LUCIANA CARLA NEVES DE BRITO

"ANÁLISE EPIDEMIOLÓGICA, MICROBIOLÓGICA E RESPOSTA IMUNE PERIAPICAL EM PACIENTES HIV POSITIVOS E NEGATIVOS PORTADORES DE INFECÇÕES ENDODÔNTICAS"

Faculdade de Odontologia
Universidade Federal de Minas Gerais
Belo Horizonte
2011

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

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Faculdade de Odontologia
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Trabalho realizado nos laboratórios: de bioquímica do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, sob a orientação da Prof[a] Leda Quercia Vieira, de microbiologia oral do The Forsyth Institute (Cambridge-MA-USA) afiliado à Harvard School of Dental Medicine, sob a orientação da Prof[a] Flávia Teles, e análise epidemiológica dos pacientes HIV+ no Centro de referência em doenças infecto-parasitárias (CTR) Orestes Diniz em Belo Horizonte-MG, sob a orientação do Prof. Antônio Paulino Ribeiro Sobrinho.

"Quando a gente acha que tem todas as respostas, vem a vida e muda todas as perguntas......"

Luís Fernando Veríssimo

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LISTA DE ABREVIATURAS

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1- AIDS: *Acquired immune deficiency syndrome*

2- ART: *Active Antiretroviral Therapy*

3- ATCC: *American Type Collection*

4- CD4: *cluster of differenciation 4*

5- CD8: *cluster of differenciation 8*

6- CCL: *Chemokine (C-C motif) ligand*

7- CCR: *chemokine (C-C motif) receptor*

8- CXCR: *C-X-C chemokine receptor type*

9- DNA: *Deoxyribonucleic acid*

10- GAPDH: *Glyceraldehyde 3-phosphate dehydrogenase*

11- HAART: *Highly Active Antiretroviral Therapy*

12- HIV: *Human immunodeficiency virus*

13- IL-: *Interleukin*

14- IFN-: *interferon*

15- MCP-1: *monocyte chemotactic protein-1*

16- MDA: *Multiple displacement amplification*

17- mRNA: *Messenger RNA*

18- OPG: *Osteoprotegerin*

19- PCR: *polymerase chain reaction*

20- RANKL: *Receptor activator of nuclear factor kappa-B ligand*

21- RANTES: *Regulated upon Activation Normal T-cell Expressed, and Secreted*

22- RNA: *Ribonucleic acid*

23- SCR: Sistema de canais radiculares

24- SD: *Standart deviation*

25- TCD4+: *Tcell CD4*

26- TCD8+: *Tcell CD8*

27- TCLE: Termo de consentimento livre e esclarecido

28- TGF: *Transforming growth factor*

29- Th: *T helper cell*

30- TNF: *Tumor necrosis factors*

31- Treg.: *Regulatory T cells*

32- UFC: unidades formadoras de colônias

33- UNAIDS: *Joint United Nations Programme on HIV/AIDS*

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RESUMO

RESUMO

As patologias pulpo-periapicais abrangem muitas áreas do conhecimento, tais quais a epidemiologia, microbiologia e imunologia. Muito estudadas em humanos e animais, pouco foi pesquisado sobre a interferência da síndrome da imunodeficiência adquirida humana sobre essas alterações. Este estudo analisou a necessidade de tratamento endodôntico em uma população HIV-positiva, e correlacionou esse achado com os dados pessoais e sistêmicos desses indivíduos. Aqueles que apresentavam necessidade de tratamento endodôntico tiveram seus canais radiculares analisados quanto ao perfil microbiológico e imunológico. Pacientes HIV negativos foram também avaliados para que se confrontassem os dados. Avaliou-se a presença de 107 espécies microbianas utilizando-se a associação das técnicas do Multiple Displacement Amplification (MDA) e da Hibridização DNA-DNA "Checkerboard". Utilizando-se o Real Time PCR, avaliou-se a expressão gênica de linfócitos $TCD4^{+}CD28^{+}$ e $TCD8^{+}$, citocinas e quimiocinas: TNF-α, IL-1β, IL-10, IL-17A, RANKL, IFN-γ, CCL2, CCL4, CCL5, CXCR4, CCR5. As amostras foram coletadas em dois momentos distintos, imediatamente após os procedimentos de limpeza e formatação e 7 dias após. Pode-se observar que a maioria dos indivíduos HIV-positivos eram mulheres, 14% apresentavam necessidade de tratamento endodôntico, 79.2% estavam sob HAART, e que existia uma correlação positiva entre a necessidade de tratamento endodôntico e uma baixa contagem de células $TCD4^{+}$. Detectou-se uma média de espécies por canal de 22.6 (± 1.4) e 21.3 (± 2.0) nos indivíduos HIV- e HIV+ respectivamente. *Dialister pneumosintes*, *Helicobacter pylori* e *Streptococcus constellatus* foram detectadas em maior frequência nos indivíduos HIV+. A

expressão gênica de células $TCD4^{+}CD28^{+}$ e $TCD8^{+}$ aumentou após a redução do conteúdo microbiano intracanal em ambos os indivíduos, HIV-positivo e negativo. Observou-se que nos indivíduos HIV-negativos houve uma redução na expressão de mediadores pró-inflamatórios com a redução do conteúdo microbiano, enquanto que nos indivíduos HIV-positivos houve um aumento na expressão desses mediadores no mesmo período, que correlacionou-se com um aumento na expressão da IL-10 nos indivíduos HIV-negativos e, do CXCR4 nos indivíduos HIV-positivos. Pode-se concluir que: a) a relação positiva observada entre a necessidade de intervenção endodôntica e a baixa contagem de células $TCD4^{+}$ se deve provavelmente a uma baixa aderência ao HAART; b) ao se comparar a microbiota de indivíduos HIV-positivos e negativos confirmou-se que a infeção é polimicrobiana com algumas espécies de importância médica dominando os SCR dos indivíduos HIV-positivos; c) a expressão dos marcadores celulares $TCD4^{+}CD28^{+}$ e $CD8^{+}$, em ambos os indivíduos, HIV-positivos e negativos, é afetada pela presença de estímulos microbianos nos SCR; d) a expressão gênica de citocinas e quimiocinas demonstra que a resposta pró-inflamatória nos pacientes HIV-positivos acontece mais tardiamente quando comparada àquela observada nos indivíduos HIV-negativos.

SUMMARY

SUMMARY

Pulpo-periapical pathologies cover many areas of knowledge, including epidemiology, microbiology and immunology. Even though they have been widely investigated in humans and animals, there is a gap in knowledge regarding the role of the human acquired immunodeficiency syndrome on these pathologies. The present study evaluated the need for endodontic treatment in an HIV-positive population and assessed its correlation with systemic and personal data of those individuals. Subjects that needed endodontic treatment had the root canals of those teeth analyzed for their microbiological and immunological profile. HIV-negative individuals (that needed endodontic therapy) were selected as controls for comparison. We evaluated the presence of 107 microbial species by combining the Multiple Displacement Amplification (MDA) technique and the checkerboard DNA-DNA hybridization technique. Using Real Time PCR, we evaluated the gene expression of lymphocytes $TCD4^{+}CD28^{+}$ and $TCD8^{+}$, cytokines and chemokines: TNF-α, IL-1β, IL-10, IL-17A, IFN-γ, CCL2, RANKL, CCL4, CCL5, CXCR4, and CCR5. Samples were collected at two times points, immediately after cleaning and shaping the root canal system and 7 days later. In the HIV-positive group, it was observed that the majority individuals were females, that 14% of the subjects needed endodontic treatment and that 79.2% of the HIV positive subjects were under the HAART regimen. There was a positive correlation between the need for endodontic treatment and a low $CD4^{+}$ T-cells count. There were, in average, 22.6 (± 1.4) and 21.3 (± 2.0) species per root canal in HIV-and HIV + respectively (mean±SD). *D. pneumosintes, H. pylori* and *S. constellatus* were the most abundant bacterial species among HIV positive individuals. The gene

expression of $TCD4^{+}CD28^{+}$ cells and $TCD8^{+}$ increased after the reduction of intracanal microbial content in both groups (HIV-positive and HIV-negative). It was observed that in HIV-negative individuals the local expression of pro-inflammatory mediators was downregulated after root canal instrumentation. Conversely, in HIV-positive individuals the expression of these mediators was upregulated in the same time point, which paralleled with an upregulation of IL-10 in HIV-negative and CXCR4 in HIV-positive individuals. It can be concluded that: a) despite the positive relationship between the need for endodontic treatment and low counts of $CD4^{+}$ T cells, this phenomenon is probably due to a low adherence to HAART regimen, b) when comparing the root canal microbiota of HIV-positive and negative individuals it was observed that these infections are polymicrobial and include medically important bacterial species, which were commonly found in the SCR of HIV-positive subjects; c) the expression of $TCD4^{+}CD28^{+}$ and $TCD8^{+}$ cell markers in both groups (HIV-positive and negative) seems to be affected by the presence of microbial stimuli inside SCR; d) the (local) gene expression of cytokines and chemokines showed that the pro-inflammatory response in HIV-positive occurs later, when compared to that observed in HIV-negative subjects.

INTRODUÇÃO E RELEVÂNCIA

1. INTRODUÇÃO E RELEVÂNCIA

As alterações pulpares e perirradiculares são normalmente resultado do envolvimento direto ou indireto de microrganismos da cavidade oral, que são essenciais à progressão e perpetuação das diferentes formas de alterações periapicais (Kakehashi *et al.*, 1965, Sundqvist, 1976, Moller *et al.*, 1981, Fabricius *et al.*, 1982).

Apesar de hoje se estimar que na cavidade oral existem mais de 500 espécies bacterianas (Paster *et al.*, 2001, Aas *et al.*, 2005), em condições normais, os sistemas de canais radiculares (SCR) não apresentam uma microbiota residente. Entretanto, nos dentes cujo suprimento vascular encontra-se comprometido, o ambiente torna-se favorável à contaminação por patógenos oportunistas (Baumgartner & Falkler, 1991, Sundqvist, 1992).

No passado, os estudos relacionados à microbiota endodôntica utilizavam técnicas de cultura pouco desenvolvidas, que favoreciam o crescimento de espécies aeróbias e/ ou facultativas (Hampp, 1957). Espécies, como os *Streptococcus*, eram favorecidas enquanto outras anaeróbias, como os *Bacteroides* (*Prevotella* e *Porphyromonas*), eram ignoradas. O *Staphylococcus aureus* e *Streptococcus* β hemolíticos eram isolados com menor freqüência (Seltzer & Farber, 1994).

Com o desenvolvimento das técnicas de coleta, transporte e cultivo dos isolados pôde-se observar que a verdadeira infecção endodôntica constituía-se, em sua maioria, de espécies anaeróbias estritas (Bergenholtz, 1974, Sundqvist, 1976, Gomes *et al.*, 1996, Lana *et al.*, 2001). A diversidade das infecções endodônticas passou a ser, em média, de 3 a 12 espécies por canal (Sundqvist, 1992, Gomes *et al.*, 1994, Lana *et al.*, 2001). Dentre elas, as mais

prevalentes pertencem aos gêneros: *Peptostreptococcus*, *Prevotella*, *Porphyromonas*, *Fusobacterium*, *Eubacterium*, *Actinomyces* e *Streptococcus* (Sundqvist, 1994). Acreditava-se que o baixo número de espécies encontradas nas infecções endodônticas, comparado ao grande número de espécies recuperadas nas bolsas periodontais, se devia às pressões seletivas que ocorrem nos SCR infectados (Sundqvist, 1992).

Recentemente, com o advento das técnicas de biologia molecular, houve uma melhora significativa na sensibilidade, especificidade e custo benefício das análises microbiológicas associadas à cavidade oral (Socransky *et al.*, 1998, Siqueira *et al.*, 2000b, Rocas *et al.*, 2001, Fouad *et al.*, 2002, Siqueira *et al.*, 2002, Kawada *et al.*, 2004, Socransky *et al.*, 2004, Siqueira *et al.*, 2005, Seol *et al.*, 2006, Brito *et al.*, 2007, Teles *et al.*, 2007b, Haffajee *et al.*, 2009, Tavares *et al.*, 2011). Muitas espécies microbianas, que acreditava-se não estarem presentes nessas infecções, são hoje detectadas e confirmadas como integrantes dessa microbiota (Paster *et al.*, 2001). Como exemplo de tal fato, a presença de espiroquetas nas infecções endodônticas, antes raramente detectadas, são frequentemente identificadas: *Treponema dentícola*, *Treponema pectinovorum*, *Treponema socranskii* e *Treponema vincentii* (Dahle *et al.*, 1996, Siqueira *et al.*, 2000a, Rocas *et al.*, 2001, Baumgartner *et al.*, 2003, Rocas *et al.*, 2003, Brito *et al.*, 2007, Sakamoto *et al.*, 2009, Montagner *et al.*, 2010, Tavares *et al.*, 2011).

Técnicas moleculares, como a reação de cadeia da polimerase (PCR) e a hibridização DNA-DNA (“checkerboard”), são utilizadas na detecção dos microrganismos presentes nos SCR infectados (Gatti *et al.*, 2000, Siqueira *et al.*, 2000a, Siqueira *et al.*, 2000b, Rocas *et al.*, 2001, Fouad *et al.*, 2002,

Siqueira *et al.*, 2002, Siqueira & Rocas, 2004a, Siqueira & Rocas, 2004b, Siqueira & Rocas, 2006, Brito *et al.*, 2007, Tavares *et al.*, 2011). Com essa última técnica ("Checkerboard") é possível analisar várias amostras e espécies bacterianas, simultaneamente, em uma simples membrana de nylon (Socransky *et al.*, 1994). Nenhum outro método apresenta o mesmo custo-benefício. Sua eficiência, porém, depende da viabilidade do DNA para que as sondas sejam preparadas, padronizadas e as amostras sejam analisadas. O tamanho da amostra bacteriana também pode ser um problema, pois o nível de detecção dessa técnica depende de amostras que contenham mais de 10^4 células.

Mais recentemente, tentando aperfeiçoar a técnica do "checkerboard", outra técnica, o "Multiple Displacement Amplification" (MDA), foi utilizada para amplificar o DNA bacteriano antes da análise pela Hibridização DNA-DNA (Teles *et al.*, 2007a). O MDA é um método que pode gerar uma grande quantidade de DNA a partir de pequenas amostras. Utiliza-se a enzima Ø29 DNA polimerase e primers randomizados para amplificar o DNA genômico total. O uso dessa enzima assegura um baixo erro de replicação, pois conserva-se uma cópia acurada da sequência do DNA original (Esteban *et al.*, 1993), além de eliminar a necessidade dos passos do método de purificação do DNA, que poderiam levar a sua contaminação (Dean *et al.*, 2002, Hosono *et al.*, 2003).

Amostras com pequenas quantidades de DNA, como as de apenas 1 ng, podem ser amplificadas 1000 vezes por essa técnica (MDA) (Brito *et al.*, 2007, Teles *et al.*, 2007a, Tavares *et al.*, 2011). Diferente do PCR, que utiliza sequências específicas, o MDA permite a amplificação do DNA da amostra de modo uniforme, com poucos desvios. A amplificação alcançada pode fornecer

material suficiente para que se realizem várias análises de uma mesma amostra (Dean *et al.*, 2002).

Pesquisas utilizando a associação do “MDA” e “Chekerboard” foram conduzidas com sucesso e permitiram que se demonstrassem a média de espécies por canal muito superior à encontrada até então (Brito *et al.*, 2007, Tavares *et al.*, 2011). Nesses estudos um número elevado de sondas bacterianas avaliadas também contribuíram para esse resultado.

Diante dos avanços no conhecimento das infecções endodônticas, pode-se hoje dizer que a propalada seletividade desse microambiente se devia às limitações das técnicas de identificação microbianas disponíveis naquele momento histórico (Brito *et al.*, 2007, Sassone *et al.*, 2007, Tavares *et al.*, 2011).

Das mais de 500 espécies microbianas que habitam a cavidade oral humana, 35% permanecem ainda hoje não cultivadas (Paster *et al.*, 2001, Paster *et al.*, 2002). Adicionalmente, bactérias orais têm sido implicadas em doenças como a endocardite bacteriana (Berbari *et al.*, 1997), as osteomielites em crianças (Dodman *et al.*, 2000), em doenças respiratórias (Scannapieco, 1999) e doenças cardíacas (Beck *et al.*, 1996). O conhecimento da microbiota presente nas infecções endodônticas, e de sua taxonomia se faz necessário para o seu diagnóstico e tratamento adequados.

A habilidade dos microrganismos em se implantar ou não em certos sítios dependerá, além de outros fatores, de seu número, virulência e da resistência do hospedeiro. Uma vez instalados nos SCR, esses microrganismos induzirão uma resposta de defesa nos arredores do ápice radicular (Stashenko, 1990). A resposta inflamatória, que aí se processa, recrutará células imunocompetentes

para conter e impedir a disseminação dessa infecção para outros sítios, culminando com a formação de uma lesão crônica e concomitante reabsorção dos tecidos de suporte periodontal adjacentes (Fukada *et al.*, 2009).

Nas últimas décadas, houve fortes evidências de que muitos dos efeitos patogênicos microbianos sobre os tecidos periapicais operam-se de forma indireta, via estimulação de mediadores solúveis derivados do hospedeiro, como as citocinas e quimiocinas. Daí o grande interesse em se conhecer esses mediadores e seus efeitos sobre as células imunocompetentes aí presentes (Silva *et al.*, 2005).

Uma grande variedade de células tem sido detectada nas lesões perirradiculares humanas, dentre elas: os linfócitos $TCD4^+$ e $TCD8^+$, macrófagos, células plasmáticas, mastócitos, eosinófilos, sendo as células T as mais numerosas nessas lesões (Colic *et al.*, 2009b).

Os linfócitos $TCD4^+$ e $CD8^+$, após o contato com antígenos ou de serem estimuladados por outras células inflamatórias, podem produzir uma grande variedade de citocinas (Marton & Kiss, 2000). As células $TCD4^+$ atualmente são subdivididas em vários subgrupos que incluem as células: Th1, Th2, Th17 e T regulatórias (T_{reg}) (McGeachy & Cua, 2008). A resposta Th1 caracteriza-se pela produção de IFN-γ, IL-12, IL-2, e TNF, envolvendo-se na progressão das lesões e destruição óssea perirradicular (Stashenko *et al.*, 1998, Colic *et al.*, 2009b). A resposta Th2 produz principalmente as citocinas IL-4, IL-5, IL-6, IL-9, e IL-13, relacionando-se com a cicatrização e regeneração dos tecidos perirradiculares (Akamine *et al.*, 1994, Stashenko *et al.*, 1998, Kawashima & Stashenko, 1999, Sasaki *et al.*, 2000, Teixeira-Salum *et al.*, 2010). O subgrupo Th17 produz a IL-17, citocina pró-inflamatória com atuação em várias células

da resposta inata, e é considerada ponte entre a resposta adaptativa e inata (Yu & Gaffen, 2008). Entretanto, as células T_{reg}, produtoras de TGF-β e IL-10 possuem um efeito inibitório sobre a reabsorção óssea durante a formação e diferenciação dos osteoclastos, além de atuarem na regulação da resposta imune contra a infecção (Colic *et al.*, 2009a).

Atualmente sabe-se que a integridade dos tecidos ósseos depende da manutenção de um delicado equilíbrio entre a reabsorção óssea promovida pelos osteoclastos e a deposição de matriz orgânica pelos osteoblastos. O principal mecanismo de regulação da atividade dos osteoclastos é dirigido por membros dos receptores da família do TNF (RANK, osteoprotegerina (OPG), e do ligante RANKL). A interação entre RANK e RANKL é necessária para a diferenciação e ativação dos osteoclastos, sendo esse fenômeno regulado pela OPG (Boyle *et al.*, 2003). Outros mediadores, como as quimiocinas, também se envolvem tanto na fisiologia como na patologia do tecido ósseo. As quimiocinas são fundamentais na atração de osteoblastos e precursores de osteoclastos, sendo moduladores potenciais da homeostase óssea (Bendre *et al.*, 2003, Wright *et al.*, 2005). A diferenciação de osteoclastos ocorreria por caminhos dependentes de RANKL (Wright *et al.*, 2005, Kim *et al.*, 2006).

Os linfócitos T expressam uma gama de quimiocinas e seus receptores, que atuam na quimiotaxia celular, e se envolvem na etiopatogênia das alterações perirradiculares (Silva *et al.*, 2005, Garlet *et al.*, 2010). Recentemente, dá-se uma atenção especial aos receptores CCR5 e CXCR4, pois durante a infecção pelo HIV, o vírus os utiliza como portais de entrada nas células alvo do hospedeiro (Alfano & Poli, 2005). O CCR5 estaria envolvido nos estágios iniciais da doença e o CXCR4 em estágios tardios do HIV/AIDS

(Mackay & Sallusto, 2006).

Sabe-se que as células $TCD4^+$ têm grande relevância no desenvolvimento, progressão e resolução das lesões periapicais (Kawashima *et al.*, 1996, Marton & Kiss, 2000, Garcia *et al.*, 2007). Paralelamente, essas células também apresentam um papel fundamental na síndrome da imunodeficiência adquirida (AIDS), elas são o alvo primário do HIV (Hunt *et al.*, 2003, Watanabe *et al.*, 2010). O conhecer, portanto, o papel da infecção endodôntica na resposta imune de pacientes HIV positivos e *vice versa*, torna-se importante.

A síndrome da imunodeficiência adquirida (AIDS) afeta atualmente 33 milhões de indivíduos em todo o mundo (UNAIDS, 2011). Sabe-se que esses pacientes possuem um alto risco de desenvolverem infecções oportunistas incluindo as lesões orais (Greenspan *et al.*, 2001, Ramirez-Amador *et al.*, 2003), por serem imunocomprometidos. A microbiota oral é uma importante via de propagação de patógenos oportunistas para os SCR que apresentam sua circulação sanguínea comprometida (Sundqvist, 1992). O conhecer, pois, a necessidade de tratamento endodôntico em pacientes HIV positivos e caracterizar a microbiota e as respostas imune perirradiculares que operam na infecção endodôntica desses pacientes e nos HIV negativos, contribuirão para a manutenção da saúde de ambos os grupos.

OBJETIVOS

2. OBJETIVOS

2.1. Objetivos gerais

- Avaliar a necessidade de tratamento endodôntico em pacientes HIV positivos, correlacionando-a aos seus dados demográficos e sistêmicos;

- Caracterizar a microbiota das infecções endodônticas de indivíduos HIV- e HIV+;

- Identificar a expressão de células $TCD4^{+}CD28^{+}$ e $TCD8^{+}$., bem como a expressão gênica de citocinas e quimiocinas nos tecidos perirradiculares de indivíduos HIV- e HIV+ portadores de infecções endodônticas.

2.2. Objetivos Específicos

- Determinar e comparar a necessidade de tratamento endodôntico, contagem de linfócitos $TCD4^{+}$ e $TCD8^{+}$, carga viral e utilização da terapia anti-retroviral em indivíduos infectados pelo HIV (agrupados por sexo e idade), monitorados em um centro de referência em doenças infecto-parasitária em Belo Horizonte, Minas Gerais;

- Comparar a composição microbiana de amostras recuperadas de indivíduos HIV-negativos e positivos, combinando as técnicas do Multiple Displacement Amplification (MDA) e de hibridização DNA-DNA (checkerboard);

- Caracterizar, por PCR em tempo real, a expressão gênica das células $TCD4^{+}CD28^{+}$ e $TCD8^{+}$, e das citocinas e quimiocinas IFN-γ, TNF-α, IL-1-β, IL-17A, IL-10, CCL2, CCL4, CCL5, CXCR4, CCR5 e RANKL no fluído intersticial periapical de dentes com infecção radicular em pacientes HIV- e HIV+.

TRABALHOS CIENTÍFICOS

3. TRABALHOS CIENTÍFICOS

Trabalho 1- " Brazilian HIV-Infected population: Assessment of the needs of endodontic treatment in the post-HAART era ".

Trabalho 2- " Microbiologic profile of endodontic infections of HIV negative and HIV positive patients using MDA and Checkerboard DNA-DNA hybridization."

Trabalho 3- "Overview of T lymphocytes and cytokine expression in human periapical tissues"

Trabalho 4- "T lymphocyte, cytokine and chemokine expression in periapical tissue from HIV-infected individuals".

Trabalho 1- “ Brazilian HIV-Infected population: Assessment of the needs of endodontic treatment in the post-HAART era “.

Trabalho 1- **“Brazilian HIV-Infected population: Assessment of the needs of endodontic treatment in the post-HAART era”**

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ABSTRACT

Introduction: The effects of HAART on the prevalence of endodontic diseases in HIV-infected individuals have not been reported.

Methods: The purpose of this study was to determine and compare gender, age, need for root canal treatment, CD4 and CD8 lymphocyte counts, HIV viral load and antiretroviral therapy in 222 HIV-infected individuals who were monitored at an HIV Reference Center in Brazil.

Results: One hundred and sixty-six patients comprised the studied population: the rate of the need for endodontic treatment was 14.5%; there was a positive correlation between the $CD4^+$ T-cells count (<500 cell/ mm^3) and the need for endodontic treatment, but there was no statistical difference between the need for endodontic treatment and the HIV viral load or the $CD4^+/CD8^+$ ratio in T cells counts. Of the population that needed endodontic treatment, 79.2% was taking the HAART regimen and the majority of the studied population was female.

Conclusion: We found that those HIV-infected individuals needing endodontic treatment had a lower $CD4^+$ T-cell count, which probably correlated with a lower adherence to the HAART regimen.

Key words: endodontic treatment, HIV, HAART, highly active antiretroviral therapy, root canal treatment.

INTRODUCTION

HIV has been estimated to infect 36.0 million individuals worldwide. The rate of new HIV infections has fallen in several countries, although, globally, these favorable trends are partially offset by increases of new infections in other countries (UNAIDS, 2008). Oral manifestations occur frequently in HIV/AIDS. Candidiasis, oral hairy leukoplakia, Kaposi´s sarcoma, non-Hodgkin´s lymphoma and periodontal disease are the most commonly studied oral lesions (Ferreira *et al.*, 2007). However, few studies have focused on endodontic diseases in HIV patients (Quesnell *et al.*, 2005, Shetty *et al.*, 2006, Alley *et al.*, 2008).

The goal of root canal therapy is to prevent or eliminate infections inside the root canal system that have the potential to induce apical periodontitis (Kakehashi *et al.*, 1965, Stashenko & Yu, 1989). In order to achieve this goal, extirpation and debridement of pulpal tissue and complete obturation of the prepared root canal system is performed (Quesnell *et al.*, 2005). It has been shown that after root canal filling, microorganisms may remain in periapical tissues (Tronstad *et al.*, 1990, Garcia *et al.*, 2007) and, in this case, the immune system is responsible for eliminating the remaining bacteria and healing the periapical tissue. To date, whether HIV infection impairs pulp and periapical immune responses to microbial infection remains to be clarified.

Several studies have shown that T-cells play an important role in the development, progression and resolution of periradicular lesions (Kawashima *et al.*, 1996, Marton & Kiss, 2000, Garcia *et al.*, 2007). In the early phases of periradicular lesion development, CD4-T-cells, which are the primary target of HIV, are more predominant. Conversely, CD8-T-cells, which are relatively

unaffected by HIV, become more prominent in the chronic phases of the lesion as the CD4-T-cells gradually decrease in number (Kawashima *et al.*, 1996, Garcia *et al.*, 2007). Patients with HIV infection are at a high risk of developing opportunistic infections, but after the introduction of highly active antiretroviral therapy (HAART) in 1996, several studies have shown an increase in the CD4 lymphocyte counts and a reduction in the plasma HIV-RNA viral load followed by a decrease in the prevalence of various oral lesions associated with HIV disease (Greenspan *et al.*, 2001, Greenwood *et al.*, 2002, Ramirez-Amador *et al.*, 2003).

The Brazilian STD/AIDS National Program is renowned worldwide for providing universal access to antiretroviral therapy. The mortality rates of AIDS patients decreased from 97.4% in 1983 to 19.8% in 2001 (UNAIDS, 2008). The number of individuals who live with HIV/AIDS is increasing as a result of both new cases of infection and the improvement in life expectancy of those already infected (Ferreira *et al.*, 2007). Knowing the benefits of such a health policy on this population, especially on its oral conditions, might lead to the implementation of strategies to promote the health of HIV-positive patients. In this regard, it is important to note that data examining the prevalence of the need for root canal treatment in an HIV population, including after the introduction of HAART, has not been reported.

The aim of this study was to compare the personal information (gender and year of birth) and the need for root canal treatment in HIV-infected patients with their systemic HIV data (CD4 and CD8 lymphocyte counts and HIV viral load), along with their use of antiretroviral therapy, at the time of initial dental presentation (first appointment).

METHODS

Sample and procedure

Data were obtained from January 2007 to July 2008 from two hundred and twenty-two HIV-infected men and women (children and adults) monitored at the Dental Care of HIV/AIDS Patients, Centro de Treinamento e Referência em Doenças Infecciosas e Parasitarias Orestes Diniz, Belo Horizonte, Minas Gerais, Brazil. Personal information (gender and year of birth) and clinical assessment of the need for endodontic treatment were obtained from patients during dental appointments, while systemic HIV data (CD4 and CD8 lymphocyte counts, HIV viral load, and antiretroviral therapy) were taken from medical records. The criteria used to determine the need for endodontic treatment was based on clinical and radiographic analyses, along with pulp vitality tests. The correlation among the collected data was assessed. In order to be included in the study, participants signed the Free Agreement Formulary. This study received approval from the Ethics Committee of the Federal University of Minas Gerais (ETIC 545/07).

Statistical Analyses

Data analyses were performed using SPSS for Windows (version 15.0). Data were subjected to the Kolmogorov-Smirnov test to characterize their normality, while Chi-square test was chosen to explore the association between the categorical variables. Mann-Whitney U test was used to examine the association between the need for endodontic treatment and viral load ($p < 0.05$). Descriptive analyses were performed to verify patient characteristics and

prevalence of oral and systemic conditions.

RESULTS

Patient characteristics

One hundred and sixty-six patients were included in this study. They were selected from the monitored population consisting of two hundred and twenty-two HIV-positive patients that attended the Dental Care of HIV/AIDS Patients (CTR/DIP Orestes Diniz) among those that presented medical records updated at the time of first appointment. The majority of the eligible patients were female (57.2%). The patients were between 5 and 66 years old, with a mean age of 33.66 years (SD=14.37). Twenty-one percent of this population was comprised of children under 15. In total, 14.5% of the study subjects were included in the group of patients who needed to receive endodontic care.

Viral load measurements

In this study 68.3% of the all patients had a viral load < 10.000 copies/mm^3, while 31.7% present a viral load >10.000 copies/mm^3. Of the patients needing root canal treatment, 70.8% had viral loads below and 29.2% had viral loads above 10.000 copies/mm^3. However, no significant difference ($p>0.05$) was observed when these data were compared to the patients with no need for root canal treatment (67.9 and 32.1%) (Fig 1 and Tab. 1).

$CD4^+$ T-cell count

Herein, 53.7% of the eligible population presented $CD4^+$ T-cell counts below 500 cell/ mm^3. In the group of patients that needed root canal treatment,

79.2% had $CD4^+$ T-cell counts below 500 cell/ mm^3. In contrast, among the patients who did not need endodontic care, only 49.3% exhibited $CD4^+$ T-cell counts below 500 cell/ mm^3. When the two groups were compared (with or without the need for root canal therapy) a statistically significant difference was detected ($p<0.05$) (Fig 1 and Tab. 1).

$CD8^+$ T-cell count

In this study, 83.2% of eligible patients presented a ratio of $CD4^+/CD8^+$ cells below 0.9. It was below 0.9 for 91.7% and 81.8% of the patients with and without the need for root canal therapy, respectively. When the two groups were compared, no statistically significant difference was observed ($p>0.05$) (Fig 1 and Tab. 1).

HAART antiretroviral regimen

Table 2 shows the HAART antiretroviral medications and the proportion of the patients who received each of the different drugs. Of the eligible population, 15.66% did not take HAART medication (Tab. 2), but for the patients that needed root canal therapy this number was 19.2%. Zidovudina and Lamivudina were the most commonly prescribed drugs (Tab. 2). When the viral loads (below 10.000 copies/mm^3) were compared between the group of patients that either had taken or not taken HAART antiretroviral medication, no statistical significance was observed ($p>0.05$) (Tab 3). In addition, no difference ($p>0.05$) was detected between these groups (taking or not HAART regimens) with regards to $CD4^+$ cell counts (below 500 cells/ mm^3) (Table 3).

DISCUSSION

Even though this study was performed only in one dental care center for HIV/AIDS Patients (CTR/DIP Orestes Diniz), this service is responsible for monitoring 60% of the HIV-positive population at the metropolitan area of Belo Horizonte, and almost all patients referred from different cities in the state of Minas Gerais. However, only multi-center studies to be performed in different states would be able to show the real prevalence of the need for endodontic treatment in the HIV-Brazilian population.

In this study, the majority of eligible patients were female (57.2%); this corresponds to a trend observed around the world. This result is in accordance with the global phenomenon known as the feminization of the HIV epidemic (Hankins, 2008). According to UNAIDS, strategies to increase women's economic independence and legal reforms to recognize women's property and inheritance rights should be prioritized by national governments to try to counter this phenomenon (UNAIDS, 2008).

According to Stoff *et al.* 2004 a reduction in HIV-related mortality and an increase in the prevalence of elderly infected by HIV have been reported after the introduction of HAART. In this study, the mean age of patients was of 33.66 years, and ranged from 5 to 66 years old. It is interesting to point out that 21.0% of this population was comprised of children under 15. The annual number of new HIV infections among children worldwide has declined since 2002 due to the expansion of services to prevent mother-to-child transmission; however, the number of children younger than 15 years living with HIV increased from 1.6 million in 2001 to 2.0 million 2007 with almost 90% of these children living in sub-Saharan Africa (UNAIDS, 2008).

Concerning the rate of 14.5% of patients needing endodontic treatments assayed in this study, it is very interesting to note that it cannot be compared to another rate of need for endodontic care from a given population, since the epidemiology of root canal diseases has not been reported. Moreover, it is interesting that this population sought assistance at a public dental health service that offers basic dental assistance to mostly low socioeconomic groups. Other parameters should be considered to analyze this result, such as stigma and discrimination, along with low budgets and policy support for dental programs. The Dental Extension School, an academic program at the Federal University of Minas Gerais, allowed all participants of this study to have their root canals treated and their other oral problems solved.

The risk for HIV disease progression should take into consideration CD4 cell counts and viral load trajectories as well as baseline viral load and medical history (Kaplan & Mounzer, 2008). Within 8 to 12 weeks after infection, HIV reaches levels as high as several million RNA molecules/mL, and afterwards, it is reduced to much lower levels (5000 to 15,000 molecules/mL) or even to an undetectable level, suggesting that an immune response can control the infection (Levy, 2006). As shown by Mellors et al. 1996, the lower the viral set point, the better the long-term prognosis for the infected person. The HIV viral load analyses help physicians make decisions concerning the patient's treatment. These analyses are useful for observing disease progression by showing the HIV quantity in the blood stream. Our findings, with respect to viral load, show that no significant difference ($p>0.05$) was observed when data from patients with or without need for root canal treatment were compared. Although these observations led to the suggestion that viral load was not related to

endodontic disease, it is important to remember that these results correspond to times of initial dental presentation, not viral load trajectories that would be correlated to the development of endodontic disease. It has been reported previously that a reduction in the plasma HIV-RNA viral load is followed by a decrease in the prevalence of various oral lesions associated with HIV (Greenspan *et al.*, 2001, Greenwood *et al.*, 2002, Ramirez-Amador *et al.*, 2003).

Several researchers suggest that the HIV-specific immune response is present early in the infection, which can be seen phenotypically as well as via the function of the responding CD4 and CD8 T cells (Day *et al.*, 2006, Zaunders *et al.*, 2006). This period is characterized by extremely high viral loads and a dramatic loss of CD4 T cells (Kassutto & Rosenberg, 2004, Zaunders *et al.*, 2006). These cells play a key role in the generation of immunity by providing appropriate stimuli for the development of humoral and cell-mediated immune responses (Betts & Harari, 2008). $CD4^+$ T cells are the primary target of HIV infection leading to a rapid depletion of the T-cell repertoire and functional impairment of $CD4^+$ T cells within weeks of infection. The absolute $CD4^+$ T-cell count is used as a marker of immunosuppression. Within the group of patients that needed endodontic treatment 79.2% had $CD4^+$T-cell counts below 500 cell/mm^3; this is statistically different from patients in the non-endodontic group ($p<0.05$). Although this suggests an association between $CD4^+$ T-cell counts and the need for endodontic treatment, it is important to note that almost all patients were under HAART regimen, and half of them (53.7%) presented $CD4^+$ T-cell counts below 500 cells/mm^3. Thus, this finding is more likely related to the patients´ adherence to the treatment than to the perceived correlation observed

in this study. Researchers have shown that a 50% adherence level to the HAART regimen is common for people living with HIV/AIDS who participate in cross-sectional studies (Godin *et al.*, 2005, Cote *et al.*, 2008). On the other hand, it has been reported that when ART is started, a significant percentage of patients develop inflammatory disease or cellular proliferative disease resulting from residual T and B cell depletion or dysfunction (French, 2007, French & Colebunders, 2008) associated with an active or resolved infection by an opportunistic pathogen (French & Colebunders, 2008). As most endodontic diseases are the outcome of pulp tissue breakdown followed by opportunistic bacterial infection (Kakehashi *et al.*, 1965, Stashenko & Yu, 1989), the association between $CD4^+$ T-cell counts and the need for endodontic treatment may be considered a consequence of this phenomenon.

A number of T cells responses, including the production and release of various cytokines and chemokines, upon antigenic stimulation have been shown to be important in antiviral immunity (Pantaleo & Koup, 2004, Betts *et al.*, 2006). $CD8^+$ T cells have been suggested to play a crucial role in controlling primary infection, exerting substantial pressure upon HIV via a multifunctional, rather than a monofunctional, response (Betts & Harari, 2008). It is likely that the depletion and dysfunction of HIV-specific $CD4^+$ T cells leads to the induction of defective CD8 T-cell responses during chronic infections, leading to the inability of the system to exert full immunological control over the virus (Williams & Bevan, 2006). In this regard, a reduction of the ratio between $CD4^+$ and $CD8^+$ cell counts to below 1 is associated with a weakening of the host's immune function. Herein, 91.7% and 81.8% of the patients from the groups with or without the need for endodontic treatment, respectively, presented with a ratio

below 0.9 ($p>0.05$). Similarly, in experimental animal models, researchers have shown that periradicular lesions that progress to a more chronic state are related to increased numbers of $CD8^+$ T lymphocytes in non-HIV (Stashenko & Yu, 1989, Stashenko *et al.*, 1994, Kawashima *et al.*, 1996) as well as in HIV-positive immune deficiency syndrome models (Levine *et al.*, 2001).

The prognosis for people living with HIV/AIDS was completely changed after the introduction of HAART in 1996, which dramatically decreased morbidity and mortality. The goal of this therapy is to achieve and maintain HIV-1 RNA below detectable levels (50 copies per milliliter) (Kaplan & Mounzer, 2008). In this study 84.3 % of the eligible population was taking antiretroviral medication (HAART), while in the group that needed endodontic treatment, the rate was lower (79.2 %). This group's patients probably did not adhere to taking the antiretroviral therapy and may have also exhibited a lower probability of seeking dental care. On the other hand, when the use of an antiretroviral regimen (HAART) was compared to $CD4^+$ T cell counts from both groups, no significant difference was observed. Different active agents were taken by the HIV-infected population, and almost all individuals used a three drug combination, with Zidovudina and Lamivudina being the drugs most commonly used (50.6 and 48.19%, respectively).

For many years, HAART has allowed for the control of HIV infection, but definitive control of this infection still requires more research. In the endodontic area, long prospective studies are needed to determine the relationship between HIV infection and root canal diseases. In addition, immune and microbiological aspects of endodontic diseases in HIV-infected individuals are remain unknown and are thus deserving of additional research.

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FIGURE AND TABLES

Fig.1

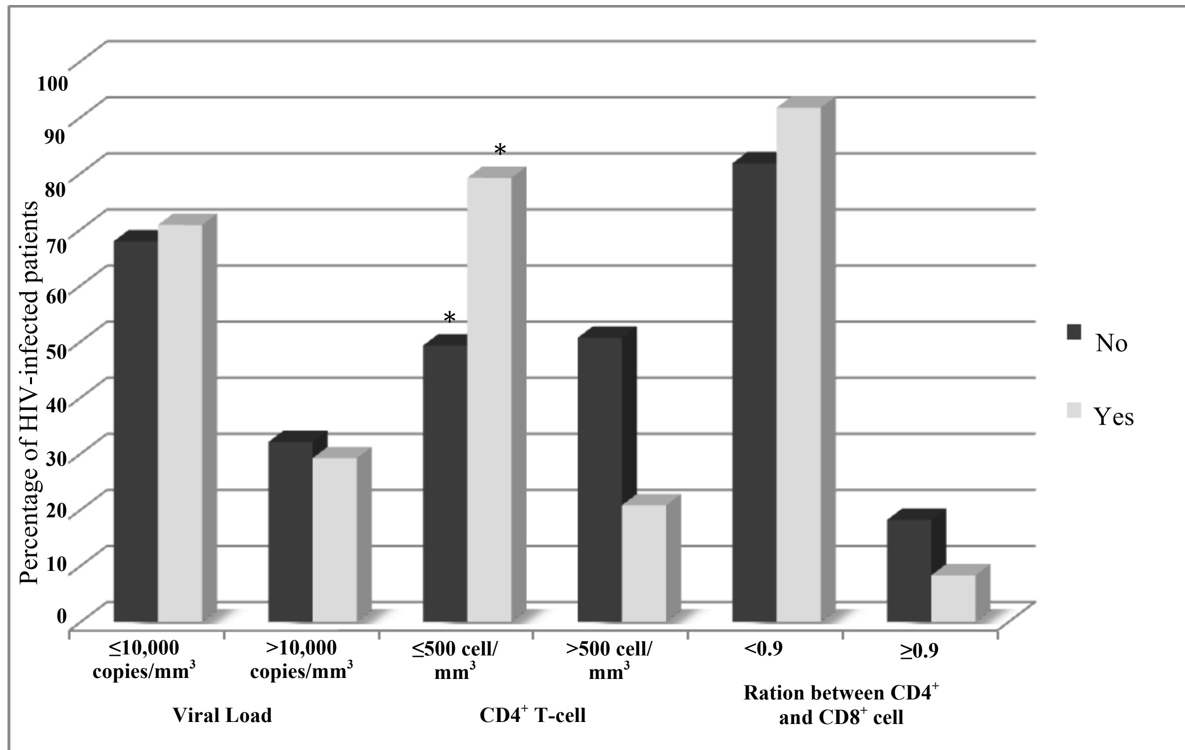

Figure 1. Percentage of patients with HIV viral load above and below 10,000 copies/mm^3, CD4$^+$ lymphocyte counts above and below 500 cells/mm^3 and CD4$^+$/CD8$^+$ T-cells ratio above and below 0.9 in the group that needed root canal treatment (yes) and in the group that did not need the treatment (no). * Indicates $p < 0.05$ by **Fisher's** exact **test.**

Table 1 - Correlation of the viral load, $CD4^+$ T-cells count and ratio of $CD4^+/CD8^+$ T-cells with the need for endodontic treatment

		Endodontic Treatment	
		Yes	No
Viral load			
	Mean	36056.43	18244.72
	Median	7300.00	350.00
	SD	72045.22	45837.02
	Min / max	50 / 275654	50 / 271931
$CD4^+$ T-cell counts			
	Mean	410.50	594.13
	Median	294.00	503.50
	SD	346.84	416.054
	Min / max	115 / 1397	3 / 2260
$CD4^+$/ $CD8^+$ T-cell			
	Mean	0.44	0.60
	Median	0.36	0.53
	SD	0.27	0.41
	Min / max	0.10 / 1.22	0.01 / 2.40

Table 2 - Percentage of patients receiving each antiretroviral medication.

Percentage/Number HIV infection sufferers		HAART	
		Yes	No
		84.3% /140	15.66%/ 26
50.6%	84	Zidovudina	
48.19%	80	Lamivudina	
30.72%	51	Efavirenz	
18.07%	30	Didanosina	
9.63%	16	Nevirapina	
17.46%	29	Biovir	
15.06%	25	Kaletra	
9.03%	15	Estavudina	
7.83%	13	Atazanavir	
10.84%	18	Nelfinavir	
8.43%	14	Tenofovir	
7.83%	13	Ritonavir	
3.61%	6	Indinavir	
10.84%	18	Nelfinavir	
2.40%	4	Lopinavir	
2.40%	4	Saquinavir	
1.20%	2	Abacavir	

Table 3 - Antiretroviral medication *versus* the viral load and $CD4^+T$ cell counts

	HAART regimen		
	Yes	No	P
Viral Load			
< 10,000 copies/mm^3	91(82.7%)	19(17.3%)	0.369
> 10,000 copies/mm^3	45(88.2%)	6 (11.8%)	
$CD4^+T$-cell counts			
< 500 cell/mm^3	74(84.1%)	14(15.9%)	0.983
> 500 cell/mm^3	64(84.2%)	12(15.8%)	

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Trabalho 2- “ Microbiologic profile of endodontic infections of HIV negative and HIV positive patients using MDA and Checkerboard DNA-DNA hybridization.”

Trabalho 2. **“Microbiologic profile of endodontic infections of HIV negative and HIV positive patients using MDA and Checkerboard DNA-DNA hybridization”**

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ABSTRACT

Although molecular techniques have allowed a better appreciation of the microbiota of endodontic infections, endodontic microbiota of HIV positive individuals remains virtually unknown.

Aim: to compare the microbiota of endodontic infections in necrotic teeth from HIV negative (HIV-) and HIV positive subjects (HIV+).

Materials and Methods: Root canal samples from necrotic teeth were collected from 40 HIV- and 20 HIV+ subjects. Samples were amplified using multiple displacement amplification (MDA). Then, checkerboard DNA-DNA hybridization was employed to assess the levels of 107 microbial taxa. The percentage of DNA the probe count of each taxon was computed for each subject and then averaged across subjects in each group separately. The percentage of teeth colonized by each test species was also computed. Significant differences between the 2 groups regarding proportions of taxa and prevalence of the test species was sought using the Mann Whitney test and the Chi-square analysis, respectively.

Results: The most prevalent taxa detected were *Prevotella tannerae*, *Abiotropia adjacens* and *Streptococcus constellatus* among HIV- subjects and *Dialister pneumosintes*, *Helicobacter pylori* and *S. constellatus* among HIV+ individuals. The most abundant species were *S. constellatus*, *A. adjacens* and *Prevotella denticola* in HIV- and *S. constellatus* and *P. tannerae* in HIV+ samples. *P. tannerae* and *Candida albicans* were significantly more abundant in HIV+ samples.

Conclusions: There were significant differences in the prevalence and proportions of specific microbial taxa between HIV- and HIV+ individuals. The

root canal microbiota may represent a reservoir of important oral and medical pathogens, mainly in HIV+ individuals.

KEY WORDS: Endodontic infections, HIV positive patients, MDA, checkerboard,

INTRODUCTION

During the past few decades, numerous studies described the microbiota of endodontic infections (Sundqvist, 1992, Siqueira *et al*., 2005b, Brito *et al*., 2007, Tavares *et al*., 2011). In particular, the use of molecular biology techniques has expanded the breadth of knowledge of this topic. They have allowed a better characterization of the microbial composition of the root canal system and have confirmed that anaerobic species predominate in endodontic infections (Brito *et al.*, 2007, Tavares *et al.*, 2011).

Despite the advances in the field, little is known regarding the microbial composition of the root canal infections in HIV positive (HIV+) patients. The HIV/acquired immunodeficiency syndrome (AIDS) affects 33.4 million individuals worldwide and remains a global pandemic disease (UNAIDS, 2011). HIV infected individuals are considered a high-risk group to develop opportunistic infections (Ramirez-Amador *et al*., 2003) including lesions of the oral cavity (Glick *et a*l., 1994, Coogan *et al*., 2005). In addition, the mouth may represent a microbial reservoir that can harbor medically important microorganisms (Nakou *et al*., 1997, de Souza Goncalves *et al*., 2009).

Checkerboard DNA-DNA hybridization is a high-throughput molecular method that allows the identification and quantification of a wide range of bacterial species in multiple samples simultaneously. This technique has been

employed in the study of the microbiota in saliva (Sachdeo *et al*., 2008), supragingival plaque (Haffajee *et al*., 2008, Uzel *et al*., 2011), subgingival plaque (Teles *et al*., 2008, Uzel *et al.*, 2011), on oral soft tissue (Mager *et al*., 2003), on dentures (Sachdeo *et al.*, 2008), from dental implants (Gerber *et al*., 2006) and in samples from root canals (Brito *et al.*, 2007, Tavares *et al.*, 2011).

The level of detection of the checkerboard DNA-DNA hybridization technique is between 10^4 and 10^7 bacterial cells of a given species in each sample. Because the microbial content of samples from root canals may be below this level, a DNA amplification step has been used to enhance detection limits (Brito *et al.*, 2007, Teles *et al*., 2007, Tavares *et al.*, 2011). Multiple Displacement Amplification (MDA) enables whole genomic amplification of DNA targets (Dean *et al*., 2002, Andres *et al*., 2006) with minimal bias (Dean *et al.*, 2002, Yan *et al*., 2004). The template is replicated again and again by a "hyperbranching" mechanism of strand displacement synthesis (Lizardi *et al*., 1998), with the polymerase laying down a new copy as it displaces previously made copies. Samples as small as 1 ng can be amplified 1000-10,000 fold (Mai *et al*., 2004). This method allows the uniform amplification of the whole genomes present in a sample and has been effectively used as an aid in Checkerboard DNA-DNA Hybridization (Brito *et al.*, 2007, Teles *et al.*, 2007, Tavares *et al.*, 2011).

The aim of the present study was to compare the microbial profile of endodontic infections in HIV- and HIV+ subjects and to seek intra-oral and medically important microbial taxa in those root canals.

MATERIALS AND METHODS

Subject population and sample collection

Forty subjects HIV- and twenty HIV+ subjects were enrolled in the study. HIV+ individuals were referred from the Reference Center of Infectious and Parasitary Diseases Orestes Diniz, Belo Horizonte, Minas Gerais, Brazil a center that specializes in HIV/Aids treatment (de Brito *et al*., 2009). HIV– subjects were recruited at the Department of Endodontics, Federal University of Minas Gerais (UFMG), Belo Horizonte, Brazil, where all study participants were examined and sample collection was performed.

To be included in the study, HIV– and HIV+ study participants had to present at least one tooth with endodontic infection and radiographically detected periradicular tissue alteration. Individuals who underwent antibiotic treatment within three months of prior to the beginning of the endodontic therapy were not eligible to participate. This study was approved by the Ethics Committee of the Federal University of Minas Gerais (ETIC 122/08).

All selected teeth had clinical crowns that permitted effective rubber dam isolation. There was no history of trauma associated with the selected teeth, periodontal involvement or previous root canal treatment. Samples from multi-rooted teeth were taken from the largest root canal always associated with the periapical lesion.

After patients signed an informed consent, microbial samples from selected root canals were collected. The selection and preparation of the teeth was performed as previously described (Brito *et al.*, 2007). In brief, samples were taken by scraping or filing the root canal walls with a #10 K-type hand file (Maillefer, Ballaigues, Switzerland). The file was introduced into the canal to a

level approximately 1 mm short of the tooth apex. After removal from the canal, the final 4 mm of the file was removed using a sterile pair of surgical scissors and placed in a microcentrifuge tube containing 20 µl of alkaline lysis buffer (400 mM KOH, 100 mM dithiothreitol, 10 mM EDTA). After 10 min of incubation on ice, 20 µl of neutralization solution (400 mM HCl, 600 mM Tris-HCl, pH 0.6) was added. Samples were kept at 4°C until analysis.

Multiple displacement amplification (MDA) of root canal samples

Multiple displacement amplification was performed as previously described (Brito *et al.*, 2007, Teles *et al.*, 2007, Tavares *et al.*, 2011). The Illustra™ GenomiPhi™ V2 DNA Amplification Kit (GE Healthcare, USA) was used for whole genomic amplification as described by the manufacturer. In brief, 1 µL of each of the DNA templates (i.e. endodontic samples) was added to 9 µL of sample buffer (50 mM Tris-HCl pH 8.2, 0.5 mM EDTA containing random hexamer primers) in 200 µL microcentrifuge tubes (Stratagene, La Jolla, CA, USA). Templates in sample buffer were heat denatured at 95°C for 3 minutes in a Perkin-Elmer Thermocycler and cooled to 4°C. One µL of phi 29 DNA polymerase mix including additional random hexamers was mixed on ice with 9 µL of reaction buffer containing dNTPs. The mixture was then added to the denatured sample to make a final volume of 20 µL and incubated at 30°C for 2 hours. Ten ng of Lambda DNA (contained in 1 µL) was used as a control. The amplification reaction was terminated by incubation of the samples at 65°C for 10 min. The amplified material was either immediately used, stored short-term at 4°C or at –20°C for longer storage.

The DNA content of the samples was measured prior to and after

amplification using the Picogreen™ dsDNA quantification assay (Invitrogen, Carlsbad, CA, USA). Picogreen™ is a fluorescent nucleic acid stain that allows the quantification of as little as 25 pg/mL of double stranded DNA in samples. The microbiological content of the amplified samples was analyzed using checkerboard DNA-DNA hybridization.

Bacterial strains and growth conditions, DNA isolation, preparation of DNA probes and Checkerboard DNA-DNA hybridization

The 107 reference strains used for the preparation of DNA probes are listed in Table 1. The grown conditions of the selected bacterial strains have been described earlier (Socransky *et al*., 2004, Brito *et al.*, 2007, Teles *et al.*, 2007, Tavares *et al.*, 2011).

Preparation of probes and standards for quantification

Checkerboard DNA-DNA Hybridization was performed as previously described (Socransky *et al*., 1994, Socransky *et al.*, 2004). To prepare probes and standards, each species listed in Table 1 was grown on agar plates (except the two spirochetes, which were grown in broth) for 3–7 days. The cells were harvested and placed in 1.5 mL microcentrifuge tubes containing 1 mL of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6). Cells were washed twice by centrifugation in TE buffer at 1300×*g* for 10 min. The cells were resuspended and lysed with either 10% SDS and Proteinase K (20 mg/mL) for Gram-negative strains or in 150µL of an enzyme mixture containing 15 mg/mL lysozyme (Sigma) and 5 mg/mL achromopeptidase (Sigma) in TE buffer (pH 8.0) for gram-positive strains. The pelleted cells were resuspended by 15 s of

sonication and incubated at 37°C for 1 h. DNA was isolated and purified using the method of Smith et al. (1989). The concentration of the purified DNA was determined by spectrophotometric measurement of the absorbance at 260 nm. The purity of the preparations was assessed by the ratio of the absorbances at 260 and 280 nm. Whole genomic DNA probes were prepared from each of the 107 test strains by labeling 1 – 3μg of DNA with digoxigenin (Boehringer Mannheim, Indianapolis, IN, USA) using a random primer technique (Feinberg & Vogelstein, 1983).

Sample preparation and microbial analysis

Following amplification and quantification, the amplified endodontic samples were boiled for 10 min. Approximately 1500 ng of DNA (5 μL) of the amplified sample were placed in a microcentrifuge tube containing 1 mL of TE buffer prior to boiling. The samples were placed into the extended slots of a Minislot 30 apparatus (Immunetics, Cambridge, MA, USA), concentrated onto a nylon membrane (Boehringer Mannheim) by vacuum and fixed onto the membrane by cross-linking using ultraviolet light (Stratalinker 1800, La Jolla, CA, USA) followed by baking at 120°C for 20 min. The Minislot device permitted the deposition of 28 different samples in individual lanes on a single membrane, as well as two control lanes containing the standards for quantification: 1 and 10 ng of DNA of each bacterial species tested, equivalent to 10^5 and 10^6 cells, respectively.

Checkerboard DNA-DNA hybridization was performed as previously described by Socransky et al., (1994, 2004). The membrane with fixed DNA was placed in a Miniblotter 45 apparatus (Immunetics) with the lanes of DNA at

90° to the channels of the device. A 30 × 45 “checkerboard” pattern was produced. Each channel was used as an individual hybridization chamber for separate DNA probes. Bound probes were detected by anti- digoxigenin antibody conjugated with alkaline phosphatase and a chemifluorescent substrate. Signal intensities of the endodontic samples and the standards (containing 10^5 and 10^6 cells of each species) on the same membrane were measured using a Storm FluorImager (Molecular Dynamics, Sunnyvale, CA, USA). Signals were converted to absolute counts by comparison with standards on the membrane (Socransky *et al.*, 2004). Failure to detect a signal was recorded as zero.

Three membranes were run for each sample: one containing the “standard” 40 DNA probes routinely used to examine periodontal samples as well as a probe to detect *Streptococcus mutans*. A second membrane employed 42 probes to species thought to be implicated in endodontic infections. A third membrane was used to assess levels of medically important microbial taxa. Sensitivity and specificity tests were performed for all probes before performing the checkerboard DNA-DNA hybridization analysis, using a protocol similar to that described by Socransky *et al,* (2004).

Data analysis

The microbial data were expressed in two ways. The prevalence of each species, reflected by presence/absence data, indicated the proportion of samples in which the species were detected at $>10^5$ cells in amplified samples. Since the sample DNA was amplified, absolute numbers could not be determined. Thus, proportions of the total DNA probe count for each species

comprised were computed for each sample and then averaged across subjects in each group separately.

Significance of differences between the prevalence of individual taxa in samples from HIV- and HIV+ subjects was sought using a Chi-square analysis. Significance of differences between the proportions of test species in samples from HIV- and HIV+ subjects was sought using the Mann Whitney test.

RESULTS

Quantification of DNA before and after MDA of endodontic samples

DNA from each root canal sample was amplified using MDA. The amount of DNA (± Standard Deviation - SD) present in the samples before the amplification averaged 6.1 (± 1.8) ng and 7.6 (± 1.2) µg after amplification, an approximately 1,000 - fold amplification.

Among the 60 samples include in this study, 28 were from single rooted and 32 from multi-rooted teeth. 56.8% of samples were from females (n= 34) and the mean age of the subjects was 34.7(±11.5) (range: 11- 64 years).

Microbial species in root canal samples.

The mean numbers of species (±standard error of the mean; SEM) detected in amplified root canal samples from HIV- and HIV+ subjects at a detection threshold of 10^5 cells was 22.6 (± 1.4) and 21.3 (± 2.0) respectively.

Figure 1 demonstrates the mean percentage of samples exhibiting counts of each of the 107 microbial species detected at the level of > 10^5 bacterial cells. The most prevalent taxa detected were *Prevotella denticola* (93.2% of samples), *Abiotropia adjacens* (93.2%) and *Streptococcus constellatus* (78.2%)

among HIV- subjects. Among HIV+ individuals *Dialister pneumosintes* (91.4%), *Helicobacter pylori* (73.7%) and *S. constellatus* (71.1%) were the most frequently detected taxa. *Corynebacterium diphtheriae*, *Enterobacter aerogenes*, *Actinomyces israelii*, *Enterobacter sakazaki*, were significantly more prevalent in the HIV- group and *D. pneumosintes* was found more frequently in samples from HIV+ subjects.

The mean proportions of the 107 microbial species in amplified root canal samples from HIV- and HIV+ patients are presented in Fig. 2. In (HIV-) samples, *S. constellatus*, *A. adjacens* and *Prevotella denticola* were the most abundant taxa, presenting % DNA probe counts (±SEM) of 13.9 (±2.0), 6.2 (±1.1) and 6.2 (±1.1) respectively. In HIV+ samples, *S. constellatus*, (15.3±2.2) followed by *Prevotella tannerae* (12.6 ±4.5) were the bacterial species detected in the highest mean proportions.

HIV+ subjects exhibited higher proportions of *Candida albicans* (2.1± 0.3) and *Candida tropicalis* (1.1 ± 0.2), in comparison with HIV- samples (1.1±0.1 and 0.8±0.1). In addition, mean proportions of *P. tannerae* and *C. albicans* were significantly higher in HIV+ positive than in HIV- patients, while *P. denticola*, *A. adjacens, C. diphtheria*, *Streptococcus sobrinus* and *H. pylori* were detected in significantly higher mean proportions in HIV- than in HIV + patients.

Campylobacter rectus, *Lactobacillus acidophilus*, *Gemella haemolysans* and *Clostridium difficile* were not detected in any of the amplified samples.

DISCUSSION

HIV/AIDS continues to be a major global health priority since it affects 33.4 million people worldwide, presenting high level of morbidity and mortality

(UNAIDS, 2008). Infected individuals are considered to be a high-risk group to develop opportunistic infections, including oral lesions (Glick *et al.*, 1994, Coogan *et al.*, 2005). However, few researchers have studied endodontic infections in HIV+ patients. Thus, the goal of the current investigation was to compare the microbial profile of endodontic infections in HIV- and HIV+ subjects and to seek intra-oral and medically important microbial taxa in those root canals using MDA and checkerboard DNA-DNA hybridization techniques.

The small size of the sample recovered from endodontic infections is a limitation in assessing the microbial content of a root canal. To overcome this limitation, the DNA content of samples can be amplified using MDA, which ensured amplification with minimal bias prior to checkerboard DNA-DNA hybridization (Teles *et al.*, 2007). It has been found that a 1000-fold amplification in sample DNA can be acheived, as demonstrated elsewhere (Brito *et al.*, 2007, Teles *et al.*, 2007, Tavares *et al.*, 2011). In this study, we assessed 60 samples recovered from root canal infections using probes to 107 different microbial taxa. Using a detection threshold of 10^5 cells, the mean number of species found per sample in the present study was 22.6 (± 1.4) and 21.3 (± 2.0) in root canal samples from HIV- and HIV+, respectively. This outcome demonstrates a somewhat lower mean number of species when compared with similar study (Brito *et al.*, 2007) but virtually the same as reported by others (Sassone et al., 2007, Tavares *et al.*, 2011), despite the higher number of taxa analyzed in the present study. This result is probably due to the use of more stringent criteria for microbial detection (10^5 cells, rather than 10^4 cells).

The endodontic samples from HIV+ and HIV- subjects exhibited similar

mean number of microbial species. Similar results were observed in samples from periodontal disease lesions when HIV-seronegative and seropositive patients were compared (Nakou *et al.*, 1997, Tsang & Samaranayake, 2001). Conversely, Gonçalves de Souza *et al*. (2007) analyzing subgingival samples from HIV+ and HIV- patients found a lower number of species in HIV positive patients. The differences found among these studies may be due to many factors, such as the method of microbial identification, the number of samples and microbial species analyzed, and the environmental features of the individuals studied (Haffajee & Socransky, 1992).

The assessment of the prevalence of the test microbial taxa revealed that *D. pneumosintes*, *H. pylori* and *S. constellatus* were the most frequently detected taxa in HIV+ subjects. *H. pylori* is a gram-negative microaerophilic organism primarily recovered from the stomach and is responsible for certain forms of gastritis or peptic ulcers. This species has been found in the oral cavity, including endodontic infections and periodontal diseases, which then could be a reservoir for *H. pylori* (de Souza Goncalves *et al.*, 2009, Silva *et al.*, 2010, Gao *et al.*, 2011, Tavares *et al.*, 2011) possibly leading to the reinfection of patients after treatment for those diseases (Karczewska *et al*., 2002, Zou & Li, 2011). Several studies have indicated that *H. pylori* infections were common in HIV-infected patients (Romanelli *et al*., 2007, de Souza Goncalves *et al.*, 2009), although many others described a low prevalence of *H. pylori* infection in such individuals (Lv *et al*., 2007, Panos *et al*., 2007). In the present investigation, *H. pylori* was more prevalent in HIV+ than in HIV- patients, as observed by previously in subgingival biofilm samples (de Souza Goncalves *et al.*, 2009).

Members of the *Streptococcus milleri* group are now separated into 3 distinct species *Streptococcus intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus* with *S. constellatus* and *S. intermedius* being more closely related to each other than to *S. anginosus*. Some studies that have detailed the site of isolation have suggested that *S. anginosus* is more likely to be isolated from blood, urogenital, and gastrointestinal specimens, whereas *S. intermedius* tends to be associated with infection of the head, neck, and respiratory tract (Whiley *et al*., 1992, Flynn & Ruoff, 1995). In the present investigation, *S. constellatus* and *S. intermedius* were among the most prevalent and abundant species in both groups, as previously demonstrated by others analyzing endodontic infections (Siqueira *et al*., 2001, Gomes *et al.*, 2004). Conversely, both strains were found in low proportions by Brito et al. (2007) and Tavares et al. (2011) using the same methodology in HIV-negative subjects. These conflicting results may be related to selective environmental pressures. *Streptococcus spp*. are facultative anaerobes and their grown will depend on clinical conditions, such as oxygen availability that are different in root canal infections presenting open or close cavities.

Prevotella and *Porphyromonas* species are among the taxa that form black- pigmented colonies. These include *P. tannerae*, *P. denticola*, *Prevotella intermedia*, *Prevotella nigrescens*, *Prevotella loeschii*, *Porphyromonas endodontalis* and *Porphyromonas gingivalis*. Such taxa have been found in high frequency in most studies of endodontic infections (Seol *et al*., 2006, Brito *et al.*, 2007, Tavares *et al.*, 2011). In the present study, *P. tannerae* and *P. denticola* were among the most prominent species in HIV+ and HIV- individuals. Interestingly, Brito *et al*. (2007), using the same methodology as the one

employed in this study, detected *P. tannerae* as the most prevalent species endodontic infections in adults, while *P. denticola* has been found in high prevalence by Tavares *et al*. (2011) in root canal samples from deciduous teeth. Taken together, those results suggest an important role for those taxa in the pathogenesis of root canal infections.

When samples from HIV+ and HIV- subjects were compared, mean proportions of *P. tannerae*, and *C. albicans* were significantly higher in HIV + than HIV- individuals. *P. tannerae*, a gram-negative, obligate anaerobic pathogen has been frequently isolated from endodontic infections (Xia *et al*., 2000, Brito *et al.*, 2007, Tavares *et al.*, 2011). *C. albicans* is a commensal yeast normally present in oral microbiota. In the presence of specific predisposing conditions, its levels may increase dramatically leading to disease states (Cannon & Chaffin, 1999). *C. albicans* is usually involved in opportunistic infections in patients with acquired immunodeficiency syndrome (AIDS) (Nguyen *et al*., 2004, Back-Brito *et al*., 2009). In this study, we detected the presence of two fungal species *C. albicans* and *C. tropicalis*. *C. albicans* was the most predominant yeast in both groups, as demonstrated elsewhere (Miranda *et al*., 2009). In addition it was present in higher mean proportions in HIV infected individuals. This finding is in accord with other studies that analyzed fungal species in oral lesions in HIV-infected patients (Baradkar & Kumar, 2009, Domaneschi *et al*., 2011).

It has been shown that bacterial communities in oral cavity, including soft tissue biofilms, supragingival and subgingival may be reservoirs of pathogens involved in pulmonary diseases (Scannapieco *et al.*, 2003, Didilescu *et al*., 2005, de Souza Goncalves *et al.*, 2009). Few researchers have examined the

role of endodontic infections as possible sources of respiratory pathogens (Chaudhry *et al*., 1997, Nandakumar *et al*., 2008). In this investigation, we sought the presence of important lung pathogens. Since HIV/AIDS patients are known to have an elevated risk of contracting serious respiratory diseases (Graham, 2007, Perello *et al.,* 2010) we compared samples from HIV-negative as well as HIV-positive patients. Among the respiratory pahogens tested, *Dialister pneumosintes*, *Enterobacter aerogenes* and *Escherichia coli* were frequently detected in samples from both groups (Fig.1), even though they were present in low levels (Fig.2), except for *D. pneumosintes*. This species was very prevalent in HIV+ samples (91.4% of samples) and abundant in both groups. *D. pneumosintes* is a nonmotile, nonfermentative, non-spore-forming, obligate anaerobe, gram-negative bacillus that has been associated with respiratory diseases. Several studies have found this species in high frequency in endodontic infections, using different methods of detection, and it was suggested that this species is an important endodontic pathogen (Siqueira & Rocas, 2002, Siqueira *et al*., 2005a, Brito *et al.*, 2007, Tavares *et al.*, 2011). In periodontal diseases, *D. pneumosintes* is also frequently detected in the subgingival microbiota (Ferraro *et al*., 2007, Colombo *et al*., 2009). However, in HIV-positive subjects, very few studies analyzed subgingival microflora seeking respiratory pathogens (Nakou *et al.*, 1997, de Souza Goncalves *et al.*, 2009).

In conclusion, the present study showed that endodontic infections in HIV+ patients present a complex microbiota. There were significant differences in prevalence and mean proportions of microbial taxa between HIV- and HIV+ subjects. In addition, many oral and non-oral pathogens were detected in both groups. Collectively, these data suggest that treatment and prevention of

endodontic infections might have a potential positive impact in clinical outcomes in HIV+ individuals.

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FIGURES

Fig.1 - Frequency of detection (%) (prevalence) of 107 test species tested microbial species

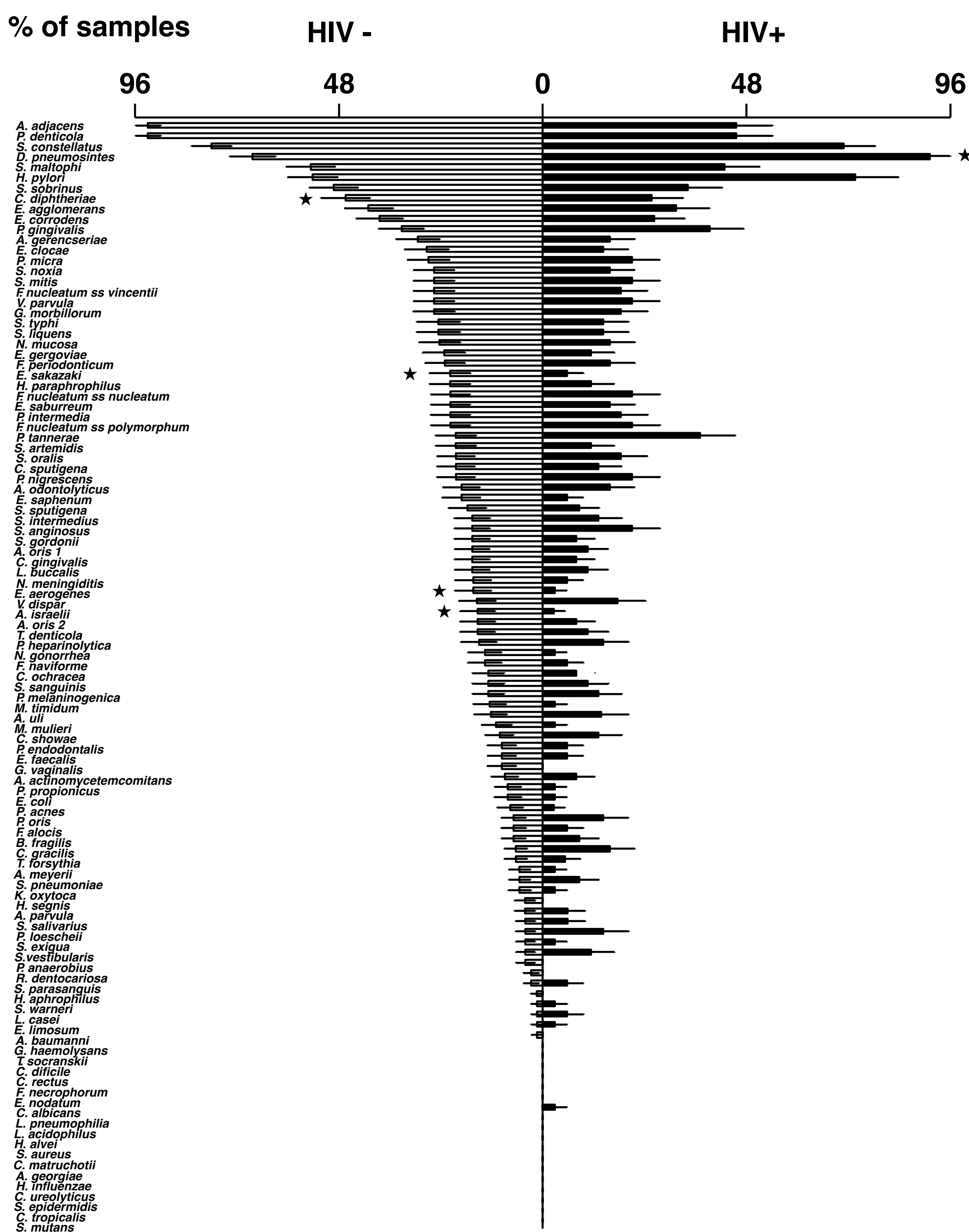
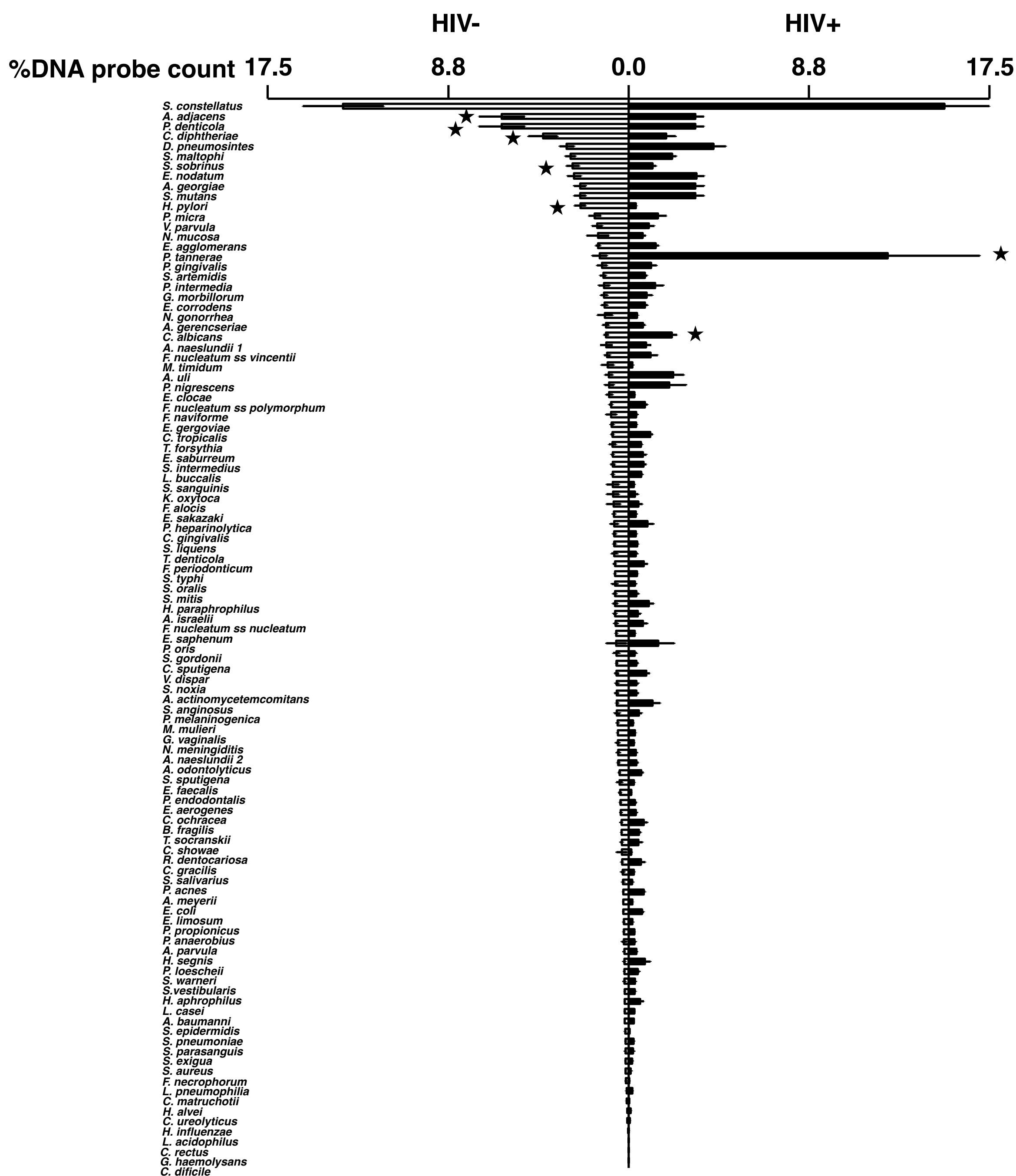


Fig. 2 - Percentage (%) of DNA probe counts of microbial species that differed significantly between HIV- and HIV+ individuals



FIGURES LEGENDS

Figure 1: Bilateral bar chart of the frequency of detection (prevalence ± SEM) of 107 microbial taxa in root canal samples taken from 40 HIV negative patients (white bars) and 20 HIV+ patients (black bars). A detection level threshold of 10^5 cells was employed. The significance of differences between groups was determined using the Chi-square analysis. The data are ordered in descending order of mean percentages of DNA probe counts detected in HIV negative samples. ss, subsp. *$p<0.05$

Figure 2. Bilateral bar chart of the mean percentages of the DNA probe counts (± SEM) for 107 microbial species in MDA-amplified root canal samples taken from 40 HIV negative patients (white bars) and 20 HIV+ patients (black bars). The proportion of each species was averaged across subjects in the two clinical groups separately. The significance of differences between groups was determined using the Mann-Whitney test. The data are ordered in descending order of mean percentages of DNA probe counts detected in HIV negative samples. ss, subsp. *$p<0.05$ Note: only taxa that comprised at least 1% of the total microbiota were eligible to be considered statistically significantly different.

TABLE

Table 1 - Microbial species tested

Bacterial strains (a)	*Leptotrichia buccalis (14201)*
Acinetobacter baumannii (19606)	*Mobiluncus mulieris (35240)*
Actinomyces georgiae (49285)	*Mogibacterium timidum (33093)*
Actinomyces gerencseriae (23860)	*Neisseria gonorrhea (21823)*
Actinomyces israelii (12102)	*Neisseria meningitidis (13077)*
Actinomyces meyeri (35568)	*Neisseria mucosa (19696)*
Actinomyces naeslundii 1 (12104)	*Olsenella uli (49627)*
Actinomyces naeslundii 2 (27044)	*Peptostreptococcus anaerobius (27337)*
Actinomyces odontolyticus (17929)	*Parvimonas micra (33270)*
Aggregatibacter actinomycetemcomitans (b)	*Porphyromonas endodontalis (35406)*
Atopobium parvulum (33793)	*Porphyromonas gingivalis (33277)*
Bacteroides fragilis (25285)	*Prevotella denticola (33184)*
Campylobacter ureolyticus (33387)	*Prevotella heparinolytica (35895)*
Campylobacter gracilis (33236)	*Prevotella intermedia (25611)*
Campylobacter rectus (33238)	*Prevotella loescheii (15930)*
Campylobacter showae (51146)	*Prevotella melaninogenica (25845)*
Capnocytophaga gingivalis (33624)	*Prevotella nigrescens (33563)*
Capnocytophaga ochracea (33596)	*Prevotella oris (33573)*
Capnocytophaga sputigena (33612)	*Prevotella tannerae (51259)*
Clostridium difficile (9689)	*Propionibacterium propionicum (14157)*
Corynebacterium diphtheriae (8024)	*Propionibacterium acnes (c)*
Corynebacterium matruchotii (14266)	*Rothia dentocariosa (17931)*
Dialister pneumosintes (GBA27)	*Salmonella typhi (27870)*

Eikenella corrodens (23834)	*Selenomonas artemidis (43528)*
Enterococcus faecalis (29212)	*Selenomonas noxia (43541)*
Enterobacter aerogenes (87423)	*Selenomonas sputigena (35185)*
Enterobacter agglomerans (27155)	*Slackia exigua (700122)*
Enterobacter cloacae (10699)	*Staphylococcus aureus (3309)*
Enterobacter gergoviae (33028)	*Staphylococcus epidermidis (14990)*
Enterobacter sakazakii (51329)	*Staphylococcus warneri (25614)*
Escherichia coli (10799)	*Stenotrophomonas maltophilia (13270)*
Eubacterium limosum (8486)	*Streptococcus adjacens (49175)*
Eubacterium nodatum (33099)	*Streptococcus anginosus (33397)*
Eubacterium saburreum (33271)	*Streptococcus constellatus (27823)*
Eubacterium saphenum (49989)	*Streptococcus gordonii (10558)*
Filifactor alocis (35896)	*Streptococcus intermedius (27335)*
Fusobacterium naviforme (25832)	*Streptococcus mitis (49456)*
Fusobacterium necrophorum (25386)	*Streptococcus mutans (25175)*
Fusobacterium nucleatum ss. *nucleatum (25586)*	*Streptococcus oralis (35037)*
Fusobacterium nucleatum ss. *polymorphum (10953)*	*Streptococcus parasanguinis (15912)*
Fusobacterium nucleatum ss. *vincentii (49256)*	*Streptococcus pneumoniae (6301)*
Fusobacterium periodonticum (33693)	*Streptococcus salivarius (27945)*
Gardinerella vaginalis (49145)	*Streptococcus sanguinis (10556)*
Gemella haemolysans (10379)	*Streptococcus sobrinus (27351)*
Gemella morbillorum (27824)	*Streptococcus vestibularis (49124)*
Haemophilus aphrophilus (33389)	*Tannerella forsythia (3037)*
Haemophilus influenzae (49766)	*Treponema denticola (B1)*
Haemophilus paraphrophilus (29242)	*Treponema socranskii (S1)*

Haemophilus segnis (33393)	*Veillonella dispar (17748)*
Hafnia alvei (13337)	*Veillonella parvula (10790)*
Helicobacter pylori (51653)	
Klebsiella oxytoca (8724)	**Fungal strains (a)**
Lactobacillus acidophilus (4356)	*Candida albicans (10231)*
Lactobacillus casei (334)	*Candida tropicalis (750)*
Legionella pneumophila (33152)	

(a) All strains were obtained from the American Type Culture Collection (ATCC number in parentheses) except for *Treponema denticola* B1 and *Treponema socranskii* S1, which were obtained from The Forsyth Institute.

(b) ATCC strains 43718 and 29523

(c) ATCC strains 11827 and 11828

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Trabalho 3- “Overview of T lymphocytes and cytokine expression in human periapical tissues”

Trabalho 3- "**T lymphocytes and cytokine expression in human periapical tissues: Overview of their immunomodulatory activity".**

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ABSTRACT

Objective: This study evaluated the mRNA expression of $CD4^{+}CD28^{+}$ and $CD8^{+}$ genes and the gene expression of IFN-γ, TNF-α, IL-1β, IL-17A, IL-10, CCL2/MCP-1, CCL4, CCL5, CXCR4, CCR5, and RANKL in periapical interstitial fluid from human root canal infections.

Study design: The samples were collected immediately after root canal cleaning and 7 days later to characterize those gene expressions during the healing process (restrained root canal bacterial load).

Results: Real-time PCR demonstrated significantly higher levels of $CD4^{+}CD28^{+}$ and $CD8^{+}$ cell markers in the former root canal condition and an increase of IL-10 and CXCR4, followed by a decrease of proinflammatory cytokines, such as RANKL, IFN-γ, IL-1β and CCL5.

Conclusions: Longitudinal analyses of root canal cleaning procedures was able to show that distinct root canal conditions may play regulatory roles in controlling local immune/ inflammatory processes.

KEY WORDS: periapical lesion, cytokines, chemokines.

INTRODUCTION

The periapical immune response is predominantly a reaction to bacterial infection present in necrotic root canals (Nair, 2004). Immune-competent cells drive and settle in periapical areas, in an attempt to prevent the dissemination of infectious microbiota (Fukada *et al.*, 2009). Among those cells, lymphocytes are the most prevalent in the periapical inflammatory infiltrate (Yu & Stashenko, 1987). The immune host response that takes place in this area is very complex and involves the recruitment of inflammatory cells, pro-inflammatory and immunoregulatory cytokines, and chemokines (Stashenko *et al.*, 1998).

Antigen-presenting cells are responsible for the polarization of T helper immune responses to Th1, Th2, Th17, or T regulatory cells (de Jong *et al.*, 2005). The Th1 response is characterized by the production of interferon-γ (IFN-γ), Interleukin-2, and TNF, whereas IL-4, IL-5, IL-6, IL-9 and IL-13 are mainly produced during the Th2 response (Mosmann & Sad, 1996, Stashenko *et al.*, 1998). The progression of lesion and the bone destruction are attributed to the Th1 response that induces differentiation and activation of osteoclasts by nuclear factor-kB ligand (RANKL) (Fukada *et al.*, 2009). Conversely, the healing process is related to the Th2 response (Kawashima & Stashenko, 1999). Th1 and Th2 responses are regulated by cells known as regulatory T (Treg) cells and by IL-10 and transforming growth factor-β (TGF-β) (Romagnani, 2006). The Th17 subset participates in the exacerbation of inflammation with potent effects on various cells of the innate immune system. IL-17 acts on stromal endothelial and epithelial cells to induce the secretion of proinflammatory mediators, promoting lymphocyte recruitment to the site of infection (Colic *et al.*, 2007).

T cells express different sets of chemokines and receptors, which are

considered important mediators of leukocyte recruitment to injured sites, including periapical lesions (Silva *et al.*, 2005). Some chemokines are constitutively secreted and regulate the trafficking of lymphocytes, whereas other chemokines are produced as a consequence of pro-inflammatory stimulation or infection, directing the migration of leukocytes and immature dendritic cells to the injured or infected site. Moreover, chemokines can also participate in wound healing (Rossi & Zlotnik, 2000).

The aim of this study was quantitatively assayed in samples collected from interstitial fluid adjacent to human root canal infections, the mRNA expression of the $CD4^{+}CD28^{+}$ and $CD8^{+}$ genes, which are expressed by T cells, as well as IFN-γ, TNF-α, IL-1β, IL-17A, IL-10, CCL2/MCP-1, CCL4, CCL5, CXCR4, CCR5, and RANKL. Additionally, we detected the same cytokines and cell markers 7 days after the root canal cleaning procedures, when root canal bacterial load was strongly reduced and the healing process had likely started. Finally, we statistically compared and analyzed both sets of data.

METHODS

Subjects

Subjects were 20 patients referred to the Dental School at the Universidade Federal de Minas Gerais (Belo Horizonte, MG, Brazil) to receive endodontic care. The exclusion criterion for this study was antibiotic therapy up to three months before starting endodontic therapy. All participants signed the Free Agreement Formulary. This study was approved by the Ethics Committee of the Universidade Federal de Minas Gerais (ETIC 122/08).

Sample collection

Clinical samples were taken from teeth with pulp necrosis that were diagnosed by clinical and radiographic analyses, in addition to pulp sensibility tests. All selected patients failed to present acute periapical symptoms at the appointment time. Teeth were isolated using a rubber dam followed by a complete asepsis, as previously described (Brito *et al.*, 2007). Cleaning and shaping of the root canals were completed using ProTaper NiTi files (Dentsply Mailleffer) in conjunction with 5.2% sodium hypochlorite. The clinical procedures were performed as follows. Briefly, the samples were collected immediately after root canal cleaning to characterize the mRNA expression profiles of cytokines, chemokines and T cell genes. After cleaning and drying, three paper points were introduced into the root canal, passing through the root apex (2 mm) for one minute. After withdrawal, the paper points were cut 4 mm from the tip and dropped into a microcentrifuge tube, and the samples were stored at -70°C. Using this procedure, RNA was extracted from the periapical interstitial fluid. No endodontic dressing was inserted into root canals. The coronal accesses of the teeth were restored with eugenol-based cement. Seven days later (Day 7), the teeth were opened and sampled to characterize the cytokine and T cell expression during the healing process (restrained root canal bacterial load). In teeth with multiple canals, the first (Day 0) and second (Day 7) samples were collected from the same canal. At this time, no teeth presented clinical signs or symptoms, and root canals were filled by lateral condensation technique.

Sample Preparation

Total RNA was extracted from each sample with TRIzol reagent (GIBCO/BRL Laboratories, Grand Island, NY, USA). Chloroform was added, the mixture was centrifuged at 12,000 x *g* at 4°C for 15 min, the aqueous phase was collected, and the RNA was precipitated by the addition of isopropanol and centrifuged at 12,000 x *g* at 4°C for 10 min. The RNA precipitate was washed once with 75% cold ethanol solution, dried, dissolved in RNase-free water and then incubated at 55°C for 10 min. The RNA was then stored at -70°C.

Real-time PCR

Complementary DNA was synthesized using 1 µg of RNA through a reverse transcription reaction as previously described (Barbosa Silva *et al.*, 2008). PCR was carried out under standard conditions: a holding stage of 95°C (10 min); a cycling stage with 40 cycles of 95°C (15 sec) followed by 60°C (1 min); and a melt curve stage of 95°C (15 sec), 60°C (1 min) and 95°C (15 sec). The primer sequences used for quantitative PCR analysis of IFN-γ, TNF-α, IL-1β, IL-17A, IL-10, CCL2/MCP-1, CCL4, CCL5, CXCR4, CCR5, and RANKL mRNA expression are shown in Table I. Additionally, mRNA expression of $CD4^{+}CD28^{+}$ and $CD8^{+}$ T-cell genes was assayed in each sample (Table 1). The sequences of human primers were designed using PRIMEREXPRESS software (Applied Biosystems, Foster City, CA, USA) based on nucleotide sequences available in the GenBank database. The real-time PCR assay was performed using the StepOne Real-time PCR System (Applied Biosystems). A Syber-Green detection system (Applied Biosystems) was used to assay primer amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization. All samples were run in duplicate.

Sequence Detection Software version v 2.0 (Applied Biosystems) was used to analyze data after amplification. Results were obtained as threshold cycle (Ct) values. Expression levels were calculated using the comparative C(T) method (Schmittgen & Livak, 2008). The values were calculated as the mean value of duplicates for each patient, and the expression levels of mRNAs in all samples were defined as the ratio of each specific primer to GAPDH expression.

Statistical Analysis

Data analysis was performed using SPSS for Windows (version 15.0; SPSS, Chicago, IL, USA). Data were subjected to the Shapiro-Wilk test to characterize their normality. The Wilcoxon test was used to determine statistical differences ($p<0.05$), because data did not present a normal distribution.

RESULTS

$CD4^+CD28^+$ and $CD8^+$ T cell gene expression

The assessment of mRNA expression of T cell genes in periapical tissues revealed significantly higher levels of $CD4^+CD28^+$ and $CD8^+$ cells at day 7 (restrained bacterial load) in relation to the sample collected immediately after root canal cleaning at day 0 (Fig. 1).

Cytokine mRNA expression

The mRNA expression of IFN-γ, IL-1β, and RANKL decreased at day 7 ($p<0.05$). TNF-α and IL-17A mRNA expressions were similar at both times ($p>0.05$). mRNA expression of IL-10 increased at day 7 ($p<0.05$) (Fig.2).

Chemokine mRNA expression

Increased mRNA expression of CXCR4 was observed at day 7 ($p<0.05$), whereas CCL-5 mRNA expression was statistically decreased at the same time ($p<0.05$) (Fig 2). CCL2, CCL4 and CCR5 did not present significant differences in mRNA expression between the two times analyzed (Fig. 3).

DISCUSSION

Periapical inflammation and alveolar bone resorption occur as a consequence of root canal infection (Kawashima & Stashenko, 1999, Colic *et al.*, 2007, De Rossi *et al.*, 2008, Fukada *et al.*, 2009, Teixeira-Salum *et al.*, 2010). Cell infiltration initiates as soon as the inflammatory process starts in an attempt to eliminate the microorganisms and to prevent their dissemination. Among those cells, lymphocytes are considered the more prevalent cells in periapical inflammatory infiltrate (Yu & Stashenko, 1987). Cell surface expression of CD4 and CD8 co-receptor molecules divides mature αβ T cells into two subsets, CD8-positive and CD4-positive T lymphocytes, which are responsible for T lymphocyte responses in connection with class I or II major histocompatibility complex (MHC), respectively, and aid in immunoglobulin production (Croft & Swain, 1991). In this study, after the root canal bacterial load was strongly diminished by cleaning and shaping procedures, the mRNA expression of both $CD4^+CD28^+$ and $CD8^+$ T-cell genes statistically increased. This may be justified by previous findings which showed that bacteria recovered from human root canal infections are able to induce cell apoptosis by tumor necrosis factor receptor p55 (TNFRp55) (Ribeiro-Sobrinho *et al.*, 2005). Hence, in the presence of an active infection, less T cells would survive.

In this study, detectable levels of IFN-γ, TNF-α, IL-1β, IL-17A, RANKL, and IL-10 were observed in the periapical interstitial fluid. These results demonstrate that proinflammatory and regulatory cytokines were detected in this inflammatory environment, as described elsewhere (Kawashima & Stashenko, 1999, Colic *et al.*, 2007, Fukada *et al.*, 2009, Teixeira-Salum *et al.*, 2010, Silva *et al.*, 2011). Susceptibility or resistance to several diseases has been found to be related to Th1 or Th2 immune responses and there is a tendency that one response predominates during a given phase of the disease (Mosmann & Sad, 1996). In this study, we characterized the cytokine profile from periapical tissues adjacent to root canal infection at two different phases of infection and compared the outcomes observed in the both times. When bacterial load of the infected root canal was diminished, the levels of mRNA expression of IFN-γ, IL-1β, and RANKL were significantly decreased ($p<0.05$), whereas the levels of TNF-α and IL-17A mRNA expression were maintained. IFN- γ upregulates both IL-1 and TNF-α expression (Ma *et al.*, 2003), IL-1 production is also upregulated by an IL-1-mediated positive feedback loop (Dinarello *et al.*, 1987). Accordingly, in this study, the IL-1β reduction observed after treatment could be related to lower expression of IFN-γ or the reduction of the IL-1 positive feedback in a TNF-α-independent manner.

The inflammatory cytokines IL-1, TNF-α, IFN-γ, IL-6 and IL-11 activate osteoclastic bone resorption by inducing the expression of cellular iNOS and RANKL (Fukada *et al.*, 2009). In agreement with these findings, it was observed here that the decrease of IFN-γ and IL-1β was paralleled with the decrease of RANKL mRNA expression.

A cross-regulation, by which Th1 cells inhibit the Th2 response and vice

versa in periapical inflammation, has been proposed (Stashenko *et al.*, 1998) and became evident here, as evidenced by the increased mRNA expression of the mediator IL-10 associated with the reduced expression of proinflammatory cytokines. Conversely, inhibitory effects on bone loss by both Th1 and Th2 signatures have been described (Alayan *et al.*, 2007). Recently, these effects were attributed to IL-10 and TGF-β regulatory phenotype (Fukada *et al.*, 2009), and it was demonstrated in periapical lesions that $CD4^{+}CD25^{+}Foxp3^{+}$ cells (Treg) expressed both IL-10 and TGF-β in higher levels than in peripheral blood (Colic *et al.*, 2009). It is believed that, although Th1 ⁄ Th2 responses are effector-type responses, both seem to be suppressed by Treg cells through contact dependent mechanisms and/or the production of IL-10 and TGF-β (Danin *et al.*, 2000). Moreover, the role of IL-10 in suppressing periodontal disease (Sasaki *et al.*, 2004) and periapical lesions (De Rossi *et al.*, 2008) was demonstrated in IL-10-knockout mice.

Another T-cell subtype (Th17), which has as a founding member the cytokine IL-17, has been evaluated in periapical lesions. IL-17 acts on stromal endothelial and epithelial cells to induce the secretion of proinflammatory mediators (Fukada *et al.*, 2009). In spite of the suggestion that Th17 cells regulate osteoclastogenesis, probably through IL-17-mediated induction of RANKL (Sato *et al.*, 2006), it was not observed in this study. Although IL-17A expression was at similar levels at both times of evaluation, significant reductions in expression of RANKL, IFN-γ, and IL-1β were detected ($p<0.05$).

Chemokines play a role in directing cell movements necessary for the initiation of T cell immune responses to different pathogens or types of stimuli, which is thought to be important in recruiting appropriate effector cells to sites of

inflammation, including the differential recruitment of Th1 and Th2 cells (Sallusto *et al.*, 2000). Here, detectable levels of CCL2/MCP-1, CCL4, and CCL5 were assayed in the periapical samples at both times. CCL2 was first characterized as a monocyte chemoattracting protein, but it not only influences inflammatory cells recruitment but also has affects on effector T cell differentiation (Chensue *et al.*, 1996). In this study, in accordance with the increased gene expression of Th(reg)-type mediator IL-10, high expression of CCL2 was observed. It is expected that CCL2 influences T cell differentiation because it leads to decreased IL-12 production by macrophages (Chensue *et al.*, 1996), suppressing Th1 responses. This anti-inflammatory activity of CCL2 has already been demonstrated in an endotoxic shock mouse model (Zisman *et al.*, 1997). The *in vivo* roles of CCL4 and CCL5 need to be better clarified, although an association between CCL5 and Th1-type cellular responses was suggested by the finding that neutralization of CCL5 reduced type 1 granuloma formation (Chensue *et al.*, 1999). Accordingly, a statistical decrease of CCL5 mRNA expression, like those of Th1 cytokines, IFN-γ and IL-1β, was observed. Th1 and Th2 cells express different sets of chemokine receptors, which allow them to migrate to different tissues (Sallusto *et al.*, 2000). In this study, the expression level of CCR5, which is expressed preferentially on Th1 cells, TNF-α, and IL-17A remained constant at both timepoints; however, CXCR4, the receptor of anti-inflammatory immune modulator ubiquitin (Majetschak, 2011), was statistically increased in addition to other Th2 mediators.

Taken together, these results demonstrate that the predicted mRNA expression of T-cell genes, cytokines, and chemokines were detected in interstitial fluid adjacent to root canal infections. However, when the root canal

bacterial load was dampening, an increase in the mRNA expression of $CD4^+CD28^+$ and $CD8^+$ T-cell genes, the Th (reg)-type mediator IL-10, and Th2 chemokine (CXCR-4), as well as a decrease in mRNA expression of proinflammatory cytokines genes, such as RANKL, Th1 cytokines (IFN-γ and IL-1β) and Th1 chemokines (CCL5) were observed. Because cytokine synthesis *in vivo* is a very complex event, the results of this study contribute to the overall understanding of the specific immune responses that predominate in the periapical area during distinct root canal infection conditions.

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FIGURES AND LEGENDS

Fig.1

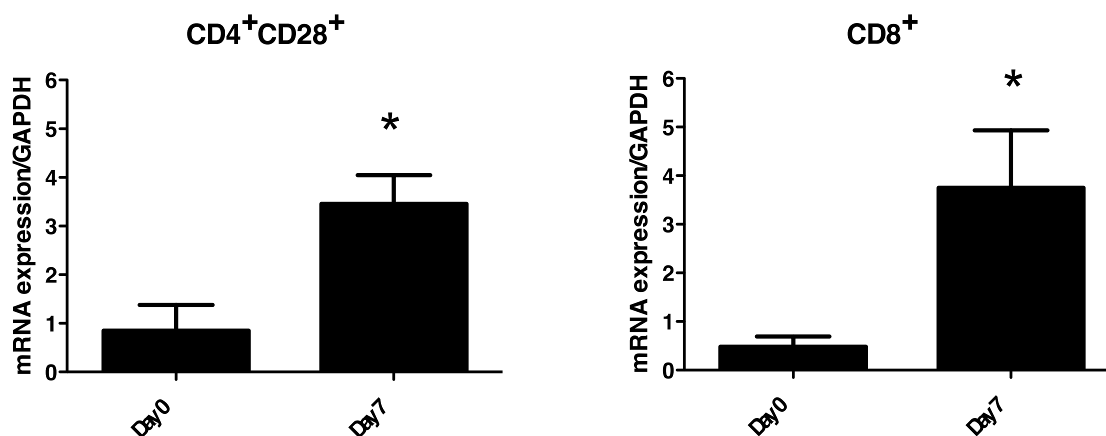

Figure 1. Expression of $CD4^+CD28^+$ and $CD8^+$ T-cell genes in perirradicular tissues of humans presenting root canal infections. Levels of expression were determined by real-time PCR and quantified by comparison with the internal control (GAPDH). Bars represent the mean values of samples recovered from 20 patients; lines represent the standard error of the mean. * Indicates $p < 0.05$ by the Wilcoxon test.

Fig.2

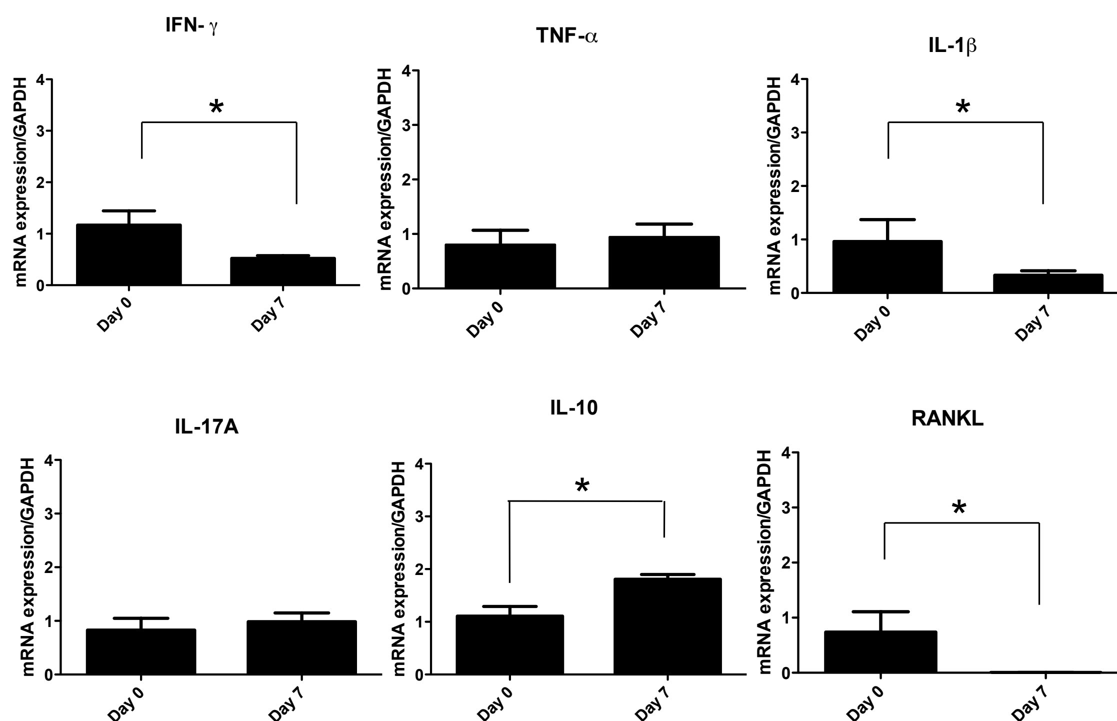

Figure 2. Expression of IFN-γ, TNF-α, IL-1-β, IL-17A, IL-10, and RANKL in perirradicular tissues of humans presenting root canal infections. Levels of expression were determined by real-time PCR and quantified by comparison with the internal control (GAPDH). Bars represent the mean values of samples recovered from 20 patients; lines represent the standard error of the mean. * indicates $p < 0.05$ by the Wilcoxon test.

Fig.3

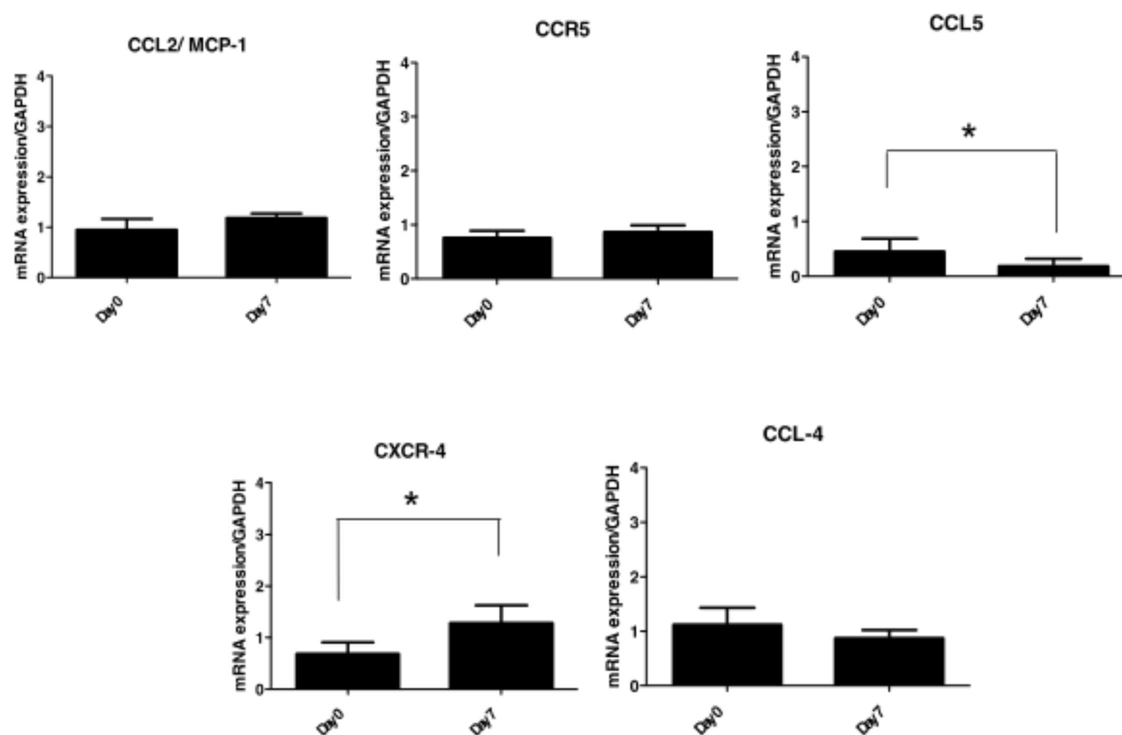

Figure 3. Expression of CCL-2/MCP-1, CCL-4, CCL-5, CXCR4, and CCR5 in perirradicular tissues of humans presenting root canal infections. Levels of expression were determined by real-time PCR and quantified by comparison with the internal control (GAPDH). Bars represent the mean values of samples recovered from 20 patients; lines represent the standard error of the mean. * Indicates $p < 0.05$ by the Wilcoxon test.

Table 1. Primer sequences

Gene	Sense and antisense	Mt* (°C)	bp*
GAPDH	5'-GCA CCA CCA ACT GCT TAG CA- 3' 5'-TGG CAG TGA TGG CAT GGA GGA- 3'	80	96
TNF-α	5'-TTC TGG CTC AAA AAG AGA ATT G- 3' 5'-TGG TGG TCT TGT TGC TTA AGG- 3'	76	73
IL-1β	5'-TGG CAG AAA GGG AAC AGA A- 3' 5'-ACA ACA GGA AAG TCC AGG CTA- 3'	73	59
IL-10	5'-GGT TGC CAA GCC TTG TCT GA- 3' 5'-TCC CCC AGG GAG TTC ACA T- 3'	81	107
IL-17A	5'-CAA TGA CCT GGA ATT ACC CAA- 3' 5'-TGA AGG CAT GTG AAA TCG AGA- 3'	70	52
IFN-γ	5'-GAA CTG TCG CCA GCA GCT AAA- 3' 5'-TGC AGG CAG GAC AAC CAT TA- 3'	80	95
CCL2	5'-AAG ACC ATT GTG GCC AAG GA- 3' 5'-CGG AGT TTG GGT TTG CTT GT- 3'	80	93
RANKL	5'-CAG AAG ATG GCA CTC ACT GCA- 3' 5'-CAC CAT CGC TTT CTC TGC TCT- 3'	73	203
CCL4	5'-TCT CCT CAT GCT AGT AGC TGC CTT- 3' 5'-GCT TCC TCG CAG TGT AAG AAA AG- 3'	78	101
CCL5	5'-CGT GCC CAC ATC AAG GAG TA- 3' 5'-CAC ACA CTT GGC GGT TCT TTC- 3'	80	91
CXCR4	5'-TGT TGG CTG AAA AGG TGG TC- 3' 5'-AAA GAT GTC GGG AAT AGT C- 3'	80	71
CCR5	5'-GGA CCA AGC TAT GCA GGT GAC- 3' 5'-TTG GCA CTG TGC TTT TGG AA- 3'	80	129
CD8	5'-CAG GCT ACC CCT TTA CAA ATA GAA TT- 3' 5'-TTC AGA GAT TCA AGA GGG CCT TA- 3'	73	70
CD28	5'-ACA GTG GTA GGA GCA ATG CTT TC- 3' 5'-AAT AGC AGC AAA TGA CAT TGT TTT CT- 3'	72	74

*Mt: melting temperature; bp: base pairs of amplicon size.

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Trabalho 4- “T lymphocyte, cytokine and chemokine expression in periapical tissue from HIV-infected individuals”.

Trabalho 4. **“T lymphocyte, cytokine and chemokine expression in periapical tissue from HIV-infected individuals”.**

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ABSTRACT

The immunological characteristics of apical periodontitis in HIV-positive individuals remain unknown. Cell surface expression of CD4 and CD8 co-receptor molecules divides mature T cells into $CD4^{+}$ and $CD8^{+}$ T lymphocytes. Both of these produce a variety of cytokines and chemokines in periapical tissues, but expression may depend on root canal infection conditions. Using real-time PCR, we characterized $CD4^{+}CD28^{+}$ and $CD8^{+}$T cell genes and the gene expression of IFN-γ, TNF-α, IL-1-β, IL-17A, IL-10, CCL2/MCP-1, CCL4, CCL5, CXCR4, CCR5, and RANKL from cells in the periapical interstitial fluid from root canal infections in HIV-positive individuals. We also compared these expression levels with those obtained from the same patients after the root canal bacterial load was diminished by cleaning procedures. Our results show significantly higher levels of $CD4^{+}CD28^{+}$ and $CD8^{+}$T cells in the former root canal condition, as well as an increase in cytokines IFN-γ, IL-1-β, TNF-α, and IL-17A, and chemokines CCL2, CXCR4, and CCR5. The chemokine CCL5 was not detected in both conditions. These findings demonstrate that a pro-inflammatory response is sustained in the periapical area after reducing the root canal bacterial load, suggesting that the expected anti-inflammatory response to these conditions may occur at later stages in HIV-positive individuals.

INTRODUCTION

Periradicular lesions are characterized by the host immune response to egress bacteria or bacterial byproducts from the infected root canal systems (Stashenko *et al.*, 1998). $CD4^{+}$, $CD8^{+}$, and $CD30^{+}$ lymphocytes, macrophages, plasma cells, mast cells, and eosinophils have been found in variable quantities

in human and experimental models of periapical lesions (Yanagisawa, 1980, Bergenholtz *et al.*, 1983, Marton & Kiss, 1993, Teronen *et al.*, 1996). Quantitative analysis of lymphocyte subpopulations consistently demonstrates an excess of T over B-lymphocytes, indicating that periapical granulomas are predominantly T cell-type lesions (Bergenholtz *et al.*, 1983, Torabinejad & Kettering, 1985, Yu & Stashenko, 1987, Piattelli *et al.*, 1991, Marton & Kiss, 1993, Sol *et al.*, 1998).

CD4$^+$ T cells, which play a role in the development, progression and resolution of periapical lesions (Kawashima *et al.*, 1996, Marton & Kiss, 2000), are the primary targets of HIV infection, which leads to a depletion of the T-cell repertoire and the functional impairment of CD4$^+$ T cells (Hunt *et al.*, 2003, Watanabe *et al.*, 2010). CD8$^+$ T cells, which are relatively unaffected by HIV, become more prominent in the chronic stages of periapical lesions (Kawashima *et al.*, 1996, Garcia *et al.*, 2007). To date, the relationship between HIV infection and periapical immune responses is unclear.

Both CD4$^+$ and CD8$^+$ T lymphocytes produce a variety of cytokines upon encountering antigens or stimulation by other inflammatory cells present in the granuloma (Marton & Kiss, 2000). According to their cytokine expression patterns, CD4$^+$ T cells are divided into at least four different Th subsets: Th1, Th2, Th17, and regulatory T cells (T_{regs}) (McGeachy & Cua, 2008). The Th1 response is characterized by the production of IFN-α, IL-12, IL-2, and TNF, while a Th2 response mainly produces IL-4, IL-5, IL-6, IL-9 and IL-13. It is believed that Th1 pro-inflammatory cytokines contribute to lesion progression and bone destruction in infected root canals (Stashenko *et al.*, 1998, Colic *et al.*, 2009b). In contrast, the immunosuppressive mechanisms mediated by Th2

anti-inflammatory cytokines are considered to be responsible for healing processes by dampening inflammatory and immune mechanisms (Akamine *et al.*, 1994, Stashenko *et al.*, 1998, Kawashima & Stashenko, 1999, Sasaki *et al.*, 2000, Teixeira-Salum *et al.*, 2010). The newly identified Th17 class of activated T cells produces IL-17, a proinflammatory cytokine with potent effects on various cells of the innate immune system. IL-17 is also considered an important bridging molecule between the adaptive and innate immune systems (Yu & Gaffen, 2008). Moreover, $CD4^{+}CD25h^{i}Foxp3^{+}$ T_{reg} cells and IL-10 are crucially important for regulating the immune response, particularly in defense against infection (Colic *et al.*, 2009a).

The ability of leukocytes to rapidly translocate where they are needed makes them among the most dynamic cells in the body. Chemokines have well-defined roles in directing the cell movements necessary for the initiation of T cell immune responses (Luther & Cyster, 2001). Several chemokines, which promote selective cell migration by binding to their specific cell surface receptors, have been identified in periapical lesions (Marton *et al.*, 2000, Kabashima *et al.*, 2001, Garlet *et al.*, 2010).

In this study, we aimed to quantitatively assay $CD4^{+}CD28^{+}$ and $CD8^{+}$ T cell gene expression, as well as the expression of cytokines and chemokines (including IFN-γ, TNF-α, IL-1-β, IL-17A, IL-10, CCL2/MCP-1, CCL4, CCL5, CXCR4, CCR5, and RANKL) in samples collected from interstitial fluid adjacent to root canal infections in HIV-infected individuals. Seven days later, we assayed these same parameters following root canal cleaning procedures, which significantly reduced root canal bacterial load. The healing process had presumably begun in the second group of samples. Data from both groups were

then compared and subjected to statistical analyses.

METHODS

Human subjects

Subjects included 23 HIV-positive patients referred to the School of Dentistry at the Universidade Federal de Minas Gerais (Belo Horizonte, MG, Brazil). Almost all patients were undergoing highly active antiretroviral therapy (HAART). Only one patient did not adhere to HAART despite universal access to the therapy in Brazil (de Brito *et al.*, 2009). All patients presented updated medical records at the first appointment. The exclusion criterion for this study included antibiotic treatment within three months of starting endodontic therapy. All participants signed the Free Agreement Formulary. This study was approved by the Ethics Committee of the Universidade Federal de Minas Gerais (ETIC 122/08).

Sample collection

Clinical samples were taken from teeth with pulp necrosis, which were diagnosed based on clinical and radiographic analyses and pulp sensibility tests. No patients presented with acute periapical symptoms at the time of the first appointment. Teeth were isolated using a rubber dam, followed by a complete asepsis as previously described (Moller, 1966). Cleaning and shaping of the root canals was completed using ProTaper NiTi files (Dentsply Mailleffer) with 5.2% sodium hypochlorite as previously described (Vieira *et al.*, 2008). The clinical procedures were performed as follows. Briefly, samples were collected immediately after root canal cleaning to characterize T cell and

cytokine/chemokine gene expression profiles. After cleaning and drying, three paper points were introduced into the root canal, passing through the root apex (2 mm) for one minute. After withdrawal, the paper points were cut 4 mm from the tip, dropped into microcentrifuge tubes, and stored at -70°C. RNA was extracted from the periapical interstitial fluid. Endodontic dressing was inserted into the root canals. The coronal accesses of the teeth were restored with eugenol-based cement. Seven days later, the teeth were re-opened and sampled, as described above, to characterize T cell and cytokine expression after the root canal bacterial load was decreased. In teeth with multiple canals, the first and second samples were collected from the same canal. At this time, no teeth presented clinical signs or symptoms, and root canals were tridimensionally sealed.

Sample Preparation

Total RNA was extracted from each sample using TRIzol reagent (GIBCO/BRL Laboratories, Grand Island, NY, USA). Following the addition of chloroform, the mixture was centrifuged at 12,000 x *g* at 4°C for 15 min, the aqueous phase was collected, and the RNA was precipitated by the addition of isopropanol and centrifugation at 12,000 x *g* at 4°C for 10 min. The RNA precipitate was washed once with 75% cold ethanol, dried, dissolved in RNase-free water, and then incubated at 55°C for 10 min. The RNA was then stored at -70°C.

Real-time PCR

Complementary DNA (cDNA) was synthesized from one microgram of

RNA by a reverse transcription reaction (Barbosa Silva *et al.*, 2008). PCR was carried out under standard conditions: a holding stage of 95°C (10 min); a cycling stage with 40 cycles at 95°C (15 sec) followed by 60°C (1 min); and a melt curve stage of 95°C (15 sec), 60°C (1 min) and 95°C (15 sec). The primer sequences used for quantitative PCR analysis of $CD4^{+}CD28^{+}$ and $CD8^{+}$ T cell gene expression, in addition to IFN-γ, IL-10, CCL2, CCR5, CCL-5 and RANKL expression, are shown in Table I. Human primers were designed using PRIMEREXPRESS software (Applied Biosystems, Foster City, CA, USA) based on nucleotide sequences available in the GenBank database. The real-time PCR assays were performed using the StepOne Real-time PCR System (Applied Biosystems). A Syber-Green detection system (Applied Biosystems) was used to assay primer amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization. All samples were run in duplicate in a 20 µL reaction volume containing 1 µg of cDNA. Sequence Detection Software version 2.0 (Applied Biosystems) was used to analyze data after amplification. The results were obtained as threshold cycle (Ct) values. Expression levels were calculated using the comparative C (T) method (Schmittgen & Livak, 2008). The results were calculated from the mean of duplicates for each patient, and the expression levels of mRNAs in all samples were defined as the ratio of each specific primer to GAPDH expression.

Statistical Analysis

Data analysis was performed using SPSS for Windows (version 15.0; SPSS, Chicago, IL, USA). Data were subjected to the Shapiro-Wilk test to

characterize normality. Because the samples did not present a normal distribution, the Wilcoxon test was used to determine statistical differences ($p<0.05$).

RESULTS

Levels of mRNA expression were determined by real-time PCR and quantified by comparison with the internal control gene GAPDH. Assessment revealed significant increases in mRNA levels of $CD4^{+}CD28^{+}$ and $CD8^{+}$ T cell genes in teeth with restrained bacterial loads (second collection) compared to the first collection (Fig. 1). Similarly, the mRNA expression of IFN-γ, IL-1-β, TNF-α and IL-17A increased in samples from the second collection ($p<0.05$). However, the mRNA expression of RANKL and IL-10 was similar at both time points ($p>0.05$) (Fig. 2). In regard to chemokines, we observed increased mRNA expression of CCL2 at the second collection ($p<0.05$); however, no significant difference was observed for CCL4 expression between the two time points ($p>0.05$) (Fig 3). In contrast, CCL5 was not detected in either the first or second samples. Importantly, a positive control for primer amplification (human PBMCs treated with LPS) was detected (data not shown). Expression of the chemokine receptors CXCR4 and CCR5 was upregulated at the second collection (Fig. 3).

DISCUSSION

Periapical lesions are characterized by an accumulation of inflammatory cells (predominately $CD4^{+}$ and $CD8^{+}$ T cells), which represents a protective host response aimed at preventing the invasion of microorganisms into the bone

surrounding the tooth apex (Marton & Kiss, 1993, Stashenko *et al.*, 1998). Cell surface expression of CD4 and CD8 co-receptor molecules divides mature αβ T cells into two subsets: $CD4^+$ (Reinherz & Schlossman, 1980, Croft & Swain, 1991) and $CD8^+$ T lymphocytes (Nabholz & MacDonald, 1983). In this study, the gene expression of $CD4^+CD28^+$ and $CD8^+$ T-cells was found in similar low proportions in periapical samples from root canal infections in HIV-positive individuals. These data are consistent with a previous study, which analyzed the proportions of these immunocompetent cells and did not find significant differences in $CD4^+$ and $CD8^+$ T cell subsets in periapical lesions (Ihan Hren & Ihan, 2009). However, when the root canal bacterial load was reduced, both $CD4^+$ and $CD8^+$ T cells were statistically increased ($p<0.05$). These results are similar to those observed in HIV-negative patients (unpublished data). These findings cannot simply be attributed to the HAART regimen that nearly all patients underwent at the time of sampling because several reports have suggested that $CD4^+$ T cell repopulation in HIV individuals may be delayed despite otherwise successful HAART (Guadalupe *et al.*, 2003, Mehandru *et al.*, 2004). However, mRNA expression in $CD8^+$ T cells, which are relatively unaffected by HIV infection, was similar to $CD4^+$ T cell mRNA expression. Given these results, the modulation of mRNA expression of T cell-specific genes in periapical tissues may be related to apoptosis. This is supported by the study showing that bacteria recovered from human root canal infections are able to induce cellular apoptosis (Ribeiro-Sobrinho *et al.*, 2005).

When T cells become activated by cytokines or antigens presented by antigen presenting cells (APCs), they rapidly secrete cytokines and differentiate into various effector cells: $CD4^+$Th cells, $CD8^+$Th cells, T_{reg} cells, or memory

cells (Hahn & Liewehr, 2007). Effector T cells are divided into at least four different Th subsets: Th1, Th2, Th17, and T_{reg} (McGeachy & Cua, 2008). Common characteristics shared by cytokines produced by T cells include effectiveness, transient secretion, and a short half-life. Thus, serum cytokine levels are generally low and often undetectable in healthy individuals (Watanabe *et al.*, 2010). Another common feature of cytokines are complicated networks or cascades, which regulate their mutual effects additively, synergistically, or antagonistically (Mosmann & Sad, 1996, Stashenko *et al.*, 1998). In this study, the expression of IFN-γ, TNF-α, IL-1-β, IL-17A, IL-10 and the bone resorption cytokine RANKL was quantitatively assayed in samples collected from periapical tissues in HIV-infected patients. Our assessment revealed that inflammatory cells from periapical tissues produced detectable levels of both proinflammatory (IFN-γ, IL-1-β, TNF-α, IL-17 and RANKL) and immunoregulatory (IL-10) cytokines. Several studies in human periapical lesions have attributed the presence of cytokines to mediating bone resorption and lesion development (Akamine *et al.*, 1994, Stashenko *et al.*, 1998, Kawashima & Stashenko, 1999, Sasaki *et al.*, 2000, Teixeira-Salum *et al.*, 2010). In addition to characterizing cytokine profiles in periapical tissues adjacent to teeth with infected root canals from HIV-positive individuals, we also investigated the effects of changing the root canal bacterial load on periapical immune responses. IFN-γ, IL-1-β, TNF-α and IL-17A mRNA expression increased 7 days after root canal cleaning; however, RANKL and IL-10 expression remained at basal levels. Interestingly, in HIV-negative individuals, the expression of INF-γ, IL-β, and RANKL mRNA were significantly decreased, and IL-10 levels increased after bacterial load was diminished (unpublished

data). Since gene expression of $CD4^+CD28^+$ and $CD8^+$ T cells increased in both HIV-positive and negative patients (unpublished data), these conflicting results may be related to the fact that abnormal T cell activation persists in patients under HAART, which is likely associated with the immunologic response to the therapy (Watanabe *et al.*, 2010). However, the relationship between the production of IFN-γ and IL-17 has been previously studied in periapical lesions (Colic *et al.*, 2009b), and the results are in agreement with those observed here. In humans, it was demonstrated that half of all $IL\text{-}17^+$ cells are also IFN- γ^+ (Wilson *et al.*, 2007). Aside from the effects of IL-17 on neutrophil activation (Kolls & Linden, 2004), this cytokine also promotes bone resorption by stimulating the production of RANKL, which affects the activity and formation of osteoclasts (Zhang *et al.*, 2007). The inflammatory cytokines IL-1, TNF- α, and IFN- γ are also capable of activating osteoclastic bone resorption. Studies by others on periapical lesions suggest that IFN- γ may interact with TNF- α and IL-1 to upregulate cellular iNOS (Chae *et al.*, 1997) and RANKL, which subsequently modulate signaling between osteoblasts and osteoclasts (Danin *et al.*, 2000, Silva *et al.*, 2011). However, in the current study, the increased expression of IFN-γ, IL-1-β, TNF-α and IL-17A was not mirrored by RANKL upregulation, perhaps due to the timing of tissue collection. IL-10 can be produced by activated B cells, Th1 cells, activated macrophages and non-hematopoietic cells (Shrestha *et al.*, 2010). IL-10 has an immunomodulatory effect, particularly in suppressing cell-mediated immunity by down regulating pro-inflammatory cytokines, costimulatory molecules, and major histocompatibility complex class II (MHC II) proteins. Studies in animal models suggest that IL-10 plays a significant role in periodontal and periapical bone

resorption (Sasaki *et al.*, 2004, De Rossi *et al.*, 2008). It was recently demonstrated in periapical lesions that CD4^{+}CD25 h^{i}Foxp3^{+} cells expressed both IL-10 and TGF-β at a higher frequency than in peripheral blood (Colic *et al.*, 2009a). In this study, it appears that the increased mRNA expression of Th1 cytokines seven days after root canal cleaning were responsible for maintaining low expression levels of IL-10.

Chemokines direct cell movements necessary for the initiation of T cell immune responses (Luther & Cyster, 2001) and recruit the appropriate effector cells to sites of inflammation, including the selective recruitment of Th1 or Th2 cells (O'Garra *et al.*, 1998, Sallusto *et al.*, 2000). Moreover, chemokines and their receptors play a central role in HIV infection and disease progression. HIV must interact with CD4 (primary receptor) and a secondary receptor or co-receptor (either CCR5 or CXCR4) (Berger *et al.*, 1999). Multiple cytokines and chemokines have been identified in periapical lesions (Marton *et al.*, 2000, Kabashima *et al.*, 2001, Shimauchi *et al.*, 2001, Silva *et al.*, 2005, Garlet *et al.*, 2010). In this study, we examined quantitative differences between chemokine (CCL2/MCP-1, CCL4, CCL5) and chemokine receptor (CXCR4 and CCR5) expression before and after decreasing the root canal bacterial load in HIV-infected individuals. Our results reveal detectable expression of CCL2 and CCL4, while CCL5 was not detected; however, it was evident in periapical samples from HIV-negative patients (unpublished data) and in a positive control by primer amplification. This former result may be related to the delayed T cell activation observed in patients under HAART (Watanabe *et al.*, 2010). CCL2 gene expression was significantly elevated after dampening the root canal bacterial load, which parallels the high expression of IL-1 and TNF-α that

promote its expression (Van Coillie *et al.*, 1999). Besides its monocyte chemoattractant activity, CCL2 also influences T cell differentiation (Chensue *et al.*, 1996) by suppressing IL-12 production, which promotes the shift from a Th1 to a Th2 response (Luther & Cyster, 2001). CCL4, a chemoattractant for natural killer cells and monocytes (along with CCL5), may also promote development of the Th1 cell population (Luther & Cyster, 2001). In this study, CCL4 mRNA expression did not change when the bacterial load was diminished, which is similar to what we observed in HIV-negative individuals (unpublished data). Upregulation of the chemokine receptor CCR5, which is expressed on Th1 cells, directly correlates with increases in the pro-inflammatory cytokines IFN-γ, IL-1-β, TNF-α and IL-17A after root canal cleaning. Previous studies have also shown increased expression of CCR5 in periapical granulomas (Marton *et al.*, 2000, Kabashima *et al.*, 2001). However, CCR5 upregulation was not observed in either human periapical granulomas (Silva *et al.*, 2005) or in periapical samples from HIV-negative individuals (unpublished data). CXCR4 is an alpha-chemokine receptor that is potently chemotactic for lymphocytes and is used by HIV to infect $CD4^+$ cells. CXCR4 expression is upregulated by the cytokines IL-2 (Glushakova *et al.*, 1999) and IL-10. Similar to our observations in HIV-negative individuals (unpublished data), we observed CXCR4 upregulation after the root canal bacterial load was diminished. The results of this study lead us to speculate that the anti-inflammatory effects of the chemokines CCL2 (Luther & Cyster, 2001) and CXCR4 (Saini *et al.*, 2010) may induce a shift from a pro- to an anti-inflammatory response in a further moment, which we observed early in HIV-negative individuals after diminishing the root canal bacterial load (unpublished data).

In summary, we have demonstrated that the gene expression of $CD4^{+}CD28^{+}$ and $CD8^{+}$ T-cells increases after bacterial load reduction in periapical samples from teeth with root canal infections in HIV-positive patients. However, we unexpectedly observed a pro-inflammatory profile, suggesting that the shift toward an anti-inflammatory profile may occur later in these patients. Finally, we propose that intervention that directly targets the determinants of T cell activation may prove useful for the health surveillance of HIV-infected individuals.

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FIGURES AND FIGURES LEGENDS

Fig.1

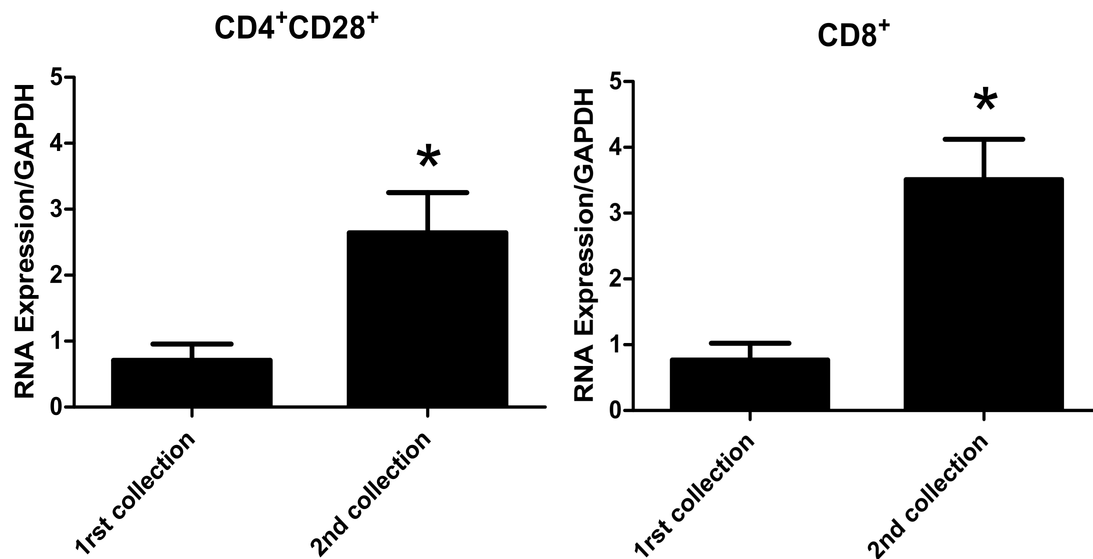

Figure 1. Expression of $CD4^{+}CD28^{+}$ and $CD8^{+}$ T cell genes in periradicular tissues from HIV^{+} patients with root canal infections. Levels of expression were determined by real-time PCR and quantified by comparison with the internal control (GAPDH). Bars represent the mean values of samples recovered from 23 patients, and lines represent the standard error of the mean. *Indicates $p < 0.05$ by the Wilcoxon test.

Fig.2

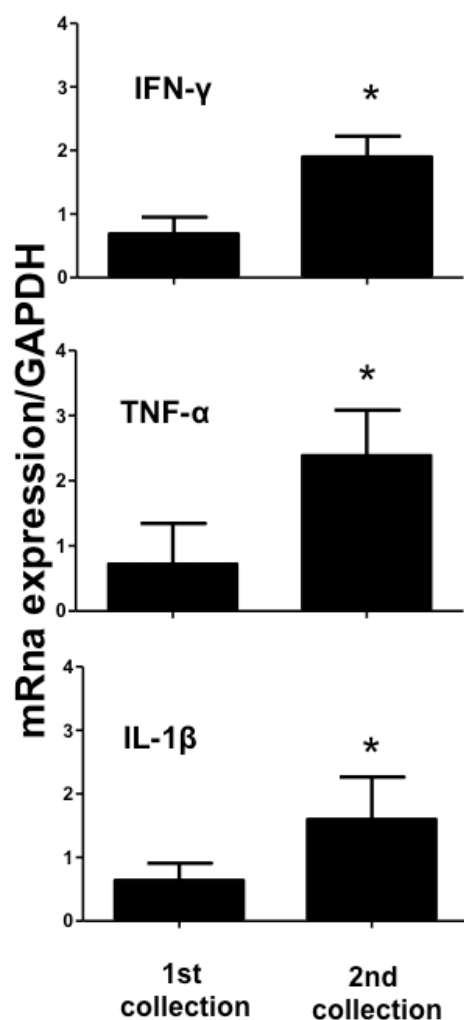

Figure 2. Expression of IFN-γ, TNF-α, and IL-1-β in periradicular tissues from HIV^+ patients with root canal infections. Levels of expression were determined by real-time PCR and quantified by comparison with the internal control (GAPDH). Bars represent the mean values of samples recovered from 23 patients, and lines represent the standard error of the mean. *Indicates $p < 0.05$ by the Wilcoxon test.

Fig.3

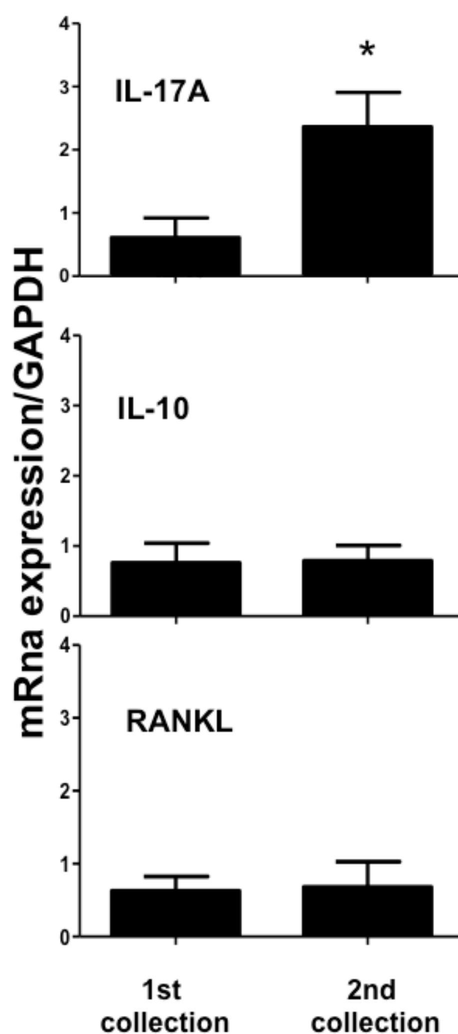

Figure3. Expression of IL-17, IL-10, and RANKL in periradicular tissues from HIV^+ patients with root canal infections. Levels of expression were determined by real-time PCR and quantified by comparison with the internal control (GAPDH). Bars represent the mean values of samples recovered from 23 patients, and lines represent the standard error of the mean. *Indicates $p < 0.05$ by the Wilcoxon test.

Fig.4

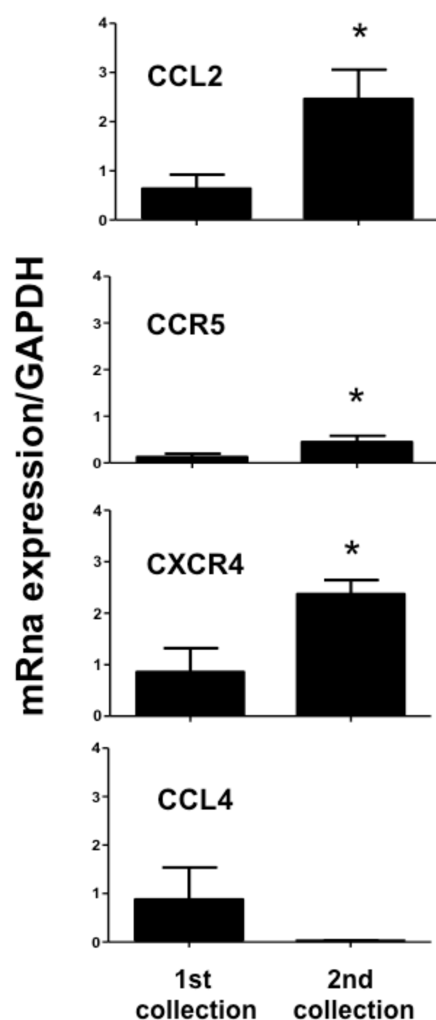

Figure 4. Expression of CCL2, CCR5, CXCR4, and CCL4 in periradicular tissues from HIV+ patients with root canal infections. Levels of expression were determined by real-time PCR and quantified by comparison with the internal control (GAPDH). Bars represent the mean values of samples recovered from 23 patients, and lines represent the standard error of the mean. *Indicates $p < 0.05$ by the Wilcoxon test.

TABLE

Table 1. Primer sequences

Gene	Sense and antisense	Mt* (°C)	bp*
GAPDH	5'-GCA CCA CCA ACT GCT TAG CA- 3' 5'-TGG CAG TGA TGG CAT GGA GGA- 3'	80	96
TNF-α	5'-TTC TGG CTC AAA AAG AGA ATT G- 3' 5'-TGG TGG TCT TGT TGC TTA AGG- 3'	76	73
IL-1β	5'-TGG CAG AAA GGG AAC AGA A- 3' 5'-ACA ACA GGA AAG TCC AGG CTA- 3'	73	59
IL-10	5'-GGT TGC CAA GCC TTG TCT GA- 3' 5'-TCC CCC AGG GAG TTC ACA T- 3'	81	107
IL-17A	5'-CAA TGA CCT GGA ATT ACC CAA- 3' 5'-TGA AGG CAT GTG AAA TCG AGA- 3'	70	52
IFN-γ	5'-GAA CTG TCG CCA GCA GCT AAA- 3' 5'-TGC AGG CAG GAC AAC CAT TA- 3'	80	95
CCL2	5'-AAG ACC ATT GTG GCC AAG GA- 3' 5'-CGG AGT TTG GGT TTG CTT GT- 3'	80	93
RANKL	5'-CAG AAG ATG GCA CTC ACT GCA- 3' 5'-CAC CAT CGC TTT CTC TGC TCT- 3'	73	203
CCL4	5'-TCT CCT CAT GCT AGT AGC TGC CTT- 3' 5'-GCT TCC TCG CAG TGT AAG AAA AG- 3'	78	101
CCL5	5'-CGT GCC CAC ATC AAG GAG TA- 3' 5'-CAC ACA CTT GGC GGT TCT TTC- 3'	80	91
CXCR4	5'-TGT TGG CTG AAA AGG TGG TC- 3' 5'-AAA GAT GTC GGG AAT AGT C- 3'	80	71
CCR5	5'-GGA CCA AGC TAT GCA GGT GAC- 3' 5'-TTG GCA CTG TGC TTT TGG AA- 3'	80	129
CD8	5'-CAG GCT ACC CCT TTA CAA ATA GAA TT- 3' 5'-TTC AGA GAT TCA AGA GGG CCT TA- 3'	73	70
CD28	5'-ACA GTG GTA GGA GCA ATG CTT TC- 3' 5'-AAT AGC AGC AAA TGA CAT TGT TTT CT- 3'	72	74

*Mt: melting temperature; bp: base pairs of amplicon size.

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DISCUSSÃO

4- DISCUSSÃO

De acordo com as últimas estimativas da UNAIDS (2008-2010), 34 a 37 milhões de pessoas vivem em todo o mundo com a Síndrome da Imunodeficiência adquirida (AIDS). Desde a descoberta da doença, há 30 anos, aproximadamente, 30 milhões de indivíduos morreram infectados pelo vírus HIV. Apesar do grande progresso conseguido nos últimos anos com o advento da terapia anti-retroviral (HAART), que reduziu drasticamente a morbidade e mortalidade da doença HIV/AIDS, o número de indivíduos vivendo com o vírus HIV continua aumentando.

Obviamente, os impactos da Síndrome da Imunodeficiência adquirida (AIDS) sobre a saúde oral desses indivíduos HIV-positivos são objeto de pesquisas em muitas instituições. Contudo, são escassos os trabalhos sobre os aspectos microbiológicos e imunológicos das infecções dos sistemas de canais radiculares nesses pacientes. Os poucos disponíveis na literatura analisam o sucesso clínico do tratamento endodôntico (Goodis & King, 1989, Shetty *et al.*, 2006, Suchina *et al.*, 2006, Alley *et al.*, 2008). Sabedores de que as infecções endodônticas são consequência da invasão dos SCR por patógenos orais oportunistas, e de que as pessoas portadoras da síndrome são susceptíveis às infecções, procuramos, neste estudo, avaliar a necessidade do tratamento endodôntico, correlacionando-o aos dados pessoais e laboratoriais sistêmicos de uma população de indivíduos HIV-positivos atendidos no maior centro de referência da Região Metropolitana de Belo Horizonte, o CTR-Orestes Dinis. Concluída essa etapa do trabalho, os pacientes foram encaminhados à Faculdade de Odontologia da UFMG, onde tiveram suas necessidades odontológicas sanadas. Especificamente, os tratamentos endodônticos foram

realizados na disciplina Optativa de Endodontia, sob a supervisão dos professores e alunos do Programa de Pós Graduação, e assistidos pelos alunos do 8º período de graduação. Nessa etapa, amostras microbiológicas e imunológicas foram coletadas dos SCR, sendo mantidas em condições ideais, para posterior análise. Adicionalmente, coletaram-se amostras de pacientes HIV-negativos para que se confrontassem os dados dos dois grupos de indivíduos.

Ao avaliarmos a necessidade de terapia endodôntica nos pacientes HIV-positivos e correlacionando-a aos dados pessoais e sistêmicos, de maneira interessante, percebemos a inexistência na literatura, de estudos sobre a necessidade de tratamento endodôntico em uma dada população. Entretanto, a percepção de que 14 % daquela população necessitava desse tratamento não nos dizia se a síndrome da imunodeficiência humana contribuiria ou não para o incremento dessa necessidade. Surpreendentemente, observamos também uma correlação estatisticamente relevante entre a necessidade de tratamento endodôntico e baixos níveis de contagem de células TCD4. Não tendo dados que nos permitissem fundamentar esse achado, levantamos as hipóteses: tal correlação provavelmente se vincularia à demonstrada baixa adesão desses pacientes ao tratamento anti-retroviral (HAART) (Godin *et al.*, 2005, Cote *et al.*, 2008) e reduzido cuidado com a saúde oral. Dois anos após a execução do tratamento endodôntico, os dados hematológicos desses pacientes foram analisados (Anexo I) e confrontados com os iniciais. Percebeu-se, nesse momento, uma correlação direta entre a redução da carga viral e o aumento da contagem de células TCD4$^+$, como se demonstrou na clínica e na literatura (Mellors *et al.*, 1996). A necessidade de tratamento endodôntico, porém,

poderia até interferir na contagem de células $TCD4^+$, mas jamais na redução da carga viral que sofre sim a interferência do HAART. Esses resultados confirmam a hipótese inicial proposta no artigo 1.

Nas últimas décadas, numerosos estudos descrevem a microbiota das infecções endodônticas (Sundqvist, 1992, Sundqvist, 1994, Siqueira *et al.*, 2000b, Lana *et al.*, 2001, Brito *et al.*, 2007, Siqueira & Rocas, 2007, Vianna *et al.*, 2007, Sakamoto *et al.*, 2009, Tavares *et al.*, 2011). Também o advento das técnicas de biologia molecular contribuiu sobremaneira para expandir esse conhecimento (Brito *et al.*, 2007, Siqueira & Rocas, 2007, Tavares *et al.*, 2011). Essa nova tecnologia permite melhor caracterização da composição microbiana dessas infecções além de confirmar o caráter polimicrobiano e anaeróbio das mesmas (Brito *et al.*, 2007, Siqueira & Rocas, 2007, Sakamoto *et al.*, 2009, Tavares *et al.*, 2011). Apesar desses reais avanços, pouco se estudou sobre essas infecções em pacientes portadores da síndrome da imunodeficiência adquirida humana (Goodis & King, 1989, Shetty *et al.*, 2006, Suchina *et al.*, 2006, Alley *et al.*, 2008). Assim, nossa próxima etapa compreenderia a caracterização da microbiota dos sistemas de canais radiculares infectados desses pacientes. E, para confrontar os resultados, analisamos também a microbiota dos não portadores do vírus. A execução dessa etapa muito se deveu à parceria promissora com o grupo de pesquisadores do The Forsyth Institute. Em trabalhos prévios, caracterizando a microbiota de infecções de dentes permanentes e decíduos (Brito *et al.*, 2007, Tavares *et al.*, 2011), demonstramos a pertinência da associação das técnicas: hibridização DNA-DNA e MDA na análise da pequena amostra tradicionalmente recuperada das infecções endodônticas. Ao amplificar em

1000 vezes a amostra inicial (Brito *et al.*, 2007, Teles *et al.*, 2007a, Tavares *et al.*, 2011), o MDA cria as condições para que elas possam ser analisadas pelo "checkerboard", que exige um conteúdo mínimo acima de 10^4 células. Esses estudos prévios do grupo (Brito *et al.*, 2007, Tavares *et al.*, 2011) identificaram uma pletora de agentes infecciosos nas infecções endodônticas. As mesmas podem albergar qualquer dos microrganismos residentes na cavidade oral, diferentemente do antigo proposto dogma de que esse ambiente seria seletivo para uma pequena amostra de microrganismos (Sundqvist, 1976, Sundqvist, 1992, Lana *et al.*, 2001). Neste estudo avaliamos 107 espécies, dentre tradicionais periodontopatógenos, microrganismos prevalentes nas infecções endodônticas, patógenos de interesse médico, tais como os presentes em infecções respiratórias, gástricas e oro-faciais. Pôde-se observar que a média de espécies encontradas, tanto nos indivíduos HIV-positivos, quanto nos HIV-negativos, foi de aproximadamente 22 espécies por canal, nos níveis maiores do que 10^5 células. As prevalentes nos indivíduos HIV-negativos foram *Prevotella denticola, Abiotropia adiacens* e *Streptococcus constellatus*. Nos indivíduos HIV-positivos foram *Dialister pneumosintes, Helicobacter pylori* e *S. constellatus*. Quanto à média de proporção do DNA da sonda, que nos dará a frequência por amostra dos microrganismos, observamos que, nos indivíduos HIV-negativos, a maior proporção encontrada foi das seguintes espécies: *P. denticola, A. adjacens, C. diphitheria* e *S. sobrinus*. Nos HIV-positivos foram: o *S. constellatus* e a *P. tannarae*. Esses pacientes exibiram uma alta proporção de *C. albicans* e *C. tropicalis* em comparação aos HIV-negativos. É interessante ressaltar que vários microrganismos de importância médica como o *H. pylori* (gástrico), *Dialister pneumosintes* (respiratória), e a *C. albicans*

(infecções oportunistas) foram espécies encontradas em proporções significativas nos indivíduos HIV-positivos em relação aos HIV-negativos. Demonstrou-se, previamente, que as comunidades bacterianas presentes na cavidade oral podem ser um reservatório de patógenos envolvidos nessas doenças (Siqueira *et al.*, 2001, Scannapieco & Rethman, 2003, Umeda *et al.*, 2003, Brito *et al.*, 2007, de Souza Goncalves *et al.*, 2009, Gao *et al.*, 2011, Tavares *et al.*, 2011). Assim, o presente estudo ajuda a demonstrar que a microbiota dos SCR de pacientes HIV-positivos é tão complexa quanto a dos HIV-negativos, mas com o predomínio de algumas espécies de importância oral e não oral. Diante do fato de que esses indivíduos apresentam um comprometimento em sua resposta às infecções, a prevenção e o tratamento endodôntico devem ser considerados nos protocolos de manutenção da sua saúde oral e geral.

A relação entre infecção dos sistemas de canais radiculares e respostas imuno-inflamatórias periapicais já está bem comprovada na literatura (Kakehashi *et al.*, 1965, Sundqvist, 1976, Moller *et al.*, 1981, Fabricius *et al.*, 1982, Teles *et al.*, 1997, Kawashima & Stashenko, 1999, Silva *et al.*, 2005, Colic *et al.*, 2009b). Com a infecção dos SCR, células imuno-competentes migram para o tecido periodontal adjacente ao ápice do dente em questão, no intuito de prevenir a disseminação microbiana. Sabedores de que: as células TCD4são as prevalentes nessas lesões (Yu & Stashenko, 1987), elas têm um papel chave no desenvolvimento e resolução das lesões periapicais (Kawashima *et al.*, 1996, Marton & Kiss, 2000), são alvos primários da infecção pelo HIV e de que havíamos observado uma correlação positiva entre a necessidade de tratamento endodôntico e baixos níveis de contagem de

células TCD4, nosso primeiro objetivo nessa fase seria quantificar a expressão gênica das células $TCD4^{+}CD28^{+}$, para determinar se o efeito sobre as mesmas se devia ao tropismo do HIV. Adicionalmente, quantificamos a expressão gênica das células $TCD8^{+}$, que são células pouco afetadas pelo HIV. Decidimos também, avaliar a expressão dessas células em dois momentos: imediatamente após os procedimentos de limpeza e formatação dos SCR (que corresponderia à sua expressão na presença da infecção nos SCR) e sete dias após esses procedimentos (quando a carga microbiana estivesse drasticamente reduzida e os processos de cicatrização se iniciassem). De forma muito interessante, observamos nos dois grupos de indivíduos, HIV-positivo e HIV-negativo, que tanto a expressão gênica das células $TCD4^{+}CD28^{+}$, quanto das $TCD8^{+}$ encontravam-se baixas na presença da infecção microbiana dos SCR, aumentando após sua remoção. Como em estudo prévio (Ribeiro-Sobrinho *et al.*, 2005), demonstramos que bactérias recuperadas de canais radiculares humanos eram capazes de induzir apoptose em linfócitos murinos, creditamos os efeitos observados sobre a expressão de ambas as células ($TCD4^{+}CD28^{+}$ e $TCD8^{+}$) à infecção dos canais radiculares. Ademais, o alvo do vírus HIV são células TCD4 e não as TCD8. Neste estudo pudemos perceber que ambas as células se comportaram similarmente, tanto nos indivíduos HIV-positivos quanto nos HIV-negativos. Os efeitos da infecção endodôntica sobre a expressão das células $TCD4^{+}CD28^{+}$ e $TCD8^{+}$ não poderiam assim explicar a correlação observada entre a necessidade de tratamento endodôntico e baixos níveis de contagem de células $TCD4^{+}$, uma vez que os efeitos sobre as células $TCD8^{+}$ não foram detectados sistemicamente. Em resumo, vimos que a necessidade de tratamento

endodôntico, entendida como a infecção dos SCR, interferiu na expressão local das células $TCD4^+$ mas não foi responsável pela correlação inicialmente encontrada, pois a análise prospectiva dos dados (Anexo I) demonstrou que a baixa na contagem de células $TCD4^+$ se correlacionou ao HAART, como acima discutido.

Os linfócitos $TCD4^+$ e $TCD8^+$ e as demais células, presentes nos sítios inflamatórios perirradiculares, produzem uma variedade de citocinas quando em contato com os antígenos microbianos advindos dos SCR infectados. Dependendo da expressão de citocinas, quatro diferentes grupos Th podem ser caracterizados: Th1, Th2, Th17 e Treg (regulatório) (McGeachy & Cua, 2008). Acredita-se que as citocinas pro-inflamatórias, Th1 e Th17, contribuam para a progressão da lesão e a destruição óssea perirradicular (Stashenko *et al.*, 1998, Yu & Gaffen, 2008, Colic *et al.*, 2009a). Os mecanismos imunosupressores, mediados pelas citocinas anti-inflamatórias (do tipo 2) e regulatórias, são responsáveis pelos processos de cronicidade e cicatrização (Akamine *et al.*, 1994, Stashenko *et al.*, 1998, Kawashima & Stashenko, 1999, Sasaki *et al.*, 2000, Teixeira-Salum *et al.*, 2010). De forma interessante, a expressão gênica das citocinas pró-inflamatórias (IFN-γ, IL-1-β, and RANKL) diminuiu após os procedimentos de limpeza do SCR, 7 dias mais tarde, nos pacientes HIV-negativos. De maneira inversa, a expressão do IFN-γ, IL-1-β, and TNF-α aumentou no mesmo período, nos pacientes HIV-positivos. Quanto a IL-10, observa-se maior expressão gênica dessa citocina após os procedimentos de limpeza dos SCR, nos pacientes HIV-negativos, a mesma permanece constante, nos dois períodos analisados, nos indivíduos HIV-positivos. Os resultados encontrados nos pacientes HIV-negativos estão

consistentes com os relatados na literatura: as citocinas pró-inflamatórias seriam expressas mais abundantemente nas fases ativas do desenvolvimento das lesões (no nosso caso na presença de infecção), como aqui demonstrado. Por outro lado, teriam sua expressão reduzida nas fases de cronicidade e cicatrização (aqui, após a reduçãoda infecção), provavelmente moduladas pelo aumento das citocinas anti-inflamatorias do tipo 2, como também se demonstrou neste estudo. Muito interessante foi o resultado encontrado nos pacientes HIV-positivos. Observamos que a fase pró-inflamatória inicia-se tardiamente, após a remoção da infecção, correlacionando-se com uma expressão basal da IL-10, nos dois períodos analisados. Demonstrou-se recentemente, que ocorre uma ativação anormal das células T em indivíduos submetidos ao HAART (Watanabe *et al.*, 2010) e essa deve ser a explicação para esse achado. Neste estudo, 79,2% dos indivíduos encontravam-se sob a terapia anti-retroviral. Cabe ressaltar que essa característica de uma resposta pró-inflamatória tardia, fundamental nos procedimentos de eliminação microbiana, deve ser levada em consideração nos resultados esperados durante o tratamento e posterior cicatrização das alterações perirradiculares, nesses pacientes.

A habilidade das células em migrarem para o periápice adjacente ao SCR infectado será de fundamental Importância para as respostas aí compreendidas. As quimiocinas têm um papel bem definido nesse aspecto, ao direcionar o movimento celular e permitir que se iniciem as respostas imunológicas locais (Silva *et al.*, 2005). Dentre os muitos efeitos das quimiocinas, destacam-se neste estudo, dois de seus receptores, CCR5 e CXCR4, uma vez que o HIV os utiliza como portal de entrada na célula alvo,

nos estágios inciais e tardios, respectivamente (Mackay & Sallusto, 2006). Ambos os receptores apresentaram um aumento em sua expressão gênica nos pacientes HIV-positivos após os procedimentos de limpeza dos SCR, enquanto que nos indivíduos HIV-negativos, ocorreu um aumento da expressão do CXCR4 e a manutenção da expressão do CCR5, nos mesmos períodos. O receptor CCR5 age sobre as células Th1, enquanto o CXCR4 tem sua expressão aumentada pelas citocinas IL-2 e IL-10 (Glushakova *et al.*, 1999). Assim, tomando juntos esses dados, nos parece plausível que o aumento e a manutenção da expressão de ambos os receptores nos pacientes HIV-positivos e negativos, pode se dever ao mesmo efeito relatado anteriormente para os marcadores celulares $TCD4^{+}CD28^{+}$ e $TCD8^{+}$, qual seja: após a remoção da infecção, houve uma maior população celular expressando esses receptores. Surpreendentemente não observamos a expressão de CCL5 nos pacientes HIV-positivos. A expressão dessa quimiocina diminuiu significantemente nos pacientes HIV-negativos após a redução da carga microbiana nos SCR. Esse achado é consistente com o observado nas demais citocinas do tipo 1, que também tiveram sua expressão reduzida no mesmo período, nos indivíduos HIV-positivos. Talvez a ausência de expressão do CCL5 nesses pacientes reforce o que anteriormente observamos: que os indivíduos HIV-positivos apresentam uma resposta pró-inflamatória tardia, uma vez que também se demonstrou que a neutralização dessa quimiocina reduz a formação de granulomas (Chensue *et al.*, 1999). Apesar de não se ter percebido variações nos níveis de expressão de CCL2 e CCL4 nos pacientes HIV-negativos, detectamos um aumento significativo na expressão de CCL2 após a redução da carga microbiana nos SCR nos pacientes HIV-positivos.

Este resultado é consistente das citocinas IL-1 e TNF-α nesses indivíduos, que sabidamente promoveram a expressão do CCL2 no mesmo período (Van Coillie *et al.*, 1999).

A síndrome da imunodeficiência adquirida humana é objeto de muitas pesquisas desde que acometeu os primeiro indivíduos, na já longínqua década de 80. Nesses 30 anos, mais de 30 milhões de indivíduos morreram infectados pelo vírus HIV e, apesar da melhoria na qualidade de vida advinda com a terapia anti-retroviral (HAART), muitos esforços ainda são necessários para que ações terapêuticas eficazes sejam aplicadas. Este estudo nos deu a oportunidade de conhecer alguns dos aspectos epidemiológicos, microbiológicos e imunológicos das infecções endodônticas nos pacientes HIV-positivos. Mais de 50% dos que foram objeto desta pesquisa eram do sexo feminino, corroborando o fenômeno da feminização da pandemia observado em todo o mundo. Alguns eram ainda crianças. Hoje, metade das novas infecções, que ocorrem pelo mundo, atingem crianças e pessoas abaixo dos 25 anos, a maioria de classes sociais menos abastada (UNAIDS, 2011).

Conforta nos saber que, graças as políticas públicas implementadas em nosso país, hoje considerado modelo mundial, todos os participantes deste estudo tiveram acesso à medicação anti-retroviral. Nesse contexto, este estudo nos traz a certeza do quão são importantes e urgentes as providências para se prevenir e tratar as patologias pulpoperirradiculares nos indivíduos HIV positivos. Só assim poderemos almejar, no âmbito da odontologia, uma assistência que elimine a presença de infecções orais e que ajude a promover a saúde desses indivíduos.

CONCLUSÕES

5. CONCLUSÕES

Conclui-se que:

a) A feminização da epidemia HIV/AIDS observada mundialmente foi também detectada neste estudo; b) apesar da relação positiva entre a necessidade de intervenção endodôntica e a baixa contagem de células $TCD4^{+}$ ter sido observada inicialmente, análise prospectiva demonstrou uma correlação direta entre a redução da carga viral e o aumento na contagem de células TCD4, sugerindo que a aderência ou não ao HAART modula a resposta sistêmica; c) ao se comparar a microbiota de indivíduos HIV-positivos com a dos negativos confirmou-se que a infecção dos SCR é polimicrobiana, com algumas espécies de importância médica dominando nos indivíduos HIV-positivos; d) a expressão local dos marcadores celulares TCD4+CD28+ e TCD8+, em ambos os indivíduos, HIV-positivos e negativos, é afetada pela presença de estímulos microbianos nos SCR; e) a expressão gênica de citocinas e quimiocinas demonstrou que a resposta pró-inflamatória nos pacientes HIV-positivos acontece mais tardiamente quando comparada à observada nos indivíduos HIV-negativos

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ANEXOS

Anexo 1: Gráfico atualizado dos dados sistêmicos dos pacientes HIV positivos.

Anexo 2: Trabalhos como colaboradora publicados durante o período de doutoramento.

Anexo 3: Certificado do Estágio no Exterior

Anexo 4: Aprovação do Comitê de Ética em Pesquisa (COEP)