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# "USO DO LASER DE $CO_2$ (λ=10,6 μm) NA PREVENÇÃO DA CÁRIE E EROSÃO DENTÁRIAS – ESTUDOS *IN VITRO*"

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Piracicaba

2009

**FICHA CATALOGRÁFICA ELABORADA PELA**
**BIBLIOTECA DA FACULDADE DE ODONTOLOGIA DE PIRACICABA**
Bibliotecária: Marilene Girello – CRB-8ª. / 6159

St35u

Steiner-Oliveira, Carolina.
Uso do laser de $CO_2$ (λ=10,6 μm) na prevenção da cárie e erosão dentárias – estudos *in vitro*. / Carolina Steiner-Oliveira. -- Piracicaba, SP: [s.n.], 2009.

Orientador: Marinês Nobre dos Santos Uchôa.
Tese (Doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Placa dentária. 2. Desmineralização. 3. Fluoretos. 4. Microbiologia. 5. Microrradiografia. 6. Reações bioquímicas. 7. Saliva artificial. 8. *Streptococcus mutans*. I. Nobre dos Santos, Marinês. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

(mg/fop)

Título em Inglês: Use of $CO_2$ laser (λ=10,6 μm) on dental caries and erosion prevention – *in vitro* studies

Palavras-chave em Inglês (Keywords): 1. Dental plaque. 2. Demineralization. 3. Fluoride. 4. Microbiology. 5. Microradiography. 6. Biochemical reactions. 7. Artificial saliva. 8. *Streptococcus mutans*

Área de Concentração: Odontopediatria

Titulação: Doutor em Odontologia

Banca Examinadora: Alberto Carlos Botazzo Delbem, Ana Cecília Correa Aranha, Maria Beatriz Duarte Gavião, Marinês Nobre dos Santos Uchôa, Mônica Campos Serra

Data da Defesa: 07-04-2009

Programa de Pós-Graduação em Odontologia

UNICAMP

**UNIVERSIDADE ESTADUAL DE CAMPINAS**
**FACULDADE DE ODONTOLOGIA DE PIRACICABA**

A Comissão Julgadora dos trabalhos de Defesa de Tese de DOUTORADO, em sessão pública realizada em 07 de Abril de 2009, considerou a candidata CAROLINA STEINER OLIVEIRA aprovada.

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## *DEDICATÓRIA*

***À DEUS,***

***PELA ILUMINAÇÃO E FORÇA DURANTE A LONGA JORNADA...***

***AOS MEUS PAIS, Haydée, Wilson e Fernando***

***Pelo amor, apoio incondicional, torcida e compreensão...***

***AO MEU IRMÃO GABRIEL,***

***Pelo carinho e admiração...***

***AO MEU AMOR Fernando,***

***Que trilhou ao meu lado e conviveu muitos momentos de alegria e preocupação, sempre com uma palavra doce e uma piada...***

## *AGRADECIMENTO ESPECIAL*

A minha orientadora, Profa. Dra. **MARINÊS NOBRE DOS SANTOS UCHÔA,**

Pela crescente amizade com o passar dos anos; nossa convivência sempre me ensinou mais um pouco. Muito obrigada por confiar tanto em mim e no meu trabalho. Isso abriu caminhos para que eu pudesse ir ainda mais longe.

Ao Prof. Dr. **ANDERSON TAKEO HARA,**

Por todos os incríveis ensinamentos. Cresci muito como pesquisadora. Agradeço também por toda ajuda e apoio na vida em outro país. É um desafio e tanto. Nossas risadas ficarão guardadas na memória.

À Profa. Dra. **LIDIANY KARLA AZEVÊDO RODRIGUES,** pela amizade maravilhosa. Por toda ajuda, incentivo e por sempre acreditar muito na minha capacidade e contar comigo.

## *AGRADECIMENTOS*

À Universidade Estadual de Campinas, na pessoa do seu Magnífico Reitor Prof. Dr. **José Tadeu Jorge**; à Faculdade de Odontologia de Piracicaba, na pessoa do seu diretor Prof. Dr. **Francisco Haiter Neto**, do Coordenador Geral da Pós-Graduação da Faculdade de Odontologia de Piracicaba-UNICAMP Prof. Dr. **Jacks Jorge Júnior** e do Coordenador do Programa de Pós-Graduação em Odontologia Profa. Dra. **Maria Beatriz Duarte Gavião**, pela participação desta conceituada instituição no meu crescimento científico, profissional e pessoal.

À **Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)** e ao **Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)** pelo apoio financeiro concedido durante o desenvolvimento destes trabalhos.

À Profa. Dra. **Cecília Gatti Guirado,** pela doçura de suas palavras e atitudes. Uma pessoa que fez muita diferença na minha vida pessoal e profissional, pois me ensinou a tratar o próximo com mais carinho. Além disso, uma das poucas pessoas que me disse olhando nos meus olhos o quanto eu era especial e competente. Muitas saudades... no entanto, grandes e maravilhosas memórias! Obrigada.

À Profa. Dra. **Maria Beatriz Duarte Gavião**, Profa. Dra. **Regina Maria Puppin Rontani,** Profa. Dra. **Regina Célia Rocha Peres** e Prof. Dr. **Érico Barbosa Lima,** pela contribuição para o meu crescimento profissional e pessoal, sempre prontos a mostrar o melhor caminho.

Aos **amigos professores do Programa de Pós-Graduação em Odontologia da FOP-UNICAMP**.

Aos Professores Dra. **Cínthia Pereira Machado Tabchoury,** Profa. Dra. **Regina Célia Rocha Peres,** e Profa. Dra. **Wanessa Christine de Souza Zaroni** membros da banca de qualificação, pelas sugestões para a realização e finalização deste trabalho.

Aos Professores Dr. **Domenick Zero**, Dr. **Armando Soto-Rojas**, Dr. **Hafsteinn Eggertsson**, Dra. **Andréa Ferreira Zandoná**, Dr. **Masatoshi Ando**, Dr. **Carlos Gonzaléz-Cabezas**, Dra. **Margherita Fontana** e Dra. **Angeles Martinez Mier** do Oral

Health Research Institute da Indiana University por terem aberto as portas de seus laboratórios, dividirem tantos conhecimentos e ainda pelas amizades.

A todos os funcionários do Oral Health Research Institute da Indiana University pela dedicação em me fazer sentir em casa e por toda ajuda durante toda a pesquisa, especialmente **Sharon E. Gwinn, Mattew Backs** e **Clif Duhn**.

Ao amigo, funcionário da Odontopediatria, **Marcelo Corrêa Maistro** pela paciência e inestimável ajuda.

Às secretárias **Maria Elisa dos Santos, Érica A. Pinho Sinhoreti** e **Raquel Q. Marcondes Cesar Sacchi** pela ajuda e atenção em todas as fases administrativas.

A equipe do **Laboratório Especial de Laser em Odontologia-LELO da Faculdade de Odontologia da Universidade de São Paulo** pela utilização do laser de $CO_2$. Especialmente à Profa. Dra. **Ana Cecília Correa Aranha**, Profa. Dra. **Patrícia Moreira de Freitas** e **Liliane Souza** pela ajuda e por sempre me manter as portas abertas.

Ao **Centro de Energia Nuclear na Agricultura**, da **Universidade de São Paulo (USP)**, pelo uso do Laboratório de Melhoramento de Plantas para esterilização dos blocos dentários, especialmente ao **Sr. Paulo Cassieri Neto**.

Às amigas e companheiras de turma da Odontopediatria **Fernanda Miori Pascon** por cada palavra e ombro amigo tanto nas horas difíceis, me compreendendo e aconselhando, como para dar muitas risadas; **Flávia Riqueto Gambareli,** pela alegria contagiante que fez cada dia que veio para Piracicaba ficar mais agradável; **Kamila Rosamilia Kantovitz,** pela habilidade em me deixar mais calma dizendo que tudo ia dar certo na sua hora; **Karlla Almeida Vieira,** que mesmo a distância sempre ofereceu ajuda e carinho; **Márcia Diaz Serra,** pela crescente amizade e momentos de longas conversas sobre a vida; **Moara De Rossi,** minha companheira de aventuras, muitas risadas e trabalho também **e Renata Andrea Salvitti de Sá Rocha,** que sempre me apoiou em todas as minhas decisões, mesmo as erradas, e permaneceu ao meu lado. Vocês trilharam comigo, durante todos esses anos, os difíceis caminhos dessa jornada e a tornou muito mais feliz e prazerosa. Eu amo muito vocês todas!

Às amigas **Fernanda Vieira Ribeiro, Maria Fernanda Peres, Vanessa Salvadego de Queiroz, Débora Cristina de Almeida Barra, Morgana Gabriotti e Sibele Pereira**

**de Oliveira**, companheiras fiéis sempre prontas para me ouvir e apoiar em casa à noite. Obrigada pela compreensão, risadas e comilanças. Eu amo vocês!

Às amigas **Cíntia Maria de Souza e Silva** e **Thaís Manzano Parisotto** pela imensa ajuda e paciência durante todas as pesquisas, viagens e madrugadas. Eu adorei conviver com vocês duas, sempre tentando apaziguar os ânimos.

Às amigas **Anna Maria Papa, Aline Castilho, Annicele Andrade, Éfani Banzi, Eliane Rodrigues, Fernanda Frasseto, Larissa Pacheco, Marcela Monteiro de Oliveira, Maria Cláudia Turelli, Maria Eliana de Almeida, Marina Leme, Renata Cerezetti, Rosane Pontes, Patrícia Sacramento** e **Taís Barbosa**, pela convivência e risadas pelos laboratórios e seminários.

Às amigas **Fabíola Galbiatti de Carvalho**, **Maria Malerba Humel, Cristiane Duque** e **Carolina Lima de Carvalho** pela amizade, ajuda e/ou aconselhamentos.

Ao Prof. Dr. **José Carlos Pettorossi Imparato**, Prof. Dr. **Fausto Medeiros Mendes**, Prof. Dr. **Marcelo José Strazzeri Bönecker**, Profa. Dra. **Daniela Prócida Raggio** e Profa. Dra. **Mariana Minatel Braga,** Profa. Dra. **Gabriela Bonini**, Profa. **Débora Moraes**, Profa. Dra. **Sandra Escheverria** e Prof. **Gabriel Politano** pela oportunidade de ter trabalhado e aprendido com todos você. Também por todo apoio, confiança e companheirismo.

Aos **alunos de graduação da FOP-UNICAMP**, a quem eu ministrei minhas primeiras aulas e orientei na clínica de Odontopediatria.

Aos **alunos do curso de extensão da Clínica de Odontohebiatria da FO-USP**, com quem compartilhei muitos bons momentos.

Às famílias **Steiner, Machado e Oliveira** pela confiança depositada em todo o meu trabalho.

Ao meu avô, Sr. **Arthur Steiner** por sempre acreditar em mim e me incentivar com muito amor, dizendo que eu era seu diamante. Aos meus primos e primas **Tati, Lelo, Fabinho, Mel, Fifo, Rubinho, Vitinho, Camila, Carol, Édric, Larissa, Alessandra, Ana Luiza, André, Maíra, Gabriela, Guilherme, Luise, Larissa e Caio** que, apesar da

distância, estiveram sempre torcendo por mim. A todos meus os **tios** e **tias** que sempre vibraram por mim.

Ao Sr. **Valdir Amaral Maciel** e Sra. **Rosa Maria Alves Maciel** por terem me acolhido como uma filha e pelo carinho.

Aos familiares **Paulo Roberto Menezes Machado, Elysiane Ornelas Machado, Andréa Alves Maciel Di Ninno, Sérgio Barbosa Di Ninno, Giulia Maciel Di Ninno, Carlos Jesus Pincerato, Anita Amaral Maciel Pincerato, Kátia Maciel Pincerato e Rita de Cássia Maciel Pincerato** por sempre torcerem com as minhas conquistas e pelo carinho.

Aos amigos **Alexandre Carlos Stievano, Carolina Rodrigues Lazzari, Roberta Duarte Vigneron, Renato Corrêa Viana Casarin** e **Vanessa Tramontino** pela torcida e amizade.

Aos **amigos da turma 44 e agregados da FOP-UNICAMP** por todos os reencontros, alegrias e diversão.

Às bibliotecárias **Marilene Girello**, pela colaboração na correção das referências bibliográficas e **Sueli Ferreira Julio de Oliveira**, pelas importantes informações.

A todos os **funcionários** da FOP pela colaboração.

A todas as pessoas que direta ou indiretamente auxiliaram na concretização deste trabalho.

**MUITO OBRIGADA!**

***"O que devemos ser? Esta é a questão essencial,***

***pois só o espírito é capaz de fertilizar a inteligência.***

***É o espírito que impregna a inteligência com a obra em***

***gestação. E a inteligência a levará a bom termo."***

***[Antoine de Saint-Exupéry]***

RESUMO

Os efeitos causados pelas modificações promovidas pela irradiação com laser de $CO_2$ podem inibir a desmineralização dos tecidos dentários e podem ser potencializados quando associados ao fluoreto. Apesar de amplo uso do fluoreto e da redução da prevalência de cárie, essa doença ainda acomete grupos de alto risco. Por outro lado, tem sido observado um aumento da prevalência da erosão dentária. Os objetivos dessa tese, composta por 4 manuscritos, foram: (1) descrever as características do laser de $CO_2$ e seus mecanismos de ação na inibição da desmineralização do esmalte; (2) desenvolver um modelo microbiológico, *in vitro,* de produção de lesão de cárie em dentina e testar duas hipóteses: (a) de que não há diferença na produção de cárie artificial em dentina utilizando um modelo microbiológico com regimes de 3 e 6 imersões ao dia em sacarose, avaliados por contagem bacteriana da dentina (UFC), análise microrradiográfica (AM) e análise de polissacarídeo insolúvel (API); (b) de que não há diferença no pH do biofilme antes e após sua imersão em sacarose; (3) avaliar, *in vitro*, a efetividade do laser de $CO_2$ ($\lambda$ = 10,6 μm) pulsado, associado ou não ao fluoreto, na redução da desmineralização da dentina radicular usando um modelo microbiológico, avaliado por AM; (4) avaliar, *in vitro*, o efeito do mesmo laser, associado ou não ao fluoreto, na redução da desmineralização do esmalte e da dentina submetidos a um desafio erosivo, pela mensuração da perda de superfície e análise da concentração de cálcio, fósforo e fluoreto das soluções desmineralizadoras. Os dados foram analisados quanto à normalidade e testes apropriados foram realizados com nível de significância de 5%. No estudo 1, os efeitos do laser no esmalte, seu mecanismo de ação na redução da desmineralização, combinados ou não ao fluoreto, foram discutidos. No estudo 2, o pH do biofilme diminuiu imediatamente após a imersão em sacarose, mas aumentou novamente 5 min depois. Lesões em dentina foram produzidas com sucesso e a adição de sacarose mostrou as maiores perdas minerais, no entanto não diferiu entre os dois regimes de sacarose. A UFC não mostrou nenhuma diferença e a API dos tratamentos foram maiores que a do grupo controle. No estudo 3, os espécimes radiculares foram tratados ou não com laser de $CO_2$ e com ou sem fluoreto antes ou após a irradiação com laser. O modelo microbiológico utilizado foi efetivo em produzir lesões dentinárias e as terapias

combinadas mostraram as lesões dentinárias mais rasas. No estudo 4, espécimes de esmalte e dentina foram tratados com fluoreto, laser e fluoreto/laser e submetidos a um desafio erosivo. Os resultados de desgaste indicaram que o tratamento combinado interferiu com as perdas minerais do esmalte e da dentina, mesmo sem mostrar efeito sinérgico. Houve uma tendência de retenção de fluoreto no esmalte pelo tratamento combinado e também de liberação de menores quantidades de cálcio, fósforo e fluoreto para as soluções desmineralizadoras. Em conclusão, o mecanismo de ação do laser de $CO_2$ na inibição da desmineralização do esmalte ainda não está completamente esclarecido e seu efeito pode ser aumentado quando associado ao fluoreto. O modelo microbiológico foi efetivo em produzir lesões de cárie dentinária. A irradiação da dentina radicular com laser inibiu a desmineralização dessa superfície apenas quando associado com o fluoreto; no entanto, não foi observado efeito sinérgico. O tratamento isolado com laser não foi capaz de prevenir a perda de superfície do esmalte e da dentina devido à erosão. Sua combinação com fluoreto mostrou alguma proteção, mas principalmente devido ao efeito do fluoreto. Não foi observada interação sinérgica significativa ou proteção duradoura com a terapia de laser.

Palavras-chave: Biofilme dentário, Desmineralização, Fluoretos, Microbiologia, Microrradiografia, Reações bioquímicas, Saliva artificial, *Streptococcus mutans*

ABSTRACT

The effects caused by the modifications promoted by the $CO_2$ laser irradiation can inhibit the dental tissues demineralization and may be enhanced when associated with fluoride. Despite the widespread use of the fluoride and the reduction of the caries prevalence, this disease still occurs in the high risk groups. On the other hand, an increase of the dental erosion prevalence was observed. This thesis, comprised by 4 manuscripts, aimed: (1) to describe the $CO_2$ laser characteristics and its action mechanisms in the enamel demineralization inhibition; (2) to develop an *in vitro* microbial model to produce dentin caries lesions and test two hypotheses - (a) that there is no difference in the artificial caries production in dentin using a microbial model with 3 and 6 sucrose bath immersions, as assessed by bacterial counts on the dentin (CFU), microradiographic analysis (TMR) and extracellular polysaccharide analysis (EPS); (b) that there is no difference in the biofilm pH before and after each sucrose bath; (3) to assess, *in vitro,* the effectiveness of a pulsed $CO_2$ laser ($\lambda$ = 10.6 μm) associated or not with fluoride, in reducing the root demineralization using a microbial model, as assessed by TMR; (4) to assess, *in vitro*, the effect of the same laser, associated or not with fluoride, on the prevention of the enamel and dentin erosions by means of surface loss measurement and analysis of the calcium, phosphorus and fluoride concentrations in the demineralizing solutions. The data were checked for normality and appropriated tests were performed with a significance level of 5%. In study 1, the laser effects on the enamel and its action mechanisms in the demineralization reduction, combined or not with fluoride, were discussed. In study 2, the biofilm pH decreased immediately after the sucrose bath but increased again after 5 min. Dentin lesions were successfully produced, and the sucrose addition showed the highest mineral losses, even though there was no difference between the sucrose regimens. The CFU did not show any difference and the EPS from the treatment groups were higher than for the control. In study 3, root specimens were treated with/without $CO_2$ laser and with/without fluoride prior or after the laser irradiation. The microbial model utilized was effective in developing dentin lesions and the combined therapies showed the shallowest dentin lesions. In study 4, specimens of enamel and root dentin were treated with fluoride, laser and fluoride/laser and

submitted to an erosive challenge. The wear results indicated that the combined treatment interfered with the enamel or dentin surface losses, although no synergistic effect was observed. There was a trend for the combined treatment to retain more fluoride in enamel and release lower amounts of calcium and phosphorus into the demineralizing solutions. In conclusion, the $CO_2$ mechanism action on the enamel demineralization reduction is still not elucidated and its effects can be increased when associated with fluoride. The microbial model was effective in producing dentin caries lesions. However, it did not reproduce the remineralizing phase of the caries process. Irradiation of the root dentin with laser inhibited the root surface demineralization only when associated with fluoride; however, no synergic effect was observed. The laser treatment alone was not able to prevent enamel or dentin surface losses due to erosion. Its combination with fluoride showed some protection, but mostly due to the fluoride effect. No significant synergistic interaction or lasting protection could be observed for the laser therapy.

Key-words: Dental biofilm, Demineralization, Fluoride, Microbiology, Microradiography, Biochemical analysis, Artificial saliva, *Streptococcus mutans*

# SUMÁRIO

## I- INTRODUÇÃO GERAL

A cárie dentária é uma doença crônica caracterizada pela destruição localizada e progressiva dos tecidos duros da estrutura dentária. No entanto, devido ao amplo uso de compostos fluoretados pela população mundial, um acentuado declínio dessa doença vem ocorrendo (Clarkson *et al.*, 2000). O principal efeito do fluoreto consiste em interferir na dinâmica da cárie dentária, reduzindo a desmineralização e aumentando a remineralização do esmalte e dentina (Tatevossian, 1990). No entanto, o fluoreto não tem sido capaz de controlar completamente o desenvolvimento das lesões de cárie, o que facilitou o aparecimento de um grupo polarizado com essa doença, ou seja, grupos de indivíduos que continuam apresentando alta atividade de cárie (Seppä, 2001).

Contrariamente à incidência da cárie dentária, tem sido observado um aumento da prevalência de outras lesões como o desgaste dentário (Imfeld, 1996). O desgaste dentário possui uma etiologia multifatorial e seu nível resulta da interação entre agentes físicos e químicos, apesar do efeito da erosão ser frequentemente dominante na ocorrência do desgaste (Hooper *et al.*, 2003). A erosão é caracterizada pela perda de tecido dentário devido à dissolução química dos dentes por ácidos de origem não bacteriana produzidos por ácidos endógenos ou fatores extrínsecos frequentemente associados ao consumo de alimentos e bebidas ácidas e pela exposição a ácidos do ambiente (Shaw & Smith, 1994; Lussi *et al.*, 2004; Putnam, 2000). Geralmente, esses grupos de indivíduos que apresentam essa condição são bem instruídos com relação à higiene bucal e, consequentemente, o risco da perda de estrutura dentária devido à limpeza é grande, já que a abrasão ou a atrição, imediatamente após o ataque ácido, é maior do que na ausência de exposição prévia dos dentes aos agentes erosivos (Hooper *et al.*, 2003; Wiegand *et al.*, 2007).

Dessa forma, enfatiza-se o aperfeiçoamento dos métodos preventivos já existentes, bem como a introdução de técnicas inovadoras que possam agir como coadjuvantes na prevenção e controle da cárie e da erosão dentárias. Neste contexto, diversos autores já relataram os efeitos do uso do laser de $CO_2$ na prevenção da cárie dentária em esmalte e dentina (Stern *et al.*, 1966; Kantola *et al.*, 1972; Nelson *et al.*, 1986; Nammour *et al.*, 1992; Westerman *et al.*, 1994; Featherstone *et al.*, 1998; Klein *et al.*, 2005; Rodrigues *et al.*, 2006; Steiner-Oliveira *et al.*, 2006; Gao *et al.*, 2006, Tagliaferro *et al.*, 2007). Outras

pesquisas também demonstraram um efeito benéfico deste laser na inibição da dissolução do esmalte dentário (Gerard *et al.*, 2005) o que poderia constituir uma ferramenta útil contra lesões de erosão. Os comprimentos de onda obtidos com os lasers de $CO_2$ ($\lambda$ = 9,3 9,6, 10,3 e 10,6 μm) são mais apropriados para a utilização na estrutura dentária, pois a radiação produzida coincide com bandas de absorção da hidroxiapatita, principalmente dos grupamentos fosfato e carbonato, presentes tanto no esmalte como na dentina. Desta forma, a efetividade na prevenção de cárie ou erosão pode ser obtida com menos efeitos deletérios aos tecidos dentários.

Acredita-se que os mecanismos de ação do laser de $CO_2$ na prevenção da cárie estejam relacionados com modificações químicas, morfológicas e estruturais, que fazem com que os substratos dentários se tornem menos susceptíveis à desmineralização. Isso pode ocorrer devido a uma redução da solubilidade do esmalte e/ou dentina ocasionada pelo derretimento e recristalização dos cristais de hidroxiapatita (Kantola *et al.*, 1972; Nelson *et al.*, 1986; Nelson *et al.*, 1987; Nammour *et al.*, 1992; Klein *et al.*, 2005; Rodrigues *et al.*, 2006; Steiner-Oliveira *et al.*, 2006). Outros autores sugerem que ocorre uma redução do conteúdo de água, proteína, carbonato e fosfato nos espécimes irradiados, diminuindo a susceptibilidade à desmineralização (Kantola, 1972; Kantola *et al.*, 1973; Steiner-Oliveira *et al.*, 2006; Tagliaferro *et al.*, 2007). Ainda, Hsu *et al.* (2000) encontraram que a decomposição parcial da matriz orgânica pode bloquear os espaços intra e interprismáticos com o comprometimento da difusão de íons e redução da desmineralização.

Diversos estudos também confirmaram que, quando associado ao fluoreto, o potencial inibitório do laser de $CO_2$ pode ser aumentado no esmalte (Featherstone *et al.*, 1991; Nobre dos Santos *et al.*, 2001; Rodrigues *et al.*, 2006; Tagliaferro *et al.*, 2007; Steiner-Oliveira *et al.*, 2008). Para a dentina, os resultados da efetividade da combinação dos tratamentos de laser com fluoreto ainda são escassos e controversos (Gao *et al.*, 2006).

A maior parte dos trabalhos foi realizada em esmalte dentário e os poucos estudos conduzidos para verificar os efeitos do laser de $CO_2$ na dentina utilizaram densidades de energia muito altas, o que poderia comprometer a segurança pulpar (Zach & Cohen, 1965; Kantola, 1972; Nelson *et al.*, 1986; Nammour *et al.*, 1992). Dessa forma, torna-se relevante

conhecer melhor os mecanismos de prevenção da cárie dentária em dentina, pois a experiência de cárie na superfície radicular tenderá a aumentar nos próximos anos. O aumento da expectativa de vida da população, associada aos conceitos da Odontologia Preventiva difundidos tanto para os cirurgiões-dentistas como para os paciente, têm contribuído para uma maior preservação dos dentes na cavidade bucal de adultos e idosos (Keltjens *et al.*, 1993). Um incremento anual de 0,47 de lesões radiculares tem sido observado nesse segmento da população (Locker *et al.*, 1989; Griffin *et al.*, 2004).

Os poucos recentes trabalhos que utilizaram dentina como substrato para irradiação com laser de $CO_2$ para prevenir cárie dentária, utilizaram modelos químicos para mimetizar o desafio cariogênico, o que não envolve microrganismos ou componentes da dieta presentes no ambiente da cavidade bucal. O desenvolvimento da cárie dentária está intimamente associado com a microbiota residente do biofilme dentário (Marsh, 1992) e a maioria dos modelos existentes utiliza imersão em caldo de cultura inoculados com microrganismos, o que apenas possibilita a obtenção da fase de desmineralização do processo de cárie. Assim, estudos *in vitro* mais relevantes sobre cárie podem ser melhor conduzidos com o emprego de modelos microbiológicos (Zanin *et al*., 2006) associados à saliva artificial (Pratten *et al*., 1998) na presença de sacarose.

## II – PROPOSIÇÃO

Os objetivos desse estudo foram:

1. Descrever as características do laser de $CO_2$, esclarecer os mecanismos de ação do laser na inibição da desmineralização do esmalte e ainda revisar a literatura a respeito de seus efeitos quando combinado ao íon fluoreto;

2. Desenvolver um modelo microbiológico, *in vitro,* de produção de lesão de cárie em dentina de forma semelhante à que ocorre na cavidade bucal e testar duas hipóteses: (a) de que não há diferença na produção de cárie artificial em dentina utilizando um modelo microbiológico com dois regimes de imersão em sacarose (3 e 6 vezes ao dia); (b) de que não há diferença no pH do biofilme antes e após sua imersão em sacarose;

3. Avaliar *in vitro,* por meio de produção de cárie em modelo microbiológico, o efeito do laser de $CO_2$ ($\lambda$ = 10,6 μm) pulsado associado ao fluoreto na redução da desmineralização da dentina radicular humana hígida;

4. Avaliar, *in vitro*, o efeito do laser de $CO_2$ ($\lambda$ = 10,6 μm) pulsado associado ao fluoreto na redução da desmineralização do esmalte e dentina submetidos a um desafio erosivo. As hipóteses testadas foram: (a) de que as modificações causadas pelo laser de $CO_2$ no esmalte e na dentina pudessem aumentar a resistência desses substratos à erosão; (b) de que essa proteção pudesse ser aumentada por um efeito sinérgico entre as terapias de laser e fluoreto; (c) de que as mudanças físicas promovidas pela irradiação com o laser de $CO_2$, associadas ou não ao fluoreto, pudessem fornecer uma proteção a longo-prazo, mesmo observada após diversos desafios erosivos.

## III – CAPÍTULOS

Esta tese está baseada na Resolução CCPG/002/06/UNICAMP que regulamenta o formato alternativo para teses de Mestrado e Doutorado e permite a inserção de artigos científicos de autoria ou co-autoria do candidato (Anexo 1). Por se tratarem de pesquisas envolvendo seres humanos, ou partes deles, os projetos de pesquisas destes trabalhos foram submetidos à apreciação do Comitê de Ética em Pesquisa da Faculdade de Odontologia de Piracicaba, tendo sido aprovados (Anexos 2,3 e 4). Assim sendo, esta tese é composta por quatro capítulos contendo artigos científicos completos e/ou em redação, conforme descrito abaixo:

✓ Capítulo 1

"O emprego do laser de $CO_2$ no controle da cárie dentária". Rodrigues LKA, **Steiner-Oliveira C**, Nobre dos Santos M. Rev Odonto Cienc 2007; 22(58):346-351.

✓ Capítulo 2

"An *in vitro* microbial-caries model associated with sucrose to produce dentin caries lesions". **Steiner-Oliveira C**, Rodrigues LKA, Carvalho CL, Kamiya RU, Hara AT, Nobre dos Santos M.

✓ Capítulo 3

"$CO_2$ laser and fluoride on the inhibition of root caries - an *in vitro* microbial model". **Steiner-Oliveira C**, Rodrigues LKA, Parisotto TM, Sousa e Silva CM, Hara AT, Nobre dos Santos M.

✓ Capítulo 4

"Effect of a pulsed $CO_2$ laser and fluoride on the prevention of enamel and dentin erosion". **Steiner-Oliveira C**, Nobre dos Santos M, Zero D, Hara AT.

CAPÍTULO 1

ARTIGO

# O EMPREGO DO LASER DE $CO_2$ NO CONTROLE DA CÁRIE DENTÁRIA

*THE USE OF $CO_2$ LASER ON DENTAL ON DENTAL CARIES CONTROL*

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**RESUMO**

O laser de dióxido de carbono atua na inibição da desmineralização do esmalte, reduzindo sua solubilidade aos ácidos e, este efeito pode ser potencializado quando associado a agentes fluoretados. Assim, o objetivo deste trabalho foi descrever as características do laser de $CO_2$, discutir os mecanismos de ação do laser na inibição da desmineralização do esmalte e ainda, revisar a literatura a respeito de seus efeitos quando combinado ao íon flúor.

**UNITERMOS:** laser de $CO_2$; flúor; desmineralização; esmalte dentário.

***SUMMARY***

*Carbon dioxide laser acts on the enamel demineralization inhibition by reducing its acid solubility and this effect can be enhanced when associated with fluoridated agents. Therefore, the aim of this paper was to describe $CO_2$ laser characteristics, discuss its action mechanisms on the inhibition of enamel demineralization and also to review the literature concerning the laser effects when combined with fluoride ion.*

***UNITERMS:*** *$CO_2$ laser; fluoride; demineralization; dental enamel.*

## INTRODUÇÃO

Em conseqüência do seu declínio, a doença cárie tornou-se fortemente polarizada (Seppä,[24] 2001). Portanto, grupos de crianças continuam apresentando alta atividade da doença. Isto enfatiza a necessidade do aperfeiçoamento dos métodos preventivos já existentes, bem como a introdução de técnicas inovadoras que possam agir como coadjuvantes na prevenção e controle da cárie dentária nestes segmentos da população.

O declínio mundial da doença cárie relatado inicialmente é atribuído ao amplo uso de compostos fluoretados (Clarkson et al.,[2] 2000). Há um consenso que o principal efeito do flúor é interferir na dinâmica da cárie dentária, reduzindo a desmineralização e aumentando a remineralização dos tecidos duros dentários (Tatevossian,[29] 1990). No entanto, o efeito do flúor é parcial já que o mesmo não consegue impedir completamente o desenvolvimento de lesões de cárie. Por conseguinte, os efeitos da associação dos lasers a compostos fluoretados poderia propiciar a obtenção de procedimentos mais efetivos na prevenção e controle da cárie (Meurman et al.,[18] 1997; Nobre dos Santos et al.,[21] 2001; Hossain et al.,[9] 2002; Rodrigues et al.,[23] 2006; Steiner-Oliveira et al.,[26] 2006b; Tagliaferro et al.,[28] 2007).

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Neste contexto, desde o desenvolvimento do laser de rubi por Maiman[17] (1960), diferentes tipos de lasers, tais como Nd:YAG, Argônio, Er:YAG e $CO_2$ têm sido estudados para uso em Odontologia com o objetivo de prevenir a cárie dentária. No entanto, o comprimento de onda ($\lambda$) dos lasers de argônio ($\lambda$ = 488-514 nm) e Nd:YAG ($\lambda$ = 1.064 nm) não são absorvidos de forma efetiva pelo esmalte dentário.

Os comprimentos de onda obtidos com os lasers de $CO_2$ ($\lambda$ = 9,3, 9,6, 10,3 e 10,6 $\mu$m) são mais apropriados para a utilização em esmalte dentário, pois produzem radiação na região do infravermelho que coincide com algumas bandas de absorção da hidroxiapatita, principalmente os grupamentos fosfato e carbonato. Desta forma, maior efetividade na prevenção de cárie pode ser obtida com menor ocorrência de efeitos deletérios aos tecidos dentários, proporcionando uma menor dissipação de raios incidentes e maior rapidez, bem como eficácia do laser. Com este laser, a maior parte da luz é absorvida nos poucos micrometros externos da superfície do esmalte e convertida em calor, causando perda de carbonato do mineral e fusão dos cristais de hidroxiapatita, tendo como conseqüência uma diminuição na reatividade ácida desta estrutura (Fried et al.,[7] 1997).

Durante os últimos 20 anos, vários estudos avaliaram os efeitos do laser de dióxido de carbono ($CO_2$) na inibição da desmineralização do esmalte e demonstraram que a irradiação do esmalte dentário com este laser promove uma redução significativa da solubilidade deste substrato aos ácidos (Featherstone et al.,[4] 1998; Kantorowitz et al.,[15] 1998; Hsu et al.,[11] 2000; Featherstone et al.,[5] 2001; Klein et al.,[16] 2005; Steiner-Oliveira et al.,[25] 2006a) e, quando associada a agentes fluoretados, o efeito de inibição de desmineralização pode ser potencializado (Featherstone et al.,[3] 1991; Hsu et al.,[10] 1998; Hsu et al.,[12] 2001; Nobre dos Santos et al.,[21] 2001; Rodrigues et al.,[23] 2006; Steiner-Oliveira et al.,[25] 2006b; Tagliaferro et al.,[28] 2007).

## REVISÃO DA LITERATURA E DISCUSSÃO

### Características do laser de $CO_2$

O laser de $CO_2$, desenvolvido por Patel et al.[22] (1964), é uma das mais conhecidas fontes de radiação eletromagnética na região infravermelha do espectro de luz. Este laser usa uma mistura de $CO_2$, $N_2$ e He, com o $CO_2$ como o meio ativo do laser. A linha laser 10,6 $\mu$m é a mais forte e a maioria dos aparelhos médicos e odontológicos disponíveis no comércio operam neste comprimento onda.

Para aplicações em Odontologia, os aparelhos de laser de $CO_2$ operam em um modo de não contato, contínuo ou pulsado (Gonzalez et al.,[8] 1996). Para tecidos duros, o laser de $CO_2$ mais eficiente é o TEA laser. O nome TEA é um acrômio para "*transversely excited atmospheric pressure*" e este laser usa um fluxo transverso de gás que opera em pressões mais altas que outros lasers de gases, geralmente em pressões próximas da pressão atmosférica. Este diferencial permite que o laser opere em um regime pulsado de poucos Hz de taxa de repetição e pulsos da ordem de 0,1 a 0,2 $\mu$s (Widgor et al.,[30] 1995).

### Mecanismo de ação do laser na inibição da desmineralização do esmalte

O emprego dos lasers de alta potência conciste no tratamento do esmalte para a obtenção de superfícies mais resistentes aos ácidos produzidos pelas bactérias cariogênicas (Stern et al.,[27] 1972; Kantola,[13] 1972; Kantola et al.,[14] 1973; Nelson et al.,[19,20] 1986, 1987; Featherstone et al.,[3,4] 1991, 1998; Kantorowitz et al.,[15] 1998; Hsu et al.,[11] 2000). A ação do laser de $CO_2$ na prevenção de lesões de cárie tem sido investigada desde a década de 60 e diversos estudos utilizaram diferentes tipos de lasers de $CO_2$, associados ou não ao uso de fluoretos. Uma compilação dos principais estudos está apresentada na Tabela 1. Esta tabela mostra o comprimento de onda e a densidade de energia empregados, bem como a maior porcentagem de inibição de desmineralização encontrada em cada estudo.

Várias são as hipóteses que tentam explicar os mecanismos pelos quais o laser de $CO_2$ inibe a desmineralização do esmalte dentário. Dentre elas podemos citar a redução da permeabilidade do esmalte a agentes químicos causada pelo derretimento da superfície do esmalte (Stern et al.,[27] 1972). No entanto, esta hipótese parece pouco provável, uma vez que o único estudo que testou a permeabilidade do esmalte irradiado demonstrou que a mesma é aumentada pela irradiação (Borggreven et al.,[1] 1980).

Técnicas como a espectroscopia infravermelha e/ou difração por raios-X evidenciaram uma redução do conteúdo de água, proteína e carbonato nos espécimes irradiados, bem como formação de compostos fosfatados no esmalte dentário, como ortofosfato $\alpha$-cálcico, fosfato $\alpha$-tricálcico e fosfato tetracálcico (Stern et al.,[27] 1972; Kantola,[13] 1972;

TABELA 1 – Comprimento de onda, densidade de energia e a maior porcentagem de inibição de desmineralização do esmalte pelo laser de $CO_2$ associado ou não a fluoretos.

| Autor | Ano | Comprimento de onda (μm) | Modo | Densidade de Energia ($J/cm^2$) | Com flúor | Porcentagem de Inibição |
|---|---|---|---|---|---|---|
| Nelson et al. | 1986 | 9,32 | Pulsado | 50 | Não | 50% |
| Nelson et al. | 1987 | 9,32 | Pulsado | 50 | Não | 50% |
| Hsu et al. | 1998 | 10,6 | Contínuo | 170 | Sim | ≈ 100% |
| Kantorowitz et al. | 1998 | 10,6 | Pulsado | 12 por pulso | Não | 87% |
| Featherstone et al. | 1998 | 9,6 | Pulsado | 2,5 por pulso | Não | 70% |
| Hsu et al. | 2000 | 10,6 | Pulsado | 0,3 por pulso | Não | 98% |
| Hsu et al. | 2001 | 10,6 | Pulsado | 0,3 por pulso | Sim | 98% |
| Nobre dos Santos et al. | 2001 | 9,6 | Pulsado | 1,5 por pulso | Sim | 76% |
| Featherstone et al. | 2001 | 9,6 | Pulsado | 1,5 por pulso | Não | 84% |
| Klein et al. | 2005 | 10,6 | Pulsado | 16 | Não | 64% |
| Rodrigues et al. | 2006 | 9,6 | Pulsado | 1,5 | Sim | 76% |
| Steiner-Oliveira et al. | 2006a | 10,6 | Pulsado | 1,5-10,0 | Não | 68% |
| Steiner-Oliveira et al. | 2006b | 10,6 | Pulsado | 10,0 | Sim | 60% |
| Tagliaferro et al. | 2007 | 10,6 | Pulsado | 5,0 | Sim | 97% |

Kantola et al.,[14] 1973). A redução do conteúdo de carbonato decorrente da irradiação com laser é um fator positivo visto que, o carbonato torna a apatita mais instável e solúvel em ácido. Por outro lado, Fowler et al.[6] (1986), salientaram que fosfato tetracálcico e fosfato α-tricálcico são substancialmente mais solúveis em ácido que o esmalte dentário e a hidroxiapatita. Deste modo, a ação do laser na superfície irradiada pode ser benéfica ou prejudicial dependendo dos parâmetros de irradiação empregados. Assim, a quantidade de energia e a forma como esta energia é depositada no substrato são particularmente importantes.

Outra explicação se baseia na redução da solubilidade do esmalte ocasionada pelo derretimento e recristalização dos cristais de hidroxiapatita ou pela mudança de solubilidade da apatita aquecida pela formação de compostos fosfatados menos solúveis (Nelson et al.[19,20] (1986, 1987). Por outro lado, análises por microscopia eletrônica de varredura demonstraram que quando presentes, as zonas de derretimento do esmalte não são homogêneas e ocorrem em áreas limitadas (Kantorowitz et al.,[15] 1998; Rodrigues et al.,[23] 2006). Por conseguinte, parece que este derretimento não é necessariamente a única causa para a diminuição da reatividade ácida do esmalte.

Finalmente, a mais recente teoria preconiza que a irradiação com laser altera a matriz orgânica presente no esmalte que normalmente já desempenha um papel protetor em situações de desafio ácido. O uso de uma baixa densidade de energia (0,3 $J/cm^2$) pode levar ao aquecimento do esmalte a temperaturas inferiores a 400ºC e isso pode causar uma decomposição parcial da matriz orgânica. Esta decomposição poderia levar a um bloqueio dos espaços intra e interprismáticos com o comprometimento da difusão de íons e redução da desmineralização do esmalte (Hsu et al.,[11] 2000).

Estudos realizados por nosso grupo de trabalho demonstraram que provavelmente o mecanismo de ação do laser de $CO_2$ na inibição da desmineralização pode ser uma combinação de alterações na estrutura do esmalte. Os primeiros estudos, realizados por Nobre dos Santos et al.[21] (2001), obtiveram resultados promissores de inibição de cárie tanto em superfície lisa quanto em superfície oclusal, quando o esmalte íntegro e desmineralizado foram submetidos a desafios cariogênicos *in vitro*. Nestes estudos, foi utilizado um TEA laser de $CO_2$ (Argus Photonics Group, Jupiter, FL) de comprimento de onda 9,6 μm com densidades de energia que variaram de 1 a 3 $J/cm^2$. Embora não tenha sido realizada nenhuma análise morfológica da superfície irradiada, a microscopia de luz polarizada mostrou indícios da ocorrência de derretimento em algumas áreas. Tais resultados foram ratificados recentemente, com a utilização de um modelo de produção de cárie *in situ* onde o efeito do mesmo laser (1,5 $J/cm^2$) foi testado. Neste estudo, zonas de derretimento e recristalização dos cristais de hidroxiapatita foram evidenciadas pela microscopia eletrônica de varredura.

Com a utilização de um laser de $CO_2$ clínico (Opus 20), com comprimento de onda 10,6 $\mu$m e operando em modo pulsado, foram realizados outros 3 estudos *in vitro*. No primeiro, Klein et al.[16] (2005) mostraram inibição da desmineralização do esmalte ao redor de restaurações de resina composta através da irradiação com 8 e 16 J/cm$^2$. Zonas de derretimento e recristalização também foram evidenciadas por imagens de microscopia eletrônica de varredura (MEV). No entanto, Tagliaferro et al.[28] (2007), também mostraram inibição de progressão da desmineralização em esmalte decíduo irradiado com 5 J/cm$^2$, porém, sem a ocorrência de alterações morfológicas da superfície do esmalte. Neste estudo, a análise da composição química do esmalte feita através de espectroscopia Raman transformada de Fourrier (FT-Raman) evidenciou diminuição do conteúdo de carbonato e alteração na matriz orgânica do esmalte (dados não publicados). Já Steiner-Oliveira et al.[25] (2006a) encontraram redução da desmineralização do esmalte dentário humano irradiado com 10 J/cm$^2$ evidenciando presença de zonas de derretimento e recristalização observados em MEV. Neste estudo também foram detectadas diminuições das bandas de carbonato e fosfato através da análise da composição química por espectroscopia FT-Raman.

Por conseguinte, o mecanismo de ação do laser de $CO_2$ na inibição da desmineralização do esmalte está intimamente relacionado com os parâmetros de irradiação usados e tipo de substrato irradiado. Mesmo em condições de irradiação similares, diferentes mecanismos podem atuar para diminuir a reatividade do esmalte aos ácidos.

## Associação do laser de $CO_2$ a compostos fluoretados

Existem dois possíveis mecanismos envolvidos no sinergismo existente quando os dois tratamentos são associados, ambos decorrentes do aumento da reatividade do esmalte aos fluoretos. Um deles sugere que o tratamento combinado flúor-laser produz, sobre o esmalte dentário, numerosos precipitados esféricos que morfologicamente lembram fluoreto de cálcio, que funcionariam como um reservatório de flúor (Hossain et al.,[9] 2002). O outro mecanismo enfatiza o papel dos lasers no aumento da incorporação de flúor dentro da estrutura cristalizada na forma de flúor fortemente ligado (fluorapatita) (Meurman et al.,[18] 1997). O primeiro mecanismo está relacionado à formação de pequenas fendas ou crateras na superfície do substrato irradiado que permitiriam maior difusão dos íons flúor no interior do esmalte. Nesta hipótese, o flúor pode ou não estar presente na ocasião da irradiação. Por outro lado, a formação de fluorapatita é dependente da presença de flúor na ocasião da irradiação para que este íon seja aprisionado dentro do cristal de hidroxiapatita.

Nossos estudos encontraram efeito sinérgico *in vitro* entre os tratamentos, tanto quando o flúor (gel de flúor fosfato acidulado – FFA) foi aplicado antes da aplicação do laser (Nobre dos Santos et al.,[21] 2001) quanto depois (Rodrigues et al.,[23] 2006). No primeiro estudo, este sinergismo foi demonstrado através de análise microrradiográfica dos espécimes de esmalte dentário humano submetidos ao tratamento de aplicação de FFA e posteriormente a irradiação laser com um TEA laser de $CO_2$ ($\lambda$ = 9,6 $\mu$m) operando com 1,5 J/cm$^2$ por pulso e submetido à ciclagem de pH e o grupo controle (apenas submetido à ciclagem). No entanto, em estudo realizado por Tagliaferro et al.[28] (2007), embora sem diferença estatística entre os grupos experimentais, a maior porcentagem de inibição de desmineralização foi encontrada quando a aplicação tópica de flúor foi realizada após a irradiação com um laser de $CO_2$ ($\lambda$ = 10,6 $\mu$m) operando com 5 J/cm$^2$. Ainda, outro trabalho realizado por Steiner-Oliveira et al.[26] (2006b) avaliou a combinação de terapias com dentifrício e enxaguatório bucal fluoretados com uso de laser de $CO_2$ ($\lambda$ = 10,6 $\mu$m) operando com 10 J/cm$^2$. Após a ciclagem de pH, os espécimes foram analisados, qualitativamente, por microscopia de luz polarizada e teste de microdureza em corte longitudinal para quantificar mudanças no conteúdo mineral. Os tratamentos foram capazes de reduzir a perda mineral do esmalte, e alguns grupos causaram remineralização do esmalte dentário. É válido ressaltar que, nesses últimos dois estudos, o efeito sinérgico entre os tratamentos não foi evidenciado, provavelmente pelas fontes de variação dos estudos tais como variabilidade do substrato dentário usado (esmalte desmineralizado), processo de irradiação e técnica de microdureza para determinação da perda mineral dos espécimes.

Por outro lado, nosso mais recente trabalho, realizado por Rodrigues et al.[23] (2006), demonstrou pela primeira vez *in situ* a existência de sinergismo entre a irradiação com laser de $CO_2$ e a utilização de dentifrício fluoretado. Neste estudo, 17 voluntários utilizaram um dispositivo intraoral, semelhante a um aparelho ortodôntico móvel,

contendo blocos de dente humano irradiados com o mesmo laser e mesmos parâmetros do estudo de Nobre dos Santos et al.[21] (2001). Os voluntários gotejavam sobre os blocos uma solução de sacarose a 20% oito vezes ao dia para proporcionar a formação de biofilme cariogênico sobre os blocos. Os autores acreditam que a potencialização dos efeitos do flúor deu-se devido à maior formação de fluoreto de cálcio decorrente da alteração tecidual ocorrida na superfície do esmalte. Os resultados deste estudo foram concordantes com aqueles encontrados por Featherstone et al.[5] (2001), que também encontraram inibição de desmineralização do esmalte, pelo uso do laser de $CO_2$, em condições intrabucais. No entanto, os efeitos da alteração da superfície sobre a adesão bacteriana ainda não foram testados, sendo objeto de estudo de pesquisas futuras.

## CONCLUSÃO

O mecanismo de ação do laser de $CO_2$ na inibição da desmineralização do esmalte ainda não está completamente esclarecido, no entanto parece estar intimamente relacionado com os parâmetros de irradiação utilizados assim como com o tipo de substrato irradiado. Também é possível concluir que a utilização do laser de $CO_2$ associado ao flúor pode permitir a redução tanto das densidades de energia quanto das concentrações de flúor utilizadas no tratamento. Além disso, melhora o desempenho dos métodos de prevenção aumentando o efeito inibidor da desmineralização.

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Recebido para publicação em: 02/05/2007; aceito em: 18/09/2007.

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CAPÍTULO 2

**An *in vitro* microbial-caries model associated with sucrose to produce dentin caries lesions**

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**Key words:** Artificial saliva, *Streptococcus mutans*, biofilm pH, microradiography

ABSTRACT

The complexity of the oral environment and the ethical issues associated with *in vivo* studies of the dental diseases in humans have prompted the development of laboratory models which simulate the oral environment. Thus, this study aimed at developing a microbial model to produce dentin caries, mimicking the conditions that closer resemble the ones that occur in the mouth. Fifty dentin specimens were randomly divided into four groups in triplicate: negative control (C), positive control (SM) (*Streptococcus mutans*), 3S (3 sucrose baths) and 6S (6 sucrose baths); an extra group was used for pH measurements of the biofilm. The experimental groups were inoculated with SM and after the microbial cariogenic challenge, dentin demineralization and lesion depth were assessed by transverse microradiography. The pH and extracellular polysaccharides in the biofilm formed over the dentin specimens were analyzed, as well as the count of microorganisms in the dentin. The data were statistically analyzed with 5% of significance level. The pH measurements decreased immediately after the sucrose bath but increased again after 5 min. Dentin lesions were successfully produced, and the sucrose addition showed higher mineral loss. The number of colony forming unities from the carious dentin have not differed amongst the groups and the extracellular polysaccharide from both 3S and 6S groups differed from the SM group. In conclusion, this *in vitro* microbial model using *Streptococcus mutans*, artificial saliva as medium and as much as 3 sucrose baths was effective in producing dentin caries lesions. However, it was not able to reproduce the remineralizing phase of the dental caries process.

INTRODUCTION

Dental caries is a chronic disease characterized by local and progressive destruction of the dental hard tissues and it is closed associated with the resident microbiota of the dental biofilm (Marsh, 1992).

After the enamel demineralization, the caries lesion can slowly progress into the dentin, initially as a wide demineralized area located under a partially and demineralized zone infected with bacteria (Kidd *et al.*, 1995). However, for esthetic or other reasons, the operative intervention should be carried out with a minimally invasive approach, even though the actual lesion is dark, discolored, arrested caries (Bjørndal, 2008). It is very critical to distinguish between the carious zones, and a major quantity of viable demineralized tissue can be removed during the cavity preparation, without need. The appropriate removal of contaminated dentin depends on the dynamics of the caries process which should consider the higher susceptibility of this substrate when compared with the dental enamel (Featherstone, 1994). This way, the use of *in vitro* models to produce caries represents a good alternative to better understand the dynamics of dentin caries.

There are currently a variety of model systems available which could be applied to the study of the process of human dentin caries, each with advantages and disadvantages. Experimental chemical models such as pH cycling and immersion in acid medium are widely used to simulate cariogenic challenges (Featherstone et al., 1986). The disadvantage of these models is that they do not simulate the real demineralization process of the oral environment due to the absence of microorganisms, consequently, concentrating on the physical-chemical aspects of enamel dissolution (Holly & Gray, 1968). Even systems such as chemostats, flowcells and constant depth film fermentors have been developed and there

have also been elaborated attempts to mimic the oral cavity (Donoghue & Perrons 1991; Zampatti *et al.*, 1994; Bradshaw *et al.*, 1996a; Bradshaw *et al.*, 1996b; Kinniment *et al.*, 1996; Bradshaw & Marsh, 1998). However, their high cost and complex apparatus may limit the use of this technology.

More relevant studies about initial and secondary caries resulted from failure in restorative marginal adaptation can be conducted using a microbial model proposed by Zanin *et al.*, 2006, associated with artificial saliva (Pratten *et al.*, 1998) and presence of sucrose. This carbohydrate can turn the biofilm microflora into a more cariogenic one (Marsh, 2003) by producing higher levels of extracellular polysaccharides (EPS). EPS may enhance bacterial adherence to the tooth surface (Schilling & Bowen, 1992) and also increase the biofilm porosity (Dibdin & Shellis, 1988) where low biofilm pH can be reached (Zero *et al.*, 1986). However, the majority of the microbial existing models use immersion in batch inoculated culture with microorganisms, which only enables the demineralizing phase of the caries process. Models that also simulate the remineralizing phase are called "artificial mouths" and along with the bacterial films fermentors, tend to require sophisticated laboratory equipment, as previously cited. Since the dynamics of the caries process consist of both phenomena (re and demineralization), an ideal situation would be the development of simpler microbial models, using immersion in microorganisms cultured batchs, in which remineralization phase was reproduced. This way, it is very important that the pH is verified in the biofilm in order to determine the Stephan's curve occurrence.

The complexity of the oral environment and the ethical issues associated with *in vivo* studies of the dental diseases such as dental caries and periodontitis in humans have

inevitably prompted the development of laboratory models which can simulate, *in vitro*, the oral environment. Thus, the purposes of this article were two-fold: the first hypothesis was that there is no difference in the artificial caries production in dentin using a microbial model with two distinct sucrose bath regimens (3 and 6 times a day) as assessed by bacterial counts on the dentin, microradiographic analysis and extracellular polysaccharide analysis; the second was that there is no difference in the biofilm pH before and after each sucrose bath, developing a microbial model to produce dentin caries, mimicking the conditions that closest resemble the ones that occur in the mouth.

## MATERIAL AND METHODS

### Experimental design

This *in vitro* study used a randomized design in triplicate. Forty-five dentin specimens were randomly allocated in four groups (n = 15): negative control (C) with no treatment, positive control (SM - *Streptococcus mutans*), 3S (3 sucrose baths) and 6S (6 sucrose baths). An extra group (n = 5) inoculated with *Streptococcus mutans* and immersed in sucrose 3 times a day was added to perform the pH measurements in the biofilm. Except for the control group, all experimental groups were inoculated with a cell suspension of SM prepared from overnight growth of a pure culture. After microbial cariogenic challenge of 7 days, dentin demineralization was assessed by transverse microradiography analysis. The concentration of water-insoluble polysaccharides in the biofilm formed over the dentin specimens was analyzed, as well as the count of viable microorganisms in the dentin.

### Specimens preparation

This study was approved by the Research and Ethics Committee of the Piracicaba Dental School at the State University of Campinas in Piracicaba, SP, Brazil (Protocol No. 79/2008). Sixty dentin specimens were prepared from 25 human unerupted third molars that had been stored in 0.1% (v/v) thymol solution at 4˚C for 30 d (Amaechi *et al.*, 1998). Fifty dentin specimens (4 x 4 x 2 mm) were obtained using a water-cooled diamond saw and a cutting machine (Isomet 1000; Buehler, Lake, Bluff, IL, USA). The specimens were coated with an acid-resistant varnish (Colorama, CEIL Coml. Exp. Ind. Ltda., São Paulo, Brazil), leaving a 16.0 $mm^2$ window of exposed occlusal dentin for the microbial cariogenic challenge (ANEXO 4). Afterwards, the surfaces to be treated were polished for 30 seconds with 1 µm alumina paste and water and sonicatted immersed in deionized water for 5 min in order to remove any residual impurities. The dentin specimens were fixed in the lids of glass containers with orthodontic wire, kept immersed in sterile distilled water, and then sterilized in a gamma radiation chamber (Gammacell 220 Excel, GC-220E; MDS Nordion, Ottowa, Canada) (Rodrigues *et al.*, 2004).

**Biofilm growth and sucrose baths**

After sterilization, the dentin specimens were removed from the distilled water and immersed in a sterile artificial saliva medium (Lab-lemco 1g/l (Oxoid), yeast extract 2 g/l, proteose peptone 5 g/l, type III hog gastric mucin (Sigma) 2 g/l, sodium chloride 0.2 g/l, calcium chloride 0.3 g/l, potassium chloride 0.2 g/l and 1.25 mL/l of a 0.2 µm filter-sterilized solution of 40% urea (Sigma), added after autoclaving) (Pratten *et al.*, 1998). Except for those in the control groups, all artificial saliva glass containers (50 mL) were inoculated with 0.2 ml (1– 2 $X10^8$ colony-forming units (CFU/mL) of an overnight culture of *Streptococcus mutans* UA 159. This procedure was performed only once, and the dentin

specimens were transferred into fresh medium every 24 h (Edmunds *et al.*, 1988). All groups were incubated for 7 days at 37°C and a partial 10% pressure $CO_2$.

The sucrose baths were set at a concentration of 40% as determined in a pilot study to allow biofilm formation in 7 days with the artificial saliva medium. The dentin specimens from groups 3S and were bathed 3 or 6 times a day, respectively, for 5 min and after this, were transferred to fresh artificial saliva media (ANEXO 5-A).

Once a day, samples of all group-cultures were streaked onto BHI agar plates and incubated at 37˚C in order to check purity. At the end of each experimental period, the biofilms were collected with sterile plastic curettes (ANEXO 5-B, C). The collected material was placed into preweighed microcentrifuge tubes and the biofilm was analytically weighed (ANEXO 5-D). The carious dentin of all groups was collected (ANEXO 5-E,F) and transferred to preweighed microcentrifuge tubes (ANEXO 5-G) and also analytically weighed.

**Biofilm Analysis**

pH measurement

The pH of the biofilm formed over five separate specimens were determined after 7 days using a pH meter (pH-boy; Camlab, Cambridge, UK) and an iridium oxide touch microelectrode (Beetrode model NMPH1, WPI, USA). The instrument was recalibrated before each sample and the measurements were taken before, immediately after, 5, 10, 15, 30 and 120 min after 40% sucrose baths (Deng & ten Cate, 2004). After the baths, the specimens were transferred to a fresh medium.

Insoluble extracellular polysaccharide (EPS) analysis

The EPS analysis was performed by adding 0.5 M hydrochloric acid solution to the biofilm (0.1 mL/mg). The samples were homogenized and kept under constant agitation for 3 h at room temperature, and then centrifuged for 3 min at 12000 *g*. Afterwards, TISAB II was added at 0.1 mL/mg and the samples were again centrifuged for 3 min at 12000 *g*. Then, 1 N NaOH was added to the first precipitate (0.1 mL/mg), and the samples were homogenized, kept under constant agitation for 3 h at room temperature and centrifuged for 3 min at 12000 *g*. The supernatant was collected, added to ethanol 75% (0.3 mL/mg) and agitated. The collected precipitate, after 12 h in freezing temperature was added at 1 N NaOH (0.1 mL/mg) and the final supernatant from groups SM, 3S and 6S was used to determine the concentration of insoluble extracellular polysaccharide (μg/mg of biofilm) using the phenol-sulfuric method (Dubois *et al*., 1956).

**Carious dentin microbial analysis**

After the biofilm removal, samples of carious dentin were collected with scalpels (0.5-1.5 mg) from the halves of each specimen, added to a 0.9% (w/v) NaCl (0.1 mL $mg^{-1}$) solution and vortexed with glass pearls for 15 s. After that, the suspensions were serially diluted (ANEXO 5-H), plated onto BHI agar, and the plates were incubated at 37˚C, at a partial pressure of 10% $CO_2$, for 48 h (ANEXO 5-I,J). After incubation, the number of *Streptococcus mutans* was determined by colony counting, and the values were expressed as colony forming units (CFU) per milligram of wet biofilm (Zanin *et al.*, 2006) (ANEXO 5-K).

**Mineral content (ΔZ) and Lesion depth (LD) analyses**

Transverse microradiography (TMR) was performed to determine the mineral content and the lesion depth of the dentin. The other half of each specimen was cut with a

Series 1000 Deluxe Silverstone-Taylor hard tissue microtome (Sci Fab, Littleton, CO, USA) to obtain sections of 160 ± 20 μm thickness. All thin sections were mounted on microradiographic X-ray plates (Kodak high-resolution plates) along with an aluminum step wedge. Then they were X-rayed using a nickel-filtered Cu (K) X-ray source (Philips) operated at 20 kV and 30 mA for 65 min. The resultant microradiograms were processed and the radiographic images were taken from the microscope (EOM, Carl Zeiss Inc., Germany) to the computer with a camera (KP-120U, Hitachi Denshi Ltd., Japan). The images were analyzed with specific computer software (TMR, transverse microradiography version 1.26; Inspektor Research Systems BV, The Netherlands). Integrated mineral loss was determined by computing the area obtained by plotting the volume percent mineral profile towards dentin depth in each dentin section, with the sound dentin set as 48 vol % mineral (van der Veen *et al.*, 1996) and this enables the analysis of both mineral loss and lesion depth.

**Statistical analysis**

Statistical analysis was performed using SAS software (SAS Institute Inc., version 8.01, Cary, NC, USA), with the significance level set to $p < 0.05$. The data were evaluated to check the equality of variances and normal distribution of errors. After data transformation (root-squared for ΔZ and LD, log for CFU counts and linear for EPS) analysis of variance (ANOVA) was used to check the significance between the response variables under study (EPS concentrations, viable microorganisms counts and mineral loss) and paired t test for the pH measures and their relations to the sucrose bath variable. The difference between treatments was assessed by the Tukey test.

RESULTS

The pH measurements have decreased immediately after the sucrose baths ($p < 0.05$). After 5 min, the values increased ($p < 0.05$) and kept soaring until it decreased again at 120 min ($p > 0.05$) (Table 1).

The mineral loss values for the negative control (C) and the positive control (SM) have statistically differed ($p < 0.05$). The sucrose addition has shown a statistically higher mineral loss ($p < 0.05$) for lesions in both experimental groups (Table 2). However, the overall quantity of mineral loss was not significantly influenced by the sucrose bath regimen of 3 or 6 times a day (Table 2). Similar results were found for the lesion depths (Table 2; Figure 1).

The colony forming unities from the carious dentin have not significantly differed ($p > 0.05$), even though a higher numerical value for group 6S could be observed (Table 2). For the extracellular polysaccharide analysis, both 3S and 6S groups have statistically differed from the SM group showing significant higher levels ($p < 0.01$), but have not shown differences between themselves (Graph 1).

Table 1. Means and standard-deviations of pH measured before and after the sucrose baths.

| Group | pH | SD | Student test |
|---|---|---|---|
| **Before sucrose** | 4.45 | 0.01 | A |
| **Immediately after sucrose** | 4.30 | 0.08 | B |
| **5 min** | 4.74 | 0.15 | C |
| **10 min** | 4.86 | 0.19 | CD |
| **15 min** | 4.89 | 0.21 | CD |
| **30 min** | 4.91 | 0.10 | D |
| **120 min** | 4.76 | 0.10 | CD |

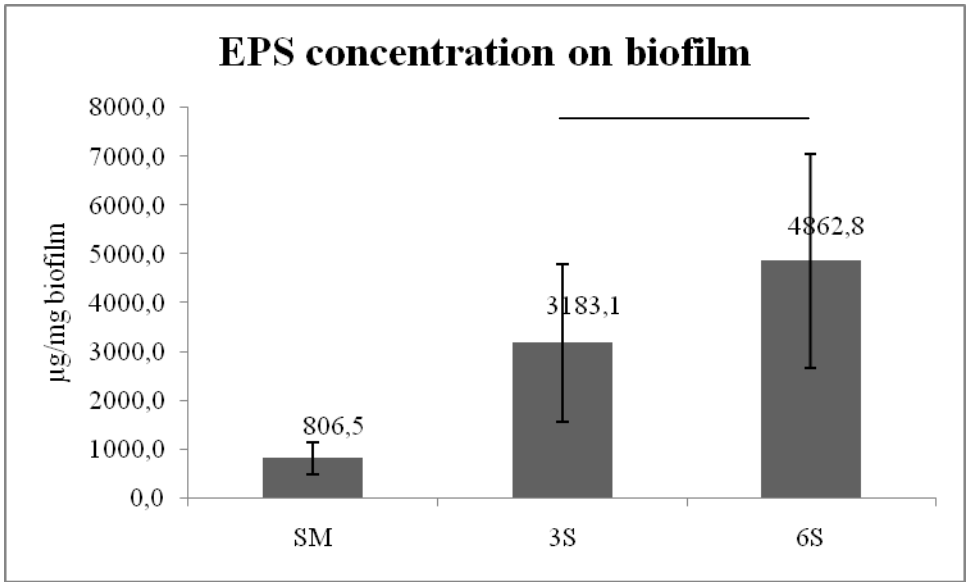
Different letters indicate significant differences among the groups

Table 2. Original means and standard-deviations of dentin mineral loss (ΔZ), lesion depths (LD) and dentin microbial counts (CFU).

| Group | ΔZ (% mineral vol/µm) | LD (µm) | CFU |
|---|---|---|---|
| **Negative Control (C)** | 185.6±139.6[a] | 12.5±9.2[a] | --- |
| **Positive Control (SM)** | 1008.1±550.4[b] | 47.6±27.5[b] | 1.1E+07±2.4E+07[a] |
| **3S** | 5148.6±2046.2[c] | 163.6±41.7[c] | 1.4E+06±3.0E+06[a] |
| **6S** | 5796.2±3097.9[c] | 161.6±69.8[c] | 4.5E+06±5.9E+06[a] |

Different letters show significant differences among the groups

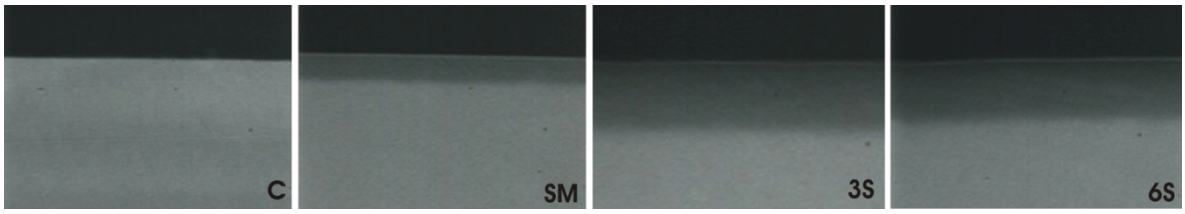
Graph 1.




Bar indicates no statistical differences on Tukey test

Original mean of EPS concentrations (µg/mg) on the biofilm formed over the dentin.

Figure 1.




Photomicrographs of the caries lesions formation on the dentin, according to the groups Control (C), *S.mutans* (SM), 3 Sucrose baths (3S) and 6 Sucrose baths (6S). The control group shows no caries lesion; the SM group has developed the shallowest dentin lesion, followed by 3S and 6S with the deepest lesion formations.

## DISCUSSION

According to our results the first hypothesis that there would be no difference in the artificial caries production in dentin using a microbial model with two distinct sucrose bath regimens (3 and 6 times a day) was accepted since microradiographic and microbiological analyses have shown no statistically significant differences between both regimens. On the other hand, the second hypothesis that stated that there would be no difference in the biofilm pH before and after each sucrose bath was rejected since the pH measurements have statistically decreased immediately after the sucrose bath.

The microbial model used in the present investigation with *Streptococcus mutans*, artificial saliva as medium and sucrose baths seemed to be suitable to study dental caries in the dentin, as showed in the photomicrographs (Figure 1). The bacterial model enabled the investigation of the etiology and related factors of the dentin caries development and the main nutrient sources in the growth medium were able to provide the primary carbon source for the bacteria culture similar to that available *in vivo* (Beighton & Hayday, 1986). Another microbial *in vitro* biofilm study has also shown the formation of lesions in the dentin (Deng & ten Cate, 2004), as also observed in the photomicroradiograph's (Figure 1), mineral loss and lesion depths values in our study (Table 2).

The influence of the presence of sucrose in the severity of the caries attack was also evidenced (Table 2) and comparable to the *in vitro* results found by Deng & ten Cate (2004) and *in vivo* findings from Øgaard *et al.* (1988). Sucrose causes major biochemical and physiological changes during the process of biofilm formation, which, in turn, enhances its caries-inducing properties (Paes Leme *et al.*, 2006). The biofilm structure was affected by the 40% sucrose baths, producing a more cariogenic environment than for the

control group. This is in agreement with animal (Hefti & Schmid, 1979) and *in situ* (Aires *et al.*, 2006) results in enamel, that investigated the influence of diets containing high sucrose concentrations, biofilm and caries development. In addition, the cariogenicity of sucrose has been shown to be dependent on the concentration and frequency of exposure (Hefti & Schmid, 1979; Cury *et al.*, 1997; Paes Leme *et al.*, 2006; Aires *et al.*, 2006). However, the present study was unable to statistically distinguish the cariogenic effects between 3 or 6 sucrose baths (Table 2). This could be explained by the high variability found in the results but also suggests that 3 sucrose baths at 40% can be used in *in vitro* studies to mimic high cariogenic challenges in the oral cavity.

Moreover, since the model was not capable of reproducing the Stephan's curve, recovering a physiological pH even after immersion in a fresh medium, not a great number of sucrose baths are necessary for producing dentin caries. Even though the biofilm pH has decreased immediately after the sucrose baths ($p < 0.05$) and after 5 min, the values increased ($p < 0.05$) (Table 1) and the buffer capacity of the saliva was not so great as to reach values higher than the critical pH of the dentin dissolution (pH ~ 6.5). Therefore, it would be necessary to increase the buffer capacity of the medium and to develop a simplified constant-flow apparatus to deliver this saliva to the specimens. It is also possible that this lack of ability of recovering the physiological pH may be related to the absence of the cleaning capacity of the saliva, which is able to dilute acids and bacterial substrates such as sucrose.

In the development of caries lesions, the EPS formation, in the presence of sucrose, is an important virulent factor to be considered (Bowen, 2002). Insoluble extracellular polysaccharides can change the properties of the biofilm matrix (Dibdin & Shellis, 1988),

enhancing bacterial adherence (Bowen, 2002) and accumulation of large numbers of cariogenic streptococci on the teeth of human subjects (Mattos-Graner *et al.*, 2000; Nobre dos Santos *et al.*, 2002). The findings of the present study (Graph 1) confirm previous results indicating that EPS is produced in larger amounts in the presence of sucrose (Cury *et al.*, 2000; Tenuta *et al.*, 2006), and are consistent with the role of this kind of polysaccharide in the caries development. Moreover, the lack of difference between the EPS production between 3 or 6 sucrose baths may infer that there is no additive effect in EPS production with more sucrose baths, using this microbial model.

The CFU counts in the carious dentin were also not statistically different between the sucrose regimens (Table 2), agreeing with Deng & ten Cate (2004) who also did not find differences on the CFU counts between 5 and 12 days of cariogenic challenge in dentin using a constant depth film fermentor with *S. mutans* and treatments of 4 or 8 sucrose pulses per day. In addition, since no differences were found in ΔZ, LD and EPS, the higher number of microorganism seems not to be so relevant for producing deeper or more demineralized dentin lesions. We believe that the number of baths and CFU counts are important factors to be considered in models where the cleansing and buffer capacity of saliva is achieved. In the present study, it may be possible that the first sucrose bath alone was capable of decreasing the biofilm pH to a point where it could not have been increased again. Consequently, one of the limitations of the proposed model was the inability of mimicking the remineralizing phase of the dental caries process.

## CONCLUSION

The *in vitro* microbial model using *Streptococcus mutans*, artificial saliva as medium and as much as 3 sucrose baths was effective in producing dentin caries lesions. However, it was not able to reproduce the remineralizing phase of the dental caries process.

## ACKNOWLEGMENTS

The first author received scholarship from CAPES and CNPq (201746/2007-7) - Brazil while she was visiting the Indiana University during her Doctorate's course in Dentistry. This paper was based on a thesis submitted by the first author to the Piracicaba Dental School, University of Campinas, in partial fulfillment of the requirements for a PhD degree in Dentistry (Pediatric Dentistry area). The authors thank the assistance given by Dr. Cíntia Maria de Souza e Silva and Thais Manzano Parisotto and also thank CENA, Esalq and LELO, FO laboratories – University of São Paulo for the use of their equipment.

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CAPÍTULO 3

## $CO_2$ laser and fluoride on the inhibition of root caries - an *in vitro* microbial model

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**Key words**: $CO_2$ laser; fluoride, microbial model, microradiography

ABSTRACT

An increase in the dental caries prevalence on root surfaces has been observed mainly in elderly. This research aimed at assessing, *in vitro,* the effectiveness of a pulsed $CO_2$ ($\lambda$ = 10.6 μm) laser associated or not with fluoride, in reducing the human root dentin demineralization in conditions that mimic an oral high cariogenic challenge. After sterilization, root dentin specimens were randomly assigned into 6 groups (n=30), in triplicate. The groups were Control (C), *Streptococcus mutans* (SM), Fluoride (F), Laser (L), Fluoride + laser (FL) and Laser + fluoride (LF). Except for the control group, all the specimens were inoculated with SM and immersed 3 times a day in a 40% sucrose bath. After the 7-day cariogenic challenge for, the mineral loss and lesion depths were evaluated by transverse microradiography. The data were statistically analyzed by variance test with 5% of significance level. The mean mineral loss (± SD) was 816.3 (± 552.5), 3291.5 (± 1476.2), 2508.5 (± 1240.5), 2916.2 (± 1323.7), 1839.7 (± 815.2) and 1955.0 (±1001.4) for C, SM, F, L, FL and LF groups, respectively. The mean lesion depth (± SD) was 39.6 (± 22.8), 103.1 (± 38.9), 90.3 (± 44.6), 91.73 (± 26.9), 73.3 (± 26.6), 75.1 (± 35.2), for C, SM, F, L, FL and LF groups, respectively. In conclusion, irradiation root dentin with a pulsed $CO_2$ laser and fluency of 6.0 J/cm$^2$ was able to inhibit root surface demineralization only when associated with fluoride. No synergic effect on the root dentin caries inhibition was provided by the combination of fluoride treatment laser irradiation.

INTRODUCTION

Root caries experience can be expected to increase in future years. The increase of the population's life expectancy associated with the widespread concepts of the Preventive Dentistry both to dentists and patients have contributed to the retention of a great number of teeth in adults and elderly (Keltjens *et al.*, 1993). For this reason, epidemiologic studies have revealed that root caries is one of the most frequent types of diseases which affect the adult and elderly population, showing an annual increment of 0.47 (Griffin *et al.*, 2004).

Due to its higher content of water and organic matrix, root surfaces are more susceptible to caries development than enamel in face of a cariogenic challenge (Keltjens *et al.*, 1993; Wefel, 1994). Even though biofilm control along with fluoride treatments are preventive measures used not only for coronary but also for root caries (Featherstone, 1994; Fejerskov & Nyvad, 1996; Wefel, 1994), they are not capable of preventing all lesions from occurring. Thus, it is necessary to develop new methods for root caries prevention, targeting this new increasing elderly population (Blinkhorn & Davies, 1996; Griffin *et al.*, 2004).

Among the methods used to prevent root caries, $CO_2$ lasers may be used to change the morphology and chemical composition both of the enamel and dentin surfaces to prevent caries (Stern *et al.*, 1966; Kantola *et al.*, 1972; Nelson *et al.*, 1986; Nammour *et al.*, 1992; Westerman *et al.*, 1994; Featherstone *et al.*, 1998; Liu *et al.*, 2006; Rodrigues *et al.*, 2006; Steiner-Oliveira *et al.*, 2006; Gao *et al.*, 2006). Moreover, it has also been demonstrated that the demineralization inhibitory effect can be increased when the $CO_2$ laser treatment is combined with fluoride (Featherstone *et al.*, 1991; Nobre dos Santos *et al.*, 2001; Rodrigues *et al.*, 2006; Tagliaferro *et al.*, 2007; Steiner-Oliveira *et al.*, 2008).

As regards to the dentin, some authors have reported demineralization inhibitory effect through melting and subsequent recristalization of the tissue due to the high temperatures achieved with the laser irradiation (Kantola *et al.*, 1972; Nelson *et al.*, 1986; Nammour *et al.*, 1992). A more recent study has revealed that laser treatment alone and fluoride treatment alone resulted in root caries inhibition of about 30%. When laser was combined with fluoride treatment, a synergistic inhibition of about 85% was achieved (Gao *et al.*, 2006). Nevertheless, these authors used a chemical model to induce the dentin caries lesion, which did not have the microbial or diet components of the oral environment and the mineral loss/gain was not quantitatively assessed.

Thus, the aim of this study was to evaluate, *in vitro,* using a microbial model, the effects of the $CO_2$ laser associated with fluoride on the root surface demineralization.

## MATERIAL AND METHODS

### Experimental design

This study was approved by the Research and Ethics Committee of the Piracicaba Dental School at the State University of Campinas in Piracicaba, SP, Brazil (Protocol No. 52/2008). The effects of two response variables, laser fluency and fluoride application on human roots were studied. One hundred eighty root specimens were randomly assigned into 6 groups (n = 30), in triplicate. The groups were Control (C), *Streptococcus mutans* (SM), Fluoride (F), Laser (L), Fluoride + laser (FL) and Laser + fluoride (LF). SM and no-laser treatment were considered as positive and negative controls, respectively. The specimens were treated and submitted to an *in vitro* caries microbial model, for 7 days with *Streptococcus mutans* in artificial saliva medium. The response variables for this study

were root subsurface mineral loss and lesion depths, measured after the cariogenic challenge with transverse microradiography analysis.

**Specimen preparation**

One hundred eighty specimens (2 x 2 x 2 mm) were obtained from fifty human unerupted third molars that had been stored in 0.1% (v/v) thymol solution at 4˚C for 30 d (Amaechi *et al.*, 1998). The specimens were cut using a water-cooled diamond saw and a cutting machine (Isomet 1000; Buehler, Lake, Bluff, IL, USA) and were randomly assigned to the 6 different groups according to the treatments. The specimens were coated with an acid-resistant varnish (Colorama, CEIL Coml. Exp. Ind. Ltda., Sao Paulo, SP, Brazil), leaving a 4.0 $mm^2$ window of exposed root for the microbial cariogenic challenge.

The root specimens were fixed in the lids of glass containers with orthodontic wire, kept immersed in sterile distilled water, and then sterilized in a gamma radiation chamber (Gammacell 220 Excel, GC-220E; MDS Nordion, Ottowa, Canada) (Rodrigues *et al.*, 2004).

**Laser Treatment**

Ninety specimens from the groups L, FL and LF were irradiated with a pulsed $CO_2$ laser at 10.6 µm wavelength (Union Medical Engeneering Co. Model UM-L30, Yangju-si, Gyeonggi-Do, Korea) (ANEXO 6-A). The parameters used were 0.8 W, 10 ms pulse duration, 10 ms of time off, 50 Hz repetition rate and a beam diameter of 0.3 mm. For these conditions, a power meter (Model - 201, Coherent Radiation, Palo Alto, CA, United States) indicated a 0.42 W peak power, thus determining an incident fluency of approximately 6.0 $J/cm^2$ per pulse (Souza-Zaroni, 2007). A-10 mm distance from the tip of the hand piece to the specimen was maintained during irradiation which was carried out through the scanning

of each specimen exposed dentin for approximately 30 s by an X-Y positioning platform, in order to provide a uniform coverage of each window.

**Fluoride treatment**

Ninety specimens from the groups F, FL and LF were received a single application of acidulated phosphate fluoride gel (Odahcam, Dentsply, Herpo, Petrópolis, RJ, Brazil) containing 1.23% F (NaF) at pH 3.5 was performed on the root specimens for 1 min in the F group, before (FL group) or after the laser treatment (LF group) (ANEXO 6-B). The gel was wiped off the specimens with paper tissue.

**Biofilm growth**

After sterilization, all specimens were removed from the distilled water and immersed in sterile artificial saliva medium (Pratten *et al.*, 1998). All artificial saliva glass containers (50 mL), except those in the control groups, were inoculated with 0.2 ml (1– 2 $X10^8$ colony-forming units (CFU)/ $mL^{-1}$) of an overnight culture of *Streptococcus mutans* UA 159. This procedure was performed only once, and the specimens were transferred into fresh medium every 24 h (Edmunds *et al.*, 1988). Groups were incubated for 7 days at 37°C and a partial 10% $CO_2$ pressure.

In order to mimic the oral conditions, the specimens were bathed with sucrose (40%) 3 times a day for 5 min and after that, they were transferred to a fresh artificial saliva media. Once a day, samples of all group-cultures were streaked onto BHI agar plates and incubated at 37˚C in order to check purity.

**Mineral Content Analysis**

Transverse microradiography (TMR) was performed to determine the mineral content and the lesion depth of all dentin specimens. The other half of each specimen was

cut with a Series 1000 Deluxe Silverstone-Taylor hard tissue microtome (Sci Fab, Littleton, CO, USA) to obtain sections of 160 ± 20 μm thickness. All thin sections were mounted on microradiographic X-ray plates (Kodak high-resolution plates) along with an aluminum step wedge. Then they were X-rayed using a nickel-filtered Cu (K) X-ray source (Philips) operated at 20 kV and 30 mA for 65 min. The resultant microradiograms were processed and the radiographic images were taken from the microscope (EOM, Carl Zeiss Inc., Germany) to the computer with a camera (KP-120U, Hitachi Denshi Ltd., Japan). The images were analyzed with specific computer software (TMR, transverse microradiography version 1.26; Inspektor Research Systems BV, The Netherlands). Integrated mineral loss was determined by computing the area obtained by plotting the volume percent mineral profile towards dentin depth in each dentin section, with the sound dentin set as 48 vol % mineral (van der Veen *et al.*, 1996) and this enables the analysis of both mineral loss and lesion depth.

**Statistical analysis**

Statistical analysis was performed using SAS software (SAS Institute Inc., version 8.01, Cary, NC, USA), with the significance level set to $p < 0.05$. The data were evaluated to check the equality of variances and normal distribution of errors. After data transformation (root-squared for $\Delta Z$ and LD) analysis of variance (ANOVA) was used to check the significance of the response variables under study (mineral loss and lesion depth). The difference between treatments was assessed by the Tukey test.

RESULTS

The microbial model utilized in this study has proven to be effective in developing dentin lesions as the values for the negative control (C) and the positive control (SM) have statistically differed ($p < 0.05$).

The groups treated with laser and fluoride (FL or LF) have evidenced the least statistically significant ($p < 0.05$) mineral loss, even though the order of these treatments did not matter (Table 1). Treatment with fluoride (F) performed separately has shown an intermediate result and treatment with laser (L) has not prevented mineral loss ($p > 0.05$) as much as for the positive control (SM) (Table 1).

Similarly, the results for lesion depth have evidenced a statistical significant difference between the negative and positive controls ($p < 0.05$) and the shallowest lesion depths were observed for the groups treated with laser and fluoride (FL or LF) (Table 1). Treatment with fluoride (F) and laser (L) alone have shown intermediate results as they did not statistically differed neither from the positive control nor from the associated treatments with laser and fluoride ($p > 0.05$).

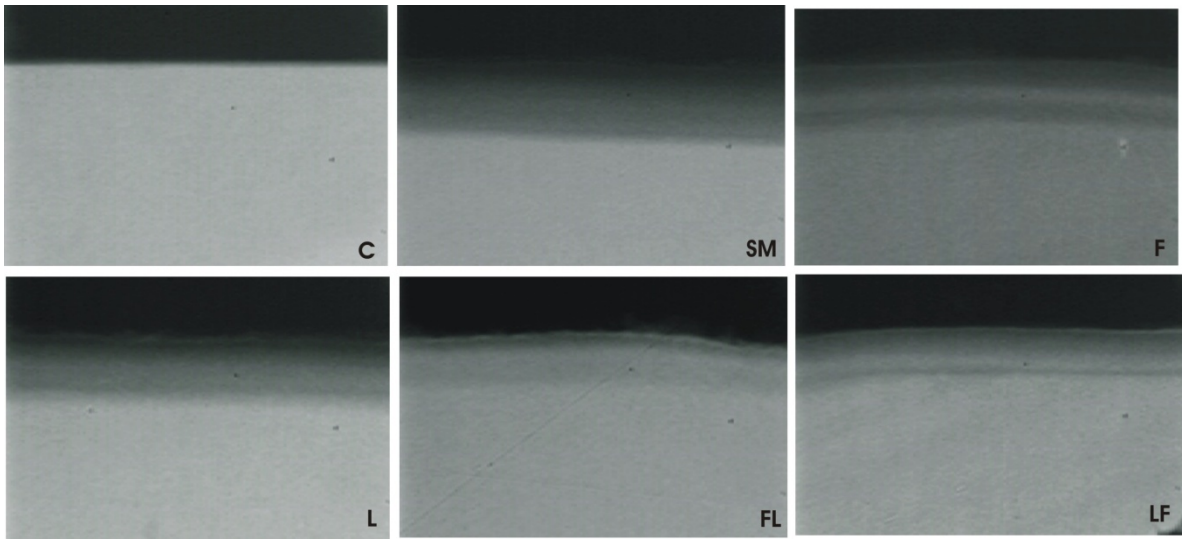
Figure 1 shows the photomicroradiographs of each group. The control group showed no caries lesion; the SM group has developed the deepest dentin lesion; F and L groups have shown similar lesion depths; FL and LF groups have developed the shallowest dentin lesions (Figure 1).

Table 1. Original means and standard-deviations of dentin mineral losses (ΔZ), dentin lesion depths (LD) and percentage of caries reduction.

| Group | ΔZ (% mineral vol/µm) | % reduction | LD (µm) | % reduction |
|---|---|---|---|---|
| **Negative Control (C)** | 816.3±552.5[a] | -- | 39.6±22.8[a] | -- |
| **Fluoride + Laser** | 1839.7±815.2[b] | 44 | 73.3±26.6[b] | 29 |
| **Laser + Fluoride** | 1955.1±1001.4[b] | 40 | 75.1±35.2[b] | 27 |
| **Fluoride** | 2508.5±1240.5[bc] | 24 | 90.3±44.6[bc] | 12 |
| **Laser** | 2916.2±1323.7[c] | 11 | 91.7±27.0[bc] | 11 |
| **Positive Control (SM)** | 3291.5±1476.2[c] | Ref. | 103.1±38.9[c] | Ref. |

Different letters indicate significant statistical differences among the groups by the Tukey test.

Figure 1.




Photomicrographs of the caries lesions formation on the dentin, according to the groups negative control (C), positive control *S.mutans* (SM), Fluoride (F), Laser (L), Fluoride+Laser (FL) and Laser+Fluoride (LF).

DISCUSSION

The microbial model utilized in the present study was capable of inducing root caries lesions. Root caries lesions can also be induced by chemical models, although they do not simulate *in vivo* caries as the bacterial model does (Gilmour *et al.*, 1997). *In vitro* procedures provide a standardized and useful approach, since they allow for greater control of variables (Cutress *et al.*, 1995). Therefore, alternatively from *in vivo* models, *S. mutans* was used to successfully produce root caries lesions (Figure 1), as the means for the dentin mineral loss ($\Delta Z$) and lesion depths (LD) for the positive control group have statistically differed from the negative control group (Table 1) ($p < 0.05$). The lesion depth of the positive control is in accordance with other *in vitro* models using *S. mutans* and sucrose (Clarkson *et al.*, 1984; Kaufman *et al.*, 1988; Shu *et al.*, 2000; Mello *et al.*, 2006) and *in situ* studies (Øgaard *et al.*, 1988; Nyvad & Fejerskov, 1990).

Root surfaces are more vulnerable to acid attack than enamel as cementum has less mineral content (Øgaard *et al.*, 1988; Wefel, 1994; Shen *et al.*, 2004). The bacteria can penetrate into the tubules and the root surface lesions produced in this *in vitro* study were similar to natural dentin lesions (Wefel *et al.*, 1985). Nyvad & Fejerskov (1990) have observed in *in vitro*-created root surface lesions, a zone of relatively high mineral content as also shown in the present investigation (Figure 1, SM). The position and width of this zone are mainly determined by the physico-chemical events taking place during demineralization and redeposition of minerals. One explanation could be the presence of the cementum layers that has proved to provide initial inhibition of the rate of demineralization of the intact root surface. The initial decrease in the subsurface rate might

occur due to the ability of cementum not only to retard the influx of acid ions, but apparently to retard the efflux of dissolution products (McIntyre *et al.*, 2000).

Some authors have demonstrated the $CO_2$ laser potential to modify the dentin structure in order to decrease the mineral loss susceptibility (Kantola, 1972; Nelson *et al.*, 1986; Nammour *et al.*, 1992; Gao *et al.*, 2006). The present investigation was unable to significantly show the isolated laser and fluoride effects in preventing root dentin caries development, with percentages of demineralization reduction of 11 and 24%, respectively (Table 1). Another study (Featherstone *et al.*, 2003) irradiated the dentin with a $CO_2$ laser, but with a different wavelength (9.6 µm), with a 20 µs pulse duration, overlapping spots, 5 pulses per spot, fluencies ranging from 0.2 to 1.0 J/cm$^2$ and a pH cycling model to produce caries lesions. Even though it is a different wavelength, they have also shown that neither low fluencies nor fluencies in the optimum range for dentin were able to significantly inhibit the lesion progression in dentin. In their study, fluoride treatment alone provided an inhibition of 33% of dentin lesion progression (Featherstone *et al.*, 2003). This lack of the isolated laser effect found in our study could be explained by the microbial model, which we believe was not able to mimic the remineralizing phase of the caries process (data not published), therefore limiting the remineralizing effect of the fluoride. However, the association of the $CO_2$ laser with highly concentrated fluoride application has proven to decrease the mineral loss, agreeing with the results found by Gao *et al.* (2006). The application of fluoride before or after laser irradiation did not provide any additional effect in the inhibition of dentin demineralization, even though it seems like the prior fluoride treatment tended to show better results ($p > 0.05$) than after the laser irradiation. Further *in*

*situ* studies with dentin as a substrate are necessary to confirm the laser and fluoride effects on the demineralization reduction.

## CONCLUSION

Irradiation of human root dentin with a pulsed 10.6-μm wavelength $CO_2$ laser and fluency of 6.0 J/cm$^2$ was able to inhibit root surface demineralization only when associated with high concentration fluoride application in an *in vitro* microbial model. The results of this study also suggest that no additional effect on the root dentin caries inhibition was provided by the combination of fluoride treatment laser irradiation.

## ACKNOWLEGMENTS

The first author received scholarship from CAPES and CNPq (201746/2007-7) - Brazil while she was visiting the Indiana University during her Doctorate's course in Dentistry. This paper was based on a thesis submitted by the first author to the Piracicaba Dental School, University of Campinas, in partial fulfillment of the requirements for a PhD degree in Dentistry (Pediatric Dentistry area). The authors thank the assistance given by Dr. Fernanda Frasseto and Dr. Carolina Lima de Carvalho and also thank CENA, Esalq and LELO, FO laboratories – University of São Paulo for the use of their equipment.

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CAPÍTULO 4

# Effect of a pulsed $CO_2$ laser and fluoride on the prevention of enamel and dentin erosion

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**Key words**: $CO_2$ laser; fluoride, profilometry, biochemical analysis

ABSTRACT

The prevalence of dental erosion has been soaring. Considering the irreversible nature of the tooth surface loss by erosion, studies should focus on preventive measures such as the possibility of increasing the resistance of enamel and dentin to erosive challenges, by treating them with a pulsed $CO_2$ laser ($\lambda$ = 10.6 μm). Sixty-four bovine specimens of each enamel and root dentin were randomly assigned to the groups (n = 8): fluoride (F), laser (L), fluoride + laser (FL) or no treatment as negative control (C). The specimens were flattened, polished, demineralized by 0.3% citric acid, pH 2.45 for 5 min and remineralized in artificial saliva for 60 min, 3 times a day, for 3 days. The surface loss was measured with a profilometer after each cycling day and calcium, phosphorus and fluoride concentrations in the demineralizing solutions were also determined at the same periods. All analyses were performed separately for enamel and dentin specimens using ANOVA and Tukey test with a significance level of 5%. The wear results have indicated that the FL treatment was able to interfere with the surface loss of either enamel or dentin, although no synergistic effect was observed. There was a trend for FL treatment to retain more fluoride in enamel and release lower amounts of calcium and phosphorus into the demineralizing solutions, mainly after the first day of the erosive challenge. Treatment with a pulsed $CO_2$ laser ($\lambda$ = 10.6 μm) alone was not able to prevent enamel or dentin surface losses due to erosion. Its combination with fluoride showed some protection, but mostly due to the fluoride effect. No significant synergistic interaction or long-term protection could be observed for the laser therapy.

## INTRODUCTION

Dental erosion is the loss of tooth substance by tribochemical processes, as result of dental surface exposure to acids of extrinsic or intrinsic origins, without the involvement of bacteria. In the modern society, the consumption of acid drinks such as soft drinks, sport drinks fruit juices and fruit teas has increased dramatically since 1950 (Shaw & Smith, 1994; Lussi *et al.*, 2004; Putnam, 2000), being potentially associated to the increase of the prevalence of dental erosion in children, adolescents and adults (Lussi & Schaffner, 2000; Ganss *et al.*, 2001). Clinical studies have reported that about 20-60% of children and adolescents (Johansson *et al.*, 1996; Shaw & Smith, 1999; Al-Malik *et al.*, 2002) and 4 to 25% of adults (Lussi *et al.,* 1991; Putz & Attin, 2002) show some sign of dental erosion. Longitudinal assessments have shown that erosion on occlusal surfaces with involvement of dentin rose up to 25% in a 6-year interval (Lussi & Schaffner, 2000).

Considering the irreversible nature of the tooth surface loss by erosion, preventive measures are extremely important and should include dietary counseling, optimization of fluoride regimens, stimulation of salivary flow rate, use of buffering medicaments and particular advice on non-destructive toothbrushing habits (Lussi & Hellwig, 2006). However, most of these measures may fail since they are heavily dependent on patients' cooperation. Therefore, research on therapies that do not depend on patient compliance are of special interest. In this study, we focused on the possibility of increasing the resistance of enamel and dentin to erosive challenges, by treating them with carbon dioxide ($CO_2$) laser.

It has been demonstrated that $CO_2$ lasers are effective in changing the chemical composition and morphology of enamel and dentin, inhibiting demineralization in both *in*

*vitro* and *in situ* conditions (Nelson *et al.*, 1987; Nammour *et al.*, 1992; Featherstone *et al.*, 1998; Hsu *et al.*, 2000; Nobre dos Santos *et al.*, 2001; Klein *et al.*, 2005; Gao *et al.*, 2006; Rodrigues *et al.*, 2006; Steiner-Oliveira *et al.*, 2006; Tagliaferro *et al.*, 2007; Esteves-Oliveira *et al.*, 2008). Carbon dioxide lasers seem appropriate for using in dental tissues, since they present high absorption in enamel and dentin substrates. This can be explained by the fact that this laser produces radiation in the infrared region, which coincides closely with some of the apatite absorption bands, mainly phosphate and carbonate groups (Featherstone *et al.*, 1998; Fried *et al.*, 1997; Takahashi *et al.*, 1998). Although the $CO_2$ laser effects in the inhibition of the dental hard tissues demineralization are well established, at least to our knowledge, no previous study has investigated the effect of this laser in the prevention of erosion.

The primary hypothesis of this study was that modifications caused by $CO_2$ laser on enamel and dentin could increase their resistances to erosion. Since fluoride seems to partially protect enamel and dentin against erosion, we also hypothesized that this protection could be enhanced by a synergistic effect of fluoride and laser therapies. Finally, considering the physical changes promoted by the laser application, our last hypothesis was that these changes either associated to fluoride or not, would provide a lasting protection, observed even after several challenges.

## MATERIAL AND METHODS

### Experimental Design

This study was conducted following a factorial 4 x 3 split plot design, with the main factor defined as treatment (plot), at four levels: fluoride (F), laser (L), fluoride + laser (FL)

or no treatment as negative control (C); and the secondary factor defined as cycling day (subplot), at three levels: 1, 2 or 3 days. Dental substrate (enamel and root dentin) was considered as an independent factor, being therefore analyzed independently. The association between the two experimental factors generated 12 groups, with sample size of 8 (n = 8). The main response variable was surface loss expressed in µm. Complementary analysis of calcium, phosphorus and fluoride concentrations (µg/mL) in the demineralizing solutions were also performed.

**Specimen preparation**

Thirty-two bovine specimens (5 x 5 x 2 mm) of each enamel and root dentin, previously stored in 0.1% thymol at 4˚C, had the pulpal and external sides flattened and were glued over 10 x 10 x 8 $mm^3$ acrylic resin blocks. The exposed surface was polished using $Al_2O_3$ papers grit # 1,200, 2,400, and 4,000 under water refrigeration and a 1-µm diamond paste polishing cloth. Specimens were sonicated in detergent solution after completion of the polishing procedures. Adhesive unplasticised polyvinyl chloride (UPVC) tapes were placed on the surface of each specimen, leaving an exposed area of 1 x 5 $mm^2$ (ANEXO 7-A). All specimens were assigned into the four experimental groups (n = 8), following a simple randomization table generated in spreadsheet software (excel 2007; Microsoft, Redmond, WA, USA).

**Fluoride treatment**

A single application of acidulated phosphate fluoride gel (One Minute APF gel, PerfectChoice, Earth City, MO, USA) containing 1.23% F (NaF) at pH 3.5 was performed on the specimens of groups F and FL, before the laser treatment (ANEXO 7-B). The gel was wiped off the specimens with paper tissue after 1 min.

**Laser treatment**

A pulsed $CO_2$ laser at 10.6 µm wavelength (Aesculight, Model AE-10, Woodinville, WA, USA) was used with the following parameters:

| Laser parameters | Enamel | Dentin |
|---|---|---|
| Fluency | 1.0 J/cm$^2$ | 0.6 J/cm$^2$ |
| Power | 3.0 W | 2.0 W |
| Pulse duration | 5 ms | 5 ms |
| Time per spot (X 2) | 10 s | 5 s |
| Pulses per spot | 200 | 20 |
| Frequency | 10 Hz | 2 Hz |
| Beam diameter | 1.4 mm | 1.4 mm |

The irradiation of each specimen exposed area was performed twice per spot using an X-Y positioning platform, in order to provide a uniform coverage of each window (ANEXO 7-C).

**Erosion cycling model**

Each specimen was immersed individually in 10 mL of 0.3% citric acid (pH adjusted to 2.45, with HCl) and statically incubated for 5 min. Following this, the specimens were rinsed in deionized water, carefully dried with absorbent paper, and immersed in 10 mL of artificial saliva (1.45 mM Ca, 5.4 mM $PO_4$, 0.1 M Tris buffer, pH 7) for 60 min. All procedures of this cycling model were conducted at room temperature (23 ±

1˚C). This acid exposure – artificial saliva incubation cycle was repeated three times each day, for a total of 3 days (ANEXO 8-A,B,C). The surfaces were scanned with a profilometer after each cycling day, after the adhesive tapes were removed.

**Surface wear measurement**

Specimens were positioned in the optical profilometer (Proscan 2000; Scantron, Venture Way, Taunton, UK) (ANEXO 9-A), having the experimental surface parallel to the horizontal plane. An area of 3 x 1 $mm^2$ covering both the two reference and treated surfaces was scanned, using horizontal resolutions of 0.02 and 0.05 µm, in the x and y directions, respectively. Surface profilometry tests were performed after the third cycle of each day. Dentin specimens were allowed to dry for 10 min before scanning, in order to reduce the possible interference caused by the shrinkage of dentin organic content. Images were analyzed using dedicated software (proscan 2000; Scantron) (ANEXO 9-B), which calculated the average height of the two reference areas and subtracted it from the experimental area. The difference, expressed in µm, was considered the response variable.

**Calcium analysis**

The calcium (Ca) content in the demineralizing solutions was analyzed by flame atomic absorption spectrometry (AAnalyst 200; Perkin-Elmer, Shelton, CT, USA) (ANEXO 10-A) at 422.7 nm. The samples were diluted with lanthanum chloride solution and calibration was performed using standard calcium solutions (1.25 to 5.00 µg Ca/mL). The values obtained were expressed as µg Ca/mL.

**Phosphorus analysis**

The inorganic phosphorus (P) concentration in the demineralizing solutions was determined spectrophotometrically (Fiske & Subbarow, 1925) using a colorimetric assay

based on the molybdenum reaction at a wavelength of 650 nm (Spectronic 601; Milton Roy, Rochester,NY, USA) (ANEXO 10-B). The reader was calibrated with standard solutions (4.0 to 16.0 µg P/mL). The results were expressed as µg P /mL.

**Fluoride analysis**

Fluoride (F) determination in the demineralizing solutions was performed using a fluoride an ion-specific electrode (Orion 96-09; Thermo Electron, Beverly, MA, USA) (ANEXO 10-C) connected to an ion meter (Orion Research Inc., Boston, MA, USA). Calibration was performed with standard solutions (0.01 to 100.00 µg F/mL). The standard solutions and samples were prepared with 1 ml of TISAB II to 1 ml of stardard/sample. The readings were expressed in millivolts (mV) and then transformed to µg F/mL through linear regression of the calibration curve.

**Statistical analysis**

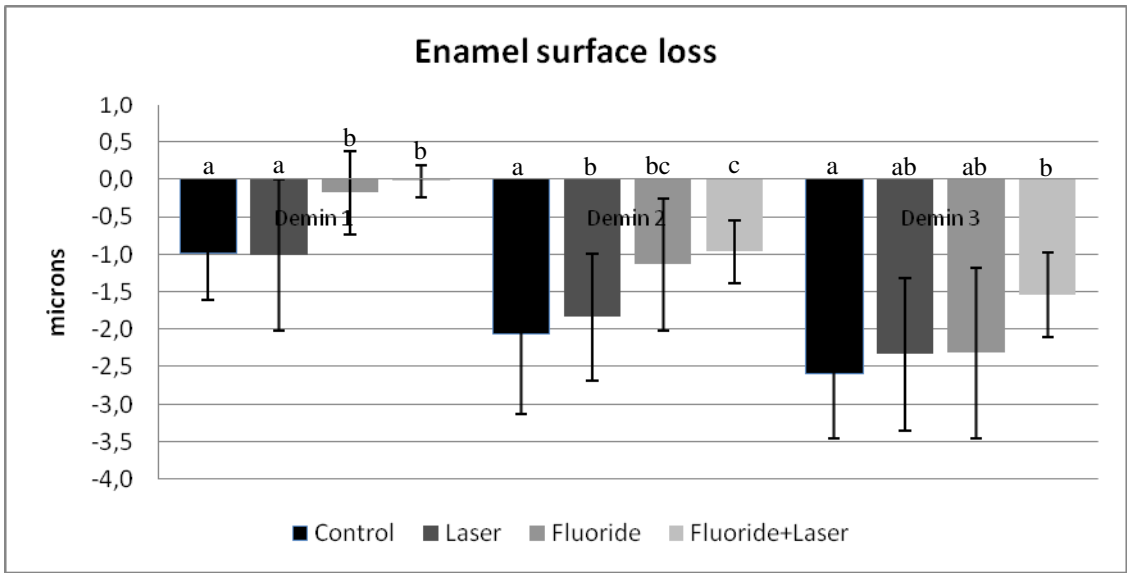
All analyses were performed separately for enamel and dentin specimens. The groups were compared for differences in surface loss, P, Ca, and F using repeated measures analysis of variance (ANOVA). The ANOVAs included terms for group, day, and the group-by-day interaction, and allowed for within-specimen correlations and different variances for the three days. Analyses were performed using the ranks of the measurements because the measurements were not normally distributed. A 5% significance level was used. Results are summarized below without any adjustment for multiple comparisons. However the unadjusted and adjusted (Tukey's method) are provided in the tables.

RESULTS

**Surface loss**

There was a significant day effect with day 1 < day 2 < day 3 ($p < 0.0001$) for both enamel and dentin analysis. The treatment effects could be best observed after the first day, followed by the second and third days. The mean values, SD (standard deviations) and statistical comparisons for wear depth are summarized in Graph 1 for the enamel and Graph 2 for the dentin.

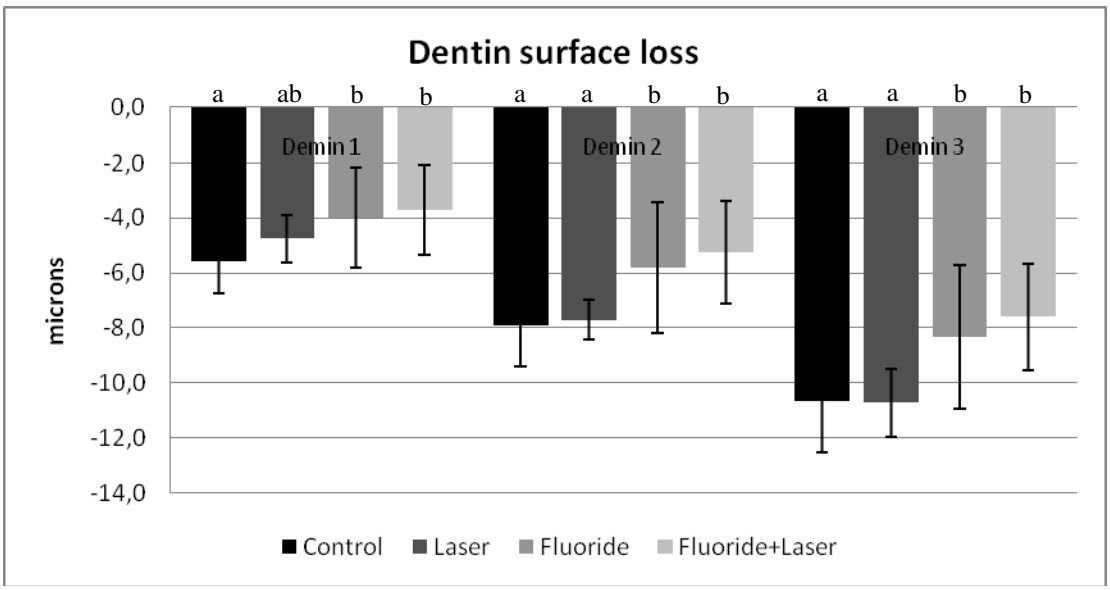
For the enamel, the Tukey test showed that surface loss was greater for the C and L groups after the first day of demineralization ($p < 0.05$). After the second day, L, F and the FL treatments showed lower values of surface loss than the C group. The latter treatment evidenced the lowest values for surface loss. After the third day, only FL group significantly differed from the C group ($p < 0.01$).




Means followed by different letters are statistically different by the Tukey test ($p<0.05$)

Graph 1. Means and SD for the cumulative enamel surface loss, after each erosive cycling day.

For the dentin, the Tukey test showed that after the first day of demineralization, the C group showed the highest surface loss, followed by the L group; F and FL treatments were able to diminish the surface loss, compared with the C group. After the second and third days of demineralization, F and FL treatments evidenced the lowest dentin surface loss.




Means followed by different letters are statistically different by the Tukey test ($p<0.05$)

Graph 2. Means and SD for the cumulative dentin surface loss, after each erosive cycling day.

**Chemical Analysis**

Calcium analysis, for the enamel, has revealed that after the first day, both L and F treatments alone have decreased the calcium loss to the demineralizing solutions. However, the FL treatment has shown the lowest values of calcium release both after the first and second days of demineralization. After the third day, F and FL treatments evidenced the

lowest calcium concentrations in the demineralizing solutions. For the dentin, the calcium analysis has revealed that only after the first day F and FL treatments decreased the calcium loss to the demineralizing solutions.

In the phosphorus analysis for the enamel, lower concentrations were found in the demineralizing solutions for the F group alone or associated with laser, for all analyzed days. For the dentin, F treatment alone or associated with laser decreased the loss of phosphorus, but only after the first day of demineralization.

The fluoride concentrations in the demineralizing solutions for the enamel, in µg/mg, after the first day were of 0.05 (±0.09), 0.03 (0.04), 0.62 (±0.57) and 0.39 (± 0.35) for the C, L, F and FL groups, respectively. The FL group released less ($p < 0.05$) fluoride than the other groups. For the dentin, the fluoride concentrations in the demineralizing solutions, in µg/mg, after the first day were of 0.01 (±0.01), 0.01, (0.01), 0.22 (±0.28) and 0.31 (± 0.23) for the C, L, F and FL groups, respectively. Both F and FL groups released less fluoride ($p < 0.05$) compared with the C and L groups. After the second and third days, the fluoride concentrations released from both enamel and dentin were too low and were out of the method sensibility curve, for all groups.

Table 1. Means and starndard-deviations (SD) values of calcium and phosphorus concentrations in the demineralizing solutions after the erosive challenges of bovine enamel/dentin specimens. Letters show statistical differences (rows) after Tukey tests.

| | **Ca, µg/mg** | | | | | | | | | **P, µg/mg** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ENAMEL | | | | | | | | | | | | | | | | | |
| | Day 1 | | | Day 2 | | | Day 3 | | | Day 1 | | | Day 2 | | | Day 3 | | |
| **Control** | 1,93 | 0,20 | a | 1,66 | 0,21 | ab | 2,05 | 0,38 | a | 1,62 | 1,20 | a | 2,30 | 3,16 | a | 1,77 | 1,42 | a |
| **Laser** | 1,65 | 0,20 | b | 1,79 | 0,27 | a | 1,89 | 0,28 | a | 0,74 | 1,22 | ab | 1,00 | 0,87 | ab | 1,84 | 2,15 | ab |
| **Fluoride** | 1,51 | 0,27 | bc | 2,10 | 1,43 | a | 1,54 | 0,16 | b | 0,70 | 0,80 | b | 0,83 | 0,96 | b | 1,73 | 2,29 | b |
| **Fluoride+Laser** | 1,36 | 0,14 | c | 1,49 | 0,25 | b | 1,70 | 0,29 | ab | 0,57 | 0,66 | b | 0,39 | 1,21 | b | 1,18 | 2,13 | b |
| | DENTIN | | | | | | | | | | | | | | | | | |
| **Control** | 1,95 | 0,18 | a | 1,66 | 0,18 | a | 2,26 | 0,39 | a | 1,06 | 1,21 | a | 0,83 | 1,25 | a | 3,24 | 2,10 | a |
| **Laser** | 2,21 | 0,43 | a | 1,85 | 0,70 | a | 2,82 | 1,42 | a | 1,78 | 1,62 | a | 1,11 | 1,90 | a | 2,57 | 2,76 | a |
| **Fluoride** | 1,38 | 0,18 | b | 1,50 | 0,24 | a | 1,49 | 0,82 | b | 0,00 | 1,25 | b | 1,20 | 2,18 | a | 3,24 | 3,86 | a |
| **Fluoride+Laser** | 1,55 | 0,19 | b | 1,78 | 0,26 | a | 2,20 | 0,45 | a | 0,47 | 1,20 | b | 1,25 | 1,22 | a | 2,82 | 2,87 | a |

## DISCUSSION

A great amount of $CO_2$ laser data on the prevention of caries is available in the literature for both enamel and dentin (Nelson *et al.*, 1987; Nammour *et al.*, 1992; Featherstone *et al.*, 1998; Hsu *et al.*, 2000; Nobre dos Santos *et al.*, 2001; Klein *et al.*, 2005; Gao *et al.*, 2006; Rodrigues *et al.*, 2006; Steiner-Oliveira *et al.*, 2006; Tagliaferro *et al.*, 2007; Steiner-Oliveira *et al.*, 2008; Esteves-Oliveira *et al.*, 2008), but only few studies have considered laser treatment as an option for erosion prevention (Vlacic et al., 2007; Magalhães et al., 2008) and none has investigated the $CO_2$ laser.

Our primary hypothesis on the laser effect preventing dental erosion could not be accepted. The wear results have indicated that the $CO_2$ laser ($\lambda$ = 10.6 µm) treatment alone did not interfere with the surface loss of neither the enamel nor the dentin (Graphs 1 and 2).

Magalhães et al. (2008) have also failed to show protective effect with a Nd:YAG laser in dentin erosion. Previous studies based on caries models have shown reduction on the demineralization of enamel (Featherstone *et al.*, 1998; Nobre dos Santos *et al.*, 2001; Rodrigues *et al.*, 2006; Steiner-Oliveira *et al.*, 2006;; Tagliaferro *et al.*, 2007; Esteves-Oliveira *et al.*, 2008) and dentin (Nammour *et al.*, 1992; Gao *et al.*, 2006). This seemed to be related to the decrease of carbonate and phosphate from the dental tissues after the laser application (Steiner-Oliveira et al., 2006). However, it seems that the more aggressive nature of the erosive lesions could have overcome any benefit provided by the laser treatment.

Similarly, our hypothesis on the synergistic effect of fluoride and laser therapies was not accepted. In the presence of fluoride, it would be expected that the hydroxyapatite phase could be transformed into fluorapatite (Meurman et al., 1997; Gao *et al.*, 2008) due to the increase of temperature induced by the laser treatment, turning the substrate less prone to erosion. The choice for the treatment with fluoride prior to the laser irradiation is in line with another study that also showed better preventive results (Nobre dos Santos *et al.*, 2001). Although there was a trend of the combined treatment to show lower surface loss than fluoride alone (Graphs 1 and 2), it did not reach statistical significance. This trend is in accordance with another study showing that the association of fluoride and laser-activated therapy conferred protection to the tooth structure (Vlacic et al., 2007). However, results can hardly be compared considering that different fluoride and laser therapies as well as experimental erosion models were used. In the current study, although similar responses were observed for enamel and dentin, in an indirect comparison between dental substrates, much higher surface loss was observed for dentin than for the enamel. This can be

explained by the higher susceptibility of dentin to the erosive challenge than for the enamel (Featherstone & Lussi, 2006).

There was a significant difference among each treatment day showing that the treatments with F or FL worked better after the first day of demineralization. After each erosive challenge day, the treatment's effects decreased because the treated surfaces were being worn and, along with them, so were their protecting capacities. Therefore, our third hypothesis based on the long-term effect of laser, either associated or not with fluoride, was rejected. Laser-treated tissues tend to be more resistant to an acidic challenge at a shallow depth (Zuerlein et al., 1999; Tsai et al., 2002), since the laser thermal changes are limited to the tooth surface area due to the absorption coefficient of the $CO_2$ laser. It is believed that the thermal changes induced by the laser irradiation promote chemical and morphological modifications either in the organic or inorganic parts of the dental substrates, making them less likely to demineralization (Nelson *et al.*, 1987; Featherstone *et al.*, 1998, Hsu *et al.*, 2000; Rodrigues *et al.*, 2006; Steiner-Oliveira *et al.*, 2006; Tagliaferro *et al.*, 2007). However, the laser treatment could not increase the protective effect of fluoride throughout the cycling days, although a numerical trend could be observed. It seems that the erosive challenge adopted was too strong, limiting the hypothetical $CO_2$ laser effect. Therefore, the protection observed in this study for FL is more likely to be due to the fluoride effect, although the laser contribution cannot be totally rejected.

It is well known that high concentrations of fluoride delivered at low pH can cause the formation of a $CaF_2$-like layer (Cruz *et al.*, 1992) and thus, protect the dental tissues from erosion (Ganss *et al.*, 2004; 2007). The fluoride deposits can also lead to a better remineralization during the periods of rinsing with artificial saliva resulting in better

prevention of further erosive softening and loss of the enamel (Lagerweij *et al.*, 2006). The laser treatment could allow the formation of microspaces in the dental hard tissue (Fowler & Kuroda, 1986; Oho & Morioka, 1990) possibly imprisoning this calcium fluoride deposition, enhancing the reservoir capacity and release of fluoride to the oral environment (Chin-Ying *et al.*, 2004, Gao *et al.*, 2006). However, it is known that lower laser fluencies may not promote such changes on the enamel surface (Esteves-Oliveira *et al.*, 2008).

The complementary chemical analyses corroborate the results observed for the surface loss tests. There was a trend for FL treatment to retain more fluoride in enamel and release lower amounts of calcium and phosphorus into the demineralizing solutions, mainly after the first day of the erosive challenge (Table 1). For the dentin, there was a similar trend for the FL group to release less fluoride after the first and second days of demineralization and the treatment was also capable of protecting more the tissues from calcium and phosphorus loss after the first day of erosive challenge. After three days of experiment, most calcium, phosphorus and fluoride concentrations did not significantly differ among the treatment groups and the control suggesting that the erosive challenge had overcome any protective effect caused by the change of the surface layers of the specimens.

## CONCLUSION

Treatment with a pulsed $CO_2$ laser ($\lambda$ = 10.6 μm) alone was not able to prevent enamel or dentin surface losses due to erosion. Its combination with fluoride showed some protection, but mostly due to the fluoride effect. No significant synergistic interaction or long-term protection could be observed for the laser therapy.

ACKNOWLEGMENTS

The first author received scholarship from CNPq (201746/2007-7)- Brazil while she was visiting the Indiana University during her Doctorate's course in Dentistry. This paper was based on a thesis submitted by the first author to the Piracicaba Dental School, University of Campinas, in partial fulfillment of the requirements for a PhD degree in Dentistry (Pediatric Dentistry area).

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## IV – CONCLUSÃO GERAL

1. O mecanismo de ação do laser de $CO_2$ na inibição da desmineralização do esmalte ainda não está completamente esclarecido. No entanto parece estar intimamente relacionado com os parâmetros de irradiação utilizados assim como com o tipo de substrato irradiado. Também é possível concluir que a utilização do laser de $CO_2$ associado ao fluoreto pode permitir a redução tanto das densidades de energia quanto das concentrações de fluoreto utilizadas no tratamento. Além disso, melhora o desempenho dos métodos de prevenção aumentando o efeito inibidor da desmineralização.

2. O modelo microbiológico *in vitro* utilizando *Streptococcus mutans*, saliva artificial como meio de cultura e pelo menos 3 imersões em sacarose foi efetivo na produção de cárie dentinária. No entanto, não foi capaz de reproduzir a fase remineralizadora do processo de cárie dentária.

3. A irradiação da dentina radicular humana com laser de $CO_2$ ($\lambda$ = 10.6-µm) pulsado e fluência de 6,0 J/cm$^2$ foi capaz de inibir a desmineralização dessa superfície apenas quando associado com aplicação de alta concentração de fluoreto em um modelo microbiológico *in vitro*. No entanto, não foi observado efeito aditivo na inibição de cárie radicular pela combinação dos tratamentos de laser e fluoreto.

4. O tratamento com laser de $CO_2$ ($\lambda$ = 10.6-µm) pulsado isolado não foi capaz de prevenir a perda de superfície do esmalte e da dentina devido à erosão. A combinação desse tratamento com fluoreto mostrou alguma proteção, mas principalmente devido ao efeito do fluoreto. Não foi observada interação sinérgica significativa ou proteção em longo prazo com a terapia de laser.

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* De acordo com a norma da UNICAMP/FOP, baseada no modelo Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

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ANEXO 1

## INFORMAÇÃO CCPG/OO2/06[6]

Tendo em vista a necessidade de revisão da regulamentação das normas sobre o formato e a impressão das dissertações de mestrado e teses de doutorado e com base no entendimento exarado no Parecer PG n° 1985/96, que trata da possibilidade do formato alternativo ao já estabelecido, a CCPG resolve:

**Artigo 1º** - O formato padrão das dissertações e teses de mestrado e doutorado da UNICAMP deverão obrigatoriamente conter:

I. Capa com formato único ou em formato alternativo que deverá conter informações relativas ao nível (mestrado ou doutorado) e à Unidade de defesa, fazendo referência à Universidade Estadual de Campinas, sendo o projeto gráfico das capas definido pela PRPG.
II. Primeira folha interna dando visibilidade à Universidade, a Unidade de defesa, ao nome do autor, ao título do trabalho, ao número de volumes (quando houver mais de um), ao nível (mestrado ou doutorado), a área de concentração, ao nome do orientador e co-orientador, ao local (cidade) e ao ano de depósito. No seu verso deve constar a ficha catalográfica.
III. Folha de aprovação, dando visibilidade à Comissão Julgadora com as respectivas assinaturas.
IV. Resumo em português e em inglês (ambos com no máximo 500 palavras).
V. Sumário.
VI. Corpo da dissertação ou tese dividido em tópicos estruturados de modo característico à área de conhecimento.
VII. Referências, formatadas segundo normas de referenciamento definidas pela CPG da Unidade ou por critério do orientador.
VIII. Todas as páginas deverão, obrigatoriamente, ser numeradas, inclusive páginas iniciais, divisões de capítulos, encartes, anexos, etc... As páginas iniciais poderão ser numeradas utilizando-se algarismos romanos em sua forma minúscula.
IX. Todas as páginas com numeração "impar" serão impressas como "frente" e todas as páginas com numeração "par" serão impressas como "verso".

§ 1º - A critério do autor e do orientador poderão ser incluídos: dedicatória; agradecimento; epígrafe; lista de: ilustrações, tabelas, abreviaturas e siglas, símbolos; glossário; apêndice; anexos.

§ 2º - A dissertação ou tese deverá ser apresentada na língua portuguesa, com exceção da possibilidade permitida no artigo 2° desta Informação.

§ 3º - As dissertações e teses cujo conteúdo versar sobre pesquisa envolvendo seres humanos, animais ou biossegurança, deverão apresentar anexos os respectivos documentos de aprovação.

**Artigo 2º** - A critério do orientador e com aprovação da CPG da Unidade, os capítulos e os apêndices poderão conter cópias de artigos de autoria ou de co-autoria do candidato, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.

§ único - O orientador e o candidato deverão verificar junto às editoras a possibilidade de inclusão dos artigos na dissertação ou tese, em atendimento à legislação que rege o direito autoral, obtendo, se necessária, a competente autorização, deverão assinar declaração de que não estão infringindo o direito autoral transferido à editora.

**Artigo 3º** - Dependendo da área do conhecimento, a critério do orientador e com aprovação da CPG da Unidade, a dissertação ou tese poderá ser apresentada em formato alternativo, desde que observados os incisos I, II, III IV, V e VII do artigo 1º.

**Artigo 4º** - Para impressão, na gráfica da Unicamp, dos exemplares definitivos de dissertações e teses defendidas, deverão ser adotados os seguintes procedimentos:

§ 1º - A solicitação para impressão dos exemplares de dissertações e teses poderá ser encaminhada à gráfica da Unicamp pelas Unidades, que se responsabilizarão pelo pagamento correspondente.

§ 2º - Um original da dissertação ou tese, em versão definitiva, impresso em folha tamanho carta, em uma só face, deve ser encaminhado à gráfica da Unicamp acompanhado do formulário "Requisição de Serviços Gráficos", onde conste o número de exemplares solicitados.

§ 3º - A gráfica da Unicamp imprimirá os exemplares solicitados com capa padrão. Os exemplares solicitados serão encaminhados à Unidade em, no máximo, cinco dias úteis.

§ 4º - No formulário "Requisição de Serviços Gráficos" deverão estar indicadas as páginas cuja reprodução deva ser feita no padrão "cores" ou "foto", ficando entendido que as demais páginas devam ser reproduzidas no padrão preto/branco comum.

§ 5º - As dissertações e teses serão reproduzidas no padrão frente e verso, exceção feita às páginas iniciais e divisões de capítulos; dissertações e teses com até 100 páginas serão reproduzidas no padrão apenas frente, exceção feita à página que contém a ficha catalográfica.

§ 6º - As páginas fornecidas para inserção deverão ser impressas em sua forma definitiva, ou seja, apenas frente ou frente/verso.

§ 7º - O custo, em reais, de cada exemplar produzido pela gráfica será definido pela Administração Superior da Universidade.

**Artigo 5º** - É obrigatória a entrega de dois exemplares para homologação.

**Artigo 6º** - Esta Informação entrará em vigor na data de sua publicação, ficando revogadas as disposições em contrário, principalmente as Informações CCPG 001 e 002/98 e CCPG/001/00.

Campinas, 13 de setembro de 2006

**Profa. Dra. Teresa Dib Zambon Atvars**
Presidente
Comissão Central de Pós-Graduação

# COMITÊ DE ÉTICA EM PESQUISA
## FACULDADE DE ODONTOLOGIA DE PIRACICABA
## UNIVERSIDADE ESTADUAL DE CAMPINAS

# CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Padronização de um modelo microbiológico para produção de lesões de cárie dentinária in vitro**", protocolo nº 079/2008, dos pesquisadores Carolina Steiner Oliveira, Carolina Lima de Carvalho e Marinês Nobre dos Santos Uchôa, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 13/08/2008.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "**Standardization of a microbiological model to produce dentinal caries lesions in vitro**", register number 079/2008, of Carolina Steiner Oliveira, Carolina Lima de Carvalho and Marinês Nobre dos Santos Uchôa, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at .

**Prof. Dr. Pablo Agustin Vargas**
Secretário
CEP/FOP/UNICAMP

**Prof. Dr. Jacks Jorge Junior**
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

**COMITÊ DE ÉTICA EM PESQUISA**
**FACULDADE DE ODONTOLOGIA DE PIRACICABA**
**UNIVERSIDADE ESTADUAL DE CAMPINAS**

# CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Efeito in vitro do laser de CO2 e fluoreto na redução de cárie em superfície radicular"**, protocolo nº 052/2008, dos pesquisadores Carolina Steiner Oliveira e Marinês Nobre dos Santos Uchôa, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 11/09/2008.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"In vitro effect of the CO2 laser and fluoride on the root surface caries reduction"**, register number 052/2008, of Carolina Steiner Oliveira and Marinês Nobre dos Santos Uchôa, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at .

**Prof. Dr. Pablo Agustin Vargas**
Secretário
CEP/FOP/UNICAMP

**Prof. Dr. Jacks Jorge Junior**
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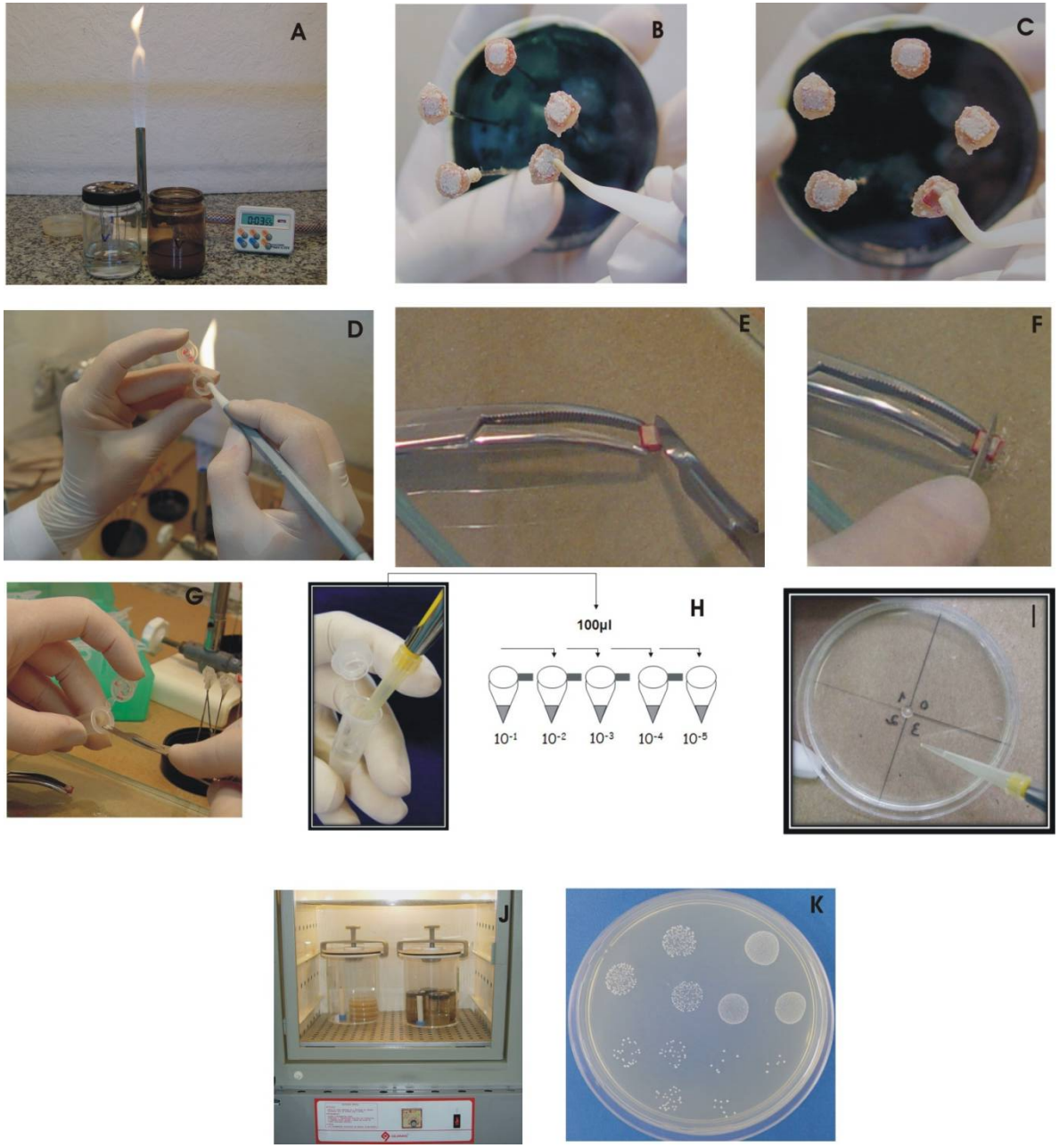
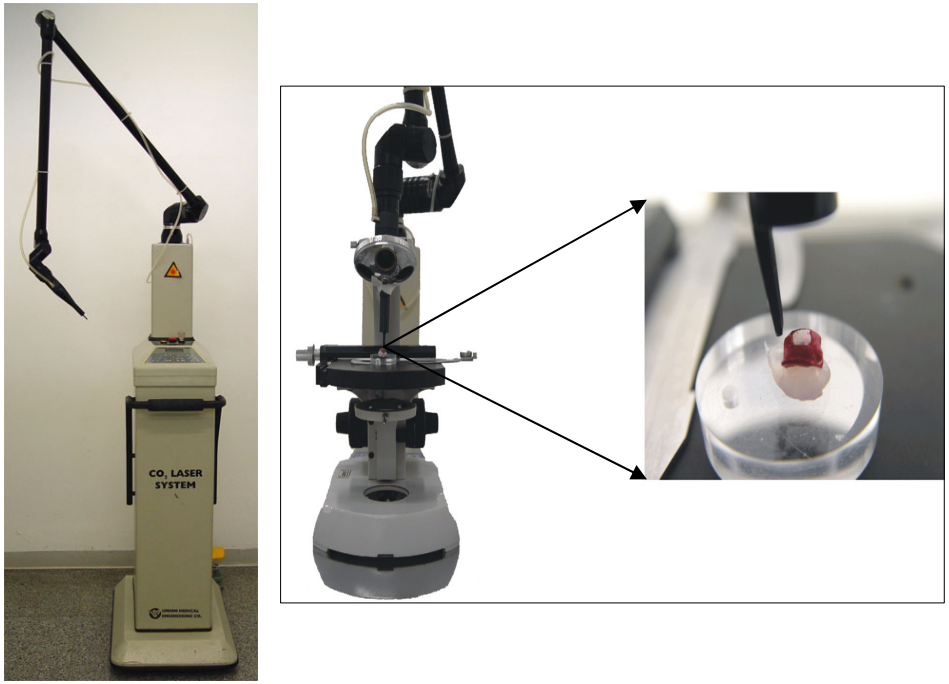
Esquema de preparo dos espécimes de dentina e modelo microbiológico. A – Desgaste oclusal dos dentes. B – Corte do fragmento dentário. C – Obtenção dos blocos. D – Isolamento da área de dentina. E – Fixação dos blocos no dispositivo. F – Inoculação da suspensão de *S. mutans* no caldo de cultura. G – Representação dos dispositivos usados para o modelo de produção de lesões de cárie *in vitro*.



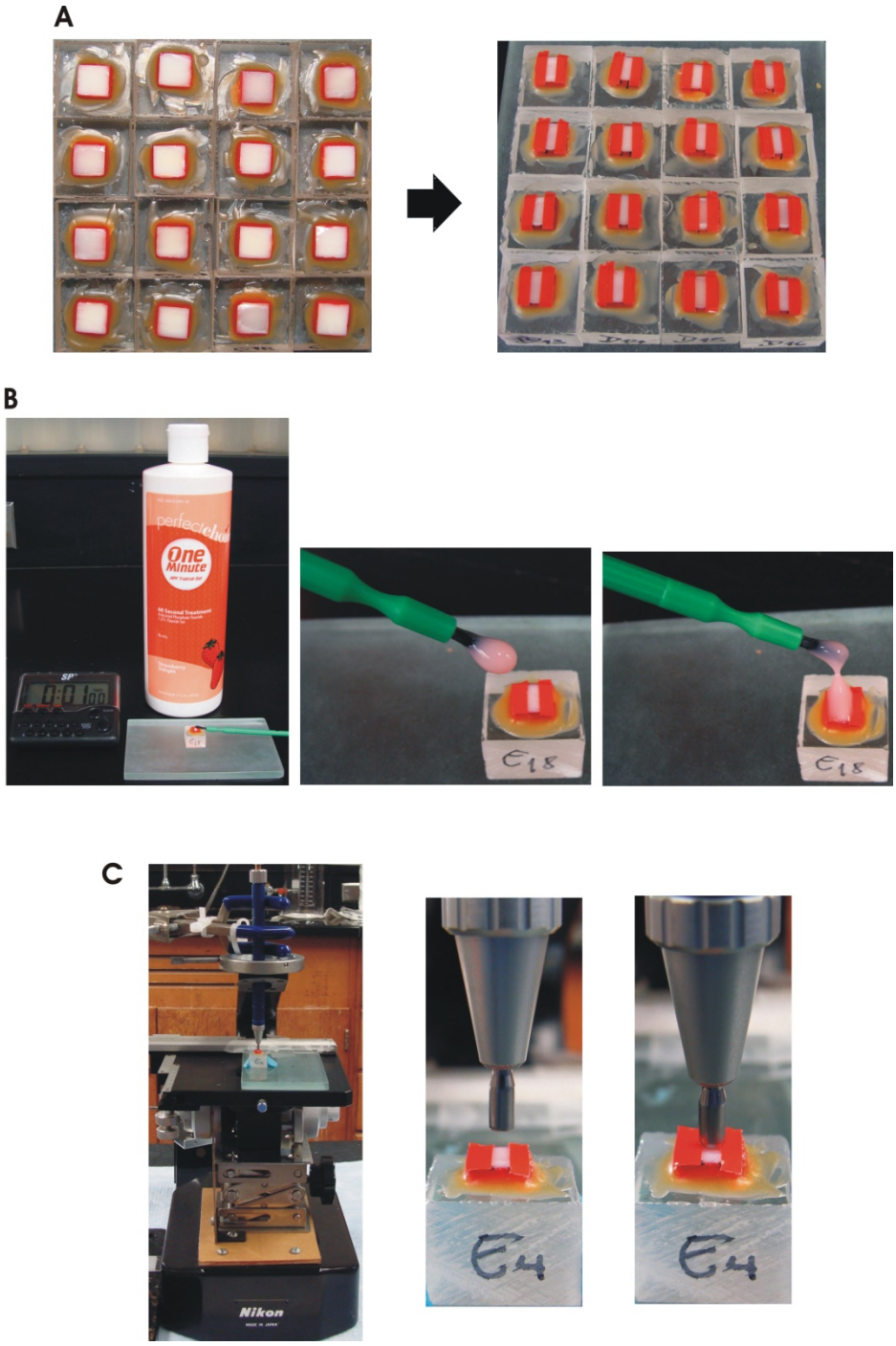

A. Imersão em sacarose 40% por 5 min; B. início da coleta do biofilme formado sobre os blocos de dentina; C. coleta do biofilme com cureta plástica; D. armazenamento do biofilme em tubos de microcentrífuga; E. coleta da dentina cariada com auxílio de lâmina de bisturi; F. raspas de dentina coletadas; G. armazenamento das raspas de dentina em tubo de microcentrífuga; H. diluição em série decimal com salina 0,9%; I. plaqueamento pela técnica das 3 gotas por diluição ($10^{-2}$ – $10^{-5}$); J. incubação dos meios de cultura e placas de petri dentro de jarras de anaerobiose em estufa a 37° C; K. crescimento bacteriano após 48 h de incubação.



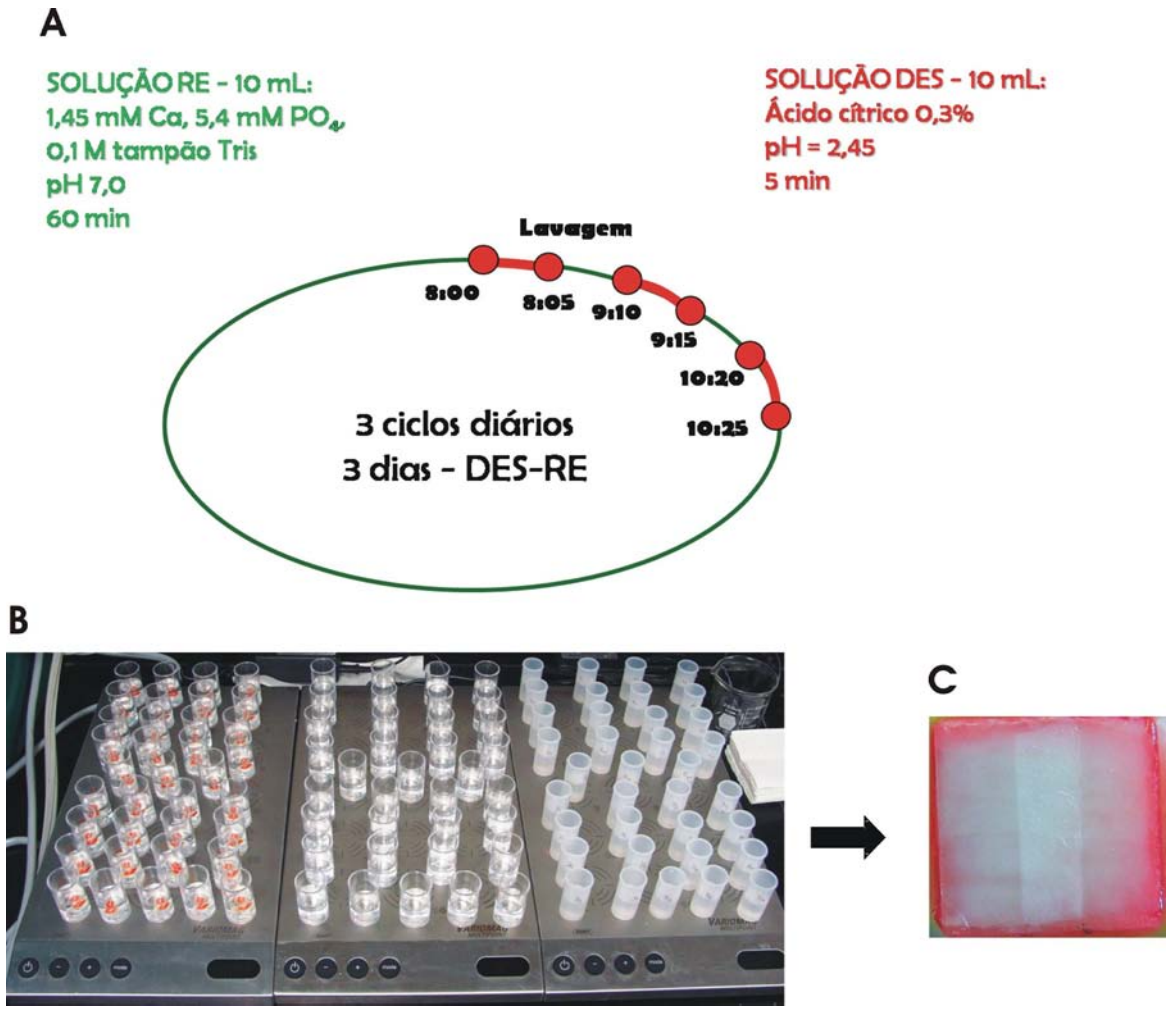

A. Laser de $CO_2$ acoplado ao microscópio para varredura da área de esmalte (4 $mm^2$) e luz guia do laser de $CO_2$ para facilitar


B. Tratamento da superfície radicular com flúor fosfato acidulado durante 1 min

**ANEXO 7**



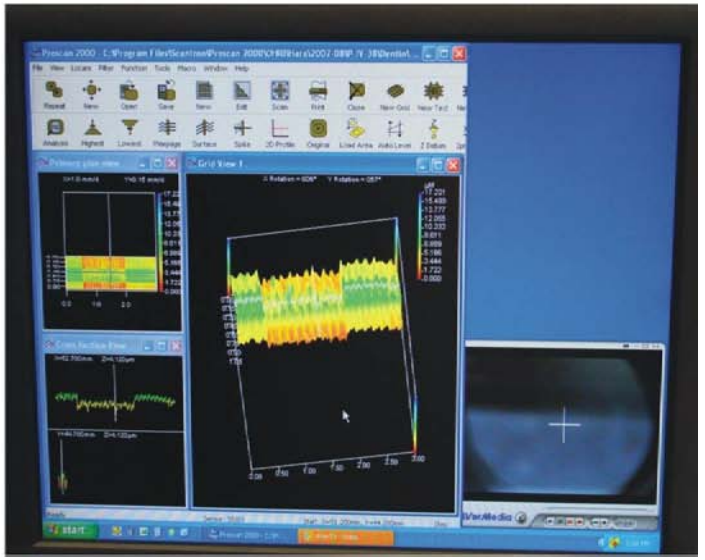

A. Espécimes com tamanho original de 5,0 X 5,0 $mm^2$; B. isolamento da área teste de 1,0 X 5,0 $mm^2$ com fita não-plástica UPVC; C. irradiação com laser de $CO_2$ utilizando mesa com eixos x e y para padronização da área irradiada.




A. Esquema de ciclagem de pH; B. Recipientes com as soluções remineralizadora, água e desmineralizadora; C. lesão produzida após o desafio erosivo.

A


B



A. Perfilômetro utilizado para análise da perda de superfície; B. programa de computador específico para análise da perda de superfície.

A

B


C


A. Espectrofotômetro de absorção atômica para análise de cálcio; B. espectrofotômetro para análise de fósforo; C. eletrodo íon seletivo para análise de fluoreto.