PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

FACULDADE DE ODONTOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

NÍVEL: DOUTORADO

ÁREA DE CONCENTRAÇÃO: ENDODONTIA

AVALIAÇÃO DO DESENVOLVIMENTO RADICULAR EM RESPOSTA ÀS PROTEÍNAS DERIVADAS DA MATRIZ DO ESMALTE E À RESOLVINA E1: ESTUDO EXPERIMENTAL EM DENTES DE RATOS COM RIZOGÊNESE INCOMPLETA E NECROSE PULPAR

ASSESSMENT OF ROOT FORMATION IN RESPONSE TO ENAMEL MATRIX DERIVATIVE AND TO RESOLVIN E1: AN EXPERIMENTAL STUDY IN RAT IMMATURE NECROTIC TEETH

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PORTO ALEGRE

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Tese apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade de Odontologia da Pontifícia Universidade Católica do Rio Grande do Sul como requisito para a obtenção do título de Doutor em Odontologia, na área de concentração de Endodontia.

Orientador: Prof. Dr. Eraldo Luiz Batista Júnior.

PORTO ALEGRE

2011

Dados Internacionais de

Catalogação na Publicação (CIP)

S286a Scarparo, Roberta Kochenborger
Avaliação do desenvolvimento radicular em resposta às proteínas derivadas da matriz do esmalte e à resolvina E1: estudo experimental em dentes de ratos com rizogênese incompleta e necrose pulpar = Assessment of root formation in response to enamel matrix derivative and to resolvin E1: an experimental study in rat immature necrotic teeth / Roberta Kochenborger Scarparo. – Porto Alegre, 2011.
92 f.
Tese (Doutorado) – Faculdade de Odontologia, Pós-Graduação Odontologia, PUCRS.

Orientador: Prof. Dr. Eraldo Luiz Batista Júnior.

1. Endodontia. 2. Infecção (Odontologia). 3. Dentes - Esmalte. I. Batista Junior, Eraldo Luiz. II. Título.
CDD 617.634

Bibliotecário Responsável

Ginamara Lima Jacques Pinto CRB 10/1204

Agradecimentos

AGRADECIMENTOS

Aos meus pais, ***Paulo Sergio Scarparo*** e ***Helena Beatriz Kochenborger Scarparo*** por todo o amor dedicado, apoio e confiança. Por serem meus mais admirados exemplos na vida.

A ***Reinaldo Benfica Neto*** pelo entusiasmo com que participa da minha vida. Pelo companheirismo, cumplicidade, por me trazer alegrias e me fazer confiar na realização dos nos nossos sonhos.

A minha avó ***Lacy Kochenborger,*** à minha madrinha ***Letícia Spano*** e aos meus irmãos ***Eduardo Kochenborger Scarparo*** e ***Marcelo Kochenborger Scarparo*** pelo carinho, amizade e presença que tanto me fazem bem.

À ***Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS)*** e ao ***Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)*** pelas oportunidades oferecidas para o desenvolvimento deste estudo.

Ao meu orientador, ***Prof. Eraldo Luiz Batista Júnior*** por toda confiança demonstrada e pelo incentivo. Meu agradecimento especial pelo convívio, excepcional aprendizado proporcionado e por compreender e apoiar as frequentes “mudanças de planos” que caracterizaram essa jornada.

Ao ***Prof. José Antônio Poli de Figueiredo*** pelos ensinamentos e oportunidades oferecidas. Especialmente pelas palavras de incentivo e pelo exemplo de dedicação ao ensino e à pesquisa.

Às queridas amigas ***Lenara Dondoni*** e ***Daiana Boettcher*** meu agradecimento especial pelo carinho, companheirismo e intensa dedicação a esse estudo. Foi maravilhoso ter dividido com vocês essa etapa da minha vida.

À professora ***Maria Ivete Bolzan Rockenbach*** pela disponibilidade e valiosa colaboração no desenvolvimento da análise radiográfica desse estudo.

A ***Tiago Giulianni*** pelos ensinamentos e pela dedicação no processamento histológico das amostras deste trabalho.

À professora ***Fernanda Morroni*** pela disponibilização do Laboratório de Farmacologia Aplicada.

À professora ***Maria Martha Campos*** pela dedicação à construção dos recursos necessários ao desenvolvimento de pesquisas nesta Universidade, e pela disponibilização do Laboratório de Toxicologia Pré-Clínica. A ela, e também às professoras ***Patrícia Poli Kopper*** e ***Fabiana Vier Pelisser,*** agradeço pelas brilhantes aulas ministradas, experiências compartilhadas e "sessões de psicoterapia de grupo".

Aos ***colegas do Mestrado e Doutorado em Endodontia da PUCRS*** pela união demonstrada e excelente convívio. Saibam que foi um imenso prazer ter contado com vocês nesses anos.

Aos queridos mestres e colegas ***Régis Burmeister dos Santos***, ***João Ferlini Filho***, ***Marcus Vinicius Reis Só***, ***Fabiana Soares Grecca*** e ***Elaine Freitas Fachin*** pelos

momentos vividos e pelo crescimento profissional proporcionado. Por manterem as portas abertas e me incentivarem a seguir lutando.

A todos vocês meus sinceros agradecimentos.

Resumo

RESUMO

Os objetivos deste estudo foram: (a) desenvolver um modelo experimental para testar estratégias de tratamento em dentes não-vitais com rizogênese incompleta, utilizando os primeiros molares inferiores de ratos; (b) avaliar, nesse contexto, o efeito da aplicação intracanal de proteínas de matriz do esmalte (EMD) e da Resolvina E1 (RVE1). Inicialmente, o método a ser utilizado para interrupção da rizogênese foi testado, comparando-se dentes hígidos e dentes que sofreram pulpectomia em estágio inicial da formação das raízes (4 semanas de idade). As avaliações radiográfica e histológica comprovaram o desenvolvimento de alterações periapicais e a interrupção da rizogênese após pulpectomia, além de permitirem a adequação de períodos apropriados para testar estratégias (3 semanas após pulpectomia) e avaliar seus resultados (3 e 6 semanas pós-tratamento). Em outro grupo de animais, após interrupção da rizogênese os canais foram irrigados com hipoclorito de sódio e solução salina e foram testadas medicações intracanal com pasta poliantibiótica, EMD ou RvE1. Para o grupo controle, os dentes foram mantidos sem tratamento e expostos ao meio oral. Os resultados radiográficos e a intensidade da inflamação foram comparados por meio de análise de variância (ANOVA) e *posthoc* de Bonferroni ($p<0,05$). Apenas a RvE1 apresentou redução significativa das lesões periapicais no primeiro período ($P<0.05$), o que foi corroborado pela menor resposta inflamatória ($P<0.05$). Já no segundo período, a pasta poliantibiótica e as EMD promoveram resultados semelhantes aos da RvE1. Ainda que algumas amostras apresentassem resultados insatisfatórios, o desenvolvimento radicular às expensas de tecido cementóide ou osteóide pode ser observado. As EMD promoveram, além da formação de tecidos mineralizados na região apical e externa das raízes, sua invaginação para o interior do canal radicular. Tanto a RvE1 como o EMD apresentaram potenciais a serem explorados para o tratamento de rizogênese incompleta e necrose pulpar. Estudos adicionais devem otimizar os protocolos, fornecer informações sobre os eventos moleculares e celulares envolvidos na formação radicular e avaliar resultados em humanos.

Palavras Chave (termos *MeSH*):

Endodontia, apicificação, apicigênese, dentes não vitais, inflamação, odontogênese, proteínas dentárias do esmalte, Resolvina E1

Palavras Chave (DeCS):

Endodontia, odonogênese, necrose da polpa dentária, inflamação, mediadores da inflamação

Abstract

ABSTRACT

The present study aimed at: (a) developing an experimental model for testing treatment strategies in nonvital immature teeth, using the lower first molars of rats; (b) evaluating the effects of intracanal medication with enamel matrix proteins (EMD) and Resolvin E1 (RvE1). At first, the method to be used for arresting root development was tested, comparing healthy teeth with teeth which underwent pulpectomy and were left open since the initial stage of root development (four weeks-age). Radiographic and histological findings proved that induction of periapical lesions and arrest of root development were achieved. Moreover, these data allowed the definition of appropriate periods for testing treatment protocols (3 weeks after pulpectomy) and for evaluating its results (3 and 6 weeks post-treatment). In another group of animals, after arresting root development, disinfection using sodium hypochlorite and saline solution was carried out and intracanal medication with either polyantibiotic paste, EMD or RvE1 was tested. At the control group, no treatment was performed and teeth cavities were left exposed to the oral environment. Radiographic and histological data were evaluated using two-way ANOVA and Bonferroni *post-hoc* ($P<0.05$). At the first time point, only the teeth subjected to RvE1 intracanal medication showed reduced periapical lesions ($P<0.05$), which was corroborated by the reduced inflammatory response ($P<0.05$). At the second time point, polyantibiotic paste, EMD and RvE1 showed similar results. Although some samples showed unsatisfactory results, root development could be observed, mainly at the expenses of cementum-like or bone-like tissues. EMD allowed, in addition to hard tissue formation at the apical and external portion of roots, its ingrowth into the root canal spaces. RvE1 as EMD presented a potential to be explored in nonvital immature teeth. Further studies should focus in the optimization protocol, cellular and molecular events that take part during root formation and treatment outcome in humans.

Keywords (*MeSH terms*):

Endodontics, apexification, apexogenesis, nonvital teeth, inflammation, odontogenesis, dental enamel proteins, Resolvin E1

Keywords (DeCS):

Endodontics, odontogenesis, dental pulp necrosis, inflammation, inflammation mediators

Sumário

SUMÁRIO

1. Introdução Geral

1. INTRODUÇÃO GERAL

Tradicionalmente, o manejo endodôntico dos dentes com rizogênese incompleta e necrose pulpar visa à apicificação por meio da utilização de hidróxido de cálcio ou selamento com agregado trióxido mineral (MTA). Embora apresentem boa previsibilidade e elevado percentual de redução de lesões periapicais, essas condutas clínicas possuem limitações importantes, como o não desenvolvimento completo da raiz dentária, mantendo essa estrutura frágil e aumentado o risco de fraturas (FRANK *et al.,* 1966; CVEK, 1992; ANDREASSEN, FARIK & MUNKISGAARD, 2002; SIMON *et al.*, 2007).

A relevância dessas limitações fez com que, já a partir da década de 60, alguns autores buscassem averiguar a possibilidade de desenvolvimento radicular em dentes imaturos com polpa necrosada (OSTBY, 1961; RULE, 1966; HAM, PATTERSON, MITCHELL, 1972). Entretanto, falhas decorrentes do pouco conhecimento disponível acerca de aspectos da regeneração, de materiais inadequados, e de desinfecção insuficiente produziram resultados insatisfatórios (OSTBY 1961; NYGAARD-OSTBY, HJORTDAL, 1971; CZVEC, NORD, HOLLANDER, 1976; HORSTED, NYGAARD-OSTBY, 1978; NEVINS *et al.*, 1976; HARGREAVES *et al*., 2008)

Atualmente, alguns relatos de casos tem afirmado a possibilidade da indução do desenvolvimento radicular em dentes que tradicionalmente seriam tratados visando à apicificação (JUNG, LEE, HARGREAVES, 2008; COTTI, MEREU, LUSSO, 2008). Provavelmente, os protocolos de tratamento descritos favoreçam a manutenção da viabilidade e a estimulação de células-tronco presentes no tecido pulpar (eventualmente remanescente), no ligamento periodontal e na região da papila apical (GRONTHOS *et al.*, 2000; GRONTHOS *et al.*, 2002; SONOYAMA *et al.*, 2008; HUANG *et al.,* 2008).

Os protocolos clínicos que visam ao desenvolvimento radicular após necrose pulpar são bastante variados. De modo geral, é indicada a irrigação com hipoclorito de sódio e a aplicação de medicação intracanal com uma pasta composta por metronidazol, ciprofloxacina e minociclina (WINDLEY *et al*., 2005; CHUEH, HUANG, 2006; BOSE, NUMMIKOSKI, HARGREAVES, 2009). Após o período de manutenção dessa medicação, alguns autores sugerem que a pasta seja removida e que sejam induzidos sangramento e formação de coágulo intracanal, por sobre o qual será realizado selamento do terço cervical das raízes (IWAYA, IKAWA, KUBOTA, 2001; BANCHS & TROPE, 2004). Por outro lado, há relatos de desenvolvimento radicular em dentes necrosados sem que seja necessária a formação do coágulo (COTTI, MEREU & LUSSO, 2008; BOSE, NUMMIKOSKI & HARGREAVES, 2009).

Os poucos estudos realizados até o momento com o intuito de testar a eficácia desses protocolos comprovam que a complementação da formação radicular após necrose pulpar é possível (SHAH *et al.*, 2008; THIBODEAU *et al.*, 2007 ; WANG *et al* 2010, DA SILVA *et al*., 2010). Por outro lado, apesar dos protocolos sugeridos visarem à revascularização do espaço endodôntico, estudos em cães comprovam que na maioria dos casos ocorre aposição de tecido cementóide ou osteóide permitindo o aumento da espessura das paredes dentárias e do comprimento radicular (DA SILVA *et al.,* 2010., WANG *et al.,* 2010).

Apesar da aposição desses tecidos aumentar a resistência radicular à fratura, cumprindo seu papel em reduzir perdas dentárias, a previsibilidade dos tratamentos sugeridos ainda é limitada, não havendo parâmetros estabelecidos para seleção de casos e percentual de sucesso clínico (DING *et al*., 2009). Sendo assim, o desenvolvimento de alternativas que favoreçam de sucesso desses procedimentos é almejado.

A aplicação de mediadores que atuam durante o desenvolvimento embriológico dos

dentes é uma hipótese ainda não testada. É sabido que a secreção de proteínas derivadas da matriz do esmalte (EMD) pela bainha epitelial de Hertwig leva à sinalização ectomesenquimal recíproca, desencadeando uma cascata de reações que conduzem à diferenciação de odontoblastos, à formação de dentina, à cementogênese e ao desenvolvimento de estruturas periodontais de suporte (LINDSKOG, 1982; BROOKES *et al*.,1995; HAMMARSTRÖM, 1997; NAKAMURA *et al.*, 2001)

Em razão de seu papel fundamental durante a embriogênese dentária, as proteínas de matriz do esmalte tem sido testadas com sucesso para diversas aplicações clínicas, tais como a de regeneração periodontal, capeamento pulpar/pulpotomia e prevenção de reabsorções dentárias e de anquilose em casos de avulsão (ZETTERSTRÖM et al.,1997; PONTORIERO, WENNSTROM, LINDHE, 1999; NAKAMURA *et al.*, 2001; FILLIPI, POHL, VON ARX, 2002; ISHIZAKI *et al*., 2003; BOSSHARDT & NANCI, 2004; OLSSON *et al.*, 2005). Entretanto, seu emprego na estimulação do desenvolvimento radicular de dentes com rizogênese incompleta e necrose pulpar até o momento não foi investigado.

Outro aspecto, ainda não explorado na Endodontia, é a aplicação de mediadores lipídicos que atuam na resolução da resposta inflamatória. Estudos prévios comprovam que a aplicação de Resolvina E1 (RvE1) suprime a resposta inflamatória e a perda óssea induzida por bactérias na doença periodontal, mesmo sem intervenção mecânica sobre o biofilme bacteriano (HASTURK *et al.*, 2006; HASTURK *et al.*, 2007; SERHAN 2007).

O princípio de atuação desses mediadores merece ser explorado em infecções endodônticas, especialmente em casos de rizogênese incompleta, quando a instrumentração do canal é limitada dada a fragilidade das paredes dentárias e pela necessidade de manter a viabilidade das células-tronco.

1.1 Proteínas derivadas da matriz do esmalte

O Emdogain (Straumann AG, Basel, Suíça) é um gel de propilenoglicol alginato

que contém proteínas derivadas da matriz do esmalte secretadas pela bainha epitelial de Hertwig durante o desenvolvimento dentário. Seu principal componente é a amelogenina, mas também contém enamelinas, tuftelinas, e ameloblastinas (HAMMARSTRÖM 1997; ZETTERSTRÖM *et al.*, 1997; PONTORIERO, WENNSTROM, LINDHE, 1999; NAKAMURA *et al*., 2001; BOSSHARDT & NANCI, 2004)

A indução promovida por essas proteínas simula parte da odontogênese normal, facilitando processos regenerativos de tecidos de origem mesenquimal. Acredita-se que as proteínas da matriz do esmalte participem da sinalização ectomesenquimal recíproca, a qual controla o desenvolvimento embrionário dos dentes (HAMMARSTRÖM, 1997). Sendo assim, desempenham papel importante na diferenciação e maturação de células odontoblásticas, na regulação da mineralização do esmalte e na formação das estruturas periodontais. Também estimulam a regeneração de tecidos periodontais, como o cemento acelular, o ligamento periodontal e o osso alveolar, simulando o desenvolvimento dentário (HAMMARSTRÖM, 1997; ZETTERSTRÖM *et al*., 1997; PONTORIERO, WENNSTROM, LINDHE, 1999; NAKAMURA *et al*., 2001; BOSSHARDT & NANCI, 2004).

Em tratamentos conservadores da polpa, as proteínas da matriz do esmalte promovem a cascata clássica de regeneração tecidual e reparo de maneira mais intensa e rápida que o hidróxido de cálcio. A polpa subjacente ao novo tecido formado apresenta-se livre de inflamação e há diferenciação de odontoblastos. Além disso, a formação de dentina inicia-se à distância do local onde a polpa foi amputada, havendo também uma marcada tendência à angiogênese nas regiões mais profundas, o que revela o aumento do nível do crescimento e metabolismo celular. O tecido duro formado inicialmente é semenlhante à osteodentina, mas torna-se semelhante à dentina secundária normal, com odontoblastos e túbulos inseridos (NAKAMURA *et al.*, 2002).

De acordo, alguns autores apontam vantagens na utilização de EMD em

detrimento do hidróxido de cálcio em tratamentos pulpares conservadores, tais como não promover a atresia dos condutos e da câmara pulpar (NAKAMURA *et al*., 2002; KAIDA *et al.*, 2008) e reduzir a sintomatologia pós-operatória (OLSSON *et al.,* 2005; KAIDA *et al.,* 2008).

1.2 - Mediadores lipídicos pró-resolução da resposta inflamatória

O início do processo inflamatório é caracterizado pelo dano tecidual seguido da liberação de mediadores químicos endógenos (como leucotrienos e citocinas) e exógenos (como mediadores químicos de origem microbiana) que agem atraindo células polimorfonucleares. Os neutrófilos atuam na fagocitose de microorganismos e degradação de restos celulares. Em algumas situações, entretanto, pode ocorrer a liberação extracelular do conteúdo de grânulos lisossomais dessas células, ricos em enzimas de degradação e espécies reativas de oxigênio, levando à amplificação do dano celular e da resposta inflamatória. Paralelamente, mediadores químicos endógenos podem, inadvertidamente, promover ativação e recrutamento excessivo de neutrófilos, contribuindo com o dano tecidual ainda que sejam produzidos como parte importante da defesa do hospedeiro (WEISSMAN, SMOLEN, KORCHACK, 1980; SERHAN, 2008).

Nesse sentido, após a remoção de materiais nocivos por meio da fagocitose, deve haver a resolução da resposta inflamatória, evitando sua ampliação e conseqüente cronificação ou manutenção de quadros patológicos. O termo “resolução” refere-se à autolimitação do quadro inflamatório agudo, o qual é caracterizado pela redução ou remoção de leucócitos e de restos celulares do sítio inflamatório, permitindo ao tecido o retorno à homeostase (WEISSMAN, SMOLEN, KORCHACK, 1980; SERHAN, 2007; SERHAN *et al.,* 2008)

Recentemente, a resolução foi caracterizada como um processo bioquímico e metabólico ativo, rapidamente iniciado por mecanismos celulares após as fases iniciais da

inflamação aguda. Esse processo se dá pela biossíntese de mediadores lipídicos “pró-resolução”, como lipoxinas, resolvinas e protectinas, os quais atuam como agonistas na redução do infiltrado inflamatório em tecidos inflamados, além de promover a eliminação de células apoptóticas e de microrganismos pelos macrófagos (LEVY *et al.,* 2001; SERHAN *et al.*, 2000; SERHAN, 2007).

Esses mediadores são derivados de ácidos graxos poli-insaturados. O ácido aracdônico (AA) dá origem às lipoxinas, o ácido eicosapentaenóico (EPA) às resolvinas da série E, e o ácido docosahexaenóico (DHA) às resolvinas da série D e às protectinas (SERHAN *et al*., 2008.).

O termo “resolvinas” foi introduzido com o intuito de explorar as características de mediadores endógenos biossintetizados durante a fase de resolução do exsudato inflamatório, os quais processam potentes ações antiinflamatórias e imunorregulatórias. No presente estudo, foi explorada a ação da Resolvina E1 (RvE1), composta de 5S,12R,18R-trihidroxi-6Z,8E,10E,14Z,16E ácido ecosapentaenóico. Resumidamente, a RvE1 é derivada da oxigenação do EPA, em um processo que leva a formação produtos intermediários como o 18R - ácido hidroperoxieicosapentaenóico (18R-HPEPE) o qual libera o 18R- ácido hidroxieicosapentaenóico (18R-HEPE). Este é rapidamente transformado pela ação de lipoxigenase-5 de neutrófilos para que seja formado o composto bioativo (SERHAN *et al*., 2000; ARITA *et al*., 2005).

Estudos prévios demonstraram que a RvE1 é um potente regulador da transmigração de neutrófilos e da inflamação, sendo também atribuído a esse composto bioativo a estimulação de fagocitose não flogística de neutrófilos apoptóticos pelos macrófagos (SERHAN *et al.*, 2000; ARITA *et al.*, 2005; ARIEL *et al.*, 2006; ARITA *et al*., 2007; SCHUWAB *et al*., 2007). Ensaios pré-clínicos revelam que a RvE1 apresenta efeito protetor na doença periodontal, com redução do infiltrado de neutrófilos, evitando a perda

óssea e de tecido conjuntivo, promovendo a cicatrização de tecidos danificados e a regeneração de tecido ósseo e do ligamento periodontal (HASTURK *et al*., 2006; HASTURK *et al*., 2007).

1.3 Objetivos

O presente estudo tem como objetivos:

- Desenvolver um modelo experimental que permita a avaliação de estratégias de tratamento para dentes com rizogênese incompleta e necrose pulpar, utilizando molares de ratos.

- Avaliar o efeito da medicação intracanal com proteínas derivadas da matriz do esmalte (Emdogain) e com o mediador lipídico pró-resolução inflamatória Resolvina E1 no reparo de lesões periapicais e no desenvolvimento radicular de dentes de ratos com rizogênese incompleta.

2. Capítulo I

2. CAPÍTULO I

Artigo 1

Response to intracanal medication in immature teeth with pulp necrosis: an experimental model in rat molars.

Submetido ao periódico Journal of Endodontics, qualis A1 e fator de impacto 2.953 (Anexo A).

Response to intracanal medication in immature teeth with pulp necrosis: an experimental model in rat molars.

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ACKNOWLEDGMENTS

This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), a Brazilian Governmental Institution. The authors are grateful to Tiago Giulianni for technical assistance. The authors deny any conflicts of interest.

Abstract

Objective: To characterize an experimental model in rats aiming at evaluating treatment strategies in necrotic immature teeth.

Methods: To define the periods to be adopted in the experimental procedures and to confirm interruption of root embryogenesis, the left lower first molars of *Wistar* rats aging 4-weeks (n=24) underwent pulpectomy and were left open to the oral environment. Vital teeth were observed on the right lower first molars. In another group of animals (n=36) the teeth were left open for three weeks, and then received interventions for disinfection. Changes in root formation were determined based on radiographic and histological evaluation.

Results: vital teeth showed an increase of root length and hard tissue thickness over the proposed experimental periods. On the other hand, induction of necrosis arrest root formation. Teeth subjected to disinfection with sodium hypochlorite and polyantibiotic paste showed significant reduction of periapical lesions, gain in root length and increased walls thickness compared to the control ($P<0.05$).

Conclusion: The protocol tested for root canal disinfection was favorable for periapical lesions reduction and root development over the experimental periods. The experimental model presented should contribute to studies that aim at improving therapeutic strategies for necrotic immature teeth using a rat model.

Keywords rat model, apexogenesis, nonvital teeth, inflammation, odontogenesis

Introduction

Pulp necrosis of immature teeth arrest tooth development, resulting in incompletely formed roots with wide open apices and fragile structure (1). Apexification is considered efficient for endodontic repair, but still fall short in inducing increase of root tickness and length (1-4).

Recently, many authors have proposed regenerative therapies that involve the preservation of stem cells from the apical papilla (5-11). Nevertheless, the ability of these strategies to promote predictable reconstruction of dental tissues has yet to be properly addressed. For these reasons, the mechanisms of root development (12-16), the definition of new treatment modalities (17-18) and the characterization of their pathways and outcomes (19) have gained great attention.

Ethical and technical issues have a role in restricting investigations using human subjects, thus requiring implementation of animal models that can reproduce clinical outcomes (19). Rat molars, including pulp and periodontal tissues, resemble in many ways those of humans (20-22,24-29). The use of rats offers economical advantages (20), availability of molecular tools and access to genetic databanks (13,29). This model has been widely used in orthodontics (21), periodontics (22-23), conservative treatment of dental pulp (20) and studies of molecular mechanisms involved in teeth embryogenesis (13), but treatment strategies for immature teeth still have been investigated mainly in dogs (17-19).

Considering the aforementioned, the present study aimed at developing an experimental model in rat molars for evaluating treatment strategies in necrotic immature teeth.

Methods

The study protocols were approved by Federal University of Rio Grande do Sul and Pontifical Catholic University of Rio Grande do Sul Institutional Animal Care and Use Committees (Protocol 10/00156 and 19001)

The sample consisted of 60 male *Wistar* rats. Twenty-four animals were used to define the periods to be adopted in the experimental procedures and to confirm induction of periapical lesions and interruption of root embryogenesis. The remaining 36 were used for testing methods for the treatment of rat immature first molars .

To perform the experimental procedures, the animals were anesthetized intraperitoneally with ketamine (0.8 ml/100g) and Xilasine (0.2ml/100g). Mouth opening was achieved by using a designed device, and the soft tissues were kept away with the aid of dental forceps. The device was made using approximately 6 cm of a 0.8 orthodontic wire. The wire was first folded in a rectangular shape with sides measuring 1 and 2 cm. Then, the central portion of both sides measuring 2 cm were bent in an angle of about 45^{o}, forming a convex arc. The sides of the arc measuring 1 cm were placed on contact with the lingual and palatal surfaces of lower and upper incisors (Figure 1A).

Definition of experimental periods and confirmation of root embryogenesis arrest

Twenty-four right mandibular first molars were used for the observation of natural embryogenesis. Pulp necrosis was induced on the left mandibular first molars during the initial stage of root development (animals aging 4-weeks old). Dental pulps were exposed by drilling cavities on the central portion of the oclusal face, with a 1011 HL round bur in high speed (KGSorensen, Cotia, SP, Brazil) to a depth nearly equal to the bur diameter (1 mm). An # 25 endodontic file (Dentsply Maillefer, Ballaigues, Switzerland) was then used to remove remmants of pulpal tissue. The teeth were left

open to the oral environment throughout the course of the experiment. The time needed for the detection of periapical lesions was confirmed by radiographs taken 1, 2 and 3 weeks after pulpectomy as previously reported (30).

Animals aging 7, 10, 13 and 16 weeks (n=6 per period) were euthanized by inhalation of isoflurane. The jaws were dissected for radiographic and histological evaluation.

Methods for implementation of treatment protocols

Pulp necrosis was induced as described above in 36 animals aging 4 weeks-old. In 18 animals teeth were left open through the course of the experiment. In the other 18, teeth were left open for three weeks, and then received intervention for disinfection of the target teeth.

Root canal disinfection

Debris were removed from the cervical third of the roots using a # 25 endodontic file, inserted to a maximum depth of 2mm to avoid injury to the apical portion of the canals. The canals were irrigated with of 2.5% sodium hypochlorite followed by 0.9% sterile saline solution, using anesthetic tubes filled with the solutions, long needles, a carpule syringe and endodontic suction apparatus. The canals were dried with absorbent paper points and filled with a polyantibiotic dressing, comprised of metronidazol, ciprofloxacin and minocycline (50 mg/ml) (Pharma&Cia, Porto Alegre, RS, Brazil) using an insulin syringe. Teeth were sealed with sterile cotton pellets and silver amalgam. The animals were divided into three experimental periods (n= 6 per group) in which the polyantibiotic paste remained for 3, 6 or 9 weeks. After euthanizia by inhalation of isoflurane, the jaws were dissected for radiographic and histological evaluation.

Radiographs and Image Analysis

The X-ray cylinder was fitted in a way as to form a perpendicular angle with the buccal surface of the first molar. A focal distance of 30 cm was observed. The X-ray unit (Gnatus, Ribeirão Preto, SP, Brazil) operated at 7 mA at 70 kVp, with a size 2 phosphor plate (Gendex, Chicago, IL, USA) and exposure time of 0.2 seconds. Digital x-ray system (Denoptix/Gendex, Chicago, IL, USA) was used to capture images scanned at the resolution of 300 d.p.i. and saved in TIFF format.

Image analysis was performed using a software (Image Tool version 3.0, UTHSCSA, USA). For root length measurements, a linear trace from the pulp chamber floor to the most apical portion of the mesial root was created. Dental wall thickness at the apical third was estimated by calculating the percentage of the linear measurement of the mesial root canal width relative to the linear measurement of the entire mesial root width. Periapical lesion area at the mesial root was demarcated and measured.

Comparisons of data obtained in each period were performed using two-way ANOVA, one-way ANOVA and Bonferroni post-hoc. Differences were regarded significant when $P<0.05$.

Sample preparation and histological analysis

Samples were fixed with buffered 10% paraformaldehyde for 24 h, decalcified in 17% EDTA for 5 weeks, dehydrated in ascending concentrations of ethanol and embedded in paraffin. Five-µm serial sections were stained with hematoxylin and eosin. Three sections were selected for each sample, so the central portion of the roots, including the apex, was visible. A histological descriptive analysis was performed.

Results

Radiographic analysis of vital teeth showed an increase of root length and thickness over the experimental periods. Pulp exposure to the oral cavity promoted development of periapical lesions, significant reduction of root length and wall thickness (Figures 1B and 1C).

Histological evaluation showed progressive apical closure of vital teeth. Root development was complete in most of the animals aging 13 weeks-old, and continuous dentin and cementum formation could be observed in internal and external root surfaces. On the other hand, pulp exposure determined intense inflammatory response and arrest of root embryogenesis, that lead to open apices and thin dental walls (Figure 2).

Due to tooth fracture and loss of coronal sealing at the third experimental period (9 weeks post treatment), teeth subjected to polyantibiotic medication were evaluated 3 and 6 weeks post treatment only. After 6 weeks, teeth presented reduced periapical lesions and increased root lengths compared to the control ($P<0.05$). Canal width was reduced compared to the first period (Figures 3A and 3B), depicting increased wall thickness ($P<0.05$).

Histological analysis showed variable inflammatory response to treatment; about half of the roots showed formation of a cementum-like tissue on its apical portion and newly formed cementum on the external surfaces (Figures 3C, 3D and 3E).

Discussion

Previous studies showed that pulp exposure of rat molars pulps promotes an inflammatory reaction identical to that observed in humans (26-28,31), which was corroborated here. Apart from similar host responses (29), the oral bacteria flora of rats is more comparable to humans than other commonly used species in research (24-26,32). Furthermore, biologically, the response progresses faster in rats (31,33), which

may be a favorable point when it comes to promptly obtaining data that enable the continuous development of therapeutic strategies (33). The application of molecular tools is a routine for this species (13,29), favoring the development of investigations that focus on biological mechanisms involved in the treatment protocols.

Contamination of root canal after pulp exposure to the oral cavity was critical in arresting root development as a response to inflammation of apical periodontal tissues. Radiographic and histological analysis of vital teeth showed that rats aging 7 weeks had incomplete root development, equivalent to nearly half of its final full length. According to these data and considering the period needed for periapical lesion development, it was established that first molars should be endodontically accessed in rats aging 4 weeks-old, and treatment protocols should be applied in animals aging 7 weeks-old.

Some technical problems had to be solved before the teeth could be used in this experimental model for treatment strategies in immature teeth. The small size of the teeth and difficulties related to access to the pulp chamber required some adaptations. Therefore, appropriate training, proper position of rat's head, mouth opening and soft tissue removal are critical aspects in order to successfully carry out the procedures (20). In the present study, complete relaxation of the animal through deep anesthesia (34), and the use of a device designed to open and stabilize the rat mouth allowed for the preparation of the operative field. Cavity preparation at the center of the oclusal surface enabled the access to the three root canals. However, it increased fragility of the dental structure. Thus, long periods favored tooth fracture, affecting coronal sealing, as observed during the third experimental period. Moreover, incomplete root development may offer some advantages related to technical issues; the larger lumen of root canals and short root length favor location of the canals, adequate irrigation and canal dressing fillings.

The treatment protocol tested promoted root development and was favorable for periapical lesions repair, similarly to the results observed in studies employing dogs (17-19). As previously reported (19), root development occurred at the expense of a cementum-like tissue, corroborating that the rat model reproduces the results observed in other animals.

On the other hand, investigation of more predictable therapeutic strategies to obtain the continuity of root development follows as a challenge to be overcome. In agreement, the experimental model presented herein should contribute to studies that aim at improving therapeutic strategies for necrotic immature teeth.

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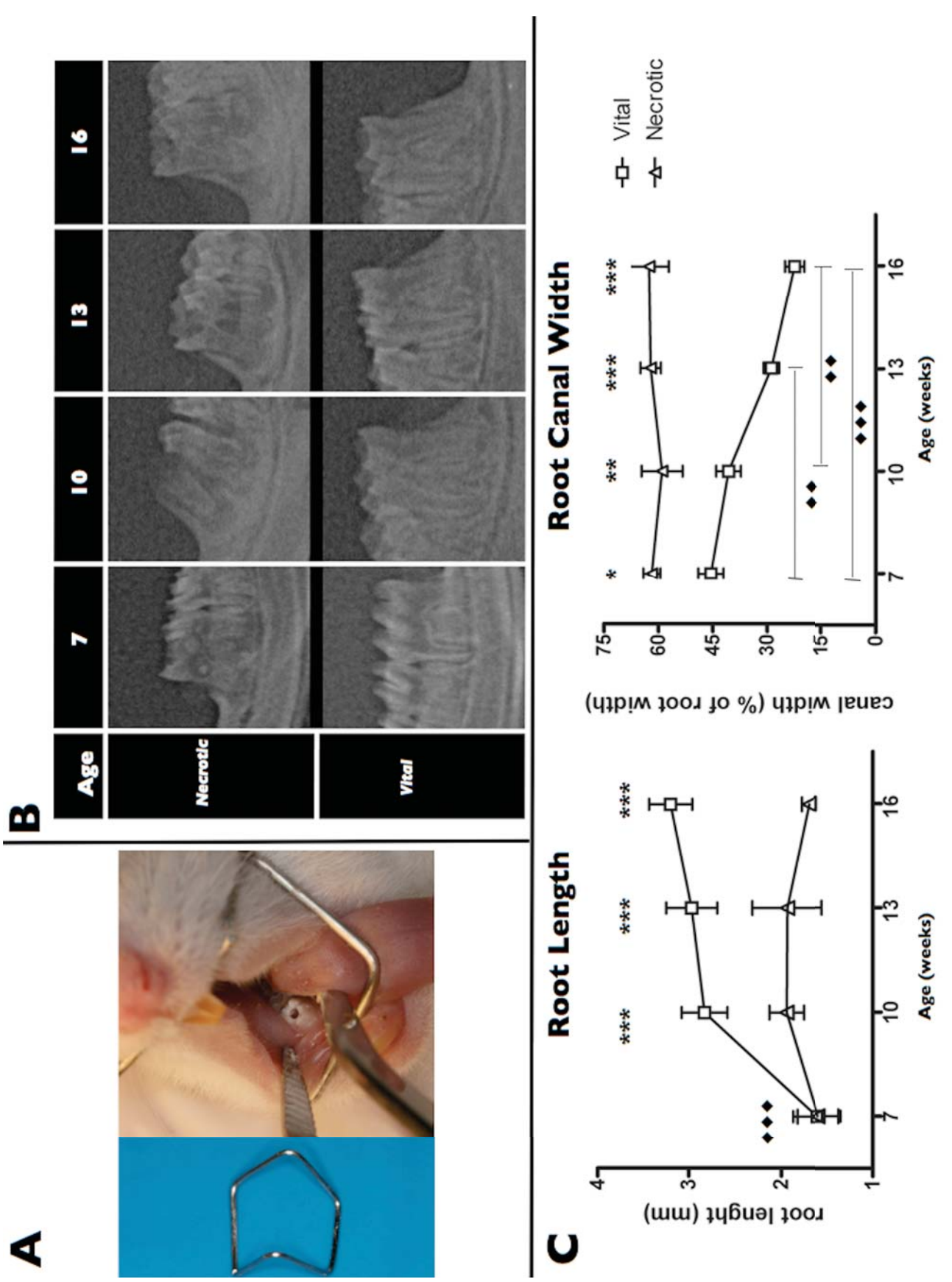
A
B
Age
7
10
13
16
Necrotic
Vital
C
Root Length
root length (mm)
Age (weeks)
Root Canal Width
canal width (% of root width)
Vital
Necrotic
Age (weeks)

Figure 1

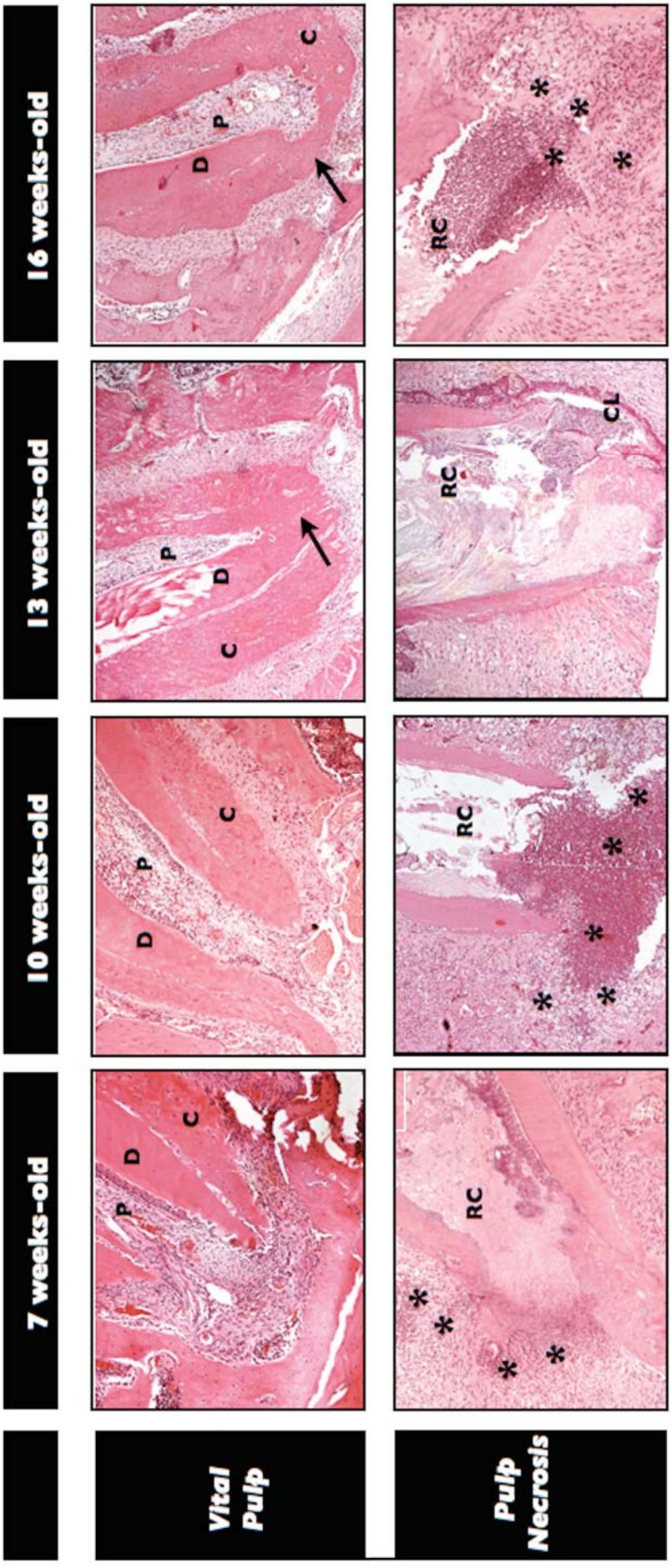
7 weeks-old
10 weeks-old
13 weeks-old
16 weeks-old
Vital Pulp
Pulp Necrosis
P
D
C
RC
CL

Figure 2

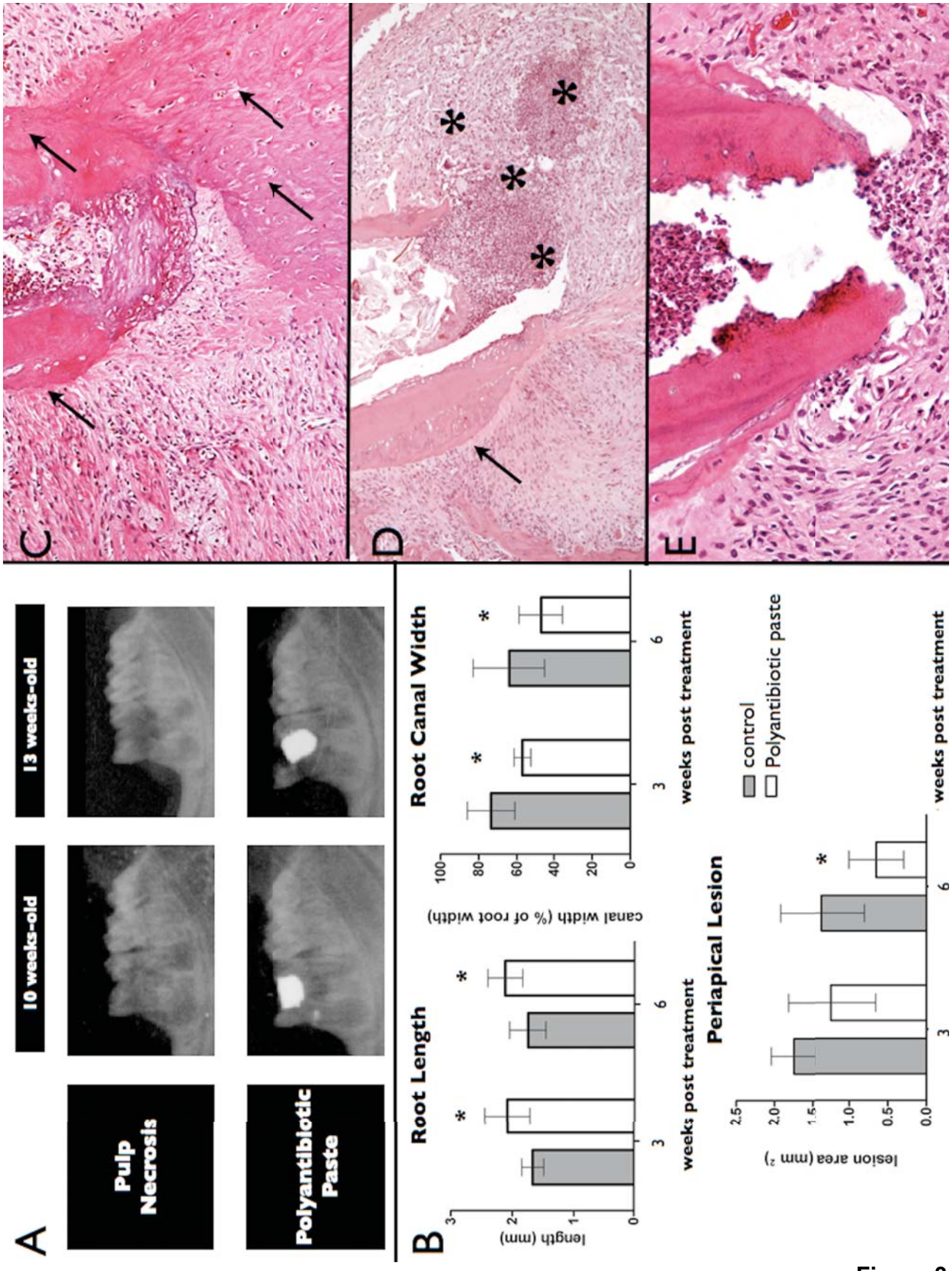
A
10 weeks-old
13 weeks-old
Pulp Necrosis
Polyantibiotic Paste
B
Root Length
length (mm)
weeks post treatment
Root Canal Width
canal width (% of root width)
weeks post treatment
Periapical Lesion
control
Polyantibiotic paste
lesion area (mm ²)
weeks post treatment
C
D
E

Figure 3

Figure Legends

Figure 1. (A) Metal device designed for animal mouth opening during experiments. (B)Radiographic aspects of necrotic and vital teeth in animals aging 7, 10, 13 and 16 weeks-old. (C) Radiographic analysis of root length and canal width showing significant differences between vital and necrotic teeth during the course of the experiment -* P<0.05; ** P<0.01; ***P<0.001. In vital teeth only, mean differences of root length (◆◆◆P< 0.001) and canal width (◆◆◆P< 0.001 ◆◆P< 0.01), were also observed among experimental periods.

Figure 2. Histological aspects of vital and necrotic teeth in animals aging 7, 10, 13 and 16 weeks-old. Vital pulp tissue (P) and the absence of periapical inflammation allowed formation of dentin (D) and cementum (C) on root internal and external surfaces. Complete apex formation was observed after 13 weeks of age (arrows). After dental pulp exposure, root canal (RC) infection lead to periapical inflammation, inducing either abscess (*) or cystic lesions (CL). Wide open apex and thin dental walls could be observed through the course of the experiment.

Figure 3. Response to canal disinfection. (A) Radiographic aspects of teeth after exposure to oral cavity (pulp necrosis) and after disinfection procedures (polyantibiotic paste). (B) Radiographic analysis of the polyantibiotic paste effects on periapical lesion area, root length and root canal width showing significant differences relative to the control infected teeth - *P<0.05. (C) Histologic evaluation showing variable healing outcomes in response to intracanal medication even after the second experimental period: formation of a cementum-like tissue on the apical portion and external surfaces (arrows); intense inflammatory infiltrate (*) and cementum formation detectable only on root external surfaces and distant from apical opening (arrows) (D); mild inflammatory infiltrate and absence of detectable hard tissue on the root apex (E).

3. Capítulo II

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Artigo 2

Assessment of root formation in response to Resolvin E1 (RvE1) and enamel matrix derivative (Emdogain®): an experimental study in rat immature necrotic teeth.

Submentido ao periodico International Endodontic Journal, quails A2 e fator de impacto 2.223

(ANEXO B)

Introduction

Traditionally, endodontic treatment of immature teeth with pulp necrosis is based on strategies as apexification with calcium hydroxide or placement of mineral trioxide aggregate apical plugs. Although considered efficient for endodontic repair, these strategies keep roots with wide open apices, reduced length and thin dental walls that are prone to fracture (Simon *et al*. 2007, Friedlander *et al.* 2009, Huang 2009).

Recently, other clinical approaches have been suggested, concerning the preservation and stimulation of stem cells from dental pulp, periodontal ligament and apical papilla (Gronthos *et al*.2002, Seo *et al*. 2005, Sonoyama *et al.* 2008). Most of them are based on root canal chemical disinfection, using sodium hypochlorite and a polyantibiotic paste comprised of metronidazole, ciprofoxacin and minocycline (Windley *et al*. 2005, Bose *et al.* 2009). After disinfection, some authors suggest that, before root formation occurs, a blood clot should be stimulated by using endodontic hand files inserted past the canal terminus into the periapical tissues (Banchs & Trope 2004). Case reports and pre-clinical studies confirm that completion of root development in nonvital teeth is possible, even in the absence of a blood clot (Bose *et al.* 2009, da Silva *et al.* 2010, Wang *et al*. 2010). Nevertheless, the suggested therapeutic strategies are limited in their predictability, and the continuous investigation of new protocols are warranted (Ding *et al.* 2009).

It is well documented that secretion of enamel matrix derivative proteins (EMD) by the Hertwig epithelial sheath triggers a cascade of reactions that stimulates odontogenesis (Sonoyama *et al*. 2007, Lyngstadaas *et al*. 2009). EMD, commercially available as Emdogain (Straumann AG, Basel Switzerland), is well recognized in periodontology for its regenerative properties (Lyngstadaas *et al*. 2009). In the conservative treatment of the dental pulp, EMD induces reparative dentin formation, also protecting the pulp tissue from inflammation (Nakamura *et al*. 2002, Igarashi *et al*. 2003, Olsson *et al.* 2005). Although the

growth factors present in EMD have a role during odontogenesis, its use to stimulate root development in immature necrotic teeth has not been investigated thus far.

Another promising approach is the use of the lipid pro-resolution compond Resolvin E1 (RvE1) to assist in the reduction of inflammatory response. Previous studies have shown that RvE1 controls the inflammatory response and bone loss in periodontal disease, even without mechanical intervention on the biofilm (Hasturk *et al*. 2006, Hasturk *et al*. 2007). The effect of RvE1 in endodontic infections warrants investigation *in vivo*, especially in immature teeth, which have root canal mechanical disinfection limited by the fragility of dental walls and the need for maintaining dental stem cells viable. The present study aimed at evaluating the effect of EMD (Emdogain) and Resolvin E1 on root development of immature teeth with pulp necrosis.

Materials and Methods

Experimental Procedures

The study protocols were approved by Pontifical Catholic University of Rio Grande do Sul Institutional Animal Care and Use Committees (Protocol 10/00156). Forty-eight (48) male *Wistar* rats were used. Experimental procedures were carried out with the animals anesthetized intraperitoneally with ketamine (0.8ml/100g) and xylasine (0.2ml/100g).

The experimental steps of the study are summarized on Figure 1. Endodontic access in the lower first molars was performed in four weeks-old animals, in order to induce periapical lesions and arrest root morophogenesis at this initial stage. Dental pulps were exposed by drilling cavities on the central portion of the occlusal surface, with a 1011 HL round bur (KGSorensen, Cotia, SP, Brazil) in high speed, to a depth nearly equal to the bur diameter (1 mm). A # 25 endodontic file (Dentsply Maillefer, Ballaigues, Switzerland) was then used to remove remnants of pulpal tissue. Periapical radiographs to verify the lesion formation were performed as previously described (Mahl & Fontanella 2008).

Periapical radiolucency was evident following three weeks of cavities exposure to the oral environment.

The animals were divided into four groups according to the treatment protocol. In the control group, teeth were left open to the oral cavity throughout the whole course of the experiment. In the other groups, teeth were left open to the oral environment for three weeks and then treatment protocols were applied (7-weeks-old animals). First, for root canal disinfection, debris was removed from the pulp chamber and cervical third of the roots using a # 25 endodontic file (Dentsply Maillefer, Ballaigues, Switzerland). During the implementation of therapeutic procedures, special care was taken in order not to traumatize the apical portion of the root canals. Canals were irrigated with 2.5% sodium hypochlorite followed by of 0.9% sterile saline solution and an endodontic suction apparatus. The pulp chamber and the canals were dried with absorbent paper points and the canals were filled with either of the following dressings: polyantibiotic paste consisting of metronidazole, ciprofloxacin and mynocicline - 50 mg of each antibiotic per ml - (Pharma & Cia, Porto Alegre, RS, Brazil); EMD - 30 mg per ml in propylene glycol alginate - (Emdogain, Straumann AG, Basel, Switzerland) or Resolvin E1. The latter preparation was delivered in ethanol and prepared from a stock solution of 50 mg/ml diluted in PBS to 1 µg/ml. The biocompounds were inserted into the canals with the aid of an insulin syringe and endodontic files until filling root canal spaces. Endodontic access was sealed with sterile cotton pellets and silver amalgam. The animals were divided into two experimental periods (n= 6 per group), being euthanized by inhalation of isoflurane at the ages of 10 and 13 weeks (3 and 6 weeks post application of treatments, respectively). The jaws were dissected for radiographic and histological evaluation.

Image Analysis of radiographs

An X-ray cylinder was fitted in a way as to form a perpendicular angle with the buccal surface of the first molar. A focal distance of 30 cm was observed. The X-ray unit (Gnatus, Ribeirão Preto, SP, Brazil) operated at 7 mA at 70 kVp, with a size 2 phosphor plate (Gendex, Chicago, IL, USA) and exposure time of 0.2 seconds. Digital x-ray system (Gendex, Chicago, IL, USA) was used to capture images scanned at the resolution of 300 d.p.i. and saved in TIFF format. Image analysis was performed by calibrated, blinded examiners (ICC>0.889 for all analysed variables) using a software (Image Tool version 3.0, UTHSCSA, USA). After a training session explaining the evaluation parameters, two examiners separately viewed the images and performed the radiographic measurements. The mean measurements were considered for statistical analysis. For root length measurements, a linear trace from the pulp chamber floor to the most apical portion of the mesial root was created. Dental wall thickness at the apical third was estimated by calculating the percentage of the linear measurement of the mesial root canal width relative to the linear measurement of the entire mesial root width. Periapical lesion area at the mesial root was measured by delineating the radiographic image to excluded teeth structure and healthy bone tissue (Figure 2A).

Sample preparation and histological analysis

The samples were fixed in 10% buffered paraformaldehyde for 24h. Then, the specimens were decalcified with 17% EDTA for 5 weeks, dehydrated in ascending concentrations of ethanol and embedded in paraffin. Five-μm serial sections were stained with hematoxylin and eosin. Three sections were selected for each sample, so the central portion of the roots, including the apex, was visible. A histological descriptive analysis was performed by blinded, calibrated examiners (Kappa=0.79; P<0.001), emphasizing the characteristics of the dental tissues and its surrounding structures. After a training session

explaining the gold standard of the evaluation parameters, two examiners separately scored the intensity of inflammatory response. When there was not agreement between both evaluators, a discussion was undertaken until a consensus was reached. Periapical inflammation was classified according to the following scores: (1) absent (inflammatory cells absent or within vessels; periodontal fibers inserted on dental tissues); (2) mild (inflammatory cells sparse or restrict to the apex; thickened periodontal ligament and few fibers arranged irregularly); (3) moderate (inflammatory cells not restricted to the vicinity of the apex, but yet not dominating the microscopic field; periodontal fibers arranged irregularly); and (4) intense (inflammatory cells present in the form of infiltrate dominating the microscopic field; disorganization of the periodontal support structures).

Statistical Analysis

Sample size estimation was obtained using SPSS® 16 for Mac (SPSS, Chicago IL, USA). Statistical analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California, USA). Radiographic and histological data were evaluated using two-way ANOVA and Bonferroni post-hoc. Differences were regarded significant when $P<0.05$.

Results

Digital radiographs showed that at the first experimental period (3 weeks), only RvE1 promoted significant reduction of periapical lesion when compared to the control; root lengths were larger for RvE1 and the polyantibiotic paste; root canal was significantly wider in the control group. At the second experimental period (6 weeks), all groups presented reduced periapical lesions, larger root lengths and narrower canals related to the control. Emdogain showed narrower canals compared to polyantibiotic paste (Figure 2B). Histological evaluation (Figure 3) showed that teeth left open to the oral environment

presented moderate or intense inflammation. At the first time point (3 weeks post treatment), periapical inflammatory reaction was significantly lower for RvE1 (inflammation was absent or mild in all specimens) compared to the other groups. Animals treated with polyantibiotic paste or EMD presented a variable inflammatory response, and about half of the roots showed moderate or intense periapical inflammation.

At 6 weeks the three test groups presented lower inflammatory response related to the control. Furthermore, in samples that presented absence or only mild inflammatory response the three treatment protocols promoted root development to some extent (Figure 4). Overall, RvE1 and EMD treated specimens presented a root morphology that closely resembled a complete root formation.The polyantibiotic paste and RvE1 promoted root formation at the expense of a bone-like and/or cementum-like tissue deposition on its apical portion, and newly formed cementum on its external surfaces. In some samples, ingrowth of periodontal ligament into root canals was detected. For EMD treated samples, the continuity of root development was mainly due to the formation of a cementum-like tissue. Additionally to the tissue deposition on root external surfaces and apical region, ingrowth of the newly formed hard tissues into root canal could be observed.

Discussion

The present study confirms that resolution of bacterial-triggered inflammation is crucial for obtaining root development in nonvital teeth, and that the population of precursor cells is able to respond even after intense bacterial challenge. Previous studies have shown that growth factors related to the inflammatory response arrest events that are necessary for root embryogenesis (Shiba *et al*.1998). In agreement, the current results show that reduction of periapical lesion was associated with gain in root length and thickness, and the absence or mild inflammation was associated with hard tissue formation. It is feasible that the treatment strategies adopted here favored the viability of

precursor cells, also stimulating their differentiation into mineralized-tissue forming cells. Apart from the intracanal medication tested, the apical portion of root canals was not instrumented, and irrigation with sodium hypochlorite was used, thus reducing infection in immature teeth. On the other hand, canal irrigation alone is unable to produce an environment that is consistently free of bacteria (Windley *et al.* 2005).

Through different pathways, the protocols tested aimed at overcoming the harm potentially caused by the maintenance of inflammatory process. Based on previous results of others, the polyantibiotic paste enhance the disinfection achieved by the irrigation regimen (Windley *et al.* 2005), RvE1 regulates host inflammatory response to microbial challenge (Serhan & Chiang, 2008) and EMD induces regenerative processes by regulating signals that are altered during infections (Suzuki *et al.* 2005). The ingrowth of connective tissue within the root canal and the stimulation of hard tissues in the apex and on external root surfaces could be observed in these groups, corroborating the results of other studies with dogs (Wang *et al.*2010, da Silva *et al.* 2010).

RvE1 lead to a significantly lower inflammatory reaction, especially at three weeks post-treatment. RvE1 was initially discovered in resolving inflammatory exudates and identified as a potent regulator of resolution of acute inflammation (Serhan *et al*. 2000, Arita *et al.* 2005). Our results showed that RvE1 reduced neutrophil infiltration, thus hastening inflammation resolution. Differently from the other groups, at the first time point, periapical lesions were reduced in teeth treated with RvE1 when compared to the control specimens, i.e., RvE1 was more effective in controlling a pre-induced inflammation faster than the other treatments. The property of rapidly downregulating inflammatory cell recruitment and activation is highly desirable since it could very effectively diminish and even abrogate the damage caused by the early inflammatory events, thus protecting and activating precursor cells. In agreement, previous studies support the effect of RvE1 in directly modulating osteoclast differentiation and consequently bone resorption, as well as

inflammatory cell recruitment (Hasturk *et al*. 2006, Herrera *et al*. 2008), which also may have an impact in the periapical lesion size.

At 6 weeks some samples treated with RvE1 showed a moderate inflammatory infiltrate, an unexpected finding given the results at three weeks, which were significantly superior to the other treatments. The single-dose regimen, the characteristics of the root canal environment and, very likely, the leakage through the occlusal access restoration, certainly played a role in this outcome. Previous studies demonstrating the beneficial effects of the topical application of RvE1 have used the drug at least daily in periodontology and ophtalmology (Hasturk *et al*. 2006, Hasturk *et al*. 2007, Li *et al.* 2010), something that was not feasible to reproduce in the protocol described herein, since repeated access to the rat teeth frequently lead to crown fracture. Another critical issue to address in order to improve its beneficial effects within the canal is the working concentration; we worked with only one concentration based on the fact that RvE1 seems to be very effective in doses as low 300 ng when used intraperitoneally in murine models (Schwab *et al*. 2007). Therefore, it was used topically in a secluded environment - tooth crown and canal - a lower concentration was chosen. Noteworthy, in the concentration used RvE1 was very effective it controlling inflammation within the first 3 weeks, with obvious advantages over the other treatments. Nevertheless, it is likely that more concentrated preparations will lead to improved results in this model. Also noteworthy, structural aspects of the endodontic environment favor the maintenance of microorganisms in empty canals (Menezes *et al.* 2004), especially in young teeth, which have a higher number of infected dentinal tubules and deeper bacterial penetration (Kakoli *et al*. 2009).

Differently from RvE1, the polyantibiotic medication and EMD have a gel-like consistency, thus, their physical characteristics seemingly allowed for better stability of the material during the course of the experiment, which may have reduced endodontic

reinfection and favored root development at the second time point. Teeth subjected to treatment with EMD presented different patterns of root formation; while the other drugs increased dentinal thickness mainly at the expense of hard-tissue deposition onto the root external surfaces, EMD promoted, additionally, the reduction of root canal width due to the ingrowth of a cementum-like tissue, which may have enhanced tooth structure resistance. These characteristics corroborate the differences of canal width observed on the radiographs. As a matter of fact, the expression of cementum attachment protein (CAP) and cementum protein-23 (CP-23), two putative cementoblast markers, has been previously detected in EMD-stimulated whole dental folicule and in cultured human dental folicle cells (Kémoun *et al*. 2007).

Several investigations confirmed the efficiency of EMD proteins in promoting osteogenesis and cementogenesis (Hammarström 1997, Boyan *et al.* 2000). The activity of growth factors including Transforming growth factor β1 (TGF-β1) and bone morphogenetic proteins (BMPs) (Suzuki *et al.* 2005), the increase of phagocytic activity of monocytic cells (Kedhmat *et al.* 2010) and the inhibition of tumor necrosis factor-α (TNF-α) (Sato *et al*. 2008) support the biological significance EMD for wound healing and periodontal regeneration (Lyngstadaas *et al.* 2009). Accordingly, these potential mechanisms may induce biological features and exclude factors that could negatively affect root development and wound healing. Although EMD have been reported to suppress the growth of microorganisms (Spahr *et al*. 2002), *Porphyromonas gingivalis* infection was found to hamper wound closure in EMD-stimulated periodontal ligament cells (Inaba *et al.* 2004). Since anaerobic microorganisms are found in endodontic infections, the hypothesis that infection has affected repair may reflect the heterogeneity of responses observed mainly during the first experimental period.

The results presented suggest that RvE1 and EMD have the potential to increase root development in necrotic immature teeth in the rat model as proposed. Further

investigations should focus in the optimization protocol for use as an intracanal medication, cellular and molecular events that take part during root formation and treatment outcome in humans.

Conclusion

RvE1 hastened periapical inflammation resolution and promoted a more favorable environment for root development earlier than other treatments. In later stages of the healing process EMD promoted, in addition to hard-tissue deposition onto the root apical portion and external surfaces, the ingrowth of a cementum-like tissue into root canal spaces. Optimization of delivery systems and concentrations of RvE1 may enhance the results presented herein.

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Acknowledgments

This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), a Brazilian Governmental Institution. The authors are grateful to Tiago Giuliani for technical assistance. Eraldo Luiz Batista Jr. is a research career awardee of the National Council for Scientific and Technological Development of Brazil (CNPq) #303175/2009-5.

Conflict of Interests

Dr. Van Dyke holds patents at Boston University that are subject to royalty payments

Figure Legends

Figure 1. Diagram summarizing the steps of experimental procedures on lower first molars of rats: at 4 weeks-old, endodontic access was performed on the central portion of oclusal surface (arrow), creating a 1mm deep cavity (*). The bur diameter and the crown length - nearly 1.5mm (**) – guided the extent of drilling (A); teeth of the control group (baseline for apical periodontitis) were left open to the oral cavity throughout the experiment (B1); At the other groups, after 3 weeks debris were removed from the pulp chamber and cervical third of the roots in an extent of about 2mm (***) and then were irrigated with NaOCl and saline solution, and filled with polyantibiotic paste, EMD or RvE1 (B2).

Figure 2. Radiographic parameters for root length, canal width (percentage of “b” relative to a”) and periapical lesion area measurements (A); Mesial roots (arrows) analysis of periapical lesion area, length and canal width for the four groups and two experimental periods. Means differ significantly related to control (*P<0.05; **P<0.01). EMD promoted narrower canals related to polyantibiotic paste (■ P<0.05) (B).

Figure 3. Histological analysis of periapical inflammation at the two experimental periods. For the three test groups, mean scores differ significantly related to control (*P<0.05; **P<0.01; ***P<0.001). At the first experimental period, Resolvin E1 promoted milder inflammation related to the other drugs (■ P<0.05). Periapical aspects after the first experimental period: teeth left open to the oral cavity during the course of the experiment showing intense periapical inflammation; Inflammatory cells were observed next to apical opening (*) and also dominating the microscopic field in distant areas (arrows) (A); Teeth subjected to intracanal medication with polyantibiotic paste (B), EMD (C) showed a variable inflammatory response to treatment. In some samples, cystic lesions (CL) and inflammatory infiltrate (*) were detected. All specimens treated with RvE1 (D) showed either absent or mild inflammatory response 3 weeks after treatmant.

Figure 4. Aspects of root development at the second experimental period. Teeth left open to the oral environment showed wide open apex (OA), inflammatory infiltrate (*) and root resorption (RR) as a consequence of root canal (RC) infection. Partial apical closure (PC) or closed apex (CA) were observed after treatment. Polyantibiotic paste and RvE1 treatment allowed the ingrowth of conective tissue (CT) into root canals. The three test groups allowed hard tissue formation at the apical portion of the roots (A) and cementum deposition on root external surfaces (E). Cementoblasts arranged in a row and newly formed cementum on root external surfaces (arrows) could be observed. EMD promoted the ingrowth of cementum on root internal surfaces (I), narrowing canals.

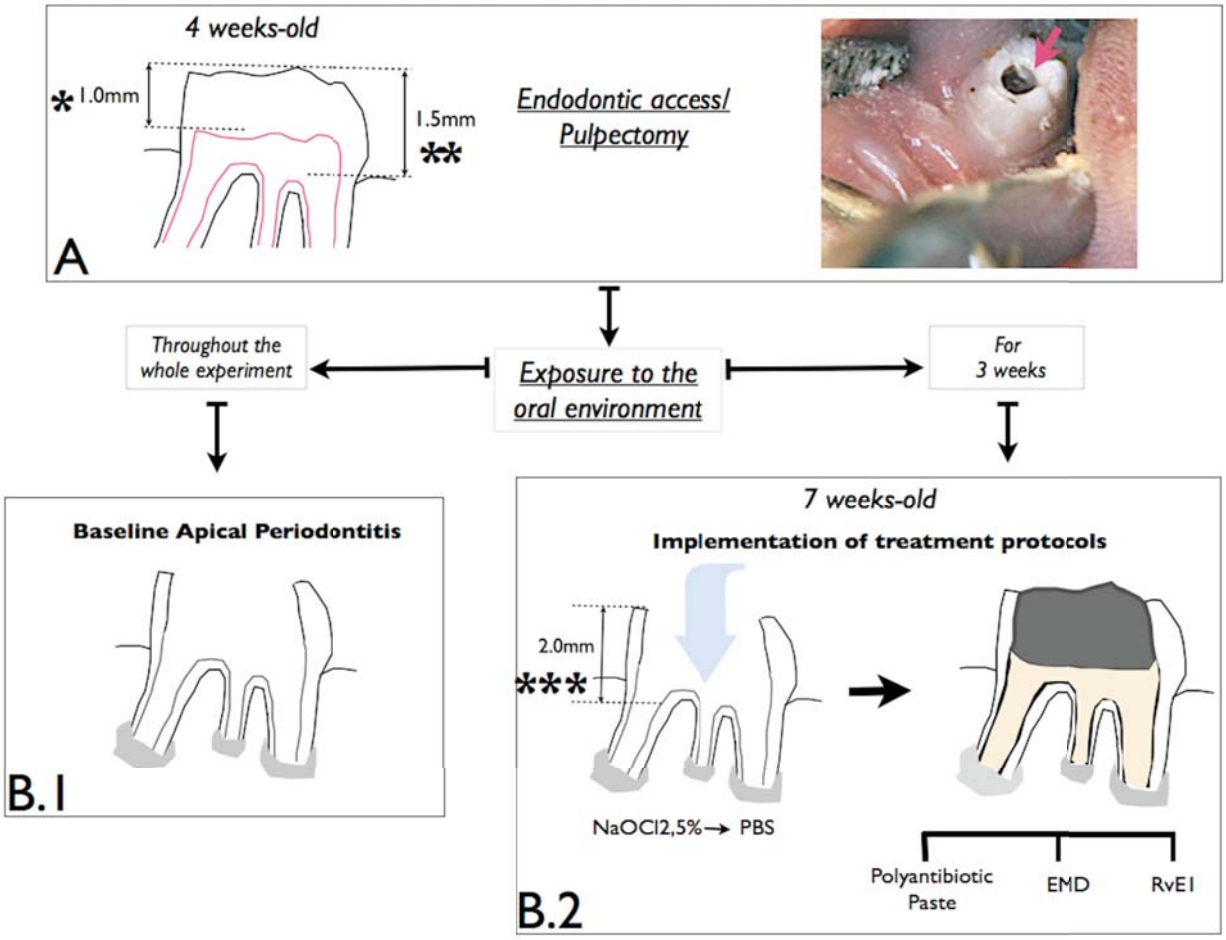

4 weeks-old
*1.0mm
1.5mm
**
A
Endodontic access/
Pulpectomy
Throughout the
whole experiment
Exposure to the
oral environment
For
3 weeks
Baseline Apical Periodontitis
B.1
7 weeks-old
Implementation of treatment protocols
2.0mm
***
NaOCl2,5%→ PBS
Polyantibiotic
Paste
EMD
RvE1
B.2

Figure 1

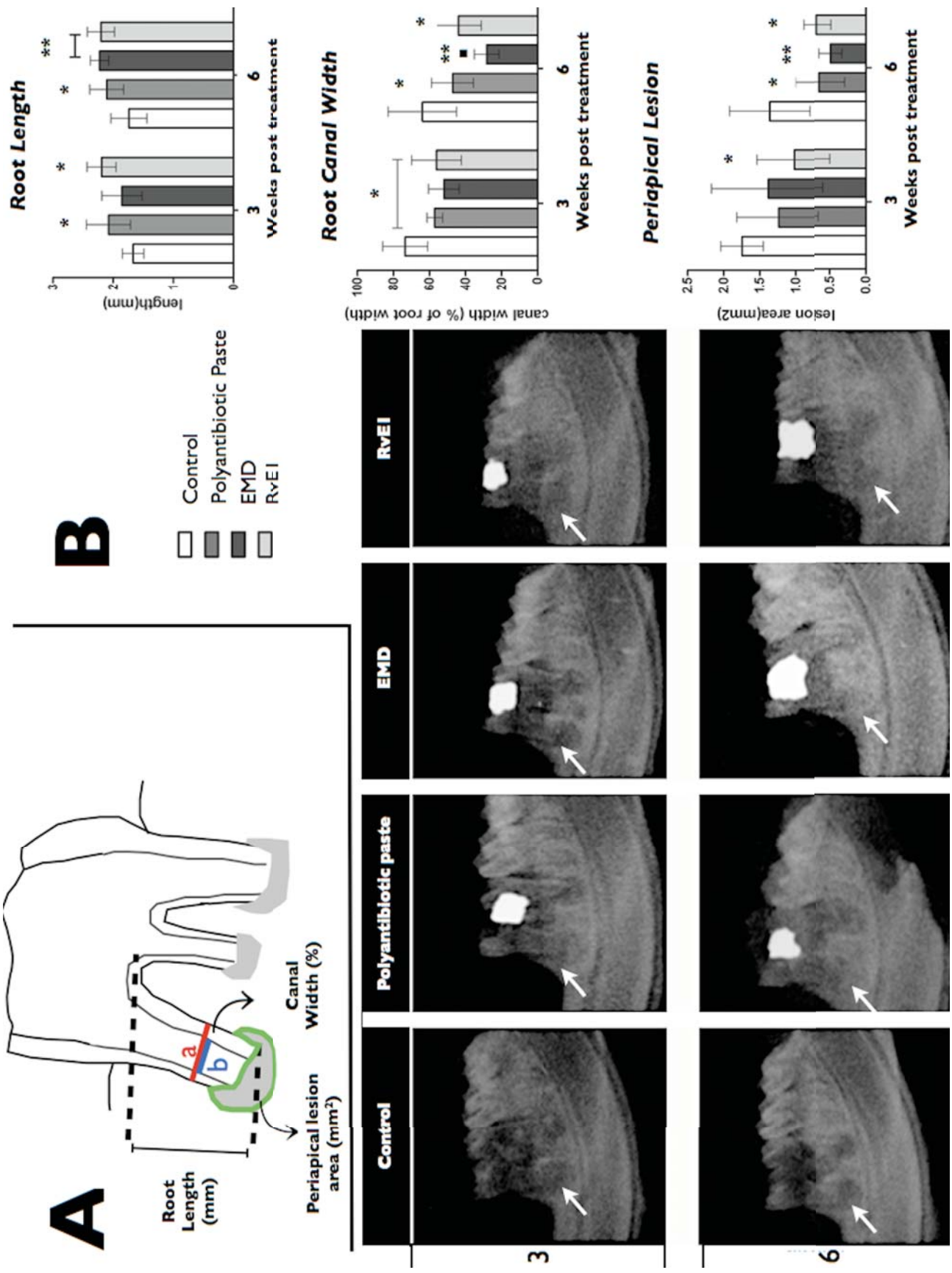

A
Root Length (mm)
Periapical lesion area (mm²)
Canal Width (%)
a
b
B
Control
Polyantibiotic Paste
EMD
RvEI
Root Length
length(mm)
Weeks post treatment
Root Canal Width
canal width (% of root width)
Periapical Lesion
lesion area(mm2)
Control
Polyantibiotic paste
EMD
RvEI
3
6

Figure 2

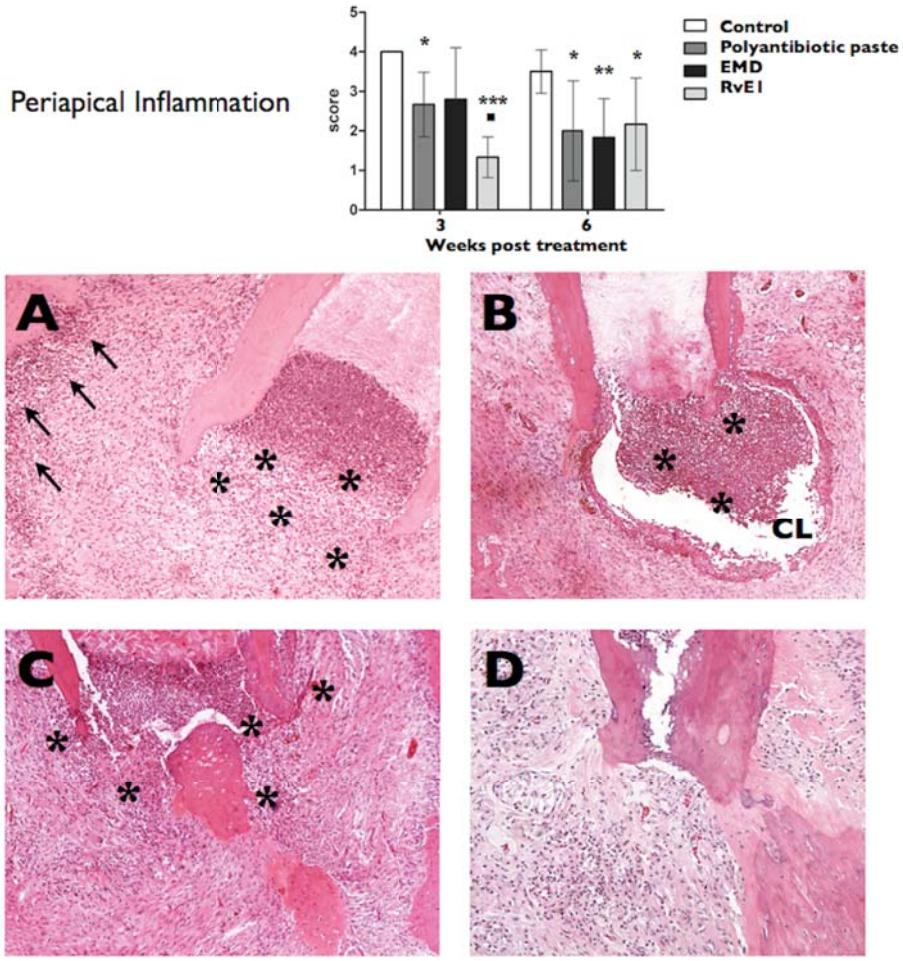
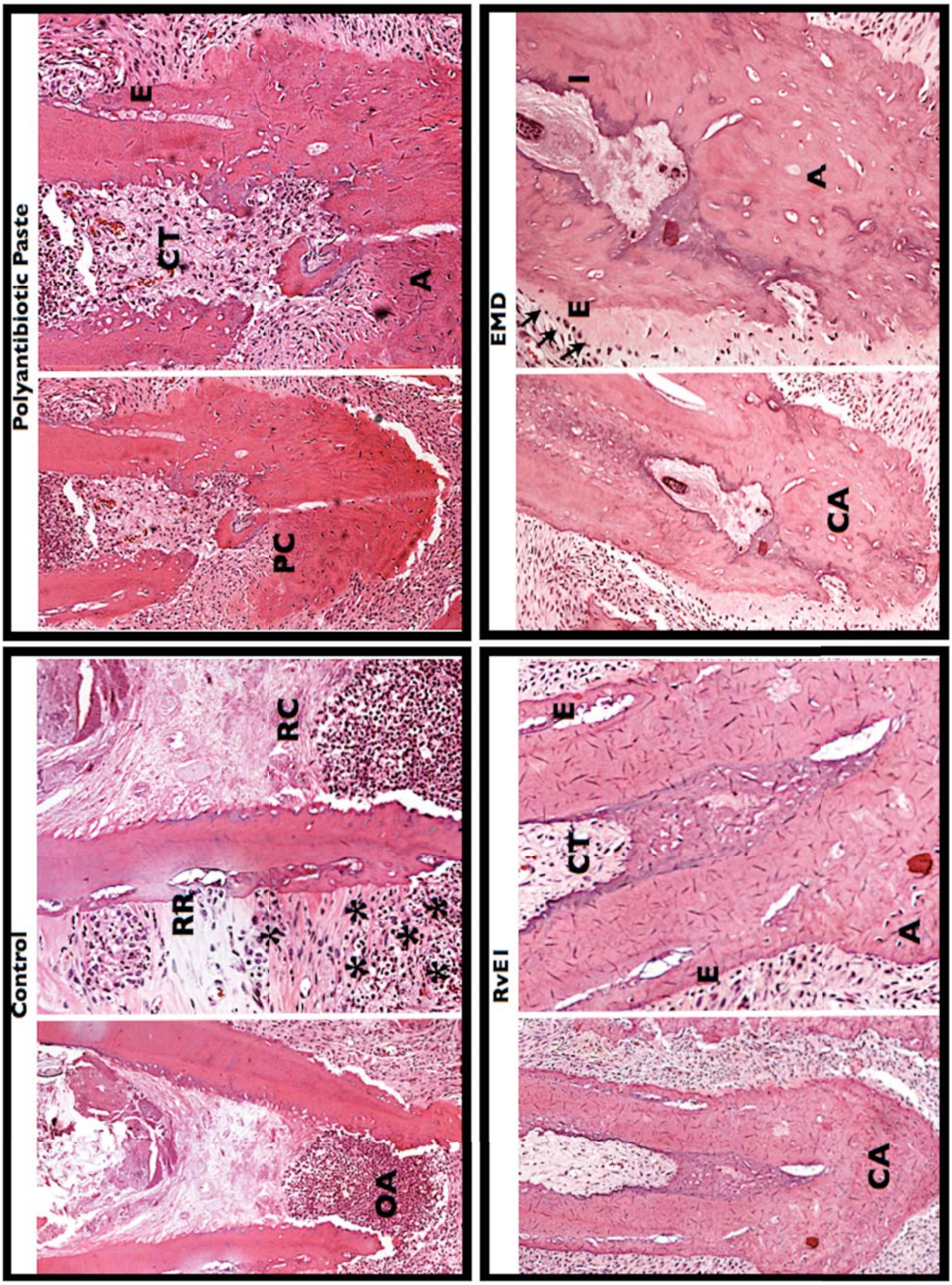

Periapical Inflammation
score
Weeks post treatment
Control
Polyantibiotic paste
EMD
RvE1
A
B
C
D
CL

Figure 3


Control
OA
RR
RC
Polyantibiotic Paste
PC
CT
E
A
RvEI
CA
E
CT
E
A
EMD
CA
E
I
A

4. Discussão Geral

4. DISCUSSÃO GERAL

O presente estudo avaliou, em ratos, alternativas de tratamento para dentes com rizogênese incompleta e necrose pulpar. Apesar de investigações nessa área comumente utilizarem cães (WANG *et al*., 2010; da SILVA *et al.* 2010), o modelo em ratos apresenta vantagens técnicas e financeiras (DAMMASCHKE, 2010), além de proporcionar maior rapidez na progressão de respostas biológicas a serem investigadas (MORETTON *et al*., 2000) e apresentar microbiota oral e respostas do hospedeiro semelhantes às observadas em humanos (KAKEHASHI *et al.*, 1965 HUXLEY, 1971; STASHENKO *et al*., 1994).

Estudos prévios demonstraram que exposições pulpares em molares de ratos produzem o desenvolvimento de alterações periapicais inflamatórias semelhantes às observadas em humanos (KAKEHASHI *et al.*, 1965; MURUZÁBAL e EURASQUIN, 1970; STASHENKO *et al*., 1994). Por outro lado, a validação do modelo para dentes com rizogênese incompleta ainda não havia sido realizada. O corrente estudo, além de confirmar o desenvolvimento de processos periapicais inflamatórios após a contaminação do sistema de canais radiculares, comprovou a interrupção do desenvolvimento radicular neste modelo animal. Contudo, as excepcionais resiliência e capacidade de reparo que caracterizam o tecido pulpar de molares de ratos (MAURICE e SCHOUR, 1955) tornam necessárias a sua desorganização por meios mecânicos e a manutenção das cavidades expostas por período não inferior a três semanas.

A avaliação das características normais do desenvolvimento embriológico em molares de ratos, somada aos aspectos técnicos necessários para indução de necrose pulpar e interrupção da formação radicular, permitiram a adequação do desenho experimental a ser executado na segunda etapa deste trabalho. Sendo assim, procedimentos para indução da necrose foram realizados em animais com quatro

semanas de idade, levando à contaminação do espaço endodôntico no início da embriogênese radicular. Além disso, a aplicação dos protocolos de tratamento sugeridos foi realizada em animais com 7 semanas de idade, proporcionando a verificação das respostas em situações de rizogênese incompleta e presença de patologias periapicais inflamatórias. Os resultados da primeira etapa do estudo também justificam os períodos pós-operatórios de três e seis semanas escolhidos, uma vez que o desenvolvimento natural das raízes está completo em grande parte dos animais com 13 semanas de idade. Por outro lado, a observação de períodos mais longos foi prejudicada pela fragilização da estrutura dentária após execução dos procedimentos, o que determinou fratura dentária e falhas no selamento coronário em animais com 16 semanas de idade, os quais foram excluídos da avaliação.

Além desta, outras dificuldades técnicas tiveram de ser superadas para a execução dos procedimentos operatórios. A dimensão reduzida e o posicionamento anatômico dos dentes tornam imprescindível, além de adequadas anestesia e posicionamento dos animais, a adaptação de meios que favoreçam o acesso ao campo operatório (MAURICE e SCHOUR, 1955, DAMMASCHKE, 2010), como o aparato desenvolvido no presente estudo para abertura de boca.

Por outro lado, características próprias da rizogênese incompleta favoreceram a aplicação técnica dos experimentos. O amplo diâmetro dos canais radiculares e o comprimento reduzido das raízes facilitaram a localização, irrigação e aplicação de medicações intracanal. Além disso, os canais não precisaram ser instrumetados e/ou obturados, proporcionando de maneira simples a padronização dos protocolos.

A aplicabilidade do modelo para avaliar estratégias de tratamento foi confirmada com base em achados semelhantes aos dos estudos que testaram a aplicação da pasta poliantibiótica em cães (THIBODEAU *et al.*, 2007; da SILVA *et al.*, 2010; WANG

et al., 2010). Conforme previamente relatado (da SILVA *et al*., 2010; WANG *et al.*, 2010), a aplicação de pasta poliantibiótica permitiu a redução do quadro inflamatório periapical em relação ao grupo controle, e o desenvolvimento radicular ocorreu principalmente às expensas de tecido osteóide ou cementóide. Também de forma semelhante aos achados da literatura (da SILVA *et al*., 2010), no segundo período experimental, aproximadamente um terços das amostras avaliadas não apresentou formação de tecidos dentários mineralizados, denotando a relevância de que outras alternativas de tratamento, como a aplicação de EMD ou RvE1, fossem investigadas.

Além da análise radiográfica quantitativa e da descrição das características histológicas dos tecidos formados, o processo inflamatório foi classificado de acordo com sua extensão e intensidade em quatro escores, os quais permitiram a comparação entre os grupos de estudo por meio de análise de variância (ANOVA). Embora este teste tenha sido inicialmente recomendado para a comparação de variáveis paramétricas, o mesmo também tem sido empregado quando do uso de valores categóricos (escores), pois, ao contrário do que se possa pensar, a inferência na análise de dados de variáveis ordinais podem envolver procedimentos paramétricos (CHILTON,1982; MONTGOMERY, 1984; ZAR, 1996). De acordo, Campbell e Machin (1993) afirmam que, se os dados são ordinais categóricos, podem ser atribuídos escores, como 1, 2, 3 e 4 e calculada a média. Essa afirmação é compartilhada por Snedecor & Cochran (1980) e Montgomery (1984) e respaldada em diversos estudos (TROIAN *et al*., 2006; GOMES *et al*., 2007; GUERRERO *et al*., 2011).

Independentemente da medicação intracanal empregada, a resolução do processo inflamatório apresentou caráter fundamental para a obtenção do desenvolvimento radicular em dentes não vitais. Por outro lado, a população de células precursoras presentes foi capaz de responder aos estímulos induzidos mesmo após intenso desafio microbiano.

Estudos prévios confirmam que fatores de crescimento relacionados com o processo inflamatório inibem ou impedem eventos indispensáveis à embriogênese dentária (SHIBA *et al*., 1998). Sendo assim, as estratégias de tratamento adotadas, além de visarem à preservação da viabilidade de células tronco remanescentes na polpa, ligamento periodontal e papila apical, buscaram reduzir o processo inflamatório decorrente da contaminação do espaço endodôntico. O regime de irrigação com hipoclorito de sódio, por si, já favorece a redução da contaminação do sistema de canais radiculares. Por outro lado, especialmente por não ser indicada a instrumentação da região apical dos canais, mantém-se algum nível de contaminação microbiana (WINDLEY *et al*., 2005).

Por diferentes meios, os protocolos testados buscaram superar o dano causado pela provável manutenção de estímulos microbianos. Estudos prévios atestam que a pasta poliantibiótica apresenta capacidade de complementar a desinfecção promovida pelo hipoclorito de sódio em dentes com rizogênese incompleta (WINDLEY *et al*., 2005); a RvE1 apresenta capacidade de regular a resposta inflamatória do hospedeiro a estímulos nocivos, como o microbiano (SERHAN e CHIANG 2008); e as proteínas derivadas da matriz do esmalte induzem processos regenerativos a partir da regulação de mecanismos alterados durante o processo inflamatório (SUZUKI *et al.*, 2005).

A invaginação de tecido conjuntivo para o interior dos canais radiculares e a estimulação da deposição de tecido mineralizado nas paredes radiculares externas e na região apical puderam ser observados em amostras submetidas à medicação intracanal com a pasta poliantibiótica. De acordo, estudos prévios confirmam que a eliminação do processo inflamatório permite que, em alguns casos, o espaço endodôntico seja ocupado por tecido conjuntivo proveniente do ligamento periodontal (WANG *et al.*, 2010; da SILVA *et al.*, 2010).

Por outro lado, a resolução do processo inflamatório foi significativamente mais rápida nas amostras tratadas com RvE1, o que fica claro nos resultados histológicos do primeiro período experimental. De acordo, estudos prévios demonstram que a RvE1 é potente reguladora da transmigração de neutrófilos e da inflamação *in vivo*, sendo também atribuído a esse composto bioativo a estimulação de fagocitose não flogística de neutrófilos apoptóticos pelos macrófagos, o bloqueio da produção de interleucina-12 por células dendríticas e a regulação da expressão de CCR5 em células T (SERHAN *et al.*, 2000; ARITA *et al.*, 2005; ARIEL *et al*., 2006; ARITA *et al.*, 2007; SCHUWAB *et al*., 2007)

Até o momento, dois receptores foram reconhecidos na atuação da RvE1. O *GPRC chemokime-like receptor* (CMKLR1) está presente em monócitos e células dendríticas e atenua a ativação de NFκB estimulada pelo TNF (mediador-chave nas fases iniciais do processo inflamatório) quando ligado à RvE1 (SERHAN *et al.,* 2000; ARITA *et al*., 2005). Por outro lado, outro receptor GPCR, o *leucotriene B4 receptor* (BLT1), se expressa em neutrófilos e interage com a RvE1 como um receptor antagonista, o qual atenua os processos inflamatórios dependentes da sinalização do leucotrieno B4 (ARITA *et al*., 2007). No presente estudo, a redução clara do infiltrado inflamatório neutrofílico pode ser observada, resultando na resolução do processo inflamatório.

Também de forma diversa dos outros grupos, no primeiro período experimental apenas a RvE1 promoveu a redução da área de lesão periapical em relação ao grupo controle. A RvE1 foi mais eficaz que os outros tratamentos no controle da inflamação previamente induzida, o que é bastante favorável por reduzir os danos causados pelo processo inflamatório. De acordo, estudos prévios confirmam o potencial deste mediador em modular a diferenciação de osteoclastos e consequentemente os processos de reabsorção óssea , assim como o recrutamento de células inflamatórias (HARSTUK *et al*., 2006; HERRERA *et al.*, 2008), o que pode ter impacto na extensão das lesões periapicais.

No segundo período experimental, algumas amostras tratadas com RvE1 apresentaram infiltrado inflamatório moderado, sendo constatado que a resposta inflamatória, a área da lesão periapical, o comprimento radicular e a espessura dos canais radiculares foram semelhantes as das amostras tratadas com os outros medicamentos. Nesse sentido, considerações acerca das características dos protocolos empregados merecem ser feitas.

Nos dentes tratados com RvE1, uma única aplicação tópica da medicação (veiculada em etanol) foi admnistrada. Este fato, somado às características do ambiente endodôntico podem ter contribuído para que no segundo período avaliado não fossem observadas as diferenças favoráveis ao mediador lipídico verificadas no primeiro período experimental. Estudos prévios que atestam os efeitos benéficos da aplicação tópica da RvE1 na periodontia e oftalmologia realizaram aplicações pelo menos a cada 24 horas, (HASTURK *et al*., 2006; HASTURK *et al.*, 2007; LI *et al.*, 2010), o que apesar de favorecer a atuação constante do mediador, não é exequível em protocolos endodônticos. Além disso, o modelo aqui adotado não permite testar protocolos que exijam repetidos acessos ao espaço endodôntico, uma vez que a fragilidade da estrutura dentária poderia resultar em fraturas e comprometer o selamento coronário.

Outro aspecto importante a ser aprimorado é a concentração de RvE1 preconizada. Com base em estudos prévios que comprovam a eficácia sistêmica desse mediador em doses reduzidas (SCHWAB *et al*., 2007), e considerando a aplicação tópica do medicamento em um ambiente isolado, no presente estudo, uma concentração bastante baixa foi escolhida. Ainda assim, os resultados do primeiro período experimental mostram clara vantagem da RvE1 em controlar o processo inflamatório em comparação aos demais medicamentos. É provável que maiores concentrações aprimorem os resultados obtidos.

Por outro lado, aspectos estruturais que caracterizam o sistema de canais radiculares podem favorecer a manutenção de microrganismos em canais vazios (MENEZES *et al.*, 2004), especialmente em dentes jovens, os quais apresentam maior número de túbulos dentinários infectados e penetração mais profunda de microrganismos (KAKOLI *et al*., 2009). Ao contrário da RvE1, a pasta poliantibiótica e as EMD apresentam consistência semelhante a de um gel. Dessa forma, suas características físicas permitem maior estabilidade do material durante o curso do experimento, o que pode ter reduzido a reinfecção do sistema de canais radiculares e favorecido o desenvolvimento radicular no segundo período experimental.

Os padrões de formação radicular nas amostras tratadas com EMD diferiram em relação aos promovidos pelas outras duas medicações. Além da deposição de tecido mineralizado na região apical e nas paredes radiculares externas, este grupo apresentou a invaginação de tecido cementóide para o interior do canal radicular, o que pode explicar a menor relação de espessura do canal radicular observada nos dados da análise radiográfica. Provavelmente, essas características contribuam com uma maior resistência da estrutura dentária, o que é desejável a fim de reduzir os riscos de fratura.

Diversos estudos confirmam a capacidade das EMD em induzir osteogênese e cementogênese (HAMMASTROM, 1997; BOYAN *et al*., 2000). Nesse sentido, a atividade de alguns fatores de crescimento podem estar relacionados com a resposta observada após medicação com EMD. Estudos prévios confirmam que essas proteínas atuam de maneira semelhante ao TGF-β1 (com efeito mitogênico sobre células do ligamento periodontal, inibição da proliferação epitelial e estímulo à diferenciação e proliferação de osteoblastos) à IL-6 (estimulando a proliferação de odontoblastos e de osteoblastos) e às BMPs (simulando a osteogênese e a cementogênese) (SUZUKI *et al*., 2005; SONOYAMA *et al*., 2007; LINDE, GOLDBERG, 1993; NAKAMURA *et al.,* 2002). Por outro lado, as EMD promovem o aumento da atividade fagocitária de monócitos (KHEDMAT *et al*.,

2010), e inibem o fator de necrose tumoral-α (TNF-α) (SATO *et al.,* 2008).

Também é atribuído às proteínas da matriz do esmalte efeito semelhante ao do TGF-β na inibição da indução de apoptose promovida pelo TNF-α em células osteoblásticas. Esses achados levam à especulação de que o mesmo efeito protetor possa ocorrer na diferenciação e cementoblastos, e nas reabsorções ósseas (HE *et al.*, 2005).

Nos últimos anos, o papel das EMD na diferenciação de cementoblastos tem sido explorado, sendo a elas atribuída a regulação da diferenciação de células do folículo dentário de camundongos em fenótipos osteo-cementoblásticos (HAKKI *et al.,* 2001). De acordo, Bosshardt *et al.*, (2005) identificaram o papel dessas proteínas na estimulação da expressão de marcadores específicos de cementoblastos, como *cementum attachment protein* (CAP) e *cementum protein-23* (CP-23). A expressão desses marcadores também foi identificada após estimulação de células do folículo dentário pelas proteínas da matriz do esmalte e pelas BMPs (*bone morphometric proteins*) 2 e 7 (KÉMOUM *et al*., 2007)

Os mecanismos acima mencionados suportam a significancia biológica das EMD para o reparo e regeneração periodontal (LYNGSTADAAS *et al.,* 2009). De acordo, esses potenciais mecanismos podem ter contribuído para a eliminação de estímulos nocivos e estimulação das respostas observadas especialmente após o segundo período. Características semelhantes as do cemento puderam ser observadas nos tecidos neoformados, o que sugere que os mecanismos já elucidados nos processos de regeneração periodontal possam estar envolvidos no desenvolvimento radicular.

Embora alguns estudos atribuam atividade antimicrobiana às EMD (SPAHR *et al.,* 2002), outros demonstram que a infecção *Porphyromonas gingivalis* inibe efeitos benéficos dessa medicação em relação a regeneração periodontal (INABA *et al*., 2004). Uma vez que as infecções endodônticas são predominantemente anaeróbias, a hipótese

de que efeito semelhante tenha retardado o reparo é consistente com a heterogeneidade das respostas observadas no primeiro período experimental.

Pelo exposto, sugere-se que tanto a RvE1 como as EMD apresentaram potenciais a serem explorados para a obtenção do desenvolvimento radicular em dentes com necrose pulpar. Estudos adicionais devem ter foco na superação das limitações dos protocolos, no esclarecimento os eventos celulares e moleculares envolvidos na formação radicular, e em resultados clínicos em humanos.

5. Conclusões

5. CONCLUSÕES

A partir dos resultados do presente estudo pode-se concluir:

- A metodologia desenvolvida permitiu a avaliação de protocolos de tratamento para casos de rizogênese incompleta e necrose pulpar, utilizando um modelo em ratos;

- O mediador lipídico Resolvina E1, aplicado como medicação intracanal, acelerou a resolução do processo inflamatório e o reparo ósseo, apresentando resultados favoráveis em todas as amostras no primeiro período experimental.

- No segundo período experimental, a medicação intracanal com EMD favoreceu o aumento da espessura das paredes radiculares, dada a invaginação de tecido cementóide para o interior do espaço endododôntico.

- Especialmente no segundo período avaliado, a desinfecção química dos canais radiculares associada à aplicação das medicações testadas promoveram o desenvolvimento radicular em dentes não vitais, estando esse condicionado à redução da inflamação periapical. Estudos adicionais devem ter foco na superação das limitações dos protocolos testados, no esclarecimento os eventos celulares e moleculares envolvidos na formação radicular, e em resultados clínicos em humanos.

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7. Anexos

Anexo A: Submissão do artigo *"**Response to intracanal medication in immature teeth with pulp necrosis: an experimental model in rat molars**"* periódico *Journal of Endodontics*

Dear Dr. Batista, Jr.,

Your submission entitled "Response to intracanal medication in immature teeth with pulp necrosis: an experimental model in rat molars." has been received by the Journal of Endodontics.

You will be able to check on the progress of your paper by logging on to the Journal of Endodontics web site as an author.

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Anexo B: Submissão do artigo "**Assessment of root formation in response to Resolvin E1 (RvE1) and Enamel Matrix Derivative (Emdogain®): an experimental study in rat immature necrotic teeth.**" ao periódico *International Endodontic Journal*

Manuscript ID: IEJ-11-00042

Title: Assessment of Root Formation in Response to Resolvin E1 (RvE1) And Enamel Matrix Derivative (Emdogain®). An Experimental Study in Rat Immature Necrotic Teeth.

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Date Submitted: 24-Jan-2011