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Avaliação do selamento do conduto radicular por materiais obturadores endodônticos resinosos e análise da expressão de metaloproteinases na matriz dentinária humana e bovina

Tese apresentada à Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas, como parte dos requisitos para obtenção do título de Doutora em Clínica Odontológica, área de Endodontia.

Orientadora: Profa. Dra. Marcela Rocha de Oliveira Carrilho
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RESUMO

Conceitos da odontologia restauradora adesiva vêm sendo empregados em endodontia na cimentação de pinos intrarradiculares e na obturação dos condutos. O primeiro estudo que compõe este trabalho teve como objetivo comparar a qualidade e durabilidade do selamento promovido por materiais obturadores endodônticos que se aderem à dentina (Epiphany/ Resilon) àquelas obtidas com materiais convencionais (AH Plus/ guta-percha) e verificar a influência da existência de uma barreira coronária neste selamento. Dentes humanos unirradiculares receberam tratamento endodôntico e obturação com uma das combinações de materiais em estudo (Epiphany/ Resilon ou AH Plus/ guta-percha). Os condutos obturados receberam selamento coronário com Coltosol, Clearfil SE Bond/ Filtek Z-250 ou foram deixados sem barreira coronária. A qualidade do selamento proporcionada por cada combinação de material foi avaliada por um método de transporte de fluidos em dois períodos: após a presa do cimento endodôntico (imediato) e depois de 180 dias de armazenamento. Espécimes obturados com Epiphany/Resilon apresentaram maior permeabilidade (selamento menos efetivo) independentemente do tipo de selamento coronário e do período de avaliação. Ambos materiais utilizados como barreira coronária (Coltosol ou Clearfil SE Bond/ Filtek Z-250) aumentaram significativamente o selamento dos espécimes. Após o armazenamento, observou-se redução nos níveis de permeabilidade dos espécimes obturados com AH Plus/guta-percha sem selamento coronário ou selados com Coltosol e dos espécimes obturados com Epiphany/Resilon que não receberam selamento coronário. Concluiu-se que a capacidade de selamento de materiais obturadores adesivos é inferior àquela dos materiais obturadores convencionais e que o armazenamento por 180 dias não interferiu negativamente nesse selamento. A presença de uma barreira coronária contribuiu para a efetividade do selamento radicular. O segundo estudo teve como

objetivo investigar a expressão de enzimas metaloproteinases da matriz (MMPs) na dentina radicular uma vez que tais enzimas têm sido implicadas na perda da estabilidade da união dente-material restaurador. Fragmentos de dentina humana coronária e radicular foram triturados e transformados em pó e as proteínas dentinárias extraídas com o uso de hidrocloreto de guanidina e EDTA. A análise por zimografia revelou atividade gelatinolítica na dentina coronária e radicular correspondente à MMP-2 e MMP-9. A metaloproteinase 2 foi mais intensamente detectada na dentina radicular desmineralizada e a MMP-9 foi, em sua maioria, recuperada do compartimento mineralizado do tecido, antes do uso do EDTA. Através do ensaio de *Western Blot* detectou-se ainda MMP-8 em ambos os substratos. Concluiu-se que metaloproteinases são expressas em dentina radicular de maneira semelhante à dentina coronária. No terceiro estudo a mesma metodologia foi empregada para a detecção de metaloproteinases no substrato dentinário bovino, uma vez que este é frequentemente utilizado como um substituto à dentina humana em ensaios de resistência de união. As proteínas da dentina bovina coronária apresentaram atividade gelatinolítica em zimografia correspondente à MMP-2 e MMP-9, enquanto a dentina radicular apresentou atividade compatível com MMP-2 somente. Pode-se concluir que o substrato dentinário bovino expressa metaloproteinases com atividade gelatinolítica e que existem diferenças no perfil de expressão entre coroa e raiz.

Palavras-chave: Obturação do Canal Radicular, Resinas, Metaloproteinases da Matriz, Dentina

ABSTRACT

Restorative strategies involving dentin bonding have been increasingly applied to endodontics. The first study evaluated the immediate and long-term sealing ability of adhesive *versus* conventional endodontic filling materials and investigated the influence of a coronal seal on leakage rates. After chemo-mechanical preparation/irrigation, human roots were filled with either AH Plus/gutta-percha or Epiphany/Resilon. Coronal sealing was performed with Coltosol or Clearfil SE Bond/Filtek Z250, and some samples received no material. The quality of root canal sealing was assessed by a fluid transport method, performed after sealer setting and 180 days of storage. Specimens filled with Epiphany/Resilon exhibited higher leakage than specimens filled with AH Plus/gutta-percha regardless of experimental condition. Coronal sealing reduced leakage significantly, but no difference was detected between Coltosol and Clearfil/Filtek Z250. After storage, significant decrease in leakage was observed in roots filled with AH Plus/gutta-percha either without coronal sealing or sealed with Coltosol, as well as in roots filled with Epiphany/Resilon without coronal sealing. It was concluded that adhesive filling materials did not provide better sealing than the conventional materials and that this sealing was not disturbed by storage of root filled specimens The presence of a coronary seal contributed to reduce leakage. Matrix metalloproteinases (MMPs) found in coronary dentin may play a role in the loss of stability of bonded restorations. The second study aimed to investigate whether these enzymes can also be detected in root dentin. Crown and root sections of human teeth were pulverized into powder and dentin proteins were extracted by using guanidine-HCl and EDTA. Zymography revealed gelatinolytic activity in both crown and root dentin samples, with bands corresponding to MMP-2 and MMP-9. MMP-2 activity was more evident in demineralized root dentin whereas MMP-9 was mostly extracted from the mineralized compartment of dentin.

Western Blot analysis was able to detect MMP-8 equally distributed in crown and root dentin. In conclusion, MMPs are expressed in radicular dentin similarly to crown dentin. Using the same methodology, the third study assessed whether metalloproteinases are expressed in bovine dentin since this substrate has been frequently used as a substitute to human dentin in bond strength evaluations. Protein extracts obtained from bovine crown dentin exhibited gelatinolytic activity in zymography corresponding to MMP-2 and MMP-9. A similar pattern could be observed in root dentin proteins regarding the expression of MMP-2 isoforms, but no gelatinolytic activity was observed in the molecular weight area corresponding to MMP-9. It could be concluded that bovine sound dentin can express MMPs with gelatinolytic activity, but differences may occur in the expression profile of crown and root dentin substrates.

Key-words: Root Canal Obturation, Resins, Matrix Metalloproteinases, Dentin

SUMÁRIO

1. INTRODUÇÃO

A obtenção de um selamento hermético é, juntamente com a limpeza e modelagem do sistema de canais radiculares, um dos determinantes para o bom prognóstico da terapia endodôntica (Schilder, 1967). Entretanto, a microinfiltração de fluidos e micro-organismos tanto via apical (Dow & Ingle, 1955) quanto via coronária (Madison & Wilcox, 1988), constitui-se ainda em um problema clínico e uma potencial causa do insucesso do tratamento (Saunders & Saunders, 1994).

Estudos *in vitro* demonstraram que, quando exposta à saliva, a obturação endodôntica sofre contaminação independentemente da técnica e dos materiais empregados (Khayat *et al.*, 1993; Brito *et al.*, 2003). Portanto, o selamento coronário dos dentes tratados endodonticamente assume importância fundamental como barreira adicional contra a microinfiltração (Madison & Wilcox, 1988). Estudos *in* vivo demonstraram que a saúde dos tecidos periapicais depende tanto da eficácia do selamento promovido pela restauração coronária quanto da qualidade do tratamento endodôntico (Ray & Trope, 1995; Hommez *et* al., 2002). Dessa forma, a literatura sugere que o prognóstico de um dente tratado endodonticamente pode ser melhorado selando-se corretamente o sistema de canais radiculares e evitando-se, imediatamente após a conclusão do tratamento endodôntico, a infiltração de fluidos orais e bactérias (Heling *et al.,* 2002; Schwartz & Fransman, 2005).

Para prevenir a ocorrência de microinfiltração, é desejável que além de um selamento coronário eficiente exista também compatibilidade entre o material obturador e o cimento endodôntico e que, preferencialmente, este último tenha ainda capacidade de adesão às paredes do canal (Ahlberg & Tay, 1998; Economides *et al.,* 2004). Conceitos da Odontologia Restauradora Adesiva vêm sendo empregados na Endodontia com o intuito de melhorar o selamento do sistema de canais radiculares, de modo a prevenir a existência de falhas entre os diferentes substratos em questão (Leonard *et al.,* 1996; Saleh *et al.,* 2002). O

fundamento para a utilização de materiais com propriedades adesivas baseia-se na premissa de que uma vez estabelecendo um íntimo contato com o substrato dentinário, tais materiais reforçam a estrutura dental e impedem a recontaminação dos condutos radiculares (Shipper & Trope, 2004).

Recentemente, um novo material para obturação endodôntica à base de polímeros de poliéster, Resilon™ (Pentron Clinical Technologies, Wallingford CT), foi desenvolvido como uma alternativa ao uso da guta-percha associada aos cimentos endodônticos . Este material é termoplástico, contém partículas de vidro bioativo, cloreto de bismuto e sulfato de bário (Shipper *et al.,* 2004; Tay *et al.,* 2005a); e é utilizado em associação ao sistema Epiphany™ (Pentron Clinical Technologies, Wallingford CT), que por sua vez é constituído por um cimento resinoso de presa dual à base de metil-metacrilato e por um *primer* que poderia ser categorizado como auto-condicionante (Tabela 1). De acordo com seu fabricante, a combinação destes materiais determina a formação de um "corpo único" ou "monobloco" através da união entre material obturador e parede do canal radicular, o que promoveria uma barreira efetiva contra a microinfiltração. De fato, inicialmente, estudos constataram que o sistema Epiphany/Resilon apresentou menor infiltração *in vitro* que os materiais obturadores convencionais, à base de guta-percha (Shipper *et al.,* 2004), enquanto um estudo *in vivo* mostrou, após três meses de avaliação, um menor grau de inflamação perirradicular em dentes de cães que tiveram seus condutos radiculares previamente contaminados e então obturados com este material (Shipper *et al.,* 2005). Entretanto, investigações subsequentes apresentaram resultados contraditórios, uma vez que alguns estudos confirmaram a boa capacidade seladora da obturação realizada com Epiphany/Resilon (Straton *et al.,* 2006; Tunga & Bodrumlu, 2006) ao passo que outros não conseguiram demonstrar que o selamento oferecido pelos materiais "adesivos" seria melhor que aquele oferecido por sistemas de obturação convencionais (Onay *et al.,* 2006; Jainaen *et al.,* 2007; Fisher *et al.,* 2007).

Tabela 1- Composição dos materiais que compõem o sistema Epiphany/ Resilon.

Material	Composição*
Epiphany Primer™	AMPS1, HEMA2, canforoquinona, água
Epiphany Sealer™	UDMA[3], PEGDMA[4], EBPADMA[5] e BISGMA[6], partículas vítreas de bário-boro-silicato silanizadas, sulfato de bário, sílica, hidróxido de cálcio, oxicloreto de bismuto com aminas, peróxidos, foto-ativador, estabilizantes e pigmentos.
Resilon™	Poliéster, metacrilato bifuncional, vidro bioativo, radiopacificadores e colorantes.

* Informaçao obtida do fabricante
[1]Ácido 2-acrilamido-2-metil-1-propenosulfônico
[2]2-hidroxi-etil-metacrilato
[3]Uretano dimetacrilato
[4]Polietilenoglicoldimetacrilato
[5]Bisfenol A dimetacrilato etoxilado
[6]Bisfenol-glicidil-dimetacrilato

Além de prevenir a ocorrência de microinfiltração, a combinação material obturador radicular/restaurador coronário deve ser capaz de manter o selamento dos condutos radiculares de forma definitiva ou, na melhor das hipóteses, por períodos efetivamente mais longos. Entretanto, no contexto da utilização de materiais com propriedades adesivas, é importante considerar que estudos têm demonstrado que a união dentina/resina apresenta durabilidade limitada (Hashimoto *et al*., 2003; Koshiro *et al.,* 2005; García-Godoy *et al.,* 2007). Muitos destes estudos têm consistentemente revelado a fragilidade físico-química e morfológica das restaurações adesivas frente às condições adversas a que estão submetidas quando encerradas na cavidade oral (Reis *et al*., 2007b,c). O comprometimento isolado, ou em conjunto, desses componentes é indicado como

responsável por abreviar a sobrevida das restaurações adesivas (Carrilho *et al.,* 2005a,b; De Munck *et al*., 2005).

As evidências científicas permitem especular que o comprometimento da interface de união dentina/compósitos de resina é provavelmente o resultado de um efeito combinado da degradação de seus componentes resinosos, que gradualmente absorvem água tornando-se cada vez mais permeáveis e susceptíveis à eluição (Malacarne *et al*., 2006; Yiu *et al*., 2006; Reis *et al.* 2007a), e da degradação da matriz dentinária exposta pelo condicionamento ácido e não protegida pela resina adesiva (Hashimoto *et al*., 2003; Pashley *et al*., 2004). A hipótese de que a desestruturação do colágeno constituinte da camada híbrida possa ocorrer em função de um mecanismo proteolítico endógeno vem sendo paulatinamente verificada por estudos conduzidos em condições *in vitro* e *in vivo* (Pashley *et al*., 2004; Hebling *et al*., 2005; Armstrong *et al*., 2006; Mazzoni *et al*., 2006; Nishitani *et al*., 2006; Carrilho *et al*., 2007a,b), que atestam a presença de atividade colagenolítica intrínseca na dentina. Tais estudos apontam para uma possível relação entre a proteólise do colágeno e a atividade de metaloproteinases, enzimas não-colagênicas que têm sido encontradas na matriz extracelular da dentina humana (Tjäderhane *et al.,* 1998; Sulkala *et al*., 2002, 2007).

As metaloproteinases da matriz (MMPs) constituem um grupo de vinte e quatro enzimas que são responsáveis pela degradação de componentes da matriz extra-celular e membrana basal (Visse & Nagase, 2003), incluindo vários tipos de colágeno. Tais enzimas participam de inúmeros processos fisiológicos, como remodelação e desenvolvimento dos tecidos (Vu & Werb, 2000) e processos reparativos como cicatrização (Steffensen *et al.,* 2001; Uitto *et al.,* 2003). Por outro lado, as MMPs podem também estar envolvidas em eventos patológicos de degradação tecidual, como condições inflamatórias e formação e invasão de neoplasias malignas (Ye 2000; Sorsa *et al.,* 2004).

Estudos reportam a participação de algumas MMPs, ao menos MMP-2, MMP-3, MMP-8, MMP-9, MMP-14 e MMP-20, nos processos de dentinogênese e amelogênese promovendo remodelação da matriz extra-celular depositada e degradação de componentes da membrana basal no germe dental (Sahlberg *et al.,* 1999; Caron *et al.,* 2001). O envolvimento das MMPs nas fases iniciais de formação do órgão dental sinaliza que elas possam permanecer inativas na matriz do esmalte e dentina após o processo de mineralização dos mesmos (Sulkala *et al*., 2002; van Strijp *et al*., 2003). De fato, estudos empregando protocolos para extração de proteínas do tecido dentinário seguidos de ensaios de zimografia e identificação antígeno-anticorpo foram capazes de identificar as metaloproteinases -2, -8, -9 e -20 em dentina coronária humana (Martin-De La Heras *et al.,* 2000; Sulkala *et al.,* 2002; Mazzoni *et al.,* 2007; Sulkala *et al.,* 2007).

Além da provável participação na degradação de fibrilas colágenas expostas na interface de união dentina-resina (Carrilho *et al.,* 2007b), a presença de MMPs no substrato dentinário está também associada à patogênese da doença cariosa (Tjäderhane *et al.,* 1998). Diante de situações de desequilíbrio metabólico, como observado no processo de cárie, foi demonstrado que a exposição das MMPs pela desmineralização ácida do esmalte e dentina as tornou novamente aptas a degradar a matriz orgânica destes substratos, sobretudo da dentina, indicando que a queda do pH exerce função na ativação enzimática (Tjäderhane *et al*., 1998; Chaussain-Miller *et al*., 2006). Desta forma, a atividade de metaloproteinases nos tecidos dentários pode também contribuir para a progressão da lesão cariosa (Sulkala *et al.,* 2001).

Apesar dos dados presentes na literatura a respeito da presença de MMPs na dentina humana e sua possível atuação em processos de degradação, os estudos disponíveis concentram-se na investigação destas enzimas na dentina coronária, não havendo dados disponíveis sobre sua expressão no substrato dentinário radicular.

À luz da crescente utilização de procedimentos restauradores adesivos na dentina da raiz (Schwartz, 2006), parece oportuna a avaliação da efetividade do selamento proporcionado por uma nova combinação de materiais resinosos para obturação de canais radiculares. Uma vez que a adesão dos materiais resinosos (adesivos, compósitos, cimentos) à dentina pode sofrer degradação resultando no comprometimento do sucesso clínico (Yourtee *et al.*, 2001; Carrilho *et al.*, 2005b), a capacidade de selamento proporcionada por esses materiais ao longo do tempo deve ser também investigada. Adicionalmente, passa a ser de grande valia a tentativa de caracterização, no substrato dentinário radicular, da presença de metaloproteinases da matriz, devido a seu possível envolvimento no processo de degradação dos componentes formadores da camada híbrida, responsáveis pela adesão.

Observa-se atualmente uma crescente utilização de dentes bovinos como substitutos para dentes humanos em estudos laboratoriais, principalmente ensaios de resistência de união. Dentre as vantagens descritas estão a possibilidade de obtenção de um número maior de amostras e padronização estrutural do substrato (Schmalz *et al.,* 2001; Wegehaupt *et al.,* 2008). A dentina bovina apresenta características morfológicas compatíveis com a dentina humana (Schilke *et al.,* 2000), o que reforça a possibilidade de sua utilização. Dentro deste contexto, é também importante verificar a eventual presença de metaloproteinases da matriz no substrato dentinário bovino, como um indicador da possibilidade de que restaurações adesivas realizadas possam ser expostas ao mecanismo de atividade proteolítica endógena já descrito na dentina humana.

O presente estudo encontra-se dividido em três capítulos principais, que abrangem a avaliação do selamento dentinário oferecido por materiais obturadores endodônticos capazes de se aderir à dentina radicular, a investigação da expressão de metaloproteinases da matriz pela dentina coronária e radicular humana sadia e adicionalmente a presença destas enzimas no tecido dentinário bovino. Os dois últimos trabalhos foram realizados na Faculdade de Odontologia

da Universidade de Helsinque, Finlândia, durante o Programa de Doutorado com Estágio no Exterior - PDEE, através de bolsa de estudos concedida pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES.

2. PROPOSIÇÃO

Os objetivos específicos de cada estudo conduzido foram:

Capítulo I

- Comparar a capacidade de selamento oferecida por materiais obturadores endodônticos convencionais, não-adesivos, àquela dos materiais à base de resina com capacidade de adesão à dentina radicular;
- Avaliar o comportamento do selamento oferecido por ambos sistemas de obturação após um período de armazenamento de 180 dias;
- Verificar a influência da presença de uma barreira coronária no selamento do sistema de canais radiculares.

Capítulo II

- Investigar o perfil de expressão de enzimas metaloproteinases na matriz da dentina coronária e radicular humana.

Capítulo III

- Investigar a presença de enzimas metaloproteinases na matriz dentinária coronária e radicular de dentes bovinos.

3. CAPÍTULOS

Esta tese está baseada na resolução CCPG/02/06 UNICAMP que regulamenta o formato alternativo para teses de Mestrado e Doutorado. Três capítulos contendo artigos científicos compõem este estudo, conforme descrito abaixo:

Capítulo I.

Dentinal sealing provided by resin-based endodontic fillings

Artigo submetido à publicação no periódico **International Endodontic Journal**

Capítulo II.

Determination of matrix metalloproteinases in human radicular dentin

Artigo submetido à publicação no periódico **Journal of Endodontics**

Capítulo III

Preliminary study of the expression of gelatinases in bovine dentin

Artigo a ser submetido à publicação no periódico **Archives of Oral Biology**

Capítulo I

Dentinal sealing provided by resin-based endodontic fillings

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Running title: Sealing ability of endodontic fillings.

Keywords: endodontic filling materials, resins, dentinal sealing, fluid filtration.

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ABSTRACT

Aim: This study evaluated the ability of conventional *versus* resin-based filling materials to provide immediate and long-term sealing of the root canal. **Methodology:** Eighty-two human roots were instrumented and obturation was performed with AH Plus/gutta-percha or Epiphany/Resilon. Root-filled teeth were coronally sealed either with Coltosol or Clearfil SE Bond/Filtek Z250 or were left unsealed. The quality of root canal sealing was assessed by a fluid transport method, performed at immediate and 180-day time intervals. **Results:** Specimens filled with Epiphany/Resilon exhibited higher leakage than specimens filled with AH Plus/gutta-percha ($p<0.05$), regardless of the coronal sealing condition and period of evaluation. No difference was detected between coronal sealing materials ($p>0.05$), while leakage in teeth without any material was significantly higher ($p<0.05$). After storage, significant decrease in leakage was observed in roots filled with AH Plus/gutta-percha either without coronal sealing or sealed with Coltosol, as well as in roots filled with Epiphany/Resilon without coronal sealing. **Conclusions:** It was concluded that AH Plus/gutta-percha provided superior immediate and prolonged root canal sealing. The presence of a coronal seal reduced leakage significantly. Storage of root-filled specimens did not disturb the sealing ability of the tested materials.

INTRODUCTION

The major goal of endodontic filling is to perfectly seal the root canal system. Along with cleaning and shaping procedures, root fillings with impermeable and biocompatible materials are thought to be essential to the long-term success of endodontic treatment (Hommez *et al.* 2002). However, once exposed to saliva and/or other oral fluids, none of the endodontic fillings can completely prevent leakage (Swanson & Madison 1987, Madison & Wilcox 1988), which seriously compromises treatment prognosis (Saunders & Saunders 1994). Therefore, the achievement of an effective coronal sealing soon after the completion of root canal treatment has been lately considered of major importance in preventing the premature failure of endodontic treatments (Ray & Trope 1995, Heling *et al.* 2002, Tay *et al.* 2005a).

Adhesive dentistry concepts have been increasingly applied to endodontics to improve the sealing ability of root canal fillings and prevent coronal leakage (Ahlberg & Tay 1998, Mannocci & Ferrari 1998, Economides *et al.* 2004). The rationale to use the so-called adhesive materials is based on the premise that as these materials can establish intimate contact with dentinal substrate, they could remain micromechanically retained to this tissue, reinforcing the tooth structure and preventing root recontamination. Undoubtedly, an effective bond between the endodontic filling materials and the root dentin would improve root canal treatment prognosis. A polymer-based root canal filling material, Resilon (Resilon™ Research LCC, Madison, CT, USA), associated to a dentin primer (Epiphany™ primer, Pentron Clinical Technologies, Wallingford, CT, USA) and a resin cement (Epiphany™ sealer, Pentron Technologies, Wallingford, CT, USA), has been introduced as a root canal obturation system that is claimed to bond to root dentin, forming a monoblock and providing an efficient dentinal sealing (Shipper *et al.* 2004, Teixeira *et al.* 2004, Shipper *et al.* 2005). However, the results related to the performance of this resin-based endodontic filling system are controversial (Tay *et al.* 2005a, Pitout *et al.* 2006, Conner *et al.* 2007), indicating

the need for additional investigation, especially concerning its ability to provide a durable dentinal sealing.

Thus, this study evaluated the ability of adhesive and non-adhesive root canal filling materials to provide immediate and long-term sealing to root dentin. In addition, the contribution of adhesive *versus* non-adhesive materials used as coronary sealants in the overall sealing of the root canal was assessed. The tested null hypotheses were: 1) the sealing ability of non-adhesive endodontic filling materials is not different from that of resin-based materials, 2) the sealing provided by both systems does not change over 180 days of *in vitro* storage and 3) the presence of a coronal seal does not affect the overall sealing of the root canal system.

MATERIALS AND METHODS

Specimen Preparation

Eighty-two extracted single-rooted human teeth were collected under a protocol reviewed and approved by the Ethic Committee for Human Studies, Piracicaba School of Dentistry /UNICAMP, Brazil. Organic debris and calculus were removed with scalers and the crowns were cut leaving uniform 13 mm root sections. A crown-down root canal preparation was performed by using Gates-Glidden drills # 5, 4, 3 and 2 (Dentsply Maillefer, Ballaigues, Switzerland) in the coronal and middle thirds of the root canal followed by step-back instrumentation of the apical third up to a 45 size K file (Dentsply Maillefer, Ballaigues, Switzerland). Root canals were irrigated during instrumentation with 5 mL of 5,25% sodium hypochlorite (NaOCl) solution and rinsed with 3 mL of 17% ethylenedyaminetetracetic acid (EDTA) for 5 minutes to remove the smear layer. Subsequently, a final flush with 10 mL distilled water was performed to wash out the EDTA solution remnants.

Obturation Procedure

Teeth with prepared root canals were randomly divided into two major experimental groups according to the obturation material employed. Roots were filled by using the cold lateral condensation technique with AH Plus® sealer (Dentsply De Trey, Konstanz, Germany) and gutta-percha points (AH Plus/gutta-percha) or Epiphany™ sealer and Resilon™ points (Epiphany/Resilon). According to the manufacturer's instructions, Epiphany primer was applied throughout root canal walls before insertion of Epiphany sealer and Resilon points. After cutting excess cones, the coronal portion of Epiphany/Resilon specimens was light-cured for 40 s, using a quartz-tungsten-halogen curing-light unit (XL 3000- 3M ESPE, St Paul, MN, USA) with an output of 500 mW/cm^2.

After filling, AH Plus/gutta-percha and Epiphany/Resilon specimens were divided into three subgroups each (n = 12), according to the coronal sealing employed. In subgroup A, the coronal portion of the roots did not receive any sealing material. In subgroup B, a temporary, non-adhesive sealing material (Coltosol, Colténe G, Altstatten, Switzerland) was condensed to form a coronal plug of 2 mm. Roots in subgroup C were sealed with a self-etching dentin adhesive (Clearfil SE Bond, Kuraray, Kurashiki, Japan), applied according to manufacturer's instructions, and resin composite (Filtek Z250, 3M/ESPE, St. Paul, MN) which was inserted in two 1-mm increments to form a coronal plug of 2 mm. Each resin increment was light-cured for 40 s using a quartz-tungsten-halogen curing-light unit with an output of 500 mW/cm^2. All specimens were stored at 37° C in a humid atmosphere for 14 days to allow the sealers to set. The positive control group consisted of five specimens that received root canal preparation and were filled with a gutta-percha cone without sealer. Another five roots were entirely coated with two layers of nail varnish and served as the negative control group. These specimens were also submitted to the same testing protocol.

Microleakage measurement

Microleakage of roots was evaluated right after the sealers were set (immediate); and after 180 days of storage in a humid environment. An *in vitro* fluid transport model was used to measure the fluid conductance induced by hydrostatic pressure, following the general guidelines reported by Pashley and Depew (1986). A connection platform was constructed by inserting an 18-gauge stainless steel tube into a center hole created in a Plexiglass slab (1.8 x 1.8 x 0.7 cm). Each root was then glued to this device with viscous cyanoacrylate cement (SuperBonder® Gel, Loctite Adesivos, Itapevi, Brazil) so that the coronal root canal orifice was centered over the metal tubing. This assembly was connected to a fluid filtration apparatus as described by Wu et al. (1993). The fluid flow in the system was recorded under a constant pressure of 10psi as the linear movement of an air bubble and converted in $\mu L\ min^{-1}$. The specimens were then removed from the fluid filtration apparatus and stored in a 100% humidity environment at 37°C for 180 days. Afterwards, they were remounted to the system and tested for microleakage as described above.

Scanning Electron Microscopy

A qualitative evaluation of surface alterations caused by moisture on sealing materials was performed by scanning electron microscopy (SEM). Each material was directly applied to the root canal space of 3-mm transversal sections of bovine teeth roots, since the greater root canal diameter would allow insertion of a higher amount of material. After insertion of AH Plus, Epiphany, Coltosol or composite resin, a glass slide covered the upper surface of the dentin discs to avoid extrusion of the material. Epiphany and composite resin specimens were light cured before storage. All filled dentin discs (n=3 /each material) were stored in a 100% humidity environment at 37°C for 30 days. After storage period, dentin

discs were polished with SiC paper (#2000), followed by soft cloth under copious water irrigation. Polyvinyl siloxane (Aquasil Ultra LV, Dentsply Caulk, Milford, USA) impressions of the dentin discs surfaces were taken to produce epoxy resin replicas (EpoxiCure®, Buehler Ltd., Lake Bluff, USA), thereby avoiding artefactual effects during preparation for SEM examination (Itthagarun & Tay 2000, Grandini *et al.* 2004). The replicas were allowed to air-dry overnight in a desiccator at 37^{o}C, sputter-coated with gold (Bal-tec SCD 050 Sputter Coater, São Paulo, Brazil), and examined with a scanning electron microscope (JEOL, JSM-5600LV, Japan) operating at 15 kV.

Statistical Analysis

Fluid flow rates obtained in both time intervals were compared between groups using two-way ANOVA and ANOVA-R followed by Tukey *post hoc* analysis. Comparison within the same specimen between the two time intervals was performed by Paired Observations tests. The level of significance was set at $p<0.05$.

RESULTS

The specimens used as controls for the method behaved as expected. Negative controls did not show any fluid movement, whereas in positive controls the fluid flow rate was too high to be recorded.

The mean fluid flow rates measured in the experimental groups at both observation time points are shown on Figure 1. The effect of root filling materials was independent upon coronal sealing condition, i.e., there was no significant interaction between them. At both immediate and 180-day measurements, roots filled with Epiphany/Resilon allowed higher fluid flow than the ones filled with AH Plus/gutta-percha (ANOVA, $p<0.05$). The differences in fluid flow values among coronal sealing conditions were statistically significant (ANOVA, $p<0.0001$) for both

root filling materials. The groups left without any coronal sealing presented higher fluid flow than the groups sealed with Coltosol or composite resin (Tukey's *post hoc,* $p<0.05$). There was no difference between the two types of sealing materials (Tukey's *post hoc,* $p>0.05$).

The paired observations test employed to compare the leakage between two time points within the same specimen showed no significant differences in immediate and 180-day fluid flow measurements of the roots filled with AH Plus/gutta-percha and sealed with composite resin (Student's t test, $p>0.05$); and the roots filled with Epiphany/Resilon and sealed with Coltosol or composite resin (Wilcoxon signed rank, Student's t test, $p>0.05$) (Figure 1). A significant decrease ($p<0.05$) in fluid flow rates after 180-day storage was observed for roots filled with AH Plus/gutta-percha either without coronal sealing (Student's t test, $p<0.05$) or sealed with Coltosol (sign test, $p<0.05$), as well as for roots filled with Epiphany/Resilon without any coronal sealing (Student's t test, $p<0.05$) (Figure 1).

Scanning electron micrographs allowed the observation of a great number of blister-like structures on the surface of Epiphany and Coltosol. These structures were much reduced on AH Plus samples and very sparse on composite resin surfaces (Figure 2).

DISCUSSION

The current results demonstrated that mean fluid flow rates of Epiphany/Resilon were higher than AH Plus/gutta-percha regardless of coronal sealing condition or period of evaluation, which incurs on the rejection of the first null hypothesis formulated for this study. The second hypothesis should be partially accepted, since the leakage rates obtained after 180 days of storage remained stable or even decreased in some groups. Finally, the third null hypothesis should be also rejected, as there were differences in fluid flow values among different coronal sealing conditions.

The described higher fluid infiltration in Epiphany/Resilon obturated specimens is in agreement with other studies that also included dye, computerized filtration, and bacterial leakage models (Onay *et al.* 2006, Munoz *et al.* 2007, Baumgartner *et al.* 2007). The general outcomes of these studies combined indicate that there is no apparent advantage of using Epiphany/Resilon over more conventional gutta-percha techniques. The highly unfavorable cavity configuration factor (C-factor) expressed inside the root canal has been consensually suggested as the main reason for the sub-optimal performance of Epipahny/Resilon (Pitout *et al.* 2006, Sagsen *et al.* 2006, Jainaen *et al.* 2007). Previous reports have already highlighted the role of C-factor in maximizing the polymerization stress of resin-based materials along the root canal walls (Goracci *et al.* 2004, Ferracane 2005, Tay *et al.* 2005b). The present results along with evidence currently available clearly demonstrate that the development of adhesive strategies for endodontic surfaces is yet to overcome the challenging influence of root canal anatomy.

The differences in fluid flow values observed among coronal sealing conditions for both root filling materials indicated that the presence of a coronal seal influenced significantly the leakage rates. These findings support the concept of immediate and effective coronal seal in prevention of leakage (Schwartz & Fransman 2005, Schwartz 2006, Leonardo *et al.* 2007). Although both temporary and resin-based materials reduced leakage, it is worthy to emphasize the limited mechanical properties of temporary restorative materials under clinical conditions (Galvan *et al.* 2002), and they should not be recommended as a long-term solution.

The finding that leakage rates obtained after 180 days of storage remained stable or even decreased may be considered somewhat unexpected. However, they are in concert with previous publications that also have failed to demonstrate a negative effect of time on sealing (Biggs *et al.* 2006, Bouillaguet *et al.* 2008).

Root-filled specimens left without coronary seal had their obturating materials directly exposed to a humid environment. Once exposed to moisture,

both AH Plus and Epiphany sealers are prone to water sorption (Versiani *et al.* 2006, Donnelly *et al.* 2007, Bouillaguet *et al.* 2008). SEM micrographs showing blister-like structures protruding from sealing materials illustrate the occurrence of water sorption during storage, which comes to the surface during the impression procedure. The phenomenon of water transudation has been previously described in resin/dentin interfaces (Tay *et al.* 2004, Bonfante *et al.* 2007). Water sorption leads to mass expansion over time (Ruyter 1995), which in turn may explain the reduced leakage observed in some groups. It may also be speculated that at the moment of the first measurement (immediate), the sealers had not reached their final set and optimal mechanical properties, meaning that the apparent set of the material may not correspond to the total completion of the process. Indeed, some studies have stated that the setting time of endodontic sealers is usually higher than what is informed by manufacturer (Allan *et al.* 2001). Besides mass increase in cement layer, expansion of gutta-percha has also been reported (Wu *et al.* 2000) and may have contributed in some extent to the decrease on leakage rates. Once again, the stability of leakage rates observed for resin-sealed root specimens, along with the very limited blistering phenomenon in SEM micrographs confirms its ability to protect the endodontic filling, as previously stated (Ozturk & Özer 2004, Imura *et al.* 2007).

Although interesting, the performance of endodontic filling materials after 180-day storage should be interpreted carefully. While being prone to water sorption, such materials are also susceptible to solubility (Donnelly *et al.* 2007). As far as longer periods of evaluation are concerned, dissolution of sealers would allow gap formation and leakage may increase (De-Deus *et al.* 2008).

CONCLUSIONS

Under the conditions and limitations of the current study, it could be demonstrated that non-adhesive sealer/gutta-percha endodontic fillings provided better immediate and long-term seal than the so-called adhesive, resin-based

endodontic system. The presence of a coronal seal reduced significantly the leakage rates of the specimens, confirming the importance of this procedure in the overall sealing ability of endodontic fillings. Storage of root-filled specimens for 180 days did not cause loss of sealing ability of the tested materials. Further evaluations for longer periods of time should be conducted to confirm the current results.

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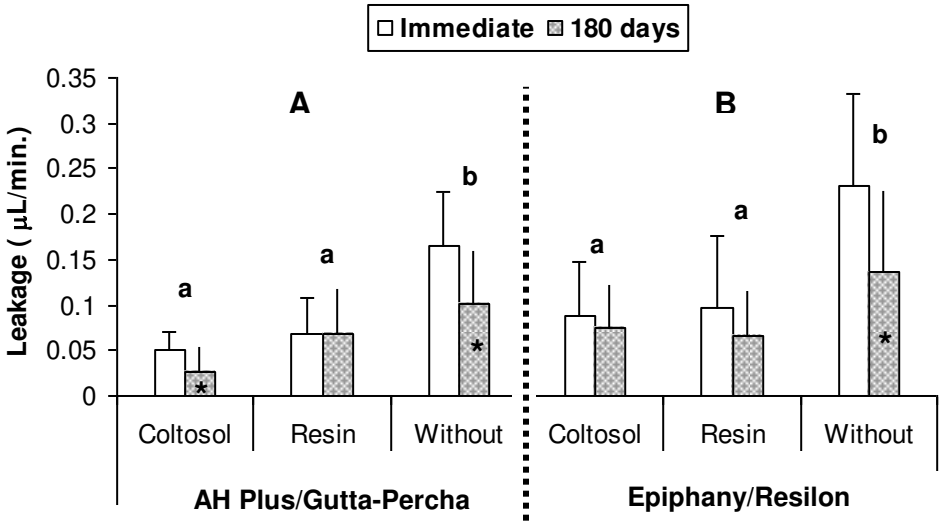

Figure 1- Fluid flow rate (µL.min^{-1}) at immediate and 180-day measurements of AH Plus/Gutta-percha and Epiphany/Resilon associated to different coronal sealing conditions. Error bars indicate standard deviations. There were no significant interactions between the root filling materials and both coronal sealing and period of evaluation. Capital letters indicate statistical differences between root filling materials (ANOVA, $p<0.05$). Lower script letters indicate statistical differences among coronal sealing conditions for each root filling material (ANOVA followed by Tukey, $p< 0.05$). Bars marked with (*) represent groups that showed a significant reduction on leakage after 180-day storage (Paired observations test, $p< 0.05$). WITHOUT = groups that received no coronal sealing.

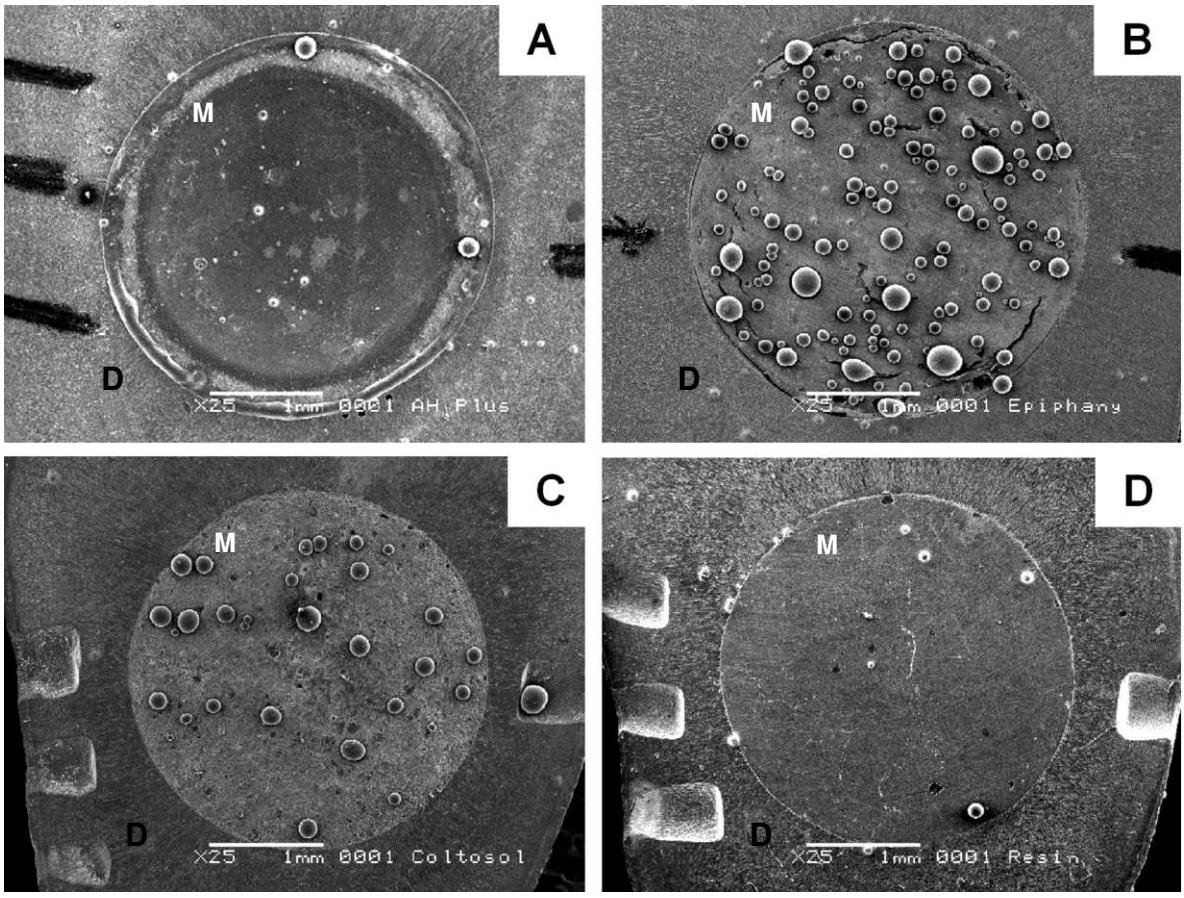

Figure 2- Scanning electron micrographs (SEM) of surfaces produced by both endodontic filling materials and coronal sealing materials. (A) SEM micrograph of AH Plus sealer (x25) showing very reduced blister-like structures. (B) SEM micrograph of Epiphany sealer surface (x25) showing great formation of blister-like structures, probably due to water sorption during storage. (C) SEM micrograph of Coltosol (x25) with the same pattern described for image (B). (D) SEM micrograph (x25) of composite resin surface showing sparse blister-like structures, indicating a higher stability of this material when exposed to moisture. **D**- radicular dentin. **M**- bulk of material.

Capítulo II

Determination of Matrix Metalloproteinases in Human Radicular Dentin

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Running title: MMPs in human dentin.

Keywords: Enzymes, zymography, human tooth, crown, root

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ABSTRACT

Introduction: Matrix metalloproteinases (MMPs) are present in sound coronal dentin and may play a role in collagen network degradation in bonded restorations. We investigated whether these enzymes can also be detected in root dentin. **Methods:** Crown and root sections of human teeth were powderized and dentin proteins were extracted by using guanidine-HCl and EDTA. Extracts were analyzed by zymography and Western blotting for matrix metalloproteinases detection. **Results:** Zymography revealed gelatinolytic activities in both crown and root dentin samples, corresponding to MMP-2 and MMP-9. MMP-2 was more evident in demineralized root dentin matrix whereas MMP-9 was mostly extracted from the mineralized compartment of dentin and presented overall lower levels. Western Blot analysis detected MMP-8 equally distributed in crown and root dentin. **Conclusions:** Since MMPs are also present in radicular dentin, their contribution to the degradation of resin-dentin bonds should be addressed in the development of restorative strategies for the root substrate.

INTRODUCTION

Current findings indicate that the loss of integrity of resin-dentin bonds with time is probably due to a combined effect of hydrolytic deterioration of resinous components following water sorption (1, 2) and degradation of denuded collagen fibrils exposed in incompletely infiltrated hybrid layers (3, 4). The latter is attributed to an endogenous proteolytic mechanism involving the activity of matrix metalloproteinases (5).

Matrix metalloproteinases (MMPs) form a structurally related but genetically distinct group of enzymes within the endopeptidase class and are mainly involved in the extracellular matrix degradation in both physiological and pathological conditions (6, 7). Collectively, these enzymes are mostly synthetized in latent zymogen forms, and they require the binding of a zinc ion in the catalytic site and the cleavage of a propeptide domain to become catalytically competent (6).

MMP-2, -8 and -9 have been detected in human crown dentin (8-10), and their release and activation may contribute to the organic matrix degradation during caries progression (11, 12) and along resin-dentin bonded interfaces (13, 14). Although the presence of MMPs in coronal dentin has been confirmed, it is not well established yet whether they are synthesized and expressed similarly in radicular dentin. Immunohistochemical localization of MMP-2 in human dentin sections showed a much less intense immunoreactivity at cementum-dentin junction when compared to dentin-enamel junction (15), and this was attributed to differences in the composition of crown and root dentin. The present study investigated whether MMPs can also be identified in root dentin. We hypothesized that the same MMPs previously observed in coronal dentin are also expressed in sound radicular dentin.

MATERIAL AND METHODS

Human dentin samples

Forty sound human third molars with complete root formation were obtained from young patients (20-30 years) at the Oulu Health Care Centre under a protocol approved by the Ethical Committee of the Northern Ostrobothnia Hospital District. After organic debris/ calculus removal, teeth were sectioned at the cementum-enamel junction. The pulp tissue was scraped off with scalers and endodontic files. Cementum and enamel were removed from the radicular and coronal teeth fragments with diamond burs operated in a high-speed handpiece under continuous water spray. Crown and root dentin fragments obtained from different teeth were pooled, cut into smaller sections (2 mm x 2 mm), frozen in liquid nitrogen and pulverized into powder in a mixer mill (Model MM301, Retsch, Haan, Germany), with coronal and root dentin being separately powderized. Then, a 2-gram aliquot was obtained from each pool of dentin powder (coronal and radicular) and stored at -20°C until further use.

Dentin proteins extraction

Extraction of dentin proteins was performed using the protocol described in detail by Martin-De Las Heras *et al.* (8). All the reagents were purchased from Sigma (Sigma Aldrich Chemie GmbH, Steinheim, Germany) unless differently specified. Briefly, crown and root dentin powder (2 g each) were treated with 4 M guanidine-HCl, centrifuged, and non-mineralized proteins were collected with supernatant (G1 extract). Dentin powder was then demineralized with 0.5 M EDTA in four cycles to extract mineral-associated proteins (E1-E4 extracts). Finally, demineralized dentin underwent a second guanidine-HCl extraction (G2 extract). The total protein concentration of extracts obtained at each step of the protocol was measured by the Lowry protein assay (8), and 60 µg aliquots were obtained and lyophilized.

Gelatin Zymography

Dentin proteins aliquots were diluted in Laemmli sample buffer in a 2:1 ratio and electrophoresed under non-reducing conditions in 11% SDS-PAGE gels containing 1mg/mL fluorescently labeled gelatin (10). Prestained low-range molecular weight SDS-PAGE standards (Bio-Rad, Hercules, USA) were used as molecular weight markers. After electrophoresis, the gels were washed for 30 min in 50 mM Tris-HCl, 2.5% Tween 80 and 0.02% (w/v) NaN_3, pH 7.5, and then for 30 min in the same buffer supplemented with 5 mM $CaCl_2$ and 1 µM $ZnCl_2$ for removal of SDS. Finally, the gels were incubated in activation solution (50 mM Tris-HCl, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, 0.02% NaN_3, pH 7.5). Proteolytic activity was monitored under long-wave UV light until judged to be in linear range and then the gels were stained in 0.2% Coomassie Brilliant Blue R-250 and de-stained in 10% acetic acid – 10% methanol in H_2O. Zymography assay of dentin proteins was performed in triplicates and repeated three times.

Western Blot

The identity of dentin proteins was further assessed by immunoblotting. Protein aliquots (60 µg each) were mixed in Laemmli buffer with and without 0.5% β-mercaptoethanol and boiled for 5 minutes before electrophoresis in 11% SDS-PAGE gels. Separated proteins were transferred to nitrocellulose membranes (Protran®, Whatman, Dassel, Germany) by means of a semi-dry apparatus (TE 77 PWR Semi-dry transfer unit, Amersham Biosciences, Piscataway, USA). Non-specific binding was blocked by TBS containing 5% non-fat dry milk. After sequential washes, the membranes were incubated with monoclonal anti-human MMP-2 (Oncogene, Boston, USA) and polyclonal anti-human MMP-8 (10) primary antibodies. Following washes, peroxidase-linked anti-mouse and anti-rabbit secondary antibodies were added and imunocomplexes were detected by a chemiluminescent method (ECL Western Blotting analysis system, GE Healthcare,

Buckinghamshire, UK). Western Blot analysis of crown and root dentin proteins was performed in duplicates and repeated twice.

RESULTS

Zymography revealed gelatinolytic bands in both crown and root dentin samples. Proteins extracted in the first guanidine cycle (G1 extracts) yielded 92 and 68 kDa bands, the molecular weights corresponding to MMP-9 and MMP-2, respectively (Fig. 1A). Relatively to crown dentin, root dentin presented stronger 92 kDa and weaker 68 kDa bands. During demineralization (EDTA extracts), enzyme activity was mainly detected as 72/68 kDa bands, corresponding to latent and active MMP-2 in crown and root dentin samples (Fig. 1B, 1C). Faint 92 kDa bands could also be observed with advancing EDTA extraction. Additionally, other lower molecular weight bands (40 kDa, 20 kDa) were detected, most likely representing the truncated forms of gelatinases (Fig. 1B, 1C). G2 extracts showed a major band of 68 kDa corresponding to MMP-2, and some truncated forms (Fig. 1D). Root dentin G2 extracts yielded stronger bands than crown dentin samples.

Western blotting analysis confirmed the presence of MMP-2 in crown and root dentin samples (Fig. 2A). Immunoreactivity was detected as bands over 104 kDa followed by bands of 68 kDa, corresponding to complexed and regular forms of the enzyme. MMP-8 was also detected both in crown and root dentin samples, with major bands corresponding to 64 kDa, and some high molecular weight bands still present in spite of reduction of samples with β-mercaptoethanol (Fig. 2B).

DISCUSSION

Previous studies have already determined the presence and distribution of MMP-2, -8 and -9 in human coronal dentin by using functional and immunological assays (8- 10, 15). This study confirmed the presence of these

enzymes in coronal dentin and additionally detected them in the radicular substrate. Since MMP-2, -8 and -9 could be identified within both crown and root dentin the anticipated test hypothesis was confirmed.

It is noteworthy to emphasize that recovery of MMP-2 from demineralized root dentin (G2 extract) was more evident than from crown dentin. The presence (8) and quantity (16) of MMP-2 in human coronal dentin is age-dependent, with decreasing presence and quantity with increasing age. Therefore, the relatively stronger presence of MMP-2 in root dentin may reflect the shorter time after dentin formation compared to coronal dentin in the third molars extracted from young patients. Even though a similar age-related variation in the expression profile of MMP-2 in root dentin is anticipated, additional confirmatory studies should be conducted. Alternatively, the lower level of mineralization of root dentin (16) could also facilitate improved extraction of these proteins from the matrix. The presence of lower molecular weight bands in gelatin zymography may represent by-products of MMP-2 activation cascade still able to degrade gelatin (17).

Odontoblasts synthesize and secrete MMP-8 (18), and in concert with the findings of Sulkala *et al.* (10), this enzyme could be detected in both mineralized and non-mineralized compartments of dentin. The main substrate of this collagenase is type I collagen (7), which corresponds to 90% of the organic component of dentin. The broad distribution of MMP-2 and -8 in coronal and radicular dentin substrate supports the previous suggestions that they are the major MMPs in this tissue (9, 10).

A common finding in the confirming Western Blotting analysis was the presence of high molecular weight immunoreactive bands against MMP-2 and MMP-8 antibodies, which remained even in some reduced samples. These complexed forms of the enzymes correspond to their status *in natura*, and can be characterized by disulfide bonds formed with other non-collagenous proteins in dentin (19). Alternatively, they may represent dimeric or multimeric forms generated upon activation (20).

MMP-9 was mainly detected in non-mineralized protein fractions (G1 extract) of both crown and root dentin, with some activity observed also in the last EDTA extracts (E3, E4). This indicates that unlike MMP-2 and -8, which were detected in all dentin compartments (8, 10), MMP-9 protein has a more specific distribution. The majority of non-mineralized MMP-9 may be present in dentinal tubules, either in odontoblast process remnants or loosely attached to dentinal tubule walls. In mineralized dentin, the enzyme may be located at the mineral-organic matrix interface, thus requiring extensive EDTA demineralization for its removal, as performed in this study (96 h extraction repeated four times). This can be supported by the previous demonstration of positive immunolabeling of both MMP-2 and MMP-9 in partially EDTA-demineralized dentin surfaces (21), in which collagen matrix was exposed but extensive demineralization was avoided to exclude the possibility for protein denaturation and loss of immunolabeling (22). Conversely, after short (24h) EDTA or EGTA extraction only very low amounts of MMP-9 could be detected in dentin matrix even after ammonium sulfate protein precipitation, while more aggressive citric and acetic acid demineralization yielded clear MMP-9 bands in zymograms of dentin proteins (9). The virtual absence of MMP-9 in G2 extracts may indicate that the enzyme was completely extracted or inhibited during EDTA treatment. Alternatively, the enzyme level in dentin organic matrix – which is reported to be low (21) – could be below detection limits. Interestingly, TGF-β1, a growth factor present in dentin and thought to be responsible for regulation of reparative dentin formation (23), induces MMP-9 mRNA expression (24) and protein synthesis (25) in mature human odontoblasts, with no apparent effect on MMP-2.

Dentin-bound MMPs may be involved in dentinal caries progression (11, 12), being responsible for dentin matrix degradation reducing the possibility for remineralization (7). Furthermore, the application of etch-and-rinse and self-etch adhesives during dentin bonding has been demonstrated to trigger collagenolytic and gelatinolytic activities in coronal dentin (26, 27) mediated by the activation of endogenous MMPs. The etching procedures employed in adhesive bonding

techniques can eventually activate latent MMPs bound to dentin matrix because low pH environments induce conformation changes in the enzyme molecule exposing their catalytic domain (11). Indeed, some adverse effects of dentinal MMPs on adhesive bonding of composites to coronal dentin have been proposed (5, 14, 28). It is possible that the same collagen degradation mechanisms described in coronal dentin may occur in the root. The treatment of root dentin powder with self-etching bonding agents increased its collagenolytic activity by 15-fold (29), and the present findings strengthen the participation of MMPs in this process. This fact raises concern about the longevity of adhesive procedures that are increasingly being applied to root substrate as bonded root fillings and post cementation with resin cements. In addition, recent studies have demonstrated that by-products of both root canal sealers (30) and bacteria related to endodontic infections (31) were able to activate at least proMMP-2 and -9, two of the dentinal MMPs that are thought to be involved with the degradation of collagen fibrils within resin-bonded dentin interfaces (9, 21).

One possibility to control the endogenous MMP activity would be the incorporation of synthetic MMP inhibitors into bonding procedures. A potential candidate for this is chlorhexidine, which is an effective MMP-2, -8 and -9 inhibitor (32). The *in vitro* and *in vivo* application of 2% chlorhexidine in cavity preparations after acid etching and prior to hybridization with adhesive monomers prevents loss of bond strength with time (14) and preserves the integrity of hybrid layer (13). Supposedly, if chlorhexidine was able to inhibit the sealer-induced activation of MMP-2 and -9 (30), it would likely improve the long-term integrity of sealer-dentin interface. In radicular dentin, one can also benefit from the use of chlorhexidine as an endodontic irrigant due to the antimicrobial and substantivity properties of this substance (33, 34), thereby it may also inhibit the bacteria-related activation of MMPs (31). Future studies should be conducted to investigate these addressed concerns.

In conclusion this study revealed, by means of gelatin zymography and Western blotting assays, the presence of the same MMPs in both coronal and root dentin of fully developed teeth of young patients (20-30 years old), although some differences in the relative amount of each enzyme may occur. The impact of the activity of MMPs in the degradation of resin-dentin bonds should therefore be also addressed in the development of restorative strategies for the root substrate.

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FIGURES

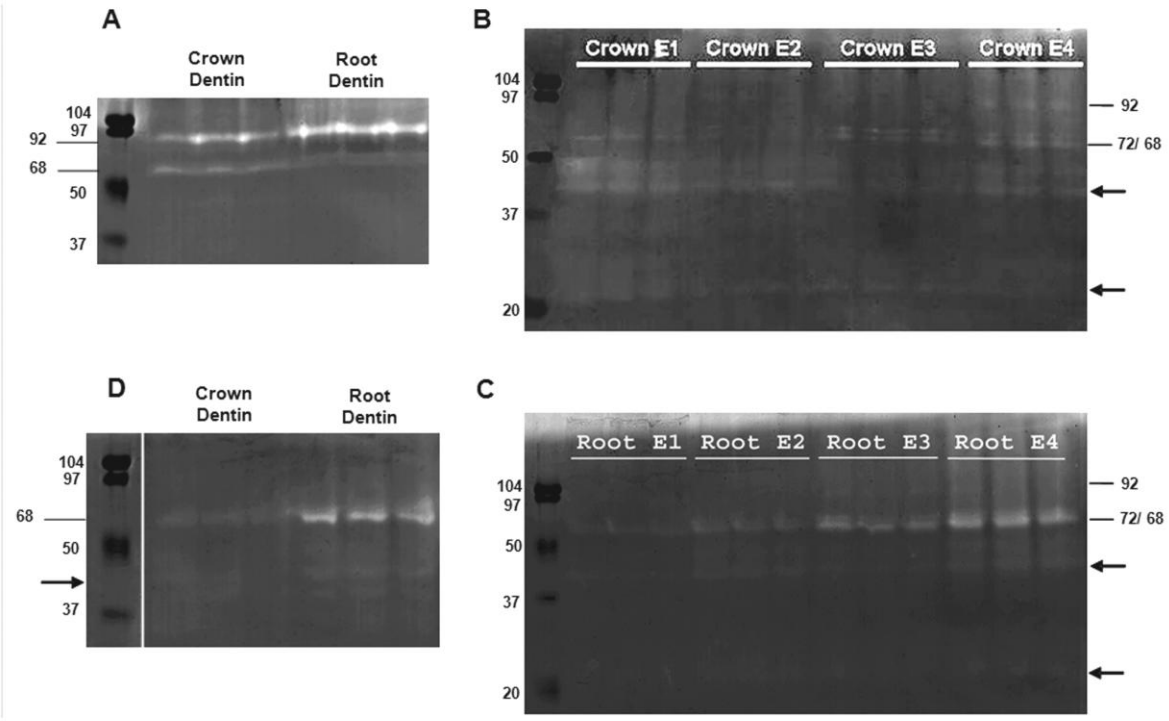

Figure 1- Gelatin zymograms of dentin proteins obtained from crown and root dentin. **(A)** Gelatinolytic activity detected in G1 extracts at approx. 92 and 68 kDa, corresponding to MMP-9 and MMP-2. **(B, C)** Crown and root dentin EDTA extracts (1-4) demonstrated 92 and 72/ 68 KDa bands, corresponding to MMP-9 and latent/ active forms of MMP-2, respectively. In coronal dentin (B), MMP-9 can be distinguished only in the last EDTA extract. Root dentin proteins (C) show increasing gelatinolytic activity with advancing demineralization steps, with MMP-9 apparent only in the 3rd and especially in the 4th extract. Low molecular weight bands (40 – 20 KDa) are also visible, most likely representing truncated forms of gelatinases (arrows →). **(D)** Crown and Root dentin samples obtained after the second guanidine extraction (G2 extracts). Stronger bands can be observed at 68 kDa range in root dentin samples. Smaller molecular weight bands presenting gelatinolytic activity can also be detected (arrow →).

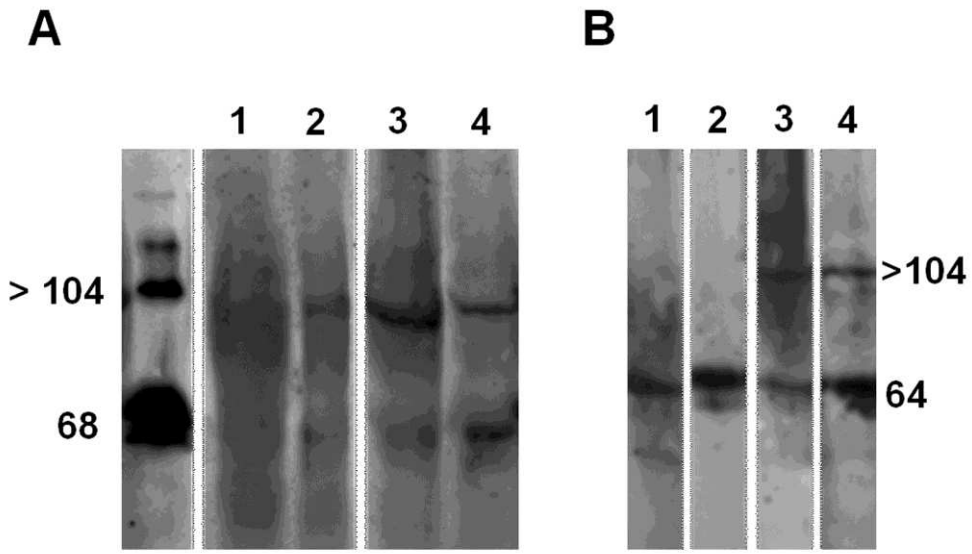

Figure 2- Western blot analysis of crown and root dentin samples. **(A)** Immunoreactivity against monoclonal anti-human MMP-2 was detected in both mineralized (G1 extract- lane 1, crown dentin and lane 2, root dentin) and demineralized compartments of dentin (G2 extract- lane 3, crown dentin and lane 4, root dentin). Proteins were mainly detected as high molecular weight complex forms, which could also be observed in purified MMP-2 (first lane on the left). **(B)** Membrane probing with polyclonal anti-human MMP-8 confirmed the presence of mesenchymal type of the enzyme at 64 kDa range in crown (lanes 1- G1 extract; lane 3- G2 extract) and root (lanes 2- G1 extract; lane 4- G2 extract) dentin samples. Complexed forms of the enzyme (>104 kDa) could also be occasionally detected (lanes 3 and 4) even in reduced samples.

Capítulo III

Preliminary Study of the Expression of Gelatinases in Bovine Dentin

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Running title: MMPs in bovine dentin.

Keywords: Enzymes, gelatinase, bovine tooth, crown, root

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ABSTRACT

Objective: Bovine dentin has been increasingly used as a substitute of human dentin for the evaluation of dental adhesives performance. Since restorative bonding procedures applied to human dental tissues are prone to loose stability due to the action of endogenous matrix metalloproteinases, this study aimed to investigate whether those enzymes can also be detected in the bovine substrate.

Design: Crown and root sections of bovine teeth were pulverized into powder and an extraction protocol was performed to remove proteins from both mineralized and demineralized compartments of dentin. Protein extracts were analyzed by zymography to identify the presence of enzymes with gelatinolytic activity.

Results: Crown dentin samples yielded bands of 92 and 72-68 kDa in all extracts, corresponding to the latent form of MMP-9 and latent/active forms of MMP-2. A similar pattern could be observed in root dentin proteins regarding the expression of MMP-2 isoforms in zymography, but no gelatinolytic activity was observed in the molecular weight area corresponding to MMP-9. Enzyme auto-activation by-products were identified as low molecular weight gelatinolytic bands in crown and root dentin samples.

C*onclusion:* It could be concluded that bovine sound dentin can express some sort of gelatinolytic activity due to the detection of MMPs in protein extracts. Therefore, the activity of matrix metalloproteinases in the degradation of resin-dentin bonds should be addressed when restorations are performed on bovine dentin as an alternative substrate.

INTRODUCTION

Although significant improvements in bonding capabilities of dental adhesives in order to hybridize the dental hard tissues have been achieved, there is growing evidence that the durability of resin-dentin bonds tends to decrease over time.[1,2] The loss of bond stability has been attributed to a combination of factors, such as hydrolysis and elution of resinous components due to water sorption[3] and degradation of the dentin substrate, mainly collagen fibrils.[4,5] The later is thought to be caused by matrix metalloproteinases (MMPs), a group of enzymes able to degrade most of the extracellular matrix components. The reported presence of some of those enzymes (MMP-2, -8, -9) in human dentin[6-8] supports the hypothesis of an endogenous proteolytic pathway for the degradation of resin-dentin bonds.

When adhesion tests are considered, bovine dentin has been increasingly used as a substitute for human dentin due to its availability and more standardized features[9,10], since animals usually come from a similar genetic lineage, have the same age at slaughter and were raised in a controlled environment (i.e. climate, diet). Despite some differences in anatomical, permeability and histochemical features[9,11], morphological studies have demonstrated that most of mammalian teeth are fundamentally alike. Bovine dentin was shown to exhibit characteristics such as number of tubules and collagen organic matrix that are similar to those of human dentin.[12] These morphological similarities have been claimed as being responsible for similar bond strength results when the same adhesives are used in human *versus* bovine dentin.[10,12] Although data on the durability of adhesives to bovine dentin are scanty, few studies reported that resin-bonded bovine dentin also degrade over time.[13,14]

While the mechanism responsible for the degradation of adhesives applied in human and bovine dentin is likely to be similar (i.e. water sorption, polymer plasticization and hydrolysis of hydrophilic components), it is not known whether resin-bonded bovine dentin could undergo degradation via the endogenous proteolytic mechanism that was described for human dentin.[5] Thus,

the aim of this study is to investigate the presence of matrix metalloproteinases in bovine crown and root dentin, as an indicator of potential proteolytic activity. It was hypothesized that bovine crown and root dentin do not have an endogenous proteolytic activity.

MATERIAL AND METHODS

Specimen preparation

Ten bovine sound lower incisors with complete root formation were used shortly after extraction. After organic debris/calculus removal, teeth were sectioned at the cementum-enamel junction. The pulp tissue was scraped off with a pair of small forceps. Cementum and enamel were removed from the radicular and coronal teeth fragments with diamond burs operated in a high-speed handpiece under continuous water spray. Crown and root dentin fragments were separately cut into smaller fragments (2 mm x 2 mm), frozen in liquid nitrogen and pulverized into powder in a mixer mill (Model MM301, Retsch, Haan, Germany). Dentin powder from crown and root dentin was equally divided in 2 g aliquots and stored at -20^{o}C until further processed.

Dentin proteins extraction

Extraction of dentin proteins was performed by using a previously described protocol.[6] Crown and root dentin powder (2 g each) were treated with 4 M guanidine-HCl, 65 mM Tris-HCl and loosely bounded proteins were extracted using centrifugation (G1 extract). Dentin demineralization was then performed by 0.5 M ethylene diamine tetracetic acid (EDTA) in four cycles to extract proteins within the mineralized matrix (E1-E4 extracts) and the remaining demineralized dentin was again extracted with 4M guanidine-HCl, 65 mM Tris-HCl (G2 extract). The protein concentration of each extraction solution obtained was measured by the Lowry protein assay[6], and lyophilized aliquots of 60 µg dentin proteins aliquots were obtained.

Gelatin Zymography

Bovine dentin proteins were subjected to electrophoresis under non-reducing conditions on 11% SDS-PAGE gels copolymerized with 1mg/mL fluorescently labeled gelatin.[8] Molecular weight markers consisted of pre-stained low-range SDS-PAGE standards (Bio-Rad, Hercules, USA). Purified commercial MMP-2 (Chemicon International, Temecula, USA) and MMP-9 (Invitek GmbH, Berlin, Germany) were loaded in the gel in a 1:10 dilution to work as positive controls. After electrophoresis, the gels were washed for 30 min in 50 mM Tris-HCl, 1% Tween 80 and 0.02% (w/v) NaN_3, pH 7.5, and then for 30 min in the same buffer supplemented with 5mM $CaCl_2$ and 1 µM $ZnCl_2$ for removal of SDS. Finally, the gels were incubated in activation solution (50 mM Tris-HCl, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, 0.02% NaN_3, pH 7.5) at 37°C. Proteolytic activity was monitored under long-wave UV light until judged to be in linear range and then the gels were stained in 0.2% Coomassie Brilliant Blue R-250 and de-stained in 10% acetic acid – 10% methanol in H_2O. Zymography assay of dentin proteins was performed in triplicates and repeated three times.

RESULTS

Zymography revealed gelatinolytic activity in both crown and root dentin samples. Proteins extracted in the first guanidine cycle (G1 extracts) from bovine crown dentin yielded 92 and 68 kDa bands, the molecular weights corresponding to MMP-9 and MMP-2 respectively (Fig. 1); while in bovine root dentin, proteins from G1 extracts yielded 68 kDa bands (Fig. 2) and there was a virtual absence of gelatinolytic bands co-migrating with MMP-9 positive control. During demineralization (EDTA extracts), enzyme activity was mainly detected as 72/ 68 kDa bands, corresponding to latent and active MMP-2 in crown and root dentin samples (Fig. 1 and 2). All extracts (crown and root dentin) exhibited markedly higher intensity for bands at 68 kDa, indicating that MMP-2 is the predominant

gelatinase form. Gelatinolytic activity was also detected at lower molecular weight range (40-20 kDa), most likely truncated forms of enzymes (Fig. 1 and 2).

DISCUSSION

This study was able to confirm the presence of gelatinolytic matrix metalloproteinases in bovine dentin as previously reported for human dentin.[6-8] Therefore, the test hypothesis set for this study that bovine crown and root dentin do not have an endogenous proteolytic activity must be rejected.

It is noteworthy to emphasize that recovery of MMP-2 from either mineralized or demineralized root dentin was the most evident for both crown and root bovine dentin, confirming that MMP-2 seems to be the major constitutive gelatinase form. The detection of faint bands corresponding to MMP-9 in crown bovine dentin is in agreement with a previously reported lower recovery of this enzyme from dentin extracts.[7] Immunohistochemical detection of MMP-2 and MMP-9 along the collagen network of coronal dentin could also demonstrate higher levels of immunocomplexes formed against MMP-2.[15] This difference is even more critical in root dentin, since no detectable amounts of MMP-9 could be extracted.

The fact that root dentin is deposited after crown dentin during tooth formation could explain the relative scarcity of MMP-9, since this enzyme is known to have minor participation in the early stages of dentinogenesis.[16] As previously described, other forms of gelatinolytic activity could also be observed in zymograms as low molecular weight bands, and they represent autolytic degradation products of the enzyme.[17] This could be related to the extraction protocol, since it may cause some extent of enzyme activation.[18]

Although previous studies have described MMPs in bovine odontoblasts[19] and induced caries lesions in bovine dentin blocks[20], the expression of those enzymes in sound bovine dentin substrate was not assessed. The current findings enhance evidence regarding similarities between bovine and human

dental substrates[21], which reinforce the possibility of using bovine dentin as a substitute for human dentin tissues in a variety of laboratory tests, and especially when one needs to study the determinant factors of the resin-dentin bonds degradation.

In this context, the presence of degrading MMPs in bovine dentin substrate is of particular importance since adverse effects of MMPs in mature human dentin have already been identified. Latent MMPs can be activated by etch-and-rinse and mild self-etch adhesives applied to dentin in bonded restorations[22-24], a mechanism related to low pH environment. Under such circumstances, a structural change may expose the zinc-dependent catalytic domain of the enzyme[25], which in turn can bind a water molecule and initiate cleavage of peptide bonds, leading to collagen fibrils degradation underneath hybrid layers. Degradation is reported to be more intense in incompletely infiltrated dentin matrices due to limited diffusion of resin monomers or elution of resinous components, since an increasing exposure of denuded collagen is expected.[4,5]

Although zymography gels detected bands with molecular weight compatible to purified MMP-2 and MMP-9, it is important to highlight that co-migration alone does not confer identity. However, the gelatinolytic bands obtained in bovine dentin samples did not show immunoreactivity in Western Blots (data not shown), probably due to a lack of specificity of the antibodies against bovine MMPs. Future confirmatory studies should therefore be considered. In addition, the presence of MMPs with collagenolytic activity in bovine dentin may also be investigated.

According to the present findings and under the constraints of this investigation it is possible to expect some sort of gelatinolytic activity in bovine dentin matrix due to the detection of MMPs in protein extracts. The intrinsic degradation of resin-dentin bonds attributed to the activity of MMPs in human dentin can be, therefore, also anticipated in the bovine substrate. This confirms the possibility of developing research on the durability of bonded restorations using

bovine dentin as an alternative substitute to human dentin when degradation of organic matrix is to be evaluated. Additionally, screening tests aiming to establish effective therapeutical procedures to inhibit the activity of matrix metalloproteinases can also be planned using bovine dentin, since one can benefit from higher availability of samples and structural standardization.

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FIGURES

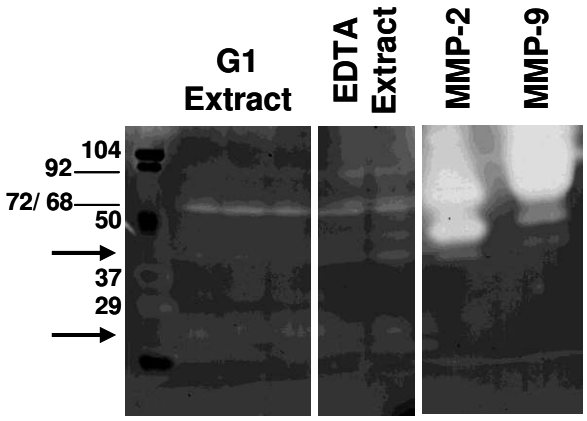

Figure 1- Gelatin zymogram of G1 extracts and EDTA extracts obtained from bovine crown dentin. Gelatinolytic activity bands could be detected at 92 kDa and 68 kDa molecular weight ranges, corresponding to latent MMP-9 and active MMP-2 as they comigrated with purified enzyme controls. Double bands at 72-68 kDa in EDTA extracts represent latent and active forms of MMP-2. Single arrows (→) indicate additional bands at lower molecular weight levels with gelatinolytic activity, probably degradation by-products of the enzyme.

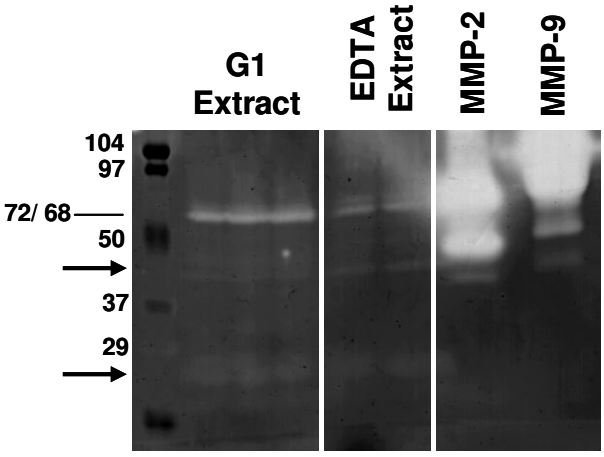

Figure 2- Zymographic analysis of bovine root dentin proteins obtained in G1 extracts and EDTA extracts. Major gelatinolytic bands can be observed at 68 kDa area, co-migrating with MMP-2 standard. Double bands at 72-68 kDa represent both latent and active forms of MMP-2 in EDTA extracts. No activity could be detected at a molecular weight range corresponding to MMP-9 standard. Single arrows (→) indicate the presence of other bands with gelatinolytic activity.

4. CONSIDERAÇÕES GERAIS

Os maiores níveis de infiltração de fluidos apresentados para o material obturador endodôntico com propriedades adesivas (Capítulo I) indicam que a hibridização do substrato dentinário radicular não ocorreu de maneira eficiente, o que provavelmente relaciona-se com as características anatômicas dos condutos radiculares (Pitout *et al.,* 2006; Sagsen *et al.,* 2006; Jainaen *et al.,* 2007), as quais propiciam o acúmulo da tensão gerada pela contração dos materiais resinosos durante a polimerização.

Tais características traduzem-se por um desfavorável fator de configuração cavitária, Fator C, que representa a relação entre as superfícies que serão submetidas à adesão e as superfícies livres, não aderidas (Feilzer *et al.,* 1987). Em um conduto de paredes longas e estreitas, como o canal radicular, esta relação é desequilibrada porque a superfície livre, sem adesão, encontra-se muito reduzida, e portanto a liberação da tensão decorrente da polimerização de materiais resinosos não ocorre de maneira suficiente. Por conseguinte, há uma grande probabilidade de ocorrerem falhas na interface de união devido à magnitude da contração de polimerização, que pode fazer com que o material se desprenda da superfície onde está aderido (Tay *et al.,* 2005b).

No contexto do sistema de canais radiculares acredita-se ser muito improvável a obtenção de um monobloco ou corpo único formado pelo material obturador e o substrato dental (Schwartz, 2006), apesar do que prometem os fabricantes dos materiais obturadores ditos “adesivos”. A fim de que funcionem como uma unidade homogênea, os materiais constituintes de um monobloco devem ser capazes de unirem-se uns aos outros e ao substrato, assim como apresentarem um módulo de elasticidade semelhante àquele da superfície à qual se encontram aderidos (Tay *et al.,* 2007). Entretanto, os valores de resistência de união relatados entre os materiais obturadores e o substrato são baixos, variando

de 1 a 3 megapascal (MPa) (Gesi *et al.,* 2005) e seu módulo de elasticidade é bem inferior àquele da dentina (Tay *et al.,* 2007).

A aparente estabilidade nas condições de selamento observada após o armazenamento dos espécimes no presente estudo pode ser atribuída às alterações dimensionais dos materiais obturadores, principalmente expansão em decorrência da absorção de água (Versiani *et al.,* 2006; Donnelly *et al.,* 2007; Bouillaguet *et al.,* 2008). Portanto, é importante interpretar com cautela tais resultados, pois a incorporação de água por um material leva, em última instância, à solubilização de seus componentes (Donnelly *et al.,* 2007), o que acarretaria em comprometimento da capacidade seladora dos mesmos quando períodos maiores de avaliação são considerados (Paqué & Sirtes, 2007).

Adiciona-se às limitações impostas pela anatomia do sistema de canais radiculares ao processo de adesão dentinária o fato de que o substrato radicular pode também apresentar potencial atividade colagenolítica intrínseca, uma vez que foi possível constatar neste estudo a presença de enzimas metaloproteinases na matriz dentinária (Capítulo II).

A detecção de MMPs na dentina radicular ganha maior relevância quando se considera a limitada eficácia do selamento promovido por materiais obturadores adesivos verificada neste estudo (Capítulo I). A pobre hibridização do tecido dentinário ou mesmo a existência de falhas entre o material obturador e as paredes do conduto radicular, além de propiciar a entrada de fluidos na interface, pode também permitir a atuação das MMPs sobre o colágeno exposto e não corretamente infiltrado por resina (Pashley *et al.,* 2004; Carrilho *et al.,* 2007a,b), deflagrando um mecanismo adicional de degradação.

A expressão de MMPs na dentina radicular não havia sido ainda confirmada. Recentemente, um estudo empregando técnicas imunohistoquímicas para a localização de MMP-2 na dentina coronária humana demonstrou intensa imuno-marcação para esta enzima na região da junção amelo-dentinária e uma diminuição da reatividade em direção à junção dentina-cemento na área cervical,

suscitando a hipótese de variações na distribuição da enzima de acordo com a localização no dente (Boushell *et al.,* 2008). Entretanto, os dados obtidos no presente estudo (Capítulo II) apontam para a presença de MMPs da matriz tanto na dentina da porção coronária quanto da porção radicular do elemento dental.

O tratamento da dentina radicular com sistemas adesivos autocondicionantes foi reportado como capaz de aumentar a atividade colagenolítica do substrato em até quinze vezes, através da provável ativação de MMPs presentes no tecido dentinário (Tay *et al.,* 2006). Este mecanismo de ativação é mediado pela queda de pH promovida pelo primer ácido dos sistemas adesivos, o que favorece a conversão de formas latentes das MMPs (zimógenos) em moléculas ativadas (Tjäderhane *et al.,* 1998; Sulkala *et al.,* 2001). Deste modo, é possível considerar que a realização de procedimentos restauradores que envolvam adesão dentinária, como a obturação endodôntica com materiais adesivos ou a cimentação adesiva de pinos retentores no interior do conduto radicular, poderia ativar as MMPs presentes no substrato. A partir de sua ativação, tais enzimas exerceriam atividade proteolítica sobre fibrilas colágenas da matriz dentinária, o que por sua vez acarretaria em implicações clínicas, como o comprometimento da união dentina-material restaurador. Tal fenômeno já foi anteriormente descrito na dentina coronária (Mazzoni *et al.,* 2006; Nishitani *et al.,* 2006) e é apontado como um dos importantes fatores que afetam a longevidade de restaurações adesivas (Carrilho *et al.,* 2007b).

Uma vez que a participação de MMPs na degradação da matriz orgânica da dentina vem sendo repetidamente confirmada, estratégias para a inibição de sua atividade são discutidas como um possível mecanismo para se alcançar maior estabilidade na união dente-material restaurador. Resultados satisfatórios foram obtidos com a aplicação de clorexidina, um conhecido inibidor de MMP-2, MMP-8 e MMP-9 (Gendron *et al.,* 1999), sobre a dentina previamente à realização de procedimentos restauradores adesivos (Hebling *et al.,* 2005; Carrilho *et al.,* 2007a,b). Nestes estudos, o tratamento da dentina com tal

substância permitiu que os níveis de resistência de união e as características morfológicas da matriz dentinária infiltrada por resina (camada híbrida) permanecessem estáveis ao longo do tempo. Dentro deste contexto é possível sugerir que a utilização da clorexidina durante a irrigação endodôntica possa trazer benefícios para a estabilidade da união de materiais restauradores à dentina radicular, hipótese que deve ser confirmada em estudos futuros.

De acordo com os dados obtidos no Capítulo III deste trabalho, que apontam para a existência de enzimas com atividade gelatinolítica no tecido dentinário bovino, espera-se que mecanismos semelhantes de degradação da matriz orgânica associados à atividade de MMPs possam ocorrer neste substrato. Isto se torna relevante perante a utilização frequente de dentes bovinos em estudos que avaliam a união de materiais restauradores à dentina (Reis *et al.*, 2004), e amplia a possibilidade de sua utilização também em estudos que acessem a longevidade da união. Do mesmo modo, uma vez que processos de degradação semelhantes à dentina humana são esperados, a investigação de medidas terapêuticas para a inibição da atividade de MMPs pode também ser planejada empregando-se o substrato dentinário bovino.

Se por um lado a presença de MMPs na dentina coronária e radicular pode ser confirmada, por outro há ainda muitas lacunas na compreensão do mecanismo exato da sua participação no processo de degradação da matriz orgânica da dentina. Estudos futuros devem ser delineados com o objetivo de esclarecer a influência dos materiais e procedimentos restauradores na atividade das MMPs. A partir do melhor entendimento destas inter-relações será possível o desenvolvimento de estratégias terapêuticas através da inibição seletiva destas enzimas.

5. CONCLUSÃO

De acordo com os resultados obtidos e dentro das limitações dos estudos realizados foi possível concluir que:

Capítulo I

- Os materiais obturadores endodônticos com capacidade de união à dentina (Epiphany/Resilon) promoveram um selamento menos efetivo do conduto radicular quando comparados aos materiais convencionais, não-adesivos (AH Plus/ guta-percha).

- O armazenamento por 180 dias não interferiu negativamente na capacidade de selamento dos materiais avaliados.

- O selamento coronário dos condutos obturados reduziu significativamente os níveis de microinfiltração.

Capítulo II

- O perfil de expressão de metaloproteinases na dentina radicular é semelhante àquele da dentina coronária, sendo possível a detecção de MMP-2, MMP-8 e MMP-9 em ambos os substratos.

Capítulo III

- O tecido dentinário bovino expressa enzimas com atividade gelatinolítica correspondente às metaloproteinases 2 e 9, entretanto há diferenças no perfil de expressão entre dentina coronária e radicular.

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APÊNDICE

1- MATERIAL E MÉTODOS – Sistema de Transporte de Fluidos

A capacidade de selamento dos materiais empregados no estudo que compõe o Capítulo I foi medida acoplando-se os espécimes a um Sistema de Transporte de Fluidos, o qual foi proposto inicialmente por Pashley & Depew (1986) e modificado por Wu *et al.* (1993) para a utilização com amostras que receberam tratamento endodôntico. Esse aparato consiste em um conjunto de capilares de polietileno, que liga uma câmara de pressão ao dispositivo para adaptação dos espécimes (Figura 1A). Os capilares são preenchidos por água destilada mantida a uma pressão constante de 10psi, controlada por um manômetro acoplado à entrada de ar comprimido. A plataforma de conexão dos espécimes ao sistema de capilares foi confeccionada inserindo-se uma agulha de 18 gauge sem bisel em um orifício preparado no centro de uma placa de acrílico de 1,8 X 1,8 X 0,7cm de dimensões, sendo essa fixada por adesivo à base de cianocrilato (Super Bonder® Gel, Loctite Adesivos, Itapevi, Brazil). Os espécimes obturados foram fixados na placa de acrílico de maneira que a porção coronária ficasse centralizada com a abertura da agulha de aço inoxidável. Externamente, além da impermeabilização prévia com esmalte para unhas, as raízes receberam vedamento adicional na porção cervical com o adesivo Super Bonder®.

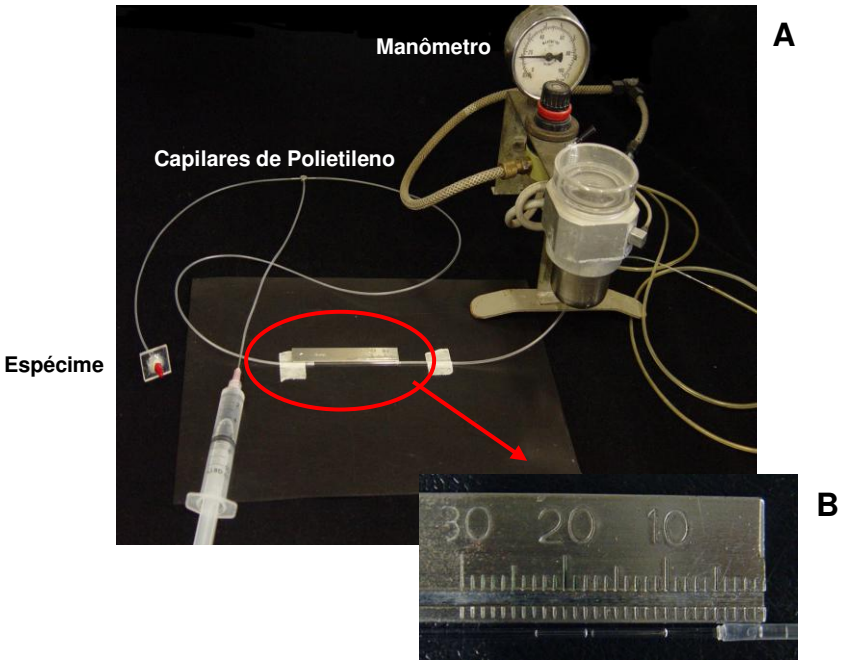


Figura 1- Ilustração do Sistema de Transporte de Fluido com seus componentes. (A) Espécime acoplado à plataforma de acrílico e ligado à câra de pressão por meio dos capilares de polietileno. (B) Bolha de ar posicionada na área de mediçao composta por um capilar de vidro justaposto a uma régua milimetrada.

A medição do transporte de fluido acontece à medida que a água destilada pressurizada penetra em eventuais falhas existentes na obturação e selamento dos espécimes. O movimento da água destilada é monitorado pelo deslocamento de uma bolha de ar inserida previamente no sistema através de uma seringa e posicionada na área de leitura da infiltração por transporte de fluido: um capilar de vidro de 65 mm de comprimento e capacidade para 25 µL, justaposto a uma escala de medição em milímetros (Figura 1B). O deslocamento linear da bolha de ar foi medido em intervalos de 2 minutos e repetidos 3 vezes para cada espécime. A partir dos valores obtidos calculou-se o Índice de Filtração de Fluido, que representa o volume de líquido deslocado em função do tempo, sendo expresso em µL/ min (Figura 2).

$$Q = \frac{25\mu l.(x)mm}{65mm\ (y)min}$$

Figura 2- Fórmula para cálculo do Índice de Filtração de Fluido (Q), onde a razão obtida ente o volume do capilar de vidro (25µL) e seu comprimento (65 mm) relaciona-se com a distância percorrida pela bolha de ar (x) e o tempo gasto para tal (y).

A qualidade do selamento foi avaliada em dois momentos distintos em um mesmo espécime: logo após a presa do cimento endodôntico e após 180 dias de armazenamento em câmara úmida a 37°C.

2- MATERIAL E MÉTODOS – Extração de Proteínas da Dentina

Protocolo de Extração sequencial, de acordo com Martin-De Las Heras *et al.* (2000)

Duas alíquotas de 2 g de dentina triturada foram processadas, sendo uma alíquota de dentina coronária e uma de dentina radicular.

Inicialmente, o pó dentinário foi lavado em 10 mL de solução de cloreto de sódio (NaCl) 2.5 M contendo inibidores de proteases (Complete Mini EDTA free, Roche Diagnostics GmbH, Mannheim, Alemanha) por 12 horas sob agitação constante para a remoção de resíduos de debris orgânicos. Ao fim da lavagem o pó dentinário foi separado da solução por centrifugação (3000 g por 5 min.) e recebeu um enxágue com água destilada.

A representação esquemática das fases que compõem o protocolo de extração está ilustrada na Figura 3.

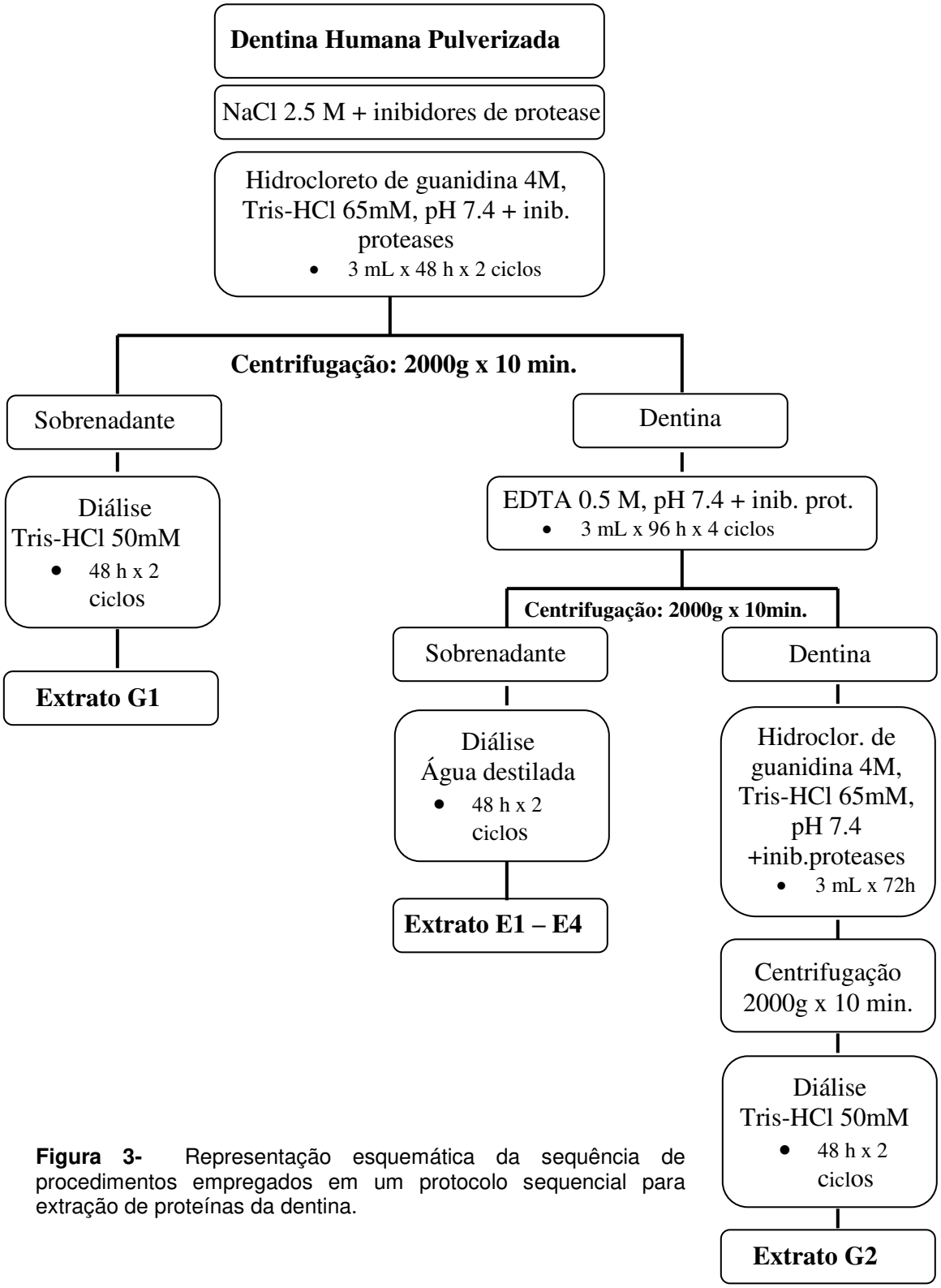


Figura 3- Representação esquemática da sequência de procedimentos empregados em um protocolo sequencial para extração de proteínas da dentina.

A primeira etapa do processo de extração foi realizada empregando-se uma solução de hidrocloreto de guanidina 4 M em Tris-HCl 65 mM, pH 7.4 acrescida de inibidores de protease. Três mililitros da solução foram adicionados a cada alíquota de pó dentinário em um béquer de vidro e mantidos sob agitação constante a 4^{o}C durante 48 h. Após centrifugação a 2000 g por 10 min. (Modelo 5810, Eppendorf, EUA) o sobrenadante foi recolhido e o pó dentinário submetido a um novo ciclo de extração com hidrocloreto de guanidina . Os sobrenadantes obtidos após centrifugação foram dialisados em uma solução de Tris-HCl 50 mM, pH 7.4 contendo inibidores de proteases durante 4 dias, com renovação da solução a cada 48 h, seguido de diálise por mais 24 h em água destilada. Ao fim do processo, o extrato obtido, denominado G1, teve seu conteúdo protéico dosado pelo método de Lowry e foi divido em alíquotas de 60 µg de proteínas, as quais foram liofilizadas e armazenadas a -20^{o}C para posterior utilização (Figura 4).

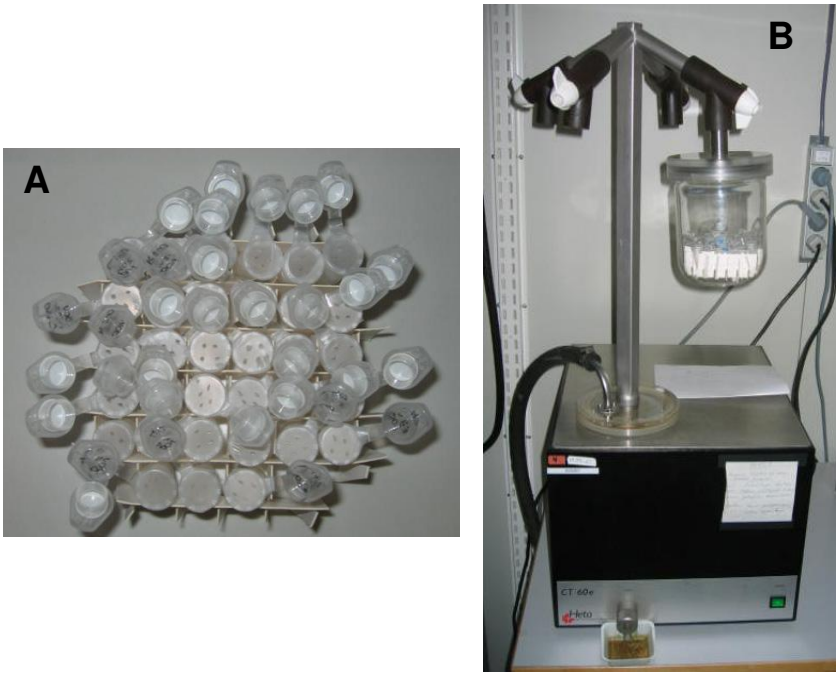


Figura 4- (A) Divisão em alíquotas da solução contendo proteínas extraídas da dentina. (B) Alíquotas posicionadas no aparato para liofilização.

O pó dentinário recuperado após os procedimentos de extração com hidrocloreto de guanidina foi submetido à segunda etapa do processo, que consiste na extração de proteínas associadas à matriz dentinária mineralizada. Para isso realizou-se a desmineralização da amostra com 3 mL de solução de ácido etilenodiaminotetracético (EDTA) 0.5 M, pH 7.4 acrescida de inibidores de proteases, a qual foi utilizada em 4 ciclos de 96 h, sob agitação constante a uma temperatura de 4^{o}C. Ao fim de cada ciclo o sobrenadante foi recuperado por centrifugação (2000 g, 10 min.), recebendo denominação extrato E1, E2, E3 ou E4 de acordo com a sequência de extração com EDTA. Cada extrato foi dialisado em água destilada contendo inibidores de proteases por 4 dias e após dosagem do conteúdo protéico procedeu-se a divisão em alíquotas de 60 µg de proteínas, liofilização e armazenagem a -20^{o}C (Figura 2).

Por fim, a dentina desmineralizada remanescente foi novamente submetida ao tratamento com solução de hidrocloreto de guanidina 4 M em Tris-HCl 65 mM, pH 7.4 para extração de proteínas associadas ao colágeno. Três mililitros da solução foram adicionados à amostra, permanecendo sob agitação constante a 4^{o}C por 72 h. Ao fim do ciclo, o sobrenadante (denominado extrato G2) foi recuperado por centrifugação e processado do mesmo modo descrito para o extrato G1.

ANEXOS

1- Certificado do Comitê de Ética em Pesquisa – FOP/UNICAMP

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 "**Análise imediata e em longo prazo da qualidade do selamento de canais radiculares obturados com um material à base de resina**", protocolo nº **119/2005**, dos pesquisadores **JULIANA NASCIMENTO SANTOS e MARCELA ROCHA DE OLIVEIRA CARRILHO**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 28/11/2005.

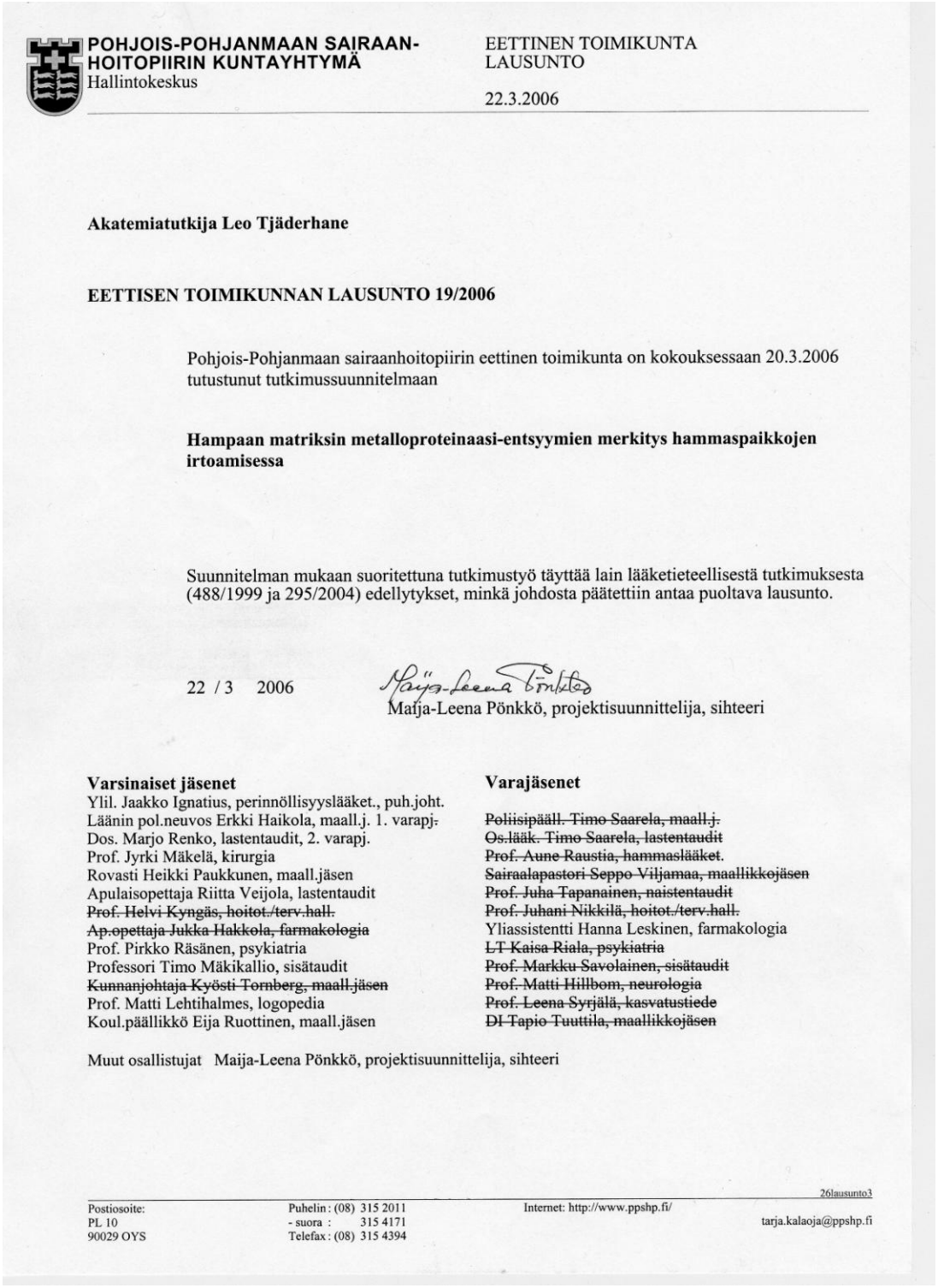
The Research Ethics Committee of the School of Dentistry of Piracicaba - State University of Campinas, certify that project "**Immediate and long-term analysis of the ability of a resin-based material to seal the root canal system**", register number **119/2005**, of **JULIANA NASCIMENTO SANTOS and MARCELA ROCHA DE OLIVEIRA CARRILHO**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for researching in human subjects and was approved by this committee at 28/11/2005.

Cinthia Pereira Machado Tabchoury
Secretária
CEP/FOP/UNICAMP

Jacks Jorge Júnior
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.

2- Certificado do comitê de Ética em Pesquisa – Northern Ostrobothnia Hospital District



POHJOIS-POHJANMAAN SAIRAANHOITOPIIRIN KUNTAYHTYMÄ
Hallintokeskus

EETTINEN TOIMIKUNTA
LAUSUNTO

22.3.2006

Akatemiatutkija Leo Tjäderhane

EETTISEN TOIMIKUNNAN LAUSUNTO 19/2006

Pohjois-Pohjanmaan sairaanhoitopiirin eettinen toimikunta on kokouksessaan 20.3.2006 tutustunut tutkimussuunnitelmaan

Hampaan matriksin metalloproteinaasi-entsyymien merkitys hammaspaikkojen irtoamisessa

Suunnitelman mukaan suoritettuna tutkimustyö täyttää lain lääketieteellisestä tutkimuksesta (488/1999 ja 295/2004) edellytykset, minkä johdosta päätettiin antaa puoltava lausunto.

22 / 3 2006

Maija-Leena Pönkkö, projektisuunnittelija, sihteeri

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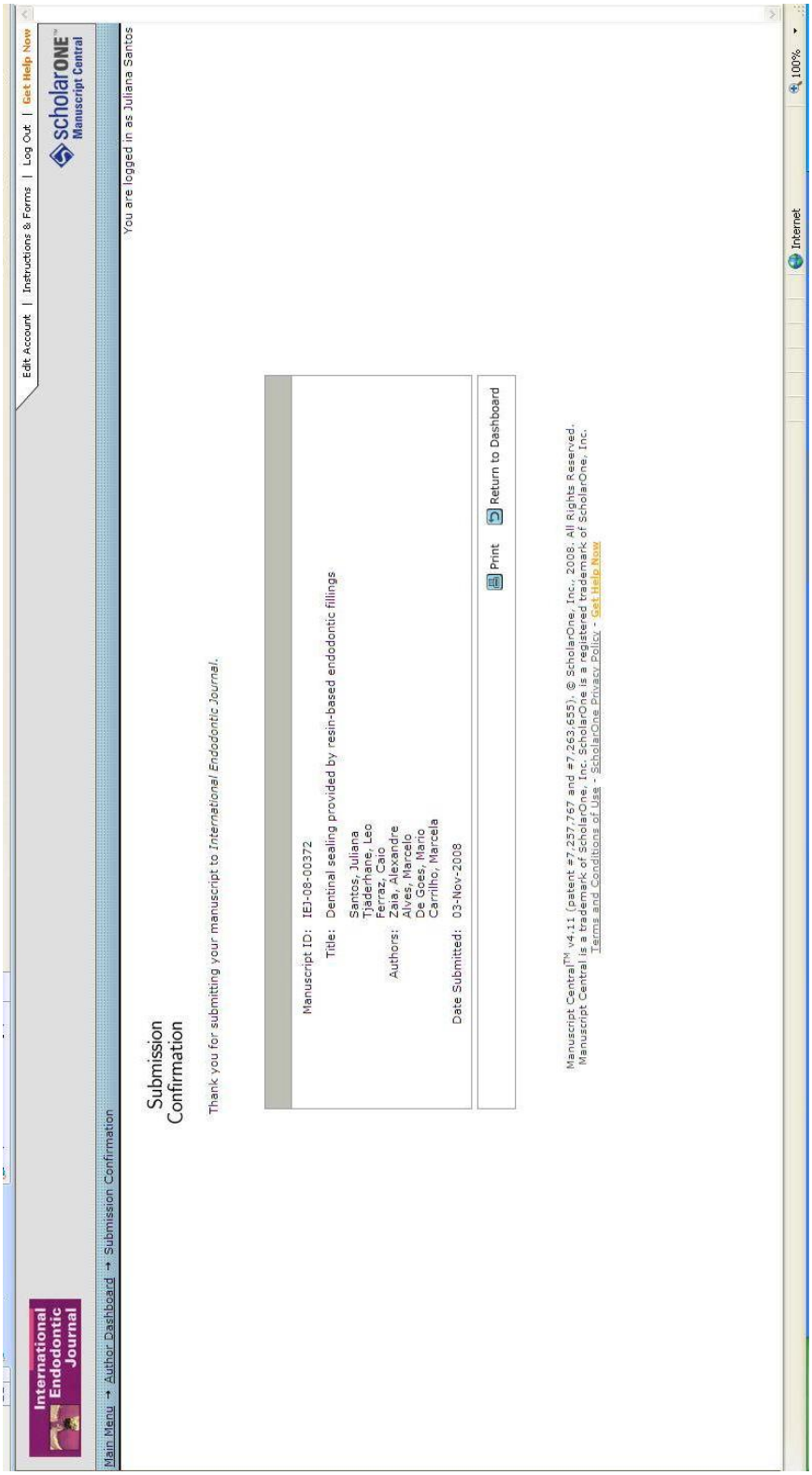
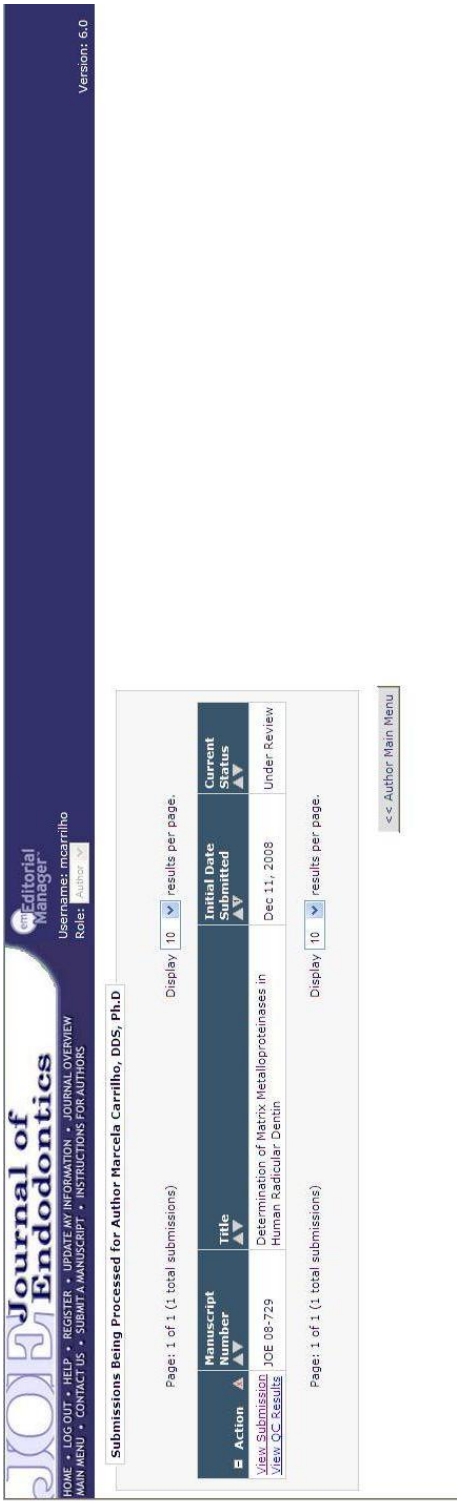
3- Comprovação da submissão do artigo referente ao capítulo I



4- Comprovação da submissão do artigo referente ao capítulo II



5- Declaração

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DECLARAÇÃO

As cópias de artigos de minha autoria ou minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Doutorado, intitulada “Avaliação do selamento do conduto radicular por materiais obturadores endodônticos resinosos e análise da expressão de metaloproteinases na matriz dentinária”, não infringem os dispositivos da Lei n° 9.610/98, nem o direito autoral de qualquer editora.

Piracicaba, 10 de Janeiro de 2009

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