IGOR VIEIRA BRACKS

PROCEDIMENTOS DE REGENERAÇÃO PULPAR:
AVALIAÇÃO LONGITUDINAL IMUNE

Faculdade de Odontologia

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AVALIAÇÃO LONGITUDINAL IMUNE

Tese apresentada ao Colegiado de Pós-Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do grau de Doutor em Odontologia – área de concentração em Endodontia

Orientador: Prof. Dr. Antôniio Paulino Ribeiro Sobrinho

Coorientador: Profa. Dra. Leda Quercia Vieira

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"Procedimentos de Regeneração Pulpar: Avaliação imune"

IGOR VIEIRA BRACKS

Tese submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em Odontologia, como requisito para obtenção do grau de Doutor, área de concentração Endodontia.

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Prof(a). Antonio Paulino Ribeiro Sobrinho - Orientador
FO-UFMG

Prof(a). Ana Cecilia Diniz Viana
FO- UFMG

Prof(a). Adriana Maria Vieira Silveira
Centro Universitário Newton Paiva

Prof(a). Érika Sales Joviano Pereira
UFBA

Prof(a). Fabiana Vargas Ferreira
FO-UFMG

Belo Horizonte, 31 de julho de 2018.

Esse trabalho é dedicado às minhas pequenas: Helena, Olívia e Cecília. Foi tudo por vocês. Filhas, o meu maior desafio é deixar para vocês o bem maior: o exemplo. Vocês deram outro significado à palavra "vida".

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"Que as coisas são inatingíveis?

Ora! Isso não é motivo para não querê-las.

Que tristes seriam os caminhos

Sem a presença distante das estrelas"

Mario Quintana

"Um passo a frente e você não está mais no mesmo lugar".

Chico Science

RESUMO

O tratamento endodôntico em dentes com desenvolvimento radicular incompleto está relacionado a algumas dificuldades e limitações. Entretanto, apesar das dificuldades técnicas, a alta susceptibilidade à fratura de dentes com rizogênese incompleta representa o fator chave para a busca de novas modalidades terapêuticas. Adicionalmente, as deficiências estéticas e funcionais apresentadas pelas terapias reabilitadoras após a perda de um elemento dentário permanente em um paciente jovem, também são fatores importantes e estimuladores. Assim, fica evidente a necessidade de pesquisas que disponibilizem novas opções terapêuticas conservadoras, com resultados previsíveis. O estudo objetivou investigar a resposta imunoinflamatória de dentes submetidos a diferentes protocolos descritos na literatura para se executar a terapia endodôntica regeneradora. Para isso, a expressão de moléculas inflamatórias e fatores de crescimento/diferenciação celular expressos nos tecidos pulpares foram analisados em diferentes intervalos de tempo, utilizando-se um modelo murino desenvolvido para a presente pesquisa. 54 Camundongos Balb/C tiveram as câmaras pulpares de seus molares superiores abertas e, subsequentemente submetidas à pulpectomia. Os animais foram então divididos em 3 grupos: grupo Sangramento (Blood) – preenchimento do espaço pulpar com coágulo sanguíneo; grupo EDTA + Sangramento (EDTA + Blood) – irrigação dos canais com solução de EDTA a 17% por 1 min, seguido do preenchimento do espaço pulpar com coágulo sanguíneo; grupo Vazio (Empty) – espaço pulpar deixado vazio. Cada grupo foi composto por 18 animais. De cada grupo, 6 animais foram sacrificados nos intervalos de 7, 14 e 21 dias após os experimentos. Utilizando-se a análise da reação em cadeia da polimerase em tempo real (Real Time PCR) avaliou-se a expressão gênica das citocinas IL-1, TNF-β, IL-10 e dos fatores de crescimento/diferenciação NGF, IGF e VEGF, comparando-se tais achados inter e extra-grupos, nos diferentes períodos de avaliação. Os resultados demonstraram as maiores expressões dos mediadores pró-inflamatórios no grupo Empty, assim como uma maior expressão de mediadores anti-inflamatórios no grupo experimental preenchido com o coágulo sanguíneo. O grupo EDTA + Blood evidenciou a maior expressão gênica de fatores de crescimento/diferenciação, em todos os períodos analisados, quando comparado aos demais grupos. Pode-se concluir que a irrigação com solução de EDTA a 17%, previamente ao preenchimento dos sistemas de canais radiculares (SCR) com o *scaffold* (coágulo sanguíneo), estimulou a expressão aumentada de mediadores relacionados ao sucesso da terapia endodôntica regenerativa. Adicionalmente, o modelo animal desenvolvido para a pesquisa mostrou-se eficaz para se analisar longitudinalmente a modulação imune que se processa nos tecidos pulpo-perirradiculares após a instituição da terapia.

Palavras-chave: Regeneração tecidual guiada. Dente não vital. Reação em cadeia da polimerase em tempo real. Vasos sanguíneos. Ácido etilenodiaminotetracético.

ABSTRACT

Pulpar regeneration procedures: Longitudinal imune assessment

Endodontic treatment in teeth with incomplete root development is related to some difficulties and limitations. However, despite the technical difficulties, the high susceptibility to fracture of teeth with incomplete rhizogenesis represents the key factor for the search for new therapeutic modalities. Additionally, the aesthetic and functional deficits presented by rehabilitation therapies after the loss of a permanent dental element in a young patient are also important and stimulating factors. Thus, it is evident the need for research that offers new conservative therapeutic options, with predictable results. Aimed to investigate the immunoinflammatory response of teeth submitted to different protocols described in the literature to perform the regenerative endodontic therapy. For this, the expression of inflammatory molecules and cell growth/differentiation factors expressed in pulpal tissues were analyzed at different time intervals using a murine model developed for the present study. 54 Balb/C mice had the pulp chambers of their upper molars opened and subsequently submitted to pulpectomy (one tooth per animal). The animals were then divided into 3 groups: Bleeding group - filling of the pulp space with blood clot; EDTA + Bleeding group (EDTA + Blood) - irrigation of the channels with 17% EDTA solution for 1 min, followed by filling of the pulp space with blood clot; Empty - pulp space left empty (negative control). Each group consisted of 18 animals. From each group, 6 animals were sacrificed at 7, 14 and 21 day intervals after the experiments. Using real-time polymerase chain reaction (RT-PCR), the cytokines IL-1, TNF-β, IL-10 and the growth/differentiation factors NGF, IGF and VEGF, comparing such inter and extra group findings in the different evaluation periods. The results showed the highest expressions of the pro-inflammatory mediators in the Empty group, as well as a greater expression of anti-inflammatory mediators in the Experimental group filled with the blood clot. The EDTA + Blood group evidenced the greater gene expression of growth / differentiation factors, in all periods analyzed, when compared to the other groups. It can be concluded that irrigation with 17% EDTA solution, prior to filling the root canals system with the scaffold (blood clot), stimulated the increased expression of detrimental mediators for the success of regenerative endodontic therapy. Additionally, the animal model developed for the research proved to be effective in longitudinally analyzing the immune modulation that occurs in octopus-periradicular tissues after the institution of therapy.

Keywords: Guided Tissue Regeneration. Tooth, Nonvital. Real-Time Polymerase Chain Reaction. Blood Vessels. Ethylenediaminetetraacetic Acid.

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

µL	microlitro
BMP	Proteína óssea morfogenética
BMSC	Célula-tronco da medula óssea
Ca (OH)$_2$	Hidróxido de Cálcio
cDNA	DNA complementar
CEBIO	Centro de Bioterismo da UFMG
CEUA	Comitê de ética no uso animal
DEPC	Dietil pirocarbonato
DPSC	Célula-tronco da polpa dental
EDTA	Ácido etilenodiamino tetra-acético
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
g	grama
IGF	Insulin-like growth factor
IL-	Interleukin
kg	quilograma
mg	miligrama
ml	mililitro
mRNA	RNA mensageiro

MSC	Célula-tronco mesenquimal
MTA	Mineral Trióxido Agregado
NaOCl	Hipoclorito de sódio
ng	nanograma
NGF	Nerve growth factor
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PDLSC	Célula-tronco do ligamento periodontal
PER	Procedimento Endodôntico Regenerador
RNA	Ácido ribonucleico
RT-PCR	PCR quantitativo em tempo real
SCAP	Célula-tronco da papila apical
SCR	Sistema de canais radiculares
SHED	Célula-tronco de dentes decíduos esfoliados
TGF-	Transforming growth factor
UFMG	Universidade Federal de Minas Gerais
VEGF	Fator de crescimento vascular endotelial

LISTA DE SÍMBOLOS

%	Porcentagem
≥	Maior ou igual
>	Maior
<	Menor
β	Beta
α	Alfa
®	Marca registrada
°C	Graus Celsius
#	Número

SUMÁRIO

1 INTRODUÇÃO

Os Procedimentos Endodônticos Regeneradores (PER) podem ser definidos como sendo procedimentos biológicos designados a repor as estruturas danificadas, incluindo dentina, estruturas radiculares, assim como as células do complexo dentina-polpa (MURRAY *et al.,* 2007). Relatos de casos clínicos têm evidenciado o sucesso desses procedimentos realizados *in vivo* (SHAH *et al.,* 2008; TORABINEJAD & FARAS, 2012). Entretanto ainda não há informação suficiente para se definir um protocolo padronizado para os REPs (AKSEL & SERPER, 2013) e, apesar das evidências publicadas, ainda se questiona a previsibilidade dos procedimentos instituídos (GEISLER, 2012).

O principal objetivo do PER é regenerar completamente, através da neoformação tecidual, a função do complexo dentina-polpa de forma que, nos dentes imaturos, esses tecidos neoformados possam dar continuidade ao desenvolvimento radicular, assim como prevenir ou reparar lesões periapicais associadas aos dentes necrosados (HARGREAVES *et al.,* 2008). Seu sucesso pode ser mensurado através da conquista de objetivos primários - eliminação dos sintomas clínicos e evidenciação de reparo de lesões ósseas; secundários - aumento da espessura das paredes radiculares e/ou aumento do comprimento radicular; e terciários - resposta positiva aos testes de vitalidade pulpar. Uma camada odontoblástica intacta, assim como a restauração das funções pulpares é o máximo que se pode desejar alcançar através do PER (HUANG, 2011).

Para que haja a regeneração dos tecidos naturais, propósito da engenharia tecidual, é necessária a presença de 3 componentes essenciais: - células indiferenciadas com capacidade de se diferenciar e formar os tecidos lesados; - sinais morfogenéticos (fatores de crescimento e/ou de diferenciação que vão modular o crescimento e diferenciação celular em linhagens específicas); e um arcabouço tridimensional (*scaffold*) capaz de atrair as células tronco e sustentar e/ou promover o

crescimento/diferenciação dessas células (NAKASHIMA & AKAMINE, 2005; ZHANG *et al.*, 2014).

O *scaffold* ideal deve ser biocompatível, biodegradável, de fácil manuseio, conter fatores de crescimento/diferenciação celular e ser capaz de sustentar o crescimento dos novos tecidos (GANESH *et al.*, 2017). Abarcando grande parte desses requisitos, o protocolo mais comumente realizado no PER utiliza-se do coágulo sanguíneo como *scaffold* (LOVELANCE *et al.*, 2011; NOSRAT *et al.*, 2011; RAJU *et al.*, 2014).

Também é descrito na literatura que há fatores de crescimento/diferenciação impregnados na matriz dentinária e que os mesmos podem ser utilizados como forma de potencializar a diferenciação das células tronco, especificamente em odontoblastos (HOWARD *et al.,* 2010). Assim, a irrigação intracanal com uma solução quelante liberaria esses fatores da matriz dentinária, aumentando a probabilidade de se conseguir a regeneração tecidual (GALLER *et al.*, 2011).

A falta de um protocolo clínico pode ser parcialmente responsável pela falta da efetiva regeneração pulpar. Os diversos materiais sugeridos para utilização, sua citotoxicidade, assim como a falta da adoção de determinados passos clínicos (como a irrigação com o EDTA) nos diversos protocolos descritos podem, eventualmente, ser uma das causas da ausência de formação dos tecidos pulpares e dentinários (BUCCHI *et al.*, 2017). Bucchi *et al* (2017), por exemplo, em revisão sistemática da literatura, encontraram que, num total de 984 artigos, apenas 6 descreveram a utilização do EDTA clinicamente.

Determinar a superioridade de um método de tratamento sobre os demais é de grande relevância clínica, já que a adoção de procedimentos mais eficazes resulta em um tratamento com maior previsibilidade de sucesso. O desenvolvimento de um modelo que permitisse a análise temporal dos mecanismos regenerativos que ocorrem após a instituição de diferentes protocolos de tratamento endodôntico regenerador foi o objetivo do presente estudo.

2 TRATAMENTO ENDODÔNTICO EM DENTES INCOMPLETAMENTE FORMADOS

O tratamento de dentes imaturos necrosados representa uma situação desafiadora na Endodontia. O amplo forame dificulta o vedamento apical pelos materiais obturadores e a presença de paredes radiculares finas limita a realização da instrumentação endodôntica. Além disso, a necrose tecidual implica no interrompimento do desenvolvimento radicular, o que faz com que estes dentes apresentem maior susceptibilidade à fratura (AKSEL & SERPER, 2014).

Historicamente, os casos de necrose pulpar em dentes incompletamente formados têm sido tratados pela técnica da Apexificação, mormente com a utilização de aplicações sucessivas de medicação intracanal à base de hidróxido de cálcio [Ca (OH)$_2$] (HARGREAVES *et al.*, 2008). Entretanto, embora produza resultados endodônticos aceitáveis, essa técnica possui importantes limitações: a formação da barreira mineralizada apical se dá de forma muito lenta, requerendo, às vezes, vários meses de uso da medicação intracanal; não há previsibilidade quanto à homogeneidade da formação da barreira mineralizada, podendo esta apresentar espessuras variadas e até mesmo fechamento incompleto; não há a continuação do desenvolvimento do comprimento radicular, assim como não há aumento na espessura das paredes radiculares, mantendo estes dentes em condição de grande susceptibilidade à fratura [aproximadamente 30% dos dentes imaturos sofrem fratura durante ou logo após o tratamento (KEREKES *et al.*, 1980)]; possiblidade de contaminação dos canais devido ao longo período de tratamento; além de representar uma grande demanda para os pacientes, tanto pelo tempo requerido para o tratamento, quanto no quesito financeiro (SIMON *et al.,* 2007; HUANG, 2009). Além disso, o uso prolongado de Ca (OH)$_2$ está associado a um aumento na fragilidade radicular (ANDREASEN *et al.*, 2006) [a resistência mecânica da dentina é determinada pela ligação entre a hidróxido apatita e as fibras colágenas e, devido a sua alta alcalinidade, o Ca (OH)$_2$ pode desnaturar os grupos carboxilatos e fosfatos, levando ao colapso da estrutura dentinária (ROSEMBERG *et al.*, 2007)].

Uma outra técnica utilizada para a Apexificação é a que se utiliza da confecção de um *plug* apical a base de Mineral Trióxido Agregado (MTA) (TORABINEJAD & CHIVAN, 1999). Com isso é possível executar o tratamento endodôntico de forma imediata, o que representa uma enorme vantagem em relação ao uso do Ca (OH)$_2$ (STEINIG *et al.*, 2003). Além disso, seu uso está associado a uma maior previsibilidade na formação de uma barreira mineralizada apicalmente (SHABAHANG *et al.*, 1999). Entretanto, como na técnica clássica, esta não é capaz de estimular o desenvolvimento radicular, tampouco o aumento na espessura de suas paredes, o que mantém o elevado risco à fratura (CVEK, 1992).

Essas limitações técnicas estimularam a busca por procedimentos que promovessem a regeneração dos tecidos pulpares, a formação continuada da dentina e a continuação do desenvolvimento radicular (WIGLER *et al.*, 2013).

A revascularização pulpar representa uma alternativa baseada em preceitos biológicos para o tratamento de dentes imaturos necrosados. Ao contrário da Apexificação e do uso de *plugs* artificiais, ela permite que haja a continuação do desenvolvimento radicular (NOSRAT *et al.*, 2011).

3 PROCEDIMENTOS ENDODÔNTICOS REGENERADORES

Os tecidos perirradiculares dos dentes imaturos são ricos em suprimento vascular e contém um *pool* de células indiferenciadas que representam um dos pré-requisitos para se instituir o procedimento regenerador. Levando em conta a presença dessas células, foi concebido o conceito da regeneração tecidual em dentes imaturos necrosados (NAGY *et al.,* 2014).

Östby, o pioneiro nos procedimentos endodônticos regeneradores, mostrou que nova vascularização tecidual poderia ser induzida no terço apical de dentes

portadores de necrose pulpar e lesão periapical (ÖSTBY, 1961). Essa neovascularização era conseguida através da formação de um coágulo sanguíneo no terço apical após os procedimentos de limpeza e descontaminação radicular. Esse, por sua vez, era produzido via introdução de uma lima endodôntica além do forame apical, até que o sangramento preenchesse todo o canal radicular. O autor propôs que, pela formação do coágulo sanguíneo (*scaffold*), a neovascularização poderia se estabelecer e suportar o crescimento de novos tecidos às partes não obturadas do canal. Além disso, o mesmo produziu evidências histológicas que suportaram seu conceito.

A revascularização com a subsequente continuação do desenvolvimento radicular e continuada deposição de tecidos mineralizados na porção interior dos canais radiculares também pode ser observada em dentes imaturos reimplantados após avulsão (WIGLER *et al.*, 2013). Isso sugere que os tecidos pulpares desvitalizados atuam com uma matriz na qual novos vasos sanguíneos e tecidos podem crescer (SKOGLUND & TRONSTAD, 1981).

Iwaya *et al.*, (2001) descreveram um procedimento (que chamaram de Revascularização) realizado em um segundo pré-molar inferior imaturo com necrose pulpar e abscesso apical crônico. 30 meses após a terapia os autores puderam observar, radiograficamente, aumento na espessura das paredes radiculares pela deposição de material mineralizado e também a continuação do desenvolvimento radicular.

Banchs & Trope (2004) publicaram relato de caso descrevendo a instituição de procedimentos de revascularização em dentes necrosados com ápice aberto e lesão periapical. Através do acompanhamento radiográfico puderam observar que o protocolo realizado pôde estimular o desenvolvimento radicular, de forma semelhante ao dente contralateral, provando que a regeneração é possível tanto em dentes avulsionados quanto em dentes infectados, desde que um ambiente favorável seja estabelecido.

Raju *et al.*, (2014) também instituíram o PER como tratamento em dentes imaturos necrosados e portadores de lesão apical. Após os procedimentos de descontaminação, procederam à criação do coágulo no interior dos canais e, em

acompanhamento de 1 ano, relataram que o paciente se encontrava assintomático, sem sinal de fístula, com imagem apical radiopaca e evidência tanto do aumento do comprimento radicular quanto do aumento da espessura das paredes dentinárias.

Murray *et al.* (2007) indicam o PER para o tratamento de dentes imaturos necrosados, reforçando que se trata de técnica relativamente simples, de baixo custo e que se utiliza de instrumentos de uso rotineiro. Peters (2014) indica como público alvo dos REPs, adolescentes que apresentam necrose pulpar e dentes com ampla abertura foraminal (≥ 0,6 mm). Mais especificamente, dentes unirradiculares com história de trauma.

A revisão sistemática da literatura (BUCCHI *et al.*, 2017) permite considerar o PER como sendo procedimento clinicamente bem-sucedido já que 84,14% dos dentes tratados (ou canais) apresentam algum grau de desenvolvimento radicular e 79,8% dos dentes evidenciam a cura de lesões periapicais associadas.

Uma explicação para a eficácia do PER foi atribuída à presença de células-tronco na papila apical e no osso medular. Provocado pela sobreinstrumentação, o sangramento que preenche o espaço radicular traz consigo células indiferenciadas capazes de se diferenciar em novos odontoblastos. Além disso, o coágulo formado serviria como um *scaffold* por onde as células podem migrar, além de conter fatores de crescimento/diferenciação que agiriam sobre essas células (ALRAHABI & ALI, 2014).

3.1 Células-tronco

Em geral, células-tronco são definidas por terem duas propriedades principais: serem capazes de se auto renovarem e, quando se dividem, algumas células filhas podem, eventualmente, ser de linhagens diferentes. Dependendo do tipo de célula-tronco e de sua habilidade e capacidade em se tornar tecidos diferentes (característica descrita como plasticidade), três categorias são citadas: células-tronco totipotentes - com capacidade de originar tanto um organismo totalmente funcional, como qualquer

tipo celular do corpo, inclusive todo o sistema nervoso central e periférico. Correspondem às células do embrião recém-formado e têm potencial para originar até mesmo as células do folheto extra-embrionário que formarão a placenta. Entretanto, estas células são efêmeras e desaparecem poucos dias após a fertilização (GAGE, 2000); pluripotentes – capazes de originar qualquer tipo de tecido sem, no entanto, originar um organismo completo, visto que não podem gerar a placenta e outros tecidos de apoio ao feto. Apesar de existirem em menor número, as células-tronco pluripotentes estão presentes, também, em indivíduos adultos (SOUZA *et al*., 2003); e multipotentes – são um pouco mais diferenciadas, presentes no infivíduo adulto, com capacidade de originar apenas um limitado número de tipos teciduais. Estas células são designadas de acordo com o órgão de que derivam e podem originar apenas células daquele órgão, possibilitando a regeneeração tecidual (GAGE, 2000). Células-tronco dentárias pertencem à terceira categoria (HUANG, 2008).

Quatro tipos de células-tronco de origem dental têm sido isolados e caracterizados: células-tronco pulpares (DPSCs); células-tronco de dentes decíduos esfoliados (SHEDs); células-tronco da papila apical (SCAPs) e células-tronco do ligamento periodontal (PDLSCs). Essas células-tronco são consideradas como células-tronco mesenquimais (MSCs) e possuem diferentes níveis de capacidade em se transformar em células formadoras de tecidos específicos (SHARMA *et al*., 2010). As SCAPs são capazes de se diferenciar em células tipo odontoblastos e produzir tecido semelhante à dentina, tanto *in vitro* quanto *in vivo* e, juntamente com as PDLSCs são capazes de formar estruturas radiculares (HUANG, 2008).

É provável que as SCAPs, residindo na papila apical, sobrevivam à infecção por sua proximidade com os tecidos periapicais. Esses tecidos são beneficiados por uma circulação colateral que os permitem sobreviver ao processo de necrose pulpar e, talvez, após a desinfecção endodôntica, essas células possam dar origem a odontoblastos que completarão a formação radicular (ESTRELA *et al.,* 2011). Em condições favoráveis (*i.e.*, eliminação da contaminação intrarradicular, dos tecidos necrosados e na presença de um *scaffold* apropriado) essas células tronco podem repovoar os espaços radiculares em dentes imaturos necrosados (HARGREAVES *et al.*, 2008).

As SCAPs são células precursoras dos tecidos pulpares, enquanto as SHEDs são células pulpares. Seguindo sua expansão, essas células podem se diferenciar em células semelhantes a odontoblastos e produzir tecido semelhante à dentina, tanto *in vitro* quanto *in vivo* (SONOYAMA *et al.*, 2008), enquanto aquelas possuem a capacidade de se diferenciar em células neurais e adiposas (YU *et al.*, 2006).

As PDLSCs são células-tronco multipotentes capazes de se diferenciar em cementoblatos e células formadoras de colágeno, adipócitos e tecidos semelhantes ao ligamento periodontal (SEO *et al.*, 2004).

Os resultados de Lovelance *et al.*, (2011) confirmaram que a manipulação dos tecidos periapicais (formação do *scaffold* de coágulo) transporta células-tronco para os espaços pulpares. Seus resultados demostraram não apenas a marcação de células-tronco no interior dos canais (concentração centenas de vezes maior que na circulação sistêmica), mas também que as mesmas são provenientes dos tecidos periapicais (os autores sugerem que sejam, muito provavelmente, SCPAs e BMSCs – células-tronco da medula óssea), e não da circulação sistêmica. Além disso, durante o período avaliado, os autores observaram que tais células indiferenciadas não se diferenciaram em linhagens osteoblástica ou odontoblástica.

Entretanto, apesar de haver uma vasta literatura corroborando a eficácia e previsibilidade do PER na continuação do desenvolvimento radicular quando do uso do coágulo sanguíneo como *scaffold* (THIBODEAU & TROPE, 2007; COTTI *et al.*, 2008; JUNG *et al.*, 2008), não há a certeza da natureza dos tecidos neoformados no interior dos canais, já que as células-tronco multipotentes podem se diferenciar em diferentes linhagens (LOVELANCE *et al.*, 2011).

3.2 Histologia dos tecidos neoformados

A completa restauração de um complexo dentina-polpa funcional não pode ser obtida pelo tratamento endodôntico convencional (NAKASHIMA & AKAMINE, 2005). Entretanto, com o advento da medicina regeneradora, tornou-se possível regenerar

os tecidos pulpares através de procedimentos endodônticos alternativos (HARGREAVES *et al.,* 2008). Esses procedimentos são baseados nos princípios da engenharia tecidual, que têm como objetivo induzir a neoformação tecidual para repor os tecidos perdidos ou comprometidos, regenerando completamente o complexo dentina-polpa (SMITH *et al.,* 2016). Para tanto, são necessárias uma fonte de células indiferenciadas, um *scaffold* tridimensional para que haja o crescimento dessas células e sinais morfogenéticos (HARGREAVES *et al.,* 2008). A técnica mais utilizada nos REPs é a que se utiliza do coágulo como *scaffold*, associado à utilização de células-tronco derivadas dos tecidos perirradiculares (WANG *et al.,* 2010).

Apesar de o PER ter eficácia clínica comprovada (MURRAY *et al.,* 2007; DING *et al.,* 2009; RAJU *et al.,* 2014) quando se avalia os objetivos primários e secundários da técnica (*i.e.*, aumento do comprimento/espessura das paredes radiculares e fechamento apical), o objetivo máximo (neoformação do complexo dentina/polpa) não pode ser alcançado de forma previsível quando do uso do coágulo como *scaffold* (GOMES-FILHO *et al.,* 2013). Resultados histológicos de dentes, tanto humanos quanto de animais falharam em mostrar deposição de novo tecido dentinário após procedimentos regeneradores (PETERS, 2014).

A natureza dos tecidos neoformados após a instituição do PER tem sido investigada em estudos tanto em animais quanto em humanos (WANG *et al.*, 2010; YAMAUCHI *et al.,* 2011; MARTIN *et al.*, 2013). Embora esses autores tenham afirmado que os novos tecidos depositados internamente nas paredes dentinárias contivessem características de cemento, osso ou ligamento periodontal, não foram observados novos tecidos semelhante à polpa dentária. Estes autores também sugerem que a invasão dos espaços pulpares pelas células-tronco se dá em quantidade insuficiente para o desenvolvimento do complexo dentina-polpa.

Mormente, apesar de promover a continuação do desenvolvimento radicular, a utilização do coágulo como *scaffold* induz a formação de novos tecidos com características histológicas de tecido conjuntivo, tecido cementário e/ou ósseo, mas não com as de tecido pulpar (GOMES-FILHO *et al.,* 2013).

Zhang *et al.,* (2014) avaliaram os tecidos neoformados em dentes de cães previamente necrosados quando da utilização de dois diferentes *scaffolds*: coágulo

sanguíneo e Plasma Rico em Plaquetas (PRP). Os autores puderam observar tanto o aumento na espessura das paredes radiculares quanto o fechamento apical em ambos os grupos. Quando avaliada as características histológicas dos tecidos neoformados pôde-se observar a presença de grande número de células inflamatórias e que os novos tecidos se assemelhavam a tecido cementário e não a tecido pulpar. Os resultados mostraram não haver diferença estatisticamente significativa entre os grupos quanto a todos os critérios analisados (*i.e.*, aumento da espessura radicular, fechamento apical, aumento no comprimento radicular, característica do tecido mineralizado depositado).

O desenvolvimento radicular se dá mais comumente através da deposição de tecido mineralizado semelhante ao cemento e tecido ósseo ao invés de dentina, e o tecido intracanal consiste em tecido conjuntivo sem a camada odontoblástica, como a do tecido pulpar original (BUCCHI *et al.,* 2017).

A revisão sistemática da literatura (ALTAII *et al.,* 2017) mostrou que, dos 110 dentes onde se utilizou o coágulo sanguíneo como *scaffold*, apenas 4% dos canais apresentaram deposição de tecido mineralizado semelhante à dentina nas paredes dentinárias, 64% continham tecido semelhante ao cemento e 10% tecido semelhante a osso. No mesmo trabalho, foi possível observar que a adoção de *scaffolds* diferentes do coágulo não trouxe benefício adicional na formação de tecido semelhante à dentina.

A diversidade na resposta celular não é surpreendente, já que as células-tronco dos tecidos dentais, classificadas como células tronco pós-natais multipotentes, retêm a capacidade de adquirir fenótipo condrogênico, odontogênico/osteogênico, neurogênico ou adipogênico, em função de sua exposição a diferentes coquetéis de fatores de crescimento e outras proteínas (Hargreaves *et al.*, 2008).

3.3 *Scaffold*

Fator integrante da engenharia tecidual, o esqueleto físico (*scaffold*) é necessário para a promoção do crescimento e diferenciação celular (SONOYAMA *et al.*, 2008). O *scaffold* provê um microambiente físico/químico/biológico tridimensional para o crescimento e diferenciação celular, promovendo a adesão celular e sua migração. Serve ainda como um carreador de fatores de diferenciação celular e de células. Deve possuir eficiência no transporte de nutrientes e de oxigênio e ser degradado gradualmente enquanto é substituído pelos tecidos regenerados, mantendo suas características até o final da estruturação dos novos tecidos. Deve ainda ser biocompatível, não tóxico e possuir apropriadas características físicas e mecânicas (NAKASHIMA *et al.*, 2005).

Para os procedimentos endodônticos regeneradores existe a possibilidade de se utilizar um dos três tipos de *scaffold* autólogo, todos eles derivados do sangue. São eles: coágulo sanguíneo, PRP e Plasma Rico em Fibrina (PRF) (HARGREAVES *et al.,* 2008; BEZGIN *et al.,* 2015; JADHAV *et al.,* 2012; KESWANI & PANDEY, 2013).

Trabalhos como os de El-Sharkawy *et al.,* (2007), Keswani & Pandey (2013), Del Fabbro *et al.,* (2016) ilustram a heterogeneidade de protocolos propostos com o mesmo intuito: promover a revascularização pulpar em dentes imaturos necrosados.

3.3.1 Coágulo sanguíneo

O *scaffold* precisa promover a adesão, proliferação e diferenciação celular, suportar nova vascularização e inervação e ser biodegradável (ALRAHABI & ALI, 2014). A formação do coágulo cria um *scaffold* tridimensional de fibrina que pode conter células indiferenciadas provenientes dos tecidos perirradiculares, assim como fatores de crescimento secretados pelas plaquetas, o que contempla a tríade exigida pela engenharia tecidual (*scaffold*, células indiferenciadas e fatores morfogenéticos) (LOVELANCE *et al.,* 2011).

A eficácia da utilização do coágulo foi comprovada por Dianat *et al.,* (2017). Os autores compararam o uso do coágulo (grupo 1) e do plasma rico em fatores de crescimento (grupo 2) como *scaffolds* em modelo animal. Seus resultados

evidenciaram 60% de reparo das áreas radiolúcidas no grupo 1 e 53,3% no grupo 2; fechamento apical foi detectado em 60% dos casos, em ambos os grupos; formação de novo tecido semelhante a cemento foi encontrada ao longo das paredes radiculares internas, assim como ilhas de tecido ostéoide junto ao tecido conjuntivo recém-formado. Os autores não encontraram diferença significativa ($p > 0,05$) entre os dois grupos em todos os quesitos avaliados, concluindo que o uso do plasma rico em fatores de crescimento não se mostra vantajoso em relação ao uso do coágulo sanguíneo.

Lovelance *et al.* (2011) demonstraram que a indução do preenchimento dos canais pelo sangramento apical resulta na entrada de células tronco nestes sítios, embora a origem de tais células não tenha sido identificada. Acreditam que o coágulo atue como *scaffold* na participação destas células nas respostas regeneradoras.

As plaquetas compõem grande parte do coágulo. Elas contêm e secretam fatores de crescimento ativos e também um grande número de proteínas séricas, tais como fibrina, fibronectina e vitronectina, que atuam como moléculas de adesão celular durante a osteocondução. Também atuam como matriz para a migração de células ósseas, conjuntivas e epiteliais durante os processos de reparo tecidual (MARX, 2004). Assim, o coágulo pode atuar como *scaffold* para que as células apicais, incluindo as células-tronco, migrem para dentro dos espaços pulpares e induzam a formação de novos tecidos (YAMAUCHI *et al.*, 2011).

Apesar de haver uma vasta literatura corroborando o uso clínico do coágulo sanguíneo como *scaffold* (JUNG *et al.*, 2008; DING *et al.,* 2009; RAJU *et al.*, 2014), existem algumas limitações inerentes ao seu uso: em alguns casos o preenchimento dos canais pode não ser conseguido via indução do sangramento periapical; além disso, a inserção do tampão de MTA sobre o coágulo pode ser uma tarefa de difícil execução (DING *et al.*, 2009).

3.4 Fatores de crescimento

Fatores de crescimento são proteínas que se ligam a receptores específicos na membrana celular e induzem a geração de novos tecidos. Eles atuam como sinais moleculares que modulam o comportamento celular. Iniciando a cascata de sinais intracelulares, atuam de maneira autócrina ou parácrina, agindo localmente sobre as células alvo (KIM *et al*., 2012).

Vários fatores de crescimento têm sido avaliados em sua capacidade de induzir e mediar a diferenciação das células-tronco em células pré-odontoblastos, em particular o TGF-β, as BMPs (proteínas ósseas morfogenéticas) e os fatores da família do FGF (GANESH *et al*., 2017). O TGF-β possui ação quimiotática e guia a diferenciação celular e a síntese da matriz extracelular, enquanto o FGF-2 aumenta a proliferação celular e exerce efeito angiogênico (embora de forma menos potente que o VEGF) (GALLER *et al*., 2015).

Nos procedimentos endodônticos regeneradores, após a entrada das células-tronco nos espaços pulpares, é imperativo que haja sua migração e diferenciação para a formação do novo complexo dentina-polpa. Howard *et al.* (2010) afirmam que 10 ng/mL de TGF-β e FGF são efetivos na indução da migração das DPSCs. Os resultados de Zeng *et al.* (2016) evidenciam que são liberadas concentrações de 2-90 ng/mL de TGF-β das paredes dentinárias pós condicionamento com EDTA, sugerindo ser essa quantidade suficiente para a indução da migração celular. Seus resultados mostram ainda que a indução da migração das DPSC pelo TGF-β é dose-dependente.

Os efeitos do TGF-β são bastante variados e dependem do tipo celular e tecidual sobre o qual está agindo. Foi demonstrada sua ação no incremento da proliferação celular e na produção da matriz extracelular em cultura de células pulpares, assim como sua indução na diferenciação destas células em odontoblastos; sua ação pode ser potencializada na presença do FGF (KIM *et al*., 2012). Além disso, em altas concentração, é capaz de induzir a apoptose (ZHANG *et al.,* 2011).

O fator de crescimento semelhante à insulina (IGF) contribui para a odontogênese e para o reparo dos tecidos dentais através do estímulo à proliferação e diferenciação celular (CAVIEDES *et al*., 2009). O estudo de seu efeito sobre as células pulpares evidenciou sua capacidade em induzir essas células em células

semelhantes a odontoblastos, assim como sugeriram seu papel na estimulação de tecidos mineralizados (KIM *et al.*, 2012).

O fator de crescimento vascular endotelial (VEGF) é um potente estimulador da angiogênese e vasculogênese, desempenhando um papel predominante no intricado processo de formação de novos vasos sanguíneos, e sua presença é necessária para iniciar e manter os sucessivos estágios da formação vascular, incluindo a remodelação da matriz extracelular, recrutamento das células endoteliais e formação do lúmen vascular (KIM *et al.*, 2012).

A dentina humana contém vários fatores de crescimento que podem promover a regeneração dos tecidos nos espaços radiculares, assim como também o coágulo sanguíneo contém fatores de crescimento derivados das plaquetas. Por isso é seguro assumir que o preenchimento destes espaços pelo coágulo cria um ambiente (*scaffold*) rico em proteínas que podem ser cruciais para o sucesso na entrada e diferenciação das células-tronco nos espaços pulpares e, em última análise, no desenvolvimento radicular (HARGREAVES *et al.*, 2011).

4 USO DO EDTA NOS PER

A dentina é tradicionalmente vista como sendo um tecido mineralizado inerte, com mínima remodelação após formada. Entretanto, o reconhecimento das propriedades bioativas da mesma já foram comprovadas (DIOGENES *et al.*, 2014). A matriz dentinária desmineralizada foi capaz de induzir o reparo pulpar e o fechamento apical em primatas, e esse efeito pró-mineralizador foi atribuído à atividade das proteínas ósseas morfogenéticas (BMP) (SMITH *et al.*, 2016). Subsequentemente, um *pool* de proteínas não-colagenosas provenientes da dentina, liberadas durante a desmineralização da mesma, foram associadas com a indução da dentinogênese (SLOAN *et al.*, 2001). Assim, a percepção da inércia da dentina reflete a imobilização

e sequestro das moléculas bioativas em sua matriz mineralizada. A dissolução da mesma é capaz de liberar esses fatores, que exercerão efeitos a nível celular (SMITH *et al.,* 2016).

O variado grupo de moléculas encontradas "fossilizadas" na matriz dentinária é composto por fatores de crescimento, quimiocinas, citocinas, moléculas da matriz extracelular e peptídeos bioativos, podendo citar o TGF-β, BMP, IGF-1, VEGF, FGF-2, PDGF e EGF (todos eles com ação necessária para a efetiva resposta dos REP), o que reflete a complexidade dos eventos celulares que podem ser induzidos (SMITH *et al.*, 2016).

Agentes quelantes, como o ácido etilenodiamino tetra-acético (EDTA), são capazes de retirar o cálcio dos cristais de fosfato de cálcio inorgânico, o que resulta na desmineralização da camada dentinária superficial. No quesito comportamento celular, essa descalcificação permite a exposição das fibrilas de colágeno da matriz orgânica, o que permite a ligação celular via receptores de integrina. Além disso, o tratamento com EDTA expõe fatores de crescimento impregnados na matriz dentinária (GALLER *et al.*, 2011). Zeng *et al.* (2016) comprovaram a liberação de significantes quantidades destes fatores das paredes dentinárias condicionadas com EDTA. Esses mediadores, por sua vez, promovem a diferenciação das células-tronco em células semelhantes a odontoblastos que, subsequentemente, depositam tecido mineralizado na face interna das paredes radiculares (PETERS, 2014).

O uso do EDTA como solução irrigadora final foi indicada por Yamauchi *et al.* (2011), que concluíram que seu uso (em modelo animal) não trouxe efeito adverso e potencializou a formação de tecidos mineralizados, o que aumentaria a resistência das paredes radiculares.

Para que o REP obtenha êxito, necessário se faz o estímulo da adesão das SCAP ou outros tipos de células-tronco à superfície radicular após o tratamento, e a presença da *smear layer* nas paredes dos canais radiculares pode inibir essa aderência, causando potenciais fracassos nos procedimentos regeneradores (RING *et al.*, 2008).

Galler *et al.* (2011), executaram estudo para verificar se o pré-tratamento da dentina poderia influenciar o comportamento celular das DPSCs na interface célula-dentina. Para isso trabalharam em cilindros de dentina irrigados com hipoclorito de sódio (NaOCl) ou condicionados com EDTA. Os autores observaram que na dentina irrigada com NaOCl, lacunas de reabsorção eram vistas, assim como células multinucleadas com atividade clástica, enquanto que na dentina condicionada com EDTA, as DPSCs estavam intimamente associadas às paredes dentinárias e se diferenciaram em células semelhantes a odontoblastos (células expressando sialoproteína da dentina e com prolongamentos celulares nos túbulos dentinários). Concluíram com isso que o condicionamento da dentina liberou os fatores de crescimento impregnados na matriz de dentina, o que influenciou favoravelmente a diferenciação celular. Além disso, Martin *et al.*, (2014) demonstraram que o uso do EDTA como solução irrigadora final é capaz de reverter os efeitos negativos do NaOCl (até a concentração de 1,5%) sobre as células-tronco, aumentando a taxa de sobrevivência das mesmas.

Almushay *et al.* (2006) demonstraram que a proteína-1 da matriz de dentina (DMP-1), uma proteína não colagenosa extraída da dentina, pode, significativamente, promover a diferenciação das DPSCs em odontoblastos e induzir a formação de dentina reparadora em tecidos pulpares expostos.

Proteínas da matriz dentinária podem induzir o recrutamento de células envolvidas na resposta inflamatória e imune. As moléculas quimiotáticas têm maior especificidade para as células-tronco. Assim, a dissolução da matriz dentinária (e liberação desses fatores) com o uso do EDTA gera um gradiente quimiotático específico para que as células-tronco migrem pelas paredes dentinárias (GALLER *et al.*, 2011; GALLER *et al.*, 2015).

Galler *et al.*, (2015) afirmam que, durante a dentinogênese, fatores de crescimento podem ficar impregnados na matriz dentinária e podem, posteriormente, ser liberados pelos processos de desmineralização. Para testar essa hipótese, os autores condicionaram discos de dentina humana com variados protocolos. Seus resultados mostraram que o condicionamento com solução de EDTA a 10% em pH 7 resultou na liberação de maior quantidade de TGF-β, e que as quantidades de FGF-2 e VEGF liberadas foram dependentes do tempo de condicionamento. Os autores

concluem que os fatores de crescimento podem ser liberados diretamente da dentina via condicionamento com EDTA.

5 HIPÓTESES

Trabalhamos com a hipótese de que, ao compararmos as expressões gênicas dos fatores de crescimento/diferenciação longitudinalmente entre os grupos, o grupo que teve seus canais irrigados com EDTA e depois preenchidos com coágulo sanguíneo evidenciariam as maiores expressões de fatores com propriedades anti-inflamatória e/ou formadora, seguido pelo grupo que teve os SCR apenas preenchidos pelo coágulo. Esperava-se que os animais que tiveram seus dentes vazios, sem o recebimento de um *scaffold*, apresentassem as maiores expressões de mediadores inflamatórios e as menores de mediadores pró-regenerativos.

6 OBJETIVOS

Levando em consideração a importância que o *scaffold* desempenha no PER, assim como o protocolo de irrigação, o objetivo deste estudo foi avaliar, por meio da resposta imuno-inflamatória estruturada nos tecidos pulpares, a eficácia dos protocolos mais comumente descritos: a) preenchimento dos espaços pulpares com

coágulo sanguíneo; ou, b) irrigação com EDTA previamente ao preenchimento com o coágulo.

6.1 Objetivos gerais

O objetivo do estudo, levando em consideração a importância atribuída aos *scaffolds*, foi avaliar o desempenho que o coágulo sanguíneo (utilizado como *scaffold)* demonstraria quanto ao seu potencial em estimular a expressão de citocinas pró e/ou anti-inflamatórias, e de fatores de crescimento/diferenciação nos tecidos pulpares. Adicionalmente, avaliar se a irrigação com EDTA exerceria algum efeito na expressão dos mediadores analisados no estudo, modulando a resposta imuno-inflamatória. O estudo visou fornecer, através da análise da resposta imunoinflamatória desencadeada nos tecidos analisados, um embasamento científico mais sólido quanto à superioridade de um método de tratamento sobre os demais, o que embasaria a indicação de seu uso clínico.

6.2 Objetivos específicos

Através do PCR em tempo real (RT-PCR), avaliar o perfil da resposta imuno-inflamatória dos tecidos pulpo-perirradiculares quando da utilização dos seguintes protocolos indicados para a terapia endodôntica regeneradora: a) preenchimento dos canais com coágulo sanguíneo; e, b) irrigação com solução de EDTA a 17% previamente à indução do preenchimento dos canais com coágulo sanguíneo. A expressão das seguintes citocinas e fatores de crescimento/diferenciação foram analisados: IL-1α, TGF-β, VEGF, IL-10, IGF e NGF.

7 METODOLOGIA EXPANDIDA

7.1 Caracterização do projeto

Este é um estudo experimental *in vivo*, em modelo animal, que avalia longitudinalmente a resposta imuno-inflamatória desencadeada em diferentes protocolos clínicos indicados e utilizados clinicamente nos procedimentos endodônticos regeneradores. Para tanto foi realizada a análise via PCR em tempo real da expressão das citocinas e dos fatores de crescimento/diferenciação celular IL-1α, IL-10, TGF-β, VEGF, NGF e IGF, que desempenham importante papel na formação de novos tecidos.

7.1.1 Plano amostral

Camundongos Balb/C foram utilizados para realizar os ensaios. Os animais tiveram seus primeiros molares superior do lado esquerdo abertos endodonticamente e os tecidos pulpares removidos. Após a pulpectomia, cada dente foi submetido a apenas um dos protocolos utilizados. Após a realização dos procedimentos, os animais foram sacrificados em intervalos de 7, 14 e 21 dias. Após o sacrifício dos animais, os dentes foram removidos com o tecido periapical e estes processados para análise via RT-PCR.

Os animais foram divididos em 3 grupos, cada um composto por 18 animais. Destes, 6 foram sacrificados em cada um dos intervalos citados.

Grupo Vazio (Empty) 7 – espaços radiculares deixados vazios após pulpectomia (controle negativo); sacrifício em 7 dias

Grupo Vazio (Empty) 14 – espaços radiculares deixados vazios após pulpectomia (controle negativo); sacrifício em 14 dias

Grupo Vazio (Emtpy) 21 – espaços radiculares deixados vazios após pulpectomia (controle negativo); sacrifício em 21 dias

Grupo Sangramento (Blood) 7 – espaços radiculares preenchidos com sangramento; sacrifício em 7 dias

Grupo Sangramento (Blood) 14 – espaços radiculares preenchidos com sangramento; sacrifício em 14 dias

Grupo Sangramento (Blood) 21 – espaços radiculares preenchidos com sangramento; sacrifício em 21 dias

Grupo EDTA (EDTA + Blood) 7 – espaços radiculares irrigados com EDTA e posteriormente preenchidos com sangramento; sacrifício em 7 dias

Grupo EDTA (EDTA + Blood) 14 – espaços radiculares irrigados com EDTA e posteriormente preenchidos com sangramento; sacrifício em 14 dias

Grupo EDTA (EDTA + Blood) 21 – espaços radiculares irrigados com EDTA e posteriormente preenchidos com sangramento; sacrifício em 21 dias

7.1.2 Animais

Camundongos com idade entre 8 e 10 semanas, de ambos os sexos, adquiridos no CEBIO (Centro de Bioterismo da UFMG, Belo Horizonte, MG, Brasil) foram utilizados. Os animais foram mantidos durante toda a fase experimental em biotério com barreiras, controle dos ciclos de luz, com comida (Nuvital, Curitiba, PR, Brasil) e água *ad libitum* e separados em gaiolas com no máximo 5 animais. Um total de 54 animais foi utilizado. O projeto de pesquisa foi aprovado pelo Comitê de Ética no Uso de Animais da UFMG (CEUA/UFMG) – protocolo CEUA n°: 255/2016.

7.1.3 Anestesia

Todos os animais utilizados receberam, antes do experimento, anestesia geral via injeção intraperitoneal de uma solução contendo 60 mg/kg Hidroclorato de Ketamina (Dopalen, Vetbrands – Divisão de Saúde Animal, Jacareí, SP, Brasil) e 20 mg/kg de Xilazina (Anasedan, Agribands do Brasil Ltda., Paulínia, SP, Brasil), diluídos em PBS. Desta solução, um volume de 0,6-0,8 mL foi administrado em cada animal.

7.1.4 Preparo dos grupos

Para a abertura coronária dos dentes, utilizou-se de brocas esféricas diamantadas ¼ (Dentsply-Maillefer, Petrópolis, RJ, Brasil), sem irrigação, acopladas em um contra-ângulo ligado a um motor elétrico com velocidade controlada (Driller, São Paulo, Brasil). Todos os procedimentos operatórios foram realizados em bancada de laboratório, com o auxílio de microscópio operatório de uso odontológico (Alliance, SP, Brasil), com aumento de 10x. Após a abertura, os tecidos pulpares foram removidos com lima tipo K #10 e #15 (Dentsply-Maillefer, Petrópolis, RJ, Brasil).

Após a pulpectomia, os dentes receberam um dos tratamentos proposto para o grupo ao qual pertencia:

- Grupo Empty: os canais foram deixados vazios e a cavidade de acesso selada com restaurador provisório Coltosol® (Coltene, RJ, Brasil)

- Grupo Blood: lima endodôntica tipo K #15 foi inserida em cada canal além do comprimento total das raízes (mais ou menos 0,5 mm) de forma a induzir sangramento que preenchesse os canais radiculares e formasse um coágulo sanguíneo. Após isso, os canais foram selados com Coltosol®.

- Grupo EDTA + Blood: a câmara pulpar foi preenchida com solução irrigadora de EDTA a 17% (Biodinâmica, RJ, Brasil), utilizando-se para isso seringa descartável de 1,0 mL (Injex, SP, Brasil) e agulha hipodérmica (0,45 x 13) (Injex, SP, Brasil). Foram padronizados o volume de 0,5 mL para cada dente, e o tempo de aplicação de 1

minuto (marcado com cronógrafo equipado com sinal sonoro). Durante o tempo de aplicação a solução foi agitada com movimentos de cateterismo feitos com lima endodôntica tipo K #10 inserida nos canais. Após a secagem da câmara pulpar/canais com cones de papel estéreis (Dentsply/Maillefer, Petrópolis, RJ, Brasil), uma lima endodôntica tipo K #15 foi inserida em cada canal além do comprimento total das raízes (mais ou menos 0,5 mm) de forma a induzir sangramento que preenchesse os canais radiculares e formasse um coágulo sanguíneo. Após isso os canais foram selados com Coltosol®.

Cada grupo do estudo foi avaliado após 7, 14 e 21 dias do experimento. Nos prazos determinados, os animais foram aleatoriamente escolhidos e sacrificados através do deslocamento cervical e o dente e os tecidos periapicais excisados com lâminas de bisturi estéreis n° 15 (Lamedid, Osasco, SP, Brasil), postos em *eppendorfs* e congelados para posterior processamento.

7.2 Avaliação da expressão das citocinas e fatores de crescimento/diferenciação

O método utilizado para avaliar a expressão gênica dos alvos foi a reação em cadeia da Polimerase em Tempo Real (RT-PCR), a qual, através da amplificação do cDNA obtido a partir do mRNA, permite quantificar exatamente o número de cópias de cada mRNA presente nas amostras.

7.2.1 Extração e quantificação do RNA

O RNA total foi obtido pelo método Trizol. Para isso, tanto os dentes quanto os tecidos periapicais de cada amostra foram submersos em *eppendorfs* contendo Trizol (GIBCO BRL Laboratories, Grand Island, NY, EUA) e macerados em aparelho elétrico (IKA T10 *basic* – Merse – SP, Brasil). Na sequência, em cada *eppendorf* foi adicionado 200µL de clorofórmio e as amostras centrifugadas durante 15 minutos a 12,000 x *g* a

4 °C. Após a centrifugação, a fase aquosa obtida foi removida e transferida para outro *eppendorf*, ao qual foi adicionado 250 µL de Isopropanol (Merck, SP, Brasil). A mistura foi incubada em estufa (Quimis®, modelo Q-315D) a 25 °C, por 15 minutos e depois centrifugada por 10 minutos a 12,000 x *g* a 4 °C, o que promoveu a precipitação do RNA. O sobrenadante foi retirado do tubo e descartado. O RNA precipitado foi lavado com 250 µL de etanol a 75%. Para obtenção do *pellet*, os *eppendorfs* foram colocados em agitador vórtex (Phoenix Instrument, Alemanha) e centrifugados a 10,000 x *g* a 4 °C por 15 minutos. O sobrenadante foi descartado e os *eppendorfs* foram emborcados sobre papel toalha para que todo o líquido remanescente escorresse. Em seguida, ao *pellet* foi adicionada água de alta qualidade tratada com DEPC (pirocarbonato de dietila, SIGMA® Chemical Co., Louis, MO EUA), para inativação da enzima RNAse.

A quantificação do RNA de cada amostra foi feita através do espectrofotômetro (Nanodrop®, modelo ND 1000, Wilmington-Delaware, EUA), e o grau de pureza das amostras obtidas determinadas pelo valor da reação 260/280.

7.2.2 Preparo do cDNA por Transcrição Reversa

A síntese do DNA complementar (cDNA) foi realizada a partir do RNA total, utilizando-se um *mix* contendo desoxirribonucleotídeos fosfatados (DNTPs), OligodT$_{15}$ (Promega Corp., Madison, WI, EUA), Dithiothreitiol (DTT), água tratada com DEPC e tampão da enzima transcriptase reversa (Promega Corp.). 12 µL do *mix* foram adicionados a 1µg de RNA de cada amostra, em *eppendorfs*, totalizando um volume final de 25 µL para cada amostra, segundo protocolo descrito por Silva *et al.,* (2008). Os tubos foram então levados a um termociclador (Thermo Hybaid – PCR Express, UK), ajustado na configuração padrão para cDNA, a saber:

1° ciclo: 70 °C por 5 minutos; 2° ciclo: 4 °C por 5 minutos; 3° ciclo: pausa para adição da enzima transcriptase reversa; 4° ciclo: 23 °C por 5 minutos; 5° ciclo: 37 °C por 1 hora; 6° ciclo: 90 °C por 5 minutos. Após os ciclos, as amostras foram recolhidas e mantidas sob refrigeração a -20 °C até sua utilização.

7.2.3 Detecção e quantificação das citocinas e fatores de crescimento/diereniação

Para análise das citocinas e dos fatores de crescimento/diferenciação, as seguintes sequências de *primers* foram utilizadas:

Tabela 1: *Sequência de primers*

Gene	Sequência (5' – 3')
TGF-β	FW: TGA CGT CAC TGG AGT TGT ACG
	RV: GGT TCA TGT CAT GGA TGG TGC
IL-10	FW: GGT TGC CAA GCC TTA TCG GA
	RV: ACC TGC TCC ACT GCC TT GCT
IL-1α	FW: CAA CCA ACA AGT GAT ATT CTC CAT G
	RV: GAT CCA CAC TCT CCA GCT GCA
IGF	FW: CCT GCT TAT GTG TCA GTC TGT
	RV: ACA GAC TGA CAC ATA AGC AGG
VEGF	FW: CTG CTC TCT TGG GTC CAC TGG
	RV: CAC CGG GTT GGC TTG TCA CAT
NGF	FW: CTG CTC TCT TGG GTC CAC TGG
	RV: CAC CGG GTT GGC TTG TCA CAT

FW: forward primer; RV: reverse primer

As reações de RT-PCR foram processadas no aparelho Step One Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), utilizando-se como método de detecção o sistema SYBR-Green (Applied Biosystems).

7.3 Análise dos dados

Após a amplificação, os dados foram analisados utilizando-se o programa Sequene Detection Software, versão 2.0 (Applied Byosystems). Os resultados foram obtidos como valores do *threshold cicle*, e a expressão dos níveis foram calculadas usando o método comparativo $2^{-\Delta\Delta CT}$ (SMITTGEN & LIVAK, 2008). Os valores foram calculados como sendo a média das duplicatas de cada amostra, e os níveis de mRNA de cada uma foram definidos baseados na sua proporção com o *primer* da β-Actina (constituinte endógeno). A análise estatística dos dados foi feita utilizando-se o programa SPSS, versão 15.0 (SPSS, Chicago, IL, EUA). Os dados foram submetidos ao teste Shapiro-Wilk para se caracterizar a normalidade. O teste de Levene foi utilizado para avaliar a igualdade de variância dos dados, enquanto o teste-*t* foi utilizado nas amostras de forma independente para se avaliar a significância nas diferenças observadas entre os grupos. Diferenças nos níveis de expressão de mRNA foram considerados estatisticamente significativos quando $p < 0,05$.

8 ARTIGOS CIENTÍFICOS

8.1 Artigo 1: Evaluation of gene expression of growth factors after regenerative endodontic treatment

Evaluation of gene expression of growth factors after regenerative endodontic treatment

Igor Vieira Bracks[1], Marcela Carvalho Espaladori[1], Luciana Carla Neves Brito[3], Leda Quercia Vieira[2] e Antônio Paulino Ribeiro Sobrinho[1]

[1]Department of Operative Denstistry, School of Dentistry, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil;

[2]Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil;

[3]Departament of Endodontics, School of Dentistry, Itaúna University, Itaúna, MG, Brazil.

[*]**Corresponding author:** Dr. Antônio Paulino Ribeiro Sobrinho – Departamento de Odontologia Restauradora, Faculdade de Odontologia, Universidade Federal de Minas Gerais, CEP 31270-901, Belo Horizonte, MG, Brazil. e-mail: sobrinho.bhz@gmail.com.

Abstract

Introduction: The treatment of immature permanent teeth with pulpal necrosis is associated with clinical challenges and results in teeth with thin, brittle and fracture-prone dentin walls. Regenerative endodontic treatment is an alternative treatment protocol that builds on the principles of tissue engineering. The success of these procedures needs an appropriate combination of a source of undifferentiated cells, morphogenetic signals and a scaffold that promotes the cells' migration/differentiation. In this process, the inflammatory mediators play a key role in cell behavior. The current study aimed to examine cytokine expression profiles during regenerative endodontic treatment using a blood clot (BC) as a scaffold in a mouse model. Methods: After pulpectomy, teeth were divided into two groups: Experimental: canals filled with blood clot and Control: canals left empty. Animals were sacrificed 7, 14 and 21 days after the operative procedures, and teeth were collected. mRNA expression of the cytokines IGF, NGF, IL-1α, IL-10, TGF and VEGF was assessed using real-time PCR. Levene's test was used to assess the equality of variance for the data, whereas a t-test for independent samples was used to evaluate the significance of the differences observed between groups (P < .05). Results: Our results show higher levels of pro-inflammatory mediators during the evaluated periods in the Control group, as well as higher levels of anti-inflammatory mediators in the Experimental group. Conclusion: The presence of a blood clot within the canals acts as an anti-inflammatory scaffold for regenerative endodontic treatment.

Regenerative endodontic treatment (RET) is defined as a biological procedure designed to replace damaged structures, including dentin and dentin-pulp complex cells (1). The goal of RET is to completely regenerate the function of the dentin-pulp complex so that, in immature teeth, these neo-formed tissues can continue root development, prevent or repair periapical lesions (2), and regain a positive response to pulp sensibility tests (3). The advantage of this therapy, in relation to other therapies, is that it promotes continued root development, increased thickness in the dentinal

walls and apical closure (3, 4). Moreover, the American Association of Endodontics considers RET a new treatment approach proposed as a conservative alternative for young permanent teeth with immature roots and pulpal necrosis (5).

A series of studies have shown the efficacy of clinical use for BC as a scaffold in RET (1, 6, 7, 8). Currently, there are growing shreds of evidence for the success of BC revascularization in immature teeth (9).

A BC consists of a fibrin matrix that traps cells necessary for tissue regeneration and it also provides a suitable pathway for cells from the periapical area to migrate into the root canals and enhance the new tissue's growth (10). Otherwise, BCs contain growth/differentiation factors (11), which may be important for the success of pulpal revascularization (12, 13). In addition, bleeding leads to the induction of angiogenesis, which may aid in the recruitment of the stem/progenitor cells necessary for the desired goals to be achieved (9).

Although regenerative endodontic treatment has been performed by several clinicians worldwide, there is no longitudinal in vivo study that investigated the immune-inflammatory response in teeth submitted to clinical regenerative endodontic protocol. The problem with this type of investigation is related to human ethical restrictions. Therefore, this study longitudinally analyzed the immune response to clinical RET performed in mice exhibiting devitalized teeth filled with blood clots. The gene expression for the growth factors IGF, NGF, and VEGF and the cytokines IL-1α, IL-10, and TGF was analyzed by RT-PCR.

Materials and methods

Animals

Balb/C mice, 6-8 weeks of age, were used, regardless of the sex [n = 36; 6 animals per time-point/group, as previously described (14); time-points were 7, 14 and 21 days after operative procedures]. During all phases of the experiment, the animals received food (Nuvital, Curitiba, PR, Brazil) and water ad libitum. Prior to all the experimental phases, the animals received general anesthesia via an intraperitoneal

injection of a solution containing 60 mg/kg of ketamine hydrochloride (Dopalen, Vetbrands - SP, Brazil) and 20 mg/kg of Xylazine (Agribands do Brasil Ltda. SP, Brazil) diluted in phosphate-buffered saline (PBS). The study was approved by the Animal Research Ethics Committee of the Federal University of Minas Gerais (CEUA Protocol n°. 255/2016).

Operative Procedures

The first upper left molar of each animal was opened endodontically with sterile 1/4 round diamond burs, non-irrigated and coupled in a contra angle connected to an electric motor (Driller, SP, Brazil). All the procedures were performed using an operating microscope (Alliance, SP, Brazil) with a 10x magnification, as demonstrated elsewhere (14, 15). After the opening, a pulpectomy was performed using type Kerr # 10 and 15 files (Dentsply-Maillefer, RJ, Brazil).

Preparation of groups

After pulpectomy, the animals were divided into two groups. In the Control group (n = 18), the root canal systems were dried with sterile paper point # 20 (Dentsply-Maillefer) and left empty, and then cavities were sealed with Coltosol® (Coltene, RJ, Brazil). In the Experimental group, an endodontic #15 file was introduced 0.5 mm beyond the apical foramen, and catheterization movements were made until all the channels were filled by the bleeding from the periapical tissues. Following BC formation, the cavities were sealed with Coltosol®.

After the 7, 14 and 21 day intervals, 6 animals from each group were sacrificed via an injection of an anesthetic solution overdose. The teeth were then surgically and aseptically removed together with the adjacent periapical tissues (representing a sample) and rinsed in PBS and flash-frozen in a mixture of dry ice and ethanol. The teeth were then frozen at -80°C until the time of processing of the mRNA samples, as previously described (14).

Real-time PCR

Total RNA from each sample was extracted using TRIzol reagent (GIBCO/BRL Laboratories, Grand Island, NY) and resuspended in 50 µL of diethylpyrocarbonate-

(DEPC) treated water (Sigma Chemical Co., Louis, MO) containing 1 mM of EDTA, as previously described (15, 16).

Total RNA was reversely transcribed at 37°C for 60 minutes in the presence of 5 µL RNA, 250 mM dNTPS, 50 mM TRIS-HCL (pH 8,3), 75 mM KCL, 3,0 mM MgCL2, 10 mM DTT (GIBCO/BRL Laboratories), 10 U of RNAsin (Promega Corp., Madison, WI) 7.0 Pm OLIGO-Dt15 (Promega Corp.) and 25 U of reverse transcriptase (Promega Corp.), and the cDNA was synthesized using 1 µg of RNA, as previously described (15, 17). PCR was performed under standard conditions, including a heating stage at 95°C (15 seconds) followed by 1 minute at 60°C and a melting stage at 95°C (15 seconds), 60°C (1 minute), and 95°C (15 seconds). The RT-PCR analyses were performed using Step One Real-time PCR Systems (Applied Biosystems, Cal, EUA), and the SYBR-Green detection system (Applied Biosystems) was used to visualize the amplification of the primers, as previously described (17). β-Actin was used with a constitutive (housekeeping) gene for normalization. All the samples were duplicated. The reactions were performed with a total volume of 25 µL and contained 1 µL of cDNA. The data were analyzed using Sequence Detection Software version 2.0 (Applied Biosystems). The results were obtained as threshold cycle values, and the expression levels were calculated using the comparative method $2\text{-}\Delta\Delta CT$ (18). The values were calculated as the average of the duplicates for each sample, and the mRNA levels in each of them were defined as their proportion with the primer of the β-Actin. The data analysis was done using the SPSS program for Windows (version 15.0; SPSS, Chicago, IL). The data were subjected to the Shapiro-Wilk test to characterize normality. Levene's test was used to assess the equality of variance for the data, whereas a *t*-test for independent samples was used to evaluate the significance of the differences observed between groups. Differences in mRNA expression levels were considered to be statistically significant when the P-value was < 0.05.

Tabela 1: P*rimers sequences*

Gene	Sequência (5' – 3')

TGF-β	FW: TGA CGT CAC TGG AGT TGT ACG
	RV: GGT TCA TGT CAT GGA TGG TGC
IL-10	FW: GGT TGC CAA GCC TTA TCG GA
	RV: ACC TGC TCC ACT GCC TT GCT
IL-1β	FW: CAA CCA ACA AGT GAT ATT CTC CAT G
	RV: GAT CCA CAC TCT CCA GCT GCA
IGF	FW: CCT GCT TAT GTG TCA GTC TGT
	RV: ACA GAC TGA CAC ATA AGC AGG
VEGF	FW: CTG CTC TCT TGG GTC CAC TGG
	RV: CAC CGG GTT GGC TTG TCA CAT
NGF	FW: CTG CTC TCT TGG GTC CAC TGG
	RV: CAC CGG GTT GGC TTG TCA CAT

FW: forward primer; RV: reverse primer

Results

Insulin-like growth factors (IGF) mRNA expression levels were similar between the Control and Experimental groups at the 7th and 14th days of evaluation (P > 0.05). However, on the 21st day, there was a reduction in the expression for this mediator in the Experimental group (EG) compared to the Control group (CG) (P < 0.05). There was also a significant decrease between the levels of IGF expression in the EG from the 14th to the 21st day of evaluation (P < 0.05) (Fig. 1).
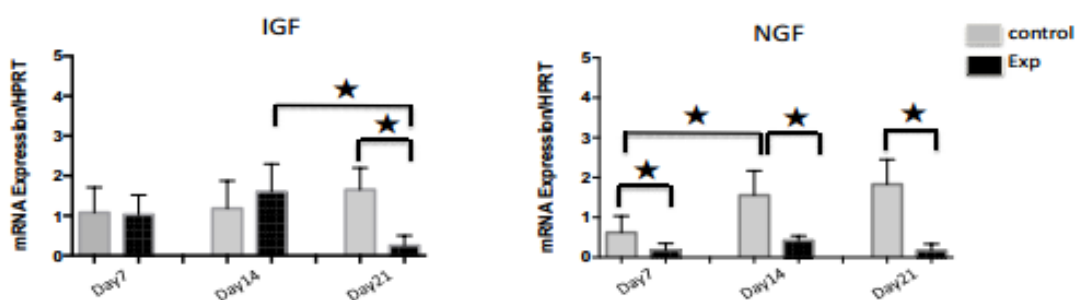
Figure 1 – Expression of the mRNA growth factors IGFand NGF in the teeth was analysed via RT-PCR. The relative expression of mRNA was compared with β-Actin as the internal Control. The expressed results represent the average values of the duplicates of each analysed factor, and each group had 6 animals. $P < 0.05$.

The gene expression for nerve growth factor (NGF) in all three evaluation periods was significantly higher in the CG than in the EG ($P < 0.05$). An increase in NGF expression from the 7th to the 14th day ($P < 0.05$) was also observed in the CG (Fig. 1).

Interleukin-10 (IL-10) expression remained constant on the periods evaluated in the CG and EG ($P > .05$). On the other hand, in the three evaluation periods, IL-10 expression was statistically higher in the CG than in the EG ($P < 0.05$) (Fig. 2).
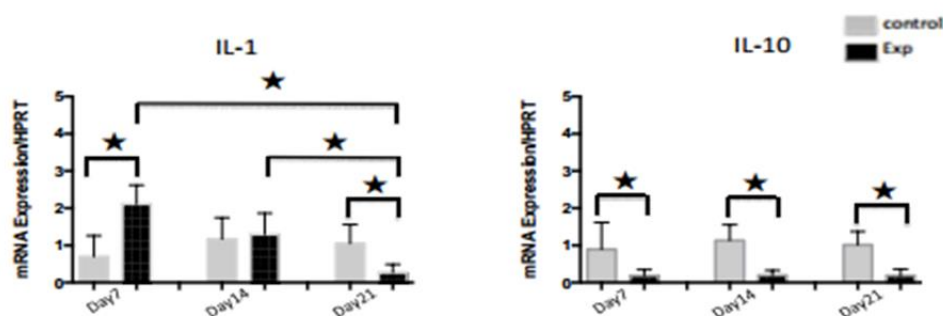

Figure 2 – Expression of the mRNA growth factors IL-1 and IL-10 in the teeth was analysed via RT-PCR. The relative expression of mRNA was compared with β-Actin as the internal Control. The expressed results represent the average values of the duplicates of each analysed factor, and each group had 6 animals. $P < 0.05$.

In comparison to the Control group, the analysis revealed an increased expression of Interleukin-1α (IL-1α) levels on the 7th day in the Experimental group (EG) ($P < 0.05$). On day 14, the levels of IL-1α were similar between the two groups, and it decreased in the EG compared to CG on the 21st day ($P < 0.05$). There was a

decrease in IL-1α expression in the EG throughout the whole period; however, with a statistically significant difference between the 7th and 21stdays and between the14th and 21st days (P < 0.05) (Fig. 2).

The transforming growth factor β (TGF-β) expression in EG was similar between days 7 and 14, with a tendency to decrease on day 21, which lacked a statistically significant difference (P > 0.05) (Fig. 3). In CG, there was an increase in TGF-β expression in all the periods with a statistically significant difference between days 7 and 21 (P < 0.05). Finally, the expression of the TGF-β gene was statistically higher in the EG compared to the CG on the 7th and 14th days (P < 0.05), and both groups equalized on the 21st day (P > 0.05).
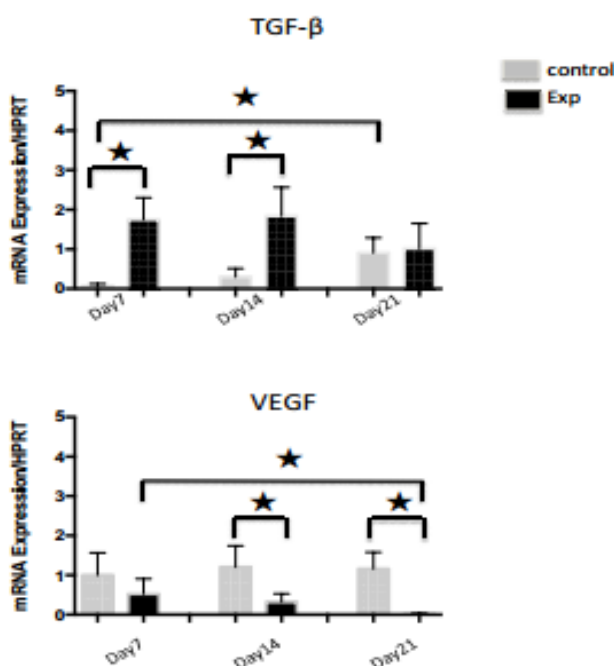

Figure 2 – mRNA expression of VEGF and TGF-β in the teeth was analysed via RT-PCR. The relative expression of mRNA was compared with β-Actin as the internal Control. The expressed results represent the average values of the duplicates of each analysed factor, and each group had 6 animals. *P* < 0.05.

The expression for vascular endothelial growth actor (VEGF) in the CG showed a similar expression in the three moments of evaluation (P > 0.05), whereas in the EG, the VEGF decreased from the 7th to the 21st day (P < .05). When comparing the two groups, a reduction of VEGF in the EG was observed in comparison to CG with statistically significant values at the 14th and 21st days (P < 0.05) (Fig. 3).

Discussion

Several clinical protocols preconize to fill root canals with a blood clot through over instrumentation when regenerative procedures are carried out in human necrotic teeth (6, 19, 20, 21). This operative technique shows advantages, in its simplicity and in the fact that it can be performed with standard endodontic instruments and with medication without expressive biotechnology. Several clinical studies have demonstrated the success of the regenerative endodontic treatment (22, 23, 24), although longitudinal immune analyses were not performed because of the limitation of human ethical concerns. To overcome this limitation, this study was first in literature that analyzed the immune responses that occurred after regeneration procedures were performed in mice exhibiting devitalized teeth filled with their own blood clot (BC).

The induction of bleeding and formation of a BC within the root canal's systems let mesenchymal stem cells regenerate pulp tissues (25), and several growth factors are also connected to this phenomenon. IGFs are important angiogenic factors related to wound healing and regeneration (26). In this study, levels of IGF mRNA expression significantly reduce from days 14 to 21 in the Experimental group but remain constant over the entire evaluated period in the control group. This result suggests that immune modulation in the Experimental group occurred faster than in the control group and that it may be associated with the presence of blood clots. In agreement, it was demonstrated that the increase in the expression for IGF was paralleled with the reduction in the pro-inflammatory response (27).

NGF is important for the development and differentiation of neural and non-neural cells (28). In addition, it has the ability to attract and develop nerve fibers in dental tissues (29). In this study, a greater NGF expression was observed in the control than

in the Experimental group in all the evaluated time-points. There was an increase in their levels between the 7th and 14th day in the control group, whereas their levels did not change throughout the entire period in the experimental group. The high levels of NGF reinforce the evidence of a more acute and prolonged inflammatory process in the control group, as demonstrated elsewhere (30).

The presence of IL-1α and IL-1α-producing cells has been demonstrated in inflamed periapical lesions (16). This cytokine initiates and exacerbates the inflammatory process possessing a strong pro-inflammatory action. In this study, the IL-1α gene expression was higher in the Experimental group than in the control group on day 7. This finding may be explained by the over instrumentation in the Experimental group, which results in a greater periapical trauma when compared to the control group, which only underwent pulpectomy. However, a full inversion of their values can be observed along the 14th and 21st days in the Experimental group. This result reinforces the evidence that there is an attenuation of the inflammatory process in this group over the assessed period, conversely from what was observed in the Control group. The blood clot seems to be protective, since the IL-1α mRNA expression in this group was similar to its expression when physical barriers filled human root canal systems (17, 31).

VEGF enhances blood vessel growth and increases vascular permeability, which is an important change observed during inflammatory processes (31). This growth factor plays a critical role in neovascularization, the stimulation of endothelial cell proliferation, the secretion of proteolytic enzymes, and the promotion of chemotaxis and cell migration, all of which are necessary for angiogenesis (33, 34). In this study, a statistically significant decrease in VEGF expression was observed between days 7 and 21 in the Experimental group, paralleled with the higher expression of this mediator observed in the control group than in the Experimental group on days 14 and 21. The higher gene expression observed in EG on the 7th day may be related to over instrumentation performed to induce blood clotting. It is important to note that both VEGF and NGF, in Experimental group, present basal levels at all the experimental time-points, inferring that VEGF depends on the ability of NGF to increase its expression, as demonstrated elsewhere (32, 34). Additionally, the greater VEGF

expression in the control group suggests that there is an ongoing inflammatory process throughout the evaluated period.

Immunosuppressive mechanisms mediated by TGF-β and IL-10 are responsible for the tissue repair processes, as well as for the restriction of the immune/inflammatory response (35). In this study, IL-10 was expressed in constant amounts throughout all analyzed periods in both groups (Fig. 2). However, its expression was statistically higher in the Control group than in the Experimental group, which suggests that blood clot down-modulates IL-10 expression. When human root canal systems were dressed with chlorhexidine (17) or calcium hydroxide (29), a reduction in IL-10 mRNA expression was observed in contrast to the empty root canals, which was similar to the results observed in this study.

On the other hand, TGF-β gene expression was expressed significantly higher in the Experimental group than the Control group, at the 7th and the 14th days. Taken together, these results suggest that the immunosuppressive response is IL-10 dependent in the control group and TGF-β dependent in the Experimental group. TGF-β regulates several biological processes such as proliferation, survival, differentiation, apoptosis and cellular migration, and extracellular matrix production, showing an important role in immunological reactions, angiogenesis, regulation of vascular tone, tissue repair and bone metabolism (35). All of these mechanisms are involved in the improvement of regenerative endodontic treatment. In other words, blood clots stimulate a regenerative treatment inducing TGF-β synthesis in those root canals.

This study is a pioneering research that evidences the immuno-inflammatory profile associated with the use of blood clots in the regenerative endodontic treatment. Taken together, the results allow us to conclude that the BC acts as an anti-inflammatory scaffold, since in the empty canals, the inflammatory process persisted throughout the analyzed period. Finally, the mouse model played an important role in allowing us to analyze, longitudinally, the immune response to clinical RET performed in teeth filled with blood clots. Moreover, despite clinical examination had show the efficacy of REP, it is the first study to show longitudinal cytokine expression after REP instituition.

Acknowledgments

References:

1. Murray PE, Garcia-Godoy F, Hargreaves KM. Regenerative Endodontics: A review of current status and a call for action. J Endod 2007; 33:377-90.

2. Hargreaves K, Geisler T, Henry M, Wang Y. Regeneration potential of the young permanent tooth: what does the future hold? J Endod 2008; 34:S51-6

3. Kontakiotis EG, Filippatos CG, Tzanetakis, Agrafioti A. Regenerative endodontic therapy: A data analysis of clinical protocols. J Endod 2015; 41:146-54.

4. Torabinejad M and Turman M. Revitalization of tooth with necrotic pulp and open apex by using platelet-rich plasma: a case report. J Endod 2011; 37:265-68.

5. Sedgley CM, Cherkas P, Chogle SMA, Geisler TM, Hargreaves KM, Paranjpe AK and Yamagishi VTK. Regenerative Endodontics. Endodontics: Colleagues for excellence newsletter. Spring 2013.

6. Ding RY, Cheung GS, Chen J, Yin XZ, Wang QQ and Zhang CF. Pulp revascularization of immature teeth with apical periodontitis: a clinical study. J Endod 2009; 35:745-49.

7. Raju SMK, Yadav SS, Kumar SR. Revascularization of immature mandibular premolar with pulpal necrosis – a case report. J Clin Diag Res 2014; 8:29-31.

8. Jung IY, Lee SJ, Hargreaves KM. Biologically based treatment of immature permanent teeth with pulpal necrosis: a case series. J Endod 2008; 34:876–87.

9. Alshehadat SA, Thu HA, Hamid SSA, Nurul AA, Rani SA, Ahmad A. Scaffolds for dental pulp tissue regeneration: a review. Int Dent Med J ADv 2016; 2:1-12.

10. Geisler, TM. Clinical considerations for regenerative endodontic procedures. Dent Clin N Am 2012; 56:603-26

11. Martin P. Aiming for perfect skin regeneration. Science 1997; 276:75-81.

12. Alrahabi MK, Ali MM. Root canal revascularization, the beginning of a new era in endodontics. Saudi Med J 2014; 35:429-34.

13. Maeda H, Wada N, Nakamuta H, Akamine A. Human periapical granulation tissue contains osteogenic cells. Cell and Tissue Res 2004; 315:203-08.

14. Maciel KF, Brito LCN, Tavares WL, Moreira G, Nicoli JR, Vieira LQ, Ribeiro Sobrinho AP. Cytokine expression in response to root canal infection in gnotobiotic mice. Int Endod J 2012; 45:354-62.

15. Silva MJB, Vieira LQ, Ribeiro Sobrinho A. The effects of mineral trioxide aggregates on cytokine production by mouse pulp tissue. Oral Surg Oral Pathol Oral Rad and Endod 2008; 105:70-76.

16. Brito LCN, Teles FRF, Teles RP, Totola AH, Vieira LQ, Sobrinho APR. T-lynphocyte and cytokine expression in human inflammatory periapical lesions. J Endod 2012; 38:481-85.

17. Tavares WLF, Brito LCN, Henriques LCF, Oliveira RR, Maciel KF, Vieira LQ, Sobrinho APR. The impact of chlorhexidine-based endodontic tratment on periapical cytokine expression in teeth. J Endod 2013; 39:889-92.

18. Schmittgen TD & Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nature Protocols 2008; 3:1101-08.

19. Ding RY, Cheung GS, Chen J, Yin XZ, Wang QQ and Zhang CF. Pulp revascularization of immature teeth with apical periodontitis: a clinical study. J Endod 2009; 35:745-49

22. Lenzi R & Trope M. Revitalization procedures in two traumatized incisors with different biological outcomes. J Endod 2012; 38:411-14.

21. Wigler R, Kaufman AY, Lin S, Steinbock N, Molina HH, Torneck CD. Revascularization: a tratment for permanent teeth with necrotic pulp and incomplete root development. J Endod 2013; 39:319-26.

22. Zhang DD, Chen X, Bao ZF, Chen M, Ding ZJ, Zhong M. Histologic comparison between platelet-rich plasma and blood clot in regenerative endodontic treatment: an animal study. J Endod 2014; 40:1388-93.

23. Altaii M, Richards L, Fedele GR. Histological assessment of regenerative endodontic treatment in animal studies with different scaffolds: a systematic review. DentTraumatol 2017; 33:235-44.

24. Lovelance TW, Henry MA, Hargreaves KM, Diogenes A. Evaluation of the delivery of mesenchymal stem cells into the root canal space of necrotic immature teeth after clinical regenerative endodontic procedure. J Endod 2011; 37:133-38.

25. Demiralp B, Keçeli HG, Muhtaroğullan M, Serper A, Demiralp B, Eratalay K. Treatment of periapical inflammatory lesion with the combination of platelet-rich plasma and tricalcium phosphate: a case report. J Endod 2004; 30:796-800.

26. Sukhanov S, Higashi Y, Shai SY, Vaughn C, Mohler J, Li Y. IGF-1 reduces inflammatory responses, suppresses oxidative stress, and decreases atherosclerosis progession in apoE-deficient mice. Arter Throm Vasc Biol 2007; 27:2684-90.

27. Schäffer M, Beiter T, Becker HD, Hunt TK. Neuropeptides: Mediators f inflammation and tissue repair? Arch Surg 1998; 133:1107-16.

28. Mitsiadis TA, Pagella P. Expression of nerve growth factor (NGF), TrKA, and p75NTR in developing human fetal teeth. Frontiers in Physiol 2016; 7:338.

29. Tavares WLF, Brito LCN, Henriques LCF, Teles FRF, Teles RP, Vieira LQ, Ribeiro Sobrinho AP. Effects of calcium hydroxide on cytokine expression in endodontic infections. J Endod 2012; 38:1368-71.

30. Woolf CJ, Garabedian BS, Ma QP, Crilly P, Winter J. Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. Neuroscience 1994; 62:327-

31. Johnson RB, Serio FG, Dai X. Vascular endothelial growth factor and progression of periodontal disease. J Periodontol 1999; 70: 848-52.

32. Yamawaki K, Matsuzaka K, Kokubu E, Inoue T. Effects of epidermal growth factor and/or nerve growth factor on Malassez's epithelial rest cells in vitro: expression of mRNA for osteopontin, bone morphogenetic protein 2 and vascular endothelial growth fator. J Perio Res 2010; 45:421-27.

33. Iijima T & Zhang JQ. Three-dimensional wall structure and the innervation of dental pulp blood vessels. Microsc Res Tech 2002; 56:32-41.

34. Colic M, Gazivoda D, Vasilijic S, Vucevic D, Lukic A. Production of IL-10 and IL-12 by antigen-presenting cells in periapical lesions. J Oral Patho Med 2010; 39:690-96.

35. Martins R, Armada L, Santos TC, Ramoa Pires F. Comarative immunoexpression of ICAM-1, TGF-β1 and ki-67 in periapical and residual cysts. Med Oral Patol Oral Cir Bucal 2017; 22:24-30.

8.2 Artigo 2: Effect of EDTA irrigation on immuno-inflammatory response in teeth submitted to regenerating endodontic therapy

Effect of EDTA irrigation on immuno-inflammatory response in teeth submitted to regenerating endodontic therapy

Igor Vieira Bracks[1], Marcela Carvalho Espaladori[1], Patricia Barros[2], Luciana Carla Neves de Brito[3], Leda Quercia Vieira[2], Antônio Paulino Ribeiro Sobrinho[1]

[1]Department of Operative Denstistry, School of Dentistry, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil;

[2]Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil;

[3]Department of Endodontics, School of Dentistry, Itaúna University, Itaúna, MG, Brazil.

[*]**Corresponding author:** Dr. Antônio Paulino Ribeiro Sobrinho – Departamento de Odontologia Restauradora, Faculdade de Odontologia, Universidade Federal de Minas Gerais, CEP 31270-901, Belo Horizonte, MG, Brazil. e-mail: sobrinho.bhz@gmail.com.

Abstract:

Introduction: The treatment of immature permanent teeth affected by pulpal necrosis, which results in teeth with thin, brittle and fracture-prone dentin walls, poses a clinical challenge. Regenerative endodontic treatment is an alternative approach base on the principles of tissue engineering. The success of regenerative procedures depends on several factors, including a source of undifferentiated cells, the presence of morphogenetic signals, and a scaffold that promotes cell migration and differentiation. During this process, inflammatory mediators play a key role in cell behavior. The goal of this study was to longitudinally analyze the immuno-inflammatory response in mice with devitalized teeth that underwent a regenerative protocol with or without the use of blood clot (BC) and ethylenediamine tetra acetic acid (EDTA) to irrigate the root canal system (RCS). Methods: After pulpectomy, teeth were divided into the following three groups: Empty - the canals were left empty; Blood - the canals were filled with a blood clot; and EDTA + Blood - the canals were irrigated with 0.06 mL of 17% EDTA for 1 minute, after which the root canals were dried and then filled with a blood clot. Then, cavities were sealed with Coltosol®. Animals were sacrificed at 7, 14 or 21 days after the operative procedures, and teeth were collected. mRNA expression of the cytokines IGF, NGF, IL-1α, IL-10, TGF and VEGF was assessed using real-time PCR. Data analysis was done using the GraphPad Prism program (version 5.01); the Kolmogorov–Smirnov test was used to characterize normality, and data were analyzed with ANOVA and the Tukey *post hoc* test. Differences in mRNA expression levels were considered to be statistically significant when the *P*-value was < 0.05. Results: Our results showed higher levels of interleukins in the EDTA + Blood group at all times analyzed, as well as a decrease in expression over time. Conclusion: The use of EDTA as an irrigation solution promotes increased expression of factors that can improve the outcome of regenerative endodontic treatment. However, the expression of these factors decreases over time.

Key words: Endodontic Regeneration Procedures; EDTA; Blood Clot; RT-PCR.

Introduction:

The treatment of immature necrotic teeth represents a clinical challenge in endodontics. The wide foramen makes difficult to seal the apical root canal, and the presence of thin root walls limits the accomplishment of endodontic instrumentation. In addition, tissue necrosis frequently disrupts root development, making these teeth more susceptible to fracture (Aksel & Serper, 2014). Regenerating Endodontic Procedures (REP), biological procedures designed to replace damaged structures, including dentin, root structures, and cells of the dentin-pulp complex (Murray *et al.,* 2007), are typically performed in such cases.

Clinical cases have demonstrated the success of these procedures (Shah *et al.*, 2008; Torabinejad & Faras, 2012). However, the available data are insufficient to standardize the protocol for such REP (Aksel & Serper, 2013). Furthermore, despite published reports detailing their success, the outcome of these procedures remains unpredictable (Geisler, 2012). The advantage of REP over other therapies is that they promote continued root development as well as increased thickness in the dentinal walls and apical closure (Kokntakiotis *et al.*, 2015; Torabinejad & Turman, 2011).

Dentin is traditionally considered an inert mineralized tissue and is thought to undergo minimal remodeling after its formation. However, bioactive properties of the dentin matrix have recently been demonstrated (Diogenes *et al.*, 2014). The dissolution of dentin results in the release of these factors, which exerts effects at the cellular level (Smith *et al.,* 2016). Bioactive molecules found in the dentin matrix include growth factors, chemokines, cytokines, extracellular matrix molecules, and bioactive peptides, such as TGF-β, BMPs, IGF-1, VEGF, FGF-2, PDGF and EGF. All of these molecules are necessary for effective REP, reflecting the complexity of the cellular events that are induced during such processes (Smith *et al.*, 2016).

The importance of EDTA, which helps to release molecules trapped in dentine, in the success of REPs is increasingly recognized (Pang *et al.,* 2014; Gonçalves *et al.*, 2016). However, few studies have described its clinical use. For example, Bucchi *et al.* (2017) found that of a total of 984 articles, only 6 described the use of EDTA in REP protocols.

There are no longitudinal studies investigating the immuno-inflammatory response in teeth submitted to different endodontic regeneration protocols. This study aimed to longitudinally analyze the immuno-inflammatory response in the devitalized teeth of mice following treatment with regenerative protocols utilizing blood clots or EDTA as an irrigant of the root canal system (RCS). The null hypothesis tested was that after EDTA irrigation, growth/differentiation factors wouldnot be released from dentin and would not increase the success of the REP.

Materials and methods:

Animals:

Balb/c mice aged 6 to 8 weeks of both sexes were used (n = 54). During all phases of the study, the animals were kept in cages maintained in an environment with controlled light and ventilation. The mice received water and feed ad libitum. The study was approved by the Animal Research Ethics Committee of the Universidade Federal de Minas Gerais (CEUA n° 255/2016). Before each phase of the experiment, the animals were submitted to general anesthesia by injection of a solution formulated with 60mg/kg hydrochloride (Dopalen, Vetbrands - SP, Brazil) and 20 mg/kg Xylazine (Agribands do Brasil Ltda. SP, Brazil) diluted in phosphate-buffered saline (PBS).

Operative Procedures:

The first upper left molar of each animal was opened endodontically with sterile 1/4 round diamond burs, was not irrigated, and was coupled to an electric motor (Driller, SP, Brazil) at a contra angle. All procedures were performed using an operating microscope (Alliance, SP, Brazil) with 10x magnification, as demonstrated elsewhere (Maciel *et al*., 2012; Silva *et al*., 2008). After opening the tooth, a pulpectomy was performed using Kerr #10 and #15 files (Dentsply-Maillefer, RJ, Brazil).

Preparation of Groups:

After pulpectomy, the animals were divided in three groups as follows: Empty group: the RCS was left empty (negative control); Blood Clot (BC) group: the RCS was

allowed to fill with blood; and EDTA + Blood group: the RCS was irrigated with EDTA and subsequently allowed to fill with blood.

In the Empty group, the root canal systems were dried with sterile paper point #20 (Dentsply-Maillefer) and left empty, and cavities were sealed with Coltosol® (Coltene, RJ, Brazil). In the Blood group, an endodontic #15 file was introduced 0.5 mm beyond the apical foramen, and catheterization movements were made until the canal was filled with blood from the periapical tissues. Following BC formation, the cavities were sealed with Coltosol®. In the EDTA + Blood group, after pulpectomy, the canals were irrigated with 0.06 mL of 17% EDTA for 1 minute. During this time, the solution was manually agitated with catheterization movements using an endodontic #10 file. After, the canal was dried with sterile paper points and was allowed to fill with blood as mentioned above. The cavities were then sealed with Coltosol®.

After each time point (7, 14, or 21 days), 6 animals of each group were sacrificed via an injection of an anesthetic solution overdose. The teeth were then surgically and aseptically removed (representing a sample) and were rinsed in PBS and flash-frozen in a mixture of dry ice and ethanol. The teeth were then frozen at -80°C for subsequent processing of mRNA samples as previously described (Maciel *et al.*, 2012).

Real-time PCR

Total RNA from each sample was extracted using TRIzol reagent (GIBCO/BRL Laboratories, Grand Island, NY) and resuspended in 50 µL of diethylpyrocarbonate (DEPC)-treated water (Sigma Chemical Co., Louis, MO) containing 1 mM of EDTA, as previously described (Silva *et al.*, 2008; Brito *et al.*, 2012.

Total RNA was reversely transcribed at 37°C for 60 minutes in the presence of 5 µL RNA, 250 mM dNTPS, 50 mM TRIS-HCL (pH 8,3), 75 mM KCL, 3.0 mM $MgCL_2$, 10 mM DTT (GIBCO/BRL Laboratories), 10 U RNAsin (Promega Corp., Madison, WI), 7.0 Pm OLIGO-Dt_{15} (Promega Corp.) and 25 U reverse transcriptase (Promega Corp.). cDNA was synthesized using 1 µg of RNA as previously described (Silva *et al.*, 2008; Tavares *et al.*, 2013). PCR was performed under standard conditions, including a heating stage at 95°C (15 seconds) followed by 1 minute at 60°C and a melting stage

at 95°C (15 seconds), 60°C (1 minute), and 95°C (15 seconds). RT-PCR was performed using Step One Real-time PCR Systems (Applied Biosystems, Cal, EUA), and the SYBR-Green detection system (Applied Biosystems) was used to visualize the amplification of the primers, as previously described (Tavares *et al.*, 2013). β-Actin was considered a constitutive (housekeeping) gene for normalization. All reactions were performed in duplicate in a total volume of 25 μL containing 1 μL of cDNA. After amplification, the data were analyzed using the GraphPad Prism program (version 5.01). The values were calculated as the average of the duplicates for each sample, and the mRNA levels in each sample were normalized to the β-actin level. The data were subjected to the Kolmogorov–Smirnov test to characterize normality and were analyzed by ANOVA and Tukey *post hoc* tests. Differences in mRNA expression levels were considered to be statistically significant when the *p*-value was < 0.05.

Results:

IL-1 mRNA expression was significantly higher in the EDTA + Blood group than in the Empty and BC groups at the 7th and 14th days of evaluation ($p < 0.05$). Moreover, when IL-1 mRNA expression in the EDTA + Blood group was compared across time, we observed that peak expression occurred at day 7 ($p < 0.05$), and decreased over the time, with a significant difference between days 7 and 21 ($p < 0.05$), presenting, in this period, similar expression to the Empty and Blood groups. The Empty and Blood Clot groups showed no significant differences in expression of these genes ($p > 0.05$) for all experimental times (Fig. 1).
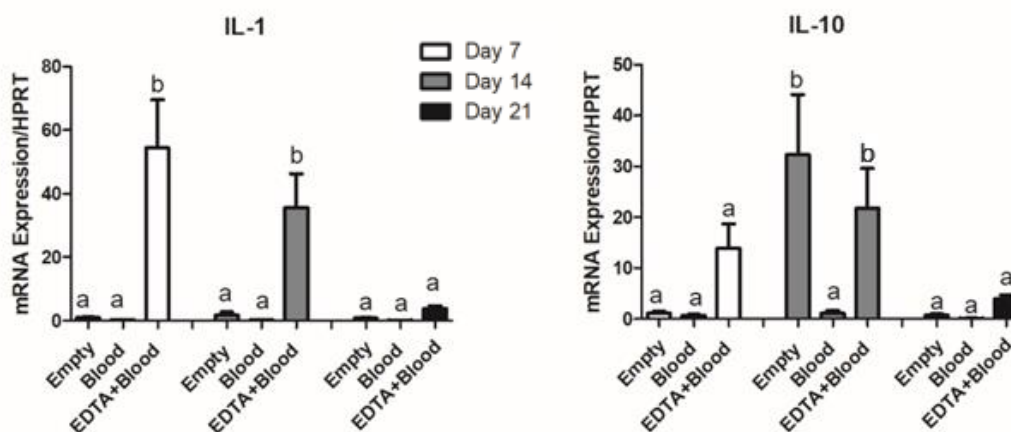
Fig. 1 – Expression of the growth factors IL-1 and IL-10 in pulpar tissues was analyzed via RT-PCR. The relative expression of mRNA was determined by normalization to the β-actin level. The results represent the average value for each analyzed factor, and each group consisted of 6 animals. $p < 0.05$. Different letters represent a significant difference ($p < 0.05$).

IL-10 mRNA expression was similar across the three groups on the 7th and 21st days of evaluation. Interestingly, for the Empty and EDTA + Blood groups on the 14th day, we observed levels of gene expression that were significantly higher than those observed on the 7th and 21st days (Fig. 1).

Fig. 2 details TGF-β mRNA expression. We found that TGF-β mRNA expression in the EDTA + Blood group was significantly higher on the 7th day than on the 14th and 21st days ($p < 0.05$). On day 7, TGF-β mRNA expression in the EDTA + Blood group was also significantly higher than that in the Empty and Blood groups ($p < 0.05$). No significant differences were observed among all groups at day 14 ($p > 0.05$). However, at day 21, TGF-β mRNA expression was similar between the Blood and EDTA + Blood groups but higher than in the Empty group ($p < 0.05$).
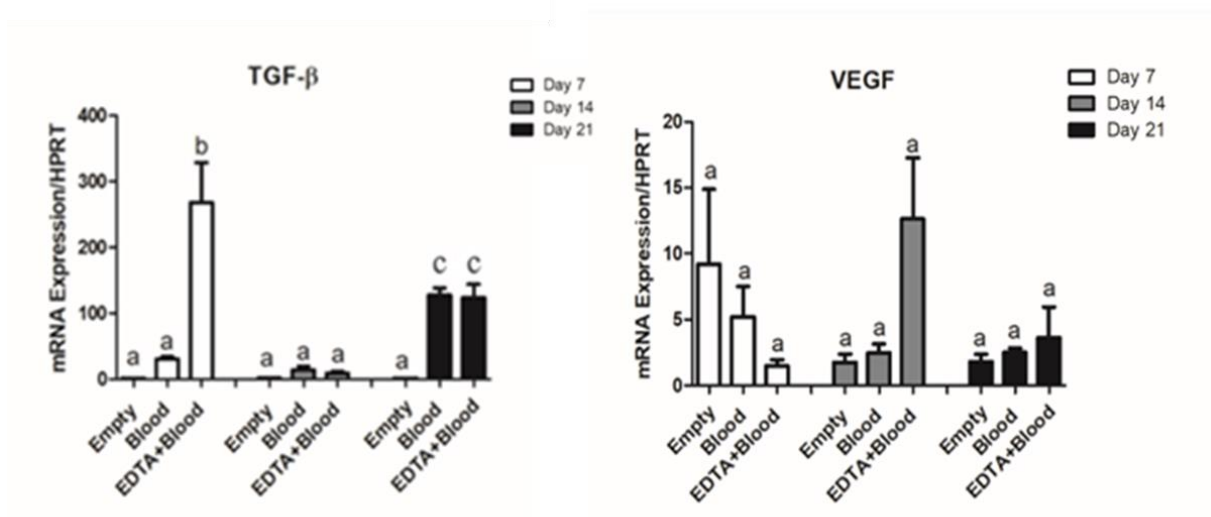
Fig. 2 – Expression of VEGF and TGF-β mRNA in pulpar tissues was analyzed via RT-PCR. Relative mRNA expression was compared to the expression of β-actin, which served as the internal control. The results shown represent the average value for each factor analyzed, and each group had 6 animals. $p < 0.05$. Different letters represent a significant difference ($p < 0.05$).

VEGF concentrations did not differ between the groups, time points, or intragroup periods; its level remained unchanged throughout the experimental period ($p > 0.05$) (Fig. 2).

Together, these results showed that IGF mRNA expression was significantly higher in the EDTA + Blood group than in the other groups (Empty and BC) on the 7th and 14th days of evaluation. Moreover, when IGF mRNA expression in the EDTA + Blood group was compared among time points, we found that its expression peaked at day 7 (p < 0.05). IGF mRNA expression in the Empty and BC groups did not differ significantly ($p > 0.05$) across experimental times (Fig. 3).
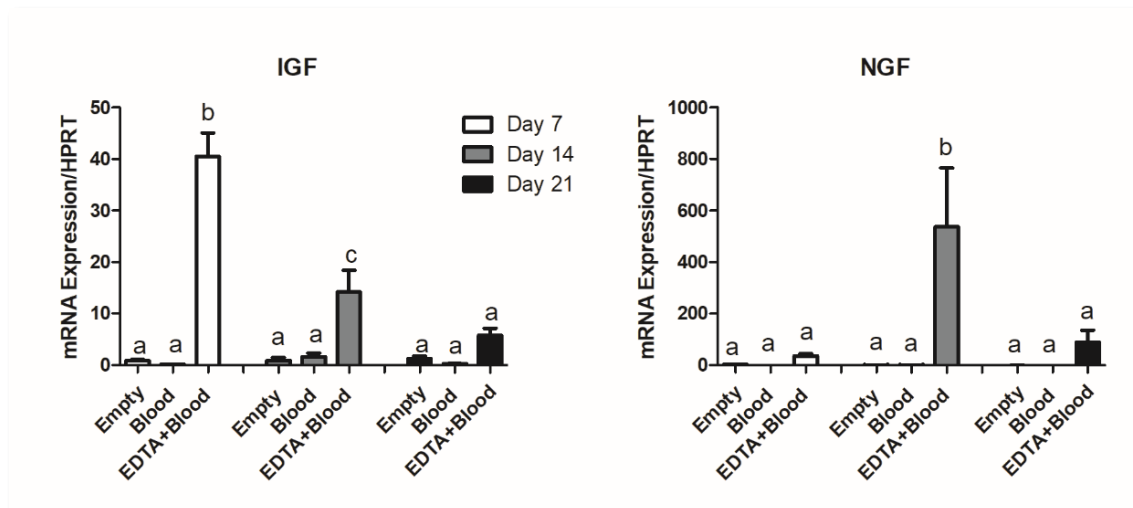
Fig. 3: The expression of IGF and NGF mRNA in pulpar tissues was analyzed via RT-PCR. The levels of IGF and NGF mRNA were normalized to the β-actin level as the internal control. The results shown represent an average value for each analyzed factor, and each group had 6 animals. $p < 0.05$. Different letters represent a significant difference ($p < 0.05$).

NGF mRNA expression was similar among all groups at the 7th and 21st days ($p > 0.05$). At the 14th day, however, there was a significant increase in NGF mRNA expression in the EDTA + Blood group ($p < 0.05$) when compared with the expression in the other groups (Fig. 3).

Discussion:

Regenerative endodontic treatment (RET) is a biological procedure designed to replace damaged structures (Murray *et al.,* 2007). To establish reliable protocols for RET, the influence of each step of RET, including irrigation, dentin treatment, and scaffold selection, on the expected outcome must be evaluated (Peters, 2014). The clinical success of RET has been described previously (Murray *et al.*, 2007; Jung *et al.*, 2008; Raju *et al.,* 2014), although few studies have attempted to analyze the longitudinal immune response to tissue engineering. Here, we evaluated the mRNA expression profiles of immune mediators across 21 days after treatment. Two different RET protocols were tested. To this end, a well-established mouse model was selected.

Soon after the teeth of mice were devitalized, root canals were or were not irrigated with EDTA and, subsequently, bleeding was induced to form a BC within the root canal systems (Shah *et al.*, 2009 Yamauchi *et al.*, 2011; Bucchi *et al.*, 2017). A group that did not receive any treatment was used as control.

IL-1 is a cytokine that exhibits potent inflammatory action and is highly associated with bone resorption (Brito *et al.*, 2012). We observed greater expression of this cytokine in the EDTA + Blood group on the 7th and the 14th days after treatment. Interestingly, IL-1 expression decreased throughout the experiment and was significantly reduced by the 21st day. It is important to point out that in addition to EDTA acting on the dentin matrix to release impregnated factors, dentin proteins also recruit cells involved in the inflammatory immune response (Galler *et al.*, 2011).

IL-10 is a cytokine that regulates the immune-inflammatory response and is synthesized by macrophages and keratinocytes (Mosmann & Sad, 1996). On the 7th day following treatment, IL-10 mRNA expression levels were not different from basal levels in all groups, but its regulatory activity was evident; on the 14th day post treatment, its expression level in the EDTA + Blood group peaked in response to increased expression of IL-1 on the 7th and 14th days. After the level of IL-1 was stabilized, IL-10 expression decreased in turn by the 21st day in the EDTA + Blood group. IL-10 and IL-1 cytokines play opposing roles in the immune-inflammatory response, which enables cross-immune regulation by type-1 cytokines of type-2 cytokines and vice versa (Mosmann & Sad, 1996). This model suggests that IL-10 plays a pivotal role in the REP by regulating the expression of IL-1 and promoting the anti-inflammatory behavior to maintain favorable conditions for pulp restoration. T regulatory (Treg) cells are defined by their ability to produce high levels of IL-10 and TGF-β; these cytokines mediate their ability to suppress pathological immune responses (Levings *et al.*, 2002)

Transforming growth factor-β (TGF-β) superfamily signaling plays a critical role in the regulation of cell growth, differentiation, and development in a wide range of biological systems (Horbelt *et al.*, 2012). Increased expression of TGF-β mRNA in the EDTA + Blood group on the 7th day post-treatment was followed by a significant decrease in its expression by the 14th day. In the next period of evaluation, its

expression was significantly increased in both the Blood and EDTA + Blood groups. The high expression of TGF-β on the 7th and 14th days in the Blood groups likely maintains ideal conditions for the promotion of regenerative procedures. In this regard, Ricucci *et al.* (2014) state that undifferentiated mesenchymal cells have the potential to regenerate new dentin tissues if an appropriate inducing stimulus is provided, such as the cell signals promoted by TGF-β.

Despite the importance of VEGF signaling in both vasculogenesis (the *de novo* formation of the embryonic circulatory system) and angiogenesis (the growth of blood vessels from pre-existing vasculature), in this study, we found that VEGF gene expression was at basal levels across all experimental time points. However, VEGF production can be induced in cells that are not receiving enough oxygen (Holmes *et al.*, 2007), and its timing of expression corresponds with endogenous re-vascularization (Mecollari *et al.,* 2014). The blood scaffold may have supplied enough oxygen to inhibit VEGF expression, since growth and differentiation factors exert their effects for short periods of time (Gazivoda *et al.*, 2009) and require a constant source of stimuli to act over long periods (Pang *et al.*, 2014).

IGF exerts a wide range of physiological actions, including the ability to induce cell proliferation, increase the number of mitotic cycles, and inhibit cellular apoptosis. Hence, IGF contributes to cell proliferation, differentiation and survival (Heemskerk *et al.*, 1999; Tsukahara *et al.,* 1994). The higher expression of IGF mRNA in the EDTA + Blood group on the 7th day compared to the expression on the 14th day was paralleled by increased IL-1 expression on the 7th compared to that on the14[th]day. Furthermore, IL-1 has been shown to be capable of suppressing the action of IGF (Lang *et al.*, 1996).

NGF acts extensively on dental and mesenchymal cells during the early stages of odontogenesis, and its expression is correlated with odontoblastic differentiation from mesenchymal cells, synthesis of the dentin matrix, and the mineralizing process (Arany *et al.*, 2009; Mitsiadis and Pagella, 2016; Mitsiadis *et al.*, 2017). In this study, the expression of NGF mRNA was only significant in the EDTA + Blood group at day 14, suggesting that the use of EDTA potentiates the release of differentiation factors that are favorable to the regeneration of pulp tissue.

Taken together, our data suggest that the use of EDTA as an irrigant solution during endodontic regenerative treatment represents an important step in surgical protocols, as has been suggested elsewhere (Pang *et al.*, 2014; Yamauchi *et al.*, 2011). EDTA promoted increased expression of factors associated with the differentiation of stem cells, thereby playing an important role in the formation of new pulp tissues. Finally, the use of an animal model allowed for the analysis of the expression of growth and differentiation factors released during regenerative pulp tissue at different time points during treatment and despite the limitations of the use of the animal model, this is the first study to longitudinally describe the expression of cytokines after the institution of REP, which is not possible to analyze in humans, with the currently available methods

References:

Aksel H, Serper A. Recent considerations in regenerative endodontic treatment approaches. J Dent Sci. 2014; 9:207-213.

Arany S, Koyota S, Sugiyama T. Nerve growth factor promotes differentiation of odontoblast-like cells. J Cell Biochem. 2009; 106:539-545.

Brito LCN, Teles FRF, Teles RP, Totola AH, Vieira LQ, Sobrinho APR. T-lynphocyte and cytokine expression in human inflammatory periapical lesions. J Endod 2012; 38:481-85.

Bucchi C, Gandur IV, Bizjak RS, Walton VT, Céspedes CM. Regenerative endodontic therapy: a systematic review of clinical protocols. Int J Clin Exp Med. 2017; 10:2006-2015.

Danin J, Linder LE, Lundqvist G, Andersson L. Tumor necrosis factor-alpha and transforming growth factor-beta$_1$ in chronic periapical lesions. OOOOE. 2000; 90:514-517.

Diogenes AR, Ruparel NB, Teixeira FB, Hargreaves KM. Translational science in disinfection for regenerative endodontics. J Endod. 2014; 40:S52-S57.

Galler KM, Buchala W, Hiller KA, Federlin M, Eidt A, Schiefersteiner M *et al*. Influence of root canal disinfectants on growth factor release from dentin. J Endod. 2015; 41:363-368.

Galler KM, D'Souza RN, Federlin M, Cavender AC, Hartgerink JD, Hecker S, *et al*. Dentin conditioning codetermines cell fate in regenerative endodontics. J Endod. 2011; 37:1536-1541.

Gazivoda D, Dzoplaic T, Bozic B, Tatomirovic Z, Brkic Z, Colic M. Production of proinflammatory and immunoregulatory cytokines by inflammatory cells from periapical lesions in culture. J Oral Pathol Med. 2009; 38:605-611.

Geisler, TM. Clinical considerations for regenerative endodontic procedures. Dent Clin N Am. 2012; 56:603-626.

Gonçalves LF, Fernandes AF, Cosme-Silva L, Colombo FA, Martins NS, Oliveira TM, et al. Effect of EDTA on TGF-β1 released form the dentin matrix and its influence on dental pullp stem cell migration. Braz Oral Res. 2016; 30:1-7.

Heemskerk VH, Daemen MARC, Buurman WA. Insulin-like growth factor-1 (IGF-1) and growth hormone (GH) in immunity and inflammation. Cytokine & Growth Fact Rev. 1999; 10:5-14.

Holmes K, Roberts OLI, Thomas AM, Cross MJ. Vascular endothelial growth factor receptor-2: Structure, function, intracellular signaling and therapeutic inhibition. Cell Signal. 2007; 19:2003-2012.

Horbelt D, Denkis A, Knaus P. A portrait of Transforming growth factor β superfamily signaling: Background matters. Int J Biochem. 2012; 44:469-74.

Jung IY, Lee SJ, Hargreaves KM. Biologically based treatment of immature permanent teeth with pulpal necrosis: a case series. J Endod. 2008; 34:876–87.

Kontakiotis EG, Filippatos CG, Tzanetakis, Agrafioti A. Regenerative endodontic therapy: A data analysis of clinical protocols. J Endod2015;41:146-54.

Lang CH, Fan J, Cooney R, Vary TC. IL-1 receptor antagonist attenuates sepsis-induced alterations in the IGF system and protein synthesis. Am J Physiol. 1996; 33: E430-E437.

Levings MK, Bacchetta R, Schulz U, Roncarolo MG. The role of IL-10 and TGF-β in the differentiation and effector function of T regulatory cells. Int Arch Allergy Immunol. 2002; 129:263-276.

Maciel KF, Brito LCN, Tavares WL, Moreira G, Nicoli JR, Vieira LQ, Ribeiro Sobrinho AP. Cytokine expression in response to root canal infection in gnotobiotic mice. Int Endod J 2012; 45:354-62.

Mattuella LG, Bento LW, Figueiredo JAP, Nör JE, Araujo FB, Fossati ACM. Vascular endothelial growth factor and its relationship with the dental pulp. J Endod. 2007; 33:524-530.

Mecollari V, Nieuwenhuis B, Verhaagen J. A perspective on the role of class III semaphoring signaling in central nervous system trauma. Frontiers in Cell Neurosc. 2014; 8:328

Mitsiadis TA, Magloire H, Pagella P. Nerve growth factor signaling n pathology and regeneration of human teeth. Scient Reports. 2017; 7:1327.

Mitsiadis TA, Pagella P. Expression of nerve growth factor (NGF), TrkA, and p75^{NTR} in developing human fetal teeth. Front Physiol. 2016; 7:1-10.

Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today. 1996; 17:138-146.

Murray PE, Garcia-Godoy F, Hargreaves KM. Regenerative Endodontics: A review of current status and a call for action. J Endod. 2007; 33:377-390.

Pang NS, Lee SJ, Kim E, Shin DM, Cho SW, Park W, et al. Effect of EDTA on attachment and differentiation of dental pulp stem cells. J Endod. 2014; 40:811-817.

Peters, OA. Translational opportunities in stem cell-based endodontic therapy: where are we and what are we missing? J Endod. 2014; 40:S82-S85.

Raju SMK, Yadav SS, Kumar SR. Revascularization of immature mandibular premolar with pulpal necrosis – a case report. J Clin Diag Res. 2014; 8:29-31.

Ricucci D, Loghin S, Lin LM, Spangberg LSW, Tay FR. Is hard tissue formation in the dental pulp after the death of the primary odontoblasts a regenerative or a reparative process? J Dentis. 2014; 42:1156-1170.

Sasaki H, Hou L, Belani A, Wang CY, Uchiyama T, Müller R, *et al*. IL-10, but not IL-4, suppresses infection-stimulated bone resorption *in vivo*. J Immunol. 2000; 165:3626-3630.

Shah N, Logani A, Bhaskar U, Aggarwal V. Efficacy of revascularization to induce apexification/apexogensis in infected, nonvital, immature teeth: A pilot clinical study. J Endod. 2008; 34:919-925.

Silva MJB, Vieira LQ, Ribeiro Sobrinho A. The effects of mineral trioxide aggregates on cytokine production by mouse pulp tissue. Oral Surg Oral Pathol Oral Rad and Endod 2008; 105:70-76.

Smith AJ, Duncan HF, Dent M, Diogenes A, Simon S, Cooper PR. Exploiting the bioactive properties of the dentin-pulp complex in regenerative endodontics. J Endod. 2016; 42:47-56.

Torabinejad M and Turman M. Revitalization of tooth with necrotic pulp and open apex by using platelet-rich plasma: a case report. J Endod 2011; 37:265-68.

Torabinejad M, Faras H. A clinical and histological report of a tooth with an open apex treated with regenerative endodontics using platelet-rich plasma. J Endod. 2012; 38:864-868.

Tsukahara H, Gordienko DV, Tonshoff B, Gelato MC, Goligorsky MS. Direct demosntration of insulin-like growth factor-1-incued nitric oxide production by endothelial cells. Kidney Int. 1994; 45:598-604.

Yamauchi N, Nagaoka H, Yamauchi S, Teixeira FB, Miguez P, Yamauchi M. Immunohistological characterization of newly formed tissues after regenerative procedure in immature dog teeth. J Endod. 2011; 37:1636-1641.

9 CONSIDERAÇÕES FINAIS

O sucesso dos procedimentos endodônticos regeneradores é dependente de uma tríade de fatores interrelacionados entre si. Dentre esses, podemos atuar clinicamente sobre dois deles: através do uso de um *scaffold* com propriedades mais compatíveis aos objetivos almejados, e/ou através do fornecimento dos fatores de crescimento e diferenciação celular necessários para a formação dos novos tecidos.

Os resultados do presente trabalho nos permitem concluir que: a) o uso do coágulo sanguíneo como *scaffold* atuou como um modulador da resposta imuno-inflamatória, agindo de forma a aumentar a expressão de mediadores anti-inflamatórios nos tecidos pulpares, assim como diminuindo a expressão dos mediadores pró-inflamatórios; b) a presença do coágulo estava associada à maior expressão de fatores promotores da diferenciação celular em odontoblastos, o que pode ser considerado como algo positivo; c) o uso do EDTA como solução irrigadora durante os procedimentos endodônticos regeneradores promoveu respostas imuno-inflamatórias mais intensas que os demais grupos; d) o uso do EDTA aumentou a liberação dos mediadores associados à proliferação e diferenciação celular; e) esse efeito foi mais intenso nos primeiros 7 dias, após o que as expressões desses mediadores sofreram significativas reduções.

REFERÊNCIAS

AKSEL, H; SERPER, A. Recent considerations in regenerative endodontic treatment approaches. **J. Dent. Sci.**, Taipei, v. 9, n.1, p. 207-213, Sep. 2014.

ALMUSHAYAT, A. *et al*. Dentin matrix protein-1 induces cytodifferentiation of human dental pulp stem cells into odontoblasts. **Gene Ther.** Houndmills**,** v. 13, n. 7, p. 611-620, Apr. 2006.

ALTAII, M. *et al*. Histological assessment of regenerative endodontic tratment in animal studies with diferente scaffolds: a systematic review. **Dent. Traumatol.,** Copenhagen, v. 33, n. 4, p. 235-244, Aug. 2017.

ALRAHABI, MK. *et al*. Root canal revascularization. The beginning of a new era in endodontics. **Saudi Med. J.,** Riyadh, v. 35, n. 5, p. 429-434, May. 2014.

ANDREASEN, JO. *et al*. Comparison of fracture resistance in root canals of immature sheep teeth after filling with calcium hydroxide or MTA. **Dental Traumatol.**, Copenhagen, v. 22, n. 3, p. 154-156, Jun. 2006.

BANCHS, F. *et al*. Revascularization of immature permanent teeth with apical periodontitis: new treatment protocol? **J Endod.**, Chicago, v. 30, n. 4, p. 196-200, Apr. 2004.

BEZGIN, T. *et al*. Efficacy of platelet-rich plasma as a *scaffold* in regenerative endodontic treatment. **J Endod.**, Chicago, v. 41, n. 1, p. 36-44, Jan. 2015.

BUCCHI, C. *et al*. Regenerative endodontic therapy: a systematic review of clinical protocols. **Int. J. Clin. Exp. Med.,** Madison, v. 41, n. 2, p. 146-154, Feb. 2015**.**

CAVIEDES, BJ. *et al*. Expression of insulin-like growth factor-1 and proliferating cell nuclear antigen in human pulp cells of teeth with complete and incomplete root development**. Int. Endod. J.**, Oxford, v. 42, n. 8, p. 686-693, Aug. 2009.

COTTI, E. *et al.* Regenerative treatment of an immature, traumatized tooth with apical periodontitis: report of a case. **J. Endod**., Chicago, v. 34, n. 5, p. 611-616, May 2008.

CVEK, M. Prognosis of luxated non-vital maxillary incisors treated with calcium hydroxide and filled with gutta-percha. A restrospective clinical study. **Endod. Dent. Traumatol.,** Copenhagen, v. 8, n. 2, p. 45-55, Apr. 1992.

DEL FABBRO, M. *et al.* Autologous platelet concentrates for pulp and dentin regeneration: a literature review of animal studies. **J. Endod.**, Chicago, v. 42, n. 2, p. 250-257, Feb. 2016.

DIANAT, O. *et al.* Endodontic repair in immature dog's teeth with apical periodontitis: blood clot vs plasma rich in growth factors scaffold. **Dent. Traumatol.**, Copenhagen, v. 33, n. 2, p. 84-90, Apr. 2017.

DING, RY. *et al.* Pulp revascularization of immature teeth with apical periodontitis: a clinical study. **J. Endod.**, Chicago, v. 35, n. 5, p. 745-749, May 2009.

DIOGENES, AR. *et al.* Translational science in disinfection for regenerative endodontics. **J. Endod.**, Chicago, v. 40, n. 4, p. S52-S57, Apr. 2014.

EL-SHARKAWY, H. *et al.* Platelet-rich plasma: growth factors and pro and anti-inflammatory properties. **J. Periodontol.**, Chicago, v. 78, n. 4, p. 661-669, Apr. 2007.

ESTRELA, C. *et al.* Mesenchymal stem cells in the dental tissues: Perspectives for tissue regeneration. **Braz. Dent. J.,** Ribeirão Preto, v. 22, n. 2, p. 91-98, 2011.

GAGE, FH. Mammalian neural stem cells.**Science**, Washington, DC, v. 287, p. 1433-1438, Feb. 2000.

GALLER, KM. *et al.* Influence of root canal disinfectants on growth factor release from dentin. **J. Endod.**, Chicago, v. 41, n. 3, p. 363-368, Mar. 2015.

GALLER, KM. *et al.* Dentin conditioning codetermines cell fate in regenerative endodontics. **J. Endod.**, Chicago, v. 37, n. 11, p. 1536-1541, Nov. 2011.

GALLER, KM. *et al.* EDTA conditioning of dentine promotes adhesion, migration and differentiation of dental pulp stem cells**. Int. Endod. J.**, Oxford, v. 49, n. 6, p. 581-590, Jun. 2016.

GANESH, M. *et al.* The regenerative endodontics – review. **Int. J. Innov. Res. Dent. Sci.**, v. 2, n. 6, p. 150-161, Jun. 2017.

GEISLER, TM. Clinical considerations for regenerative endodontic procedures. **Dent. Clin. N. Am.**, Philadelphia, v, 56, n. 3, p. 603-626, Jul. 2012.

GOMES-FILHO, JE. *et al.* Histologic characterization of engineered tissues in the canal space of closed-apex teeth with apical periodontitis. **J. Endod.**, Chicago, v. 39, n. 12, p. 1549-1556, Dec. 2013.

HARGREAVES, K. *et al.* Regeneration potential of the young permanent tooth: what does the future hold? **J. Endod.**, Chicago, v. 34, n. 7, p. S51-S56, Jul. 2008.

HOWARD, C. *et al.* Dental pulp stem cell migration. **J. Endod.**, Chicago, v. 36, n. 12, p. 1963-1966, Dec. 2010.

HUANG, GTJ. Apexification: the beginning of its end. **Int. Endod. J.**, Oxford, v. 42, n. 10, p. 855-866, Oct. 2009.

HUANG, GTJ. Dental pulp and dentin tissue engineering and regeneration: advancement and challenge. **Front. Biosci.**, Searington, v. 1, n. 3, p. 788-800, Jan. 2011.

HUANG, GTJ. *et al.* The hidden treasure in apical papilla: the potential role in pulp/dentin regeneration and bioroot engineering. **J. Endod.**, Chicago, v. 34, n. 6, p. 645-651, Ju. 2008.

IWAYA, SI. *et al.* Revascularization of an immature permanent tooth with apical periodontitis and sinus tract. **Dent. Traumatol.**, Copenhagen, v. 17, n. 4, p. 185-187, Aug. 2001.

JADHAV, G. *et al.* Revascularization with and without patelet-rich plasma in nonvital, immature, anteriior teeth: a pilot clinical study. **J. Endod.**, Chicago, v. 38, n. 12, p. 1581-1587, Dec. 2012.

JUNG, IY. *et al.* Biologically based treatment of immature permanent teeth with pulpal necrosis: a case series. **J. Endod.**, Chicago, v. 34, n. 7, p. 876-887, Jul. 2008.

KEREKES, K. *et al.* Follow-up examination of endodontic treatment in traumatized juvenile incisors. **J. Endod.**, Chicago, v. 6, n. 9, p. 744-748, Sep. 1980.

KESWANI, D. *et al.* RK. Revascularization of an immature tooth with a necrotic pulp using platelet-rich fibrin: a case report. **Int. Endod. J.**, Oxford, v. 46, n. 11, p. 1096-1104, Nov. 2013.

KIM, SG. *et al.* Effects of growth factors on dental stem/progenitor cells. **Dent. Clin. N. Am.**, Philadelphia, v. 56, n. 3, p. 563-575, Jul 2012.

LOVELANCE, TW. *et al.* Evaluation of the delivery of mesenchymal stem cells into the root canal space of necrotic immature teeth after clinical regenerative endodontic procedures. **J. Endod.**, Chicago, v. 37, n. 2, p. 133-138, Feb. 2011.

MARTIN, DE. *et al.* Concentration dependente effect of sodium hypochlorite ons tem cells of apical papilla survival and differentiation. **J. Endod.**, Chicago, v. 40, n. 1, p. 51-55, Jan. 2014.

MARTIN, G. *et al.* Histological findings of revascularized/revitalized immature permanente molar with apical periodontitis using platelet-rich plasma. **J. Endod.**, Chicago, v. 39, n. 1, p. 138-144, Jan. 2013.

MARX, RE. Platelet-rich plasma: evidence to support its use. **J. Oral Maxillofac. Surg.**, Philadelphia, v. 62, n. 4, p. 489-496, Apr. 2004.

MURRAY, PE. *et al.* Regenerative Endodontics: A review of current status and a call for action. **J. Endod.**, Chicago, v. 33, n. 4, p. 377-390, Apr. 2007.

NAGY, MM. *et al.* Regenerative potential of immature permanent teeth with necrotic pulps after different regenerative protocols. **J. Endod.**, Chicago, v. 40, n. 2, p. 192-198, Feb. 2014.

NAKASHIMA, M. *et al.* The application of tissue engineering to regeneration of pulp and dentin in endodontics. **J. Endod.**, Chicago, v. 31, n. 10, p. 711-718, Oct. 2005.

NOSRAT, A. *et al.* Regenerative endodontic treatment (revascularization) for necrotic immature permanent molars: a review and report of two cases with a new biomaterial. **J. Endod.**, Chicago, v. 37, n. 4, p. 562-567, Apr. 2011.

ÖSTBY, NB. The role of the blood clot in endodontic therapy: an experimental histologic study. **Acta Odontol. Scand.**, Stockholm, v. 19, p. 324-353, Dec. 1961.

ÖSTBY, NB. *et al.* Tissue formation in the root canal following pulp removal. **Scand. J. Dent. Res.**, Copenhagen, v. 79, n. 5, p. 333-339, 1971.

PETERS, OA. Translational opportunities in stem cell-based endodontic therapy: where are we and what are we missing? **J. Endod.**, Chicago, v. 40, n. 4, p. S82-S85, Apr. 2014.

RAJU, SMK. *et al.* Revascularization of immature mandibular premolar with pulpal necrosis – a case report. **J. Clin. Diag. Res.**, India, v. 8, n. 9, p. 29-31, Sep. 2014.

RING, KC. *et al.* The comparison of the effect of endodontic irrigation on cell adherence to root canal dentin. **J. Endod.**, Chicago, v. 34, n. 12, p. 1474-1479, Dec. 2008.

ROSEMBERG, B. *et al.* The effect of calcium hydroxide root filling on dentin fracture strength. **Dental Traumatol.**, Copenhagen, v. 23, n. 1, p. 26-29, Feb. 2007.

SEO, BM. *et al.* Investigation of multipotent postnatal stem cells from human periodontal ligament. **Lancet**, Boston, v. 364, n. 9429, p. 149-155, Jul. 2004.

SHABAHANG, S. *et al.* A comparative study of root-end induction using osteogenic protein-1, calcium hydroxide, and mineral trioxide aggregate in dogs. **J. Endod.**, Chicago, v.25, n. 1, p. 1-5, Jan. 1999.

SHAH, N. *et al.* Efficacy of revascularization to induce apexification/apexogensis in infected, nonvital, immature teeth: A pilot clinical study**. J Endod.** 2008; 34:919-925.

SHARMA, S. *et al.* Regeneration of tooth pulp and dentin: trends and advances. **Ann. Neurosci.**, Lucknow, v. 17, n. 1, p. 31-43**,** 2010.

SILVA, MJB. *et al.* The effects of mineral trioxide aggregates on cytokine production by mouse pulp tissue. **Oral Surg. Oral Pathol. Oral Radiol. Endod.**, St. Louis, v. 105, n. 5, p. 70-76, May 2008.

SIMON, S. *et al.* The use of mineral trioxide aggregate in one visit apexification treatment: a prospective study. **Int. Endod. J.**, Oxford, v. 40, n. 3, p. 186-197, Mar. 2007.

SOUZA, VF. *et al.* Células-tronco: uma breve revisão. **R. Ci. Méd. Biol**., Salvador, v. 2, n. 2, p. 251-256, Jul/Dez. 2003.

SKOGLUND, A. *et al.* Pulpal changes in replanted and autotransplanted immature teeth of dogs. **J. Endod.**, Chicago, v. 7, n 7, p. 309-316, Jul. 1981.

SMITH, AJ. *et al.*Exploiting the bioactive properties of the dentin-pulp complex in regenerative endodontics. **J. Endod.**, Chicago, v. 42, n. 1, p. 47-56, Jan. 2016.

SLOAN, AJ. *et al.* Expression of TGF-β receptors I and II in the human dental pulp by *in situ* hybridization. **Adv. Dent. Res.**, Washington, v. 15, p. 63-67, Aug. 2001.

SONOYAMA, W. *et al.* Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. **J. Endod.**, Chicago, v. 34, n. 2, p. 166-171, Feb. 2008.

STEINING, TH. *et al.* The use and predictable placement of mineral trioxide aggregate in one-visit apexification cases. **Aust. Endod. J.**, Melbourne, v. 29, n. 1, p. 34-42, Apr. 2003.

THIBODEAU, B. *et al.* Pulp revscularization of a necrotic infected immature permanent tooth: a case report and review of the literature. **Pediatr. Dent.**, Chicago, v. 29, n. 1, p. 47-50, Jan-Feb. 2007.

TORABINEJAD, M. *et al.* Clinical applications of mineral trioxide aggregate. **J. Endod.**, Chicago, v. 25, n. 3, p. 197-205, Mar. 1999.

TORABINEJAD, M. *et al.* A clinical and histological report of a tooth with an open apex treated with regenerative endodontics using platelet-rich plasma. **J. Endod.**, Chicago, v. 38, n. 6, p. 864-868, Jun. 2012.

WANG, X. *et al.* Histologic characterization of regenerated tissues in canal space after the revitalization/revascularization procedures of immature dog teeth with apical periodontitis. **J. Endod.**, Chicago, v. 36, n. 1, p. 56-63, Jan. 2010.

WIGLER, R. *et al.* Revascularization: a treatment for permanent teeth with necrotic pulp and incomplete root development. **J. Endod.**, Chicago, v. 39, n. 3, p. 319-326, Mar. 2013.

YAMAUCHI, N. *et al.* Immunohistological characterization of newly formed tissues after regenerative procedure in immature dog teeth. **J. Endod.**, Chicago, v. 37, n. 12, p. 1636-1641, Dec. 2011.

YU, J. *et al.* Differentiation of dental pulp stem cells into regular-shaped dentin-pulp complex induced by tooth germ cell conditioned médium. **Tissue Eng.**, New York, v. 12, n. 11, p. 3097-3105, Nov. 2006.

ZENG, Q. *et al.* Release of growth factors into root canal by irrigations in regenerative endodontics. **J. Endod.**, Chicago, v. 42, n. 12, p. 1760-1766, Dec**.** 2016.

ZHANG, DD. *et al.* Histologic comparison between platelet-rich plasma and Blood Clot in regenerative endodontic treatment: an animal study. **J. Endod.**, Chicago, v. 40, n. 9, p. 1388-1393, Sep. 2014.

ZHANG, R. *et al.* Angiogenic activity of dentin matrix componentes. **J. Endod.**, Chicago, v. 37, n. 1, p. 26-30, Jan. 2011.

ANEXO A - Atividades desenvolvidas durante o curso de doutorado

Atuação profissional

Centro de Especialização em Odontologia – CESO

2014 – atual: Vínculo: Professor. Enquadramento funcional:

Professor nos cursos de Especialização em Ortodontia. Carga horária: 16 horas/mensais

Universidade Federal de Juíz de Fora – Campus Governador Valadares

2017: Vínculo: Professor Substituto, Enquadramento funcional:

Professor de Cirurgia e Pacientes com Necessidades Especiais. Carga horária: 20 horas/semanais.

Faculdades Doctum – Campus Teófilo Otoni

2017: Vínculo: Professor convidado, Enquadramento funcional:

Professor convidado a compor o Núcleo Docente Estruturante para a implementação do curso de Odontologia. Carga horária: 40 horas/mês.

Artigos completos publicados em periódicos:

- Rôças IN, Lima KC, Assunção IV, Gomes PN, Bracks IV, Siqueira Jr JF. J Advanced caries microbiota in teeth with irreversible pulpitis. J Endod. 2015; 41:1450-1455.

Artigos completos submetidos:

- Igor Vieira Bracks, Marcela Carvalho Espaladori, Leda Quercia Vieira e Antônio Paulino Ribeiro Sobrinho.Evaluation of gene expression. of growth factors after regenerative endodontic treatment.

Orientação de Trabalho de Conclusão de Curso:

- Impacto dos agravos em saúde bucal de pacientes cardiopatas internados em enfermarias do Hospital Municipal de Governador Valadares. Aluna Ismênia Edwirges Bernades. Faculdade de Odontologia da UFJF – Campus Governador Valadares. 2017.

- Odontologia Hospitalar: Panorama atual. Aluno Guilherme Freitas Menezes. Faculdade de Odontologia da UFJF – Campus Governador Valadares. 2017.

Cursos e Aulas Ministradas

- Atualização em Instrumentos de Níquel-Titânio na Endodontia. CESO. 60 horas/aula. Setembro de 2016

- Atualização em Instrumentos de Níquel-Titânio na Endodontia. CESO. 60 horas/aula. Setembro de 2017

- Estética Rosa. CRO (Delegacia de Teófilo Otoni). 4 horas/aula. Outubro de 2017

- Tratamento Endodôntico Regenerador: Da necrose à vitalidade pulpar. 4 horas/aula. Março de 2017

- Atualização em Cirurgia Plástica Periodontal e Periimplantar. CESO. 36 horas/aula. Março de 2018

ANEXO B - Parecer do comitê de ética

UNIVERSIDADE FEDERAL DE MINAS GERAIS

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

UFMG

Prezado(a):

Esta é uma mensagem automática do sistema Solicite CEUA que indica mudança na situação de uma solicitação.

Protocolo CEUA: 255/2016
Título do projeto: Avaliação do Perfil Inflamatório Perirradicular em Tratamento Endodôntico Regenerador
Finalidade: Pesquisa
Pesquisador responsável: Antonio Paulino Ribeiro Sobrinho
Unidade: Faculdade de Odontologia
Departamento: Departamento de Odontologia Restauradora

Situação atual: Decisão Final

Aprovado na reunião do dia 21/11/2016.

Belo Horizonte, 01/12/2016.

Atenciosamente,

Sistema Solicite CEUA UFMG
https://aplicativos.ufmg.br/solicite_ceua/

Universidade Federal de Minas Gerais
Avenida Antônio Carlos, 6627 – Campus Pampulha
Unidade Administrativa II – 2º Andar, Sala 2005
31270-901 – Belo Horizonte, MG – Brasil
Telefone: (31) 3409-4516
www.ufmg.br/bioetica/ceua - cetea@prpq.ufmg.br

ANEXO C - Artigo científico

Clinical Research

Advanced Caries Microbiota in Teeth with Irreversible Pulpitis

Isabela N. Rôças, DDS, MS, PhD, * *Kenio C. Lima, DDS, MS, PhD,[†]*
Isauremi V. Assunção, DDS, MS, PhD,[†] Patrícia N. Gomes, DDS, MS,[†]
Igor V. Bracks, DDS, MS, * *and José F. Siqueira, Jr, DDS, MS, PhD* *

Abstract

Introduction: Bacterial taxa in the forefront of caries biofilms are candidate pathogens for irreversible pulpitis and are possibly the first ones to invade the pulp and initiate endodontic infection. This study examined the microbiota of the most advanced layers of dentinal caries in teeth with irreversible pulpitis. **Methods:** DNA extracted from samples taken from deep dentinal caries associated with pulp exposures was analyzed for the presence and relative levels of 33 oral bacterial taxa by using reverse-capture checkerboard hybridization assay. Quantification of total bacteria, streptococci, and lactobacilli was also performed by using real-time quantitative polymerase chain reaction. Associations between the target bacterial taxa and clinical signs/symptoms were also evaluated. **Results:** The most frequently detected taxa in the checkerboard assay were *Atopobium* genomospecies C1 (53%), *Pseudoramibacter alactolyticus* (37%), *Streptococcus* species (33%), *Streptococcus mutans* (33%), *Parvimonas micra* (13%), *Fusobacterium nucleatum* (13%), and *Veillonella* species (13%). *Streptococcus* species, *Dialister invisus*, and *P. micra* were significantly associated with throbbing pain, *S. mutans* with pain to percussion, and *Lactobacillus* with continuous pain (*P* < .05). Quantitative polymerase chain reaction revealed a mean total bacterial load of 1×10^8 (range, 2.05×10^5 to 4.5×10^8) cell equivalents per milligram (wet weight) of dentin. Streptococci and lactobacilli were very prevalent but comprised only 0.09% and 2% of the whole bacterial population, respectively. **Conclusions:** Several bacterial taxa were found in advanced caries lesions in teeth with exposed pulps, and some of them were significantly associated with symptoms. A role for these taxa in the etiology of irreversible pulpitis is suspected. (*J Endod 2015;41:1450–1455*)

Pulpitis is the inflammation of the dental pulp and is commonly a sequel to caries (1). Bacteria located in the advanced frontline of the caries biofilm are directly involved in inducing damage and consequent inflammation in the pulp tissue (2–4). The bacterial effects on the pulp are caused either by bacterial virulence factors and antigens that diffuse through the dentinal fluid or by the bacterial cells themselves, which may reach the pulp via dentinal tubules, especially in very profound caries cavities (5, 6).

Pulpitis can be clinically classified as reversible or irreversible (7). In the former condition, removal of the causative agent usually permits the pulp to return to normality, whereas in the latter condition, direct intervention in the pulp tissue may be required for improved treatment outcome (8). Irreversible pulpitis usually develops when the pulp is frankly exposed to the caries biofilm (6).

Many studies evaluating the microbiota associated with deep dentinal caries revealed that the bacterial composition is substantially different from enamel caries (4, 9–11). This is highly likely to be a result of different ecological conditions associated with these lesions. In addition to lactobacilli, which are very prevalent in dentinal caries (4, 12–14), asaccharolytic and/or proteolytic anaerobic bacteria have been frequently detected (9–11, 13–15). Most of the species in carious dentin have also been detected in infected root canals (11, 14, 16–22), suggesting that in addition to being involved with pulpal damage, these dentinal lesions might well be the primary source of bacteria that initiate endodontic infections. However, there are not many studies evaluating the microbiota of advanced caries lesions in association with pulp conditions. A study identified bacteria isolated from carious lesion biofilms and vital carious exposures of pulps of deciduous teeth and observed that the microbiota of the cariously exposed pulps were similar in composition to those of carious lesion biofilms except that fewer species/taxa were identified from the pulps (23). *Actinomyces* and *Selenomonas* species were associated with carious lesions, whereas *Veillonella* species were associated with pulps. Other studies have reported a close association between pain and the presence of *Prevotella*, *Porphyromonas*, and *Fusobacterium* species in deep dentinal caries (3, 4). Black-pigmented anaerobic bacteria and *Streptococcus mutans* have been positively related to pulpal pain triggered by heat, whereas *Fusobacterium nucleatum* and *Actinomyces viscosus* have been associated with cold sensitivity (24). Positive associations between *Parvimonas micra* and *Porphyromonas endodontalis* detection in carious dentin and irreversible pulpitis have been found (2).

Bacterial taxa present in the forefront of deep dentinal caries biofilms are candidate pathogens for irreversible pulpitis and are possibly the first ones to invade the pulp and initiate endodontic infection. The present study used reverse-capture checkerboard assay to evaluate the prevalence of several caries and endodontic bacterial pathogens in the most advanced layers of dentinal caries in teeth with the clinical diagnosis of irreversible pulpitis. Quantification of total bacteria, streptococci, and lactobacilli was also performed by using real-time quantitative polymerase chain reaction (qPCR). Associations between the presence and levels of the target bacterial taxa and clinical symptoms of irreversible pulpitis were also evaluated.

Materials and Methods

Subject Population

This study included 30 patients (23 female and 7 male) with deep occlusal caries in permanent maxillary or mandibular molars diagnosed with irreversible pulpitis. Patients ranged in age from 12 to 33 years. Each patient contributed 1 tooth. Medical history revealed no significant systemic condition or disease. Ethical approval for the study was granted by the Ethics Committee of the Federal University of Rio Grande do Norte, and informed consent was obtained from all subjects or their parents/guardians.

The diagnosis of irreversible pulpitis was based on clinical and radiographic findings and following the reports of the American Association of Endodontists Consensus Conference on diagnostic terminology (7). All cases had extensive caries lesions that led to pulp exposure. Intensity of pain was evaluated by using a visual analogue scale ranging from 0 (no pain) to 170 mm (severe pain). This scale permitted pain intensity to be ranked as mild, moderate, or severe. If present, pain was also recorded as provoked or spontaneous, intermittent or continuous, cold- or heat-evoked, localized or diffuse, and throbbing or after physical efforts. Pulp status was evaluated by thermal sensibility tests. Radiographic analysis involved extent of the caries lesion, presence of coronal restoration, stage of apical root formation, and conditions of the apical periodontal ligament space. Teeth with necrotic pulps or treated root canals and teeth with no evidence of pulp exposure after dentinal caries removal were excluded from the study.

Sample Taking and DNA Extraction

Selected teeth were cleaned with pumice, and the patient was anesthetized. Undermined enamel, superficial carious tissue, and debris were removed by using high-speed burs under water cooling. The target tooth was isolated with rubber dam, and the operative field, including the tooth, was cleaned with 6% hydrogen peroxide and disinfected with 2.5% sodium hypochlorite (NaOCl). The latter solution was inactivated with 10% sodium thiosulfate. The superficial layers of the caries lesion were removed by using a sterile spoon excavator and dismissed. Another sterile excavator was used to collect the deepest layer of dentinal caries in direct contact with the pulp, which was then transferred to cryotubes containing Tris-EDTA buffer. Transference of the material to the flasks in the clinical setting was always performed in the aseptic zone around a flame. Samples were immediately frozen at $-20°C$.

Caries dentin samples were weighed (wet weight), and DNA was extracted by using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA), following the protocol recommended by the manufacturer for tissues. DNA from a panel of several oral bacterial species was also prepared to serve as controls (25).

Reverse-capture Checkerboard Assay

The reverse-capture checkerboard assay used in this study was as described previously (26–28). DNA extracted from clinical samples was used as template in a 2-step 16S rRNA gene-based PCR protocol. In the first step, a practically full-length 16S rRNA gene fragment was amplified by using universal primers 8f and 1492r (26, 29, 30). In the second step, the resulting PCR product from each sample was used as template to run 2 sets of partial 16S rRNA gene amplification; one set used primers digoxigenin-8f and 519r, and the other set used primers digoxigenin-515f and 1492r (27). PCR amplifications were performed in 50 μL reaction mixture containing 1 μmol/L of each primer, 5 μL of 10× PCR buffer (Fermentas, Burlington, ON, Canada), 3 mmol/L MgCl$_2$, 2 U Taq DNA polymerase (Fermentas), and 0.2 mmol/L of each deoxyribonucleoside triphosphate (Invitrogen Life Technologies, Carlsbad, CA). Negative controls consisted of sterile ultrapure water instead of sample and were included with each batch of samples analyzed. Temperature profile for the first PCR reaction was 95°C/1 min, 26 cycles at 94°C/45 s, 50°C/45 s, 72°C/1.5 min, and 72°C/15 min, and for the second step it was 95°C/5 min, 28 cycles at 94°C/30 s, 55°C/1 min, 72°C/1.5 min, and 72°C/20 min. PCR products were separated by electrophoresis in agarose gels, which were then stained with GelRed (Biotium, Hayward, CA) and visualized under ultraviolet transillumination.

Labeled PCR products were mixed by using equal proportions of each (40 μL) and used in the checkerboard assay to determine the presence and levels of 33 bacterial taxa by using probes described and validated previously (26, 27, 31). In addition to the taxon-specific probes, 2 universal probes were included in each checkerboard membrane to serve as controls. Two lanes in the membrane contained extracted DNA standards at the concentration of 10^5 and 10^6 cells, which were treated the same way as the clinical samples. The reverse-capture checkerboard assay was performed by using the Minislot-30 and Miniblotter-45 system (Immunetics, Cambridge, MA). First, 100 pmol of probe in TE buffer (10 mmol/L Tris HCl, 1 mmol/L EDTA, pH 8.0) was introduced into the horizontal wells of the Minislot apparatus and cross-linked to the Hybond- N+ nylon membrane (AmershamPharmacia Biotech, Little Chalfont, Buckinghamshire, UK) by ultraviolet irradiation by using a Stratalinker 1800 (Stratagene, La Jolla, CA) on autocross-link position. Each probe has a polythymidine tail that is preferentially cross-linked to the nylon and leaves the specific probe available for hybridization. The membrane was then prehybridized at 55°C for 1 hour. Subsequently, 80 μL of the labeled PCR products mixed with 60 μL of 55°C preheated hybridization solution was denatured at 95°C for 5 minutes and loaded on the membrane by using the Miniblotter apparatus. Hybridization was carried out at 54°C for 2 hours.

After blocking in a buffer with casein, the membrane was incubated in antidigoxigenin antibody conjugated with alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany) and then in ultrasensitive chemiluminescent substrate CDP Star (Roche Molecular Biochemicals). Finally, a square of x-ray film was exposed to the membrane in a cassette to detect the hybrids.

Quantitative Real-time PCR

To quantify the total bacterial load and levels of streptococci and lactobacilli in caries samples, 16S rRNA gene-targeted qPCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on an ABI 7500 Real-time PCR instrument (Applied Biosystems) in a total reaction volume of 20 μL. The primers used were as described and validated elsewhere (32–36) (Table 1).

Primers in a concentration of 0.5 μmol/L each and DNA extract volume of 2 μL were added to the PCR master mix in MicroAmp Optical

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TABLE 1. Primers Used for Bacterial Quantification in Samples from Advanced Caries Lesions in Teeth with Irreversible Pulpitis by Using Real-time PCR

Taxa	Primer sequences	Annealing temperature (°C)	Fragment length (base pairs)	Reference
Universal 16S rRNA gene	5′ – GAT TAG ATA CCC TGG TAG TCC AC – 3′ 5′ – TAC CTT GTT ACG ACT T – 3′	52	733	(32, 33)
Streptococcus species	5′ – AGA TGG ACC TGC GTT GT – 3′ 5′ – GCT GCC TCC CGT AGG AGT CT – 3′	60	~120–130	(34, 35)
Lactobacillus species	5′ – TGG AAA CAG RTG CTA ATA CCG – 3′ 5′ – GTC CAT TGT GGA AGA TTC CC – 3′	62	223	(36)

(Life Technologies) 96-well reaction plates. Plates were sealed, centrifuged, and then subjected to amplification. Cycling conditions for universal bacteria and streptococci included 95°C/10 min and 40 repeats of the following steps: 95°C/1 min, annealing for 1 min (temperature shown in Table 1), and 72°C/1 min. The temperature profile for lactobacilli quantification was 50°C/2 min, 95°C/10 min, 40 cycles at 95°C/15 s, and 62°C/1 min. At each cycle, accumulation of PCR products was detected by monitoring the increase in fluorescence of the reporter dye (dsDNA-binding SYBR Green). All measurements were done in triplicate for samples and standards. In all experiments, triplicates of appropriate negative controls containing no template DNA were subjected to the same procedures. After amplification, melting curve analysis of PCR products was performed to determine the specificity of the amplified products. Melting curve was obtained from 60°C to 95°C, with continuous fluorescence measurements taken at every 1% increase in temperature. Data acquisition and analysis were performed by using the ABI 7500 software v2.0.6 (Applied Biosystems).

Standard curves for quantification of streptococci and lactobacilli were constructed with known concentrations of genomic DNA extracted from *S. mutans* ATCC 25175 and *Lactobacillus casei* ATCC 393, respectively. *S. mutans* DNA was also used for total bacteria quantification by using the pair of universal primers. Because the levels of total bacteria cannot be precisely calculated because of the differences in numbers of *rrn* operons among oral bacteria, *S. mutans*, which contains 5 copies of the 16S rRNA gene, was used, with 5 being regarded as the approximate average copy number in the range of most oral bacteria (http://www.cbs.dtu.dk/services/GenomeAtlas-3.0). DNA extracts were 10-fold diluted from 10^7 to 10^2 cells in ultrapure water and used for making the standard curves. Relative amounts were calculated as the percentage of streptococci or lactobacilli out of the total bacterial load.

Data Analysis

Data from the checkerboard assay were evaluated as the prevalence of the target taxa in the samples examined. The presence/absence of bacteria was analyzed in relation to clinical conditions by using the χ^2 or the Fisher exact test, and prevalence ratio and confidence interval were calculated. The chemiluminescent signals were also analyzed with ImageJ (http://rsb.info.nih.gov/ij/) and converted to counts by comparison with standards at known concentrations run on each membrane. Because of the recognized difficulties in inferring absolute counts for samples amplified by end-point PCR used in the checkerboard assay and because estimates had to be made for counting as-yet-uncultivated phylotypes, counts were transformed into semiquantitative data and categorized as follows: a level below detection (or absence), a level $<10^5$ bacteria, a level $= 10^5$ bacteria, a level $>10^5$ to $<10^6$ bacteria, a level $= 10^6$ bacteria, and a level $>10^6$ bacteria. Data from qPCR quantification of total bacteria, streptococci, and lactobacilli levels per milligram (wet weight) of dentin were transformed into log numbers for statistical analysis. The Mann-Whitney and

Kruskall-Wallis tests were used to compare the semiquantitative data from checkerboard and the absolute bacterial counts from qPCR with the clinical parameters. For comparisons between groups after the Kruskal-Wallis test, the Mann-Whitney test with Bonferroni correction was used. Significance level was set at 5% ($P < .05$).

Results

Clinical conditions are shown in Table 2. All sample extracts were positive for the presence of bacterial DNA as demonstrated by pre-checkerboard PCR amplification and qPCR by using universal 16S rRNA gene primers. This also indicates that significant inhibitors of the PCR reaction were not present. Negative controls for PCR yielded no amplification.

Reverse-capture Checkerboard Assay

The results of the reverse-capture checkerboard analysis revealed that 15 of the 33 oligonucleotide probes tested were reactive with 1 or more dentinal samples. All samples but 2 were positive for at least 1 taxon-specific probe. The number of target taxa per sample ranged from 1 to 8.

The most frequently detected taxa were *Atopobium* genomospecies C1 (16 samples, 53%), *Pseudoramibacter alactolyticus* (11 samples, 37%), *Streptococcus* species (10 samples, 33%), *S. mutans*

TABLE 2. Description of Independent Clinical Variables

Variables	Categories	n	%
Coronal restoration	Yes	12	40
	No	18	60
Provoked pain	Present	14	47
	Absent	16	53
Spontaneous pain	Present	16	53
	Absent	14	47
Intermittent pain	Present	7	23
	Absent	23	77
Continuous pain	Present	9	30
	Absent	21	70
Localized pain	Present	21	70
	Absent	9	30
Diffuse pain	Present	2	7
	Absent	28	93
Cold-evoked pain	Present	25	83
	Absent	5	17
Heat-evoked pain	Present	7	23
	Absent	23	77
Pain to percussion	Present	5	17
	Absent	25	83
Throbbing pain	Present	12	40
	Absent	18	60
Pain after physical efforts	Present	0	0
	Absent	30	100
Visual analogue scale	Absent to mild	6	20
	Moderate	12	40
	Severe	12	40

(10 samples, 33%), *P. micra* (4 samples, 13%), *F. nucleatum* (4 samples, 13%), and *Veillonella* species (4 samples, 13%) (Fig. 1). Associations were found between some target taxa and clinical conditions. As for the presence/absence data, associations were found for *S. mutans* with pain to percussion and *P. micra* with throbbing pain. As for the semiquantitative data, the significant associations observed are depicted in Table 3.

Quantitative Real-time PCR

Analysis by using universal primers revealed a mean total bacterial load of 1×10^8, ranging from 2.05×10^5 to 4.5×10^8 per mg (wet weight) of dentin. Streptococci were detected in 28 of the 30 cases, whereas lactobacilli occurred in 29. The average numbers of cells of streptococci and lactobacilli were 3.6×10^5 and 2.32×10^6, respectively. As for proportion, streptococci comprised from 0.0004% to 91.5% of the total bacterial counts (mean, 4.3%; median, 0.12%), whereas lactobacilli comprised from 0.01% to 98.1% of the total bacteria (mean, 8.2%; median, 2%). The only significant association observed for the qPCR data was between lactobacilli and continuous pain ($P = .04$). Quantitative data are summarized in Table 4.
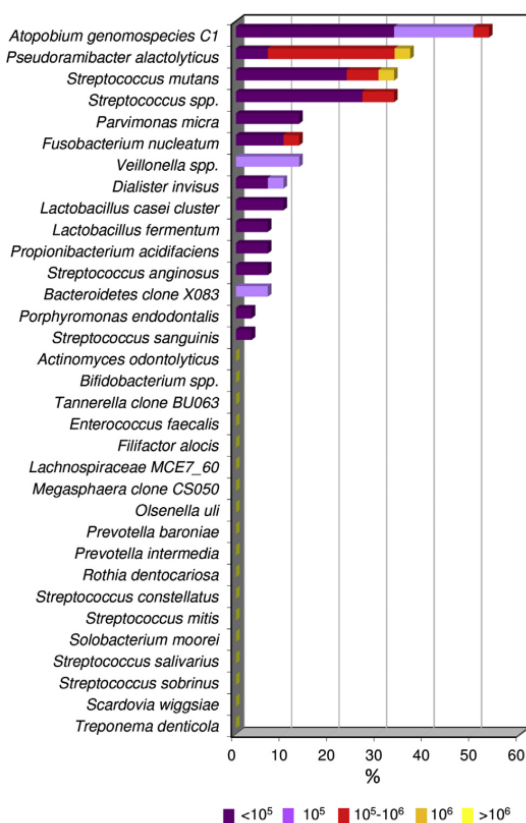

Figure 1. Stacked bar chart of frequency of detection and levels of bacterial species/phylotypes in deep dentinal caries lesions associated with pulp exposure and irreversible pulpitis. Total length of each bar stack indicates percentage of positive samples (ie, prevalence of bacterial species/phylotypes). Different colors within each bar indicate percentage of samples containing different levels of the species.

TABLE 3. Bacterial Taxa Significantly Associated with Demographic or Clinical Conditions Based on Semiquantitative Data from Reverse-capture Checkerboard Hybridization Assay

Bacterial taxa	*P* value	Variable
Streptococcus species	.03	Provoked pain
	.03	Intermittent pain
	.04	Throbbing pain
Dialister invisus	.03	Throbbing pain
	.03	Presence of restoration
Parvimonas micra	.01	Throbbing pain

Discussion

Bacteria present in the advanced front of carious dentin can be considered as etiologically significant in the development of pulpitis. In the present study, the checkerboard DNA hybridization method was used to evaluate the prevalence of several bacterial taxa commonly found in both deep dentinal caries lesions and in endodontic infections. There are numerous studies evaluating the microbiota associated with these 2 conditions separately (11, 14, 16–22). Because endodontic infections are usually a sequel to caries, studies evaluating the process in between (ie, initial pulp invasion by caries bacteria) may provide valuable information about the bacteria that cause irreversible pulpitis and participate in the early phases of pulp colonization preceding endodontic infections. There are not many studies in this regard, and the present one was intended to help bridge this gap in knowledge.

Of the taxa targeted in checkerboard, the most prevalent were *Atopobium* genomospecies C1, *P. alactolyticus*, *Streptococcus* species, *S. mutans*, *P. micra*, *F. nucleatum*, and *Veillonella* species. *Atopobium* genomospecies C1, *S. mutans* and other streptococci, and *Veillonella* species have been very frequently associated with advanced caries, whereas *P. alactolyticus*, *P. micra*, *F. nucleatum*, and *Streptococcus* species are commonly associated with endodontic infections. Therefore, it seems that there is a coexistence of caries and endodontic pathogens in very advanced caries lesions. This may indicate a shift in the microbiota as the caries process advances to the pulp. Shifts are expected to be governed by ecological changes in the affected tissues, including presence of inflammation and the dominant species in the consortium. For instance, inflammation may change the ecology by making some nutrients more abundant, especially glycoproteins from the inflammatory exudate, and/or may exert a selective pressure as a result of the host defense attack. Moreover, the most dominant species may set the stage for establishment of appropriate partners for metabolic interactions (37). Longitudinal studies in animals evaluating the microbiota shifts from initial caries to endodontic infection are required to confirm these assumptions and reveal the most significant species occurring in different phases of the pathologic process.

Some differences in the composition of the caries microbiota can be observed when comparing the present reverse-capture checkerboard findings with a previous study that used the very same method to evaluate the microbiota of the deepest layers of dentinal caries lesion with no pulp exposure (15). For instance, fewer species were found in the present study, and there were some subtle differences in prevalence of some taxa. Although several of the most prevalent taxa occurred in both studies, *P. micra* and *P. alactolyticus* were more commonly found in deep dentin layers associated with pulp exposures than in cases with no exposure. *P. micra* has been previously shown to be associated with irreversible pulpitis (2). Whether the high prevalence of these species in irreversible pulpitis cases represents a random finding or a shift in the biofilm composition remains to be determined. The

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TABLE 4. Prevalence and Quantitative Data for Total Bacteria, Streptococci, and Lactobacilli in Advanced Caries Lesions Associated with Irreversible Pulpitis as Determined by Quantitative Real-time PCR

Target	Prevalence (%)	Absolute counts per mg dentin			% Total bacteria		
		Mean	Median	Range	Mean	Median	Range
Total bacteria	30/30 (100)	1.00×10^8	3.07×10^7	2.05×10^5 to 4.50×10^8	100	100	100
Streptococcus species	28/30 (93)	3.60×10^5	2.89×10^4	1.32×10^2 to 2.63×10^6	4.3	0.12	0.0004–91.5
Lactobacillus species	29/30 (97)	2.32×10^6	5.66×10^5	1.85×10^2 to 1.26×10^7	8.2	2	0.01–98.1

reduced number of species and the appearance of new species may be a result of the ecological changes in the environment. Species more frequently found in advanced caries lesions in association with pulp exposures are candidate pathogens for irreversible pulpitis.

Some species were significantly associated with signs/symptoms of pulpitis. For instance, *Streptococcus* species, *Dialister invisus*, and *P. micra* were more frequent in cases with throbbing pain, whereas *S. mutans* was associated with pain to percussion. *Lactobacillus* species occurred in significantly higher levels in cases with continuous pain. Previous studies have described some species associated with pulpal symptoms, including black-pigmented anaerobic bacteria, *Fusobacterium* species, *S. mutans*, and *A. viscosus* (3, 4, 24). Some bacterial metabolic products have been demonstrated to be involved in the development of symptoms. Ammonia and indole, which are produced by many anaerobic bacteria found in deep caries, can make intradental sensory nerves more susceptible to stimuli that evoke pain (38, 39). The amount of lipopolysaccharide in caries was positively associated with pulpal pain (40). Lipopolysaccharide-mediated pain may be related to the proinflammatory effects of this bacterial component (41) or its direct effects on sensory nerve fibers (42).

Quantitative PCR analysis was also carried out to evaluate the presence and levels of streptococci and lactobacilli, which are bacterial groups very frequently associated with caries (13–15, 31, 36, 43). Both *Streptococcus* species and *Lactobacillus* species were much more prevalent in the qPCR analysis as compared with the checkerboard approach, and this can be explained by the higher sensitivity of the former. The high prevalence of these bacterial groups, especially lactobacilli, in deep caries lesions is in agreement with several previous reports in the literature (4, 12–15, 36). Counts of lactobacilli were significantly increased in cases of continuous pain. Although very frequent, both streptococci and lactobacilli were rarely found in high relative abundance. These bacterial groups corresponded to more than 10% of the total population in only 2 cases each. Further quantitative studies should reveal the bacterial taxa that dominate the microbiota in advanced caries lesions.

Knowledge of the microbiota involved in irreversible pulpitis may be invaluable for the development of therapeutic approaches to improve the predictability of vital pulp therapy. For instance, the use of antimicrobial agents that selectively target the species associated with irreversible pulpitis may open new perspectives on the conservative treatment of teeth with this condition.

In conclusion, this study identified some bacterial taxa associated with advanced caries lesions in teeth with irreversible pulpitis. Some species were more frequently detected in the presence of symptoms. Bacteria found in high prevalence in the forefront of caries lesions that resulted in pulp exposure may be important pathogens in evoking pulp inflammation. Also, they may be pioneer species in the pulp colonization process to initiate endodontic infections.

Acknowledgments

References

1. Bergenholtz G. Inflammatory response of the dental pulp to bacterial irritation. J Endod 1981;7:100–4.
2. Martin FE, Nadkarni MA, Jacques NA, Hunter N. Quantitative microbiological study of human carious dentine by culture and real-time PCR: association of anaerobes with histopathological changes in chronic pulpitis. J Clin Microbiol 2002;40:1698–704.
3. Massey WL, Romberg DM, Hunter N, Hume WR. The association of carious dentin microflora with tissue changes in human pulpitis. Oral Microbiol Immunol 1993;8:30–5.
4. Hahn CL, Falkler WA Jr, Minah GE. Microbiological studies of carious dentine from human teeth with irreversible pulpitis. Arch Oral Biol 1991;36:147–53.
5. Hoshino E, Ando N, Sato M, Kota K. Bacterial invasion of non-exposed dental pulp. Int Endod J 1992;25:2–5.
6. Ricucci D, Loghin S, Siqueira JF Jr. Correlation between clinical and histologic pulp diagnoses. J Endod 2014;40:1932–9.
7. Levin LG, Law AS, Holland GR, et al. Identify and define all diagnostic terms for pulpal health and disease states. J Endod 2009;35:1645–57.
8. Ricucci D, Siqueira JF Jr. *Endodontology: an integrated biological and clinical view.* London: Quintessence Publishing; 2013.
9. Edwardsson S. Bacteriological studies on deep areas of carious dentine. Odontol Revy 1974;32(Suppl):1–143.
10. Hoshino E. Predominant obligate anaerobes in human carious dentin. J Dent Res 1985;64:1195–8.
11. Aas JA, Griffen AL, Dardis SR, et al. Bacteria of dental caries in primary and permanent teeth in children and young adults. J Clin Microbiol 2008;46:1407–17.
12. Callaway A, Kostrzewa M, Willershausen B, et al. Identification of Lactobacilli from deep carious lesions by means of species-specific PCR and MALDI-TOF mass spectrometry. Clin Lab 2013;59:1373–9.
13. Chhour KL, Nadkarni MA, Byun R, et al. Molecular analysis of microbial diversity in advanced caries. J Clin Microbiol 2005;43:843–9.
14. Munson MA, Banerjee A, Watson TF, Wade WG. Molecular analysis of the microflora associated with dental caries. J Clin Microbiol 2004;42:3023–9.
15. Lima KC, Coelho LT, Pinheiro IV, et al. Microbiota of dentinal caries as assessed by reverse-capture checkerboard analysis. Caries Res 2011;45:21–30.
16. Schulze-Schweifing K, Banerjee A, Wade WG. Comparison of bacterial culture and 16S rRNA community profiling by clonal analysis and pyrosequencing for the characterization of the dentine caries-associated microbiome. Front Cell Infect Microbiol 2014;4:164.
17. Gross EL, Beall CJ, Kutsch SR, et al. Beyond *Streptococcus mutans*: dental caries onset linked to multiple species by 16S rRNA community analysis. PLoS One 2012;7:e47722.
18. Santos AL, Siqueira JF Jr, Rôças IN, et al. Comparing the bacterial diversity of acute and chronic dental root canal infections. PLoS One 2011;6:e28088.
19. Munson MA, Pitt-Ford T, Chong B, et al. Molecular and cultural analysis of the microflora associated with endodontic infections. J Dent Res 2002;81:761–6.
20. Sakamoto M, Rôças IN, Siqueira JF Jr, Benno Y. Molecular analysis of bacteria in asymptomatic and symptomatic endodontic infections. Oral Microbiol Immunol 2006;21:112–22.
21. Hong BY, Lee TK, Lim SM, et al. Microbial analysis in primary and persistent endodontic infections by using pyrosequencing. J Endod 2013;39:1136–40.
22. Siqueira JF Jr, Rôças IN. Diversity of endodontic microbiota revisited. J Dent Res 2009;88:969–81.
23. Chalmers NI, Oh K, Hughes CV, et al. Pulp and plaque microbiotas of children with severe early childhood caries. J Oral Microbiol 2015;7:25951.
24. Hahn CL, Falkler WA Jr, Minah GE. Correlation between thermal sensitivity and microorganisms isolated from deep carious dentin. J Endod 1993;19:26–30.

Clinical Research

25. Siqueira JF Jr, Rôças IN. Uncultivated phylotypes and newly named species associated with primary and persistent endodontic infections. J Clin Microbiol 2005;43: 3314–9.
26. Paster BJ, Bartoszyk IM, Dewhirst FE. Identification of oral streptococci using PCR-based, reverse-capture, checkerboard hybridization. Methods Cell Sci 1998;20: 223–31.
27. Rôças IN, Siqueira JF Jr. Root canal microbiota of teeth with chronic apical periodontitis. J Clin Microbiol 2008;46:3599–606.
28. Siqueira JF Jr, Rôças IN. The microbiota of acute apical abscesses. J Dent Res 2009; 88:61–5.
29. Edwards U, Rogall T, Blöcker H, et al. Isolation and direct complete nucleotide determination of entire genes: characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res 1989;17:7843–53.
30. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 1991;173:697–703.
31. Becker MR, Paster BJ, Leys EJ, et al. Molecular analysis of bacterial species associated with childhood caries. J Clin Microbiol 2002;40:1001–9.
32. Asai Y, Jinno T, Igarashi H, et al. Detection and quantification of oral treponemes in subgingival plaque by real-time PCR. J Clin Microbiol 2002;40:3334–40.
33. Lyons SR, Griffen AL, Leys EJ. Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria. J Clin Microbiol 2000;38:2362–5.
34. Rudney JD, Pan Y, Chen R. Streptococcal diversity in oral biofilms with respect to salivary function. Arch Oral Biol 2003;48:475–93.
35. Dalwai F, Spratt DA, Pratten J. Use of quantitative PCR and culture methods to characterize ecological flux in bacterial biofilms. J Clin Microbiol 2007;45:3072–6.
36. Byun R, Nadkarni MA, Chhour KL, et al. Quantitative analysis of diverse *Lactobacillus* species present in advanced dental caries. J Clin Microbiol 2004;42: 3128–36.
37. Grenier D, Mayrand D. Nutritional relationships between oral bacteria. Infect Immun 1986;53:616–20.
38. Olgart LM. Pain research using feline teeth. J Endod 1986;12:458–61.
39. Panopoulos P, Mejare B, Edwall L. Effects of ammonia and organic acids on the intradental sensory nerve activity. Acta Odontol Scand 1983;41:209–15.
40. Khabbaz MG, Anastasiadis PL, Sykaras SN. Determination of endotoxins in caries: association with pulpal pain. Int Endod J 2000;33:132–7.
41. van Amersfoort ES, van Berkel TJC, Kuiper J. Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. Clin Microbiol Rev 2003; 16:379–414.
42. Wadachi R, Hargreaves KM. Trigeminal nociceptors express TLR-4 and CD14: a mechanism for pain due to infection. J Dent Res 2006;85:49–53.
43. Corby PM, Lyons-Weiler J, Bretz WA, et al. Microbial risk indicators of early childhood caries. J Clin Microbiol 2005;43:5753–9.