JOSE ROSA GOMES

MT1-MMP, MMP-2, MMP-9, TIMP-1,-2,-3 E A PROLIFERAÇÃO CELULAR DA REGIÃO ODONTOGÊNICA DE DENTES INCISIVOS DE RATOS ADULTOS: EFEITO DA DOXICICLINA EM CONDIÇÃO ACELERADA DE PROLIFERAÇÃO CELULAR

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para a obtenção do Título de Doutor em Biologia Buco Dental, área de concentração Histologia e Embriologia.

Orientador: Prof. Dr. Pedro Duarte Novaes

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RESUMO

Metaloproteinases (MMPs) são enzimas que degradam os componentes da matriz extracelular e são reguladas por moléculas denominadas inibidores teciduais de metaloproteinases (TIMPs). São poucos os relatos da função dessas moléculas nos tecidos que sustentam, constituem e dão origem aos dentes. Entretanto, já foi mostrado que antibióticos como a doxiciclina podem reduzir a atividade dessas enzimas. Considerando que o incisivo de rato apresenta crescimento e erupção continuados ao longo da vida do animal assegurada por constante proliferação e diferenciação das células localizadas na região apical (odontogênica) em esmalte, dentina e ligamento periodontal, este se torna um excelente modelo para o estudo da atividade de MMPs e seus inibidores sobre o processo proliferativo e eruptivo do mesmo. Assim, os objetivos deste estudo foram: 1-Avaliar, na região odontogênica, o efeito da doxiciclina e da hipofunção sobre a expressão e a atividade da MMP-2 e MMP-9; a expressão dos TIMP-1,-2,-3 por meio das técnicas de zimografia, zimografia reversa, imunohistoquimica e a expressão de MT1-MMP pela técnica de Western Blot, 2- Avaliar o efeito da doxiciclina e a condição hipofuncional sobre a taxa de erupção dos dentes incisivos, 3- Avaliar a proliferação celular da região odontogênica, na presença de doxiciclina e hipofunção, por meio de marcador de proliferação celular, 4- Estabelecer relação entre os efeitos da doxiciclina, a expressão das MMPs, TIMPs, a proliferação celular da região odontogênica e o processo de erupção. Foram usados de 5 a 7 ratos machos adultos divididos em 4 grupos: controles - normofuncional (NF) e doxinormofuncional (DNF) e tratados - hipofuncional (HP) e doxhipofuncionais (DHP). Os grupos HP e DHP tiveram os dentes esquerdos inferiores cortados com broca de alta rotação e medidos a cada dois dias durante 12 dias. Nos dentes dos grupos NF e DNF uma marca foi feita para estimar a taxa de erupção realizada da margem gengival até a marca ou até o final do dente nos grupos HP e DHP. Os grupos DNF e DHP receberam dose diária matutina, de 80mg/kg de doxiciclina diluída em água por agulha de gavagem durante 14 dias. Os ratos foram sacrificados por deslocamento cervical e as hemimandibulas foram processadas para os métodos de imunohistoquimica, zimografia e Western Blot. Os resultados mostraram que os tratamentos não alteraram a atividade e expressão de MMP-2 e MMP-9 e nem de TIMP-1 e TIMP-3. Entretanto, a hipofunção aumentou a taxa de erupção, a expressão de MT1-MMP, TIMP-2 e a proliferação celular quando comparadas com os grupos controles. Conclui-se que: 1- MMP-9 não participa da remodelação da matriz extracelular na região odontogênica, 2- A doxiciclina não altera a erupção, atividade e expressão das moléculas avaliadas e nem a proliferação celular; 3- A condição hipofuncional altera; aumentando a erupção, a expressão de MT1-MMP, TIMP-2 e a proliferação celular, 4- Existe relação entre o aumento da erupção e proliferação celular bem como entre a expressão de MT1-MMP/TIMP-2 e a proliferação celular sugerindo papel importante dessas moléculas nos processos de proliferação e erupção.
Palavras chaves: proliferação celular, incisivo, erupção, metaloproteinase, doxiciclina

Abstract:

Metalloproteinases (MMPs) are enzymes that degrade extracellular matrix components and are regulated by molecules called tissue inhibitors of metalloproteinases (TIMPs) and there is few reports of the function of these molecules in the tissues that support and give rise to teeth. However, it has been shown that antibiotics such as doxycycline can reduce the activity of these enzymes. Whereas the rat incisor has erupted and continued growth over the life of the animal provided by continuing proliferation and differentiation of cells in the apical region (odontogenic) on enamel, dentin and periodontal ligament, it becomes an excellent model for studying the activity of MMPs and their inhibitors on the proliferative and eruption process. Thus the aims of this study were: 1-To study in odontogenic region, the effects of doxycycline and hypofunctional condition on the expression and activity of MMP-2 and MMP-9, the expression of TIMP-1, -2, -3, using techniques of zymography, reverse zymography, immunohistochemstry and the expression of MT1-MMP by Western Blot; 2 - To evaluate the effect of doxycycline and hypofunctional condition on the eruption rate of incisors. 3 - To estimate the cellular proliferation of odontogenic region in the presence of doxycycline and hypofunctional eruption condition, using cell proliferation marker, 4 - Establish relationship between the effects of doxycycline, the expression of MMPs, TIMPs, the cellular proliferation of the odontogenic region and the process of eruption. Were used 5 to 7 adult male rats divided into 4 groups: control-functioned normally (NF) and doxinormofuncional (DNF) and treated-hipofuncional (HP) and doxihipofuncional (DHP). The groups HP and DHP had left lower teeth cut with high-speed drill every two days until for 12 days. In the teeth of groups NF and DNF a mark was made to estimate the rate of eruption of the gingival margin held up to the mark or until the end of the tooth in groups HP and DHP. Groups DNF and DHP received daily morning dose of 80mg/kg of doxycycline diluted in water by needle gavage for 14 days. The rats were sacrificed by cervical dislocation, and the hemimandibles were processed for immunohistochemical methods, zymography and Western Blot. The results showed that the treatments did not alter the activity and expression of MMP-2 and MMP-9 nor TIMP-1 and TIMP-3. However, the hypofunction increased the rate of eruption, the expression of MT1-MMP, TIMP-2 and cell proliferation when compared with control groups. We conclude that: 1 - MMP-9 does not participate in remodeling the extracellular matrix in the odontogenic region 2 - Doxycycline does not alter the eruption, activity and expression of the molecules assessed and not cell proliferation, 3 - The condition hipofuncional changes, increasing the eruption, the expression of MT1-MMP, TIMP-2 and cell proliferation, 4 - There is a relationship between the eruption and increased cell proliferation and between the expression of MT1-MMP/TIMP-2 and cell proliferation suggesting an important role of molecules in the cell proliferation and eruption processes.

Key words: cell proliferation, incisor, eruption, metaloproteinases, doxycicline

SUMÁRIO

INTRODUÇÃO

Durante o desenvolvimento de qualquer órgão, as populações celulares passam por processos cinéticos como a proliferação, a migração, a diferenciação e a morte celular. A regulação destes processos geralmente ocorre pela interação molecular entre tecidos próximos, através da indução ou inibição recíproca.

Um exemplo marcante desta interação tecidual é a que ocorre entre epitélio e o mesênquima e que determina o aparecimento das fases de lamina dentário, botão, capuz, campânula e finalmente a forma anatômica e funcional dos dentes. Nesta interação, todos os processos cinéticos que ocorrem no epitélio e no mesênquima, são rigorosamente controlados através de diferentes e complexas vias de sinalização molecular (Jernvall & Theslef, 2000) e, entre os diferentes processos que ocorrem durante a formação do dente está o de remodelação da matriz extracelular através da ação das metaloproteinases (MMPs).

As MMPs são enzimas proteolíticas, capazes de degradar todos os componentes da matriz extracelular. Entretanto, o controle da atividade das MMPs é realizado pelos inibidores teciduais das metaloproteinases (TIMPs) que são em número de quatro e denominados como TIMP-1,-2,-3 e -4.

As metaloproteinases são sintetizadas sob a forma de pró-enzimas (pró-MMPs), por diferentes tipos celulares presentes nos tecidos conjuntivos, e possuem sítios catalíticos zinco e cálcio dependentes (Stamenkovic, 2003). Existem as metaloproteinases de matriz (MMPs) e as de membrana celular (MTs-MMP). Dentre as MMPs destacam-se a MMP-2, também denominada gelatinase A e a MMP-9, denominada como gelatinase B (Sahlberg *et al.*, 1999) e, entre as de membrana destacamos a MT1-MMP (Beertsen *et al.,* 2002).

MMP-2 e 9 são chamadas de gelatinases porque finalizam a digestão do colágeno inicialmente clivado por outras MMPs, entre elas estão as MMPs-1,-13 e 18 (Sahlberg *et. al.*,

1999). MMP-2 e 9 também atuam sobre o colágeno do tipo IV encontrado principalmente nas membranas basais (Sahlberg *et al.*, 1999; Yoshiba *et al.*, 2003). O colágeno IV apresenta sítios de ligação com outras importantes moléculas como a laminina 5 e fibronectina também presentes nas membranas basais. Esses componentes da matriz mantêm contato funcional com diferentes integrinas e assim, podem controlar diferentes processos celulares (Ryan *et al*., 1999).

Já entre as metaloproteinases de membrana, destaca-se o papel da MT1-MMP nos processos proteolíticos pericelulares (Barbolina *et al.,* 2008) em diferentes tecidos conferindo às células ou populações celulares a habilidade invasiva, no caso dos tumores (Egeblad & Werb, 2002) mas também a migração das células em tecidos normais para substituir aquelas perdidas da população ou mesmo durante a morfogênese.

Apesar de existir um amplo conhecimento das funções das metaloproteinases em diferentes tecidos sob diferentes tratamentos, ainda são poucos os relatos do papel funcional das MMP-2 e 9 e seus inibidores (TIMPs) em tecidos que dão origem ou mesmo sustentam os dentes no alvéolo dentário na vida pós-natal.

Nos tecidos dentários Beertsen *et al.* (2002) mostraram, que em dentes molares de ratos mutantes para o gene da MT1-MMP, houve uma inibição severa tanto da erupção quanto do crescimento da raiz e um acúmulo de fagolisossomas nos fibroblastos em função da idade dos ratos. Eles sugerem que esta metaloproteinase é importante no processo final de fagocitose das fibrilas colágenas e, conseqüentemente no processo de remodelação do ligamento periodontal.

Entretanto, são recentes as descrições da localização e da expressão das MMPs e TIMPs, e se resumem, principalmente durante o desenvolvimento de dentes molares e incisivos de ratos, sendo poucas as conclusões sobre o papel funcional tanto das MMPs como

dos TIMPs, nos processos de remodelação da matriz extracelular nos tecidos envolvidos na formação, erupção e manutenção dentária.

Sahlberg *et al.*, (1999) estudaram a expressão das TIMPs-1,-2 e 3 e MMP-2 e –9 em molares de camundongos, a partir do 12º dia de gestação até 11º dia de vida pós-natal. Os autores mostraram que a expressão dessas moléculas, no início da formação do dente, é marcante no mesênquima dentário, com exceção apenas para TIMP-3 que também foi expresso no epitélio dentário. Em estágios mais avançados do desenvolvimento, MMP-2 e TIMP-1 são expressos pelos odontoblastos em diferenciação, indicando que a membrana basal entre odontoblastos e ameloblastos sofre degradação, para em seguida ser depositada a dentina e que, a presença de TIMP-1 pode controlar a atividade de MMP-2 durante este processo. Já MMP-9 e TIMP-2 foram observadas no mesênquima dentário que formará o tecido ósseo adjacente. TIMP-3, em estágios mais avançados e no pós-natal, foi encontrado nos ameloblastos, no estrato intermediário e retículo estrelado bem como no folículo dentário.

Yoshiba *et al.*, (2003) mostraram a presença de MMP-2,-9 e MT1-MMP na membrana basal do epitélio dentário e no mesênquima, até o início da fase de campânula. Também foram detectadas na região apical de ameloblastos e odontoblastos em diferenciação. A expressão de TIMP-1 foi observada na membrana basal do epitélio dentário nas fases de botão, capuz e início de campânula, porém, na fase final de campânula desaparece da membrana basal na região de diferenciação de odontoblastos e ameloblastos. Após o nascimento, TIMP-1 reaparece na membrana do epitélio da alça cervical e persiste durante o desenvolvimento pós-natal. Já TIMP-2 foi expresso no mesênquima, durante as fases de botão até o início da fase de campânula, na membrana basal da lâmina dentário e no órgão primário do esmalte na fase final de campânula. Durante o desenvolvimento pós- natal, TIMP-2 foi observado na membrana basal da gengiva, na matriz do esmalte, e no mesênquima. Já TIMP-3, segundo os

autores, apresentou uma distribuição em função do tempo/espaço durante o desenvolvimento do dente. Este foi detectado na membrana basal e nas células endoteliais na fase de botão. Desaparece gradualmente da membrana basal do epitélio dentário interno durante a fase de capuz e, reaparece em pré-ameloblastos na fase final de campânula. Os autores sugerem que TIMP-3 pode estar diretamente envolvido nos processos de proliferação e diferenciação tanto de odontoblastos como de ameloblastos.

Já, durante o desenvolvimento de dentes incisivos Yoshiba *et al.*, (2006) demonstraram a existência diferencial da expressão das TIMPs-1,-2 e -3. TIMP-1 foi detectado de forma mais intensa nas regiões de interface entre o epitélio e o mesênquima, preferencialmente nas membranas basais. Também foi observado na alça cervical, porém TIMP-1 desaparece na membrana basal da região de pré ameloblastos e odontoblastos em diferenciação e, reaparece nos ameloblastos e na matriz do esmalte. TIMP-2, no início do desenvolvimento do dente predomina no mesênquima dentário e mais tarde está presente no folículo dentário, permanecendo nesta região, durante o desenvolvimento pós-natal. Também é detectado durante a mineralização, nos ameloblastos. TIMP–3, diferentemente do que ocorre durante o desenvolvimento dos molares, foi detectado no mesênquima associado à alça cervical, durante todo o desenvolvimento do incisivo. Também foi evidenciado nas células endoteliais, nas células epiteliais da alça cervical, na camada subodontoblástica e entre os odontoblastos. Com dois dias de vida pós-natal foi fortemente detectado ao longo da alça cervical, porém desaparece da membrana basal da zona de citodiferenciação e, reaparece mais tarde, na região distal dos ameloblastos.

O papel dos TIMPs em inibir a atividade das MMPs nos processos de morfogênese ou de remodelação da matriz é evidente. Sabe-se que TIMP-1 inibe a atividade de muitas MMPs com exceção da MT1-MMP e MMP-2 enquanto que TIMP-2 também inibe a atividade de

várias MMPs com exceção da MMP-9 (Yoshiba *et al.*, 2003). Durante a mineralização do dente a MMP-9, que é secretada pelos ameloblastos, é inibida por TIMP-1 (Ogata *et. al.*, 1995). TIMP-3 inibe a atividade de MMP-1-2-3-9 e MT1-MMP (Yoshiba *et al.*, 2003).

Entretanto, vários trabalhos têm mostrado que os TIMPs possuem outras funções além de inibir as MMPs e assim controvérsias aparecem na literatura sobre os demais papeis realizados pelas TIMPs, em função do tipo celular e situações experimentais estudadas.

Hayakawa *et al.*, (1992, 1994) e Yan *et al.*, (1992), mostraram que TIMP-1 e TIMP-2 possuem função de promover o crescimento celular enquanto que Liu *et al.,* (2003) demonstraram que TIMP-1 possui efeitos anti-apotóticos. A ação de TIMP-1 sobre a diferenciação de eritrócitos e linfócitos B também já foi mostrada (Petifrere *et al.*,2000) bem como a contribuição de TIMP-1 na diferenciação de odontoblastos (Yoshiba *et al.*, 2003).

Hoegy *et al.*, (2001) mostraram que TIMP-2 inibe a proliferação celular e Hayakawa *et al.*, (1992, 1994) e Yang *et al.*, (1992) mostraram que esse inibidor promove o crescimento celular. Yoshiba *et al.*, (2003) evidenciaram a presença de TIMP-2 na membrana basal da região externa da alça cervical durante o desenvolvimento de incisivos, entretanto, observaram que nesta região não existe marcação para o antígeno Ki-67, (que é expresso em células em processo proliferativo), levando os autores a sugerir que TIMP-2 tem papel de inibir a proliferação celular.

As mesmas controvérsias envolvem a ação de TIMP-3 onde foi sugerido que este desempenha um papel na regulação da progressão do ciclo celular e diferenciação celular por Wick *et al.*, (1994) enquanto que Baker *et al.*, (1998) e Bond *et al.*, (2000 e 2002) descreveram um papel pró-apoptótico para TIMP-3. Já Yoshiba *et al.,* (2003), mostraram que TIMP-3 predomina no mesênquima associado ao epitélio da alça cervical durante o desenvolvimento de incisivos e, portanto, teria uma função de estimular a proliferação celular.

Por outro lado, Lu *et al.,* (2005), mostraram que a expressão de TIMP-3 parece estar na dependência da indução do fator de crescimento de fibroblasto do tipo dez (FGF10). Já o papel do FGF10 na manutenção das células tronco do retículo estrelado do dente incisivo e, conseqüentemente em estimular a proliferação das células do epitélio interno do esmalte foi demonstrado por Harada *et al.* (2002) e Tamaki *et al.*, (2006). Entretanto outras moléculas também podem estar envolvidas no processo de proliferação celular da região odontogênica, como é o caso do fator de crescimento epidermal (EGF) que está aumentado em condições aceleradas de erupção do dente incisivo (Shore *et al.*, 1992). Percebe-se que inúmeras moléculas estão envolvidas no processo de desenvolvimento do dente e entre elas algumas já estão com suas funções mais bem definidas como é o caso do FGFs. Entretanto, a relação entre as MMPs e TIMPs no processo proliferativo do dente incisivo ainda precisa ser avaliada em virtude das controvérsias apresentadas anteriormente.

Por outro lado, apesar dos TIMPs terem a função de inibir as MMPs durante a morfogênese, em estados patológicos como a doença periodontal e nas metástases, entre outras, onde a degradação da matriz extracelular pelas MMPs é um fator importante para a progressão da doença. Nestes estados patológicos o controle da atividade proteolítica das MMPs é realizado por drogas sintéticas que atualmente são classificadas em três categorias: a) os peptídeos que mimetizam o colágeno (derivados do hidroximato) que possuem sítios onde se ligam as MMPs que, uma vez ligadas sofrem clivagem ; b) os bifosfanatos, cuja ação ainda não está totalmente elucidada, porém sabe-se que ao se ligarem na matriz inorgânica de tecidos mineralizados, inibem a atividade de MMPs secretadas pelos osteoclastos e c) os derivados de tetraciclinas como a minociclina e doxiciclina que inibem, não apenas a atividade mas também a síntese de MMPs por diferentes mecanismos ainda não bem elucidados (Hidalgo & Eckhardt, 2001).

A doxiciclina, por exemplo, é uma tetraciclina muito utilizada e estudada como um agente antitumoral exercendo funções de inibição de síntese e atividade das MMPs. Em cultura de células tumorais, a doxicilina inibe a produção de MMP-2 e MMP-9, a proliferação de linhagens de osteosarcomas, e induz a apoptose de linhagens de melanoma. *In vivo*, a doxicilina quando associada à um peptídeo mimético inibe a progressão de câncer de mama (Hidalgo & Eckhardt, 2001). O uso das tetraciclinas inibindo a atividade das MMPs, também está entre as terapias empregadas no controle da doença periodontal (De Souza *et al.*, 2005).

A partir das considerações feitas anteriormente percebe-se que o estudo das MMPs e TIMPs é de fundamental importância para se entender as relações que se estabelecem entre epitélio e mesênquima durante o desenvolvimento do dente, após sua completa formação bem como em estados patológicos.

Dessa forma, como o dente incisivo do rato é de crescimento contínuo, por conter um compartimento de células tronco e um compartimento proliferativo, este se apresenta como um interessante modelo para o estudo das relações entre a expressão de MMPs, TIMPs, FGF10 e outras moléculas envolvidas em processos cinéticos como a proliferação, migração, diferenciação e morte celular. Neste sentido, avaliações da cinética celular da região odontogênica podem ser realizadas utilizando tratamentos que estimulem tais processos, seguido de outros que interfiram na atividade das MMPs e ou TIMPs.

Entre os métodos utilizados para acelerar a proliferação celular da região odontogênica está o de desimpedir o dente incisivo continuamente. Este método estimula a proliferação da região odontogênica, reduzindo o tempo do ciclo celular e aumentando conseqüentemente, a taxa de erupção do dente (Zajicek *et al.*, 1972). Aliado ao uso de drogas como a doxicilcina que interfere, segundo a literatura, na atividade das MMPs (Hidalgo & Eckhardt, 2001) estes

tratamentos pode fornecer informações sobre a relação entre as MMPs, TIMPs, a proliferação celular da região odontogênica e o processo eruptivo.

Considerando que o dente incisivo apresenta uma região odontogênica responsável pela formação contínua da estrutura dentária, através da proliferação celular que ocorre no epitélio da alça cervical, e conseqüentemente da migração e diferenciação das células ameloblásticas e odontoblásticas em direção incisal;

Considerando que estas características morfológicas, presentes no dente incisivo, permitem a manutenção durante estes processos, de uma relação direta entre o epitélio da alça cervical com o mesênquima associado, isto é uma relação de interdependência funcional entre epitélio e matriz extracelular para a renovação contínua dos tecidos dentários que são desgastados na região incisal;

E finalmente, considerando que alterações que ocorrem na estrutura da matriz extracelular podem perturbar a relação funcional entre epitélio e matriz extracelular este projeto propôs aprofundar o estudo desta inter-relação buscando entender melhor o papel das metaloproteinases e seus inibidores teciduais presentes na região odontogênica dos incisivos de ratos.

Dois tratamentos foram utilizados: o desimpedimento; que acelera os processos de erupção e proliferação celular e a administração da doxiciclina que segundo a literatura, interfere na atividade das MMPs.

Portanto, tentou-se neste projeto, provar a hipótese da existência de uma relação entre a atividade, expressão da MMP- 2, -9, MT1-MMP, os TIMP-1,-2,-3, com a proliferação celular e o processo eruptivo, uma vez que tais informações ainda não existem. Além disso, os resultados deste projeto permitirão visualizar quais as moléculas possuem potencial envolvimento funcional nos processos de remodelação e proliferação celular da região

odontogênica do incisivo de ratos e que podem consequentemente estar envolvidas na erupção do dente.

Dessa forma os objetivos que nortearam o presente trabalho foram:

a- Avaliar, na região odontogênica, o efeito da doxiciclina e da hipofunção sobre a expressão e a atividade da MMP-2 e MMP-9; a expressão dos TIMP-1,-2,-3 por meio das técnicas de zimografia, zimografia reversa, imunohistoquimica e a expressão de MT1-MMP pela técnica de Western Blot,

b- Avaliar o efeito da doxiciclina e a condição hipofuncional sobre a taxa de erupção dos dentes incisivos,

c- Avaliar a proliferação celular da região odontogênica, na presença de doxiciclina e hipofunção, por meio de marcador de proliferação celular,

d- Estabelecer relação entre os efeitos da doxiciclina, a expressão das MMPs, TIMPs, com a proliferação celular da região odontogênica e o processo de erupção.

CAPÍTULO I

Doxycycline and hypofunction condition effects on the eruption rate, activity and proteins expressions of MMP-9, MMP-2, TIMP-1, TIMP-2 and TIMP-3 from odontogenic region of rat incisor tooth

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Abstract: Doxycycline can inhibit the metaloproteinases activity in different systems however in tooth, submitted to altered eruption condition, it is little known. Thus, doxycycline and hypofunction condition effects on the eruption rate, activity and proteins expressions of MMP-9, MMP-2, TIMP-1, TIMP-2 and TIMP-3 from odontogenic region of rat incisor tooth were evaluated. Doxycycline (80mg/kg/day) were gavages in male Wistar rats during 14 days. Two days after the beginning the treatment, left lower incisors were shortened every two days using a drill high rotation producing hypo-functional (HP) or doxycyline hypo-functional (DHP) groups. Rats with intact lower teeth were considered to undergo normal eruption and were called normofunctional (NF) or as doxycyline normofunctional (DNF) groups. Eruption rate and TIMP-2 were doubled in HP and DHP and MMP-9/MMP-2 expression/activity did not changed as well as TIMPs 1 and 3. We conclude that TIMP-2 may be related with eruption rate.

1. Introduction

Matrix metalloproteinases (MMPs) constitute a large family of endopeptidase proteins that degrade all components of the extracellular environment. The most studied MMPs are MMP-9 and MMP-2, found in the matrix compartment however MMPs are also present in the cell membrane which is denominated matrix membrane metaloproteinases (MT-MMP).[1]

MMP-9 and MMP-2 are denominated as gelatinases since their substrate is degraded collagen. MMP-9 is synthesized by cell types such as macrophages and neutrophils; furthermore, its presence indicates tissue inflammation. MMP-2 is produced by resident cells of connective tissues such as fibroblasts and condroblasts.

On the other hand regulation of endogenous MMP activity involves complex interactions among MMPs and different molecules types that are not yet completely understood. However, it has been demonstrated that proteins denominated tissue inhibitory of metalloproteinases (TIMPs) perform this regulation. There are four TIMPs; TIMP-1 inhibits MMP-9 activity and TIMP-2 inhibits MMP-2, TIMP-3 inhibits MMP-1,3,7,13 and TIMP-4 inhibits MMPs 1,2,3,7,9. However the association betweenTIMP-2 with MMP-2 and both establishing contact with the cellular membrane metalloproteinase denominated MT1-MMP promotes activation of MMP-2.[2,3]

Conversely, in a disease status when there is a higher degradation of connective tissues by MMPs, such as periodontitis, cancer, arthritis and other degenerative diseases, tetracyclines including doxycycline are often used to inhibit the MMP activity. The effects of tetracyclines on the reduction of MMP-9 and MMP-2 activity is already known *in vivo* as well as *in vitro.* [4,5,6,7,8,9,10]

Thus, matrix extracellular remodeling is a normal or inductive process that is present in all organs. The interference of exogenous inhibitors of MMPs activity such as chemically modified tetracyclines is little known in rat incisor tissues.[5,10] However in teeth, the presence of MMPs and TIMPs has been demonstrated during the morphogenetic process of molar and incisors. [11,12,13,14]

On the other hand in the odontogenic region of the rat incisor, stem cells are observed in the cervical loop, surrounded by the dentário follicle region, in which the cells have mesenchimal characteristics. The molecular signaling interaction between these two compartments maintains the cell population of the enamel organ in continuous maturation to produce the tooth and consequently assure, in addition to surround connective tissues, its eruption to oral cavity. [15] Therefore it is expected that, during the eruptive process, an intense remodeling may exist in the extracellular matrix of odontogenic region. In spite of this, none reported was found about the roles of MMPs and TIMPs in odontogenic region tissue, such as dentário follicle and enamel organ in a altered eruption condition.

Conversely, the eruption of the rat incisor can be easily altered experimentally by immobilization of the tooth or shortening treatments.[16,17,18] Therefore, we hypothesized that these alterations may also alter the responsiveness of the molecules involved in the remodeling process. As such, the present study aims to investigate the possible role of MMPs and TIMPs in the odontogenic region of the rat incisor in an altered eruption condition. Thus, the effects of the hypofunction condition and doxycycline on eruption rate, protein expressions of TIMP-1, TIMP-2, TIMP-3 and activity of MMP-9 and MMP-2 were determined to improve the knowledge regarding the proteins involved in the remodeling process of the extracellular matrix and, in particular, in odontogenic region.

2. Material and Methods

2.1 Animal experimental groups: Male adult Wistar rats were divided into four groups of five up to seven rats, according to the eruption condition and methods employed: normofunctional (NF); doxycycline normofunctional (DNF); unimpeded/hypofunctional (HP) and doxycycline unimpeded/hypofunctional (DHP).

2.2 Treatments: The DNF and DHP groups received a doxycycline daily dose at 80mg/kg (dissolved in water) orally by blunt tip needle for 14 days. The NF and HP groups received only water and their left lower teeth were considered as control (figure 1A). Two days after the beginning of the doxycycline treatment, until day 12, the HP and DHP groups had their left lower incisors shortened every two days using a high rotation drill to produce the unimpeded, that is, hypofunctional eruption condition (figures 1 B). Doxycycline and shortened treatments as well as the collect of hemimandibles were performed always in the morning to avoid circadian rhythmic variation. The total duration times to the hypofunctional and doxycycline treatments were 12 and 14 days respectively.

2.3 Eruption estimative: The eruptions were always measured in the morning, every two days until the 14^{th} day using a millimetric ocular, from the gingival margin to the incisal end in the HP and DHP groups, and until the mark made on the tooth in the NF and DNF groups, to obtain the eruption rate mean as indicated in the figure 1 (A, B) .

2.4 Hemimandibles processing: After cervical dislocation, the hemimandibles of 5 rats per group were removed and were immersed in 4% paraformaldehyde for 48h. Hemimandibles were then demineralized in 4.12% EDTA for one month and were then reduced to small fragments from the distal level of the third molar to the end of the mandible as indicated in the figure 2. After this, samples were dehydrated and included in paraplast to obtain cross sections of 5 μm from apical end of the incisor to the incisal direction according

showed in figure 2. Sections were mounted on appropriate slides; underwent dewaxing and rehydrating steps and then followed to immunohistochemistry methods.

2.5 Immunohistochemistry method: Endogenous peroxidadse activity was quenched three times with 2% hydrogen peroxide for 10 min each, and slides washed in water and PBS pH 7.4. Sections (3 per group) were immunostained with primary antibodies (2µg/ml purchased from Chemicon), as follows: MMP-9 (1:200), MMP-2 (1:250), TIMP-1, TIMP-2 and TIMP-3 (1:20) in PBS-1% BSA, overnight. After washing in PBS, the sections were incubated with biotinylated secondary antibody (LSAB2 system, DAKO), followed by streptavidin–peroxidase complex (LSAB2 system, DAKO) for 30 min at 37°C each one. Brown staining was produced by sections treatment with diaminobenzidine solution (3,3diaminobenzidine; Sigma, St. Louis, MO, USA) and counterstaining was produced with haematoxylin on three sections of each animal for qualitative and quantitative analysis. Control sections were carried out by either omitting or substituting the primary antisera with non-immune serum.

2.6 Zymography method: Three pools per group containing 5 rats were used. In each hemimandibles (left) a window was opened in the bone to collect the odontogenic tissues as demonstrated in the figure 3 which were then placed in DMEN culture medium (Dubelcco's modified Eagle medium) purchased from SIGMA without addition of fetal bovine serum but containing 100µg/ml garamicyn antibiotic. The samples were incubated at 37°C for 12 hours (this time was enough to the MMPs secretion and produces the conditioned media). After this time the odontogenic tissues were discarded and conditioned media were submitted to quantification of total protein by the Bradford method.

The zymography method is very sensitive to minimum changes in the volume of sample applied. This way to avoid errors during analysis of results were used in each pool

samples three applied concentrations of total protein 0.2, 0.1 and 0.05 μg (where the second lane had half of the first and third lanes half of the second lane). Samples were electrophoresed in a 10% polyacrylamide gel, containing 0,06% gelatin, for 4 hours. Gels were rinsed twice (30min) in 2.5% Triton X-100, under shaking, and then immersed in incubation buffer (1M CaCl; 1M Tris-HCl) for 16h at 37°C to activate MMPs from samples protein applied. After this period, gels were stained with Comassine Blue until the degraded bands could be seen.

2.7 ***Inhibiton of proteinases test:*** Inhibition assays were carried out by incubating the zymogram gels in 0.5mM of specific protease inhibitor proteases, such as 1,10-phenantroline (PHE), N-ethylmaleimide (NEM) and phenylmethanesulphonylfluoride (PMSF) which are a metalloproteinase, serinase and cysteinase specific inhibitors to confirm the nature of the metalloproteinases MMP-2 and MMP-9 in the samples in each group studied.

2.8 Image analysis: The images of sections immunostained to MMP-9, MMP-2, TIMP-1, TIMP-2 and TIMP-3 were taken at a magnification of 50X using microscopy and software (Leicca). The images from sections staining were analyzed by Image J software 1.42 using the deconvolution pluggin, HDAB. This pluggin converts the intensity of immunostaining to pixel values from zero (0) up to 255.

Thus, in the immunohistochemistry interpretation results, values close to zero represent greater staining and values close to 255 represent slower staining.

The images of zymography bands were taken with a photodocumenting equipment and the areas of band degradation were converted to pixels using Image J 1.42 software (NIH) to obtain the density of area degrade.

2.9 Statistical analyses: The results were analyzed with ANOVA with Tukey post test at ($P<0.05$) using the graphpad prism software 3.0.

3. Results

3.1 Eruption rate: The estimated eruption rate, measured every two days, in all teeth of the groups studied is demonstrated in Figure 4. Data demonstrate that there was a significant increase ($P<0.05$) in the eruption rate in teeth submitted to hypofunctional (HP) conditions and also in HP teeth that received doxycycline (DHP). The eruption rate values, calculated daily in millimeters ± standard error, were (0.99 ±0.08) for NF, (1.05± 0.13) for DNF, (1.97 ±0.14) for HP and (1.91±0.10) for DHP. Results show that the eruption rates of the HP and DHP groups were almost doubled, compared to the NF and DNF groups. In spite of this, during all periods of doxycycline administration, the eruption rate of the DHP group showed a tendency towards reduction, but this was not significant compared to the HP group, that is, doxycycline at a dose of 80mg/Kg did not have an effect on the eruption rate.

3.2 Immunohistochemistry: Immunostaining for MMP-2 and MMP-9 are presented in figure 5 and for TIMPs in figure 6. In the odontogenic region, MMP-9 was stained only in nervous structures. It is probable that MMP-9 was localized in the membrane of Schwann cells in all groups studied and, therefore, statistical analysis was not carried out. None of the immunological lineage cells were seen to incorporate the antibody to MMP-9, indicating that the shortening treatment did not induce an inflammatory state in the odontogenic region.

MMP-2 and TIMPs staining was detectable in all cells of the dentário follicle and enamel organ epithelium. An intense staining was also observed in the preodontoblast and preameloblast cells and in dentário follicle in cells localized on the surface of the alveolar bone on the lingual side. In contrast, a weaker staining was seen in cells of the papilla region in all studied groups.

3.2.1 ***Statistical analysis of immunohistochemistry:*** The Image J software transforms the intensity of the staining in pixel values where the optical density (near to 255) indicate slower staining while low values (close to zero) indicate an intense staining. Thus, figure 7 demonstrated that MMP-2 expression did not change under any of the eruption conditions or even following doxycycline treatment. However figure 8 shows to TIMP-2 lower values to density optical ($P<0.05$) in the HP and DHP groups, that is, an intense staining indicating that the expression of TIMP-2 was increased in the HP and DHP groups, independently of the drug effect.

3.3 Zimography: The zymographic analysis (Figure 9, A) and zymographic inhibitory tests (Figure 9,B), particularly those carried out with phenantroline (a specific inhibitor of MMP) confirmed the presence of MMP-9 and MMP-2 from 3 pools of the odontogenic region in each group studied. Figures demonstrate gelatin band degradation, corresponding to MMP-9 and MMP-2 activity, at 92 and 72kDa respectively. However the MMP-9 bands observed were produced by nervous structures existent into the dentário follicle according to observed by immunohistochemistry and therefore the MMP-9 bands were not considered to statistical analysis

3.3.1 Statistical analysis of zimograms: Figure 10 present the optical density of gelatin degraded bands and demonstrated that there was no changed in the MMP-2 activities after the shortening treatment, as well as after doxycycline treatment among the studied groups in none of three different concentration of total protein samples analised.

4. Discussion

Doxycycline may inhibit MMPs in different tissues by several mechanisms, both direct and indirect. Doxycycline can bind to the Zn^{2+} or Ca^{2+} associated with the enzyme, thus blocking the active site or inducing conformational changes that render the pro-enzyme

susceptible to fragmentation during activation.[19,20] Tetracyclines can also prevent the oxidative activation of pro-MMP zymogen in vitro.[21,22,23]

After an inductive injury, there is an increase in tissue MMP-2, as well as TIMP-1, activity/expression, indicating that injury can activate molecules involved in the remodeling process. However, if doxycycline is administered, the activities of MMPs and TIMPs, as measured by zymogram and reverse zymogram methods, are down regulated, showing the inhibitory effect of doxycycline on the MMP-2 activity and a reduction in birefringence of collagen fibers was also detected.[6] These authors suggested that doxycycline can also change the collagen fiber organization present in the aorta wall after inducible injury following by doxycycline treatment. However, TIMP-2 expression was not altered by any of the situations of injury or doxycycline treatments.

In human endothelial cells, an indirect MMP inhibitory effect of doxycycline on phorbol-12-myristate-13-acetate (PMA) and TNFα, which mediated the induction of MMP-8 and MMP-9 was also demonstrated.[24] The inhibitory effects were measured by zymogram activity and also by mRNA synthesis level, although no effects were observed on MMP-2 or even on TIMP-1 and TIMP-2. The authors suggest that endothelial cells may display a specific, doxycycline-sensitive regulation of metalloproteinase synthesis. An indirect inhibitory effect of doxycycline were observed on TGFβ, which induced MMP-9 via Smad and the MAPK pathways in human corneal epithelial cells, reducing MMP-9 synthesis and activity.[8]

Studies in sulfur mustard-induced respiratory lesions in guinea pigs, which are characterized by epithelial damage associated with inflammatory cell infiltration, showed that MMP-2 and MMP-9 activities were increased after analyses of zymography and in situ

zymography, but no changes were observed in TIMP-1 or TIMP-2. However, after doxycycline treatment, there was a reduction in MMPs activity without alteration in TIMPs.[4]

Numerous studies are available regarding the effects of doxycycline on MMPs and TIMPs in several different normal and abnormal tissues; however, although doxycycline is routinely used for the treatment periodontitis [25,26] there is still little reported about its effects in dentário tissues *in vitro* and *in vivo*. One report, employing modified tetracyclines and observing the MMP-2 production by periodontal ligament cells *in vitro,* demonstrated that a lower concentration of tetracycline stimulated MMP-2 production while a higher concentration inhibited the activity of MMP-2 and MMP-9.[10] On the other hand, a collagen-mimicking peptide, which can inhibit MMP activity, was administrated during mouse molar development and found to alter the morphogenesis and mineralization of the tooth germs, demonstrating the critical role of MMPs in the processing and maturation of the dentário matrix.[5]

On the other hand we hypothesized, as previously found for other systems, that doxycycline could also have an inhibitory effect on MMP-2 and MMP-9 activity from the odontogenic region, as well as on the expression of these MMPs and their tissue inhibitors (TIMPs). In contrast to results already described in literature, regarding doxycycline, in the odontogenic region of the rat incisor, a daily dose of 80mg/kg doxycycline did not change the eruption rate or even protein expression/activity of either MMP-2 or MMP-9 measured by immunohistochemistry or zymography methods.

In general, MMP-9 is associated with the presence of inflammatory cells[26]; however, we found MMP-9 in association with nervous structures, probably in the membrane of axons or Schwann cells entering and leaving the odontogenic region. The presence of MMP-9 has been previously described in Schwann cells and a relationship between adhesion molecules and MMPs was also observed in central nervous system lymphomas.[27,28] We suggest that

MMP-9 does not have any role in remodeling of the extracellular matrix in the odontogenic region under normal or altered eruption conditions. In spite of this, the present results from zymogram methods showed a presence of degraded bands at 92kDa, corresponding to MMP-9. Since zymogram is a highly sensitive method, we conclude that the degraded bands observed at 92kDa came from the nervous structures into dentário follicle surrounding the odontogenic region.

On the other hand, our results also demonstrate that, in the odontogenic region, there were no changes in the expressions of MMP-2, TIMP-1 and TIMP-3, as well as the MMP-2 activity, under hypofunctional eruption conditions or following doxycycline treatment, as studied by zymography. However, an increase in TIMP-2 expression was observed only in groups in which the tooth was submitted to hypofunctional conditions (HP and DHP) concomitantly with the increase in the eruption rate produced by shortened treatment.

Although the complete knowledge regarding the role of TIMP-1 and TIMP-3 is still lacking, it is known that, in general, TIMP-1 has an inhibitory effect on MMP-9, and that TIMP-2 inhibits MMP-2, while TIMP-3 can inhibit other MMPs. Our results are therefore consistent with these notions. MMP-9 was not present in the dentário follicle, except in the nervous structure and, therefore, TIMP-1 and TIMP-3 could be expressed in its physiological state. MMP-2 activity may be inhibited by TIMP-2, whose expression was increased after the shortening treatment, as observed in the HP and DHP groups.

In contrast to TIMP-1 and TIMP-3, the functional role of TIMP-2 is well established. TIMP-2 not only inhibits MMP-2 activation, in some conditions, it also promotes its activation. When TIMP-2 is bound to MMP-2, they form a complex with MT1-MMP present in the cell membrane leading to the MMP-2 activation.[29,30] Thus, we can suggest that the hypofunctional condition increased TIMP-2 expression in the HP and DHP groups, which may

be related to the increase in eruption rate observed in these groups although the exact relationship for these observation need to be evaluated yet. One possibility is that TIMP-2 could be related to the presence of MT1-MMP improving the cells of the enamel (odontoblast and ameloblast) from differentiation to maturation compartments, along the odontogenic region, since a functional role of MT1-MMP on the molar eruption was already demonstrated.[11]

Another experiment realized in our laboratory, under the same conditions of this present study, evaluated the MMP-2 activity in the periodontal ligament of the rat incisor. In the periodontal ligament, MMP-2 activity was significantly decreased by doxycycline (measured by immunohistochemistry and zymography) under normal and hypofunctional conditions (data not published), indicating that MMP-2 from the periodontal ligament may have some role in the remodeling process of the periodontal ligament during the eruption of the rat incisor, in contrast to the results observed in the odontogenic region in the present study.

Conversely, the effects of doxycycline on TIMP expression support observations in other systems, where doxycycline had no effect on the expression of some TIMPs [4,6,24] showing that this drug may have or not some role on expression/ function of TIMPs.

Although, in general, the inhibition of MMPs by doxycycline to be one of the major finding reported in the literature, from our results in the odontogenic region and the periodontal ligament and other studies, we suggest that doxycycline may show different responses depending on the system studied or the experimental treatment given (lesion injury), that is the inhibitory effects of doxycycline may be effective, or not, indicating the existence of different metabolic pathways implicated in inhibitory effects at several levels such as gene translation or direct activity of MMPs site.[23]

On the other hand, the odontogenic region of rat incisor is the most important cell niche for dentário tissue formation and significant change may be more enhanced at the enamel organ level, as governed by the dentário follicle.[29] In addition molecules present in dentário follicle could be induce the proliferation and differentiation of the odontoblasts and ameloblasts to produce the tooth. As such, TIMP-2 may be involved in all these processes, as indicated by the finding that when eruption is stimulated by the shortening treatment, TIMP-2 also increases at the same time. Since TIMP-2 and MT1-MMP interact, we suggest that these proteins may have a relationship with some of the cell kinetic processes such as proliferation, migration and differentiation in the odontogenic region, which is a new approach to be investigated.

Thus, since the odontogenic region is formed by dentário follicle cells surrounding the enamel organ, we conclude that the remodeling process of extracellular components in dentário follicle is limited in the odontogenic region, even under the accelerated eruption conditions produced by the shortening treatment, suggesting that TIMP-2 may have some role in kinetics process of enamel organ epithelium.

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FIGURES AND LEGENDS – CAPÍTULO I

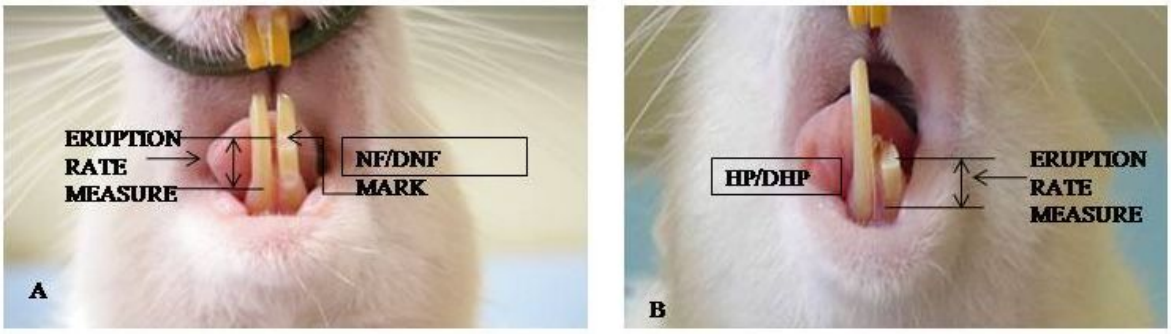


Figure 1- Rat incisor under (A) NF/DNF and (B) HP/DHP eruption conditions. Figure also illustrates the procedures carried out to estimate the eruption rate from gingival margin up to reference marks made on the NF/DNF tooth or up to the shortened tooth in the HP/DHP groups.

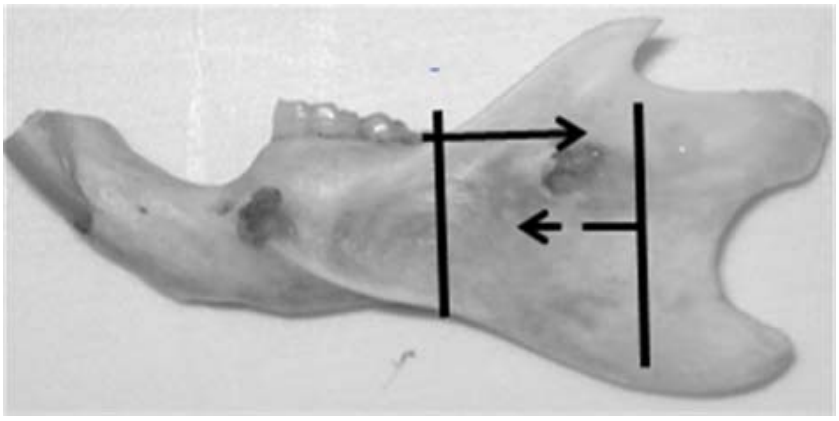


Figure 2- The vertical lines delimit the hemandible fragments used and the full line arrow points to the end of the odontogenic region, while the broken line arrow indicates the direction in which the sections were obtained for immunohistochemistry methods.

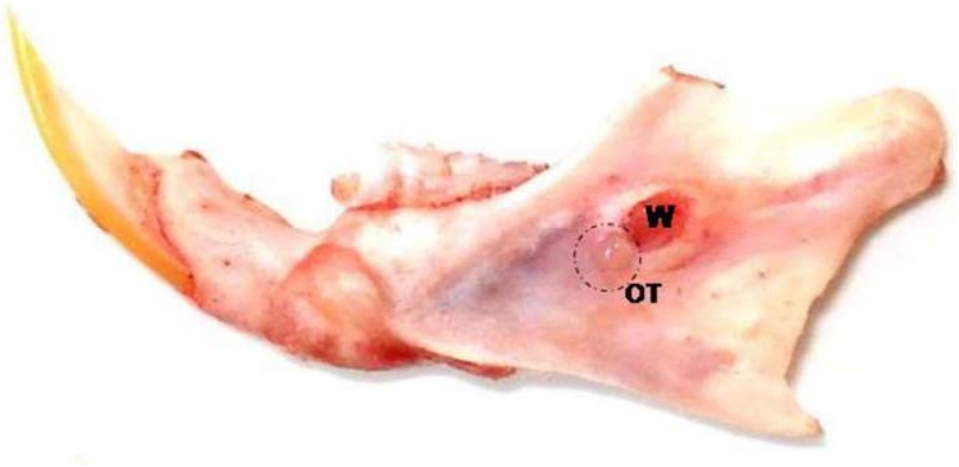


Figure 3- Odontogenic tissue (OT) collected from a window (W) opened on hemimandible bone surface used in Zymography method.

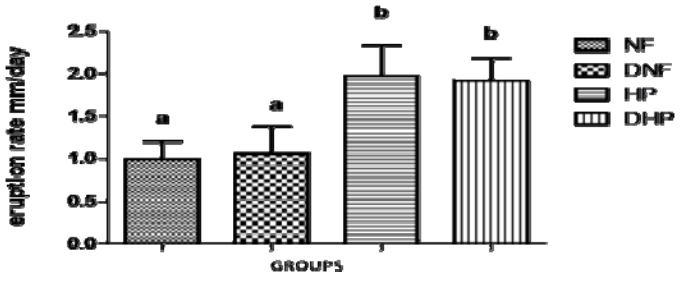


Figure 4- Mean ± standard deviation of the eruption rate measured in the incisor tooth during 12 days of shortening treatment and 14 days of doxycycline treatment (b=P<0.5)

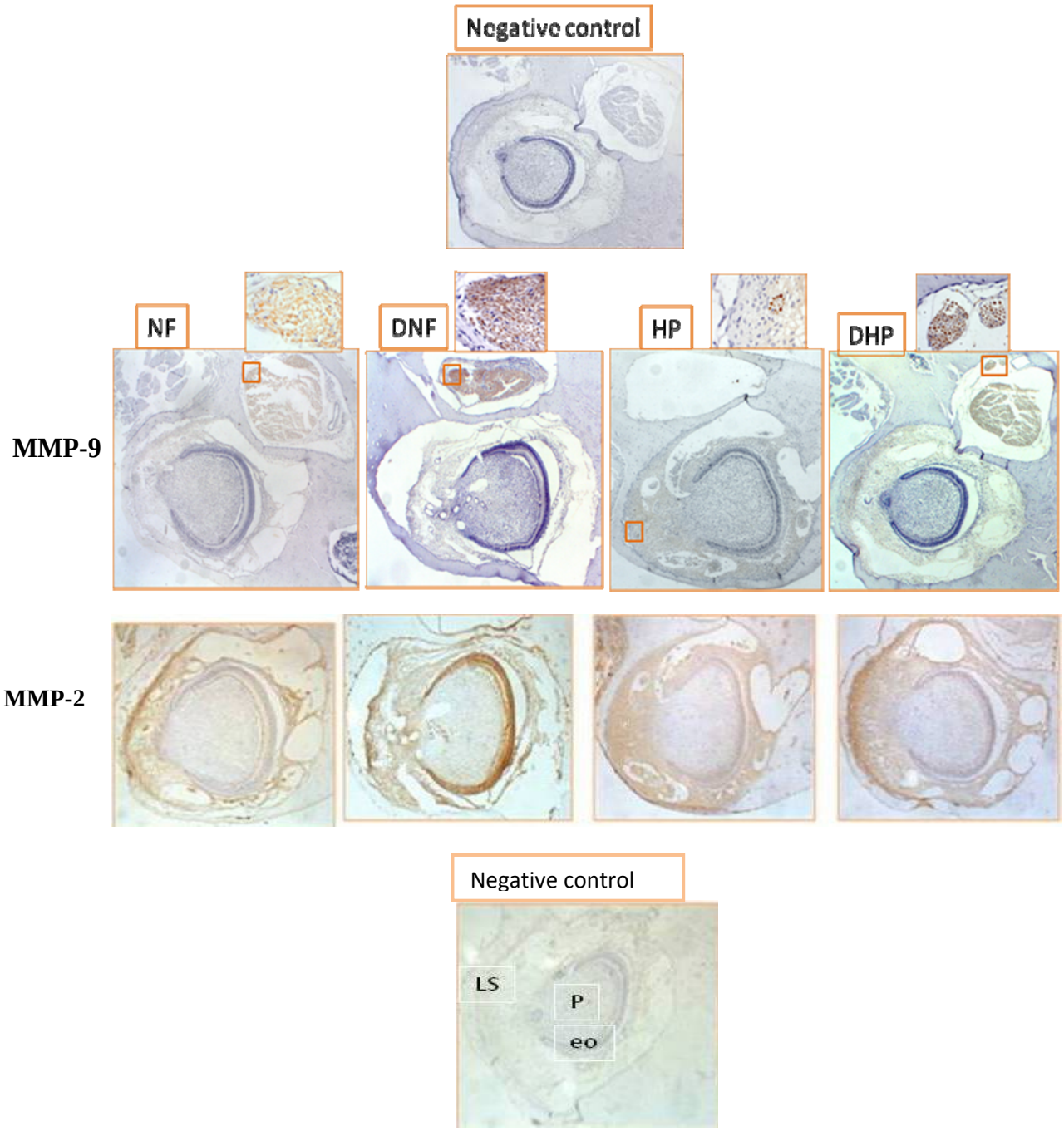


Figure 5- Immunohistochemical staining of MMP-9 and MMP-2 in the odontogenic region of the incisor tooth, after 12 days of shortening and 14 days of doxycycline treatment. Observe in all groups an intense staining to MMP-9 only in the nervous structures. An intense staining to

MMP-2 on the bone surface in the labial side (LS) was observed. Papilla (P), enamel organ (eo), PN (periferic nervous). 50X magnification.

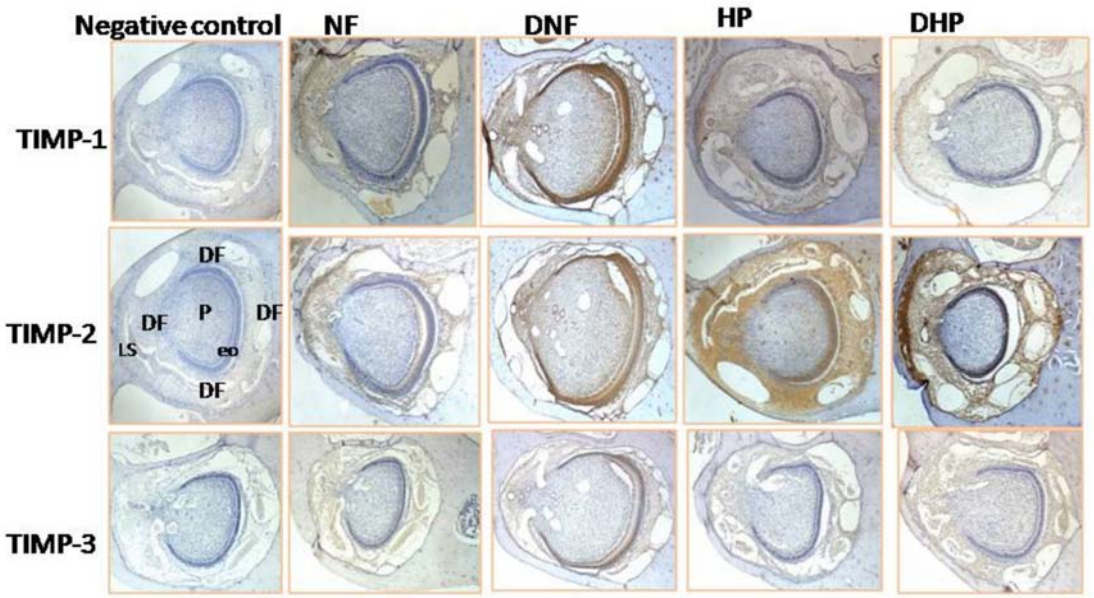


Figure 6- Immunohistochemical staining to TIMP-1, TIMP-2, TIMP-3 in the odontogenic region of the incisor tooth, after 12 days of shortening and 14 days of doxycycline treatment. Observe in HP and DHP groups images the intense staining to TIMP-2 in all dentário follicle (DF) as well as in the enamel organ (eo) epithelium and a weak staining in the papilla region (P). Labial side (LS). 50X magnification.

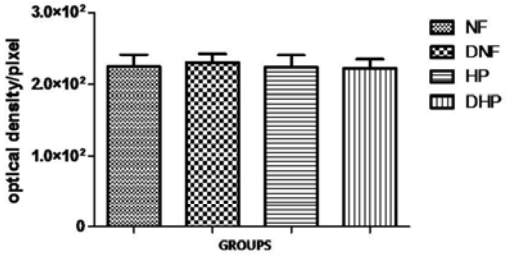


Figure 7– Mean ± standard deviation of the optical density to MMP-2 measured in odontogenic region of the incisor tooth during 12 days of shortening treatment and 14 days of doxycycline treatments with P>0.05.

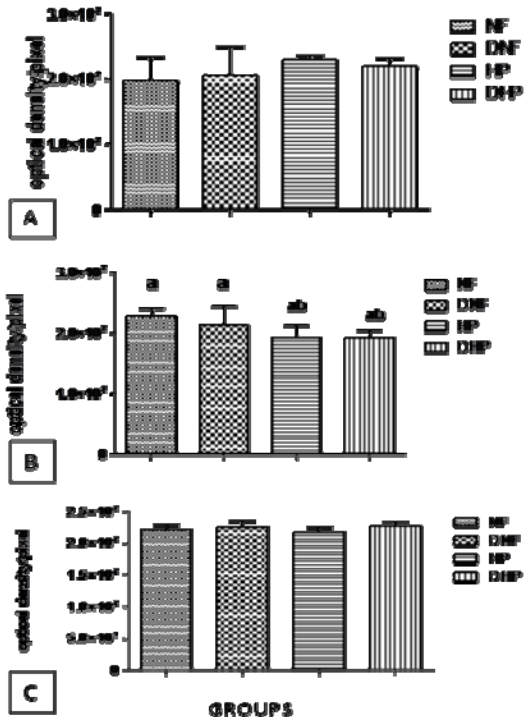
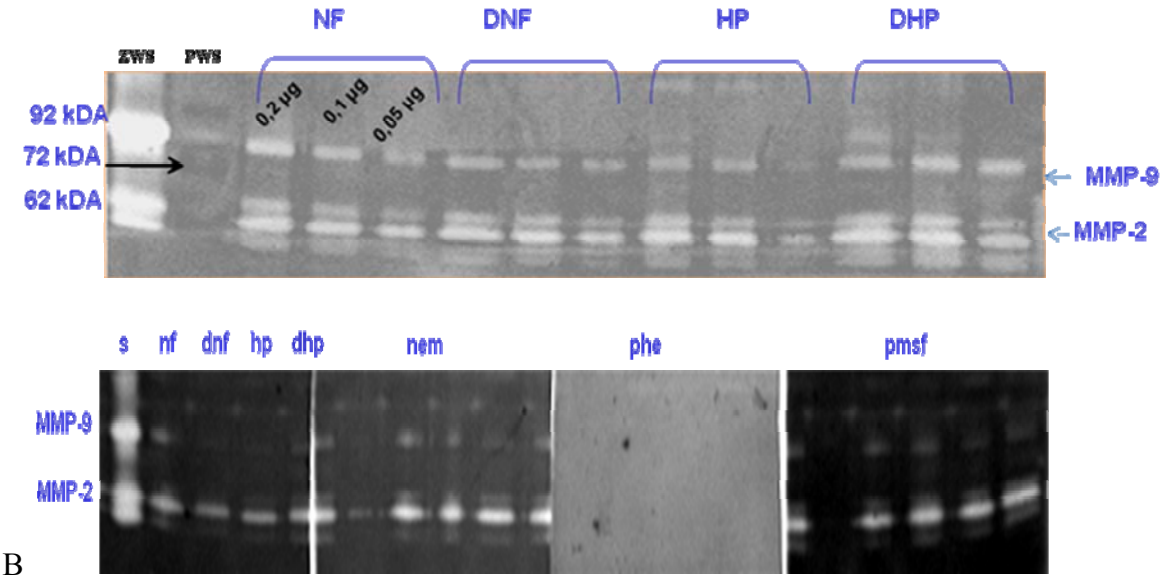


Figure 8– Mean ± standard deviation of the optical density to TIMP-1 (A), TIMP-2(B), TIMP-3(C) measured in odontogenic region of the incisor tooth during 12 days of shortening treatment and 14 days of doxycycline treatments where ab=P<0.05 to the TIMP-2 in HP and DHP groups.



B

Figure 9- In (A) Zymograms from 0.2; 0.1 and 0.05µg of total protein from NF, DNF, HP and DHP samples groups and in (B) inhibitory assay test to proteinase using 0,05mM of NEM,

PHE and PMSF. Observe that there were no bands in samples incubated with PHE, a MMP inhibitor. Zimography weight standard (zws); protein weight standard (pws).

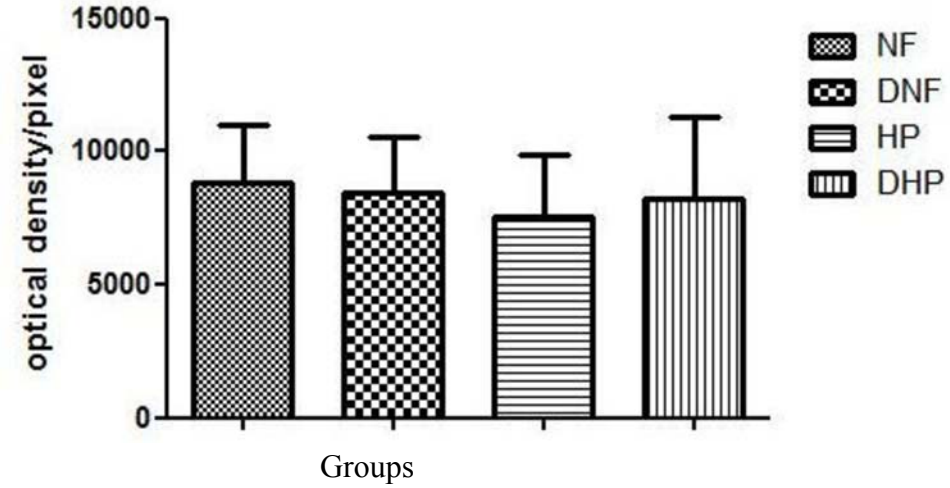


Figure 10- Representative optical density analysis of degraded bands of MMP-2 from 0.2 µg of sample applied in zymograms.

Capítulo II

Membrane type-1 matrix metalloproteinase, inhibitor of metalloproteinase 2 and cell proliferation in the odontogenic region of the rat incisor during accelerated eruption conditions and doxycycline treatments

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Key words: incisor tooth, matrix metalloproteinase, tetracycline, Ki-67

Abstract:

Objectives: MT1-MMP and TIMP-2 are well known for their roles in the degradation of matrix components. However, new reports are emerging on the involvement of these molecules in cell kinetics. Doxycycline can inhibit metalloproteinase activity in different systems, but in tooth, under accelerated eruption and cell proliferation conditions, the effects of doxycycline have not been well explored. In this study, doxycycline and hypofunction were used to study the relationships between eruption rate, MT1-MMP and TIMP-2 protein expression as well as cell proliferation in the odontogenic region of the rat incisor tooth.

Materials and methods: Male Wistar rats were treated with doxycycline for 14 days. Two days after the beginning the treatment, rats underwent tooth shortening to produce hypo-functional (HP) and doxycycline hypo-functional (DHP) groups. Rats with intact lower teeth maintained normal eruption and were called normofunctional (NF) or doxycycline normofunctional (DNF). In each group, eruption rates were measured, and the levels of MT1-

MMP, TIMP-2 and Ki-67 were measured in different regions using immunostaining and western blotting.

Results and conclusions: The increase of MT1-MMP and TIMP-2 expression allowed us to conclude that there is a relationship between cell proliferation and eruption rate in the HP and DHP groups, suggesting that MT1-MMP and TIMP-2 may have direct roles in increasing cell proliferation in the rat incisor tooth, as observed in other study models.

Introduction

In the incisor tooth, the dental epithelium invaginates into the mesenchyme and forms the tooth bud. Thereafter, the epithelium grows in a posterior direction and surrounds the dental mesenchyme. A cervical loop of the labial epithelium protrudes as a bulbous structure known as the apical loop and contains epithelial stem cells. In this region, continuous cell proliferation in the cervical loop directs the preameloblast to differentiate into the enamel-producing ameloblast, while the preodontoblast differentiates from the ectomesenchyme to produce dentine (1,2). In rat, these tooth formation processes start at around the 13th day of intrauterine life, with the eruption of the incisor reaching the oral cavity by the time of birth. The processes of growth and eruption remain unlimited and persist throughout the life of the animal.

Since the incisor undergoes continuous growth and eruption, when the odontogenic organ (3), is sectioned from apical end to the incisal edge, it is possible to observe all stages of odontogenesis, including amelogenesis and dentinogenesis (2,3). Because of these characteristics, the incisor tooth is an excellent model for the study of eruption and kinetics processes and may have implications for other systems that replace their cell populations, such as the small intestine epithelia (4,5).

One experimental advantage of using the rat incisor tooth is that the treatment used to change the kinetics process, the shortening and immobilization of the tooth, is not an invasive

procedure (6,7). It is well established in the literature that a continuous shortening treatment of the incisor produces a hypofunctional state and significantly increases cell proliferation as well as the eruption rate (8,9,10).

The presence of matrix metalloproteinases, MMP-2 and MMP-9, and their tissue metalloproteinase inhibitors (TIMP-1, 2 and 3) has been described during the morphogenesis of tooth, suggesting a role for these proteins in the remodelling of the extracellular matrix during tooth growth and differentiation (11,12). While a role for these proteins in eruption is also possible, there has been little reported about the functions of MMPs and TIMPs in the incisor tooth during its normal, or even in altered, eruption in adult rats.

Matrix metalloproteinases have an important role in tumour cell invasion and also during the tissue repair process, which indicates the influence of these proteins on cell physiological behaviour (13). A metalloproteinase bound to cell membrane, MT1-MMP (14), was the first MMP to be described as indispensable for normal growth and development. Mice deficient in MT1-MMP exhibit a variety of connective tissue pathologies and a short life span (15). In tooth, it was demonstrated that MT1-MMP is important for the eruption process and root growth (16), but no consistent reports have been found on the functions of MT1-MMP or TIMPs in dental tissues of adult rats.

TIMP-2 maintains a functional relationship with MT1-MMP, as demonstrated by the fact that TIMP-2 can inhibit MT1-MMP and also that is able to promote the activation of MMP-2 when connected to MT1-MMP (17,18). Although there have been some descriptions of the role of TIMP-2 during tooth morphogenesis, the relationship between MT1-MMP and TIMP-2 in incisor tooth tissues has not been described.

In disease states such as periodontitis, cancer, arthritis and other degenerative diseases, when there is higher degradation of connective tissues by MMPs, tetracyclines such as doxycycline or chemically-modified variants are often used to inhibit the MMP activity. The effects of tetracyclines on reducing MMP-9 and MMP-2 activity have been shown in different organs in vivo and in vitro. Although there have been reports of the effect of tetracyclines on MMPs during the development of tooth (19,20), these effects remain to be studied in adult dental tissues.

Other than the inhibition of metalloproteinase activity, other functions of TIMPs 1, 2 and 3 have been proposed, such as the stimulation of cell proliferation (21,22), regulation of cell cycle progression and terminal differentiation (23). Also, specific signalling pathways and cell surface binding partners for members of the TIMP and MT1-MMP family have been identified. In addition to its function in regulating MMP-2 when linked to MT1-MMP, a suggested function of TIMP-2 is to regulate responses to growth factors. The first description of an interaction of a cell surface receptor with a TIMP family member was TIMP-2 binding to the integrin $\alpha 3\beta 1$. TIMP-2 has been shown to induce gene expression, promote G1 cell cycle arrest and inhibit cell migration in different models studied (24,25). In addition to extracellular proteolysis, it had been demonstrated that MT1-MMP and TIMP-2 could control cell proliferation and migration through a non-proteolytic mechanism (26).

These last findings gave new insights about the biological functions of MT1-MMP and TIMP-2 in the proliferation and migration of the cell. Because the rat incisor shows continuous cell proliferation, migration and differentiation from its odontogenic organ to replace tooth lost at the incisal edge, we hypothesised that MT1-MMP and TIMP-2 might be involved in cell proliferation and eruption rate. We tested this hypothesis using two treatments: accelerating cell proliferation by shortening the incisor and administrating a

tetracycline called doxycycline to try to inhibit the MMP activity. The results obtained are consistent with newly described relationships between MT1-MMP, TIMP-2 and cell proliferation.

Materials and Methods

Animal experimental groups: Adult male Wistar rats were divided into four groups of five to seven rats each according to treatment conditions: normofunctional (NF); doxycycline normofunctional (DNF); hypofunctional (HP); and doxycyclinehypofunctional (DHP).

Treatments: The DNF and DHP groups received doxycycline daily for 14 days at a dose of 80 mg/kg (dissolved in water), given orally by a blunt tip needle. The NF and HP groups received only water, and their left lower teeth were considered as control (fig. 1A). To produce the hypofunctional eruption condition, the HP and DHP groups had their left lower incisors shortened using a high rotation drill starting two days after the beginning of doxycycline treatment and continuing every two days until day 12 (fig. 1 B). Doxycycline and shortening treatments as well as the collection of hemimandibles were always performed in the morning to avoid variation due to circadian rhythm (27). The total durations of the shortening and doxycycline treatments were 12 and 14 days, respectively.

***Eruption rate measurement*:** The eruption rates were measured using a ocular (Ernest Leitz wetzlar Germany 12,5x) with a millimeter rule (Carl Zeiss 5+ $^{100}/_{100}$ mm) every two days, always in the morning, until the 14th day. To obtain the eruption rate mean, as indicated in figure 1 (A, B), the measurement was taken from the gingival margin to the incisal end in the HP and DHP groups and until the mark made on the tooth in the NF and DNF groups.

Processing of hemimandibles: After cervical dislocation, the hemimandibles of five rats per group were removed and were immersed in 4% paraformaldehyde for 48 h.

Hemimandibles were then demineralised in 4.12% EDTA for one month and were then reduced to small fragments from the distal level of the third molar to the end of the mandible as indicated in figure 2. After this, samples were dehydrated and placed into paraplast to obtain 5 μm cross-sections from the apical end of the incisor in the incisal direction. Sections were mounted on slides for immunohistochemistry procedures.

Method for estimating cell proliferation: Semi-seriated cross-sections were taken from the apical end of the incisor in the incisal direction according to the broken arrow (fig. 2). Sections were selected to represent the following phases of odontogenesis: region 1 (initial bud stage), region 2 (end bud stage) and region 3 (early bell stage). At least two sections were selected in each of the five hemimandibles per group and examined to obtain the proliferative indexes. After dewaxing and rehydrating the sections, they were immersed in citrate buffer pH 6.0 for 30 min at 98°C for antigen retrieval of the Ki-67 protein (28).

Immunohistochemistry: After dewaxing and rehydrating three sections for each of five rats per group of five rats per group that corresponded to odontogenic region 3, sections were quenched three times with 2% hydrogen peroxide for 10 min each to the inhibit endogenous peroxidase activity. Slides were washed in water and PBS pH 7.4 and were immunostained with primary antibodies (2 μg/ml, purchased from Chemicon) as follows: MT1-MMP (1:1000) and TIMP-2 (1:20). MIB5 (1:20), purchased from DAKO, for Ki-67 protein was used to stain sections from regions 1, 2 and 3, selected according to figure 3, in PBS containing 1% BSA overnight. Sections were then washed in PBS and incubated with secondary antibody using the DAKO (Universal LSAB kit) for 30 min at 37°C in each solution. For the Ki-67 protein staining, the DAKO ABC kit was used according to the recommendations of the manufacturer. After washing in PBS, sections were incubated with the DAB reagent (SIGMA) prepared in PBS in the presence of hydrogen peroxide. Staining controls were performed for each

molecule by either omitting the primary antisera or substituting the primary antisera with non-immune serum.

Western blot: In 84 rats, hemimandibles from each of the four treatment groups were divided into three groups of seven . In each left hemimandible, a window was opened on the surface bone to collect the odontogenic tissue as demonstrated in figure 4. Odontogenic regions from each group of seven were pooled and homogenised in 500 µl of an ice cold buffer (50 mM Tris-HCl pH 7.5 containing 5 mM $CaCl_2$, 0.9% NaCl and 0.05 mM PMSF). The resulting homogenate was then briefly sonicated on ice (3 x 50 sec), cleared by centrifugation at 15.000 g for 15 min at 4°C and submitted to quantification of total protein by the Bradford method. The volumes of the homogenates were recorded and were concentrated in a speed vacuum overnight. Samples (7 µg) from each pool were resuspended and briefly sonicated in Lammelli buffer under reducing conditions then subjected to SDS-PAGE in a 10% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (Millipore) overnight, and blots were blocked with 5% BSA in TBST (100 mM Tris-HCl pH 7.5, 150 mM NaCl containing 0.1% Tween-20) for 8 h. Membranes were incubated overnight with anti-human MT1-MMP (1:500) and with beta actin (1:10.000) , purchased from Millipore. Membranes were then washed in TBST (3 x 5 min) and were incubated for 2 hr at room temperature with a secondary antibody from the Western Blot Detection System Kit code 2620 (purchased from Millipore) according to the manufacturer's instructions. Control blots were performed by either omitting the primary antisera or by substituting the primary antisera with non-immune.

Image analysis: After staining with hematoxylin, images of sections immunostained with MT1-MMP or TIMP-2 were taken at a magnification of 50X and sections stained with Ki-67 were taken at 1000X using a microscope and software from Leica. The images from

MT1-MMP and TIMP-2 staining were analysed by Image J software 1.42 (public domain) using the deconvolution plug-in, HDAB. This plug-in converts the intensity of immunostaining to pixel values from zero (0) to 255. Thus, in the immunohistochemistry interpretation results, values close to 0 represent greater staining and values close to 255 represent slower staining.

The cell counter plug-in of Image J was used to obtain the proliferating cell number by calculating the ratio of Ki-67-positive cells to the total number of cells counted (about 1000 cells) in each section.

The images of bands obtained from western blotting were scanned and converted to pixels using the Image J 1.42 software (NIH). Bands were normalised to the bands for beta-actin. The results were expressed as percentages, setting the normofunctional group to 100.

Statistical analyses: The results for cell proliferation and immunostaining for MT1-MMP and TIMP-2 were analysed using the Graphpad Prism software 3.0 using ANOVA with the Tukey post test at $P<0.05$.

Results

The estimated of continuously eruption rate the incisors of each of the groups studied was measured every two days and is demonstrated in fig. 5. The data demonstrate that there was a significant increase ($P<0.05$) in the eruption rate of teeth subjected to hypofunctional (HP) conditions and also in HP teeth that received doxycycline (DHP). The eruption rate values, calculated in millimetres ± standard error for each day of treatment, were 0.99 ±0.08 for NF, 1.05± 0.13 for DNF, 1.97 ±0.14 for HP and 1.91±0.10 for DHP. Results show that the eruption rates of the HP and DHP groups were almost doubled in comparison to the NF and DNF groups. During all periods of doxycycline administration, the eruption rate of the DHP

group showed a tendency towards reduction compared to the HP group, but this was not significant. Thus, doxycycline, at a dose of 80 mg/kg, did not have a significant effect on the eruption rate.

Immunohistochemical results are presented in figure 6 (A, B) and statistical analyses are shown in figure 7 (A, B). These results show that in the odontogenic region, MT1-MMP and TIMP-2 were co-expressed in odontogenic region 3 (bell stage). In all studied groups, intense staining for both molecules was observed in odontoblast and ameloblast cells, and a weaker staining was seen in cells of the papilla region. In all studied groups, intense staining for both molecules was detectable in all cells of the dental follicle, particularly those localised near the alveolar bone surface on the lingual side.

The Image J software transforms the intensity of staining into pixel values, where optical densities closer to 255 indicate slower staining while low values (close to 0) indicate an intense staining. figure 7 (A, B) demonstrates lower pixel values for MT1-MMP and TIMP-2 in the HP and DHP samples, confirming the visual observation of intense staining for these two molecules in the odontogenic regions of HP and DHP groups when compared to NF and DNF, respectively. These results indicate that the shortening treatment increases the expression of MT1-MMP and TIMP-2.

Western blotting detected three fragments of MT1-MMP in the pooled samples, as shown in figure 8 (A). The fragments corresponded to molecular weights of 44 kDa, 57 kDa and 64 kDa. After density analysis, the bands from each pool were normalised to beta actin bands using the Image J software. The pixel area of the NF group was set to 100% and results from other groups demonstrated an increase in expression of 52%, 56% and 12% in the DNF, HP and DHP groups, respectively (fig.8 B). These findings indicate that both doxycycline and the hypofunctional condition increase MT1-MMP expression over the normofunctional

group. However, within the HP groups, treatment with doxycycline decreases the level of MT1-MMP expression over control treatments. figure 9 A (1, 2, 3) shows representative sections of odontogenic regions stained with the cell proliferation marker, Ki-67. In all samples, few Ki-67-positive cells were observed in the dental follicle and in the papilla compartment. However, in odontogenic region 2, which corresponds to the transition between the end bud stage to the early bell stage, we observed a more intense Ki-67 staining. This was observed in both internal and external epithelia and adjacent cell layers, as well as in the cervical loop epithelium.

The data above suggest that odontogenic region 2, situated immediately before the bell stage end, correspond to the cell proliferation compartment of the incisor tooth. The beginning of the cell polarity of odontoblasts and ameloblasts have been observed in this region indicates the differentiation and maturation process. In addition, the statistical analysis (fig. 9 B) demonstrated that the shortening treatment (HP and DHP groups) significantly increased the amount of cell proliferation in the odontogenic region independently of doxycycline treatment.

Discussion

The continuous growth and eruption of the rat incisor tooth permits its use as a model to study tooth eruption and the kinetic process of cell proliferation. In addition, when the incisor tooth is subjected to a shortening treatment, that is, an elimination of the occlusal contact, the incisor assumes a hypofunctional state in which the eruption rate is around twice that of normal eruption. This observation was already well established, has been demonstrated in the literature for decades (29,30,31,32,7) and was also observed in the present study. Twelve days of shortening treatment produced an increase in the eruption rate in the HP and DHP groups. However, we did not observed the expected reduction in the eruption rate upon

administration of doxycycline (80 mg/kg), and only a discrete trend toward reduction of the eruption rate was observed in the DHP group. These results allow us to conclude that doxycycline does not interfere with the mechanisms that govern the eruption process. However, western blotting showed that doxycycline increased the expression of MT1-MMP in all treated groups compared to the NF group and decreased expression in the DHP group compared to HP group. Although it has been reported that doxycycline can affect the expression of different genes (33,34), the effect of doxycycline on expression of MMP and TIMP genes has not been examined. The results presented here generate a new hypothesis about doxycycline and gene activation that needs to be better evaluated in our model in the future.

The hypofunctional condition of the odontogenic region of the rat incisor produces an increase in cell proliferation that has been demonstrated using different methods than those used in our studies (3,8,9,35). In our study, we measured proliferation of the odontogenic region of incisor teeth that had been subjected to shortening treatment (HP and DHP) by immunohistochemistry for Ki-67 protein expression. The results showed that in all groups studied, cells have proliferative activity in the three regions selected from odontogenic sections following the bud to bell stage divisions proposed by Ohshima et al. (36). These results are consistent with previous reports about proliferative cells in the apical end of rat incisors (9,37,38.39). However, an intense staining was observed in epithelia corresponding to preodontoblast, preameloblast and intermedium stratum cells in the tissue sections from region 2 (bud end stage). Although all regions contained cells in a proliferative state, we defined region 2 as the proliferative cell compartment because of its high level of staining. This region could correspond to previously described regions where there are an an intermediate population of cells , known as transit-amplifying cells, that originate from stem cells. In these

regions, there is intense cell proliferation leading to the generation of daughter cells and continuation through the differentiation process (40).

Although the increase in cell proliferation in the rat incisor tooth after shortening treatment has been confirmed by our results and previous data, the relationship between cell proliferation indexes and eruption rate have not been well explored in this study model. Bar-Lev et al. (10) demonstrated that the incisor, under conditions of high impediment, showed a decrease in eruption rate and production of enamel epithelium as well as an increase of generation time. These authors were the first to suggest a linear relationship between cell production and tooth eruption, where cell production could be estimated from eruption rate measurements. In the present study, using the shortening treatment, we could observe the same measures as the Bar-Lev (10) study. When the incisor was subjected to a shortening treatment, we observed an increase in the eruption rate and in Ki-67 expression, indicating that there is a direct relationship between increased eruption rate and increased cell proliferation. An estimate of eruption rate (whether it is increasing or decreasing) is directly dependent on whether there is an increase or decrease in cell proliferation in the apical end of the incisor.

Besides eruption and cell proliferation, our results show an in vivo increase of MT1-MMP and TIMP-2 expression. These two molecules are well known to function in the remodelling of extracellular matrix (14). The increased expression of these molecules yields new insights about their involvement in cell proliferation in tissues, such as the incisor tooth, that have constant replacement of cell populations.

Previous research demonstrated that TIMP-2 binding to MT1-MMP induces MAPK activation and cell growth, controlling cell proliferation and migration by a non-proteolytic mechanism. These findings illustrate a novel role for the MT1-MMP/TIMP-2 interaction in cell kinetic processes by a mechanism independent of extracellular matrix degradation (21,22,23,40,26). It has been demonstrated that TIMP-2 induces gene expression, promotes

G1 cell cycle arrest and inhibits cell migration (24,15). Furthermore, results from this study are consistent with these previous reports, and we conclude that there may be a direct relationship between the increase in Ki-67 protein and the increases in MT1-MMP/TIMP-2 in teeth subjected to shortening treatments, indicating a possible role of MT1-MMP and TIMP-2 on cell proliferation in the odontogenic region of the rat incisor. However, new approaches are still necessary to investigate what extracellular and intracellular pathways are involved in cell proliferation, migration and differentiation using the rat incisor tooth as a model.

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FIGURE LEGENDS

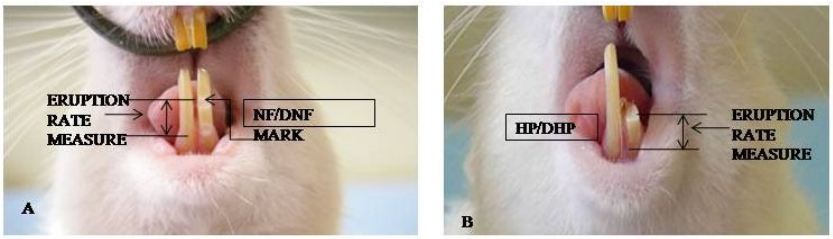


Figure 1- Rat incisor under (A) NF/DNF and (B) HP/DHP eruption conditions. Figure also illustrates the procedures carried out to estimate the eruption rate from the gingival margin up to the reference marks made on the NF/DNF tooth or up to the shortened tooth in the HP/DHP groups.

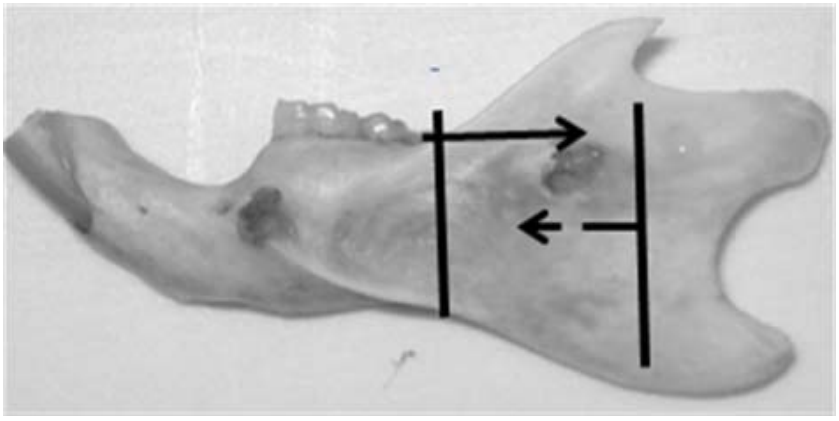


Figure 2- The vertical lines delimit the fragments used. The full line arrow points to the end of the odontogenic region, while the broken line arrow indicates the direction in which the sections were obtained for immunohistochemistry.

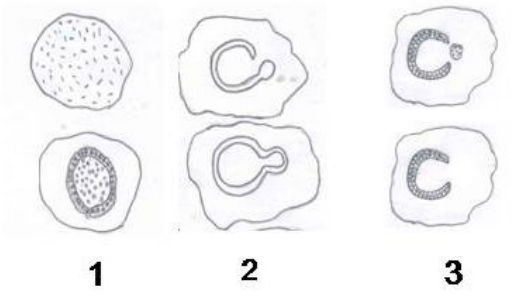


Figure 3- Illustrations of odontogenic cross-sections used to estimate the cell proliferation, obtained from the apical end of the rat incisor in the direction of the incisal edge. Sections 1 and 2 indicate the early and end bud stages and 3 indicates the bell stage.

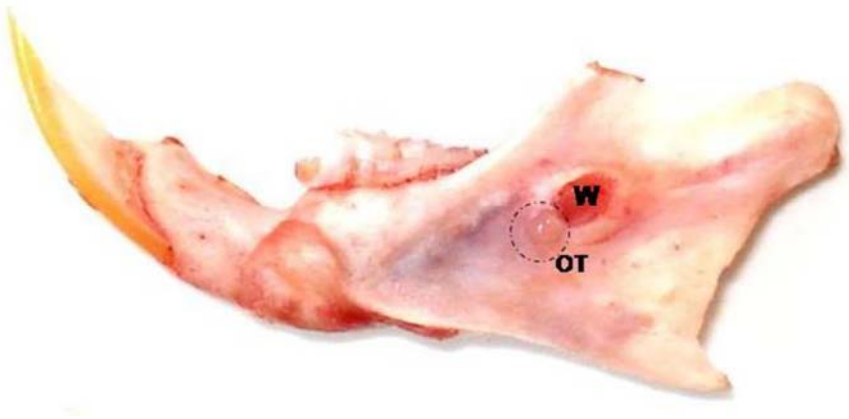


Figure 4- Odontogenic tissue (OT) collected from a window (W) opened on the hemimandible bone surface. This was used for western blotting.

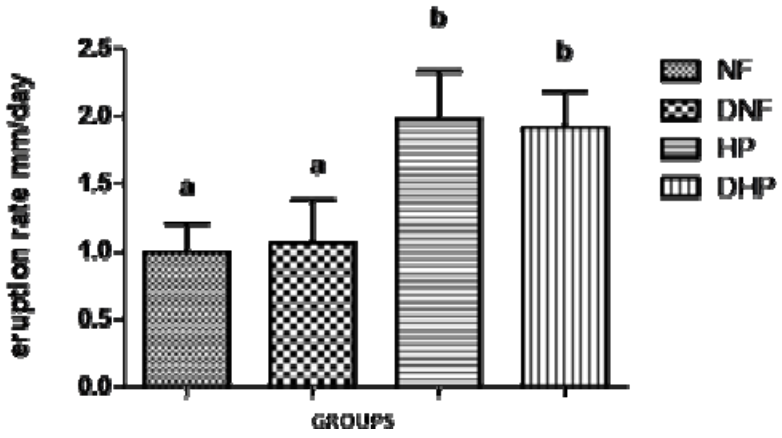


Figure 5- Mean ± standard deviation of the eruption rate measured in the incisor tooth during the 12 days of shortening treatment and 14 days of doxycycline treatment (b=P<0.5)

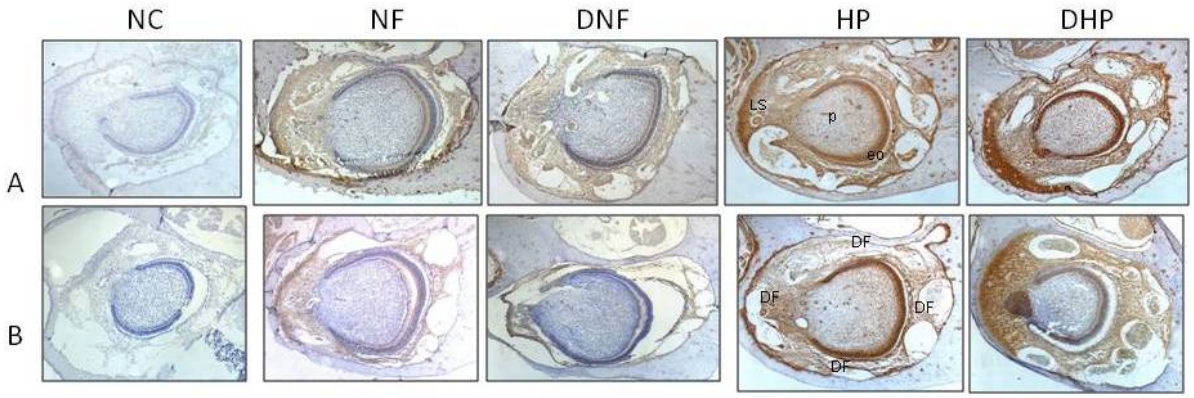
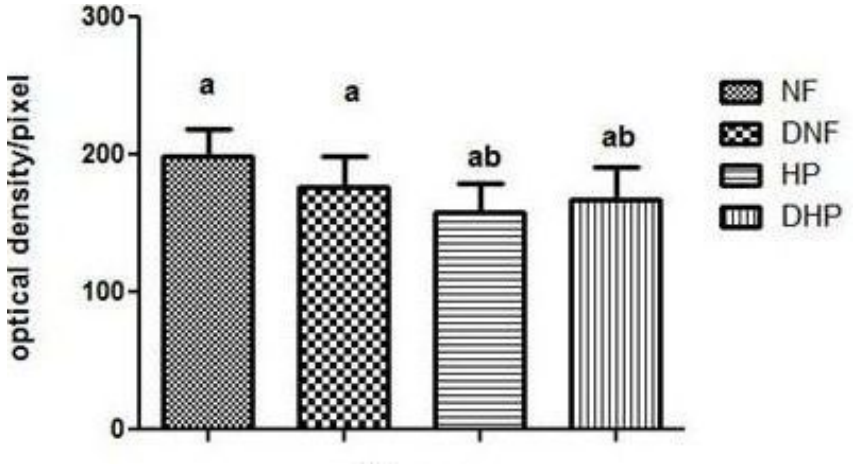
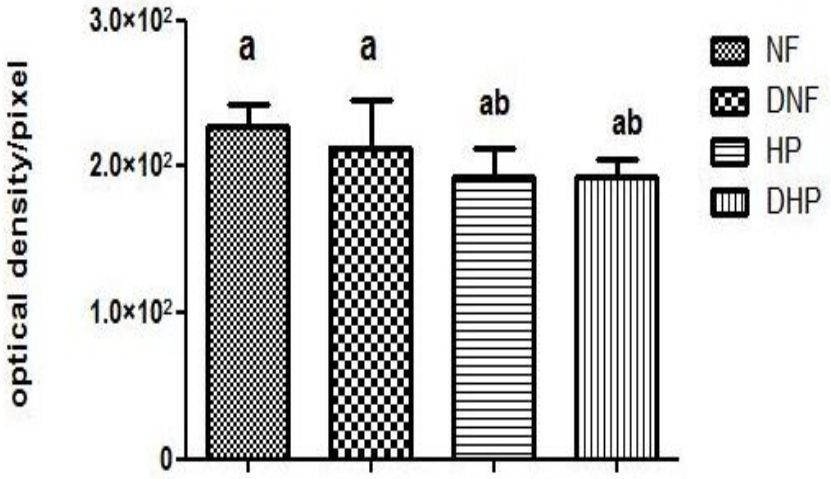


Figure 6 - Immunohistochemical staining of MT1-MMP (A) and TIMP-2 (B) in the odontogenic region of the incisor tooth after 12 days of shortening and 14 days of doxycycline treatment. HP and DHP cross-sections demonstrate greater staining in all dental follicles (DF) and the enamel organ (eo), as well as in cells next to the alveolar bone surface on the labial side (LS). (P = papilla region).



A

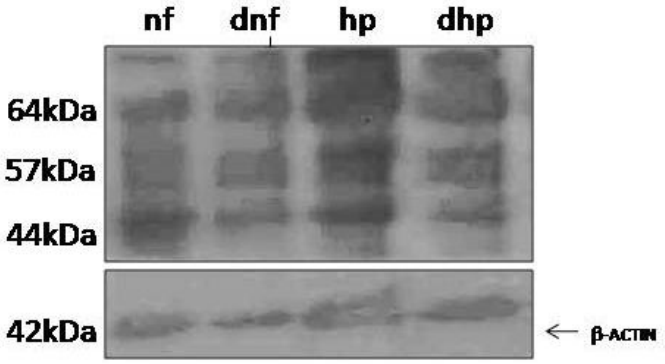
Groups



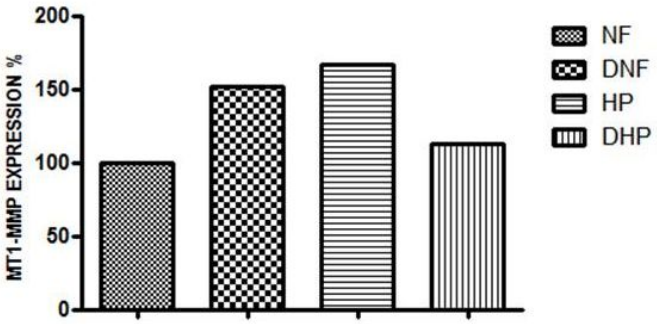
B

Groups

Figure 7– Mean ± standard deviations of the optical densities for MT1-MMP (A) and TIMP-2 (B),measured in cross-sections of the odontogenic region 3 of the incisor tooth after 12 days of shortening treatment and 14 days of doxycycline treatment. The HP and DHP groups (ab=$P<0.05$) demonstrate an increase in expression of these molecules following Image J pixel conversion.

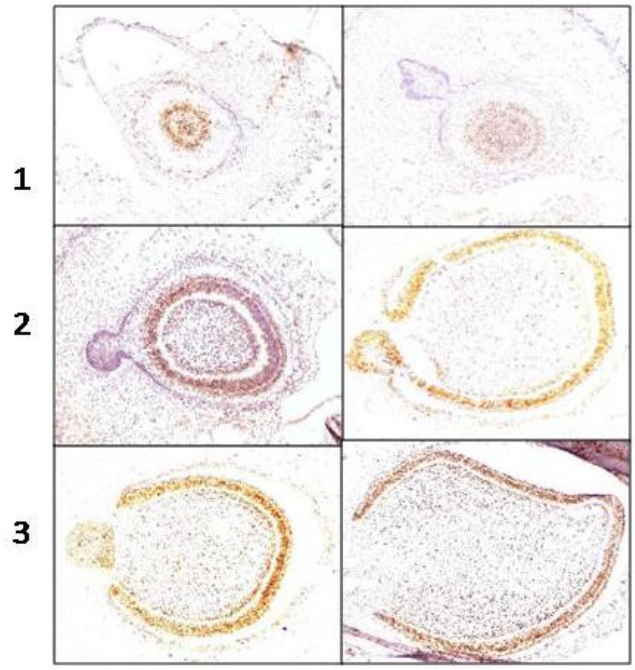


A

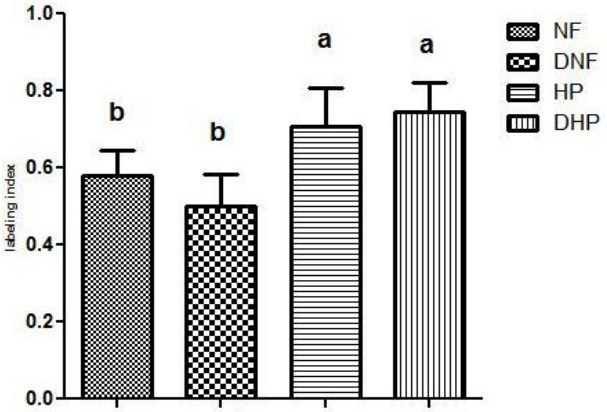


B

Figure 8 – Immunoblotting for MT1-MMP in samples containing 7 µg of total protein from NF, DNF, HP and DHP groups after 12 days of shortening and 14 days of doxycycline treatments (A). Percentage analysis of 3 pools of samples (7 rats per pool) in the groups studied (B), where NF=100% and DNF, HP and DHP correspond to 152%, 156% and 112% of NF MT1-MMP expression.



A



B

Figure 9- (A) Representative sections of odontogenic regions 1, 2 (early and end bud stages, respectively) and 3 (early bell stage) with Ki-67-positive cells, and (B) the statistical analysis of the number of Ki-67-positive cells. The HP and DHP groups demonstrate an increase in cell proliferation. (a=P<0.05).

CONSIDERAÇÕES GERAIS

Apesar das idéias novas sobre TIMP-2 e MT1-MMP colocadas no primeiro artigo assim como no segundo e que pelos resultados podemos elegê-las como moléculas importantes e envolvidas tanto no processo de proliferação quanto no processo de erupção dentário, ainda precisamos discutir nossos resultados em relação à técnica de zimografia reversa cuja finalidade é a de demonstrar a presença de TIMPs e que ainda não foram satisfatórios. Uma das dificuldades foi a de que não tínhamos um protocolo definido e demoramos para receber a MMP pura e iniciar a padronização da técnica.

Apesar disso, utilizamos neste estudo, para o ensaio da zimografia reversa amostras controles de músculo esquelético foram processadas de duas formas: na primeira usamos o mesmo tampão de lise utilizado para analisar, por Western blot, a MT1-MMP e a segunda foi da forma como nossas amostras experimentais foram processadas, isto é, coletadas em meio de cultura. Como fonte de metaloproteinases utilizamos no gel uma MMP-2 comercial e fizemos vários testes para se chegar aos resultados que são apresentados na figura 1 (A,B) deste tópico.

Também utilizamos como fonte de metaloproteinase meio de cultivo onde fragmentos de intestino condicionaram este meio por 12 horas a 37^{o} C. Após isso foram testadas várias concentrações do meio condicionado em tampão de revelação escolhendo a de 75% como sendo a melhor. Assim os testes com as amostras controles e experimentais foram realizadas incubando os géis no meio condicionado ou adicionando no gel MMP-2 purificada. A figura 1-A,B mostra que no gel onde foram utilizados 2 e 4µg de proteína total das amostras e 15 µl de MMP-2 existem poucos rastros de proteínas na altura das TIMPs enquanto que no gel que foi incubado em meio condicionado de intestino houve uma degradação da gelatina, como no gel com MMP-2, entretanto apareceram bandas de proteínas correspondentes às alturas dos TIMPs apenas nas canaletas das amostras controles de músculo. Desta forma estes resultados

obtidos embora ainda não conclusivos, sugerem que a quantidade de TIMPs existentes nas amostras experimentais foram insuficientes para serem detectadas por ambos os métodos, entretanto, sugere-se também que o método de incubação das amostras em intestino condicionado parece ser menos exigente quanto a quantidade de TIMPs, isto é, ele é mais eficiente quando comparado com o método tradicional usando MMP-2 no gel onde, a presença de MMP-2 pode exigir uma quantidade maior de proteína total e por conseguinte mais TIMPs para que a ligação entre MMP-2 e TIMPs resulte na inibição da primeira evidenciando dessa forma os TIMPs das amostras. Essas observações também sugerem que o meio condicionado de intestino parece ser uma grande fonte de proteases.

Assim, apesar da técnica de imunohistoquímica utilizada neste trabalho para a localização de TIMPs com uma concentração alta de anticorpo tenha gerado ótimos resultados, principalmente em relação à TIMP-2, as observações feitas pelos métodos de zimografia reversa alternativo (usando meio condicionado de intestino) e o tradicional (com MMP pura) ainda precisam ser confirmadas. Assim pretendemos insistir em novos testes para confirmá-las uma vez que o meio condicionado de intestino parece ser uma técnica viável para a detecção de TIMPs pelo método de zimografia reversa e principalmente se mostra mais econômica em termos financeiros se comparada com a que usa MMP-2 purificada.

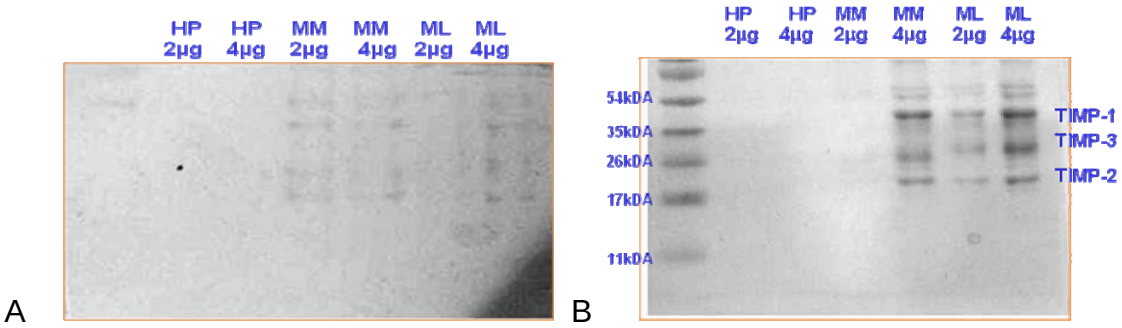


Figura 1- Em A, gel de zimografia contendo 15µl de MMP-2 purificada incubada em tampão de revelação. Em B gel contendo as mesmas amostras incubadas apenas em meio condicionado de intestino 75% em tampão de revelação com tempo de 10 horas para ambos os géis.HP (hypofuncional) MM(músculo em meio condicionado) e ML (músculo coletado em tampão de lise).

CONCLUSÃO

Baseado em todos os resultados apresentados e posterior análises podemos obter deste trabalho as seguintes conclusões:

a- Na região odontogênica, a doxiciclina não teve efeito sobre a expressão de MMP-2, MMP-9, dos TIMPs 1,2,3 e MT1-MMP, entretanto, a hipofunção aumentou a expressão de TIMP-2 e MT1-MMP.

b- A doxiciclina não teve efeito sobre a erupção, entretanto, a condição hipofuncional dobrou a taxa de erupção do dente incisivo.

c- A doxiciclina não teve efeito sobre a proliferação celular da região odontogênica.

d- Apenas a condição hipofuncional aumenta a taxa de erupção, a proliferação celular e a expressão de MT1-MMP da região odontogênica mostrando que existe uma relação entre as funções de MT1-MMP e TIMP-2 nos processos de proliferação e, conseqüentemente com o processo de erupção do incisivo.

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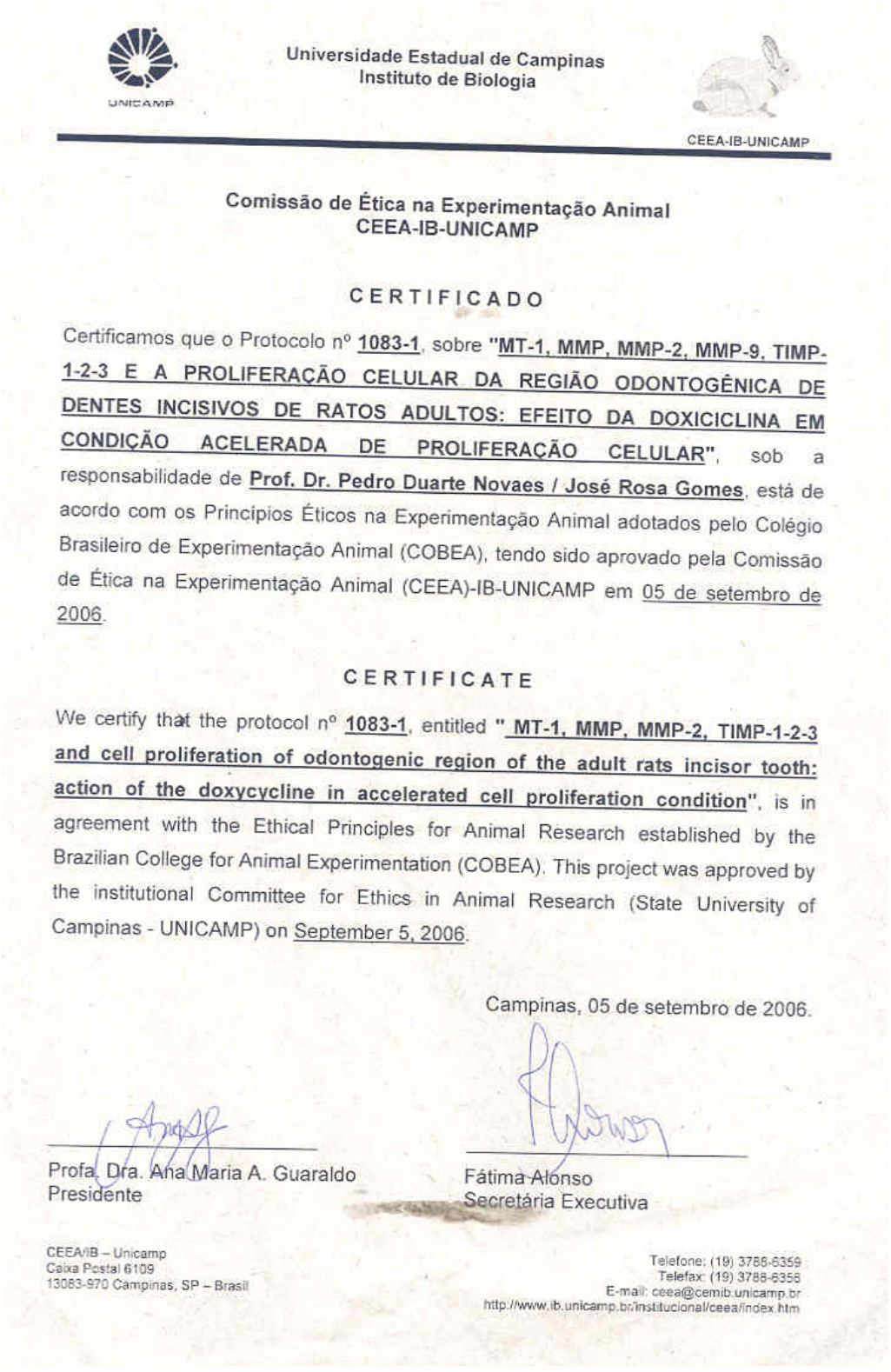
ANEXOS



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Instituto de Biologia

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Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

CERTIFICADO

Certificamos que o Protocolo nº **1083-1**, sobre **"MT-1, MMP, MMP-2, MMP-9, TIMP-1-2-3 E A PROLIFERAÇÃO CELULAR DA REGIÃO ODONTOGÊNICA DE DENTES INCISIVOS DE RATOS ADULTOS: EFEITO DA DOXICICLINA EM CONDIÇÃO ACELERADA DE PROLIFERAÇÃO CELULAR"**, sob a responsabilidade de **Prof. Dr. Pedro Duarte Novaes / José Rosa Gomes**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em 05 de setembro de 2006.

CERTIFICATE

We certify that the protocol nº **1083-1**, entitled **" MT-1, MMP, MMP-2, TIMP-1-2-3 and cell proliferation of odontogenic region of the adult rats incisor tooth: action of the doxycycline in accelerated cell proliferation condition"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on September 5, 2006.

Campinas, 05 de setembro de 2006.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

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Coordenadoria Geral da Universidade
Comissão de Ética Ambiental

GERENCIAMENTO DE RESÍDUOS BIOLÓGICOS, QUÍMICOS E RADIOATIVOS

Cidade Universitária "Zeferino Vaz", 22 de novembro de 2007.

Of. CGU/Resíduos/Comissão de Ética Ambiental 265/2007

Ao
Prof. Dr. Pedro Duarte Novaes
Departamento de Morfologia
Faculdade de Odontologia de Piracicaba - FOP

Referência: PARECER SOBRE RESÍDUO DE PROJETO FAPESP 2007.

Prezado Prof. Dr. Pedro Duarte Novaes,

Segue, em anexo, o parecer sobre a destinação final dos resíduos a serem gerados no Projeto: "MT1-MMP, MMP-2, MMP-9, TIMP-1,-2,-3 E A PROLIFERAÇÃO CELULAR DA REGIÃO ODONTOGÊNICA DE DENTES INCISIVOS DE RATOS ADULTOS: EFEITO DA DOXICICLINA EM CONDIÇÃO ACELERADA DE PROLIFERAÇÃO CELULAR", conforme informações e resumo do projeto enviados à Comissão de Ética Ambiental da UNICAMP.

Recomendamos a aprovação da destinação dada ao resíduo: incineração para o resíduo químico perigoso e tratamento em microondas, seguido de destinação final em aterro sanitário, para as cobaias (resíduo biológico infectante).

Atenciosamente,

Fernando Coelho
Presidente da Comissão de Ética Ambiental
Universidade Estadual de Campinas

Archives of Oral Biology

mostrar detalhes 02/12/09

Archives of Oral Biology

Ref: AOB-D-09-00387

Title: Doxycycline and hypofunction condition effects on the eruption rate, activity and proteins expressions of MMP-9, MMP-2, TIMP-1, TIMP-2 and TIMP-3 from odontogenic region of rat incisor tooth

Authors: José R Gomes, Prof; josé rosa gomes, PhD; Nádia F Omar, MsC; Juliana S Neves, MsC; Eliene O Narvaes; Pedro D Novaes, prof

Article Type: Original Paper

Dear José,

Your submission entitled "Doxycycline and hypofunction condition effects on the eruption rate, activity and proteins expressions of MMP-9, MMP-2, TIMP-1, TIMP-2 and TIMP-3 from odontogenic region of rat incisor tooth" has been assigned the following manuscript number: AOB-D-09-00387.

You may check on the progress of your paper by logging on to the Elsevier Editorial System as an author. The URL is http://ees.elsevier.com/aob/.

Thank you for submitting your work to this journal. Please do not hesitate to contact me if you have any queries.

Kind regards,

Paul Crabtree

Journal Manager

Archives of Oral Biology