UNIVERSIDADE SAGRADO CORAÇÃO

BRUNO LARA ZARELLA

PERFIL E ATIVIDADE DAS METALOPROTEINASES DA MATRIZ EM DENTINA HUMANA E BOVINA

BAURU

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Monografia de iniciação científica apresentada ao centro de ciências biológicas e profissões da saúde como parte dos requisitos para obtenção do título de bacharel em ciências biológicas, sob orientação de Profa. Dra. Marília Afonso Rabelo Buzalaf.

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Prof. Ms. Alexandre Bechara

Bauru, 03 de dezembro de 2010.

Dedico este trabalho aos meus pais e meus irmãos, os quais sempre me apoiaram e acreditaram em meus sonhos e meu potencial.

AGRADECIMENTOS

Primeiramente queria agradecer a minha banca, e dizer que para mim é um privilégio ter profissionais de tal importância fazendo minha avaliação. Profa. Dra. Dulce Constantino além de minha professora minha enorme amiga, me orientando, ensinando, brigando, e elogiando. Prof. Ms. Alexandre Bechara, meu amigo com a maiúsculo, o qual me encaixou em sua amizade e turma quando entrei na faculdade e não conhecia ninguém, me dando apoio, e muitos momentos felizes, jamais irei deixar de ter você em meu coração meu amigo “Pelex”. Minha querida orientadora Profa. Dra. Marília Afonso Rabelo Buzalaf, você imagina a importância que tem em minha vida? Dando uma oportunidade a um estagiário qualquer, o transformando em um pesquisador, você é muito importante para mim, e sempre que tenho oportunidade eu tenho que dizer, você é uma das mulheres mais importantes em minha vida e para mim é um prazer tê-la como minha orientadora, digo isso com a boca cheia e com o peito estufado. Muito obrigado a todos vocês.

Meus agradecimentos são divididos em alguns grupos, grupo dos quais jamais iria conseguir atingir tal momento sem suas presenças.

O primeiro grupo é o familiar, pai, mãe, Felipe, Camila, muito obrigado por existirem em minha vida, como é bom ter vocês como minha família, como é bom saber que irei chegar em casa e encontrar vocês. Mãe, você é minha heroína, um exemplo de mulher, sempre forte, sempre disposta a me ajudar, um doce, uma maravilha, uma mão com qual sempre pude e sei que sempre poderei contar. Pai, o que falar de você meu pai, você é o melhor ser que já pude conhecer! Inteligente, elegante, justo, trabalhador e meu herói, espelho no qual quero sempre me refletir. Felipe, Camila, que irmãos maravilhosos que ganhei, brigas existem, claro, mas só servem para nos unir mais ainda. Queria muito agradecer vocês quatro primeiro, vocês são a razão do meu viver. Também agradeço a todos os meus familiares nos quais enchem os olhos de brilho quando conto minhas conquistas e me dão apoio quando conto alguma coisa que está dando errado. Meus tios, meus primos, minhas primas, muito obrigado, agradeço demais a todos vocês, sempre estarão em meu coração e em meus pensamentos.

O segundo grupo eu junto o profissional com a amizade. Meus amigos e minhas amigas, alguns deles presentes em minha apresentação, outros infelizmente não puderam estar, mas sei que estão torcendo demais por mim, e como sempre, estão me apoiando. O que é da vida sem os amigos? Pra mim não seria nada, com eles que adquiri forças para agüentar os momentos mais difíceis e com eles que comemoro as vitórias. Citar nome neste caso ficaria complicado, pois com toda a certeza algum nome irá escapar, as mesmo assim alguns nomes tenho que citar: Léo, Gui, Gutão, Rafa, Renan, Yury, vocês são amigos dos quais jamais esquecerei. Também faço um agradecimento a todos que sabem que são meus amigos. Profissional, o porquê juntar profissional com amigos, minhas queridas co-orientadoras Aline, Angélica, Melissa, Angela, Larissa? Porque vocês não são apenas colegas de trabalho, vocês são minhas amigas de trabalho, minhas anjas da guarda que tenho o prazer de trabalhar ao lado de vocês, obrigado por tudo que me ensinaram e muito obrigado por terem estendido a mão para mim, vocês são as responsáveis pelo garoto Bruno se tornar o homem Bruno, muito obrigado.

Claro que não poderia deixar de falar dela, a mulher na qual convivo à três anos e espero viver para o resto de minha vida. Tatiane, você sabe o quanto você é especial para mim certo? Você é uns dos pilares no qual me sustento. Sempre me apoiando, brigando comigo na hora que tem que brigar, chorando comigo nos momentos tristes e rindo comigo nos momentos certo, você é um anjinho que apareceu em minha vida! Obrigado minha linda, muito obrigado por tudo o que você fez por mim. E claro, ao falar de você eu também tenho que agradecer seus pais, os quais considero meus pais também! Seres maravilhosos sempre prontos a me ajudar, então Dona Sônia e Senhor Bigoto, vocês jamais iriam passar em branco em meus agradecimentos.

Para encerar, eu tinha que falar de duas pessoas, as quais infelizmente não estão mais ao meu lado. A minha anja da guarda Juracy, minha avó da qual sinto muita falta, mas sei que ela esta do meu lado me protegendo e me orientando nas melhores escolhas e sem ela muita coisa não seria possível, umas das mãos que foram estendidas a minha família no momento mais difícil de nossas vidas, Jura, sem você eu não estaria aqui! E o meu tio Plínio, esse tio maravilhoso que sempre me ensinou tudo do melhor, sempre acreditando em seu sobrinho e dando muita força para todas as minhas escolhas, me

ensinando até mesmo na hora de sua morte! Saudades de vocês dois, mas sei que vocês estão do meu lado neste momento, então agradeço demais a vocês, vocês são muito especiais para mim e por isso sinto tanta falta. Obrigado meu tio querido, obrigado minha avó maravilhosa meu anjinho da guarda, saudades de vocês!

“Toda ação humana, quer se torne positiva ou negativa, precisa depender de motivação.” (Dalai Lama)

RESUMO

As metaloproteinases de matriz são endopeptidases responsáveis pela degradação ou reabsorção de todos os compostos (componentes) da matriz extracelular. Elas estão envolvidas no desenvolvimento do esmalte dentário humano e remodelamento da matriz orgânica da dentina. As MMPs -2,-8,-9 já foram identificadas em dentes humanos. Desde que, a dentina bovina é frequentemente utilizada em estudos *in vitro* e *in situ* como substituta para dentina humana, o objetivo deste estudo foi avaliar a expressão e atividade das MMPs-2 e MMPs-9 presentes em dentina humana e bovina. Blocos de incisivos bovinos e molares humanos foram obtidos de raízes e coroas. Foram, então, triturados utilizando-se um moinho criogênico. O pó de dentina foi desmineralizado em ácido cítrico a 0,87M pH 2,3 por 24 h a 4° sob agitação. Após a centrifugação, o pó desmineralizado de dentina foi suspendido em um tampão de extração e sonificado três vezes. As amostras foram incubadas por 24 h no tampão de extração. Após a centrifugação, o sobrenadante foi coletado e o total de concentração de proteínas foi medido pelo método de Lowry. O gel de zimografia foi realizado. O total de MMPs 2 e 9 ativas foi determinado através do teste ELISA. Atividades gelatinolíticas das MMP-2 e MMP-9 foram detectadas nos extratos de dentina da coroa e da raiz tanto de origem humana quanto bovina. O total de MMP-2 ativa foi 14,85 ng/ml para raiz bovina, 20,16 ng/ml para raiz humana, 14,71 ng/ml para coroa bovina e 22,92ng/ml coroa humana. Os valores correspondentes para MMP-9 foram 16.71ng/ml para raiz bovina, 19,95 ng/ml para raiz humana, 21,97 ng/ml para coroa bovina e 19,46 ng/ml para coroa humana. Os resultados indicam que os tipos de substratos de dentina testados podem ser utilizados em estudos envolvendo o desempenho das MMPs 2 e 9.

Palavras chaves: dente bovino. dente humano. dentina. matriz metaloproteinases.

ABSTRACT

Matrix metaloproteinases (MMPs) are endopeptidases responsible for the degradation or resorption of all components of the extracellular matrix (ECM). They are involved in the development of human dental enamel and remodeling of dentin organic matrix. MMPs -2, -8 and -9 have already been identified in human dentin. Since bovine dentin is frequently employed in *in vitro* and *in situ* studies as surrogate for human dentin, the purpose of the present investigation was to evaluate the expression and activities of MMP-2 and -9 present in human and bovine dentin. Blocks from bovine incisors and human molars were obtained from roots and crowns. They were pulverized using a cryogenic mixer mill. Dentin powder was demineralized in 0.87 M citric acid pH 2.3 for 24 h at 4℃ under stirring. After centrifugation, the demineralized dentin powder was suspended in extraction buffer and sonicated 3 times. Samples were incubated for 24 h in extraction buffer. After centrifugation, the supernatant was collected and total protein concentration was measured by Lowry's Method. Gelatin zymography was performed. Total activity of MMPs -2 and -9 was determined by ELISA. Gelatinolytic activities identified as MMP-2 and MMP-9 were detected in extracts of crown and root dentin samples both from human and bovine origin. Total activities of MMP-2 were 14.85, 20.16, 14.71 and 22.92 ng/mL for bovine root, human root, bovine crown and human crown, respectively. The correspondent figures for MMP-9 were 16.71, 19.95, 21.97 and 19.46 ng/mL, respectively. The results indicate that all kinds of dentin substrates tested can be used in studies involving the performance of MMPs -2 and -9.

Key Words: Dentin; MMP; Bovine tooth; Human tooth

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SUMÁRIO

1 INTRODUÇÃO, JUSTIFICATIVA E OBJETIVO

As metaloproteinas de matriz (MMPs) constituem uma importante família de endopeptidases metalodependentes e representam a maior classe de enzimas responsáveis pela degradação ou reabsorção de todos os componentes da matriz extracelular (MEC) (Souza; Line, 2002). Essas enzimas regulam a maior parte dos processos de desenvolvimento, homeostase e doença (Sternlicht; Werb, 2001), sendo estas sintetizadas como zimogênios inativos que requerem ativação (Harper et al., 1971). As MMPs apresentam propriedades estruturais funcionais diferentes, porém todas exibem capacidade de degradar uma ou mais moléculas constituintes da matriz extracelular. Normalmente, estas moléculas são secretadas como zimogênios e encontram-se desativadas por inibidores específicos (Nagase e Woesner, 1999).

As MMPs estão envolvidas tanto em eventos fisiológicos como patológicos e estão amplamente ativas nos processos biológicos. As colagenases (MMP-1, -8 e -13) possuem a função de degradar colágeno intersticial dos tipos I, II e III, sendo também capazes de digerir outras moléculas da MEC. Já as gelatinases (MMP-2 e -9) promovem a desnaturação da gelatina de matriz (Massova et al., 1998).

Muitos estudos indicam que as MMPs possuem um papel fundamental no desenvolvimento e remodelação dos tecidos dentários. De acordo com Bartlett et al. (2004), as MMPs estão envolvidas no desenvolvimento de esmalte dentário, bem como no processo da fluorose dentária. As MMPs também estão associadas com a remodelação da matriz orgânica da dentina (Hall et al., 1999, Martin de Las Heras, 2000, Sulkala et al., 2002). Tem sido demonstrado que a ativação das MMP-2 e MMP-9 desempenha um papel crucial na degradação de colágeno nas lesões cariosas (Tjäderhane et al., 1998, Sulkala et al., 2002). As MMPs têm sido identificadas em processos inflamatórios da polpa e região apical (Wahlgren et al., 2002, Gusman et al., 2002) e doenças periodontais (Van Der Zee et al., 1996, Ejeil et al., 2003). Elas são componentes essenciais no crescimento e invasão de tumores da boca (Sorsa et al., 2004). E, finalmente, as MMPs podem ser importantes na perda da adesão de restaurações adesivas com o decorrer do tempo (Pashley et al.,

2004, Tay, Pashley, 2004, Hebling et al., 2005, Carrilho et al., 2007a,b, García-Godoy et al., 2007).

A matriz dentinária é uma completa rede de fibrilas e estruturas globulares composta principalmente por colágeno tipo I, além de proteoglicanas e outras proteínas não colagenosas, formando assim, um arcabouço para a deposição mineral (Marshall et al., 1997, Linde and Goldberg, 1993; Butler, 1995; Embery et al., 2001; Goldberg, Smith, 2004). A MMP-2 foi isolada a partir da matriz dentinária humana (Martin-De Las Heras et al., 2000) e sua atividade foi verificada em dentina desmineralizada (van Strijp et al., 2003). Mais recentemente, MMP-8 e MMP-9 também foram caracterizadas a partir da dentina humana (Sulkala et al. 2007, Mazzoni et al. 2007).

Devido à dificuldade crescente para a obtenção de dentes humanos, dentes bovinos têm sido freqüentemente utilizados em estudos *in vitro* e *in situ* para simular o comportamento de dentes humanos. A comparação das forças de ligação promovidas por um sistema adesivo ao esmalte e dentina humana e bovina tem mostrado que os dentes bovinos podem ser possíveis substitutos para os dentes humanos, tanto em testes de adesão em esmalte e dentina (Reis et al., 2004; Krifka et al., 2008). Dentes bovinos têm sido também utilizados extensivamente em estudos de erosão (Rios et al., 2006a,b; Vieira et al., 2006; Francisconi et al., 2008). O uso de incisivos bovinos como substitutos para dentes humanos para investigações em estudos envolvendo erosão/abrasão é bem aceito, uma vez que não há diferenças significativas no desgaste de dentina de terceiros molares humanos quando comparados com incisivos bovinos (Wegehaupt et al., 2008). Quando a radiodensidade e a dureza de esmalte e dentina humana e bovina foram comparadas e avaliadas em relação à idade do dente bovino, observou-se que a radiodensidade é similar para o esmalte, mas a dentina bovina apresentou uma radiodensidade maior que a humana, independentemente dos grupos etários, o que poderia ser atribuído à maior quantidade de dentina peritubular observada nos dentes bovinos (Fonseca et al., 2008). Tem-se demonstrado ainda que a dentina coronária bovina é similar à dentina humana em relação ao número e diâmetro dos túbulos dentinários (Schilke et al., 2000). Com relação às características de permeabilidade transdentinária, a dentina bovina próxima à junção amelo-

cementáriapara ser uma alternativa viável para substituir a dentina coronária humana para testes *in vitro* (Schmalz et al., 2001).

Entretanto, pouco se sabe acerca de outras características dos dentes bovinos, como os componentes da matriz orgânica e quanto diferentes ou similares estes componentes são em relação aos dentes humanos. Até o momento, há poucos estudos comparando o perfil de MMPs em dentes humanos e bovinos. A atividade gelatinolítica tem sido detectada em tecido radicular bovino em reabsorção (Linsuwanont et al., 2002). Sabe-se ainda que o processamento proteolítico mediado pela MMP-2 é um importante passo na aceleração do processo de maturação da matriz de dentina bovina, que inclui a forforilação e subseqüente mineralização (Satoyoshi et al., 2001). Apesar de as MMPs-20 de várias espécies apresentarem uma homologia de aproximadamente 80% com a humana (Caterina et al., 2000) e de as MMPs-20 recombinante humana e bovina possuírem muitas similaridades, algumas diferenças existem entre as mesmas, como a velocidade de auto-ativação e a preferência pelo substrato (Zhu et al., 2008).

Tem sido desenvolvido uma série de estudos envolvendo a utilização de inibidores de MMPs para redução da progressão de erosão em dentina (Kato et al., 2009 e 2010a,b; Magalhães et al., 2010) A realização destes estudos seria bastante facilitada se utilizássemos dentes bovinos nesses protocolos, mas ainda não se sabe sobre a similaridade ou não entre as MMPs presentes em ambos os substratos. O presente projeto visa a contribuir neste sentido, tendo como objetivo avaliar o perfil e atividade das MMPs-2 e -9 presentes em dentina humana e bovina. Se houver similaridades nos perfis observados para ambos os substratos dentários, então os dentes bovinos poderão ser utilizados com segurança como substitutos para os humanos em estudos envolvendo a atividade de MMPs dentinárias.

2 MATERIAL E MÉTODO

2.1 Procedimentos realizados na FOB - USP

2.1.1 Obtenção e preparo da amostra

Para a realização deste estudo foram utilizados incisivos bovinos obtidos do Frigorífico Vangélio Mondelli Ltda (Bauru-SP) e terceiros molares obtidos nas clínicas de cirurgia da FOB-USP, após aprovação pelo CEP Institucional (Proc. 128/2008). Após obtenção, os dentes permaneceram armazenados em recipientes de vidro, contendo solução de timol, em temperatura ambiente por no mínimo 6 meses.

Os dentes foram fixados com godiva termoativada (Kerr Corporation, EUA) numa placa de acrílico (40 mm X 40 mmm X 5 mm) para facilitar a adaptação posterior na máquina de corte. A placa de madeira foi parafusada em um aparelho de corte de precisão ISOMET Low Speed Saw (Buehler Ltd., Lake Bluff, IL, EUA) e com auxílio de dois discos diamantados (Isomet 1000; Buehler, Lake Bluff, IL, EUA) e um espaçador de 4 mm, foram obtidas fatias com a mesma espessura, tanto da raiz quanto da coroa de dentes humanos (RH e CH, respectivamente) e bovinos (RB e CB, respectivamente), como demonstrado na figura 1a. Em seguida, a superfície de esmalte foi totalmente removida, utilizando-se uma lixa (figura 1b). Os fragmentos de dentina foram então triturados em um moedor convencional de bolas (VEB Leuchtenbau 4600, Alemanha) (figuras 1c e 1d) e o pó de dentina congelado até sua utilização.

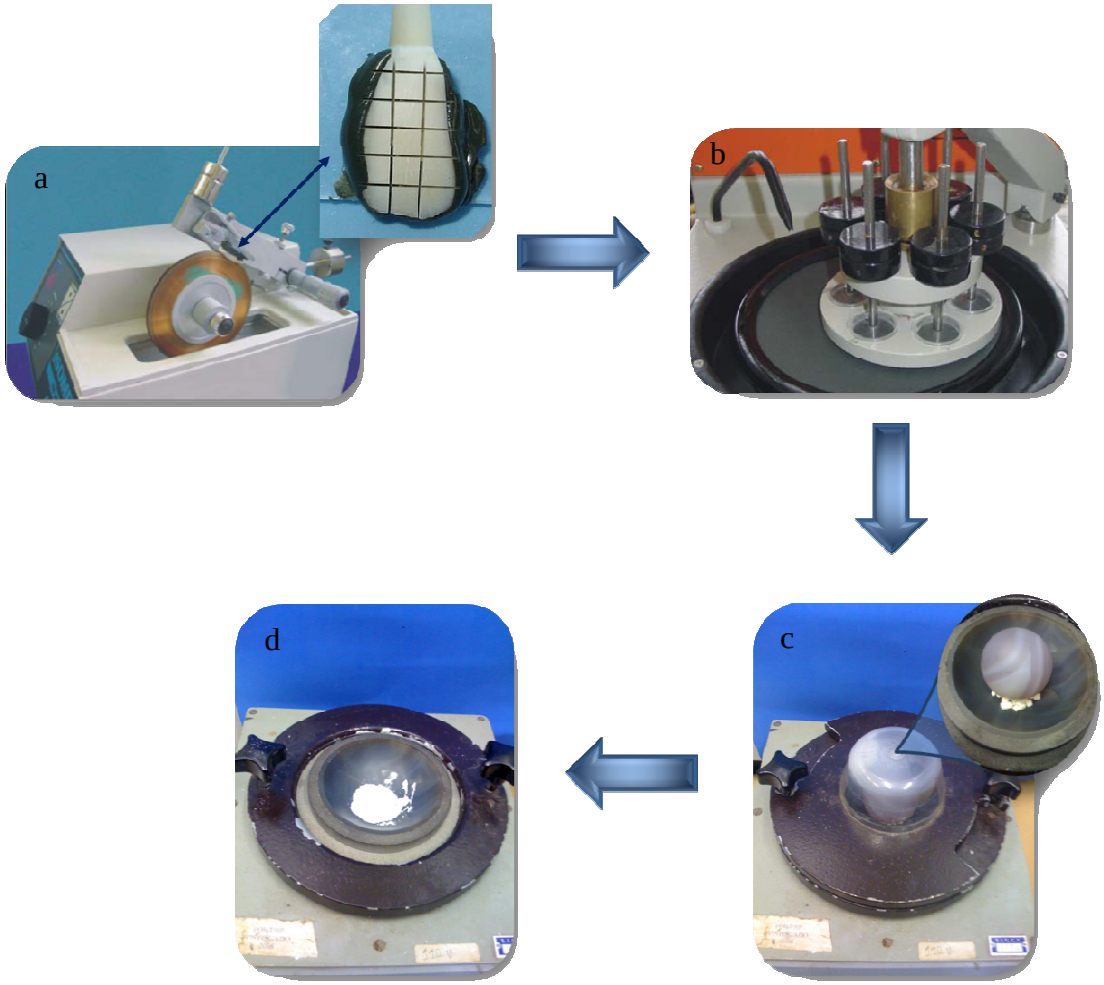


Figura 1 Resumo das etapas realizadas para obtenção do pó da dentina.

2.1.2 Extração das proteínas

Aproximadamente 1 g de pó de dentina de raiz ou coroa, humana e bovina foram desmineralizados com 10 mL de solução de ácido cítrico 0,26 M a 4℃ por 24 h, sob agitação (Mazzoni et al., 2007). Em seguida, as amostras foram centrifugadas a 8000 rpm (Sorval RC2-B, Waltham, MA, USA) e o pó de dentina desmineralizado lavado duas vezes em água gelada sob agitação em vórtex e centrifugado a 2000 rpm, por 1 min. Após as lavagens, o pó foi ressuspenso em tampão de extração contendo Tris-HCl a 50 mM pH 6,0, $CaCl_2$

a 5 mM, NaCl a 100 mM, Triton X-100 a 0,1%, NONIDET P-40 0,1%, $ZnCl_2$ a 0,1 mM, NaN_3 0,02% e inibidores de protease.

As amostras foram então submetidas ao ultrassom a 30-40 W (Branson Sonic Power Co e Smithkline company, Modelo Sonifier Cell Disruptor, Danbury, USA), sendo feitas 3 exposições de 20 s cada a 4℃, com intervalo de 2 min. Em seguida, os tubos foram centrifugados a 18000 rpm por 30 min a 4℃ e o sobrenadante foi coletado. Para precipitação das proteínas foi adicionado ao sobrenadante sulfato de amônio para a concentração final de 85%, incubando-se por 30 min a 4℃. Após este procedimento, as amostras foram centrifugadas a 18000 rpm por 30 min a 4℃. A figura 2 demonstra os passos citados. Ao final deste procedimento, um *pellet* deveria se formar, o que não aconteceu.

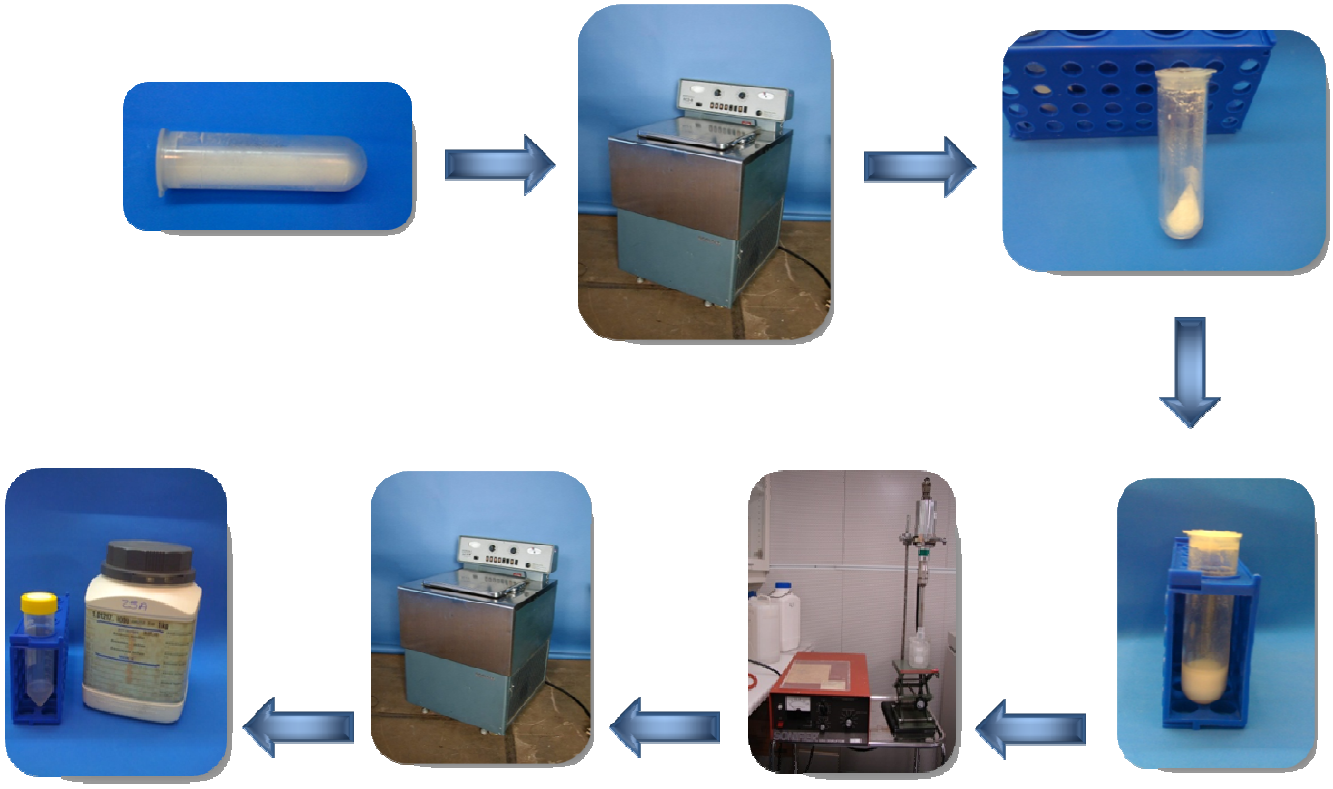


Figura 2 Procedimentos para obtenção do pellet.

A hipótese para tal foi que o moinho utilizado não foi adequado, pois o mesmo não apresentava controle de temperatura, e o calor gerado no atrito poderia ter resultado na desnaturação das proteínas. Um dos pontos importantes no preparo da amostra para a técnica de zimografia é que as amostras jamais devem ser extraídas sob condições redutoras, pois isso acarreta na perda da estrutura nativa da proteína, impedindo que a mesma seja ativada. O calor é uma condição redutora e por esse motivo decidimos repetir o experimento utilizando um moinho criogênico (Fisher Scientific, Modelo Spex SamplePrep 6770, EUA), Além desta alteração, o artigo de Mazzoni et al. (2007), há uma informação contraditória, onde na seção de materiais e métodos dizia-se que a concentração do ácido cítrico era 0,26 M enquanto que na tabela fornecida nos resultados, a informação dada era que a concentração do ácido era de 0,87 M. Diante disto, novamente foi repetido o experimento com o ácido cítrico a 0,87 M. Porém, a formação do *pellet* novamente não aconteceu.

Diante destas tentativas infrutíferas, suspeitou-se que as amostras poderiam ser o problema, já que tinham sido preparadas a partir de dentes antigos (~1 ano de armazenamento), e como relatado na literatura, a quantidade da MMP-2 em dentina é reduzida com o tempo (Martin de Las Heras et al., 2000). Outra hipótese foi que as amostras poderiam não ter sido corretamente conservadas, uma vez que foram mantidas em timol e à temperatura ambiente por longo tempo (6 meses no mínimo), o que poderia ter causado degradação das proteínas.

O próximo passo foi, então, a busca por amostras frescas, que foram coletadas diretamente do frigorífico citado anteriormente. Cinquenta incisivos bovinos frescos foram obtidos de animais jovens, os quais são usualmente abatidos no frigorífico Mondelli. Os dentes foram coletados, limpos e imediatamente armazenados em azida sódica 0,2% a 4 ℃. Da mesma maneira, cerca de 50 terceiros molares humanos foram obtidos de voluntários jovens, com idade entre 18 e 25 anos, da clínica de cirurgia da FOB-USP. No dia após a coleta, os dentes eram cortados e lixados para remoção do esmalte, como feito para os blocos anteriores. Os novos fragmentos de dentina foram

então triturados em moinho criogênico (Fisher Scientific, Modelo Spex SamplePrep 6770, EUA) e o pó de dentina foi liofilizado para conservação da amostra até o dia da sua utilização. As amostras foram divididas em quatro grupos: raiz bovina (RB), raiz humana (RH), coroa bovina (CB) e coroa humana (CH).

Os procedimentos de extração e quantificação foram realizados na Universidade de Oulu, Finlândia, sob supervisão do prof. Leo Tjäderhane. O aluno de iniciação científica Bruno Zarella (FAPESP, Proc. 2008/08379-0) teve a oportunidade de se deslocar até esta universidade junto com a co-orientadora deste projeto e descrito a seguir.

2.2 Procedimentos realizados na Universidade de Oulu, Finlândia

2.2.1 Extração das proteínas a partir de amostras recém preparadas

De posse de amostras novas, cerca de 1 g de pó de dentina de raiz ou coroa, humana ou bovina foi desmineralizado com 10 mL de solução de ácido cítrico ou ácido acético a 0,26 M e 0,87 M pH 2,3 a 4℃ por 24 h, sob agitação (Mazzoni et al., 2007), pois as informações referentes às concentrações dos ácidos testados eram contraditórias. A formação de *pellet* obtida a partir da desmineralização por ácido cítrico a 0,87 M foi a que apresentou os melhores resultados. Sendo assim, para o protocolo experimental foi utilizado o ácido cítrico a 0,87 M para desmineralização do pó de dentina.

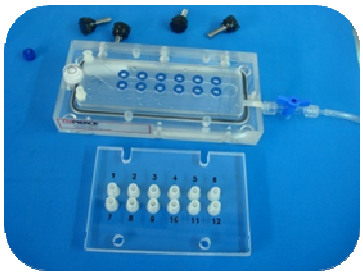
O protocolo experimental foi, portanto, adaptado de Mazzoni et al., 2007. O pó de dentina desmineralizada foi suspenso em tampão de extração (Tris-HCl a 50 mM e pH 6,0, $CaCl_2$ a 5 mM, NaCl a 100 mM, Triton X-100 a 0,1%, NONIDET P-40 0,1%, $ZnCl_2$ a 0,1 mM, NaN_3 0,02%) e coquetel de inibidores de proteases livre de EDTA (Roche Diagnostics, Indianapolis, IN, EUA). As amostras foram tratadas com ultrassom a 30-40 W (Branson Sonic Power Co e Smithkline company, Modelo Sonifier Cell Disruptor, Danbury, USA), sendo feitas 3 exposições de 20 s cada a 4℃, com interva lo de 2 min. Os tubos foram centrifugados a 18000 rpm (19900 X g) por 30 min a 4℃ e o sobrenadante foi coletado. A este sobrenadante foi adicionado sulfato de amônio 85% para a

precipitação das proteínas e feita incubação *overnight* (4℃). A seguir, as amostras foram centrifugadas a 18000 rpm (19900 X g) por 30 min a 4℃. O sobrenadante foi descartado e o *pellet* foi redissolvido em um tampão de extração diluído 10X, e dialisado através de uma membrana para microdiálise de 50 kDa (Thermo Scientific, Rockford, IL, EUA) por 24 h (Esquema 1), e por fim foram as amostras foram estocadas a 4℃ até aná lise.

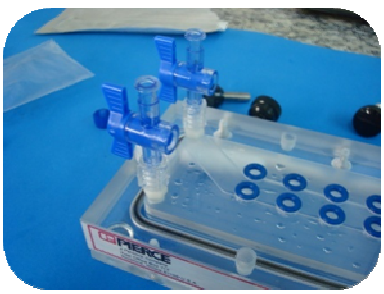
Esquema 1 - Protocolo para diálise

1 –Anexar uma válvula borboleta na porta de saída (fica sozinha), e então anexar um adaptador nesta mesma borboleta, para poder encaixar a mangueira. Encaixar a mangueira e certificar que a válvula está fechada.

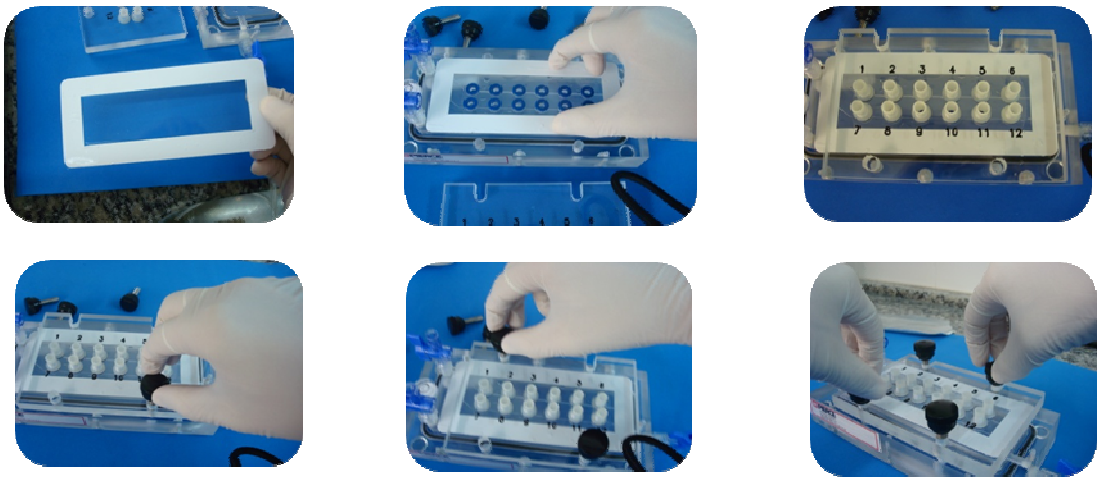
2- Remover a tampa de acrílico e os parafusos, em seguida retirar com muito cuidado a *sample well* (placa de poços numerados) para não remover os anéis de silicone.



3 – Encaixar as outras duas borboletas nos dois encaixes, situado perto da roda de nível, mantendo-as fechadas.

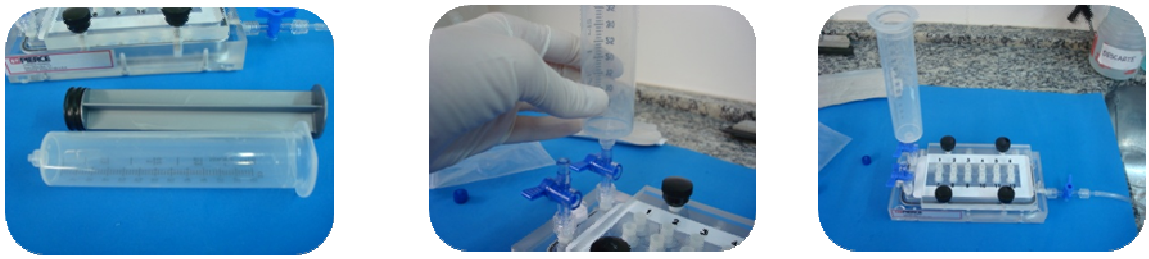


4 – Remover a membrana do pacote e lave-as em H_2O deionizada e destilada. Colocar a membrana com a face voltada para baixo, sobre os anéis de silicone (rodinhas azuis) e recolocar a *sample well* com cuidado para não danificar a membrana, e então os parafusos. Aperte-os levemente de dois em dois na diagonal, alternadamente.



Apertar dois parafusos em diagonal de cada vez.

5 – Retirar o embolo da seringa e anexá-la em uma borboleta de entrada. Em seguida colocar a solução a ser utilizada na diálise. A solução deve estar na mesma temperatura em que as amostras serão dialisadas.



6 – Posicionar um objeto embaixo do equipamento para que ele fique com um ângulo de 30-45°. Coloque então um Becker ao fim da mangueira para recolher o resíduo da diálise e abra a válvula de saída.

7 – Abrir a válvula de entrada, que está conectada à seringa e deixar a solução fluir devagar para a cavidade do sistema. O objetivo é preencher a cavidade do sistema sem deixar formar bolhas de ar. Não pode deixar a seringa esvaziar, sendo que o sistema estará cheio quando não mais sair bolhas de ar na mangueira.

8 – Nivelar o sistema novamente, tendo certeza de que não há bolhas embaixo da membrana. Em seguida, aplicar 20-100 µL de amostras com uma pipeta nos poços da *sample well* e em seguida com os pocinhos com parafilme. Por último, colocar a tampa de acrílico e deixar que a diálise ocorra de acordo com os padrões da amostra.

Figura 3 Protocolo para diálise.

2.3 Quantificação de proteínas

A concentração total de proteína foi mensurada pelo método de Lowry et al. (1951), utilizando o kit DC Protein Assay Reagent albumina como padrão. A leitura da absorbância foi realizada por espectofotometria (EGeG Wallac,

Modelo Victor 1420 Multilabel Counter, Turku, Finlândia) com comprimento de onda 660 nm, utilizando-se o software Wallac 1420 Victor, versão1.00. Foram feitas determinações proteína total em triplicata e em três diferentes estágios de preparo das amostras: antes da precipitação protéica, após a precipitação proteica mas antes da diálise ou após a diálise.

2.4 Zimografia

Para a realização da zimografia, as amostras obtidas da extração da dentina bovina (CB, CH, RB e RH) foram imersas em um tampão não redutor (Tris HCl/SDS 0,1M pH 6,8) na proporção de 2:1 (v/v) antes da aplicação no "poço" correspondente do gel. Após a aplicação de todas as amostras, o sistema de placas que suporta o gel foi mantido a uma temperatura de 4ºC, ajustando a mili-amperagem para 20 mA, enquanto as amostras passavam pelo gel de largada e, em seguida, ajustou-se a amperagem para 10 mA para o restante da corrida.

Ao final da corrida, o sistema foi desmontado e o gel separado e colocado em recipiente plástico para a substituição do tampão de corrida por Tampão I de enxágue (Tris HCl, Tween 80 2,5%, NaN_3 0,02% (p/v), pH 7,5 a 22ºC) e Tampão II (Tris HCl, Tween 80 2,5%, NaN_3 0,02% (p/v), $ZnCl_2$ 10 mM, $CaCl_2$ 100 mM). As soluções foram descartadas após a agitação por 30 min e substituídas por Tampão III (Tris HCl, NaN_3 0,02% (p/v), $ZnCl_2$ 10 mM, $CaCl_2$ 100 mM, pH 7,5, solução de incubação). Nessa etapa de incubação, o gel foi mantido em estufa a 37ºC durante 24 h (Souza et al., 1999). Terminado o período de incubação, a solução de Tampão III foi substituída pelo Corante de Coomassie Blue G-250 (0,5%). O gel ficou nesse corante por um período de 30 min. Por fim, a solução corante foi substituída por uma solução descorante (metanol 30% + ácido acético 10%). Para confirmação dos resultados, o protocolo de preparo e corrida do gel de zimografia foi realizado em triplicata. Os géis foram escaneados, utilizando o Image Scanner, Amersham Biosciences. As áreas ocupadas pelas bandas referentes às MMPs 2 e 9 e suas pró-enzimas foram analisadas.

2.5 Determinação do perfil de atividade das MMP-2 e -9

A atividade total das MMPs 2 e 9 foi determinada com auxílio do kit ELISA (Matrix Metalloproteinases, MMP-2 e 9, Activity Assay Biotrak^{TM} System, GE Healthcare, Reino Unido), de acordo com as instruções do fabricante. Os experimentos foram feitos em triplicatas.

3 RESULTADOS E DISCUSSÃO

Os dados de quantificação de proteína obtidos em absorbância foram tabulados em uma planilha de Excel (Microsoft Office 2007, EUA) para conversão linear (Gráfico 1), sendo transformados em mg/mL (Tabela 1). Como pode ser observado na Tabela 1, a concentração de proteínas obtida foi maior quando se fez apenas a extração, sem realização de precipitação ou diálise. Assim, esta condição foi escolhida para a realização do experimento (obtenção de amostra para realização de zimografia e determinação de atividade das MMP-2 e -9). Em adição, a concentração de proteínas foi similar para os 4 grupos.

Gráfico 1 – Dados da leitura de absorbância (660 nm) dos padrões de albumina

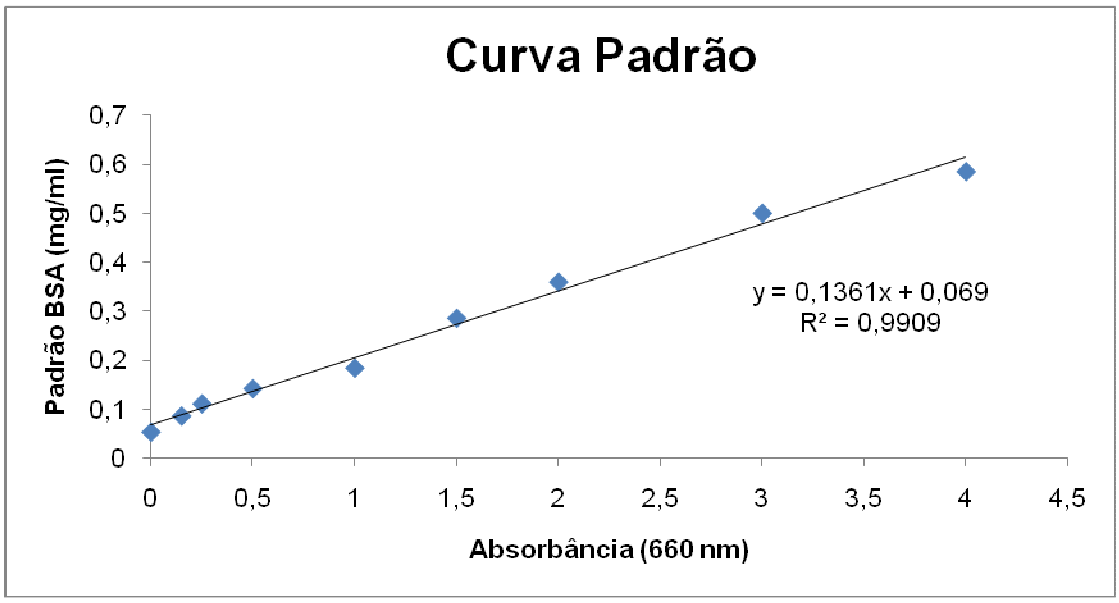


Figura 4 Gráfico leitura de absorbância dos padrões de albumina.

Quadro 1 –Quadro de quantificação protéica (mg/mL±DP) para os diversos grupos, após extração por diferentes metodologias

Grupos	*Diálise* mg/ml*	*Com precipitação** mg/ml*	*Sem precipitação*** mg/ml*
Raiz bovina (RB)	0,083±0,004	0,078±0,000	0,109±0,002
Raiz humana (RH)	0,087±0,001	0,078±0,000	0,108±0,001
Coroa bovina (CB)	0,081±0,000	0,077±0,000	0,114±0,002
Coroa humana (CH)	0,086±0,000	0,077±0,000	0,106±0,001

* Extração + precipitação com sulfato de sulfato de amônio a 85% + diálise.
** Extração + precipitação com sulfato de sulfato de amônio a 85%
*** Extração apenas

Figura 5 Quadro de valores quantificação protéica.

O gel de zimografia representa a presença tanto das formas da pró-enzima, quanto da enzima ativa, de ambas as metaloproteinases testadas (MMP-2 e MMP-9) .

Figura 4 - Atividade gelatinolítica das enzimas formas ativa e pró de MMP-2 e -9. Coluna 1 – MWM, padrão de peso molecular; Colunas 2 e 3 – Controle de MMPs recombinantes humanas (MMP-2 e MMP-9, respectivamente); Colunas 4 a 7: amostras de MMPs extraídas de dentina da coroa bovina (CB), coroa humana (CH), raiz bovina e raiz humana (RH), respectivamente.

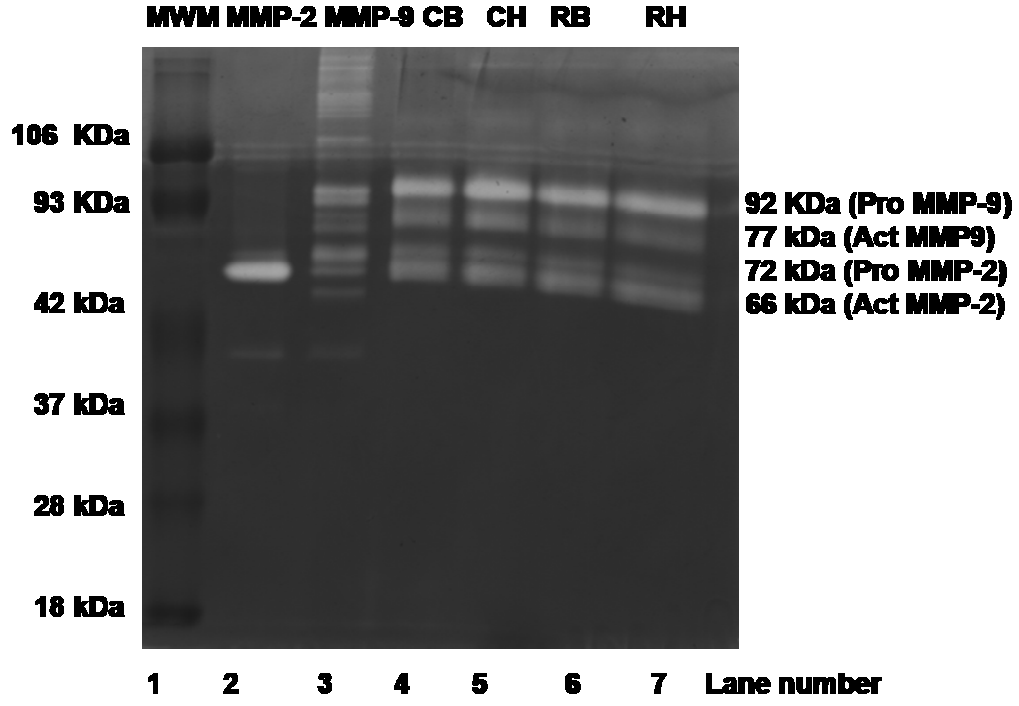


Figura 6 Gel de zimografia.

Foi examinada a presença e atividade de MMPs em dentina bovina e humana e foi possível identificar e quantificar duas das mais importantes MMPs presentes na dentina, as gelatinases A e B (MMP-2 e -9). Os gráficos 2 e 3 mostram os dados da leitura de absorbância para os padrões de MMP-2 e -9, respectivamente, e a Tabela 2 reporta a quantificação da atividade das MMP-2 e -9 após extração para os diferentes grupos. Os dados na ausência de APMA indicam a enzima ativa, enquanto que na presença de APMA representam a pró-enzima ativada. Portanto, a atividade enzimática total é obtida pela soma das duas condições anteriores.

Gráfico 2 – Dados da leitura de absorbância (405 nm) dos padrões de MMP-2

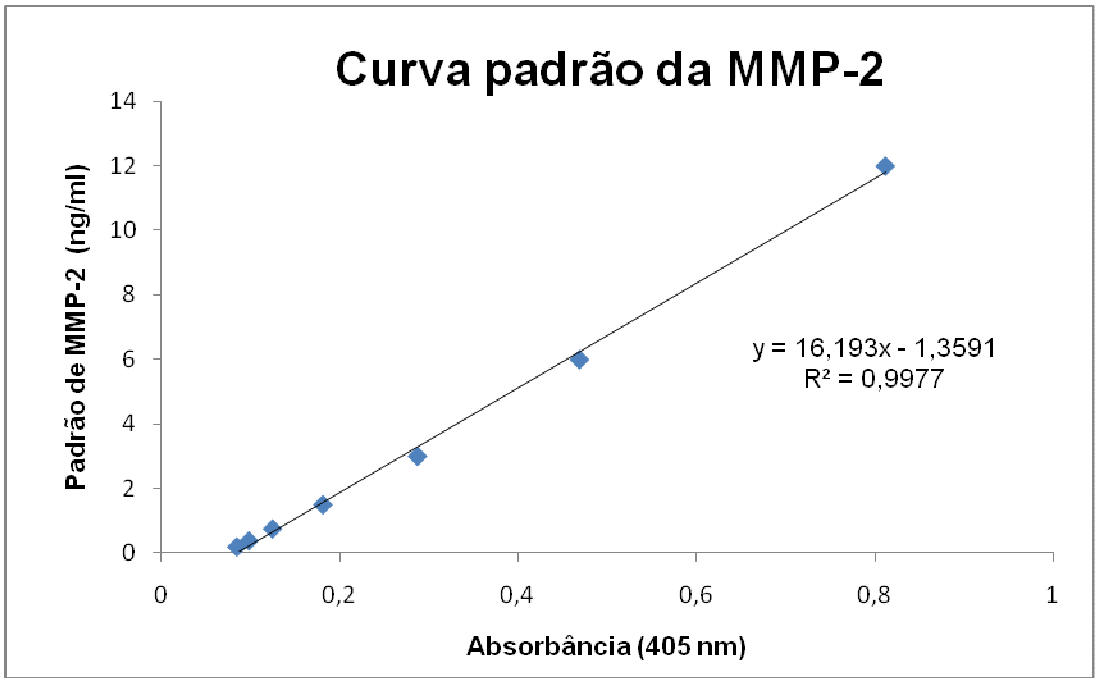


Figura 7 Gráfico leitura de absorbância dos padrões de MMP-2.

Gráfico 3 – Dados da leitura de absorbância (405 nm) dos padrões de MMP-9

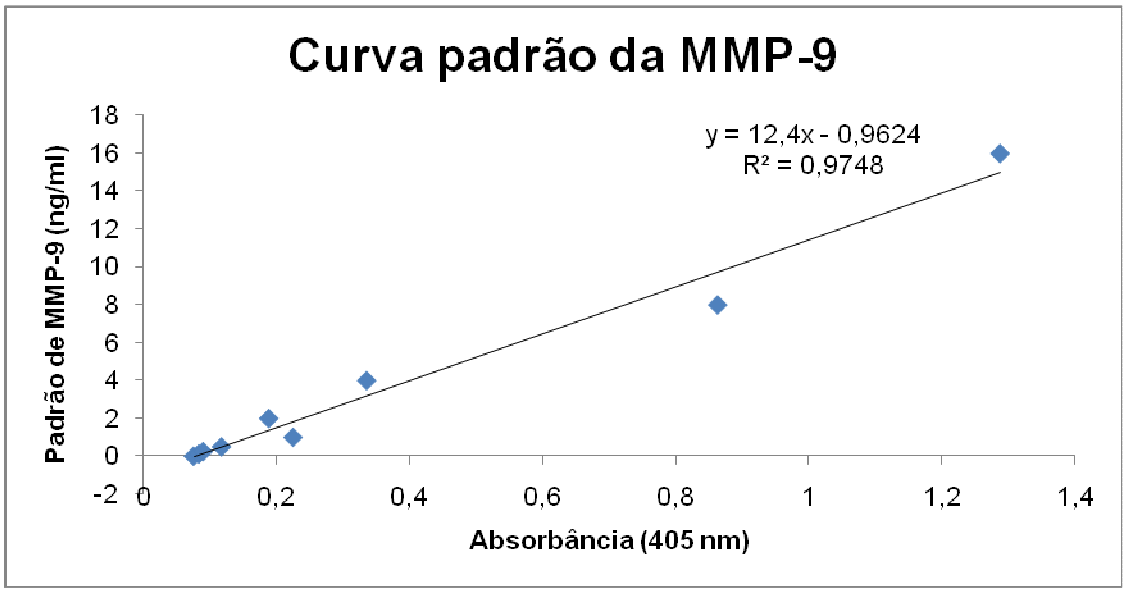


Figura 8 Gráfico leitura de absorbância dos padrões de MMP-9.

Quadro 2 – Quantificação da atividade das MMP-2 e -9 (ng/mL±DP) para os diferentes grupos

Grupos	MMP-2 ng/mL	MMP-9 ng/mL
Blanck (tampão de extração)	0,14±0,00	0,05±0,00
RB	3,42±0,51	1,80±0,02
RB +APMA	11,43±2,21	14,91±2,02
RB total	**14,85**	**16,71**
RH	5,59±0,00	4,61±0,02
RH +APMA	14,57±2,01	15,34±1,31
RH total	**20,16**	**19,95**
CB	5,06±1,70	6,61±0,06
CB +APMA	9,65±1,16	15,36±1,32
CB total	**14,71**	**21,97**
CH	12,35±0,93	4,00±0,05
CH+APMA	10,57±1,35	15,46±1,32
CH total	**22,92**	**19,46**

*** RB – Raiz bovina; RH – raiz humana; CB – coroa bovina; CH – coroa humana**

Figura 9 Quadro com valores da quantificação da atividade das MMPs-2 e MMPs-9.

MMP-2, -8 e -9 tinham sido detectadas em dentina de coroa humana (Martin-De Las Heras et al., 2000; Mazzoni et al., 2007; Sulkala et al., 2007b) e em dentina de raiz humana (Santos et al., 2009). Embora a presença de MMPs na dentina coronária e radicular humana tenha sido confirmada, nenhum desses estudos estabeleceu o perfil de atividade destas enzimas, bem como, não havia registro anterior de localização, síntese e expressão destas enzimas em dentina coronária e radicular bovina.

O presente estudo investigou a presença de MMP-2 e -9 na dentina coronária e radicular de dentes bovinos, bem como, o perfil de atividade destas enzimas, em dentinas humana e bovina. Como pode ser observado, as atividades das MMP-2 e -9 foram similares quando comparadas a dentina da coroa bovina (CB) com a da coroa humana (CH), bem como a da raiz bovina (RB) com a da raiz humana (RH), mostrando que existe a presença tanto da forma pró-enzima quanto da forma ativa. Este trabalho é de fundamental importância, já que foi o primeiro a conseguir a quantificação dessas enzimas nas dentinas humana e bovina. A análise comparativa entre dentina humana e bovina confirmou que a dentina bovina é similar à dentina humana em relação ao perfil de MMP-2 e -9. Desde que a dentina bovina é amplamente utilizada em estudos de erosão, cárie e sistemas adesivos, e levando-se em consideração o papel e função das gelatinases nas condições acima mencionadas, os resultados obtidos justificam a utilização de dentina bovina nos experimentos subsequentes.

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APÊNDICE

- ***Manuscrito a ser submetido ao periódico Matrix Biology.***

Expression and activity profiles of matrix metalloproteinases in bovine and human dentin using different protocols of extraction

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Abstract

Matrix metaloproteinases (MMPs) are endopeptidases responsible for the degradation or resorption of all components of the extracellular matrix (ECM). They are involved in the development of human dental enamel and remodeling of dentin organic matrix. MMPs -2, -8 and -9 have already been identified in human dentin. Since bovine dentin is frequently employed in *in vitro* and *in situ* studies as surrogate for human dentin, the purpose of the present investigation was to evaluate the expression and activities of MMP-2 and -9 present in human and bovine dentin. Blocks from bovine incisors and human molars were obtained from roots and crowns. They were pulverized using a cryogenic mixer mill. Dentin powder was demineralized in 0.87 M citric acid pH 2.3 for 24 h at 4℃ under stirring. After centrifugation, the demineralized dentin powder was suspended in extraction buffer and sonicated 3 times. Samples were incubated for 24 h in extraction buffer. After centrifugation, the supernatant was collected and total protein concentration was measured by Lowry's Method. Gelatin zymography was performed. Total activity of MMPs -2 and -9 was determined by ELISA. Gelatinolytic activities identified as MMP-2 and MMP-9 were detected in extracts of crown and root dentin samples both from human and bovine origin. Total activities of MMP-2 were 14.85, 20.16, 14.71 and 22.92 ng/mL for bovine root, human root, bovine crown and human crown, respectively. The correspondent figures for MMP-9 were 16.71, 19.95, 21.97 and 19.46 ng/mL, respectively. The results indicate that all kinds of dentin substrates tested can be used in studies involving the performance of MMPs -2 and -9.

Key Words: Dentin; MMP; Bovine tooth; Human tooth

Introduction

Matrix metalloproteinases (MMPs), collectively known as matrixins, form a multigene family within the metalloproteinase class of endopeptidases that mediate the degradation of practically all extracellular matrix (ECM) molecules, including native and denatured collagen (Birkedal-Hansen, 1993; Visse and Nagase, 2003). It is now recognized that MMPs usually degrade multiple substrates, with considerable substrate overlap between individual MMPs (Hannas et al., 2007).

MMPs can contribute to both normal and pathological events. They are widely active on the biological processes. Collagenases (MMP-1, -8 and 13) have the ability to cleave interstitial collagen types I, II, and III, and they are capable of digesting other ECM and non-ECM molecules. Gelatinases degrade the denaturated collagen molecules (gelatin).

There are several studies indicating that MMPs have a fundamental role in oral tissue development and remodeling. MMPs are involved in the development of enamel and enamel fluorosis (Bartlett et al., 2004; Caterina et al., 2000; DenBesten et al., 2002). They are also associated with remodeling of the organic matrix of dentin (Hall et al., 1999; Martin-De Las Heras et al., 2000; Sulkala et al., 2002). The activation of MMP-2 and MMP-9 has been shown to have a crucial role in dentin collagen breakdown in caries lesions (Sulkala et al., 2002; Tjäderhane et al., 1998). MMPs have been identified in both pulpal and periapical inflammation (Gusman et al., 2002; Wahlgren et al., 2002) and periodontal diseases (Ejeil et al., 2003; van der Zee et al., 1996). MMPs are essential components in the growth and invasion of oral tumors (Sorsa et al., 2004). Finally, MMPs may be important in the time-dependent loss of composite restoration adhesion (Carrilho et al., 2007a; Carrilho et al., 2007b; Caterina et al., 2000; Chaussain-Miller et al., 2006; Chen et al., 2008; Curzon et al., 1970; Curzon and Losee, 1977a; b; 1978; Curzon et al., 1978; Garcia-Godoy et al., 2007; Hebling et al., 2005; Pashley et al., 2004; Tay and Pashley, 2004).

Dentin matrix is a complex network of fibrillar and globular structures constituting the organic scaffold of dentin (Marshall et al., 1997). Type I collagen is the main component of the dentin matrix, while proteoglycans and other minor non-collagenous proteins complete its organic portion (Butler et al., 2003;

Embery et al., 2001; Goldberg and Smith, 2004; Linde and Goldberg, 1993). MMP-2 was isolated from mature human mineralized dentin matrix (Martin-De Las Heras et al., 2000) and zymographically identified in demineralized dentin (van Strijp et al., 2003). Also, MMP-8 and -9 from human sound dentin have been recently characterized (Mazzoni et al., 2007; Sulkala et al., 2007b).

Due to the increasing difficulty to obtain human teeth, bovine teeth have been frequently used in *in vitro* and *in situ* studies to simulate the human tooth behavior. The comparison of the bond strength promoted by an adhesive system to human and bovine enamel and dentin has shown that bovine teeth can be possible substitutes for human teeth in adhesion tests (Krifka et al., 2008; Reis et al., 2004). Bovine teeth have also been used extensively in studies involving dental erosion and abrasion (Francisconi et al., 2008; Rios et al., 2006a; Rios et al., 2006b; Vieira et al., 2006). The use of bovine incisors as substitute for human teeth in studies involving erosion/abrasion is well accepted, once no significant differences in dentin wear has been found in human third molars when compared to bovine incisors (Wegehaupt et al., 2008). When the radiodensity and the hardness of human and bovine enamel and dentin have been compared and evaluated in relation to the age of the bovine tooth, it was observed that the radiodensity was similar for the enamel, but the bovine dentin presented a higher radiodensity than the human one, independently of the groups ages, what could be attributed to the increased amount of peritubular dentin observed in bovine teeth (Fonseca et al., 2008). It has also been demonstrated that bovine coronary dentin is similar to the human one in relation to the number and diameter of tubules (Schilke et al., 2000). With regard to dentin permeability, the bovine dentin close to the amelo-cement junction was shown to be a viable alternative substitute to the coronary human dentin tested *in vitro* (Schmalz et al., 2001). However, little is known concerning other characteristics of bovine teeth, such as the components of the organic matrix and how similar these components are in relation to human teeth. Until the moment, few studies have compared the profile of MMPs in human and bovine teeth. Gelatinolytic activity has already been detected in radicular bovine tissue (Linsuwanont et al., 2002). Besides, the proteolytic processing mediated by MMP-2 is an important step in the acceleration of the process of maturation of the bovine dentin matrix that includes the fosforilation and subsequent

mineralization (Satoyoshi et al., 2001). Although MMP-20 in some species presents a homology of approximately 80% with the human MMP (Caterina et al., 2000) and that the recombinant human and bovine MMP-20 present many similarities, some differences exist between them, such as the speed of auto-activation and the substratum preference (Zhu et al., 2008).

A series of studies involving the use of MMP inhibitors in the reduction of the progression of erosion in dentin have been recently carried out (Kato et al.; Kato et al., 2009; Magalhães et al., 2009). If similarities in the profiles of MMPs in bovine and human teeth really exist, then bovine dentin can be safely used as substitute for human dentin in studies involving the activity of MMPs. Therefore, the profile of MMP content in both dentin substrata should be assessed. Thus, the aim of the present study was to evaluate the expression and activity of MMPs-2 and -9 content in human and bovine dentin using two different extraction protocols. The null hypothesis tested was that there are no differences in the expression and activity of MMPs-2 and -9 in human and bovine dentin.

Materials and Methods

Protocol 1

1.1 Specimen preparation

Ten bovine lower incisors with complete root formation were employed shortly after extraction. After removal of organic debris, calculus and pulp tissue crowns were separated from the roots at the cementum-enamel junction. Dentin was exposed upon removal of enamel and cementum by a diamond bur operated in a high-speed hand piece under copious water irrigation. Crown and root dentin samples 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 both crown and root samples was equally divided in 2 g aliquots and stored at -20℃ until being further processed.

1.2 Dentin proteins extraction

Extraction of dentin proteins was performed by using a protocol first described by Martin-De Las Heras et al. (Martin-De Las Heras et al., 2000). Briefly, crown and root dentin powder (2 g each) was 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 mineral-associated proteins (E1-E4 extracts) and finally demineralized dentin underwent a second guanidine extraction (G2 extract). The protein concentration of extracts produced was measured by the Lowry protein assay, and 60 µg aliquots were obtained and lyophilized.

1.3 Gelatin Zymography

Bovine dentin proteins were mixed to Laemmli sample buffer at a 2:1 ratio and electrophorezed under non-reducing conditions in 11% SDS-PAGE gels containing 1 mg/mL fluorescently labeled gelatin (Sulkala et al., 2007b). 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 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) at 37℃. Proteolytic activity was monitor ed 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 a aqueous solution of 10% acetic acid and 10% methanol. Zymography assay of dentin proteins was performed in triplicates and repeated three times.

Protocol 2

2.1 Bovine and human dentin powder preparation

Bovine incisors or human molars were stored in 0.2% sodium azide and 0.9% sodium chloride solution at 4℃ (Carrilho et al., 2005) and used until one month after extraction. Human third molars were obtained from young patients, aged from 18 to 25 years, after approval of the Institutional Review Board of Bauru Dental School, University of São Paulo, Brazil (Proc. 128/2008). Likewise, bovine incisors were taken from young cattle, which are usually abated. The care of taking both human and bovine teeth from corresponding ages is due to the fact that the quantity of MMP-2 in dentin decreases over time (Martin-De Las Heras et al., 2000). Using one diamond disk (Isomet 1000; Buehler, Lake Bluff, IL, USA) the crowns were sectioned from the roots. Next, using three parallel diamond disks separated by two 4-mm spacers, the fragments were cut from the crown of each bovine incisor. Their enamel surfaces were flattened with 300 and 600 of AL_2O_3 papers. Dentin fragments were ground in a cryogenic mixer mill (Fisher Scientific, Modelo Spex SamplePrep 6770, EUA) and dentin powder was obtained.

1 g of bovine and human dentin powder from crown and root samples was demineralized in 10 mL of 0.87 M citric acid, pH 2.3 at 4℃ for 24 hours, under constant stirring (Mazzoni et al., 2007). In sequence, the samples were centrifugated at 1500 rpm for 2 min.

2.2 Enzyme Extraction of Demineralized Dentin Specimens and Sample conditioning

The citric acid was discarded and the demineralized dentin powder was suspended in 10 mL extraction buffer (50 mM Tris-HCl, pH 6.0, containing 5 mM $CaCl_2$, 100 mM NaCl, 0.1% Triton X-100, 0.1% NONIDET P-40, 0.1 mM $ZnCl_2$, 0.02% NaN3) and EDTA-free protease inhibitor cocktail (Sigma Chemical, St. Louis, MO, USA) as described by Mazzoni et al. (Mazzoni et al., 2007). The samples were ultrasonically treated at 30-40 W (Branson Sonic Power Co e Smithkline company, Model Sonifier Cell Disruptor, Danbury, USA) output for 3

bursts of 20 sec each at 4℃. The vials were centrifuged at 18,000 rpm (19,000G) for 30 min at 4℃, and the supernatants were collected.

Furthermore, it was also assessed whether precipitation and dialysis would provide good extraction of MMPs. All the proteins present in the supernatants were precipitated or not at 4℃ by the addition of powdered ammonium sulphate (w/v) to achieve a final concentration of 85%, pH 7.0. The precipitated samples were collected by centrifugation at 24,000 rpm for 30 min at 4℃, redissolved in a 10-fold dilution in extraction buffer, dialyzed through a 30-kDa membrane against extraction buffer overnight, and stored at 4℃ until analyzed.

2.3 Protein Content Determinations

Total protein concentration in the demineralized dentin extracts were measured by Lowry's method (Lowry et al., 1951), using the DC Protein Assay Reagent kit (Amersham-Pharmacia, Milan, Italy), according to manufacturer's instructions. Absorbance reading was performed using a spectrophotometer (EGeG Wallac, Model Victor 1420 Multilabel Counter, Turku, Finland) with 660 nm wavelenght using the software Wallac 1420 Victor, version1.00. Total MMP-2 and -9 activities were determined in dentin extracts with the use of immunoassay kits (Matrix Metalloproteinases, MMP-2 and -9, Activity Assay Biotrak™ System, GE Healthcare, UK). The specific immunoassay recognizes both pro and active forms. Detection limits were estimated at 0.5 and 0.25 µg/L for MMP-2 and -9, respectively. Total protein content was determined in triplicates and in three stages of sample preparation: before protein precipitation, after protein precipitation but before dialysis and after dialysis.

2.4 Gelatin Zymography

Aliquots of dentin powder (in a volume 2:1 sample/sample buffer) were dissolved in non-reducing sample buffer at room temperature. The samples were underplayed into the wells using a fine-tipped microsyringe and were separated after 11% SDS-PAGE electrophoresis at 110 V for approximately 2 h and 15 min. The gels were run in triplicate. Then they were incubated for 30 min with wash buffer I (50 mmol/L Tris HCl, 2.5% Tween 80, 0.02% (w/v) NaN_3, pH

7.5 at 22^{0}C) and buffer II (50 mmol/L Tris HCl, 2.5% Tween 80, 0.02% (w/v) NaN_3, 1 μm $ZnCl_2$, 5 mmol/L $CaCl_2$). Next, the gels were incubated at 37^{0}C for 18 h in buffer III (50 mmol/L Tris HCl, 5 mmol/L $CaCl_2$, 1 μM $ZnCl_2$, 0.02% (w/v) NaN_3). MMPs were incubated in 3 mmol/L 1,10 phenanthroline, which is a specific inhibitor. Proteolytic activity was monitored under long-wave UV light until judged to be in linear range and the gels were incubated with the staining 0.1 % Coomassie Blue solution for 60 min at 22^{0}C on a shaker. After de-staining, gelatinolytic activity was detected as clear bands in the background of uniform staining. Electrophoretic bands were scanned (Image Scanner, Amersham Biosciences, Upsala, Sweden).

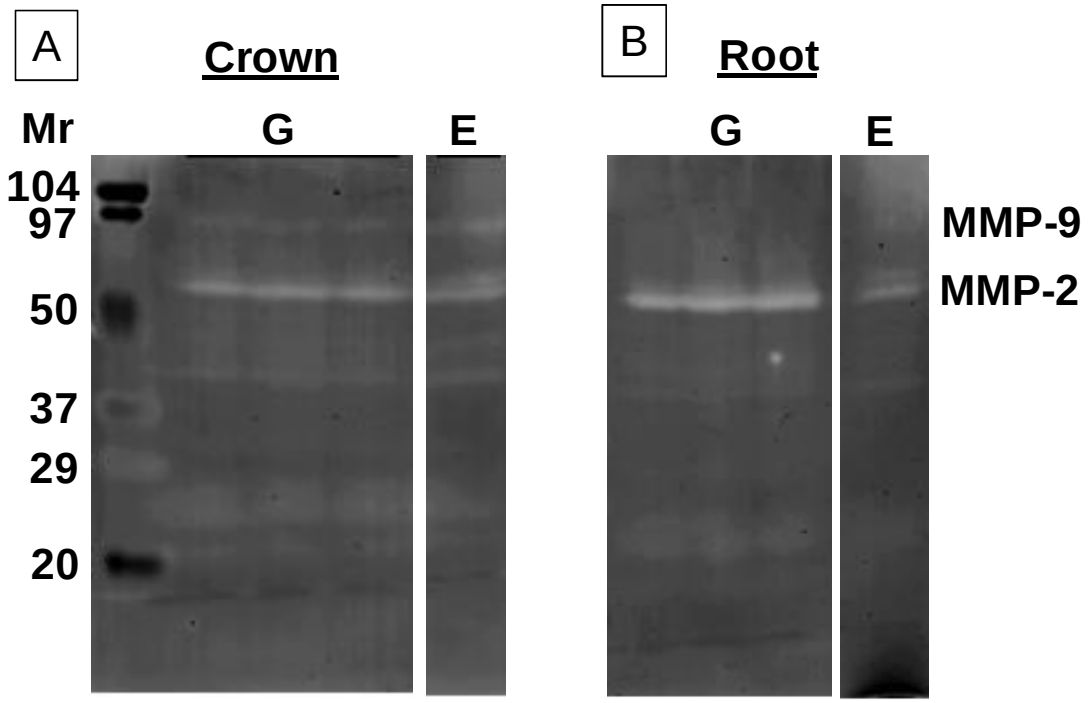
Results and Discussion

Protocol 1

Zymography analysis of powdered bovine crown and root dentin proteins detected gelatinolytic activity in both substrates. The bands created by purified MMP-2 and MMP-9 positive controls were larger than expected due to the long incubation time needed to detect dentin gelatinolytic activities.

Proteins extracted in the first guanidine cycle (G1 extracts) from bovine crown dentin yielded 92 and 68 kDa bands which are the molecular weights corresponding to MMP-9 and MMP-2, respectively (Fig. 1A). Bovine root dentin proteins from G1 extracts yielded 68 kDa bands (Fig. 1B) 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. 1A and B). All extracts (crown and root dentin) exhibited markedly higher intensity for bands at 68 kDa, indicating MMP-2 as the predominant gelatinase form. Gelatinolytic activity was also detected at lower molecular weight range (40-20 kDa), most likely truncated forms of enzymes (Fig. 1A and B).

Figure 1: Gelatinolytic activities observed with proteins extracted with guanidine-HCL (G) before demineralization. Distinct bands corresponding to MMP-2, as well as fainter lower molecular weight bands corresponding to truncated forms, are present both in crown and root dentin samples. In crown samples, also faint but clear bands corresponding to MMP-9 can be seen. Respective gelatinolytic activities are also present in EDTA (E) extracts of mineralized dentin.



Protocol 2

Absorbance results from protein quantification were converted to mg/mL (Table 1). Increased protein concentration was obtained after MMP extraction, without further precipitation or dialysis. Therefore, this condition was chosen to carry out the experiment (MMP extraction, zymography and MMP-2 and -9 activity detection). In addition, protein concentration was similar for all groups.

Table 1 – Protein quantification (mg/mL± SD) for the groups after different protein extraction protocols

Groups	***Dialysis**** ***mg/ml***	***With precipitation***** ***mg/ml***	***Without precipitation****** ***mg/ml***
Bovine root (BR)	0.083±0.004	0.078±0.000	0.109±0.002
Human root (HR)	0.087±0.001	0.078±0.000	0.108±0.001
Bovine crown (BC)	0.081±0.000	0.077±0.000	0.114±0.002
Human crown (HC)	0.086±0.000	0.077±0.000	0.106±0.001

* Extraction + precipitation with 85% ammonium sulphate + dialysis.
** Extraction + precipitation with 85% ammonium sulphate
*** Only extraction

Zymography gel shows the presence of both pro and active enzyme forms for both MMPs tested (MMP-2 and MMP-9) (Figure 2).

When the presence and activity of MMPs from bovine and human dentin was compared, it was possible to identify and to quantify two of the most important MMPs present in dentin, gelatinases A and B (MMP-2 and -9). Quantification of the activity of MMP-2 and -9 was reported after protein extraction for the different groups (Table 2). Data in the absence of APMA indicate the active enzyme, while data in the presence of APMA represent the proenzyme that was activated. Thus, total gelatinolytic activity was calculated by the addition of the two previously mentioned conditions.

Figura 3 - Gelatinolytic activity of both pro and active MMP-2 and -9. Lane 1 – MWM, molecular weight marker; Lanes 2 and 3 – Positive controls from human recombinant MMPs (MMP-2 e MMP-9, respectively); Lanes 4 to 7: MMP samples extracted from bovine dentin crown (BC), human crown (HC), bovine root (BR) and human root (RH), respectively.

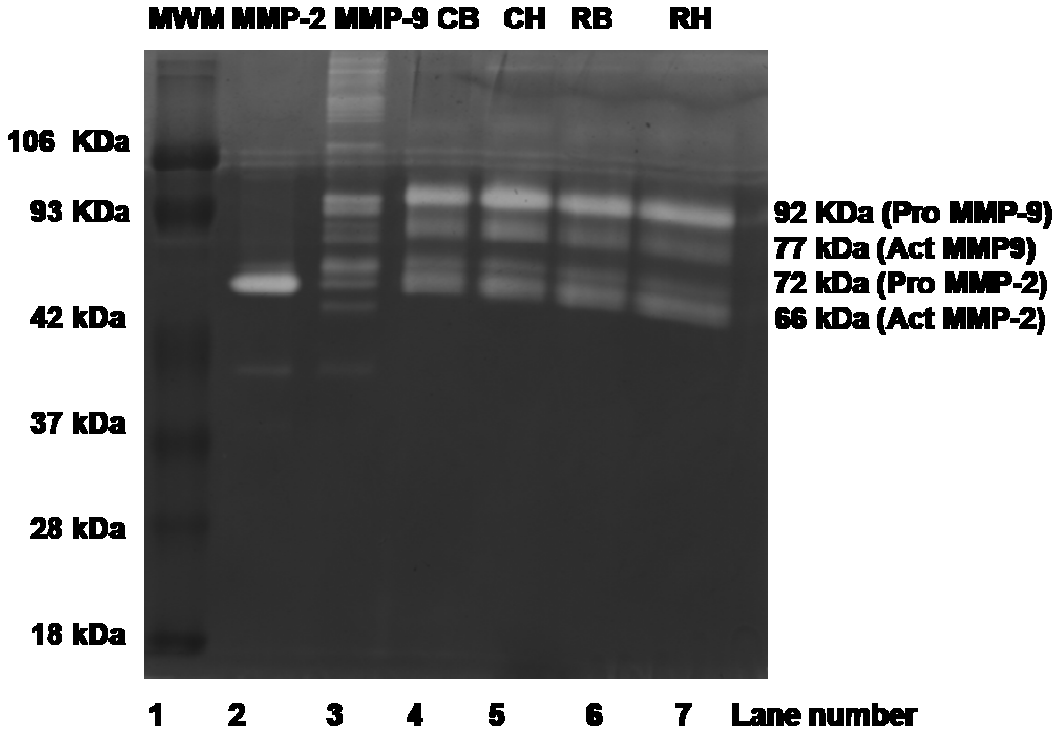


Table 2 – MMP-2 and -9 activity quantification for the different groups (ng/mL±SD)

Groups	MMP-2 ng/mL	MMP-9 ng/mL
Blanck (extraction buffer)	0.14±0.00	0.05±0.00
BR	3.42±0.51	1.80±0.02
BR +APMA	11.43±2.21	14.91±2.02
BR total	**14.85**	**16.71**
HR	5.59±0,00	4.61±0.02
HR +APMA	14.57±2,01	15.34±1.31
HR total	**20.16**	**19.95**
BC	5.06±1.70	6.61±0.06
BC +APMA	9.65±1.16	15.36±1.32
BC total	**14.71**	**21.97**
HC	12.35±0.93	4.00±0.05
HC+APMA	10.57±1.35	15.46±1.32
HC total	**22.92**	**19.46**

*Samples: BR – Bovine root; HR – Human root; BC – Bovine crown; HC – Human crown
Samples + APMA represent the pro-enzyme that was activated.

Since this study confirmed the expression and activity of MMP-2, and -9 within crown and root dentin from both human and bovine samples, the test hypothesis was confirmed.

The protein analysis in mineralized substrates is only possible by using extraction protocols to release them from the tissue. Both protocols employed in this study were able to remove proteins out of dentin properly. Even though the extraction procedures described by Mazzoni et al. (Mazzoni et al., 2007) are less labor-intensive and are claimed to enhance protein recovery by means of ammonium sulfate precipitation, the gelatinolytic profile obtained may not be comparable to the one obtained with samples extracted by the protocol described by De La Heras et al. (Martin-De Las Heras et al., 2000). While the first protocol promotes extraction of all protein content at once, it preserves only the enzymes bound to the dentin extracellular matrix, and analyzing the

enzymes in non-mineralized protein component is not possible. The other protocol allows sequential extraction in different steps, including the proteins extracted prior to demineralization (Martin-De Las Heras et al., 2000; Santos et al., 2009; Sulkala et al., 2007b).

In the second protocol carried out in the present study, the first efforts to extract the MMPs from dentin were not successful in the initial experiments due to important factors. First, it is essential to ground the dentin blocks in a cryogenic mill. Initially, the samples might have been denaturized due to the temperature increase during grounding. Therefore, new samples were subsequently ground in a cryogenic Mill (Fisher Scientific, Modelo Spex SamplePrep 6770, EUA) and lyophilized until the experiment. The second factor that may have interfered in the extraction of MMPs was the storage time and media. According to De Las Heras, the MMP content in dentin may decrease over time (Martin-De Las Heras et al., 2000). Only when completely fresh bovine incisors and human third molars that had been stored in 0.2% NaN_3 and 0.9% NaCl solution and kept at 4^0C for less than 1 month before being tested were used, it was possible to achieve better results. The third alteration in the extraction protocol was carried out after it was realized that there was a contrasting experimental step in the materials and methods in Mazzoni`s protocol (Mazzoni et al., 2007). In that paper, even though in the materials and methods section it was described that a 0.26 M citric acid was used, in the table results a 0.87 M citric acid concentration was reported. Thus, the extraction protocol was repeated using the 0.87 M citric acid. The extraction of MMPs was only successful with the protocol that involves incubation of the demineralized powder in extraction buffer, sonication and centrifugation. The extraction was not successful after precipitation with ammonium sulphate and dialysis, what is in contrast with the findings reported by Mazzoni (Mazzoni et al., 2007).

MMP-2, -8 e -9 had been detected in human crown dentin (Martin-De Las Heras et al., 2000; Mazzoni et al., 2007; Sulkala et al., 2007a) and in human and bovine root dentin (Santos et al., 2009). The present study corroborates the results found by Santos et al. (Santos et al., 2009) which demonstrated the presence of MMP-2 and -9 in both human and bovine crown and root dentin. However, that study indicated that the recovery of MMP-2 from demineralized root dentin was more evident than in crown dentin. In the present study, MMP-2

was more predominant in crown dentin from both human and bovine powder. Nevertheless, increased MMP-9 activity was detected in human root when compared to bovine one, but bovine crown showed stronger presence of MMP-9 than human crown.

The presence of both pro and active forms of the gelatinases provides indirect evidence for *in vivo* activity of gelatinases during dentin matrix remodeling before and during mineralization (Martin-De Las Heras et al., 2000). It also shows that gelatinase was deposited in the dentin matrix during tooth development before, or during mineralization. This emphasizes the stability of the enzyme when embedded in mineral (Martin-De Las Heras et al., 2000).

In spite of the substantial developments with regard to the potential relationship between host-derived MMPs and caries progression (Tjäderhane et al., 1998), some of the fundamental issues such as the location of MMPs and their association with collagen matrix are not totally understood. It was immunohistochemically demonstrated that MMP-2 is concentrated in the predentin and the inner dentin area adjacent to the predentin, which is consistent with the hypothesis that MMP-2 is actively involved in the organization of pre-mineralized matrix formation as well as in its subsequent mineralization (Boushell et al., 2008). This is in line with the observation that MMP-2 and MMP-9 are intrinsic constituents of the fibrillar network of the human dentin organic matrix (Mazzoni et al., 2009), thus supporting the hypothesis that these enzymes may play important roles in the degradation of the dentin organic matrix.

In conclusion, the demonstration of the similarity of bovine dentin to the human one in relation to MMP-2 and -9 profiles is of great relevance for future investigations. Considering the fact that bovine dentin is largely used in experiments in the Dentistry field, especially those involving erosion, caries and restorative protocols and that gelatinases play an important role in collagen degradation, the results obtained in the present study justify the use of bovine dentin as a substrate for future studies assessing the influence of MMPs in different situations.

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- ***Manuscrito a ser submetido ao periódico Cells Tissue Organs.***

Activity of MMPs in bovine *versus* human dentin

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Short title: Activity of MMPs in bovine and human dentin

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Keywords: Dentin; MMP; Bovine tooth; Human tooth; Gelatinase

Abstract

Metalloproteinases (MMPs) have been implicated with metabolism of collagen in physiological and pathological processes in human dentin. As bovine teeth have been used as substitute of human teeth in laboratorial analysis, this study evaluated the activity of MMP-2 and -9 in bovine *versus* human dentin. Bovine and human dentin fragments, from crowns and roots, were powderized. Protein extraction was performed by two protocols: a neutral extraction with guanidine-HCl/EDTA (pH 7.4) and an acidic extraction with citric acid (pH 2.3). Gelatinolytic activities of extracts were revealed by zymography. MMP-2 and -9 were detected in crown and root dentin from bovine (BC, BR) and human (HC, HR) teeth. Total activities of MMP-2 were 14.85, 20.16, 14.71 and 22.92ng/mL for BR, HR, BC and HC. Corresponding activities for MMP-9 were 16.71, 19.95, 21.97 and 19.46ng/mL. Bovine dentin showed to be a reliable substrate for studies involving the activity of MMPs -2 and -9.

Introduction

Matrix metalloproteinases (MMPs), collectively known as matrixins, form a multigene family of endopeptidases that mediate the degradation of practically all extracellular matrix (ECM) molecules, including native and denatured collagen [Visse and Nagase, 2003; Sorsa et al., 2004]. MMPs are widely active on the biological processes and can contribute to both normal and pathological events [Hannas et al., 2007].

MMP-2, -8 and -9 have been identified in human dentin [Martin-De Las Heras et al., 2000; Sulkala et al., 2007; Santos et al., 2009]. They are also present in carious dentin both in the latent pro- and active forms [Tjäderhane et al., 1998; van Strijp et al., 2003]. In addition, MMP-20 was shown to be present at least in dentinal fluid [Sulkala et al., 2002].

Due to the increasing difficulty to obtain human teeth, bovine teeth have been frequently used in *in vitro* and *in situ* studies to simulate the human tooth behavior [Rios et al., 2006; Vieira et al., 2006a; Francisconi et al., 2008]. Although a number of comparisons have been performed in order to characterize similarities/dissimilarities in morphology [Schilke et al., 2000; Reis et al., 2004; Fonseca et al., 2008], physiology [Schmalz et al., 2001] and mechanical structure [Hara et al., 2003; Wegehaupt et al., 2008] between bovine and human dentin, very few studies have dedicated considerable attention to disclose how similar/dissimilar is the biochemical behavior of organic components of human *versus* bovine dentin matrices. For instance, only a few studies have compared the profile of MMPs in human and bovine enamel

matrix [DenBesten et al., 1989; Caterina et al., 2000; Zhu et al., 2008], with non-conclusive findings.

A series of recent studies using MMP inhibitors demonstrate the reduction of the progression of erosion in dentin [Kato et al., 2009; Magalhães et al., 2009; Kato et al., 2010a; Kato et al., 2010b], indicating the presence of collagenolytic/gelatinolytic MMPs in bovine dentin. If similarities in the profiles of MMPs in bovine and human teeth really exist, then bovine dentin can be safely used as substitute for human dentin in all kinds of studies involving the activity of MMPs.

Therefore, the aim of the present study was to evaluate the profile and activity of MMP-2 and -9 in human and bovine dentin. The null hypothesis tested was that there are no differences in the profile and activity of MMP-2 and -9 in human and bovine dentin.

Materials and Methods

Specimen Selection and Preparation

Human third molars were obtained from young patients, aged from 18 to 25 years, after approval of the Institutional Review Board of Bauru Dental School, University of São Paulo, Brazil (Proc. 128/2008)**.** Likewise, bovine incisors, with complete root formation, were taken from slaughtered young animals in order to minimize the possible variation in the amount of MMP-2 in dentin due to aging [Martin-De Las Heras et al., 2000]. After removal of organic debris, calculus and pulp tissue, crowns were separated from the roots at the cementum-enamel junction. Dentin was exposed by a diamond bur operated in a high-speed hand piece under copious water irrigation. Crown and root dentin

samples were separately cut into smaller fragments (2x2 mm), frozen in liquid nitrogen and powderized in a mixer mill (Model MM301, Retsch, Haan, Germany).

Dentin proteins extraction

Protocol I: Neutral Extraction

Dentin powder from both crown and root samples was equally divided into 2-g aliquots. Extraction of dentin proteins was performed using the protocol described by Martin-De Las Heras et al. [Martin-De Las Heras et al., 2000]. Briefly, crown and root dentin powder was treated with 4 M guanidine-HCl in 65 mM Tris-HCl and loosely bound 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 mineral-associated proteins (E extracts).

As MMP-9 was not detected in bovine root dentin using this protocol, we decided to test another extraction protocol involving demineralization with citric acid for further analysis of all substrates.

Protocol II: Acidic Extraction

One-gram aliquots of bovine and human dentin powder, from crown and root samples, were demineralized in 10 mL of 0.87 M citric acid, pH 2.3 at 4℃ for 24 hours [Mazzoni et al., 2007]. Briefly, after demineralization the citric acid was discarded and dentin powder was suspended in 10 mL extraction buffer (50 mM Tris-HCl, pH 6.0, containing 5 mM $CaCl_2$, 100 mM NaCl, 0.1% Triton X-100,

0.1% NONIDET P-40, 0.1 mM $ZnCl_2$, 0.02% NaN_3) and EDTA-free protease inhibitor cocktail. Samples were ultra-sonicated (Branson Sonic Power Co. and Smithkline Co., Sonifier Cell Disruptor, Danbury, USA) at 30-40 W output for 3 bursts of 20 s each at 4℃ and centrifuged at 19,900 X *g* for 30 min at 4℃. The supernatants were collected and concentrated 4 times in a refrigerated protein concentrator (Savant Speedvac System , UVS400A, Arizona, USA). It was observed higher protein concentration for extracts that were not further precipitated with ammonium sulphate and dialyzed (Table 1). Thereby, in the present study, it was decided to exclude these steps of Mazzoni's protocol.

Protein Content Determinations

The protein concentration in all extracts, obtained from both protocols of extraction, was measured in triplicates by the Lowry protein assay [Lowry et al., 1951]. Aliquots (60 µg) were obtained and lyophilized for zymography analysis.

Gelatin Zymography

Bovine dentin proteins were mixed with non-reduced sample buffer at a 2:1 ratio and electrophoresed under in 11% SDS-PAGE gels containing 1 mg/mL fluorescently labeled gelatin [Sulkala et al., 2007]. 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 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 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) at 37℃. Proteolytic activity was monitored un der long-wave UV light until judged to be in linear range. The gels were stained in 0.2% Coomassie Brilliant Blue R-250 and de-stained in an aqueous solution of 10% acetic acid and 10% methanol. Zymography assay of dentin proteins was performed in triplicates and repeated three times.

Activity profile of MMP-2 and -9

For enzymes extracted with the acidic protocol, total MMP-2 and -9 activities were determined in dentin extracts with the use of immunoassay kits (MMP Activity Assay Biotrak™ System, GE Healthcare, UK). Detection limits were estimated at 0.5 and 0.25 µg/L for MMP-2 and -9, respectively. Detection of enzyme activity was performed with or without the addition of APMA, according to the manufacturer's instructions.

Results

Protocol I: Neutral Extraction

Zymography analysis revealed gelatinolytic activity for root and crown bovine dentin extracts. G1 extracts from bovine crown dentin yielded 68 and 92

kDa bands corresponding likely to MMP-2 and MMP-9, respectively (Fig. 1A). Bovine root dentin proteins from G1 extracts yielded 68 kDa bands (Fig. 1B) with virtual absence of gelatinolytic bands co-migrating with MMP-9 positive control. EDTA extracts revealed mainly 68/72 kDa bands corresponding to active and latent MMP-2 in crown and root dentin samples (Fig. 1A and B). All extracts exhibited markedly higher intensity for bands at 68 kDa, indicating MMP-2 as the predominant gelatinase form. Gelatinolytic activity was also detected at lower molecular weight range (40-20 kDa), most likely truncated forms of enzymes (Fig. 1A and B).

Protocol II: Acidic Extraction

Zymography analysis showed the presence of both pro and active enzyme forms for both gelatinases (MMP-2 and MMP-9) in all tested substrates (Figure 2).

Quantification of the activity profiles of MMP-2 and -9 after protein extraction for the different substrates is presented in Table 2. Data in the absence of APMA indicated the endogenously active enzyme, while data in the presence of APMA represented the proenzyme that was activated. Thus, total gelatinolytic activity was calculated by the addition of the two previously mentioned conditions. It was possible to observe that the activity of both enzymes was similar for all tested substrates.

MMP-2 total activity was 25% higher in human than in bovine dentin. There was no difference in total activity detected in crown *versus* bovine dentin. Conversely, the activity detected in human crown dentin was 10% higher than in

human root dentin. The total activity for MMP-9 was slightly different. When bovine and human dentins were compared, the activity observed in human root was 15% higher than that in bovine root. However, for crown dentin the total activity was 10% higher for bovine substrate. MMP-9 total activity was similar for human root and crown dentin, but it was 25% higher in bovine crown when compared to bovine root.

Discussion

MMP-2, -8 and -9 have been detected in human crown [Martin-De Las Heras et al., 2000; Mazzoni et al., 2007; Sulkala et al., 2007] and root dentin [Santos et al., 2009]. Results of the present study corroborate with previous findings and strongly indicate that gelatinases embedded in the dentin matrix can be partially in active form. Partial activation e.g. by dentin cysteine cathepsins [Tersariol et al., 2010] during extraction could not be absolutely ruled out. However, as protein extractions were performed with protease inhibitor cocktail containing cysteine cathepsin inhibitor E-64 (an effective irreversible inhibitor of cysteine proteases), the activation of gelatinases during extraction is less likely. Since the MMPs have been embedded into the mineralized dentin even years before the extraction, the presence of active gelatinases in mineralized dentin emphasizes the incredible stability of these enzymes when embedded in mineralized dentin. This is supported by the previously demonstrated ability of dentinal gelatinases to withstand excessive heat without loss of activity [Sulkala et al., 2007].

Despite the substantial evidence on the potential relationship between host-derived MMPs and caries progression [Tjäderhane et al., 1998], some of

the fundamental issues about dentinal MMPs, such as their location and their association with collagen matrix, are not totally understood. It has been immunohistochemically demonstrated that MMP-2 is concentrated in the predentin and in the inner dentin area adjacent to the predentin [Boushell et al., 2008], which is consistent with the hypothesis that MMP-2 is actively involved in the organization of pre-mineralized matrix formation as well as in its subsequent mineralization and the regulation of peritubular dentin formation [Boushell et al., 2008]. However, MMP-2 and -9 are intrinsic constituents of the fibrillar network of human dentin organic matrix [Mazzoni et al., 2009], thus supporting the hypothesis that these enzymes may play important roles in the degradation of the dentin organic matrix in caries lesions [Tjäderhane et al., 1998; Sulkala et al., 2002], during erosion [Kato et al., 2009; Magalhães et al., 2009; Kato et al., 2010a; Kato et al., 2010b] and in the degradation of composite adhesive hybrid layers [Pashley et al., 2004; Tay and Pashley, 2004; Hebling et al., 2005; Carrilho et al., 2007a; Carrilho et al., 2007b; Pashley et al., 2007].

Protein analysis in mineralized substrates is only possible by using extraction protocols to release them from the tissue. The extraction procedures involving citric acid demineralization [Mazzoni et al., 2007] are less labor-intensive and are claimed to enhance higher protein recovery. While this protocol promotes extraction of all proteins at once, it permits only the analysis of enzymes that at the end of the extraction process remained bound to the dentin extracellular matrix. The neutral HCl/EDTA protocol [Martin-De Las Heras et al., 2000], conversely, allows determination of gelatinases in loosely bound/unbound protein fraction and sequential extraction in different steps, including the proteins extracted prior to and during demineralization [Martin-De

Las Heras et al., 2000; Sulkala et al., 2007; Santos et al., 2009]. Having such different processes to expose and extract enzymes from mineralized dentin, direct comparisons between the performances of one protocol over the other would not be realistic. However, it is interesting to reflect on the fact that only the acidic protocol was able to reveal the activity of MMP-9 in bovine dentin. Although we can just speculate on this issue, it is possible to consider two aspects: 1) the residual presence of EDTA (used in the neutral protocol), a well-known MMP-inhibitor, may have affected preferentially the activity of bovine MMP-9; 2) the acidic environment provided when running the acidic protocol may have stimulated the activity of MMP-9. Actually, by investigating the activity of human salivary MMPs, it was demonstrated that the exposure of MMP-2 and -9 to low pH (between 2.3 to 4.5) followed by neutralization (i.e. a sequence typical in dental plaque after the ingestion of sugars), caused a greater than four-fold increase in the gelatinolytic activity of those enzymes, being it perceptually more significant for MMP-9 [Tjäderhane et al., 1998]. Additionally, after acidic activation, it was also observed by zymography a gradual time-dependent shift of MMP-9 from 92 kDa to a lower molecular mass of about 82 kDa [Tjäderhane et al., 1998], which corresponds to the functionally active form of MMP-9 [Ogata et al., 1992]. Thus, it seems to be reasonable to consider that the acidic protocol may provide better conditions for MMPs to exhibit their gelatinolytic potential.

To the best of our knowledge, this was the first study wherein comparisons of the profile and activity of MMPs in bovine *versus* human dentin were performed at once. For MMP-2, the activities in crown and root human dentin were consistently higher than in their respective counterparts in bovine

dentin. For MMP-9, however, total activity was higher in human root when compared to bovine root, but lower in human crown when compared to bovine crown. Nevertheless, differences between respective human and bovine dentin were within reasonable limits, varying between 11 and 35%. Also, the relative presence of pro and active forms of both enzymes seemed to be fairly constant. Together, these findings indicate that substrates from both origins can be used in experiments where the activity of MMP-2 and MMP-9 is for any reason investigated, such as protocols involving erosion, caries and dental restorations.

Acknowledgements: This study was supported by FAPESP (Proc. 07/08389-3, 07/04209-0, 08/09857-3 and 07/54618-4) and CNPq (300615/2007-8). The authors thank Ms. Andrieli Silva for the technical assistance.

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Figure legends

Figure 1 - Gelatinolytic proteinases observed with proteins extracted with guanidine-HCl (G) before demineralization. Distinct bands corresponding to MMP-2, as well as fainter lower molecular weight bands corresponding to truncated forms, are present both in crown and root dentin samples. In crown samples, also faint but clear bands corresponding to MMP-9 can be seen. Respective gelatinolytic activities are also present in EDTA (E) extracts of mineralized bovine dentin.

Figure 2 - Gelatinolytic proteinases of both pro and active forms of MMP-2 and -9. Lane 1 – MWM, molecular weight markers; Lanes 2 and 3 – Positive controls (human recombinant MMP-2 and -9, respectively); Lanes 4 to 7: samples extracted from bovine dentin crown (CB), human crown (CH), bovine root (RB) and human root (RH) dentin, respectively.

Table 1 – Total protein quantification (mg/mL± SD) for the different substrates after different protein extraction protocols

Substrates	Dialysis[a]	With precipitation[b]	Without precipitation[c]
Bovine root (BR)	0.083±0.004	0.078±0.000	0.109±0.002
Human root (HR)	0.087±0.001	0.078±0.000	0.108±0.001
Bovine crown (BC)	0.081±0.000	0.077±0.000	0.114±0.002
Human crown (HC)	0.086±0.000	0.077±0.000	0.106±0.001

[a] Extraction + precipitation with 85% ammonium sulphate + dialysis.
[b] Extraction + precipitation with 85% ammonium sulphate
[c] Extraction only

Table 2 – Activity quantification of MMP-2 and -9 for the different substrates (ng/mL±SD)

Substrates	MMP-2	MMP-9
Blanck (extraction buffer)	0.14±0.00	0.05±0.00
BR	3.42±0.51	1.80±0.02
BR +APMA	11.43±2.21	14.91±2.02
BR total	**14.85**	**16.71**
HR	5.59±0.00	4.61±0.02
HR +APMA	14.57±2.01	15.34±1.31
HR total	**22.92**	**19.46**
BC	5.06±1.70	6.61±0.06
BC +APMA	9.65±1.16	15.36±1.32
BC total	**14.71**	**21.97**
HC	12.35±0.93	4.00±0.05
HC+APMA	10.57±1.35	15.46±1.32
HR total	**20.16**	**19.95**

*Samples: BR – Bovine root; HR – Human root; BC – Bovine crown; HC – Human crown
Samples + APMA re

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UNIVERSITY of OULU
OULUN YLIOPISTO

Leo Tjäderhane
DDS, Ph.D., Professor
Department of Cariology, Endodontology, and Operative Dentistry

CERTIFICATE

This is to certify that

Bruno Zarella

has worked as a visiting scientist in the Dentin-Pulp Research Group in the Institute of Dentistry, University of Oulu, for four weeks between 1st and 31st June, 2009.

During the visit, he worked in two projects:

1) **Expression and Activity Profiles of Matrix Metalloproteinases (MMPs) in Human and Bovine Dentin;**

2) **The Role of Cola Soft Drink on Activation of Human Salivary Matrix Metalloproteinases (MMPs).**

The projects are part of the research collaboration between the research groups of Professora Marília Afonso Rabelo Buzalaf (Bauru Dental School, University of São Paulo, Brazil) and our group, aiming to clarify the role and importance of MMPs in dental erosion.

Project #1
Project #1 examined the presence of MMPs in human and bovine dentin, identifing and quantitating two most important MMPs in dentin, the gelatinases A and B (MMP-2 and -9). The laboratory procedures used in the work are presented in the end of the certificate in detail.

The work is of importance, since this is the first time that the exact quantitation of these enzymes in human dentin has been performed. Moreover, the comparative analysis between human and bovine dentin confirm that bovine dentin is comparable to human dentin in respect to MMP-2 and -9. Since bovine dentin is widely used to study e.g. erosion and dentin adhesives, the results justify its use in the experiments, where the role and function of gelatinases in the above-mentioned conditions, and others, is used.

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Project #2

Project #2 examined the potential for acidic beverage to activate human gelatinolytic MMPs present in human saliva. The laboratory procedures used in the work are presented in the end of the certificate in detail.

The results indicate that acidic beverages may activate salivary MMP-9. Acidic drinks are important in erosion pathogenesis, and the mechanism of action has so far been thought to be based only on the acidity of the drinks. Since the previous work in Professora Buzalaf's laboratory has demonstrated the role of MMP inhibition in the progression of erosive lesions in dentin, the mechanism of salivary MMP activation by acidic beverages offers a new and unique alternative mode for erosion pathology. Moreover, MMPs have been shown to be important also in caries progression. Therefore, activation of salivary MMPs by acidic beverages may also contribute to the progression of dentinal caries lesions. While the results are still preliminary, the concept of role of MMP activation in erosion and caries pathogenesis is attractive, and the results provide a strong scientific basis for further research.

Bruno Zarella's work in the project was of high importance and significantly contributed to the successful outcome. His visit has therefore been highly successful, and I am confident that the results will attract the related scientific community worldwide.

Oulu, 31st June, 2009

Leo Tjäderhane

The laboratory procedures in the projects:

Project #1:

- Demineralization of dentin
- Enzymatic extraction
- Precipitation of the sample proteins
- Dialyse of samples
- Protein quantitation
- Gelatin zymography
- Gelatinase activity determination with MMP-2 and -9-specific microplate assays

Project #2:

- Sample collection and handling
- Protein quantitation
- pH-controlled activation of sample gelatinases
- Gelatin zymography