Universidade Estadual de Londrina

TÂNIA MARIS PEDRINI SOARES DA COSTA

# ANTICORPOS E IMUNOCOMPLEXOS AOS ANTÍGENOS DENTINÁRIOS EM RATOS E ANTICORPOS SALIVARES E RESPOSTA LINFOPROLIFERATIVA PARA FRAÇÕES DE DENTINA HUMANA ASSOCIADOS À REABSORÇÃO DENTÁRIA NO TRATAMENTO ORTODÔNTICO

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Tese apresentada ao Programa de Pós Graduação em Patologia Experimental da Universidade Estadual de Londrina como requisito para obtenção do título de Doutor.

Orientadora: Prof. Dra. Eiko Nakagawa Itano

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*De tudo ficam 3 coisas:*

*A certeza que estamos sempre*

*começando,*

*A certeza de que precisamos continuar,*

*A certeza de que seremos sempre,*

*interrompidos.*

*Portanto devemos fazer da interrupção,*

*um caminho novo.*

*Da queda, um passo de dança.*

*Do medo, uma escada.*

*Do sonho, uma ponte.*

*Da procura, um encontro*

*(Fernando Pessoa)*

COSTA, Tânia Maris Pedrini Soares da. **Anticorpos e imunocomplexos aos antígenos dentinários em ratos e anticorpos salivares e resposta linfoproliferativa para frações de dentina humana associados à reabsorção dentária no tratamento ortodôntico.** 2015. 74 fls. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2015.

## RESUMO

O potencial imunogênico da dentina tem sido atribuído a antígenos que estariam presentes na dentina, os quais poderiam ser expostos ao sistema imune durante os processos de reabsorção radicular como consequência do tratamento ortodôntico. Este estudo teve como objetivo investigar em modelo animal, a resposta imune humoral sistêmica e local aos antígenos dentinários, a formação de imunocomplexos e os níveis séricos de antígenos dentinários solúveis; e em humano, os níveis de IgG salivar, a resposta linfoproliferativa à frações de dentina humana e a correlação entre os dados encontrados. Em modelo experimental, quarenta ratos Wistar machos escolhidos aleatóriamente foram submetidos ao movimento de inclinação mesial do primeiro molar superior direito, por aplicação de uma força ortodôntica de 55 cN por 3, 7, 14 e 21 dias ou deixado sem tratamento (controle). Após cada periodo experimental, amostras de saliva, sangue e a maxila direita de cada animal foram coletadas para análise. O estudo humano constituiu-se de 3 grupos. Grupo I, formado por pacientes adolescentes (n=10) onde amostras de saliva foram coletadas em 4 periodos: antes (T0), 3 (T3), 6 (T6) e 12 (T12) meses de tratamento ortodôntico, para análise dos níveis de reatividade da IgG salivar para as frações FI, FII ou FIII de extrato dentinário humano. No grupo II (n=30), amostras de soro e células mononucleares do sangue periférico de pacientes adolescentes com 6 meses de tratamento ortodôntico foram coletadas para análise dos níveis de IgG sérica e resposta linfoproliferativa às frações de extrato dentinário humano FI ou FIII. No terceiro grupo (n=16), voluntários adolescentes que nunca haviam se submetido à tratamento ortodôntico e sem sinais radiográficos de reabsorção radicular, serviram de controle para o segundo grupo de estudo. O extrato dentinário total foi obtido à partir de incisivos de rato Wistar ou de dentes terceiros molares humano, respectivamente, sendo que em humano o extrato dentinário foi submetido à cromatografia de gel filtração, Sephadex G-120-150. A reabsorção radicular foi avaliada por análise histopatológica em microscópio óptico em modelo animal e através do método de subtração radiográfica digital dos incisivos superiores, em humano. Os níveis de anticorpos, imunocomplexos e antígenos dentinários solúveis no soro foram determinados por ensaio imunoenzimático (ELISA). Para o estudo da resposta linfoproliferativa, células mononucleares de sangue periférico (PBMC) humano estimuladas com frações dentinárias foram analisadas por ensaio MTT. Níveis mais elevados de IgG sérica em ratos foram detectados em 14 e 21 dias em relação ao controle ($p < 0.05$). No entanto, em saliva foram detectados um aumento dos níveis de IgG salivar em 3 e 7 dias em comparação com o controle ($p < 0.001$) e uma diminuição de seus níveis em 21 dias em relação a 3 e 7 dias ($p < 0.05$). Níveis mais elevados de imunocomplexos no soro foram detectados em 14 e 21 dias ($p < 0.001$) e na

saliva em 14 dias ($p < 0.05$) em relação ao controle, com uma diminuição em 21 dias em relação à 14 dias ($p < 0.05$). No grupo I humano foi observado uma maior reactividade da IgG salivar para a fração FII em relação à FIII em T6 e T12 ($P < 0.05$) e no grupo II houve uma correlação positiva significante entre proliferação de linfócitos e níveis de IgG sérica para a fração III ($r = 0.508$). Em conclusão, houve imunomodulação na resposta de anticorpos IgG salivar a antígenos dentinários durante o tratamento ortodôntico em ratos que diferem secreção sistêmica, da externa (saliva). Os níveis de imunocomplexos em soro ou saliva tem potencial para servir de marcadores biológicos, por indicar a fase ativa da reabsorção radicular. Em humanos, mudança na reatividade IgG salivar para FII no decorrer do tratamento ortodôntico também sugerem o potencial desses anticorpos como marcadores biológicos para a detecção precoce da reabsorção radicular.

**Palavras-chaves:** marcadores biológicos; antígenos dentinários; reabsorção radicular; imunomodulação; imunocomplexo.

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## ABSTRACT

The immunogenic potential of dentin has been attributed to antigens that would be present into dentin, which could be exposed to the immune system during the root resorption processes as a result of orthodontic treatment. This study aimed to investigate in animal model the systemic and local humoral response to dentinal antigens, forming immune complexes and serum soluble dentinal antigens; and in human the salivary and serum IgG levels, the lymphoproliferative response to human dentin fractions and the correlation between the data found. In experimental model, forty Wistar rats, male, were chosen randomly, in which the upper right first molar of each animal was moved mesially by applying a orthodontic force of 55 cN for 3, 7, 14 and 21 days or untreated (control). After each experimental period, saliva and blood samples and right maxilla were collected for analysis. The human study comprised three groups: Group I (n=10), consisted of adolescent patients in which saliva samples were collected in four periods: before (T0), 3 (T3), 6 (T6) and 12 (T12) months of orthodontic treatment for analysis of reactivity salivary IgG levels to FI, FII or FIII human dentinal extract fractions. In Group II (n=30), serum and peripheral blood mononuclear cells from adolescent patients with 6 months of orthodontic treatment were collected for analysis of serum IgG levels and lymphoproliferative responses to FI or FIII human dentinal extract fractions. In the third group adolescent volunteers (n=16), who had never undergone orthodontic treatment and no radiographic signs of root resorption, served as control for the second study group. The total dentinal extract was obtained from the Wistar rat incisor teeth or human third molars teeth, respectively and in human it was subjected to gel filtration chromatography, Sephadex G-120-150. In animal model, the root resorption was evaluated through histopathological analysis by light microscope and by digital subtraction method of upper incisors, in human. The antibodies levels, immune complexes, serum soluble dentinal antigens in were determined by enzyme-linked immunosorbent assay (ELISA). To study the lymphoproliferative response, peripheral blood mononuclear cells (PBMC) stimulated with dentinal fractions were analyzed by MTT assay. Statistical difference were detected in rat serum IgG levels in 14 and 21 day group compared to control ($p < 0.05$). However, in saliva were detected higher salivary IgG levels in the 3 and 7 day groups when compared to the control ($p < 0.001$) and a decrease in their levels in the 21 days group in relation to 3 and 7 day groups ($p < 0.05$) . Higher levels of immune complexes in serum were detected in the 14 and 21 day groups ($p < 0.001$) and in saliva in the 14 days group ($p < 0.05$) in relation to the control and a decrease in the 21 days group compared to 14 days group ($p < 0.05$). In the human group I was observed higher reactivity of salivary IgG to FII fraction compared to FIII at T6 and T12 ($P < 0.05$) and in group II there was a significant positive correlation between lymphocyte proliferation and serum IgG levels to FIII fraction, r =

0.508. In conclusion, there was immunomodulation in salivary IgG antibody response to antigens dentinal during orthodontic treatment in rat which differ systemic and external secretion (saliva). The immune complexes formed in serum or saliva has the potential to serve as biological markers indicating the active phase of root resorption. In human, changes in salivary IgG reactivity to FII fraction during orthodontic treatment also suggest the potential of these antibodies as biological markers for early detection of root resorption.

**Key words:** biological markers; dentinal antigens; root resorption; immunomodulation; immune complex.

## LISTA DE ILUSTRAÇÕES

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## LISTA DE TABELAS

**Artigo 2**

## LISTA DE ABREVIATURAS E SIGLAS

**APCs** - células apresentadoras de antígeno

**Arg** - arginina

**Asp** - aspartato ou ácido aspártico

**BCR** - receptor de linfócitos B

**BSA** - albumina de soro bovino

**BSP** - sialoproteína óssea

**cN** - cNewton - unidade de força ortodôntica 1cN= 1g

**CNPs** - proteínas não colagenosas

**DGP** - glicoproteína dentinária fosforilada

**DMP1** - proteína da matriz dentinária 1

**DMSO** - sulfóxido de dimetilo

**DPP** - fosfoproteína dentinária

**DRI** - imunodifusão radial dupla

**DSP** - sialoproteína dentinária

**DSPP** - sialofosfoproteína dentinária

**EDTA** - ácido etileno diamino tetra acético

**ELISA** - ensaio imunoenzimático

**ERR** - reabsorção radicular externa

**EARR** - reabsorção radicular apical externa

**Gly** - glicina

**Gp 130** - glicoproteína transmembranosa

**$H_2O_2$** - água oxigenada

**$HaSO_4$** - ácido sulfúrico

**HDE** - extrato dentinário humano

**IC** - imunocomplexo

**IDRD** - imunodifusão radial dupla

**Ig** - imunoglobulina

**IgA** - imunoglobulina A

**IgE** - imunoglobulina E

**IgG** - imunoglobulina G

**IgM** - imunoglobulina M

**IL-1** - interleucina 1

**IL-1β** - interleucina 1 beta

**IL-2** - interleucina 2

**IL-6** - interleucina 6

**IL-8** - interleucina 8

**IL-10** - interleucina 10

**IL-12** - interleucina 12

**IL-17** - interleucina 17

**INF-Y** - interferon gama

**Inh ELISA** - ensaio imunoenzimático de inibição

**LB** - linfócito B

**LT** - linfócito T

**MHC** - complexo de histocompatibilidade principal

**MHC II** - complexo de histocompatibilidade principal classe II

**mRNA** - RNA mensageiro

**MTT** - dimetil tiazol difenil tetrazolato de bromo

**NCPs** - proteínas não colagenosas

**NFkB** - fator nuclear kappa B

**OPD** - orto-fenilenodiamino

**OPG** - osteoprotegerina

**PBMC** - células mononucleares de sangue periférico

**PBS** - tampão fosfato salino

**PBS/T** - PBS, Tween 20

**PDL** - ligamento periodontal dental

**$PGE_2$** - prostaglandina $E_2$

**PGs** - prostaglandinas

**PMSF** - fluoreto fenilmetanosulfonado

**PTH** - paratormônio

**RANK** - receptor ativador do fator de transcrição NFkB

**RANK-L** - receptor ligante de RANK

**RDE** - extrato dentinário de rato

**RGD** - sequência específica de aminoácidos Arg-Gly-Asp

**RNA** - ácido ribonucleico

**RNAm** - RNA mensageiro

**RPMI** - meio de cultura Roswell Park Memorial Institute

**RRAE**- reabsorção radicular apical externa

**RRE** - reabsorção radicular externa

**SIBLING** - glicoproteínas que se ligam à integrinas

**TCR** - receptor de linfócitos T

**TGF- β** - fator transformador de crescimento beta

**TMB** - tetrametil benzidina

**TNF-α** - fator de necrose tumoral alfa

**TRAP** - tartarato fosfatase ácido-resistente

**d** - dia

**g** - giro

**kDa** - quilodalton

**ml** - mililitro

**mm** - milímetro

**mM** - milimolar

**MM** - massa molecular

**µg** - micrograma

**µl** - microlitro

**O.D**. - densidade óptica

**pH** - potencial hidrogeniônico

## SUMÁRIO

**Artigo 2 Distinct human salivary IgG reactivity and lymphoproliferative response to antigenic fractions of dentin**

# 1 CONTEXTUALIZAÇÃO

De acordo com Bittencourt e Machado (2010), ao investigarem 4.776 crianças entre 6 e 10 anos provenientes de 18 estados brasileiros, concluíram que 85,17% das crianças apresentaram algum tipo de alteração oclusal significativa. Se essas crianças não forem diagnosticadas e tratadas eficazmente no momento oportuno, elas se tornarão adultos com má oclusão e sem tratamento, condição essa que uma vez negligenciada será responsável pela manutenção desse problema de saúde pública. Para deixar o cenário mais crítico, o estudo epidemiológico nacional sobre a prevalência da má-oclusão, divulgado pelo relatório do Ministério da Saúde 2010, apresentou discrepâncias e falhas em seus resultados que mascaram a alta incidência e prevalência das patologias oclusais no Brasil. O tratamento ortodôntico tem como principais objetivos a restauração da função mastigatória com consequente restabelecimento da harmonia oclusal e facial, a saúde dos tecidos periodontais e a estabilidade dentária após o tratamento. No entanto, apesar de seus benefícios, o tratamento ortodôntico apresenta como sequela comum e indesejável a reabsorção radicular apical externa, um processo patológico que resulta em perda irreversível de estrutura dentária com diminuição do comprimento radicular. Casos graves de reabsorção radicular podem ocorrer em até 10% dos pacientes submetidos ao tratamento ortodôntico (MAKEDONAS, 2012), e podem colocar em risco a estabilidade e longevidade do dente afetado. O uso de animais no estudo do movimento dentário induzido possibilita uma maior compreensão dos mecanismos imunológicos e as reações teciduais envolvidas no processo da reabsorção radicular externa, transpondo dificuldades inerentes às pesquisas com seres humanos.

## 1.1 MOVIMENTO ORTODÔNTICO

A movimentação dentária induzida é um processo biológico múltiplo caracterizado por reações inflamatórias do ligamento periodontal em resposta às forças biomecânicas que induzem alteração em duas regiões do tecido periodontal: o lado da pressão e o lado da tensão (SANTAMARIA JR, 2009; GIANNOPOULOU et al., 2008).

A pressão induzida pela força ortodôntica gera um estresse local que acarreta alterações bioquímicas e metabólicas nas populações celulares presentes nesse microambiente. Com a diminuição do suprimento vascular haverá hipóxia ou anóxia e subsequente morte e necrose de células do ligamento periodontal (GAENGLER; MERTE, 1983) com a formação de uma zona hialina, livre de células (MABUCHI et al., 2002). Simultaneamente, ocorre a liberação de mediadores inflamatórios principalmente $PGE_2$ (SAITO et al., 1991) os quais possibilitam a migração de leucócitos dos vasos sanguíneos em direção às áreas hialinas. Estudos têm investigado a expressão de várias citocinas durante o movimento ortodôntico como IL-1α e IL-1β (DADOVITCH et al., 1988), TNF-α (ALHASHIMI et al., 2001), IL-2 (BASARAN et al., 2006), TGF-β (GARLET et al., 2007), IL-6 (YANG et al., 2013) IL-8 (TUNCER et al., 2005), INF-Y (ALHASHIMI et al., 2000), IL-4 (MORENO et al., 2003), IL-10, IL-12, IL-17 (LIMA et al., 2014), RANK/RANKL/OPG (YAMAGUCHI, 2009). Concomitantemente, ocorre a formação de unidades osteorremodeladoras (UORs) compostas por osteoblastos, clastos e macrófagos, reabsorção osteoclástica do osso alveolar no lado da pressão e aposição óssea no lado da tensão (ABUABARA, 2007), possibilitando o deslocamento do dente para uma nova posição.

### 1.1.1 Reabsorção Óssea na Remodelação Óssea

A remodelação óssea tem como mediadores sistêmicos o paratormônio (PTH), estrogênio, esteróides sexuais e vitamina $D_3$ (BOBAID et al., 2004).

O PTH estimula de forma aguda as reabsorções ósseas porém, forma osso quando injetados em pequenas doses diárias. Sugere-se que isto ocorra porque o PTH reduz a apoptose osteoblástica, prolongando a vida do osteoblasto, possibilitando a potencialização da sua função na síntese de colágeno. Este efeito protetor é mediado pela regulação da atividade osteoblástica, com secreção de RANKL e OPG (BARROS et al., 2003; BOBAID et al., 2004). O PTH liga-se a receptores nos osteoblastos, que então emitem o sinal hormonal aos osteoclastos, desde que somente os primeiros possuem receptores para o PTH. Tanto os linfócitos B quanto os T possuem receptores

para o PTH, sugerindo que o hormônio possa atuar diretamente sobre ambas as células (ROSKAMP et al., 2006).

As células efetoras responsáveis pelo evento da reabsorção dos tecidos mineralizados são células gigantes multinucleadas que são formadas pela fusão de células mononucleares pertencentes à linhagem de macrófagos/monócitos e provenientes do sistema hematopoético. Estas células são denominadas clastos e podem ser identificadas sob o microscópio óptico por causa de seu tamanho (50 a 100 µm) e a multinucleação (2 a 10 núcleos por célula). Os clastos ocupam depressões superficiais conhecidas como lacunas de Howship (THEILL et al., 2002).

A inflamação instalada no local por ação da força ortodôntica mobiliza os clastos principalmente via TNF-α, IL-2 e $PGE_2$. O TNF- α, cujos efeitos são indiretos e mediados pelos osteoblastos, estimulando a reabsorção óssea dependente da síntese de prostaglandinas (KATAGIRI et al., 2002). A IL-1 estimula o crescimento e a diferenciação das células precursoras dos osteoclastos e a atividade dos osteoclastos maduros através de sua ligação à glicoproteína transmembranosa chamada gp 130. A IL-1 β é a citocina mais ativa envolvida no processo de reabsorção óssea. É produzida por monócitos, macrófagos e osteoblastos, servindo de mensageiro para comunicar sinais de reabsorção aos osteoclastos (NUKAGA et al., 2004). O fator transformador de crescimento beta (TGF-β) facilita a adesão leucocitária às paredes dos vasos e à matriz extracelular nos sítios inflamatórios, por aumento da expressão de integrinas (SILVA et al., 2004a). Em sequência a essa cascata de eventos, os osteoclastos dissolvem a matriz mineralizada e degradam a matriz orgânica mediante ação de proteinases cisteína lisossomais (catepsina K) e metaloproteinases 1, 2 e 9. Após fazer endocitose os clastos transportam e liberam continuamente os componentes da matriz óssea durante a reabsorção. As moléculas liberadas passam a ter função quimiotática e de sinalização para osteoblastos, células do ligamento periodontal, macrófagos e neutrófilos, alimentando o processo (HILL, 1998; NESBITT; HORTON, 1997).

### 1.1.2 Diferenciação Osteoclástica

RANK, RANKL, OPG possuem efeitos reguladores no metabolismo ósseo. RANK é o receptor de ativação do fator de transcrição NF-kB e está expresso em altos níveis em precursores osteoclásticos, osteoclastos e cementoclastos, sendo exigido para a ativação e diferenciação dos osteoclastos. O seu ligante, o RANKL (proteína transmembranosa, membro da família dos ligantes associados à membrana do TNF) é expresso em vários tipos celulares, especialmente em osteoblastos e linfócitos T ativados. A interação desse ligante ao RANK de precursores dos osteoclastos e cementoclastos induz a reabsorção de tecido duro (KANZAKI et al., 2001). A osteoprotegerina (OPG), que é um membro secretado da família do TNF, expresso por osteoblastos, cementoblastos, fibroblastos e linfócitos T, inibe a reabsorção de osso ou dente se ligando com grande afinidade ao seu ligante RANKL, prevenindo assim que este se ligue ao seu receptor RANK. OPG e RANK-L competem pelo mesmo receptor RANK. Células que apresentam RNA mensageiro (mRNA) para RANKL com estímulo de citocinas como IL-1α, TNF α, IL-17, promovem inflamação e reabsorção. A OPG e IL-4 inibem a diferenciação dos osteoclastos a partir dos precursores da medula óssea de uma maneira irreversível, também inibindo a capacidade reabsortiva dos osteoclastos maduros. A OPG suprime a expressão de RANK no desenvolvimento dos precursores celulares e promove a geração de células gigantes multinucleadas tartarato fosfatase ácido-resistente (TRAP) negativas, como via alternativa de diferenciação (MORENO et al., 2003).

## 1.2 REABSORÇÃO RADICULAR

É um fenômeno biológico complexo que resulta na degradação dos tecidos mineralizados do dente, cemento e dentina. É um processo fisiológico e necessário durante a fase de exfoliação dos dentes decíduos e sua substituição pelos dentes permanentes. No entanto, fora desse período, todas as reabsorções radiculares tanto em dentes decíduos como em permanentes são consideradas patológicas. Várias são as etiologias que levam a reabsorção radicular, entre elas: trauma dental, infecções periapicais, infecções

periodontais, dentes impactados, clareamento dental, cistos, tumores, anquilose, idiopática e tratamento ortodôntico (FUSS et al., 2003).

### 1.2.1 Reabsorção Radicular Apical Externa (RRAE)

A reabsorção radicular externa é um processo patológico inflamatório com características autoimunes, que pode ocorrer em consequência do tratamento ortodôntico. Essa patologia tem como resultado a perda permanente de estrutura mineralizada (cemento e dentina), com consequente encurtamento da raiz dentária (RAMANATHAN; HOFMAN, 2006; KRISNAN; DAVIDOVITCH, 2006). É a sequela mais comum advinda de procedimentos odontológicos relacionados ao tratamento ortodôntico (HARRIS, 2000; KILLIANY, 2004). A reabsorção radicular ocorre quando a pressão no cemento excede sua capacidade reparativa (SODAWALA; REDDY, 2012) e está relacionada com a morte dos cementoblastos no terço apical (JONES; BOYDE, 1994) e com a exposição e degradação da dentina por odontoclastos (LARA et al., 2003; SELOW et al., 2006). O curso da RRAE dependerá de uma complexa interação entre células inflamatórias, ósseas e dentárias (SILVA et al., 2004b).

## 1.3 DENTINA

A dentina é um tecido conjuntivo especializado, é o maior componente estrutural do dente. Ela é constituída de 65% de matéria inorgânica, sendo a maior parte hidroxiapatita e pequenas quantidades de fosfatos, carbonatos e sulfatos. A matéria orgânica corresponde à 20% onde, 85% são de colágeno, principalmente do tipo I, e em pequena quantidade os tipos II, IV, V, XI e XII. Água corresponde a 13% e os 2% restantes são formados por proteínas não colagenosas, proteoglicanas, fatores de crescimento, proteínas derivadas do soro, citrato, lactato e lipídios (GARANT, 2003; PARK et al., 2009). A dentina similarmente ao osso é um reservatório de polipeptideos e citocinas potencialmente ativos (SILVA et al., 2004b).

### 1.3.1 Dentina e Reabsorção Radicular

A exposição da dentina *in vivo* é geralmente acompanhada pela colonização de clastos e reabsorção da superfície radicular (NE et al., 1999). As proteínas dentinárias, uma vez liberadas de sua matriz mineral, pela RRAE

podem contribuir com a resposta imune inata (LARA et al., 2003; SILVA et al. 2004a). Adicionalmente, vários trabalhos têm confirmado o potencial imunogênico da dentina (KING; COURTS 1988; 1989; NG et al.,1990). Wheeler; Stroup (1993), hiperimunizaram camundongos com extrato de dentina e provocaram reabsorção radicular traumática. O procedimento da hiperimunização parece ter protegido da reabsorção radicular. Hidalgo et al., (2005), mostraram que o soro de pacientes com reabsorção dentária por substituição continha maior nível de IgG e menor nível de IgM humana anti-extrato dentinário total ou frações em relação aos controles sem tratamento. Ramos et al., (2011), demonstraram um decréscimo nos níveis de IgG anti-extrato dentinário total no soro de pacientes com reabsorção radicular grau 2 e os mesmos pacientes tiveram maiores níveis de IgA anti-extrato dentinário total. Lima (2013), investigou os níveis de anticorpos IgG e IgE e não encontrou diferença significativa no extrato dentinário total em relação ao controle; no entanto, foi observado diminuição nos níveis de anticorpos no decorrer do tratamento quando utilizado frações de extrato dentinário humano.

### 1.3.2 Proteínas Não Colagenosas (NCPs) da Dentina

As proteínas não colagenosas correspondem a 5-10% da matriz extracelular da dentina. Normalmente elas se encontram presas dentro da matriz mineralizada da dentina (BUTLER, 1998). Como NCPs são incluídas: proteoglicanas (tais como decorina, biglicana, fibromodulina, lumican, osteosderina e versican) e várias glicoproteínas como osteocalcina, proteínas SIBLING, osteopontina, sialoproteina óssea, proteína da matriz dentinária 1, sialofosfoproteína dentinária e fosfoglicoproteína da matriz extracelular (ORSINI et al., 2012). A maioria das NCPs da dentina são encontradas com as mesmas características no osso, no entanto a dentina apresenta uma diferença quantitativa para a sialofosfoproteína (DSPP). O potencial imunogênico da dentina têm sido atribuído às NCPs.

### 1.3.3 Sialofosfoproteína Dentinária (DSPP)

DSPP é a NCP mais abundante da dentina e também é encontrada no tecido ósseo (QIN et al., 2002). Na dentina, a DSPP é expressa e secretada

por odontoblastos e clivada por proteases extracelulares em 3 partes: sialoproteína dentinária (DSP), fosfoproteína dentinária (DPP) e glicoproteína dentinária fosforilada (DGP) (YAMAKOSHI, 2005). Como os locais proteolíticos de DSPP são altamente conservados, foi hipotetizado que a conversão de DSPP para DSP e DPP representam um evento de ativação, convertendo o precursor inativo para a forma ativa de DPP (BUTLER, et al., 2002). DSPP está envolvida no processo de desenvolvimento e mineralização dentária e sua importância na dentinogênese é apoiada em experimentos que demonstram a associação de mutações do gene DSPP e a dentinogênese imperfeita (XIAO et al., 2001; ZHANG et al., 2001).

DSPP é um membro da SIBLING, uma família de glicoproteínas que se ligam à pequenas integrinas (FISHER et al., 2001). Essas proteínas têm características comuns como presença de fosforilação, glicosilação e sequência de ligação celular com aminoácidos Arg-Gly-Asp (RGD), bem como semelhança na organização genômica e localização (QIN et al., 2002). As proteínas SIBLING propagam sinais biológicos e iniciam a sinalização através das integrinas e sequestro de outras proteínas através da superfície celular. Elas podem se ligar à metaloproteinases e ao fator H do complemento, interferindo com a cascata do complemento ou mesmo extinguindo as respostas inflamatórias que envolvem a via alternativa do complemento (BELLAHCÉNE et al., 2008).

### 1.3.4 Fosfoproteína Dentinária

DPP corresponde a 50% das NCPs, são extremamente ácidas, ricas em ácido aspártico e serina, além de possuírem alta afinidade por cálcio e colágeno. Tem função reguladora na nucleação de cristais de hidroxiapatita sobre a matriz de colágeno da dentina e subsequente crescimento dos cristais de hidroxiapatita (GEORGE et al., 1996), estando envolvida com o estágio de maturação da dentina mineralizada (SUZUKI et al., 2009). Também apresentam potencial para induzir a migração de neutrófilos (SILVA et al., 2004a). Em ratos a DPP foi identificada com uma massa molecular de 90 a 95 kDa (BUTLER, 1983). Chang et al. (1996), identificaram DPP humana em eletroforese de gel de poliacrilamida 7,5% com SDS e encontraram bandas de

140, 60, 50 e 34 kDa em extrato dentinário de dentes com ápice incompleto. Clarkson et al., (1998), extraíram DPP humana a qual apresentou massa molecular de 140 kDa em eletroforese SDS-PAGE 7,5% e observaram que a MM variava provavelmente em decorrência da degradação da amostra.

### 1.3.5 Sialoproteína Dentinária

DSP corresponde a 5% das NCPs (BUTLER, 1998; RITCHIE et al., 1994) e parece estar relacionada com o início da mineralização da dentina (SUZUKI et al., 2009). Estudos de caracterização da DSP, em ratos, indicaram que ela é altamente glicosilada, contendo 29,6% de carboidrato, incluindo 9% de ác.siálico. Apresenta 13 potenciais locais de fosforilação de caseína quinase (RITCHIE et al., 1994). Gel de eletroforese em poliacrilamida 5-15%, identificaram DSP em rato com uma banda de 53 kDa (BUTLER et al., 1992). Qin et al. (2003), identificaram em rato, uma DSP de alto peso molecular com diferenciação na migração em diferentes frações de 125 kDa até 210 kDa. Pode haver diferentes espécies moleculares de DSP devido à diferença na quantidade ou comprimento da cadeia de carboidrato.

### 1.3.6 Glicoproteína dentinária fosforilada

DGP é o terceiro domínio da DSPP, tem quatro resíduos de serina fosforilada (Ser453, Ser455, Ser457 e Ser462) e uma asparagina glicosilada (Asn397). Tem um peso molecular aparente de 19 kDa, em SDS-PAGE e quando reduzido por glicopeptidase- A tem 16 kDa. Uma variedade de glicanos podem estar ligados a Asn397. As massas moleculares calculadas das diferentes formas glicosiladas da fosfoproteína DGP são: 10.523 e 10.670 kDa, 10.815 e 10.961 kDa e 11,106 e 11.252 kDa. DGP pode ter uma importante função na biomineralização (YAMAKOSHI, 2005).

### 1.3.7 Dificuldades na purificação de CNPs

Reações que induzem modificações pós-translacionais podem ser responsáveis em parte pela heterogeneidade da fosfoproteínas humanas. Devido à longevidade e a estabilidade metabólica das proteínas da dentina humana, reações não enzimáticas podem alterar o tamanho e as

características das mesmas. Entre as reações envolvendo aminoácidos está deamidação, desfosforilação, cross-linking, hidrólise e racemização (MASTER, 1983).

### 1.3.8 NCPs como marcadores de RRAE

Balducci et al. (2007), detectaram DMP1, DPP e DSP no sulco crevicular de pacientes que apresentaram reabsorção radicular pós-tratamento ortodôntico de graus leve e grave. Análise por Western blot identificou DMP1 com MM de 55 e 66 kDa, DPP com MM de 55-60 kDa e DSP com MM entre 50 e 70 kDa. No entanto, não houve diferença entre os grupos controle e os grupos investigados. Contudo, em análise por ELISA, método mais sensível, foi demonstrado diferença quantitativa entre os três grupos, sugerindo que a diferença de níveis entre reabsorção leve e grave pode demonstrar que diferentes quantidades de DPP e DSP são liberadas de acordo com o seu grau. Como DPP e DSP são mais específicas para a dentina elas são as mais indicadas como possíveis marcadores.

Kereshanan et al. (2008), identificaram DSP em sulco crevicular durante a fase de reabsorção radicular fisiológica e sugeriu o uso da DSP como biomarcador para detectar e quantificar reabsorção radicular durante situações patológicas resultantes de tratamento ortodôntico ou dentes impactados.

## 1.4 RESPOSTA IMUNE HUMORAL

A imunidade humoral tem como principal molécula efetora o anticorpo. Linfócitos B (LB) específicos reconhecem o antígeno, na forma solúvel ou não, através do seu receptor (BCR). Dessa forma, os linfócitos B são ativados transformando-se em plasmócitos capazes de secretar anticorpos. Diferentemente dos linfócitos T, os linfócitos B maduros reconhecem o antígeno estranho na sua forma solúvel ou nativa e são capazes de reconhecer antígenos proteicos e não proteicos, como polissacarídeos, ácidos nucléicos e glicolipídios. O desenvolvimento da resposta imune humoral para antígenos proteicos é dependente do processamento dos mesmos para que possam ser apresentados para os linfócitos T na forma de peptídeo associado à molécula de histocompatibilidade principal (MHC) classe II. Entre as principais células

apresentadoras de antígenos (APCs) estão as células dendríticas, os macrófagos e os LB, que uma vez ativados pelo antígeno passam a expressar em sua superfície moléculas de MHC classe II, além de uma variedade de moléculas co-estimulatórias e de adesão. Os anticorpos desempenham diversas funções efetoras de defesa como neutralização, opsonização, ativação do complemento e citotoxicidade celular dependente de anticorpo (ABBAS, 2011).

### 1.5 AUTOANTICORPOS

Embora o sistema imune tenha a capacidade de distinguir aquilo que é próprio daquilo que não é próprio, a presença de autoanticorpos é observada em indivíduos saudáveis e em fenômenos de autoimunidade. Autoanticorpos são anticorpos formados contra ou para antígenos próprios do organismo. Mecanismos para inibir a auto-reatividade, denominados tolerância, operam em vários níveis. A tolerância central serve para deletar linfócitos T e B auto-reativos e a tolerância periférica inativa linfócitos auto-reativos (KINDT et al., 2008). A autoimunidade natural difere da doença autoimune porque é um processo fisiológico relacionado com a homeostasia de um sistema de auto reconhecimento molecular. A capacidade de autoanticorpos inibirem funções de certos autoantígenos sugere a existência de uma conservação evolutiva de autoepítopos entre várias espécies (TAN, 1994). O que caracteriza um organismo sadio não é a ausência de autoanticorpos (COUTINHO et al., 1995) ou de linfócitos T reativos a peptídeos autólogos (PEREIRA et al., 1986), mas sim uma discreta reatividade que é robustamente estável, que não varia embora os linfócitos estejam continuamente expostos aos auto-antígenos correspondentes (VAZ et al., 2006). Em estudos com modelos experimentais têm sido observado que antígenos próprios expressos por longo período ou pela vida inteira, são capazes de se ligar à receptores de antígeno por períodos prolongados e são normalmente expostos a linfócitos sem inflamação ou imunidade natural (ABBAS, 2011). Na periferia sob condições normais, células B de memória auto-reativas $IgG^+$ podem ser anérgicas. Alternativamente, pode existir mecanismos regulatórios ainda não definidos, capazes de controlar a diferenciação de células B de memória auto-reativas $IgG^+$ em plasmócitos

(KLINMAN, 1996; SHOKAT et al., 1995). Contudo, falhas na regulação ou ativação desordenadas de células B de memória auto-reativas podem contribuir com o desenvolvimento de autoimunidade em indivíduos susceptíveis (KANEKO et al., 2006; RADBRUCH et al., 2006).

### 1.6 RESPOSTA LINFOPROLIFERATIVA

A proliferação clonal de linfócitos naive ou de memória *in vivo* ocorre após apresentação e reconhecimento do antígeno pelo LT via interação TCR-peptideo MHC, na presença de moléculas co-estimulatórias. O ensaio da proliferação de linfócitos mede a capacidade de proliferação clonal de linfócitos quando estimulados *in vitro* por um antígeno ou mitógeno. Linfócitos T $CD4^+$ proliferam em resposta à peptídeos antigênicos em associação com MHC classe II. A resposta de linfócitos *in vitro* reflete a competência imune *in vivo* (ELVES et al., 1963).

Considerando que os mecanismos imunológicos envolvidos no processo da reabsorção radicular associado ao tratamento ortodôntico ainda não são completamente entendidos, o diagnóstico geralmente tardio dessa patologia e a importância de se buscar marcadores biológicos para a sua detecção precoce, este trabalho teve como objetivo investigar a reatividade de anticorpos séricos e salivares e a formação de imunocomplexos aos antígenos dentinários em rato, assim como os anticorpos salivares e a resposta linfoproliferativa à frações de dentina associados à reabsorção dentária no tratamento ortodôntico em humano.

# OBJETIVOS

## 2.1 OBJETIVO GERAL

Este estudo teve como objetivo investigar a reatividade de anticorpos séricos e salivares e a formação de imunocomplexos aos antígenos dentinários em rato, assim como os anticorpos salivares e a resposta linfoproliferativa à frações de dentina associados à reabsorção dentária no tratamento ortodôntico em humano.

## 2.2 OBJETIVOS ESPECÍFICOS

- Induzir reabsorção radicular externa em ratos;
- Obter extrato dentinário total de rato e humano;
- Obter frações dentinárias de extrato dentinário humano;
- Obter soro imune anti-extrato dentinário de rato em coelho;
- Obter IgG policlonal purificada anti-extrato dentinário de rato;
- Determinar os níveis de anticorpos reativos ao extrato dentinário e imunocomplexos em soro e saliva de ratos ou à frações de dentina em soro e saliva humana no decorrer do tratamento ortodôntico;
- Determinar os níveis de antígenos dentinários solúveis em soro de ratos no decorrer do tratamento ortodôntico;
- Avaliar o reconhecimento de antígenos dentinários por pool de amostras de soro de ratos submetidos ao tratamento ortodôntico por Immunoblotting;
- Avaliar a resposta linfoproliferativa aos antígenos proteicos de extrato dentinário humano em pacientes com reabsorção dentária e controles sem tratamento ortodôntico;
- Correlacionar a resposta linfoproliferativa com os níveis de IgG sérica para antígenos proteicos de extrato dentinário humano.

# Antibodies and immune complexes to dentinal antigens in rats with external root resorption after orthodontic treatment

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## ABSTRACT

The immunogenic potential of dentin has been demonstrated in human and animal dental root resorption. Here, we used an experimental model to investigate the different responses of dentin-reactive antibodies within systemic and external secretions and the immune complex levels. Forty male Wistar rats received orthodontic treatment in which the maxillary right first molars were mesially moved by applying 55 cN of force for 3, 7, 14, or 21 days or left untreated (control), corresponding to five study groups (n=8 for each group). Antibodies to rat dentin extract and immune complex levels in serum and saliva samples were determined by immunoenzyme assays. Higher serum IgG and immune complex levels were detected in the 14 and 21 day groups ($p < 0.05$ and $p < 0.001$ respectively) as compared to the control group. However, in saliva, we detected increased IgG levels in 3 and 7 days ($p < 0.001$) in relation to the control with a decrease in 21 days in relation 3 and 7 days ($p < 0.05$) and increased immune complex levels in the 14 day group ($p < 0.05$) as compared control. In conclusion, we observed immunomodulation of IgG antibodies in response to dentinal antigens exposure during orthodontic treatment, and these response differed within systemic and external secretions. Immune complex levels may be used as biological markers of active root resorption.

Key words: biological markers; root resorption; immune complex; dentinal antigen; immunomodulation.

External root resorption (ERR) as a consequence of orthodontic treatment is a local and acquired pathological process of an inflammatory nature (1) with autoimmune characteristics (2). This type of resorption causes root shortening through the degradation of dental tissues (cement, dentin) by clastic cells (3).

Orthodontic force generates mechanical and cellular stress on periodontal tissues that results in events that are necessary to allow for tooth movement such as inflammation, bone resorption, and bone apposition (4). However, local compression of microcirculatory vessels and subsequent tissue damage and necrosis may expose critical auto-antigens and allow immune competent cells access to antigens that are normally isolated (5).

Dentin contains numerous multifunctional proteins and signaling molecules incorporated in a mineralized matrix (6). After root injury with inflammation, these dentin components may be released and potentially trigger the migration and activation of several cell types including macrophages, inflammatory cells, osteoclasts, osteoblasts, and periodontal cells (7, 8, 9).

The presence of dentin-reactive antibodies has been detected in human root resorption after accidental trauma (10), during orthodontic treatment (2), and by studying a traumatic experimental model in animals (11, 12, 13). These studies confirm the immunogenic potential of dentin and highlight the potential beneficial role of this immunological response in the dental resorption process. However, there are few studies that investigate dentin-reactive antibodies and their relationship with ERR.

The present study aimed to investigate the different responses of dentin-reactive antibodies within systemic and external secretions and the immune complex levels during orthodontic treatment in a Wistar rat animal model.

## Material and methods

### Animals

Forty male 10-week-old Wistar rats (*Norvegicus albinus*) weighing an average of 260 g each, were used for this experiment. The animals were housed in separate cages (four rats/cage) under climate-controlled conditions (12 hour light / 12 hour dark) at 23ºC and provided with rat standard chow and water ad libitum.

**Experimental Model**

The animals (n=40) were divided into five groups (n=8 for each group). In the experimental groups, animals received orthodontic treatment and were euthanized by anesthetic overdose after the following experimental periods: 3, 7, 14, and 21 days. As a control, 8 animals were housed under identical conditions and two were euthanized at each of the same timepoints. All experimental procedures used in this study were performed in accordance with and approved by the Ethics Committee of State University of Londrina (n. 7534.2011.83).

**Orthodontic device**

An orthodontic device, similar to the model proposed by Heller and Nanda (14), was used to mesially tilt the maxillary right first molar (Fig. 1) of each treated rat. Experimental tooth movement was performed with a stainless-steel closed-coil spring (0.06 X 0.22 inch) connected to the maxillary first molar by a ligature wire (0.12 mm). The other side of the coil spring was attached to the maxillary right incisor by a stainless steel wire (0.25 mm) threaded through small holes drilled into the tooth. A controlled force of 55 cN was applied and, checked for precision by a tensiometer (Zeusan Exporting Ltda, Campinas, São Paulo, Brazil). Chemically actived composite resin in both maxillary incisors, increased the anchoring and protected the anterior ligature wire. Prior to the procedure, the animals were intramuscularly anesthetized with a mix of 0,8 mL/Kg ketamine cloridrate (Dopalen, Vetbrands, São Paulo, Brazil) and 0,8 mL/kg of xilazine cloridrate (Anasedan, São Paulo, Brazil).

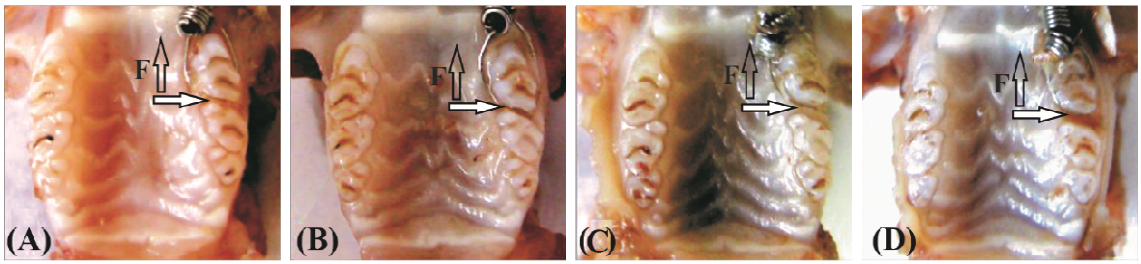


**Figure 1 Mesial tooth movement.** Mesial tooth movement in rats by applying a orthodontic force of 55 cN. **(A)** Control without orthodontic treatment **(B)** 3d **(C)** 7d **(D)**14d **(E)** 21d. The F arrow represents the direction of the mesial movement and the white arrow indicates the tooth movement.

**Serum samples**

After each experimental period, the animals were anesthetized, and blood samples from each animal were collected by cardiac puncture. The blood was centrifuged at 1.100g for 10 min, and the serum was stored at -20ºC until use.

**Saliva samples**

After the experimental period, taking account the influence of circadian cycle in saliva production, whole saliva samples were collected from each animal between 10:00 and 16:00, using three circles of sterile filter paper (5 mm diameter). Next, the samples were eluted into eppendorf tubes with 100 µL of PBS (phosphate buffered saline) and 0.1 mM phenyl metil sulfonyl fluoride (PMSF). Then, they were vortexed two times for 30s each and centrifuged at 1.100g for 6 min. The supernatants were stored at - 80°C until use.

**Histological preparation and histopathological analysis**

After euthanasia, the maxilla of each animal was removed and fixed with 4% paraformaldehyde for 24h. The specimens were demineralized with 10% ethylene diamine tetra acetic acid (EDTA) solution (pH 7.4) for 4 weeks. Next, were histologically processed and transverse sections were stained with hematoxylin and eosin. The histopathological analysis concerning root resorption (Fig. 2) was performed by a light microscope (Eclipse E200, Nikon, Tokyo, Japan). The images were captured by a Moticam 5.0 camera (Toronto, ON, Canada).

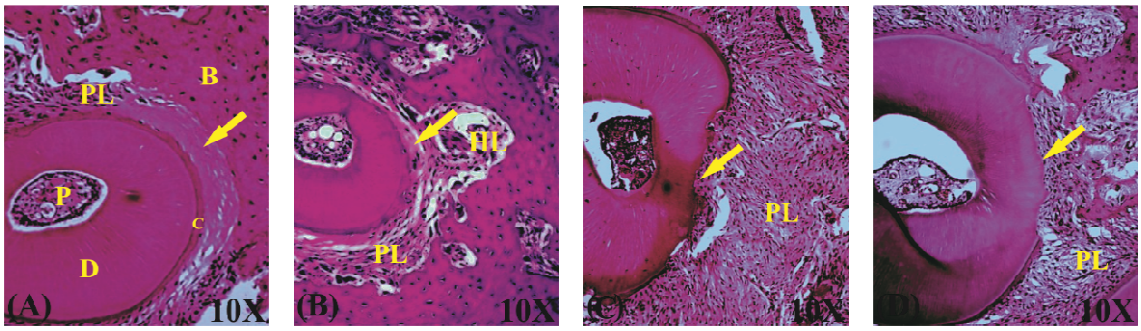


**Figure 2 Histopathological analysis.** Histopathological analysis of periodontal and tooth tissues in rats after orthodontic treatment. **(A)** Hyaline area in the periodontal ligament observed in 3 days of orthodontic treatment **(B)** Hyaline area already dissipating in 7 days of treatment **(C)**-External root resorption in 14 days and in 21 days of treatment **(D).** (B- bone, D- dentin, P- dental pulp, PL- periodontal ligament, HL- Howship's lacunae).

**Rat dentinal extract (RDE)**

The rat dentin extract was obtained through of technique described by Wheeler and Stroup (21) and modified by Hidalgo et al. (10). Incisor teeth from Wistar rats were extracted and placed in cold PBS, pH 7.4. After to remove the enamel and dental pulp the teeth were demineralized using solution of guanidine-HCl 5M containing 10% EDTA pH 5.0 (10, 13) and 1µm of phenylmethylsulfonyl fluoride (PMSF) for 14 days at 4°C. Then, the extract was centrifuged twice at 1.100g, dialyzed against distilled water, lyophilized and stored at -80°C. In this study, total dentinal extract containing non-collagenous proteins was used as antigen.

**Rabbit immune serum and polyclonal anti-RDE IgG**

A white rabbit (*New Zealand*), 24 weeks old, was initially immunized subcutaneously with a total volume of 1.5 mL of a mixture of 750 µL of rat dentin extract (200 µg) and 750 µL of Freund's complete adjuvant. A second and third dose were given and were similar to the first dose but using Freund's incomplete adjuvant, at an interval of 15 days between each immunization. Control serum was collected from the rabbit prior to immunization.The presence of anti-RDE IgG antibody in rabbit immune serum was checked by Double Radial Immunodiffusion (DRI). For IgG purification, the immune serum was

subjected to affinity chromatography using Affi-prep Protein A (156-0006 BIORAD, Hercules, CA, USA). IgG purified was lyophilized and stored at -80°C.

**Serum and salivary IgG antibodies**

Dentin-reactive IgG antibody levels were evaluated by ELISA. Polystyrene microtitre plates were coated with 10 µg/mL of rat dentinal extract diluted in 0.06 M carbonate-bicarbonate buffer (pH 9.6, 100 µL/well for serum or 50 µL/well for saliva) and incubated for 1 h at 37°C and overnight at 4°C. Plates were washed in PBS-Tween 20 and blocked for 60 min at room temperature. The plates were then incubated for 120 min at 37°C with serum samples from each rat at a 1:40 dilution (100 µL/well) or with saliva samples at a 1:6 dilution (50 µL/well). Next, the plates were incubated with rabbit anti-rat Fc-IgG (SAB 3700546 – Sigma Chemical Co., St. Louis, MO, USA), at a dilution of 1:2000. After incubation, the plates were incubated with a secondary antibody, peroxidase-labeled goat anti-rabbit IgG (A1949-Sigma, Chemical Co., St. Louis, MO, USA), at a dilution of 1:4000 for 90 min at 37°C. The reaction was induced by adding 0.03% H2O2 and o-phenylenediamine (OPD) in 0.1 M citrate-phosphate buffer (pH 5.0) and incubated for 15 min at room temperature. The reaction was stopped by adding $H_2SO_4$. The optical density (OD) was determined at 492 nm using a plate reader (Multiskan; Flow Laboratories, McLean, VA, USA). All samples were tested in duplicate.

**Soluble dentinal antigens in serum**

Soluble dentinal antigen levels were determined in the serum by inhibition ELISA (inh-ELISA). Serum aliquots from each rat (150 µL) were diluted 1:2 in PBS mixed with an equal volume of 0.1 M EDTA pH 7.2 (Sigma Chemical Co., St. Louis, MO, USA) and boiled at 100ºC for 5 min. The tubes were cooled and centrifuged at 1.100g for 40 min. The resulting supernatant was used for the ELISA. The diluting buffer added to the immune serum and used to determine the inhibition curve consisted of a pool of normal rat serum diluted 1:10 in 0.05% PBS and 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA). All samples were tested in duplicate. In an inhibition plate previously

blocked with 200 µL of 5% BSA/PBS-Tween 20 for 120 min at 37°C, an inhibition standard curve was constructed by adding different concentrations of RDE (from 0.11 µg/mL to 5 µg/mL) in diluting buffer up to 150 µL/well (Fig. 4A). Serum samples were plated (75 µL/well) and rabbit polyclonal anti-RDE IgG at 10 µg/mL (75 µL/well) was added. Plates were mixed in a shaker for 30 min at room temperature and then incubated overnight at 4ºC. To perform the reaction, the reaction plate was first coated with 2.5 µg/mL of RDE in 0.06 M bicarbonate buffer pH 9.6 (100 µL/well). The mixture of antibody, immune complexes, and free antibody on each inhibition plate was then transferred to the respective wells on the reaction plate. Next, the reaction plate was incubated for 120 min at 37ºC. After 100 µL of peroxidase-labeled goat anti-rabbit IgG (A1949-Sigma, Chemical Co., St. Louis, MO, USA) was added, the plate was incubated for 90 min at 37ºC. The reaction was detected as previously described above.

**Serum and saliva immune complexes**

Polystyrene microtitre plates (Zurich, Switzerland) were coated with 100 µL/well of rabbit polyclonal anti-RDE IgG (10 µg/mL), in duplicate. Rat serum samples, diluted 1:10, or saliva samples, diluted 1:6 (50µL/well), were added and incubated for 120 min at 37°C. Peroxidase-labeled goat anti-rat IgG (sc 3823 - St. Cruz Biotechnology, Dallas, TX, USA) was added at a dilution of 1:4000. The reaction was incubated for 90 min at 37°C and detected as described above.

**Electrophoresis (SDS-PAGE)**

The protein content of the RDE was determined using a NanoDrop Lite spectrophotometer (Thermo Scientific, Rockford, IL, USA). The samples were first loaded onto a 5-15% gradient polyacrylamide gel and run at 90 V along with a molecular weight marker (Kaleidoscope, cat. #161-0375, Bio-Rad, Hercules, CA, USA). The gel was stained by silver staining (Bio-Rad, Hercules, CA, USA).

**Immunoblotting**

After rat dentin extract was electrophoresed (SDS-PAGE) the dentinal components were transferred to a nitrocellulose membrane. The membrane was blocked with PBS-T and 5% skim milk for 1 h at room temperature. Next, the membrane was incubated for 120 min at 37°C with a 1:50 dilution of rat serum pooled from 3, 7, 14, and 21 day treated rats or control untreated rats. The membrane was then washed and processed with peroxidase-labeled goat anti-rat IgG (sc 3823 - St. Cruz Biotechnology, Dallas, TX, USA), diluted 1:2000, for 90 min at 37ºC. The reaction was performed in TMB (tetramethyl benzidine, Thermo Scientific, Rockford, IL, USA) and stopped with distilled water.

**Statistical Analysis**

The data were analyzed by One-Way ANOVA and unpaired Student's t test was used to detect differences in ELISA absorbance (antibody levels expressed as (OD) inter-groups. *p* values less than 0.05 were considered to be statistically significant. Statistical analysis was performed using GraphPad Prism 5.0 for windows (San Diego, California, USA).

**Results**

**Serum and salivary IgG levels**

Serum dentin-reactive IgG was detected at higher levels in the 14 day (0.754 ± 0.115) and 21 day (0.756 ± 0.153) groups as compared to the control group (0.566 ± 0.058), with $p < 0.05$ (Fig. 3A). In contrast, higher salivary dentin-reactive IgG levels was detected in the 3 day (0.414 ± 0.025) and 7 day (0.408 ± 0.018) groups as compared to the control (0.350 ± 0.029); $p < 0.001$ (Fig. 3B). Also, in the 3 and 7 day groups salivary IgG levels were higher in relation to the 21 days group (0.364 ± 0.038); $p < 0.05$ (Fig. 3B).

**Serum soluble dentinal antigen concentration**

Although serum-soluble dentin antigens were detected in all groups, there was no statistically significant difference in the concentration (µg/mL) of serum soluble dentinal antigens in the 3, 7, 14, and 21 day groups as compared to the control (Fig. 4B).

**Serum and saliva immune complex levels**

The serum immune complexes formed by specific IgG / dentinal antigens were detected at higher levels in the 14 day (1.528 ± 0.200) and 21 day (1.471 ± 0.241) groups as compared to the control (1.064 ± 0.131); $p < 0.001$ (Fig. 5A). In saliva was observed higher levels of immune complexes in the 14 days group (0.203 ± 0.086) as compared to the control (0.101 ± 0.037); $p < 0.05$ (Fig. 5B).

**Electrophoresis SDS-PAGE analysis**

SDA-PAGE analysis identified proteins of approximately 250, 180, 160, 150, 120, 77, 62 and 40 kDa, in rat dentin extract (data not shown).

**Immunoblot analysis**

All serum samples from rats that had undergone tooth movement for 3, 7, 14, or 21 days and from control (untreated) showed more intense bands corresponding to the ~120 kDa and less intense bands corresponding to the dimer 150 kDa, as detected by immunoblotting (Fig. 6A).

**Discussion**

Root resorption may be affected by the magnitude and duration of an applied orthodontic force. In our orthodontic movement study in rats in which was applied a heavy force of 55 cN, greater root resorption was detected in rats treated for 14 and 21 days than in those treated for shorter periods of time. These results were similar to those found by Gonzales et al. (15) in which a force of 50 cN was applied.

The higher serum IgG levels reactive to dentin were also detected in the 14 and 21 day groups, which are those with a higher root resorption period. These results suggest that root resorption in consequence of orthodontic treatment may contribute to an exposition of antigens from dentin matrix with activation and proliferation of specific B lymphocytes, which in turn product dentin-reactive antibodies.

B cells expressing autoreactive tendencies may be found in the peripheral B cell repertoire of healthy individuals. However, the recognition of antigens by activated lymphocytes or tolerance is determined by properties of

the antigen, the state of maturation of antigen-specific lymphocytes, and by stimuli received when these lymphocytes encounter self-antigens (16, 17, 18).

Investigations using a traumatic root resorption model were also performed in dogs and mice, where the presence of serum dentin-reactive antibodies and their subsequent decrease at 10 to 14 days post treatment were detected during the active root resorption phase (11, 12, 13). Additionally, Ramos et al. (2) reported a significant decrease in serum IgG levels in humans after 6 months of orthodontic treatment, with a grade 2 root resorption severity. However, in our study, we did not observe decreased serum IgG levels, which was consistent with the results of Hidalgo et al. (10) that found increased dentin-reactive IgG levels in patients with ERR. These variations may be due to differences in the time intervals used, methodology, or factors such as the formation of immune complexes.

Serum IgG recognized two dentin antigenic components presenting ~120 kDa and 150 kDa in all tested groups. However, there was a quantitative difference in antibodies levels to these antigens between the treated and untreated groups by ELISA. Due to small amount of saliva samples obtained, was not possible investigate the recognition of dentinal antigens by salivary IgG.

This study demonstrated that soluble dentinal antigens exposed to the immune system in consequence of the external root resorption may be complexed to specific antibodies with increased immune complex levels during active phase of root resorption. Thus, immune complex levels could be used as biological markers during orthodontic treatment for monitoring the active phase of the root resorption.

Unlike in the serum samples, higher levels of IgG to dentinal antigens were detected in saliva samples in the initial phase of orthodontic treatment, followed by a reduction at the active phase of resorption. According to Ramos et al. (10), variations may occur in systemic and local immune responses to dentinal antigens during orthodontic treatment. IgG class antibodies may diffuse from plasma to saliva via the gingival crevicular epithelium or be locally produced by plasmatic cells from salivary glands or tonsils (19). Palatine tonsils in humans, may be an important inductive site for memory/effector B cells destined to salivary glands (20). Therefore, the presence of higher levels of

salivary IgG antibodies at the initial stage of orthodontic treatment may indicate a local humoral response independent of the systemic response. For the other hand, the decreased antibodies levels found within our saliva samples, in 21 days group, suggests local immunomodulation.

According to Wheeler and Stroup (21), anti-dentin autoantibodies did not mediate tooth resorption in mice and were considered to be a protective factor against root resorption, as determined by dentin extract immunization results.

Our investigation also demonstrated that the soluble form of dentinal antigens can be detected in serum and may be found in the control group. This data could explain the dentin-reactive antibodies presence in subjects who have never been submitted to orthodontic treatment. Foo et al. (22), through Micro Computerized Tomography analysis detected root resorptions in control rats, which could be attributed to normal physiological tooth drift. Other studies in humans reported that dental resorption may naturally occur in 81% of cases within the general population (23) and investigations using Micro Computerized Tomography confirmed the microscopic areas of ERR presence in non-orthodontically treated permanent teeth, which have been reported at baseline levels of resorption (24). Thus, the micro-resorptions commonly present in human or animal teeth, may indicate periods of immunogenic induction, which may occur constantly, as proposed by Hidalgo et al. (10).

Surprisingly, in this study were observed similar levels of serum soluble dentinal antigens in control and treated groups, suggesting the presence of regulatory mechanisms. However, the true biological meaning of these small root resorptions is still not understood. In conclusion, immunomodulation of IgG antibodies occurred in response to dentinal antigens exposure during orthodontic treatment in rats, but differences were detected within systemic or external secretions. Immune complex levels could be used as biological markers during orthodontic treatment for monitoring the active phase of the root resorption. More studies are necessary to corroborate these findings.

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## FIGURES

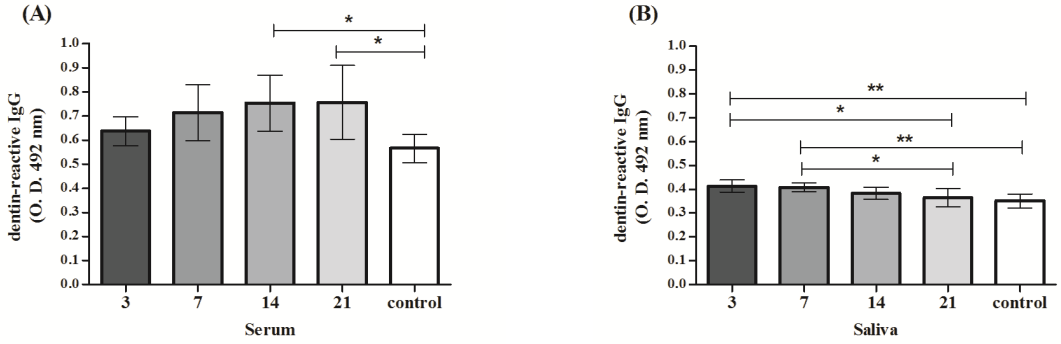


**Figure 3 Serum or salivary dentin-reactive IgG levels.** Dentin-reactive IgG levels in rats undergoing orthodontic treatment for 3, 7, 14 or 21 days and control **(A)** Serum **(B)** Saliva. Optical densities values (OD) 492nm, by ELISA. *$p < 0.05$ ** $p < 0.001$.

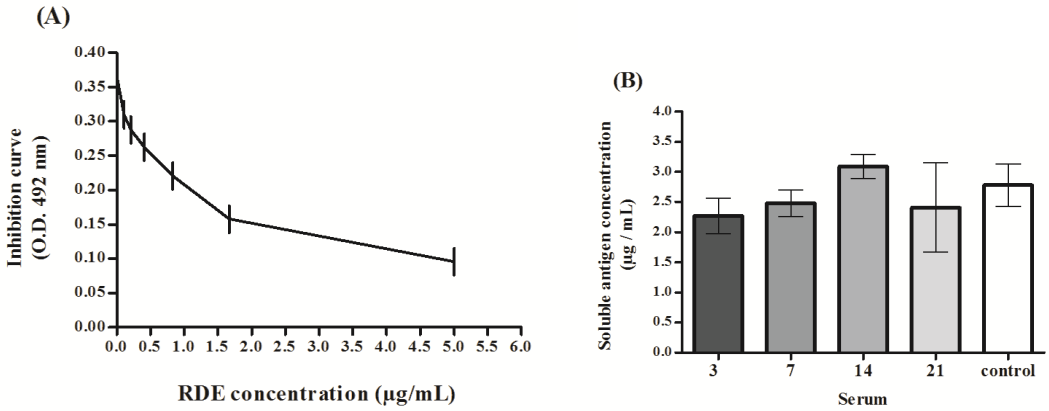


**Figure 4 Serum soluble dentinal antigen.** Serum soluble dentinal antigen in rats undergoing orthodontic treatment for 3, 7, 14 or 21 days and control. **(A)** Inhibition standard curve was obtained with known quantities of rat dentinal extract: 0.11; 0.21; 0.41; 0.83; 1.67; 5 µg/mL. Optical densities values (OD) 492nm, by ELISA. **(B)** Serum soluble dentin antigen concentration, inh-ELISA.

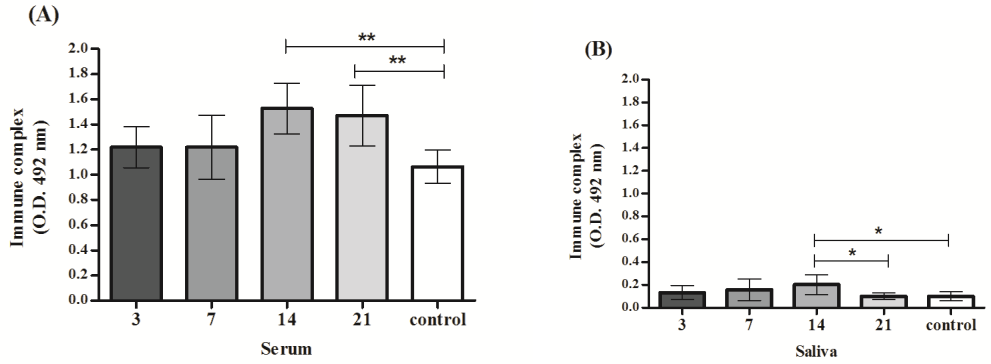


**Figure 5 Serum or salivary immune complex levels.** Immune complex levels in rats undergoing orthodontic movement for 3, 7, 14 or 21 days and control. **(A)** Serum **(B)** Saliva. Optical densities values (OD) 492nm, by ELISA. *$p$ < 0.05 **$p$ < 0.001.

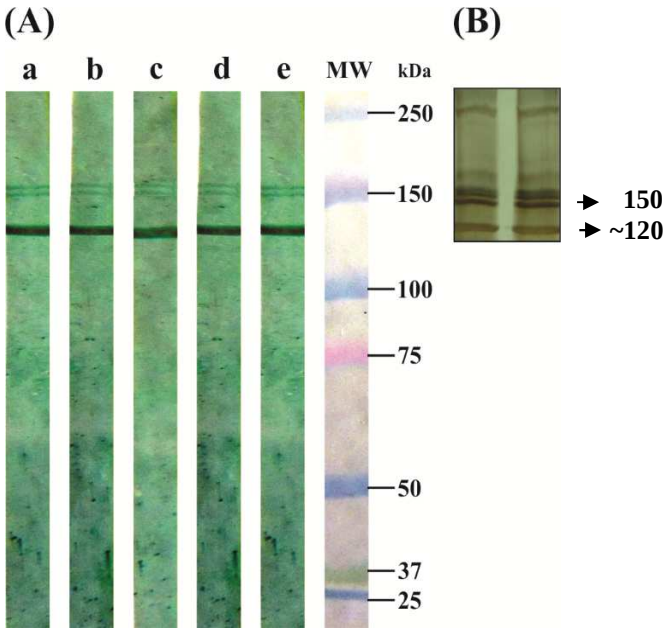


**Figure 6 Immunoblot. (A)** Dentin proteins recognized by pool of rat serum by immunoblot. A- 3d, B- 7d, C- 14d, D- 21d, E- control. MM- molecular mas marker. **(B)** Dentinal antigens ~120 and 150 kDa demonstrated in Electrophoresis SDS-PAGE in 5% and 15% gel gradient.

# Distinct human salivary IgG reactivity and lymphoproliferative response to antigenic fractions of dentin

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## Abstract

Dentinal antigens are exposed during apical external root resorption in consequence of orthodontic treatment and may induce immune response. This study aimed to evaluate the salivary IgG levels and lymphoproliferative response to fractions of human dentin extract during orthodontic treatment. The study comprised three groups: Group I consisted of adolescent patients (n = 10), where saliva samples were collected in four periods: before (T0), 3 (T3), 6 (T6) and 12 (T12) months of orthodontic treatment for analysis of reactivity salivary IgG levels to FI, FII or FIII human dentinal extract fractions. Group II (n=30), serum and peripheral blood mononuclear cells from adolescent patients, with 6 months of orthodontic treatment, were collected for analyzes of serum IgG levels and lymphoproliferative responses to FI or FIII human dentinal extract fractions. The third group, participated adolescent volunteers (n=16) who had never undergone orthodontic treatment and no radiographic signs of root resorption, served as control for the second study group. The total dentinal extract obtained from human third molar teeth, was fractioned by gel filtration chromatography, Sephadex G-120-150. The root resorption analysis was performed by digital subtraction method of upper incisors. Salivary and serum IgG levels were determined by enzyme-linked immunosorbent assay (ELISA) and the lymphoproliferative response was analyzed by MTT assay. The results showed similar salivary IgG levels and lymphoproliferative response to dentinal fractions between the groups evaluated. However, it was observed higher IgG reactivity to FII in relation to FIII in T6 and T12 ($P < 0.05$) and in Group II there was a significant positive correlation between lymphoproliferative response and serum IgG levels to FIII fraction (r = 0.508). In conclusion, changes in salivary

IgG reactivity to FII fraction during orthodontic treatment suggest the potential of these antibodies as biological markers for early detection of root resorption. More studies are necessary to corroborate these findings.

Key words: biological markers; non-collagenous proteins; dentinal antigens; lymphoproliferative response; root resorption.

External apical root resorption (EARR) is a pathologic process, that result in loss of cementum and dentin (RAMANATHAN, 2006), and may occur after tooth orthodontic movement. Its nature is inflammatory (TROPE, 1998; BREZNIAKI; WASSERSTEIN, 2002) with autoimmune characteristics (RAMOS, 2011) and, commonly, affect the root apex.

Dentin is a specialized connective tissue that contains several multifunctional polypeptides and cytokines, potentially active, hidden in its mineralized matrix (BUTLER, 1998). Some growth factors and their receptors expressed during embryonic tooth development remain sequestered in mineralized tissues and are re-expressed in mature dental and periodontal tissues under normal and pathological conditions (PARKAR et al., 2001).

A number of non-collagenous proteins (NCPs) have been identified from dentin and bone, and may be important to several processes during dentinogenesis and osteogenesis (LINDE, 1989; BUTLER, 1992; XIAO et al., 2001; ZHU et al., 2012).

Non-collagenous proteins, when released of dentin for clasts action, after the root injury and inflammation generated by orthodontic force, have potential to interfere with the root resorption course (SILVA et al., 2004). Dentin extracts triggered a leukocyte migration, in a time and dose dependent *in vivo*, and also the expression of inducible nitric oxide synthase, TNFα e IL-1β for macrophages *in vitro* (LARA et al., 2003).

Given the paucity in literature about immunogenic dentin components related with root resorption and the need of biological markers for this pathology associated to orthodontic treatment, this study aimed to investigate salivary antibodies levels and the lymphoproliferative response to dentinal fractions and the correlation between the data found.

## Material and Methods

*Subjects*

The study comprised three subjects groups (Table 1). Group I (n=10), mean age 15 ± 1,75 years, 2 females and 8 males, in which saliva samples were collected for analysis in different times: before (T0), 3 months (T3), 6 months (T6) and 12 months (T12) of orthodontic treatment. Group II (n=30), mean age 13 ± 2,5 years, 20 females and 10 males with 6 months of orthodontic treatment, in which peripheral blood mononuclear cells (PMBC) were collected to lymphoproliferative response analysis and its correlation with serum IgG levels. Group III (n=16), mean age 14 ± 2 years, 10 females and 6 males who have never undergone orthodontic treatment was used as control of the group II. Inclusion criteria - all participants in the study not having undergone orthodontic treatment previously. Exclusion criteria – clinical or radiographic signal of root resorption , periapical lesions or periodontal disease, previous trauma of the primary or permanent dentition, autoimmune or chronic inflammatory disease, use of steroidal and non-steroidal anti-inflammatory drugs. All procedures were performed after informed consent was given by parent/legal guardian and were approved by Research Ethics Committee for Human Experiments at Londrina State University (n. 7534.2011.83).

**Table 1. Study groups**

| | GROUP I | GROUP II | GROUP III |
|---|---|---|---|
| **Samples** | **Saliva (n=10)** | **Serum (n=30 )** | **Serum n=16** |
| **Time** | **T0, T3, T6, T12** | **T0, T6** | **Control** |
| **Samples** | | **PBMC (n=30)** | **PBMC (n=16)** |
| **Time** | | **T6** | **Control** |

*Saliva and serum samples*

Saliva samples were collected at T0, T3, T6 and T12. To avoid the effect of the circadian circle in IgG secretion into saliva, samples were collected between 10:00 to 16:00. Unstimulated whole saliva sample was collected by

expectoration into sterilized vial. After, the samples were centrifugated at 1.100g for 10 min and the supernatants were stored at - 80°C. Peripheral blood samples were collected at T0, T6 and control group, leaving them clot at room temperature. Next, the serum was obtained by centrifugation at 200g for 15 min at 4°C, aliquoted and stored at –20º C.

*Peripheral Blood mononuclear cells (PBMC)*

For PBMC, blood samples from patients with 6 months of treatment orthodontic (T6) and from a control group were collected into tubes containing sodium heparin and centrifuged at 600g for 4 min. The buffy coat was collected, re-suspended in PBS (0.15M, pH 7.2) and transferred to tube containing Ficoll-Hipaque gradient, D- 1.076 (Amersham Biosciences, Piscataway, New Jersey, USA) and centrifuged at300 g, for 30 min at 4°C. An hemolytic solution (tris hidroximetil aminometano-tris-base 0,206%; 0,77% ammonium chloride) was used to lysis the remaining red blood cells. After washing, PBMC were resuspended in RPMI with dimethyl sulfoxide (DMSO) with 50% fetal bovine serum. Finally, they are frozen in liquid nitrogen.

*Human dentin extract (HDE)*

Extract of human dentin containing NCPs was used as the antigen. HDE was obtained through modification of the technique described by Wheeler Stroup (1993) from 30 third molars donated by patients for whom the extraction were indicated. The dentin was drilled out using high-speed bit. After, the precipitate was demineralized using solution of guanidine-HCl 5M containing 10% EDTA pH 5.0 and 1µm of phenylmethylsulfonyl fluoride (PMSF) for 14 days at 4°C. Next, the extract was centrifuged twice at 1.100g for 40 min at 4°C, dialyzed against distilled water, lyophilized and stored at - 80°C.

*Radiographs*

For periapical radiographs were used radiographic films (Kodak speed - Eastman Kodak Co. - Rochester - NY) and Spectro X-ray apparatus (Dabi Atlante - Ribeirão Preto - SP - Brazil) operating at 70kV and 10 mA and exposure time 0.7 seconds. The revelation was performed in developing

solution (Kodak, Eastman Kodak Co- Rochester, NY) following the manufacturer's instructions in the criterion temperature / time. The radiographs were digitalized through HP Scanjet G4050 scanner (Hewlett-Packard - Palo Alto - CA) with transparency adapter and its associated software for image capture, with fixed resolution of 300 dpi and 100% scale. For analysis of the root resorption was used digital subtraction method and to severity resorption degree analysis was used Levander and Malmgren classification (1988) in which mild or 1 grade (0 to 0.5 mm); moderate or 2 grade (> 0,5 - 2 mm); severe or 3 grade (> 2mm < 1/3 root); extreme or 4 grade (> 1/3 root). In group I, n=10, root resorption was higher in 12 months of treatment orthodontic (T12) in relation to control (T0), presenting resorption severity grade I and II. Group II patients also presented radicular resorption grade I and II after 6 months of orthodontic treatment.

*Gel filtration chromatography*

For obtainment of dentinal fractions, the crude dentin extract was first desalinate in a Sephadex G-25 gel fitration column (Sigma, St. Luis, USA). The automatic fraction collector (FC 203B, Gilson, Middleton, USA) was maintained at 4°C. Next, the fraction obtained was re-fractionated in a Sephadex G-120-150 (Sigma, St. Louis, USA). The fractions were eluted in PBS, and the absorbance was read in a spectrophotometer (Pharmacia Biotech, Sweden) at 280 nm. In the same column were eluted IgG (150kDa), albumin (69kDa) and ovalbumin (45kDa) as (MM) marker. MM fractions greater than 300.000 kDa were excluded from the inner volume of the gel particles. The elution profiles of desalinate extract dentin from a Sephadex G-120-150 column with three absorbing peak at 280 nm were denominated FI with MM (< 300kDa and > 69), FII with MM (69 to 45 KDa) and FIII with MM (< 45kDa), (Fig. 1).

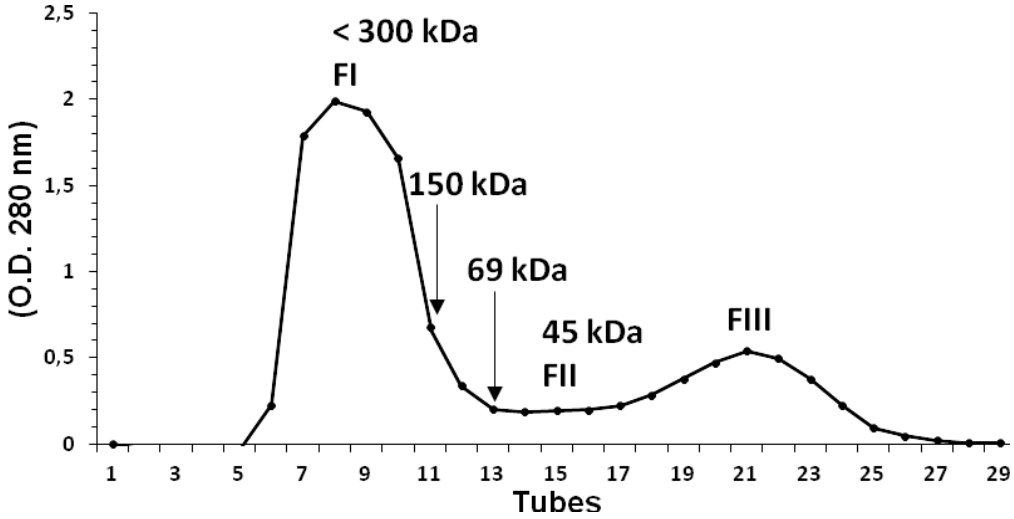


**Figure 1 Spectrophotometric profile of human dentin extract.** Total human dentin extract was fracionated in Sephadex G-120-150 column for obtain FI, FII and FIII dentin fractions. In the same column were eluted IgG (150kDa), albumin (69kDa) and ovalbumin (45kDa) as (MM) marker. MM fractions greater than 300.000 kDa were excluded from the inner volume of the gel particles. Values optical density (OD) 280 nm.

*Enzyme-linked immunosorbent assay (ELISA)*

ELISA immunoplates were sensitized with 10 µg/mL of FI or FII or FIII fractions in carbonate-bicarbonate buffer ($Na_2Co_3$ 1.59 g, $NaHCO_3$ 2.93 g, distilled water, qsp 1000 mL, pH 9.6), incubate for 60 min at 37°C and overnight at 4°C. The plate was washed four times with phosphate buffered saline (PBS) containing 0.05 per cent Tween 20 (PBS-T), blocked with 5 per cent skimmed milk for 60 min at room temperature. After washing, the serum samples (1:40 in PBS) or saliva no diluted wereincubated with goat anti-human IgG labelled with peroxidase (A8775, Sigma-Aldrich, St Louis, USA), diluted 1: 4000, for 90 min at 37°C. After washing, 100 µL of substrate solution was added (5 mg orthophenylenediamine, 10 mL of 0.1 M citrate buffer, pH 4.5, and 5 µl $H_2O_2$). After 15 min, the reaction was stopped with 50 µL of $H_2SO_4$, 4 N and absorbance was read in a Multiskan EX reader (Lab Systems, Helsinki, Finland) at 492 nm. Antibody levels were expressed as absorbance in optical density (OD) units.

*Lymphoproliferation assay*

In 96-well flat-bottom culture plates, 100 µL of the defrosted and washed PBMC ($1x10^5$ cells/mL) and 100 µL of the FI or FIII dentin fractions (20µg/mL) were cultured in triplicate wells in RPMI 1640 (containing L-glutamine, 10% fetal bovine serum). As a control, the dentin fraction was replaced by RPMI medium. The cells were cultured for 120 h at 37ºC with 5% $CO_2$, and then lymphocyte proliferation was evaluated by using the MTT detection kit (Trevigen, R & D Systems Inc., Minneapolis, Minnesota, USA). The formazan crystals were solubilized by adding 100 µL DMSO. OD was measured by a Multskan EX microplate reader (Labsystems, Helsinki, Finland), at 550 nm.

*Statistical Analysis*

Non-parametric tests (ANOVA Friedman/Dunn's test, Wilcoxon) were applied to detect absorbance differences by ELISA. Parametric test (ANOVA/Tukey ) was applied to detect absorbance differences in lymphoproliferation by MTT. Spearman's rank correlation coefficient was applied to detect correlations between lymphoproliferative response (MTT) and serum IgG antibodies to dentin fractions in patients presenting root resorption after 6 months of treatment orthodontic. Significant correlation was considered when $r \geq 0.50$. *P* values less than 0.05 were considered to be statistically significant. Statistical analysis was performed using GraphPad Prism 5.0 for windows (San Diego, California, USA).

## Results

*Salivary dentin fractions-reactive IgG levels*

There was no statistical difference in the salivary IgG levels reactive to FI, FII or FIII at (T3), (T6) or (T12) in relation to control (T0) (Fig. 2). However, when the three fractions were compared in each time (Fig. 3), the salivary IgG reactivity levels were statically different to FII at (T6) and (T12) in relation to FIII, $P < 0.05$ (Fig. 3 C and 3D).

*Serum dentin fractions-reactive IgG levels*

Serum FI or FII-reactive IgG levels decreased at (T6) in relation to control, $P < 0.0001$ (Fig. 4A and 4B).

*Lymphoproliferative response analysis*

The results of lymphoproliferative response by MTT method expressed in OD at 550 nm, showed higher level of absorbance for the fraction FI than FIII in both groups, orthodontically treated group and in control group, $p < 0.05$ (Fig. 5).

*Linear correlation*

Spearman's correlation test between lymphoproliferative response and serum IgG antibodies in patients presenting root resorption after 6 months of Orthodontic treatment shows statistical significance to FIII ($r^2 = 0.508$) (Fig. 6), but, there was no correlation to fraction I ($r^2 = -0.223$). In addition, in patients control also there was no correlation between lymphoproliferative response and serum IgG to FI ($r^2 = -0.325$) or to FIII ($r^2 = -0.072$).

**Discussion**

The dentin exposure and the release of their components during Orthodontic treatment, would be evaluated by an indirect method as the detection of specific immune response, using antibodies formed against these antigens. The use of saliva samples instead of serum has great advantage to be a simple and noninvasive method and have better compliance by patients. This study showed that the higher molecular mass HDE fractions may be recognized by IgG salivary both in patients undergoing Orthodontic treatment as those who received no treatment. This suggests that may be occurring some sensitization to these antigens due to the presence of small root resorption observed in the general population, detected by computed tomography micro-micro and histological analysis (MASSLER, 1954; WIERZBICKA, 2009). The dentin-reactive antibodies in human and animal fluids have been described in dental resorption studies, demonstrating that orthodontic treatment induces both a systemic and local humoral immune response to dentinal antigens (COSTA et al., 2014; HIDALGO et al., 2005; NG et al., 1990; RAMOS et al., 2011).

This study investigated salivary IgG levels to three distinct HDE fractions and it was observed similar levels between samples collected before and after orthodontic treatment. As the salivary IgG may be derived by local production and by blood diffusion, we expected the higher IgG levels to HDE fractions. it is possible that this result is due to the lesser degree of resorption of patients evaluated in this study and or also due to low sampling, which requires further study.

Indeed, when salivary IgG reactivity was evaluated to different fractions in each time of treatment, it was detected changes in IgG recognition to FII (MM intermediate ~ 69 to ~45kDa) in relation to fraction III (< 45kDa) in (T6) and (T12). Change in salivary IgG levels to distinct human dentin fractions suggests that, at some stage of orthodontic treatment, these antibodies may to detect an increased exposition of the FII indicating the possibility of early detection of external root resorption for these antibodies.

For the other hand, in serum samples from group II there was a decrease in IgG levels at (T6) to FI and FIII fractions suggesting that the formation of immune complexes may be responsible for such difference.

Several researchers have associated the root resorption with specific sequestered dentin antigens that would activate the immune system after dentin exposure (HIDALGO et al., 2005; KING & COURTS, 1988, 1989; NG, 1990; RAMOS et al., 2011; SILVA et al., 2004). The dentin-reactive antibodies, detected in patients with root resorption associated with trauma or orthodontic treatment, belong mainly to IgG class (HIDALGO et al., 2005, RAMOS et al., 2011). This IgG isotype require $CD4^+$ T lymphocytes activation and all antigens that use T lymphocytes for antibodies production are denominated T-dependent antigens (ABBAS et al., 2011).

Comparing the dentinal fractions, higher lymphoproliferative response to FI in relation to FIII dentin fraction may be due to greater complexity and immunogenicity of antigens of great molecular mass. Large molecules are generally good immunogens and more easily phagocytozed, processed, linked to the MHC molecule and presented to T cells (KINDT et al., 2008).

Considering that the IgG response is dependent on T lymphocytes, it was expected that serum IgG to FI should correlate with a lymphoproliferative

responses to the both studied groups. However, it was not detected. The lack of correlation to FI may be due to an immunomodulation of the immune response such as participation of regulatory T cells specific, which requires further study. On the other hand, as expected, there was a positive correlation between the lymphoproliferative response and IgG levels to FIII, suggesting collaboration between specific T and B lymphocytes for these antigens.

The occurrence of root resorption is usually monitored through X-rays and this method is not able to detect the resorption in its early stages. In conclusion, this study showed that the changes in salivary IgG reactivity levels during orthodontic treatment to FII suggest o potential these antibodies for early detection of root resorption, which require further study.

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## FIGURES

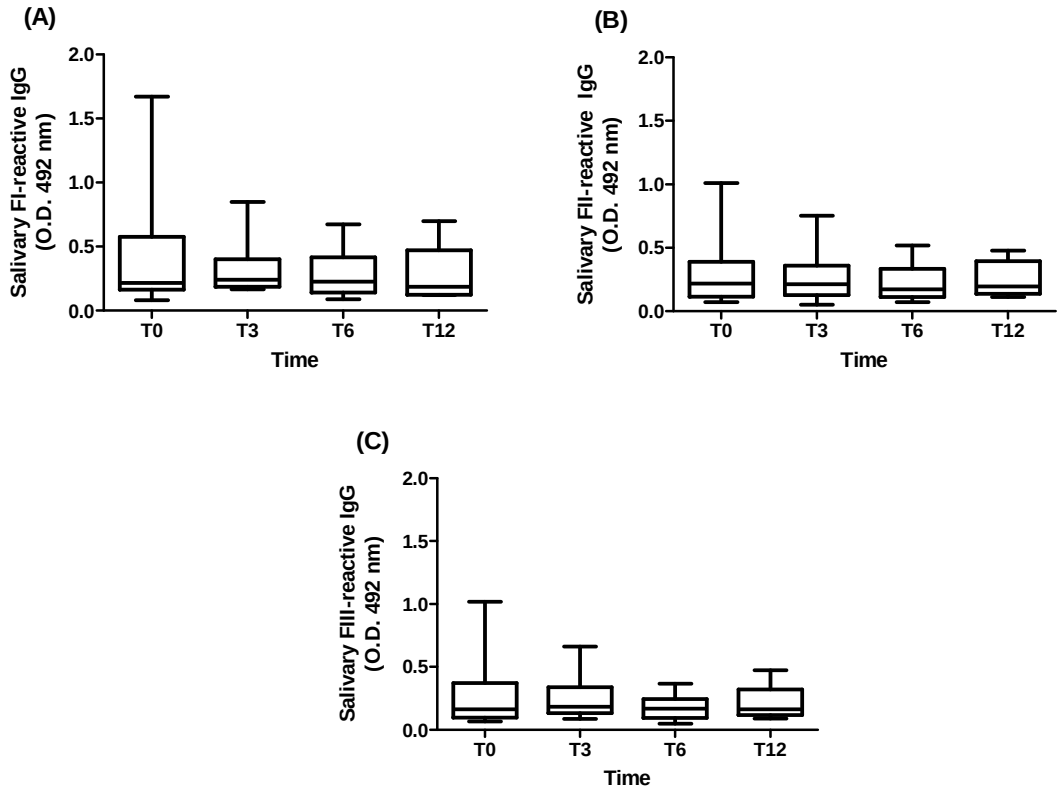


**Figure 2 Salivary dentin fractions-reactive IgG levels .** Salivary FI, FII or FIII-reactive IgG levels: before (T0), 3 (T3), 6 (T6) and 12 months of orthodontic treatment. (A) Salivary FI-reactive IgG levels. (B) Salivary FII-reactive IgG levels. (C) Salivary FIII-reactive IgG levels, (n=10). Difference between IgG levels with different times was tested by paread Friedman test. The box represents 25-75 per cent of the values of optical densities (OD) at 492 nm and horizontal bar represents the median.

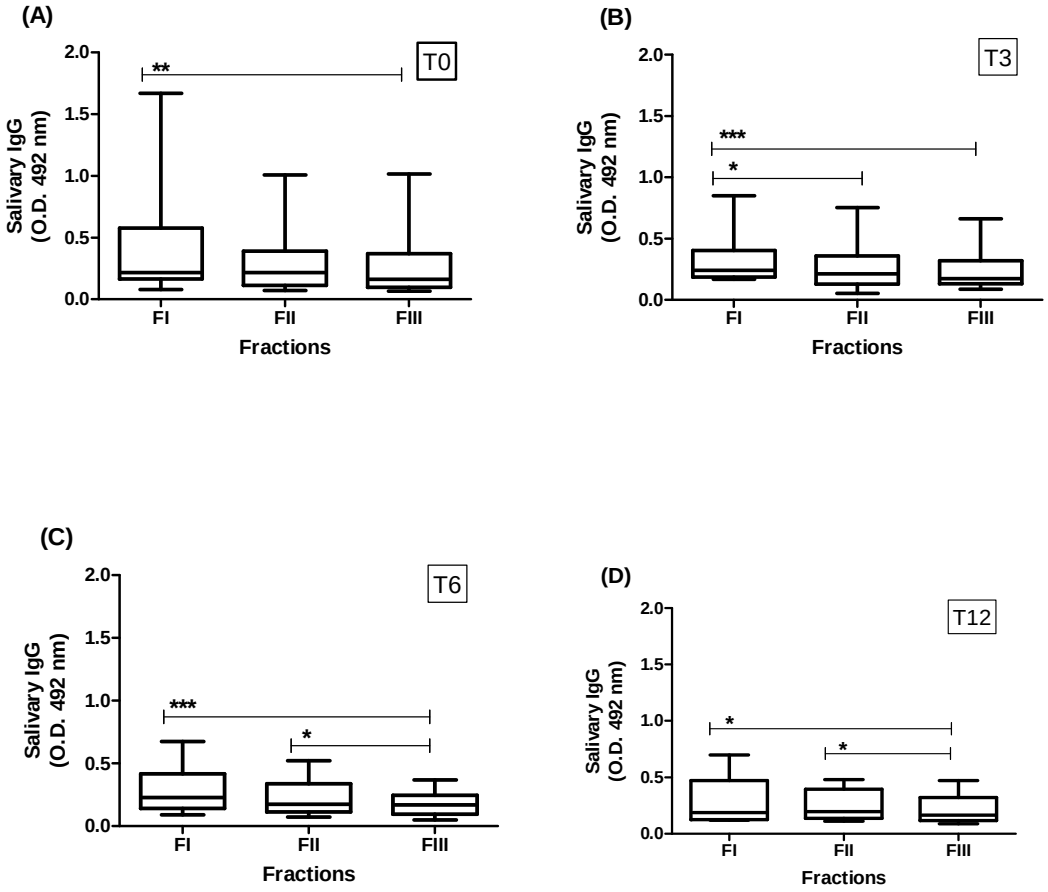


**Figure 3 Difference in salivary IgG reactivity to dentin fractions.** Salivary IgG reactivity to FI, FII and FIII dentin fractions. A- Before of the treatment (T0). B- 3 (T3). C- 6 (T6) and D- 12 months of orthodontic treatment (T12), (n=10). ). Differences between IgG levels were tested by Friedman test, * $P < 0.05$ ** $P < 0.001$ *** $P < 0.0001$. The box represents 25-75 per cent of the values of optical densities (OD) at 492 nm and horizontal bar represents the median.

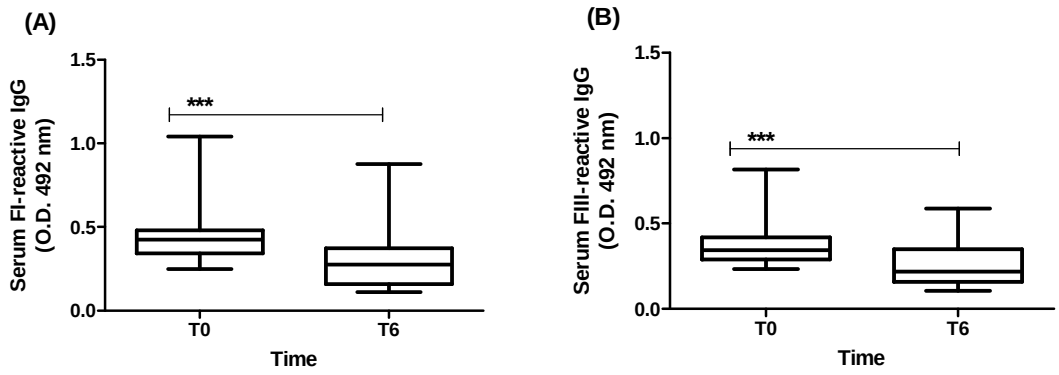


**Figure 4 Serum dentin fractions-reactive IgG levels**. (A) FI dentin fraction. (B) FII dentin fraction, n=30. IgG levels difference was tested by Wilcoxon test,*** *P* < 0.0001. The box represents 25-75 per cent of the values of optical densities (OD) at 492 nm and horizontal bar represents the median.

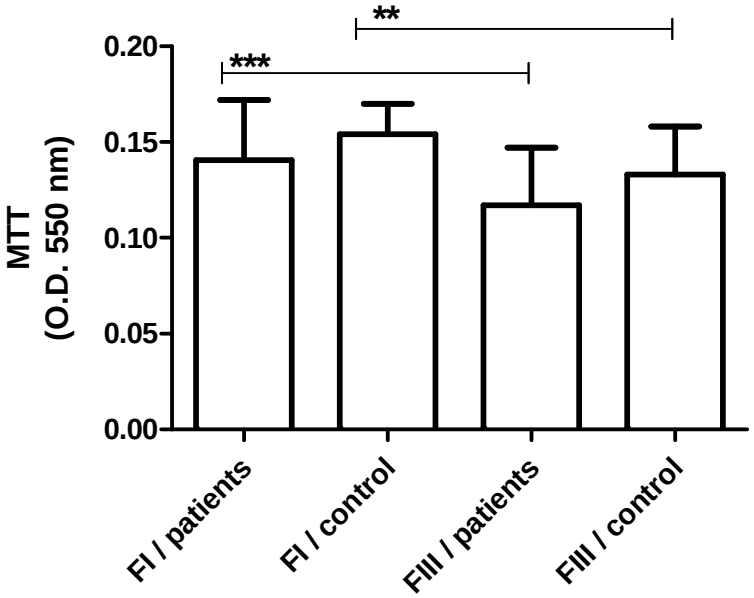


**Figure 5 Lymphoproliferative response analysis.** Absorbance difference (MTT) between lymphocytes stimulated with FI or FIII from orthodontic patients or control without orthodontic treatment. Limphoproliferation was tested by ANOVA/Tukey test, ** $p < 0.001$ *** $p < 0.0001$.

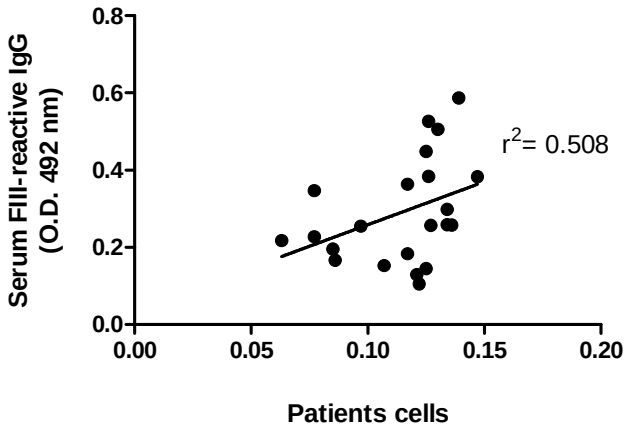


**Figure 6 Linear correlation.** Correlation between lymphoproliferative response and serum IgG levels to FIII fraction of human dentin extract in patients with 6 months of orthodontic treatment, n=30. Correlation was tested by Spearman test.

## CONCLUSÃO GERAL

Através desse estudo propomos duas novas opções de marcadores biológicos para a reabsorção radicular como consequência do tratamento ortodôntico: níveis de imunocomplexos em soro ou saliva para indicar a fase de reabsorção radicular ativa e IgG salivar reativa à FII de extrato dentinário humano para monitoramento e prevenção da reabsorção radicular.

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