S, Ekstrand KR, Ellwood R. Efficacy of sealing proximal early active lesions: an 18-month clinical study evaluated by conventional and subtraction radiography. *Caries Res* 2006;40:382-8.
27. Oliveira EF, Carminatti G, Fontanella V, Maltz M. The monitoring of deep caries lesions after incomplete dentine caries removal: results after 14-18 months. *Clin Oral Investig* 2006;10:134-9.
28. Ismail AI, Sohn W, Tellez M, Amaya A, Sen A, Hasson H, et al. The International Caries Detection and Assessment System (ICDAS): an integrated system for measuring dental caries. *Community Dent Oral Epidemiol* 2007;35:170-8.

29. Bjorndal L, Larsen T, Thylstrup A. A clinical and microbiological study of deep carious lesions during stepwise excavation using long treatment intervals. *Caries Res* 1997;31:411-7.
30. Braga MM, Mendes FM, Ekstrand KR. Detection activity assessment and diagnosis of dental caries lesions. *Dent Clin North Am* 2010;54:479-93.
31. Barnes DM, Blank LW, Gingell JC, Gilner PP. A clinical evaluation of a resin-modified. Glass ionomer restorative material. *J Am Dent Assoc* 1995;126:1245-53.
32. Martignon S, Ekstrand KR, Gomez J, Lara JS, Cortes A. Infiltrating/sealing proximal caries lesions: a 3-year randomized clinical trial. *J Dent Res* 2012;91:288-92.
33. Carneiro LS, Nunes CA, Silva MA, Leles CR, Mendonca EF. In vivo study of pixel grey-measurement in digital subtraction radiography for monitoring caries remineralization. *Dentomaxillofac Radiol* 2009;38:73-8.
34. Jung MS, Lee SP, Kim GT, Choi SC, Park JH, Kim JW. Three-dimensional analysis of deciduous maxillary anterior teeth using cone-beam computed tomography. *Clin Anat* 2012;25:182-8.
35. Wenzel A, Anthonisen PN, Juul MB. Reproducibility in the assessment of caries lesion behaviour: a comparison between conventional film and subtraction radiography. *Caries Res* 2000;34:214-8.
36. Massara ML, Alves JB, Brandao PR. Atraumatic restorative treatment: clinical, ultrastructural and chemical analysis. *Caries Res* 2002;36:430-6.
37. Hickel R, Manhart J. Longevity of restorations in posterior teeth and reasons for failure. *The Journal of Adhesive Dentistry* 2001;3:45.
38. Ritter AV, Browning WD, Swift J, Edward J. Partial Caries Excavation. *Journal of Esthetic and Restorative Dentistry* 2012;24:148-52.
39. Mejàre I, Axelsson S, Davidson T, Frisk F, Hakeberg M, Kvist T, et al. Diagnosis of the condition of the dental pulp: a systematic review. *International Endodontic Journal* 2012; 45:597-613.
40. Al-Zayer MA, Straffon LH, Feigal RJ, Welch KB. Indirect pulp treatment of primary posterior teeth: a retrospective study. *Pediatr Dent* 2003;25:29-36.
41. Hayashi M, Fujitani M, Yamaki C, Momoi Y. Ways of enhancing pulp preservation by stepwise excavation--a systematic review. *J Dent* 2011;39:95-107.
42. Ferreira JM, Pinheiro SL, Sampaio FC, de Menezes VA. Caries removal in primary teeth--a systematic review. *Quintessence Int* 2012;43:e9-15.

Acknowledgements: This work was supported by the brazilian research agency Fundação Araucária - Apoio ao Desenvolvimento Científico e Tecnológico do Paraná (grant # 525/10). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Table 1 - Criteria and codes for clinical direct assessment of the restorations

Criteria	Code*	Definition
Cavosurface marginal discoloration	A	There is no visual evidence of marginal discoloration.
	B	There is visual evidence of marginal discoloration at the junction of the tooth structure and the restoration, but the discoloration has not penetrated along the restoration in a pulpal direction.
	C	There is visual evidence of marginal discoloration at the junction of the tooth structure and the restoration that has penetrated along the restoration in a pulpal direction.
Recurrent caries	A	Absent
	B	Present
Loss of substance (wear)	A	The restoration is a continuation of existing anatomic form or is slightly flattened.
	B	A surface concavity is evident.
	C	A surface concavity is evident, and the dentin is exposed.
Marginal integrity	A	There is no visible crevice along the periphery of the restoration; no explorer catch at the margin.
	B	There is visible evidence of a crevice, which the explorer penetrates, but dentin is not exposed.
	C	The explorer penetrates into a crevice that is of a depth that exposes dentin
Surface Texture	A	Surface of restoration is smooth
	B	Surface texture is gritty or similar to a surface subject to a white stone
	C	Surface is deeply pitted, with irregular grooves

* A = alfa (1); B = bravo (2); C = charlie (3)

Table 2 – Number of restorations rated/total number of restorations (percent) for each USPHS criteria in each assessment period (n=30).

	Score	Baseline	10-13-month	p-value*
Marginal discoloration	A	30/30 (100)	22/30 (73.3)	0.008 ^s
	B	0/30 (0)	8/30 (26.7)	
Recurrent caries	A	30/30 (100)	30/30 (100)	1 ^{ns}
	B	0/30 (0)	0/30 (0)	
Wear	A	30/30 (100)	12/30 (40)	0.001 ^s
	B	0/30 (0)	18/30 (60)	
Marginal Integrity	A	30/30 (100)	26/30 (86.7)	0.125 ^{ns}
	B	0/30 (0)	4/30 (13.3)	
Surface Texture	A	30/30 (100)	7/30 (23.3)	0.001 ^s
	B	0/30 (0)	23/30 (76.7)	

*Mc-Nemmar test (alfa = 0.05); ^{ns}Nonsignificant; ^sSignificant.

Table 3 - Means (standard deviations) of gray levels values of caries-affected and sound dentin from primary teeth at baseline and 10-13-month radiographs (n=30).

	Baseline	10-13-month
Cariou dentin	93.7 (15.3) a	120.3 (15.7) c
Sound dentin	102.6 (15.6) b	121.0 (14.9) c

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

FIGURE LEGENDS

Figure 1 - Illustrative images obtained after image subtraction routine. There is too much structural noise on A and B. On C, the structural noise have been reduced and image 3 could be registered.

Figure 2 - Illustrative images of the post-processing routine of digitalized dental radiographs. Baseline and 10-13-month images are shown in A and B. In C, the subtracted image (image A - image B) can be seen. The focus is on tooth 85. Subtraction process was sucessful because structural noise is not seen. However, a brighter area (arrow) can be noted under restorative material, indicating mineral gain. In D, the registered image obtained after computerized alignment of digital images. Mean gray levels will be measured comparing image A (baseline) and D (10-13-month with adjusted geometric projection).

Figure 3 - SEM photographs of baseline and 60-day samples. At baseline, exposed collagen network can be seen, with big gaps between the fibers and bacterial invasion. After 60 days of cavity sealing, dentin samples showed an organized tissue and remineralization is evident.

FIGURES

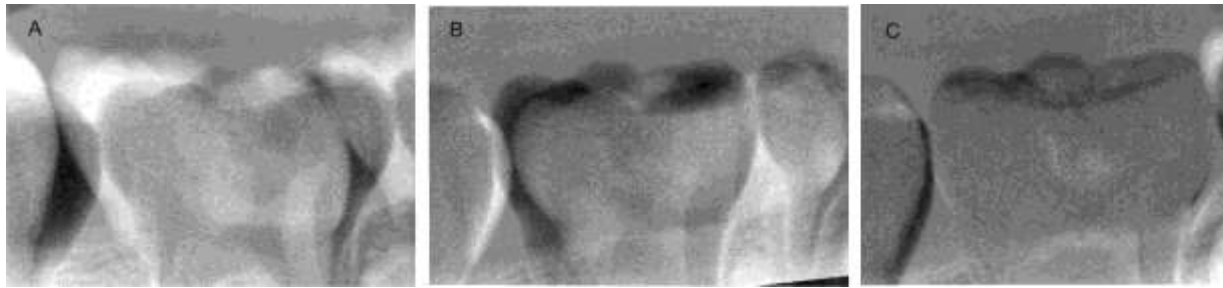


Figure 1 - Illustrative images obtained after image subtraction routine. There is too much structural noise on A and B. On C, the structural noise have been reduced and image 3 could be registered.

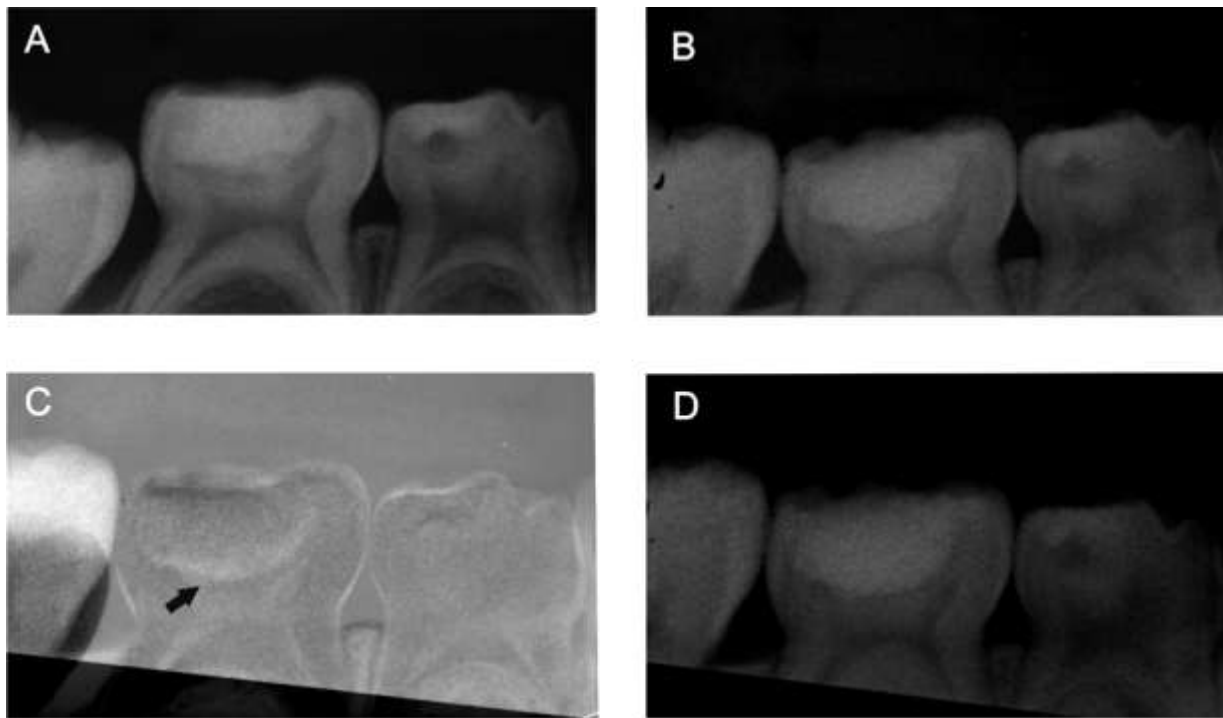
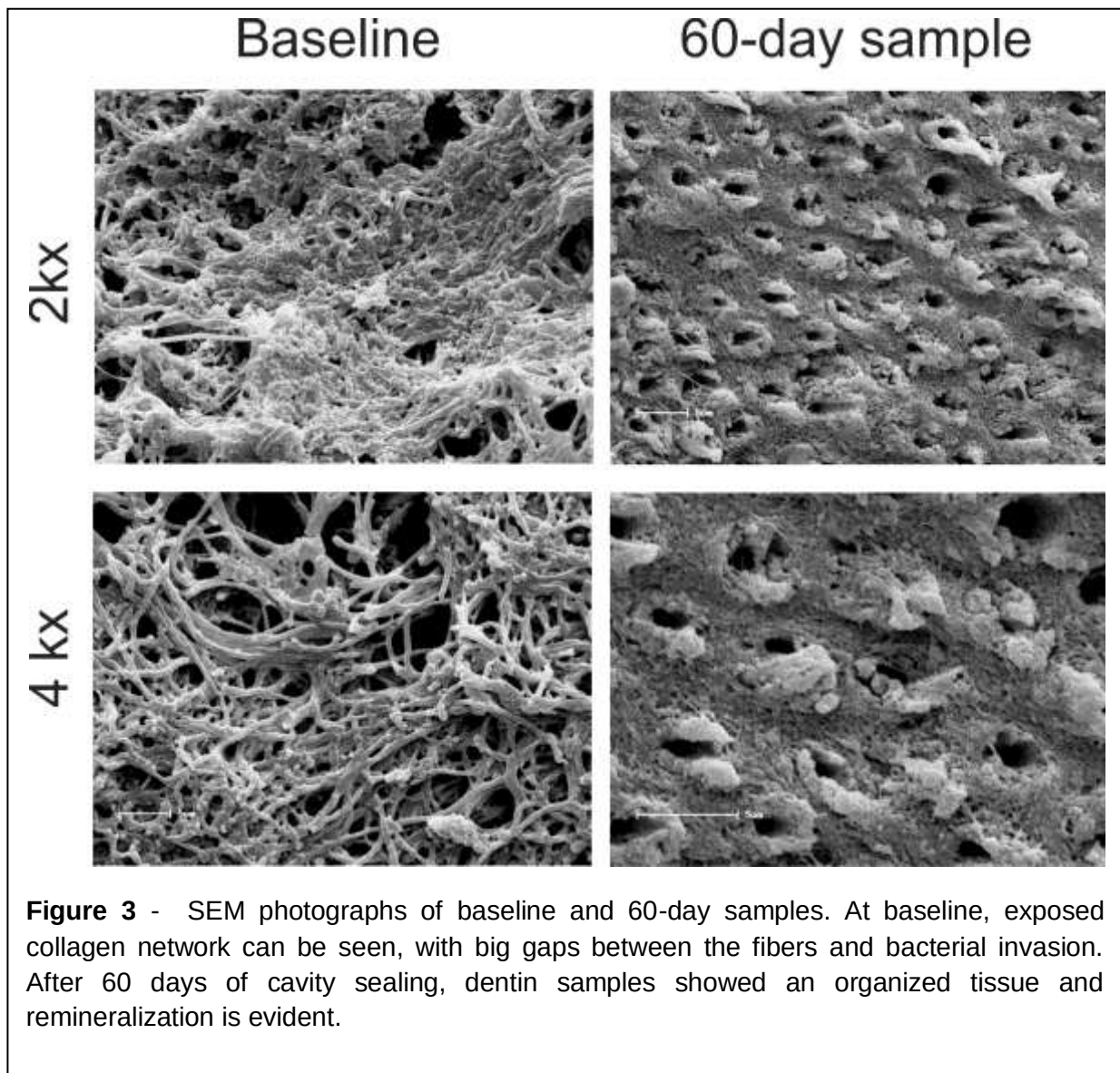


Figure 2 - Illustrative images of the post-processing routine of digitalized dental radiographs. Baseline and 10-13-month images are shown in A and B. In C, the subtracted image (image A - image B) can be seen. The focus is on tooth 85. Subtraction process was sucessful because structural noise is not seen. However, a brighter area (arrow) can be noted under restorative material, indicating mineral gain. In D, the registered image obtained after computerized alignment of digital images. Mean gray levels will be measured comparing image A (baseline) and D (10-13-month with adjusted geometric projection).



5 DISCUSSÃO

A literatura odontológica contemporânea é vasta quando se trata de remoção parcial de tecido cariado e unânime ao afirmar que mudanças positivas são alcançadas quando a dentina afetada é mantida na cavidade sob uma restauração adequadamente selada (MALTZ et al., 2002; MASSARA; ALVES; BRANDÃO, 2002; PINTO et al., 2006; WAMBIER et al., 2007; CASAGRANDE et al., 2009; DUQUE et al., 2009; FRANZON et al., 2009; LULA et al., 2009; LULA et al., 2011), seja em dentes decíduos ou permanentes. De um modo geral, pode-se dizer que as evidências apontam para a inatividade do processo carioso no interior da cavidade (ORHAN et al., 2008).

Portanto, o que tornou este trabalho singular foi a hipótese de que a dentina infectada, tal qual a afetada, poderia ser mantida no interior da cavidade e ainda assim, resultados similares seriam atingidos. Ao longo dos três artigos aqui compilados e que tiveram origem num mesmo projeto de pesquisa, observa-se que tal hipótese foi comprovada. O selamento cavitário de lesões de cárie em dentes decíduos favoreceu a reorganização tecidual mesmo com a manutenção de dentina infectada na cavidade.

A análise ultra-estrutural da dentina infectada sintetiza visualmente as características observadas nas diferentes avaliações clínicas e laboratoriais que foram conduzidas e as mudanças motivadas pelo selamento cavitário. É importante que se enfatize que a metodologia empregada permitiu que as amostras dentinárias controle e experimental fossem coletadas de um mesmo dente e paciente, eliminando assim variações individuais que poderiam comprometer a comparação final de maneira fidedigna. Da mesma forma, todos os dentes selecionados apresentavam lesões de cárie profundas, com envolvimento de pelo menos 2/3 da espessura da dentina, mas sem sinais ou sintomas de patologia pulpar detectados em exame clínico-radiográfico.

No momento inicial, a amostra dentinária veio de uma cavidade aberta, não tratada, banhada pela saliva e coberta pelo biofilme dental e de consistência macia: nitidamente uma lesão de cárie ativa.

A fotomicrografia obtida em MEV corroborou o diagnóstico clínico. Tratava-se de um tecido que, ainda que apresentasse atributos característicos da dentina, mostrava-se altamente desorganizado (dentina infectada). Os túbulos dentinários apresentavam grandes diâmetros, resultado da dificuldade do complexo dentino-pulpar de conter o avanço rápido do processo carioso. Condizente com este quadro agudo, aglomerados bacterianos eram facilmente identificados na estrutura dentinária, o que também foi observado nas elevadas contagens de microorganismos detectados na análise microbiológica. Acrescente-se ainda, como comprovação adicional da colonização do tecido, o aumento da fluorescência dentinária, resultado da detecção das porfirinas, um sub-produto metabólico bacteriano.

Todavia, os produtos metabólicos bacterianos que apresentam participação mais ativa no processo carioso são os ácidos. Sua ação modificou drasticamente o tecido, resultando numa dentina nitidamente desmineralizada, com exposição da rede de fibrilas colágenas nas regiões intertubular e até mesmo peritubular, que com o avanço do processo carioso começa a perder mineral, aumentando o diâmetro dos túbulos e facilitando a difusão dos ácidos e bactérias (GONZALEZ-CABEZAS, 2010). Tal desmineralização também foi comprovada por análise em EDS, que demonstrou porcentagens em peso reduzidas dos principais minerais constituintes da porção inorgânica da dentina: cálcio e fósforo.

A ação das MMPs endógenas é responsável pela continuidade do processo, devido à desnaturação da matriz orgânica da dentina, representada principalmente pelo colágeno tipo I (MAZZONI et al., 2012). Nas amostras de dentina infectada no momento inicial, foram detectadas as expressões de MMP-8 (colagenase) e MMP-2 e MMP-9 (gelatinases), ou seja, enzimas capazes da degradação do colágeno *in natura* e também das gelatinas (SHIMADA et al., 2009).

Com os ensaios imunohistoquímicos utilizados, não se pode afirmar que tais enzimas estavam em sua forma ativa. No entanto, como o pH ácido presente nas lesões de cárie é um mecanismo ativador dos zimógenos (CHAUSSAIN-MILLER et al., 2006), e também em função das outras

características avaliadas na dentina infectada pré-selamento, supõe-se que as enzimas liberadas do tecido após a desmineralização ácida (TOLEDANO et al., 2010) estejam ativas. Logo, a consequência natural é o processo de degradação, aumentando progressivamente a perda tecidual.

Num segundo momento, as cavidades foram devidamente seladas e acompanhadas por um período de 60 dias, quando foram reabertas e novas amostras de dentina infectada foram coletadas. Ao interromper o contato com o biofilme e estabilizar as flutuações de pH, o selamento cavitário deu condições para que o processo carioso estacionasse e a resposta biológica do dente se efetivasse (KIDD, 2010), como observado nas microfotografias pós-selamento. A neutralização do pH também é condição fundamental para que as MMPs se mantenham em atividade (ORSINI et al., 2012), numa ação conjunta de collagenases e gelatinases essencial para a remodelação do tecido (TOLEDANO et al., 2010).

Agora, a estrutura da dentina se mostrou mais organizada, com túbulos dentinários de menor diâmetro e ausência de fibras colágenas expostas, reflexo da mineralização tecidual. De acordo com estes achados, laboratorialmente verificou-se uma redução marcante no total de microorganismos viáveis, bem como um aumento no conteúdo de cálcio e fósforo, e, clinicamente, aumento na consistência do tecido e fluorescência dentinária reduzida.

Observou-se ainda um aumento nas expressões de MMP-8, colágeno tipo I e BSP. Ao se constatar uma estrutura dentinária mais organizada nas microfotografias, tem-se a certeza de que uma resposta positiva do complexo dentino-pulpar estava se processando. Ao mesmo tempo em que novas fibrilas colágenas eram secretadas, a MMP-8 trabalhava remodelando o tecido e dando condições para que a BSP efetivamente fizesse a ligação entre a rede colágena e o cálcio disponível.

A maioria das características descritas, seja no momento pré-selamento ou 60 dias após, são similares às alterações verificadas nos estudos sobre dentina afetada e representam evolução favorável nos diferentes aspectos avaliados: consistência (PINTO et al., 2006; MARCHI et al., 2008;

DUQUE et al., 2009; FRANZON et al., 2009; LULA et al., 2011), total de microorganismos viáveis (PINTO et al., 2006; WAMBIER et al., 2007; DUQUE et al., 2009; LULA et al., 2009; LULA et al., 2011), conteúdo mineral (MASSARA; ALVES; BRANDÃO, 2002).

No entanto, diferentemente do relatado por outros autores ao avaliarem a dentina afetada selada (MALTZ et al., 2002; PINTO et al., 2006; LULA et al., 2011), no presente estudo não se observou alteração significativa na coloração da dentina infectada após selamento cavitário. Todavia, uma tendência da dentina infectada a tornar-se mais escura foi observada, o que indica provavelmente que as alterações de cor necessitam de um tempo maior de acompanhamento para serem detectadas estatisticamente.

Por outro lado, diferenças significativas foram observadas nos valores de fluorescência dentinária antes e após selamento cavitário. É interessante observar que o DIAGNOdent - equipamento periférico para medir a fluorescência - foi utilizado aqui de maneira distinta da sua aplicação precípua, que é o diagnóstico de lesões de cárie. A fluorescência foi considerada como um indicador a mais das modificações induzidas pelo selamento na dentina infectada. Apesar das médias de fluorescências serem menores após selamento cavitário, estas ainda se mantiveram muito superiores às da dentina normal (0-13) ou aos limites de corte para cárie de dentina propostos na literatura (>38) (DINIZ et al., 2009). Acredita-se que tais valores estão relacionados à difusão das porfirinas bacterianas na matriz desmineralizada da dentina cuja fluorescência ainda é detectada pelo aparelho mesmo após remineralização.

O aumento na consistência da dentina (critérios táteis) associada a uma maior porcentagem em peso de cálcio e fósforo são dois achados condizentes com a remineralização dentinária, assim como o aumento na expressão de BSP. Observou-se também que este é um processo contínuo, envolvendo não apenas a dentina infectada, mas toda estrutura dentinária após selamento cavitário, o que foi comprovado no acompanhamento radiográfico via subtração radiográfica por um período médio de 11 meses.

Uma vez que a dentina tem um elevado conteúdo orgânico, o processo de remineralização dentinária torna-se mais complexo do que no esmalte (BERTASSONI et al., 2010). A reposição mineral na dentina, entre outros fatores, é mediada por proteínas não colagênicas, como a BSP, mas depende do arcabouço colágeno para dar sustentação aos cristais de apatita (ZHANG et al., 2012).

A apatita está localizada em duas regiões específicas: dentro das fibrilas colágenas (mineral intrafibrilar) e entre as fibrilas colágenas (mineral extrafibrilar) (BERTASSONI et al., 2010). São os cristalitos remanescentes na dentina cariada que atuam como facilitadores da remineralização por serem sítios para a nucleação da apatita (BERTASSONI et al., 2011; ZHANG et al., 2012), tão logo haja biodisponibilidade de cálcio e fosfato (GONZALEZ-CABEZAS, 2010).

Portanto, pode-se supor que, mesmo na dentina infectada analisada, havia regiões onde o colágeno mantinha suas ligações cruzadas intactas e apresentava cristalitos suficientes para estimular a remineralização tão logo o desafio cariogênico fosse estacionado. Em nível molecular, observou-se também uma expressão aumentada da BSP, que é uma proteína aniônica, capaz de se conectar ao colágeno e à hidroxiapatita (BOUSHELL; NAGAOKA; YAMAUCHI, 2011), atuando como um elo de ligação entre as fibrilas colágenas e os minerais. O aumento da expressão de MMPs observado após selamento também faz parte do processo, já que as MMPs são elementos essenciais na remodelação de tecidos conectivos em condições fisiológicas ou patológicas (CHARADRAM et al., 2012) e necessitam do cálcio para cumprir esta função (TJADERHANE et al., 1998; TEZVERGIL-MUTLUAY et al., 2010).

O aumento no conteúdo de cálcio e fósforo na dentina infectada 60 dias após o selamento cavitário poderia estar relacionado à difusão de íons que ocorre entre o cimento de ionômero de vidro (CIV) utilizado como material restaurador e o tecido desmineralizado (BEZERRA et al., 2012). No entanto, ao longo do período de acompanhamento de 10-13 meses, alterações radiográficas compatíveis com um aumento no conteúdo mineral foram observadas na dentina infectada mantida abaixo do CIV, por meio de

dados quantitativos. Este achado aponta para o fato de que, ainda que o CIV seja responsável pela liberação dos íons inicialmente, é a capacidade reacional do dente que mantém constante a remineralização da dentina infectada que permaneceu na cavidade. Trata-se da formação de dentina terciária, desencadeada por eventos inflamatórios inerentes a atividade aguda de cárie, que estimularam os mecanismos de defesa pulpares e visam preservar a vitalidade do dente (ALVES et al., 2010), num processo lento e contínuo de deposição mineral como o que foi observado neste estudo.

Cabe aqui um comentário a respeito de um outro íon, constantemente relacionado à remineralização dentinária: o flúor (EXTERKATE; DAMEN; TEN CATE, 2005). Neste estudo, não foram observadas diferenças significativas no conteúdo de íons flúor após selamento cavitário. A literatura mostra, em trabalhos *in vitro* e lesões de cáries artificiais (NGO et al., 2011) ou em estudos *in vivo* (NGO et al., 2006) que o cimento de ionômero de vidro é capaz de liberar flúor para a dentina desmineralizada num padrão compatível com remineralização. No entanto, apesar deste elemento mostrar um leve aumento em relação a amostra inicial, este não foi significativo, fato também verificado em outro estudo clínico em dentes decíduos (MASSARA; ALVES; BRANDÃO, 2002). Portanto, a influência do flúor na remineralização da dentina infectada ainda é um tópico a ser estudado.

A remineralização dentinária também depende do selamento cavitário adequado, que reduza a um mínimo possível a microinfiltração (GRUYTHUYSEN; VAN STRIJP; WU, 2010). Portanto, procedeu-se a avaliação das restaurações em ionômero de vidro ao longo do tempo (índice USPHS). Critérios que efetivamente interfeririam de maneira negativa no selamento marginal da restauração como a presença de cáries recorrentes ou *gaps* na interface dente-restauração não diferiram significativamente entre a avaliação de 10-13 meses e o momento de avaliação inicial.

Três dentes foram excluídos da amostra devido a complicações endodônticas após 10-13 meses. Considerando-se a amostra disponível neste momento (n=30), a taxa de sucesso do tratamento proposto é de 90%. Índices semelhantes foram relatados por revisões sistemáticas (HAYASHI et al., 2011;

FERREIRA et al., 2012) ou em estudos clínicos (BJORNDAL; LARSEN; THYLSTRUP, 1997; AL-ZAYER et al., 2003; GRUYTHUYSEN; VAN STRIJP; WU, 2010). Sendo assim, o nível de sucesso alcançado mantendo-se dentina infectada na cavidade foi compatível com outros estudos que investigaram a manutenção da dentina afetada.

A partir dos resultados obtidos, pode-se afirmar que a preocupação de muitos autores (GURBUZ; YILMAZ; SENGUL, 2008; IWAMI et al., 2008; NEVES et al., 2011; ALLEMAN; MAGNE, 2012; ALMAHDY et al., 2012) em diferenciar as duas camadas "teóricas" da dentina - dentina infectada e afetada - que têm sido usadas como um parâmetro para remoção de tecido cariado (GONZALEZ-CABEZAS, 2010), pode não ter tamanha relevância clínica, uma vez que o reparo tecidual tende a se processar em elementos dentais vitais devidamente selados mesmo na presença de dentina infectada na cavidade.

Ao indicar a manutenção de uma maior quantidade de dentina cariada em lesões profundas de cárie, o protocolo proposto neste trabalho aumentará as chances do dente decíduo cumprir integralmente seu ciclo biológico, sem necessidade de intervenção endodôntica ou exodontia, procedimentos de rotina em tal situação. Esta releitura do tratamento restaurador minimamente invasivo oferece uma nova opção ao profissional que se dedica ao atendimento de crianças com aplicabilidade clínica direta nos serviços odontológicos privados e, principalmente, em Saúde Pública.

6 CONCLUSÕES

A partir dos dados obtidos neste trabalho, é possível concluir que, após o selamento cavitário de lesões de cárie ativa em dentes decíduos, a dentina infectada mantida na cavidade:

- tornou-se mais consistente e menos contaminada, porém sem alteração significativa na coloração;
- apresentou menores valores de fluorescência dentinária;
- mostrou-se com melhor organização tecidual, redução de diâmetro de túbulos dentinários e evidência de mineralização;
- exibiu conteúdo aumentado de cálcio e fósforo;
- intensificou as expressões de MMP-8, colágeno tipo I e sialoproteína óssea;
- ao longo do tempo, apresentou evidências radiográficas de remineralização.

Portanto, o protocolo investigado pode ser considerado uma opção de tratamento para dentes decíduos vitalizados com lesões de cárie profunda, o que, na prática diária, reduzirá a possibilidade de exposições pulpares e a necessidade de intervenções endodônticas.

REFERÊNCIAS

- Al-Zayer MA et al. Indirect pulp treatment of primary posterior teeth: A retrospective study. *Pediatr Dent*, 2003, 25:29-36.
- Alleman DS; Magne P. A systematic approach to deep caries removal end points: The peripheral seal concept in adhesive dentistry. *Quintessence Int*, 2012, 43:197-208.
- Almahdy A et al. Microbiochemical analysis of carious dentine using raman and fluorescence spectroscopy. *Caries Research*, 2012, 46:432-40.
- Alves LS et al. Qualitative and quantitative radiographic assessment of sealed carious dentin: A 10-year prospective study. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, 2010, 109:135-41.
- American academy of pediatric dentistry reference manual 2010-2011. *Pediatr Dent*, 2010, 32:1-334.
- Barnes DM et al. A clinical evaluation of a resin-modified. Glass ionomer restorative material. *J Am Dent Assoc*, 1995, 126:1245-53.
- Bertassoni LE et al. Biomechanical perspective on the remineralization of dentin. *Caries Res*, 2009, 43:70-7.
- Bertassoni LE et al. Mechanical recovery of dentin following remineralization in vitro--an indentation study. *J Biomech*, 2011, 44:176-81.
- Bertassoni LE et al. Evaluation of surface structural and mechanical changes following remineralization of dentin. *Scanning*, 2010, 32:312-9.
- Bertassoni LE et al. The dentin organic matrix - limitations of restorative dentistry hidden on the nanometer scale. *Acta Biomater*, 2012, 8:2419-33.
- Bezerra AC et al. Ion concentration adjacent to glass-ionomer restorations in primary molars. *Dent Mater*, 2012, 28:e259-63.
- Bjorndal L; Larsen T; Thylstrup A. A clinical and microbiological study of deep carious lesions during stepwise excavation using long treatment intervals. *Caries Res*, 1997, 31:411-7.
- Bonecker M et al. Clinical, histological and microbiological study of hand-excavated carious dentine in extracted permanent teeth. *SADJ*, 2003, 58:273-8.
- Bourd-Boittin K et al. Matrix metalloproteinase inhibition impairs the processing, formation and mineralization of dental tissues during mouse molar development. *Exp Cell Res*, 2005, 304:493-505.

Boushell L; Nagaoka H; Yamauchi M. Increased matrix metalloproteinase-2 and bone sialoprotein response to human coronal caries. *Caries Research*, 2011, 45:453-9.

Braga MM; Mendes FM; Ekstrand KR. Detection activity assessment and diagnosis of dental caries lesions. *Dent Clin North Am*, 2010, 54:479-93.

Butler WT et al. Extracellular matrix proteins and the dynamics of dentin formation. *Connective tissue research*, 2002, 43:301-7.

Buyukgural B; Cehreli ZC. Effect of different adhesive protocols vs calcium hydroxide on primary tooth pulp with different remaining dentin thicknesses:24-month results. *Clin Oral Investig*, 2008, 12:91-6.

Carneiro LS et al. In vivo study of pixel grey-measurement in digital subtraction radiography for monitoring caries remineralization. *Dentomaxillofac Radiol*, 2009, 38:73-8.

Casagrande L et al. Effect of adhesive restorations over incomplete dentin caries removal: 5-year follow-up study in primary teeth. *J Dent Child*, 2009, 76:117-22.

Charadram N et al. Regulation of reactionary dentin formation by odontoblasts in response to polymicrobial invasion of dentin matrix. *Bone*, 2012, 50:265-75.

Chaussain-Miller C et al. The role of matrix metalloproteinases (MMPS) in human caries. *J Dent Res*, 2006, 85:22-32.

Christgau M et al. Accuracy of quantitative digital subtraction radiography for determining changes in calcium mass in mandibular bone: An in vitro study. *J Periodontal Res*, 1998, 33:138-49.

Coll JA. Indirect pulp capping and primary teeth: Is the primary tooth pulpotomy out of date? *J Endod*, 2008, 34:S34-9.

Corbel M; Boichot E; Lagente V. Role of gelatinases mmp-2 and mmp-9 in tissue remodeling following acute lung injury. *Braz J Med Biol Res*, 2000, 33:749-54.

Diniz MB et al. In vivo evaluation of laser fluorescence performance using different cut-off limits for occlusal caries detection. *Lasers Med Sci*, 2009, 24:295-300.

Duque C et al. Clinical and microbiological performance of resin-modified glass-ionomer liners after incomplete dentine caries removal. *Clin Oral Investig*, 2009, 13:465-71.

Ericson D. What is minimally invasive dentistry? *Oral health & preventive dentistry*, 2004, 2:287.

Ersin NK et al. A clinical evaluation of resin-based composite and glass ionomer cement restorations placed in primary teeth using the art approach: Results at 24 months. *J Am Dent Assoc*, 2006, 137:1529-36.

Exterkate R; Damen J; Ten Cate J. Effect of fluoride-releasing filling materials on underlying dentinal lesions in vitro. *Caries Research*, 2005, 39:509-13.

Fedarko NS et al. Three small integrin binding ligand n-linked glycoproteins (SIBLINGS) bind and activate specific matrix metalloproteinases. *The FASEB journal*, 2004, 18:734-6.

Ferracane JL; Cooper PR; Smith AJ. Can interaction of materials with the dentin-pulp complex contribute to dentin regeneration? *Odontology*, 2010, 98:2-14.

Ferreira JM et al. Caries removal in primary teeth--a systematic review. *Quintessence Int*, 2012, 43:e9-15.

Frank RM. Structural events in the caries process in enamel, cementum, and dentin. *J Dent Res*, 1990, 69 Spec No:559-66; discussion 634-6.

Franzon R et al. Dentin rehardening after indirect pulp treatment in primary teeth. *J Dent Child*, 2009, 76:223-8.

Gomes JR et al. Immunolocalization and activity of the mmp-9 and mmp-2 in odontogenic region of the rat incisor tooth after post shortening procedure. *Journal of molecular histology*, 2011, 42:153-9.

Gonzalez-Cabezas C. The chemistry of caries: Remineralization and demineralization events with direct clinical relevance. *Dent Clin North Am*, 2010, 54:469-78.

Gormez O; Yilmaz HH. Image post-processing in dental practice. *Eur J Dent*, 2009, 3:343-7.

Gruythuysen RJ; van Strijp AJ; Wu MK. Long-term survival of indirect pulp treatment performed in primary and permanent teeth with clinically diagnosed deep carious lesions. *J Endod*, 2010, 36:1490-3.

Gurbuz T; Yilmaz Y; Sengul F. Performance of laser fluorescence for residual caries detection in primary teeth. *Eur J Dent*, 2008, 2:176-84.

Hannas AR et al. The role of matrix metalloproteinases in the oral environment. *Acta Odontol Scand*, 2007, 65:1-13.

Hayashi M et al. Ways of enhancing pulp preservation by stepwise excavation--a systematic review. *J Dent*, 2011, 39:95-107.

Hibst R; Paulus R; Lussi A. Detection of occlusal caries by laser fluorescence: Basic and clinical investigations. *Medical Laser Application*, 2001, 16:205-13.

Hickel R; Manhart J. Longevity of restorations in posterior teeth and reasons for failure. *The Journal of Adhesive Dentistry*, 2001, 3:45.

Innes NP; Evans DJ; Stirrups DR. Sealing caries in primary molars: Randomized control trial, 5-year results. *J Dent Res*, 2011, 90:1405-10.

Ismail AI et al. The international caries detection and assessment system (ICDAS): An integrated system for measuring dental caries. *Community Dent Oral Epidemiol*, 2007, 35:170-8.

Iwami Y et al. Relationship between the color of carious dentin with varying lesion activity, and bacterial detection. *J Dent*, 2008, 36:143-51.

Iwami Y et al. Relationship between bacterial infection and evaluation using a laser fluorescence device, diagnodent. *Eur J Oral Sci*, 2004, 112:419-23.

Iwami Y et al. Relationship between laser fluorescence and bacterial invasion in arrested dentinal carious lesions. *Lasers Med Sci*, 2011, 26:439-44.

Jung MS et al. Three-dimensional analysis of deciduous maxillary anterior teeth using cone-beam computed tomography. *Clin Anat*, 2012, 25:182-8.

Kidd EA. Clinical threshold for carious tissue removal. *Dent Clin North Am*, 2010, 54:541-9.

Lee YL et al. Dentin-pulp complex responses to carious lesions. *Caries Res*, 2006, 40:256-64.

Leonardi R; Loreto C. Immunohistochemical localization of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in human carious dentine. *Acta Histochemica*, 2010, 112:298-302.

Lula EC et al. Partial caries removal in primary teeth: Association of clinical parameters with microbiological status. *Caries Res*, 2011, 45:275-80.

Lula EC et al. Microbiological analysis after complete or partial removal of carious dentin in primary teeth: A randomized clinical trial. *Caries Res*, 2009, 43:354-8.

Maltz M et al. A clinical, microbiologic, and radiographic study of deep caries lesions after incomplete caries removal. *Quintessence Int*, 2002, 33:151-9.

Maltz M et al. Deep caries lesions after incomplete dentine caries removal: 40-month follow-up study. *Caries Res*, 2007, 41:493-6.

Marangos O et al. Physico-mechanical properties determination using microscale homotopic measurements: Application to sound and caries-affected primary tooth dentin. *Acta Biomater*, 2009, 5:1338-48.

Marchi JJ et al. Analysis of primary tooth dentin after indirect pulp capping. *J Dent Child*, 2008, 75:295-300.

Martignon S; Ekstrand KR; Ellwood R. Efficacy of sealing proximal early active lesions: An 18-month clinical study evaluated by conventional and subtraction radiography. *Caries Res*, 2006, 40:382-8.

Martignon S et al. Infiltrating/sealing proximal caries lesions: A 3-year randomized clinical trial. *J Dent Res*, 2012, 91:288-92.

Massara ML; Alves JB; Brandão PR. Atraumatic restorative treatment: Clinical, ultrastructural and chemical analysis. *Caries Res*, 2002, 36:430-6.

Mazzoni A et al. A review of the nature, role, and function of dentin non-collagenous proteins. Part ii: Enzymes, serum proteins, and growth factors. *Endodontic Topics*, 2012, 21:19-40.

Mazzoni A et al. Zymographic analysis and characterization of mmp-2 and-9 forms in human sound dentin. *Journal of Dental Research*, 2007, 86:436-40.

Mazzoni A et al. Immunohistochemical identification of mmp-2 and mmp-9 in human dentin: Correlative fei-sem/tem analysis. *Journal of Biomedical Materials Research Part A*, 2008, 88:697-703.

Mejàre I et al. Diagnosis of the condition of the dental pulp: A systematic review. *International Endodontic Journal*, 2012, 45:597-613.

Mendes FM et al. Performance of diagnodent for detection and quantification of smooth-surface caries in primary teeth. *J Dent*, 2005, 33:79-84.

Mertz-Fairhurst EJ et al. Ultraconservative and cariostatic sealed restorations: Results at year 10. *J Am Dent Assoc*, 1998, 129:55-66.

Neves AA et al. Does diagnodent provide a reliable caries-removal endpoint? *J Dent*, 2011, 39:351-60.

Ngo HC et al. Chemical exchange between glass-ionomer restorations and residual carious dentine in permanent molars: An in vivo study. *J Dent*, 2006, 34:608-13.

Ngo HC et al. An in vitro model for the study of chemical exchange between glass ionomer restorations and partially demineralized dentin using a minimally invasive restorative technique. *J Dent*, 2011, 39 Suppl 2:S20-6.

Niu L et al. Localization of MMP-2, MMP-9, TIMP-1, and TIMP-2 in human coronal dentine. *Journal of dentistry*, 2011, 39:536-42.

Oliveira EF et al. The monitoring of deep caries lesions after incomplete dentine caries removal: Results after 14-18 months. *Clin Oral Investig*, 2006, 10:134-9.

Ono E et al. Evaluation of simulated external root resorptions with digital radiography and digital subtraction radiography. *Am J Orthod Dentofacial Orthop*, 2011, 139:324-33.

Orhan AI; Oz FT; Orhan K. Pulp exposure occurrence and outcomes after 1- or 2-visit indirect pulp therapy vs complete caries removal in primary and permanent molars. *Pediatr Dent*, 2010, 32:347-55.

Orhan AI et al. A clinical and microbiological comparative study of deep carious lesion treatment in deciduous and young permanent molars. *Clinical Oral investigations*, 2008, 12:369-78.

Orsini G et al. A review of the nature, role, and function of dentin non-collagenous proteins. Part 1: Proteoglycans and glycoproteins. *Endodontic Topics*, 2012, 21:1-18.

Palosaari H et al. The expression of MMP-8 in human odontoblasts and dental pulp cells is down-regulated by TGF-beta1. *J Dent Res*, 2000, 79:77-84.

Peters MC et al. In vivo dentin remineralization by calcium-phosphate cement. *J Dent Res*, 2010, 89:286-91.

Petrikowski CG et al. Interobserver variability in pediatric radiographic quality assessment. *J Can Dent Assoc*, 1998, 64:36-41.

Pinheiro SL et al. Repairing collagen in dentin carious lesions. Influence of sealing the material: A morphometric study. *Journal of Clinical Pediatric Dentistry*, 2010, 34:223-8.

Pinheiro SL et al. Antibacterial activity of glass-ionomer cement containing antibiotics on caries lesion microorganisms. *Am J Dent*, 2005, 18:261-6.

Pinto AS et al. Clinical and microbiological effect of calcium hydroxide protection in indirect pulp capping in primary teeth. *Am J Dent*, 2006, 19:382-6.

Ribeiro CC et al. Rationale for the partial removal of carious tissue in primary teeth. *Pediatr Dent*, 2012, 34:39-41.

Ricketts DN et al. Complete or ultraconservative removal of decayed tissue in unfilled teeth. *Cochrane Database Syst Rev*, 2006, CD003808.

RITTER AV et al. Partial caries excavation. *Journal of Esthetic and Restorative Dentistry*, 2012, 24:148-52.

Santiago BM et al. Microhardness of dentine underlying art restorations in primary molars: An in vivo pilot study. *Br Dent J*, 2005, 199:103-6.

Selwitz RH; Ismail AI; Pitts NB. Dental caries. *Lancet*, 2007, 369:51-9.

Shimada Y et al. Localization of matrix metalloproteinases (MMPS-2, 8, 9 and 20) in normal and carious dentine. *Aust Dent J*, 2009, 54:347-54.

Silva NR et al. Evaluation of a self-limiting concept in dentinal caries removal. *J Dent Res*, 2006, 85:282-6.

Smith A et al. Dentine as a bioactive extracellular matrix. *Archives of Oral Biology*, 2012, 57:109-21.

Sulkala M et al. Matrix metalloproteinase-8 (MMP-8) is the major collagenase in human dentin. *Arch Oral Biol*, 2007, 52:121-7.

Tezvergil-Mutluay A et al. The requirement of zinc and calcium ions for functional mmp activity in demineralized dentin matrices. *Dental Materials*, 2010, 26:1059-67.

Thompson V et al. Treatment of deep carious lesions by complete excavation or partial removal: A critical review. *J Am Dent Assoc*, 2008, 139:705-12.

Tjäderhane L et al. Dentin basic structure and composition—an overview. *Endodontic Topics*, 2012, 20:3-29.

Tjaderhane L et al. The activation and function of host matrix metalloproteinases in dentin matrix breakdown in caries lesions. *J Dent Res*, 1998, 77:1622-9.

Toi CS; Bonecker M; Cleaton-Jones PE. Mutans streptococci strains prevalence before and after cavity preparation during atraumatic restorative treatment. *Oral Microbiol Immunol*, 2003, 18:160-4.

Toledano M et al. Differential expression of matrix metalloproteinase-2 in human coronal and radicular sound and carious dentine. *Journal of Dentistry*, 2010, 38:635-40.

Van Strijp A et al. Host-derived proteinases and degradation of dentine collagen in situ. *Caries Research*, 2003, 37:58-65.

Wambier DS et al. Ultrastructural and microbiological analysis of the dentin layers affected by caries lesions in primary molars treated by minimal intervention. *Pediatr Dent*, 2007, 29:228-34.

Weber CM; Alves LS; Maltz M. Treatment decisions for deep carious lesions in the public health service in southern brazil. *J Public Health Dent*, 2011, 71:265-70.

Wenzel A; Anthonisen PN; Juul MB. Reproducibility in the assessment of caries lesion behaviour: A comparison between conventional film and subtraction radiography. *Caries Res*, 2000, 34:214-8.

Zavgorodniy AV; Rohanizadeh R; Swain MV. Ultrastructure of dentine carious lesions. *Arch Oral Biol*, 2008, 53:124-32.

Zhang X et al. Remineralization of partially demineralized dentine substrate based on a biomimetic strategy. *J Mater Sci Mater Med*, 2012, 23:733-42.

Zheng X et al. Real-time enzymatic degradation of human dentin collagen fibrils exposed to exogenous collagenase: An AFM study in situ. *J Microsc*, 2011, 241:162-70.

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

COEP

COMISSÃO DE ÉTICA EM PESQUISA

PARECER Nº 03/2010
Protocolo: 15821/09

No dia 25 de Março de 2010, a Comissão de Ética em Pesquisa, **APROVOU** o protocolo de pesquisa intitulado **"Avaliação in vivo das alterações em dentina cariada de dentes decíduos após selamento cavitário em crianças de diferentes faixas etárias"** de responsabilidade do pesquisador Denise Stadler Wambier.

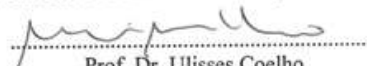
Conforme Resolução CNS 196/96, solicitamos que sejam apresentados a esta Comissão, relatórios sobre andamento da pesquisa, conforme modelo (<http://www.uepg.br/coep/>).

Data para entrega do relatório parcial: 25 de Fevereiro de 2011

Data para entrega do relatório final: 25 de Março de 2012.

Ponta Grossa, 29 de Março de 2010.

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


Prof. Dr. Ulisses Coelho
Coordenador

ANEXO B - Carta de aceite do artigo 3

 **Ana Claudia Rodrigues Chibinski** [Adicionar a contatos](#)
Para anachibinski@hotmail.com

14/10/2012 
[Responder](#)

----- Mensagem encaminhada -----

Remetente: rgillmeister@aapd.org
Data: 14/10/2012 14:58 (05:39 horas atrás)
Assunto: Pediatric Dentistry Manuscript PediaD-2012-05-2257.R6
Para: anachibinski@uepg.br, mauchibinski@gmail.com
14-Oct-2012

Dear Prof. Chibinski:

We are pleased to inform you that your manuscript "Evaluation of primary carious dentin after cavity sealing in deep lesions: a 10-13 month follow-up" (PediaD-2012-05-2257.R6) has been accepted for publication in Pediatric Dentistry.

We will be touch as we near publication, which generally occurs within 10-12 months of acceptance.

Authors of studies funded by the National Institutes of Health (NIH) whose papers are accepted for publication are reminded that they must submit the study as published to NIH for inclusion in the PubMed Central digital archive. Prior to submitting to the archive, authors must request permission from the American Academy of Pediatric Dentistry, who holds the copyright to all material published in the journal. It is the author's responsibility to request the permission and to submit the published version of the article to NIH. Instructions on submitted the paper to NIH can be found at: "http://publicaccess.nih.gov/submit_process.htm".

Thank you for the opportunity to publish your manuscript.

Sincerely,
Dr. N. Sue Seale
Editor-in-Chief, Pediatric Dentistry

*