WILSON BAMBIRRA JÚNIOR

AVALIAÇÃO DA EXPRESSÃO DE INTEGRINA ALFA2, PROTEÍNA DE CHOQUE TÉRMICO 47, E MEDIADORES PRO-INFLAMATÓRIOS E IMUNORREGULATÓRIOS EM RESPOSTA À INFECÇÃO ENDODÔNTICA.

FACULDADE DE ODONTOLOGIA

UNIVERSIDADE FEDERAL DE MINAS GERAIS

BELO HORIZONTE

2014

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Orientador: Prof. Dr. Antônio Paulino Ribeiro Sobrinho

Co-orientador (a): Prof (a). Dr (a). Leda Quercia Vieira

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PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

FOLHA DE APROVAÇÃO

Avaliação da expressão de integrina alfa 2, proteína de Choque Térmico 47, e mediadores proinflamatórios e imunorregulatórios em resposta a infecção endodôntica.

WILSON BAMBIRRA JÚNIOR

Dissertação submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ODONTOLOGIA, como requisito para obtenção do grau de Mestre em ODONTOLOGIA, área de concentração ENDODONTIA.

Aprovada em 28 de agosto de 2014, pela banca constituída pelos membros:

Prof(a). Antonio Paulino Ribeiro Sobrinho - Orientador
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Prof(a). Evandro Neves Abdo
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Belo Horizonte, 28 de agosto de 2014.

UNIVERSIDADE FEDERAL DE MINAS GERAIS

PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

ATA DA DEFESA DA DISSERTAÇÃO DO ALUNO
WILSON BAMBIRRA JÚNIOR

Realizou-se, no dia 28 de agosto de 2014, às 08:30 horas, Sala 3403, Faculdade de Odontologia da Universidade Federal de Minas Gerais, a defesa de dissertação, intitulada *Avaliação da expressão de integrina alfa 2, proteína de Choque Térmico 47, e mediadores proinflamatórios e imunorregulatórios em resposta a infecção endodôntica.*, apresentada por WILSON BAMBIRRA JÚNIOR, número de registro 2012733063, graduado no curso de ODONTOLOGIA, como requisito parcial para a obtenção do grau de Mestre em ODONTOLOGIA, à seguinte Comissão Examinadora: Prof(a). Antonio Paulino Ribeiro Sobrinho - Orientador (UFMG), Prof(a). Leda Quercia Vieira (UFMG), Prof(a). Luciana Carla Neves de Brito (FOUI), Prof(a). Evandro Neves Abdo (UFMG).

A Comissão considerou a dissertação:

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Finalizados os trabalhos, lavrei a presente ata que, lida e aprovada, vai assinada por mim e pelos membros da Comissão.
Belo Horizonte, 28 de agosto de 2014.

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Prof(a). Leda Quercia Vieira (Doutora)

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DEDICATÓRIA

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“Tenha coragem de seguir o seu próprio coração e a sua intuição. Eles de alguma maneira já sabem o que você quer se tornar. Todo o resto é secundário.”

STEVE JOBS.

RESUMO

Objetivo: Analisar a expressão de integrina alfa2, mediadores moleculares, citocinas e quimiocinas, a partir de células presentes no líquido intersticial periapical adjacente a dentes portadores de infecção dos canais radiculares.

Metodologia: Os 13 pacientes incluídos no estudo foram encaminhados à Faculdade de Odontologia da Universidade Federal de Minas Gerais (Belo Horizonte, MG, Brasil). As amostras foram retiradas de dentes com necrose pulpar e nenhum paciente apresentou sintomas periapicais agudos no momento das coletas. Após a limpeza e formatação dos sistemas de canais de radiculares (SCR), 3 cones de papel absorventes foram introduzidos no SCR. De forma passiva, o cone de papel ultrapassou o ápice radicular em 2 mm e permaneceu por 1 minuto. As amostras foram coletadas imediatamente após a limpeza do canal radicular e 7 dias após, para caracterizar as expressões dos genes ITGA2, Hsp47, FAK, OPN, IL-1β, TGF-β,IL-17A , IL-10, IFN-γ, IL-8, CCL2/MCP-1, CCL5, utilizando-se a PCR em Tempo Real.

Resultados: Observaram-se níveis significativamente mais baixos de TNF-α, CCL5, CCL2 / MCP-1 e IL-8 em dentes com cargas bacterianas reduzidas (segunda coleta), quando comparada à primeira coleta. Do mesmo modo, a expressão do mRNA das proteínas SSP1 / OPN e FAK diminuiu nas amostras coletadas na segunda amostragem (7 dias). A expressão gênica da IL-10 foi significativamente superior nas amostras coletadas 7 dias após a limpeza e formatação dos SCR quando comparada àquela coletada no dia 0. As expressões gênicas da IL-1β, IL-17A, IFN-γ, ITGA2 e Hsp47 / SERPINH1 foram semelhantes nos dois momentos avaliados.

Conclusão: Estes resultados sugerem que após a redução da carga bacteriana do SCR uma resposta anti-inflamatória se instala no periápice, aparentemente mediada pela IL-10.

Palavras-chave: integrina, SSP1 / OPN, FAK, ITGA2, Hsp47 / SERPINH1, periodontite apical, citocinas e quimiocinas.

Abstract

Aim: To examine alpha 2 integrin, molecular mediators, cytokines, and chemokines from cells in periapical interstitial fluid from root canal infections.

Methodology: Subjects included 13 patients referred to the College of Dentistry at the Universidade Federal de Minas Gerais (Belo Horizonte, MG, Brazil). Clinical samples were taken from teeth with pulp necrosis and no patients had acute periapical symptoms at the time of the appointments. After cleaning and drying, 3 paper points were introduced into the root canal, passing passively through the root apex (2 mm) into the periapical tissues for 1 minute. The samples were collected immediately after root canal cleaning and 7days later (restrained root canal bacterial load) to characterize ITGA2, Hsp47, FAK, OPN, IL-1β, TGF-β,IL-17A , IL-10, IFN-γ, IL-8, CCL2/MCP-1, CCL5 gene expressions using Real Time PCR.

Results: Significantly lower levels of TNF-α, CCL5, CCL2/MCP-1, and IL-8 in teeth with restrained bacterial loads (second collection) compared to the first collection were observed. Similarly, the mRNA expression of the proteins SSP1/OPN and FAK decreased in samples from the second collection. The regulatory cytokine IL-10 mRNA was significant higher in the samples from the second collection compared to the first collection. mRNA expression of IL-1β, IL-17A, IFN-γ, ITGA2 and Hsp47/SERPINH1 were similar at both time points.

Conclusion: These findings suggest that after reducing the root canal bacterial load an anti-inflammatory response take place in periapical area regulated by the Treg IL-10 cytokine.

Key words: Integrin, SSP1/OPN, FAK, ITGA2, Hsp47/SERPINH1, apical periodontitis, cytokine and chemokine.

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LISTA DE ABREVIATURAS E SIGLAS

1. CCL2/MCP-1: *monocyte chemotactic protein 1*
2. CCL5: *Chemokine ligant 5*
3. FAK: *focal adhesion kinases*
4. HSP47: *heat shock protein 47*
5. IFN: *interferon gamma*
6. IL: *interleukin*
7. ITGA2: *integrin alpha 2*
8. OPN: *osteopontin*
9. pH: potencial hidrogênionico
10. RANK: *receptor activator of nuclear factor Kappa B*
11. RANKL: *receptor activator of nuclear factor Kappa B ligant*
12. SCR: sistema de canais radiculares
13. TGF*: transforming growth factor*
14. Th: T *helper*
15. TNF: *tumor necrosis factor*

SUMÁRIO

SUMÁRIO

Introdução e relevância

1 INTRODUÇÃO E RELEVÂNCIA

As patologias pulpares e perirradiculares iniciam-se no momento em que microorganismos oportunistas violam a estrutura dental através de lesões cariosas, traumas, restaurações mal adaptadas e defeitos estruturais, como trincas e fissuras produzidas pelo estresse mastigatório (37). Isso permite que agentes patogênicos penetrem na estrutura dental provocando uma resposta inflamatória no hospedeiro frente aos agentes agressores que infectam e colonizam o Sistema de Canais Radiculares (SCR).

A resposta inflamatória contra os patógenos provenientes do sistema de canais radiculares (SCR) infectados induz a liberação de mediadores inflamatórios que, ao conter a invasão dos agentes agressores aos tecidos perirradiculares, promovem destruição localizada no próprio tecido do hospedeiro (50). Mediadores químicos como a Interleucina-1β (IL-1β), Fator de Necrose Tumoral alfa (TNF-α), Interferon gama (IFN-γ) promovem interações entre o receptor ativador do fator nuclear Kappa B (RANK) e o seu ligante (RANKL) e consequente ativação e diferenciação dos pré-osteoclastos em osteoclastos. Essa interação provocará uma reabsorção óssea periapical proporcionando a instalação de um tecido imunocompetente que visa impedir a disseminação de microrganismos para tecidos periapicais e para os demais tecidos do hospedeiro. (7,10,13).

No decorrer entre a penetração e a colonização dos SCR, inter-relações ecológicas microbianas ocorrem mudando o perfil e a patogenicidade microbiana. Fatores como: ambiente específico; pressões seletivas existentes nos canais radiculares (48); disponibilidade de nutrientes; o pH; temperatura e baixa tensão de oxigênio

(potencial redox) (43,48) podem determinar estas interações como sinérgicas ou antagônicas (33,34,45,46,48). Essas relações influenciam de forma decisiva na colonização e no estabelecimento da microbiota infectante do SCR (3, 48), culminando na instalação da infecção endodôntica composta principalmente por espécies anaeróbias (2,7,11,21,31,34,43,44,52), e por conseguinte podem produzir respostas agudas ou crônicas aos tecidos periapicais do hospedeiro. Assim a virulência e o número de espécies bacterianas determinarão os efeitos patogênicos microbianos que atuam de forma direta, através da produção de enzimas e de toxinas sobre os tecidos que circundam o dente; e indireta, via estimulação e liberação de mediadores solúveis derivados do hospedeiro, como as citocinas e quimiocinas (42).

A patogenicidade da microbiota infectante determinará o recrutamento de células inflamatórias, liberação de citocinas e quimiocinas pró-inflamatórias e imunorregulatórias responsáveis pela polarização da resposta imune. De acordo com o conhecimento atual, os Linfócitos T *helper* (Th) ativados se subdividem em subgrupos Th1, Th2, Th17 e Treg que se caracterizam principalmente pelo perfil de citocinas produzidas e seus efeitos sobre o organismo (25). A resposta imune do tipo 1 (Th1) caracteriza-se principalmente pela produção de TNF-α, IFN-γ e IL-1β que induzirão a diferenciação e ativação dos pré-osteoclastos pelo ligante do receptor ativador do fator nuclear Kappa B (RANKL) e consequente progressão da infecção (10,51). Os osteoclastos se ligam a hidroxiapatita, através da Osteopontina, que é uma proteína fosfoproteica biosintetizada por células do sistema imune, como macrófagos, células T e B, neutrófilos e células dendríticas. A OPN atua como imunomodulador ao promover o recrutamento de células inflamatórias ao sítio da inflamação, influenciando a imunidade celular com funções de citocinas pró-inflamatórias ao estimular a produção de IFN-γ, IL-12 e IL-17 e inibir a produção de IL-10 (35,41). A OPN age também como proteína

adesiva, na ligação entre células e átomos de cálcio, disponíveis em tecidos ósseos e dentes, através do fosfato de cálcio atuando como remodelador ósseo e inibidor de crescimento de cristais de cálcio (26,56).

A resposta celular do tipo 2 (Th2) produz IL-4, IL-5, IL-6, IL-10 e IL-13 que estão relacionadas aos processos de cura e reparo da área afetada (19,53). Esses dois subgrupos, Th1 e Th2, apresentam regulação cruzada, através de liberação de citocinas antagônicas como a IL-10 e o fator de crescimento tumoral (TGF-β). Ou seja, as células Th1 inibem a resposta Th2 pela produção de IFN-γ e IL-12, enquanto as células Th2 inibem a geração de uma resposta Th1 produzindo citocinas como a IL-4 e IL-10 (28).

Os linfócitos T reguladores (Treg) atuam como agentes imunossupressores e reestabelecem o equilíbrio entre a progressão da infecção e a resposta imunológica do hospedeiro restringindo uma reação inflamatória mais intensa e danosa ao próprio organismo (10,38,58).

Células Th17 são caracterizadas por expressarem a IL-17 e IL-22 e estão relacionadas à exacerbação de processos inflamatórios em lesões perrirradiculares, através da indução de secreção de mediadores pró-inflamatórios como a IL-8, TNF-α, IL-1β, e IL-6 que promovem rapidamente o recrutamento de neutrófilos para o local da infecção (6), sendo consideradas pontes entre a resposta adaptativa e inata (59), através de estímulo para produzir outras citocinas pró-inflamatórias que atuam na progressão de lesões periapicais (6,57).

Quimiocinas possuem um papel de destaque no processo inflamatório uma vez que funcionam como agentes quimiotáticos e promovem uma resposta imune inicial para diferentes tipos de patógenos, através do recrutamento de células efetoras apropriadas para o sítio inflamatório, incluindo o recrutamento de diferentes células Th1 e Th2. CCL2/MCP-1 tem sido associado ao recrutamento de células inflamatórias e tem

ação sobre a diferenciação de células T efetoras, uma vez que leva a diminuição da produção da IL-12 pelos macrófagos e a supressão da resposta Th1. CCL-5 possui funções quimiotáticas para células T, eosinófilos, e basófilos, desempenhando um papel ativo no recrutamento de leucócitos, além de induzir a expressão de moléculas de adesão celular (7,15).

Os granulomas e cistos radiculares são lesões perirradiculares crônicas, consideradas como sequelas de processos infecciosos resultantes da necrose pulpar. Os linfócitos T CD4+, LT CD8+, neutrófilos, macrófagos, mastócitos e eosinófilos são as principais células presentes nestes sítios e, juntos, formam um intenso infiltrado inflamatório circundado por uma cápsula de tecido conectivo (32).

De todas as células presentes na lesão perirradicular os macrófagos tem merecido destaque na função fagocitária, desempenhando o papel de células apresentadoras de antígenos e secretando fatores de crescimento que estimularão o recrutamento de fibroblastos, proliferação e deposição de colágeno. Com isso tem papel fundamental na coordenação da reparação tecidual (1,9,23).

O processo de reparação da área afetada é ativado por fatores de crescimento, moléculas de adesão celulares, citocinas, quimiocinas, enzimas remodeladoras e componentes da matriz extracelular (MEC) (5,20).

As células produtoras de colágenos sempre são acompanhadas pela expressão de proteínas do choque térmico 47 (Hsp47) que são essenciais para a síntese e processamento de vários tipos de colágenos em vários tecidos e em algumas condições patofisiológicas (29,30). Hsp47 são chaperones moleculares localizados no retículo endoplasmático que se ligam transitoriamente ao pró-colágeno durante seu dobramento, montagem e transporte para o meio extracelular, assegurando a correta maturação de

vários tipos de moléculas de colágeno e impedindo a formação de agregados celulares (30,49).

Integrinas são glicoproteínas localizadas na membrana plasmática e estão envolvidas em interações entre células, células-matriz extracelular e célula-patógeno, por meio de ligações a ligantes distintos ou a combinação de ligantes (47). Essas atuam como mediadores no processo inflamatório, uma vez que exercem papel importante nas etapas de adesão leucocitária, quimiotaxia, fagocitose, adesão endotelial e, por fim, na agregação celular (4).

A classificação das integrinas é feita de acordo com a subunidade β em comum (17,40). Sua afinidade se dá pelo ligante na matriz extracelular que é conferida pela cadeia α (54).

As principais integrinas que se ligam ao colágeno na célula são α1β1 e α2β1. Essas integrinas se ligam ao colágeno tipo I, muito presente no ambiente perirradicular e também em lesões periapicais (8).

A matriz extracelular é constituída por diferentes combinações de colágenos, proteoglicanos, ácido hialurônico e várias glicoproteínas como a laminina e fibronectina, que atuam de forma a preencher os espaços intercelulares (14,39). A integrina α2β1 interage com o colágeno e a laminina, atuando não apenas como receptor para essas proteínas, mas também como organizadora da matriz extracelular, moduladora da migração celular e organizadoras do citoesqueleto e da produção de metaloproteinases (16,60).

A subunidade α2 (ITGA2) é expressa tanto por células epiteliais quanto por fibroblastos, células endoteliais, plaquetas e células T ativadas. Tem como principal função estimular a expressão gênica de colágeno e colagenase em duas e três

dimensões, e assim reparar o arcabouço da matriz de colágeno na região afetada pela infecção (18,22,36).

Os sinais iniciados pela interação das proteínas de matriz com a integrina devem ser transduzidos no interior da célula por proteínas citosólicas denominadas quinase de adesão focal (FAK). Essas proteínas formam um elo entre a matriz extracelular, o citoesqueleto de actina e integrinas (12,27,55). Essa interação atua diretamente na plasticidade da estrutura do citoesqueleto e confere a habilidade da célula em migrar e ou evadir para os sítios de reparo em resposta aos fatores de crescimento.

Os tecidos possuem a capacidade de restaurar sua estrutura original após a remoção do agente agressor por meio de processos reparativos, regulados através de sinalizações e liberação de mediadores químicos. Essas sinalizações ocorrem entre célula-célula e célula-matriz com uma variedade de citocinas e fatores de crescimento que promoverão mitoses celulares e apoptoses, que restabelecem os tecidos afetados pela infecção (24). O conhecimento desses processos de inter-relações celular e tecidual vem desvendar, cada vez mais, a patogênese e a cura das doenças endodônticas, e, tem ainda, a capacidade de desenvolver novos métodos para a prevenção, diagnósticos e tratamento da periodontite apical.

OBJETIVOS

2 OBJETIVOS

OBJETIVO GERAL:

Avaliar a expressão de integrinas, chaperona molecular, citocinas e quimiocinas em dentes portadores de necrose pulpar, por meio do PCR em tempo real.

OBJETIVO ESPECÍFICO:

Avaliar a expressão de ITGA2, Hsp47, OPN, FAK, IL-1β, IFN-γ, TNF-α, CCL2/MCP-1, CCL5, IL-17A, IL-10 e IL-8 nos tecidos perirradiculares, imediatamente após a limpeza e formatação dos SCR (na presença de infecção) e 7 dias após (na ausência de infecção).

TRABALHO CIENTÍFICO

Assessment of apical expression of alpha2 integrin, heat shock protein, proinflammatory and immunoregulatory cytokines in response to endodontic infection.

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Abstract

Aim: To examine alpha 2 integrin, molecular mediators, cytokines, and chemokines from cells in periapical intersticial fluid from root canal infections before and after the reduction of the bacterial load by cleaning procedure.

Methodology: Subjects included 13 patients referred to the School of Dentistry at the Universidade Federal de Minas Gerais (Belo Horizonte, MG, Brazil). Clinical samples were taken from teeth with pulp necrosis and no patients had acute periapical symptoms at the time of the appointments. After cleaning and drying, three paper points were introduced into the root canal, passing passively through the root apex (2 mm) into the periapical tissues for 1 minute. The samples were collected immediately after root canal cleaning and seven days later (restrained root canal bacterial load) to characterize those gene expressions using Real Time PCR.

Results: Significantly lower levels of TNF-α, CCL5, CCL2/MCP-1, and IL-8 in teeth with restrained bacterial loads (second collection) compared to the first collection were observed. Similarly, the mRNA expression of the integrins SSP1/OPN and FAK decreased in samples from the second collection. The mRNA for the regulatory cytokine IL-10 was significant higher in samples from the second collection compared to the first collection. mRNA expression of IL-1β, IL-17A, IFN-γ, ITGA2 and Hsp47/SERPINH1 were similar at both time points.

Conclusion: These findings suggest that after reducing the root canal bacterial load an decrease in the inflammatory response took place in the periapical area.

Key words: Integrin, SSP1/OPN, FAK, ITGA2, Hsp47/SERPINH1, apical periodontitis, cytokine and chemokine

Introduction

Apical periodontitis is an inflammatory disease of periradicular tissues caused by the host immune response to root canal infection, characterized by localized inflammation concomitant with bone resorption. The pathogenesis of periapical disease involves degradation of extracellular matrix components, including laminin, fibronectin, collagen and proteoglycans (13, 21, 24, 27).

Lymphocytes are the most prevalent cells in the periapical inflammatory infiltrate (46). These cells are associated with the production of cytokines and chemokines that restrain the root canal infection (31, 38), but also lead to alveolar bone destruction.

The antigen-presenting cells, especially dendritic cells and macrophage, are responsible, at least, for the polarization of four different Th subsets (28). Inflammatory response is related to Th1 subset which produces cytokines like IFN-γ, TNF-α and IL-1, which are involved in the progression, bone destruction and remodeling of periapical lesions (39). Conversely, the healing process is related to the Th2 subset (21). Th17 subset may play a role in exacerbating inflammation by stimulating the secretion of pro-inflammatory mediators, such as IL-8, TNF-α, IL-6 (20). Regulatory T (Treg) cells maintain normal homeostasis and reduce the over-activity of Th1, Th2 and Th17 response. IL-10 exhibits strong anti-inflammatory properties and are produced by Th1, Th17, Th2 and Treg cells (4).

Cleaning and shaping procedures play a role in the reduction of root canal microbiota and its byproducts, allowing the proper healing of apical tissues (14). This process is tightly regulated through cell-cell interactions and cell-matrix signaling,

promoted by integrins, a variety of cytokines and growth factors, as well as by cell mitosis and apoptosis (6,16).

Integrins are a large family of heterodimeric membrane glycoproteins involved in cellular processes mediated by cell to cell and cell to extra-cellular matrix interactions. Alpha-2 integrin mediates induction of collagenase MMP-1 in fibroblasts within collagen gels (22).

Heat Shock proteins (HSPs) exert a protective role against harmful environmental conditions and pathogens. They are characteristically induced by stress signals such as elevated temperature, infectious agents and inflammatory mediators participating in the folding and translocation of polypeptides across the cell membrane (11). Hsp47 is the major collagen-binding heat inducible glycoprotein expressed in fibroblast (30,43).

The aim of this study was to quantitatively assay the mRNA expression of the alpha 2 integrin (ITGA2), heat shock protein 47 (Hsp47/SERPINH1), osteopontin (OPN/SSP1), focal adhesion kinase FAK/PTK2, as well as TNF-α, IFN-γ, IL-1β, IL-17, IL-10, CCL2/MCP-1, CCL5, and IL-8 in samples collected from interstitial fluid adjacent to human root canal infections. In addition, we detected the same integrins and cytokines seven days later, after the root canal cleaning procedures, when root canal bacterial load was strongly reduced, providing conditions for healing process. Finally, we statistically compared and analyzed both sets of data.

Materials and Methods

Study Participants

Subjects were 13 patients referred to the Dental School at the Federal University of Minas Gerais (UFMG), Belo Horizonte, Brazil to receive endodontic care, 18 to 80 years old. The exclusion criterion for this study was anti-inflammatory and antibiotic therapy up to 3 months before starting endodontic therapy. All participants signed the informed consent formulary. This study was approved by the Ethics Committee of the Federal University of Minas Gerais (CAAE 20383914.0.0000.5149).

Sample Collection

Clinical samples were taken from 13 teeth (single and multirooted) presenting pulp necrosis and apical periodontitis, diagnosed by clinical and radiographic analyses and pulp sensibility tests. All selected patients failed to present acute periapical symptoms. Teeth were isolated by using a rubber dam followed by complete asepsis of the isolated crown with 30% hydrogen peroxide, followed by 5% iodine and by inactivation with 5% sodium thiosulfate as proposed by Möller. Cleaning and shaping of the root canals were completed by using ProTaper nickel-titanium files (Dentsply Maillefer, Ballaigues, Switzerland) in conjunction with 5.2% sodium hypochlorite. Collections were performed as previously described (19). Briefly, the samples were collected using paper points immediately after root canal cleaning to characterize the mRNA expression profiles of cytokines, chemokines, and integrins. After cleaning and drying, three paper points were introduced into the root canal through the root apex (2 mm) into the periapical tissues, for 1 minute (day 0). The 4 mm tip of the paper points were dropped into a microcentrifuge tube, and the samples were stored at -70°C. By

using this procedure, RNA was collected 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 reopened, and the periapical interstitial fluid was sampled again to characterize the expressions of cytokines and integrins in teeth after instrumentation. In teeth with multiple canals, the first (day 0) and second (day 7) samples were collected from the same canal. No clinical signs or symptoms were present at the time of the second collection, 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). Briefly, chloroform was added to the tubes containing the paper points, and the mixture was centrifuged at 12,000g at 4°C for 15 minutes; the aqueous phase was collected, and RNA was precipitated by isopropanol, 75% cold ethanol was added and samples were centrifuged again at 12,000g at 4°C for 15 minutes. Ethanol was discarded, samples were dried, dissolved in RNase-free water, and then incubated at 55°C for 10 minutes. The RNA was then stored at -70°C.

Real-time Polymerase Chain Reaction

Complementary DNA was synthesized by using 1 µg of RNA through a reverse transcription reaction as described by Barbosa Silva *et al*. Polymerase chain reaction (PCR) was carried out under standard conditions: a holding stage of 95°C (10 minutes), a cycling stage with 40 cycles of 95°C (15 seconds), followed by 60°C (1minute) and a melt curve stage of 95°C (15 seconds), 60°C (1 minute), and 95°C

(15 seconds). The primer sequences used for quantitative PCR analysis of IFN-γ, TNF-α, IL-1β, IL-17, IL-10, CCL2/MCP-1, IL-8, CCL5, ITGA2, Hsp47, OPN, FAK, and GAPDH mRNA expression are shown in Table 1. The sequences of human primers were designed by using PRIMEREXPRESS software (Applied Biosystems, Foster City, CA) based on nucleotide sequences available in the GenBank database. The real-time PCR assay was performed by 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 System Software version v 2.4.1 (Applied Biosystems ®) was used to analyze data after amplification. Results were obtained as threshold cycle (C_T) values. Expression levels were calculated by using the comparative $2^{-\Delta\Delta C_T}$ method (25). The values were calculated as the mean value of duplicates for each patient, and the expression levels of mRNAs in all samples were expressed as the ratio of each specific primer to GAPDH expression.

Statistical Analysis

Data analysis was performed by using SPSS for Windows (version15.0; SPSS, Chicago, IL, USA). Data were subjected to the Shapiro-Wilk test to characterize their normality. Because data did not present a normal distribution, the Wilcoxon test was used to determine statistical differences in samples from the same groups ($P < 0.05$).

Results

mRNA expression was determined by real-time PCR and quantified by comparison with the internal control gene GAPDH. Assessment revealed significant decreases in TNF-α, CCL5, CCL2/MCP-1, and IL-8 mRNA levels on day 7 when compared to day 0 ($p < 0.05$) (Fig. 1). Similarly, the mRNA expression of the integrins SSP1/OPN and FAK decreased in samples from the second collection ($p < 0 .05$) (Fig. 2). IL-1β, IL-17A, IFN-γ and Hsp47/SERPINH1 and ITGA2 gene expression was similar at both time points ($p > 0.05$, Fig 1 and 2). mRNA expression of IL-10 significant increased at day 7 ($P < 0.05$ Fig. 1).

Discussion

The presence of microorganisms and their byproducts inside infected root canals evokes host immune response aimed at avoiding dissemination through the periapical foramen. Cleaning and shaping procedures strongly diminish the root canal bacterial load and, afterwards, periapical healing starts. This process involves overlapping phases of inflammation, proliferation and remodeling, each characterized by dynamic interactions among components of the extra-cellular matrix, growth factors, and cells (2, 33). In this study, expressions of inflammatory and regulatory genes were investigated in periapical lesions immediately after root canal cleaning procedures and 7 days later.

The identification of novel targets that restrain the progression of periapical lesions might improve diagnostic procedures and the development of individualized treatment plans and regenerative procedures. In this regard, this study investigated ITGA2 and Hsp47 gene expression in active lesions and seven days after root canal cleaning, since several periapical cellular processes involve cell-extra-cellular matrix and cell-cell interactions (43). Integrins are critical components of the cell attachment

machinery. Most normal vertebrate cells cannot survive unless they are anchored to the extra-cellular matrix. ITGA2 and Hsp47 are major collagen-binding heat-inducible glycoproteins in fibroblast (13), which are expressed associated with collagen in various tissues during pathophysiological conditions (30). In this study, ITGA2 and Hsp47 mRNA were present at similar levels in both conditions evaluated. This result suggests that the healing process is still in course seven days after root canal instrumentation. Recently, it was shown that ITGA4 mRNA expression was significantly higher in inactive compared with active periapical granulomas (16). However, no data for ITGA2 were found in the literature.

Hsp44 genes were significantly increased in a pool of periapical granulomas compared with a pool of healthy periodontal ligaments (17). These authors indicated that HSP40 gene family plays a crucial role in the maintenance of infection and tissue destruction in apical periodontitis. Here, we investigated the levels of Hsp47 in endodontic lesions. We found that Hsp47 was expressed in active lesions and seven days after cleaning, at similar levels, again indicating that seven days after root canal instrumentation tissue re-shaping was still in course.

Recently, it was shown that FAK expression is associated with cell motility, invasiveness, proliferation and apoptosis (18, 23, 47). In the present study, after cleaning procedures FAK mRNA expression was significantly down-regulated ($p < 0.05$) indicating that microbial infection stimulates its expression, as demonstrated elsewhere (32).

OPN regulates adhesion and recruitment of neutrophils and macrophages, and T cell binding to integrins (34). It has been associated with Th1 responses by increasing IL-12 and diminishing IL-10 production (5, 45). In this study, OPN mRNA expression was significantly decreased at day 7 after cleaning procedures ($p < 0.05$), which

mirrored the decrease of type 1 cytokines at the same time, as demonstrated by others (10, 40, 41).

The inflammatory cytokines IL-1β, IL-17, IFN-γ, IL-8, and TNF-α were detected in periapical interstitial fluid, as observed elsewhere (10, 15, 35, 40, 42). In this study, mRNA expression of the inflammatory cytokines TNF-α, CCL5, CCL2/MCP-1, and IL-8 decreased at day 7 after cleaning and shaping procedures, in agreement with the findings previously observed (10, 40, 41).

IL-1 β stimulates bone resorption by osteoclasts, by inducing IL-17 synthesis (28). Moreover, IL-17 induces the secretion of IL-8, TNF-α and IL-1β by monocytes. These cytokines promote quick neutrophil movement to the site of the infection (8, 20). In this study mRNA expression of IL-1β and IL-17 were similar at both times analyzed. Recently, similar results were found concerning IL-17 but not in regards to IL-1β, which was expressed in similar levels during the observation time (10, 40, 41). In an animal model, it was demonstrated that both cytokines (IL-1β and IL-17) were detected in higher levels during the active phase when compared with chronic phase of lesion development (21, 44). Thus, it is possible that, despite the cleaning procedure, some inflammation was still in course in our patients, as suggested by these cytokines. However, mRNA expression of TNF-α and IL-8 were significantly lower seven days after root canal cleaning ($p < 0.05$), suggesting resorption and neutrophil recruitment decrease after this procedure. Other researchers did not observe any significant differences in mRNA TNF-α expressions, neither in HIV negative (10, 40, 41) nor in HIV positive individuals (9).

CCL2/MCP-1 and CCL5 are chemotactic for monocytes, T cells, eosinophils, and basophils, and play an active role in recruiting leukocytes into inflammatory sites (12, 26, 36). Association between CCL5 and Th1-type cellular responses was suggested

by the finding that neutralization of CCL5 reduced type 1 granuloma formation (7). In this study, CCL2 and CCL5 mRNA expressions were significantly decreased at day 7 after cleaning procedures ($p < 0.05$). Previously it was demonstrated similar results concerning CCL5, but not in regards to CCL2, which was found at similar levels on days zero and seven after root canal cleaning (10). These contradictory results may the due to differences in the efficiency of the cleaning procedures. We suggest that the process of control of inflammation has started at this time point and if investigated later would reveal a more clear-cut non-inflammatory scenario. In addition, different inicial infections might account for the variability encountered. Further evidence of the healing and immunoregulatory process at day 7 in our patients is the higher leves of IL-10 found seven days after root canal cleaning.

In conclusion, our results demonstrate a decrease in mRNA expression of proinflammatoy mediators such as TNF-α, IL-8, OPN, FAK, CCL5 and an increase in the mRNA expression of immune regulator cytokine (IL-10) when the root canal bacteria load was reduced. Fibroblast and endothelial cells are expected to migrate into the healing tissue. Alpha 2 integrin and heat shock protein 47 had similar expression before and after cleaning procedures, probably indicative of the healing process. Further comprehensive clinical studies should be conducted in the periapical area, given the fact that several other players are involved in the inflammatory and healing processes. However, expression of the genes described here suggested an on-going healing process after reduction of bacterial load by root canal cleaning.

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References

1. Alayan J, Ivanovski S, Farah CS. Alveolar bone loss in T helper 1/T helper 2 cytokine-deficient mice. J Periodontal Res 2007, v.42, p.97-103.
2. Bakhshayesh M,Soleimani M, Mehdizadeh M ,et al. Effects of TGF-β and b-FGF on the potential of peripheral blood-borne stem cells and bone marrow derived stem cells in wound healing in a murine model. Inflammation 2012, v.35 (1), p.138-142.
3. Barbosa Silva MJ, Vieira LQ, Sobrinho AP. The effects of mineral trioxide aggregates on cytokine production by mouse pulp tissue.Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008, v.105, p.e70-6.
4. Bluestone Ja, Mackay CR, O'Shea JJ, Stockinger B. The functional plasticity and auto-immunity. Nat Immunol 2009, v. 9, p811-6.
5. Buback F, Renkl AC, Schulz G, Weiss JM. Osteopontin and the skin: multiple emerging roles in cutaneous biology and pathology. Exp.Dermatol 2009, v.18, p.750-759.
6. Chen L, Arbieva ZH, Guo S, et al. Positional differences in the wound transcriptome of skin and oral mucosa. BMC Genomics 2010, v.11, p.471.
7. Chensue SW, Warmington KS, Allenpach EJ, et al. Differential expression and cross-regulatory function of RANTES during mycobacterial (type 1) and schistosomal (type 2) antigen-elicited granulomatous inflammation. J Immunol 1999, v.163, p.165-73.
8. Colic M, Gazivoda D, Vucevic D, Sasa V, Rudolf R, Lukic A. Proinflammatory and immunoregulatory mechanisms in periapical lesions. Mol Immunol 2009, v.47, p. 101-13.

9. de Brito LCN, Teles FR, Teles RP, Nogueira PM, Vieira LQ, Ribeiro Sobrinho AP. Immunological profile of periapical endodontic infections from HIV- and HIV+ patients. Int Endodon J 2014, epub ahead of print.
10. de Brito LCN, Teles FRF, Teles RP, Totola AH, Vieira LQ, Sobrinho APR. T-Lymphocyte and cytokine expression in human inflammatory periapical lesions. J Endodon 2012, v.38(4), p.481-485.
11. De Maio A. Heat shock proteins: facts, thougts, and dreams. Shock 1999, v.11 (1), p.1-12.
12. De Rossi A, Rocha LB, Rossi MA. Interferon-gamma, interleukin-10, intercelular adhesion molecule-1, and chemokine receptor 5, but not interleukin -4, attenuate the development of periapical lesions. J Endodon 2008, v.34, p.31-8.
13. Delzangles B, Boy-Lefevre ML, Forest N. Glycoproteins expression in apical pathologic tissue: clinical incidences. J Endodon 1997, v.23, p.565-8.
14. Fabricius L, Dahlen G, Sundqvist G, et al. Influence of residual bactéria on periapical tissue healing after chemomechanical treatment and root filling of experimentally infected monkey teeth. Eur J Oral Sci 2006, v.114, p.278-85.
15. Fukada SY, Silva TA, Garlet GP, Rosa AL, da Silva JS, Cunha FQ. Factors involved in the T helper type 1 and type 2 cell commitment and osteoclast regulation in inflammatory apical diseases. Oral Microbiol Immunol 2009, v.24, p.25-31.
16. Garlet GP, Horwat R, Ray HLJ, Garlet TP, Silveira EM, Campanelli AP, Trombone APF, Letra A, Silva RM. Expression analysis of wound healing genes in human periapical granulomas of progressive and stable nature. J Endodon 2012, v.38, p.185-90.

17. Goodmam SC, Letra A, Dorn S, Araujo-Pires AC, Vieira AE, Chaves de Souza L, Yadlapati M, Garlet GP, Silva RM. Expression of heat shock proteins in periapical granulomas. J Endodon 2014, v.40 (6), p.830-6.
18. Hauck CR, Hsia DA, Schlaepfer DD. The focal adhesion kinase- a regulator of cell migration and invasion. IUBMB Life 2002, v.53, p.115-119.
19. Henriques LC, de Brito LC, Tavares WL, Vieira LQ, Ribeiro Sobrinho AP. Cytokine analysis in lesions refractory to endodontic treatment. J Endodon 2011, v.37, p.1659-62.
20. Jovanovic DV, Di Battista JA, Martel-Pelletier J, Jolicoeur FC, He Y, Zhang M, Mineau F, Pelletier JP. IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. J Immunol 1998, v.167, p.3513-3521.
21. Kawashima N, Stashenko P. Expression of bone-resorptive and regulatory cytokines in murine periapical inflammation. Arch Oral Biol 1999, v.44, p.55-66.
22. Langholz O, Röckel D, Mauch C, Kozlowska E, Bank I, Kieg T, Eckes B. Collagen and collagenase gene expression in three-dimensional collagen lattices are differentially regulated by alpha 1 beta 1 and alpha 2 beta 1 integrins. J Cell Biol 1995, v.131 (6 Pt 2), p.1903-15.
23. Li J, Meng Q, Sun Y, Qing H. Inhibition of focal adhesion kinase induces apoptosis in human gastric carcinoma cells. Mol Biol Rep 2013, v.40 (1), p.401-6.
24. Liapatas S, Nakou M, Rontogianni D. Inflammatory infiltrate of chronic periradicular lesions: an immunohistochemical study. Int Endodon J 2003, v.36, p.464-71.

25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001, v.37, p.1659-62.

26. Marçal JRB, Samuel RO, Fernandes F, et al. T-helper cell type 17/regulatory t-cell immunoregulatory balance in human radicular cysts and periapical granulomas. J Endodon 2010, v.36, p.995-9.

27. Martón IJ, Kiss C. Protective and destructive immune reactions in apical periodontitis. Oral Microbiol Immunol 2000, v. 115, p.139-50.

28. McGeachy MJ, Cua DJ. Th17 cell differentiation: the long and winding road. Immunity 2008, v.4, p.445-53.

29. Möller AJ. Microbiological examination of root canals and periapical tissues of human teeth. Methodological studies. Odontol Tidsks 1966, v.74 (5), Suppl.1-380.

30. Nagata K. Expression and function of heat shock protein 47: a collagen-specific molecular chaperone in the endoplasmic reticulum. Matrix Biol. 1998, v. 16, p.379-86.

31. Nair PN. Apical periodontitis: a dynamic encounter between root canal infection and host response. Periodontol 2000 1997, v.13, p.121-48.

32. Owens LV, Xu L, Marston WA, Yang X, Farber MA, Iacocca MV, Cance WG, and Keagy BA. Overexpression of the focal adhesion kinase (p125FAK) in the vascular smooth muscle cells of intimal hyperplasia. J Vasc Sur 2001, v.34, p. 344-349.

33. Ricucci D, Lin LM, Spangberg LS. Wound healing of apical tissues after root canal therapy: a long-term clinical, radiographic, and histopathogic observation

study. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009, v.108, p.609-21.

34. Salvi V, Scutera S, Rossi S, Zucca M, Alessandria M, Greco D, Bosisio D, Sozzani S, Musso T. Dual regulation of osteopontin production by TLR stimulation in dendritic cells. J Leukoc Biol 2013, v.94(1), p.147-58.

35. Silva TA, Garlet GP, Fukada SY, Silva JS, Cunha FQ. Chemokines in Oral Inflammatory Diseases: Apical Periodontitis and Periodontal Disease. J Dent Res 2007 v. 86(4), p.306-319.

36. Silva TA, Garlet GP, Lara VS, et al. Differential expression of chemokines and chemokine receptors in inflammatory periapical diseases, Oral Microbiol Immunol 2005, v.20, p.310-6.

37. Stashenko P, Teles R, D'Souza R. Periapical inflammatiry responses and their modulation. Crit Rev Oral Biol Med 1998, v.9, p.498-521.

38. Stashenko P. The role of immune cytokines in the pathogenesis of periapical lesions. Endod Dent traumatol 1990, v.6, p.89-96

39. Takeichi O, Saito I, Tsurumachi T, Moro I, Saito T. Expression of inflammatory cytokine genes in vivo by human alveolar bone-derived polymorphonuclear leukocytes isolated from chronically inflamed sites of boné resorption. Calcif Tissue Int 1996, v.4, p.244-8.

40. Tavares WL, de Brito LC, Henriques LC, Oliveira RR, Maciel KF, Vieira LQ, Sobrinho AP. The impact of chlorhexidine-based endodontic treatment on periapical cytokine expression in teeth. J Endodon 2013, v.39 (7), p.889-92.

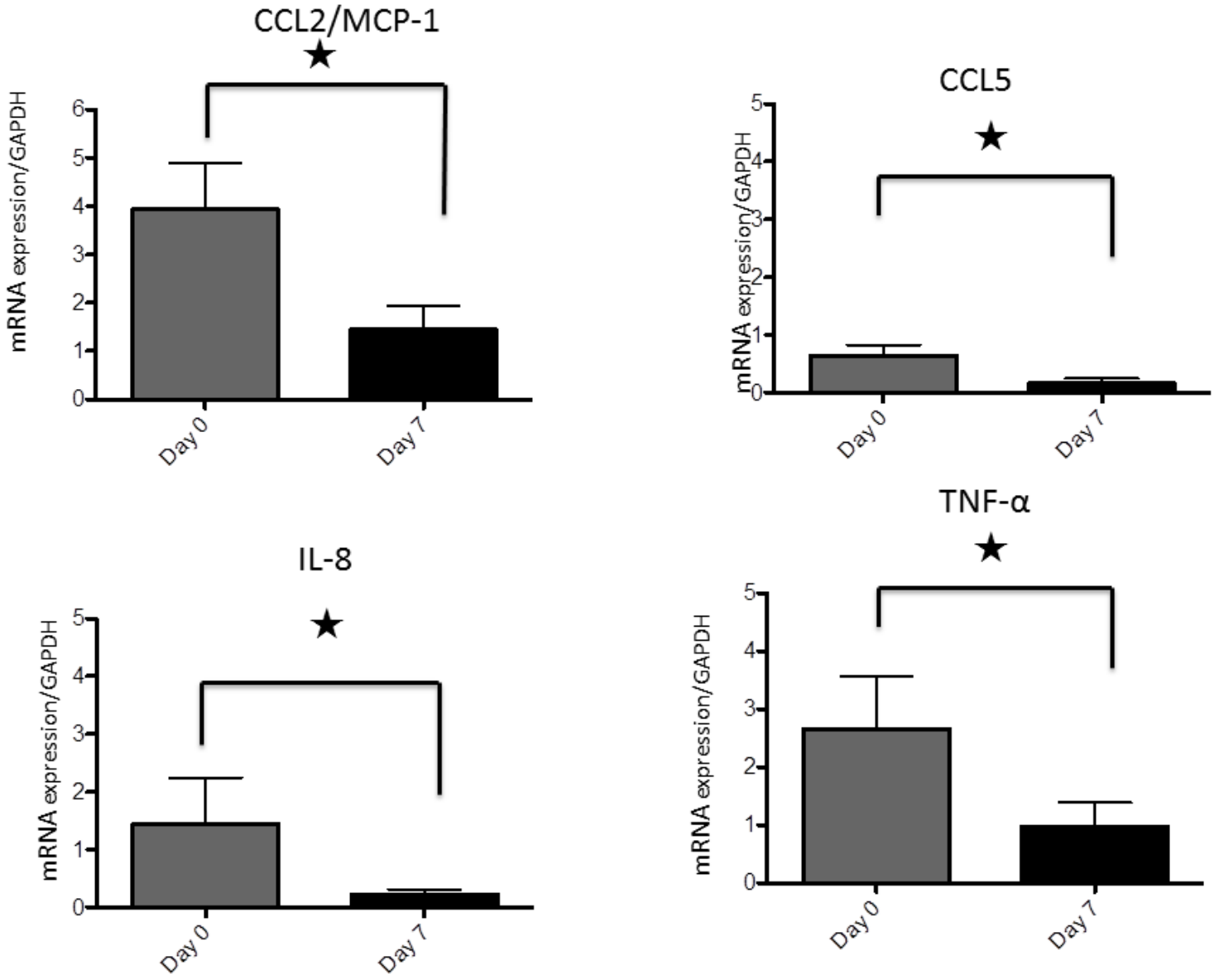
41. Tavares WL, de Brito LC, Henriques LC, Teles FR, Teles RP, Vieira LQ, Ribeiro Sobrinho AP. Effects of calcium hydroxide on cytokine expression in endodontic infections. J Endodon 2012, v.38 (10), p.1368-71.
42. Teixeira-Salum TB, Rodrigues DB, Gervasio AM, Souza CJ, Rodrigues V Jr, Loyola AM. Distinct Th1, Th2 and Treg cytokines balance in chronic periapical granulomas and radicular cysts. J Oral Pathol Med 2010, v.39, p.605-11.
43. Vieira-Júnior JR, de Oliveira-Santos C, Della-Coletta R, Cristianismo-Costa D, Paranaíba LMR, Martelli-Júnior H. Immunoexpression of α2-integrin and Hsp47 in hereditary gingival fibromatosis and gingival fibromatosis associated dental abnormalities. Med Oral Patol Oral Cir Bucal 2013, v. 18(1), p. e45-8.
44. Wang CY, Stashenko P. Kinects of bone-resorbing activityin developing periapical lesions. J Dent Res 1991, v. 70, p.1362-6.
45. Wang KX, Denhart DT. Osteopontin:role in immune regulation and stress responses.Cytokine Growth Factor Rev 2008, v. 19, p.333-345.
46. Yu SM, Stashenko P. Identification of inflammatory cells in developing rat periapical lesions. J Endodon 1987, v.12, p.535-40.
47. Zagzag D, Friedlander DR, Margolis B, Grumet M, Semenza GL, Zhong H, Simons JW, Holash J, Wiegand Sj, Wancopoulos GD. Molecular events implicated in brain tumor angiogenesis and invasion. Pediatr Neurosurg 2000, v.33, p.49-55.

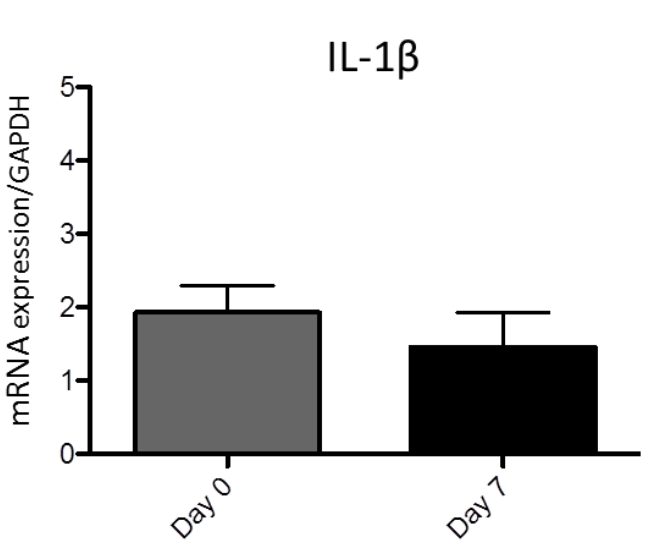
Gene	Sense and antisense	Mt* (^{o}C)	bp*
GAPDH	5’-GCA CCA CCA ACT GCT TAG CA- 3’	80	96
	5’-TGG CAG TGA TGG CAT GGA GGA- 3’		
TNF-α	5’-TTC TGG CTC AAA AAG AGA ATT G- 3’	76	73
	5’-TGG TGG TCT TGT TGC TTA AGG- 3’		
IL-1β	5’-TGG CAG AAA GGG AAC AGA A- 3’	73	59
	5’-ACA ACA GGA AAG TCC AGG CTA- 3’		
IL-10	5’-GGT TGC CAA GCC TTG TCT GA- 3’	81	107
	5’-TCC CCC AGG GAG TTC ACA T- 3’		
IL-17	5’-CAA TGA CCT GGA ATT ACC CAA- 3’	70	52
	5’-TGA AGG CAT GTG AAA TCG AGA- 3’		
IFN-γ	5’-ACT GAC TTG AAT GTC CAA CGC A- 3’	61	101
	5’-ATC TGA CTC CTT TTT CGC TTC CC- 3’		
ITGA2	5’-GCA GAT GGA CCA CAC TTT GA- 3’	60	115
	5’-TGT CTG TGC CCT TTT CCT CT- 3’		
HSP47	5’-TGC TGA GCC CGG AAA CTC- 3’	62	63
	5’-TTC AGG GCA GGC AGA ATG- 3’		
IL-8	5’- GAA TGG GTT TGC TAG AAT GTG ATA- 3’	60	129
	5’- CAG ACT AGG GTT GCC AG ATT TAA C - 3’		
CCL5	5’-CGT GCC CAC ATC AAG GAG TA- 3’	80	91
	5’-CAC ACA CTT GGC GGT TCT TTC- 3’		
OPN	5’-GCC GAG GTG ATA GTG TGG TT- 3’	62	101
	5’-TGA GGT GAT GTC CTC GTC TG- 3’		
FAK	5’-CAA CAG GTG AAG AGC GAT TA- 3’	58	99
	5’-CCA GTA TGA TCG CCG TAT TT- 3’	81	93
CCL2	5’-AGG ACC ATT GTG GCC AAG GA-3’		
	5’-CGG AGT TTG GGT TTG CTT GT-3’		

Table 1. Primer sequences

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

Figure 1. Expression of CCL2/MCP-1, CCL5/RANTES, IL-8, TNF, IL-1β, IFN-γ IL-17A and IL-10 in infected root canal periradicular tissues. Expression was determined by real-time PCR and quantified by comparison with internal control (GAPDH). Bars represent mean values of samples recovered from 13 patients; lines represent standard error of mean.* indicates p <0.05.




IL-1β
mRNA expression/GAPDH
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1
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4
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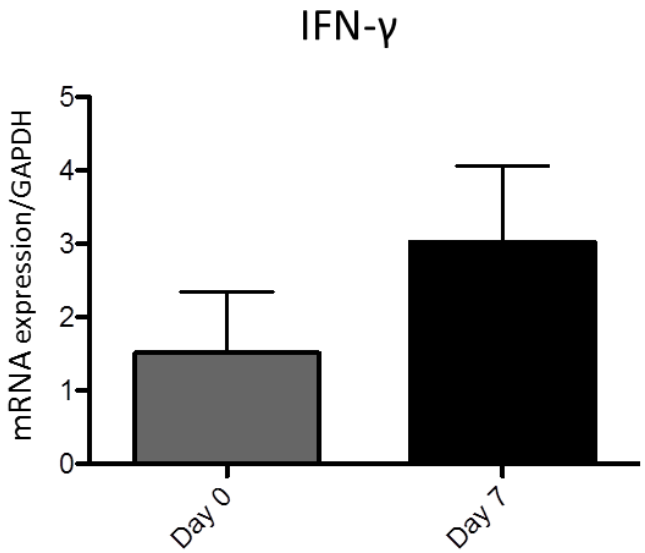
Day 0
Day 7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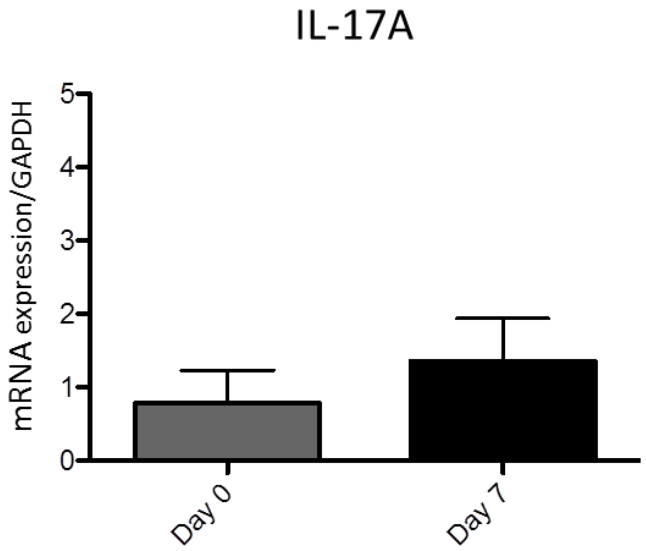
IL-17A
mRNA expression/GAPDH
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Day 0
Day 7

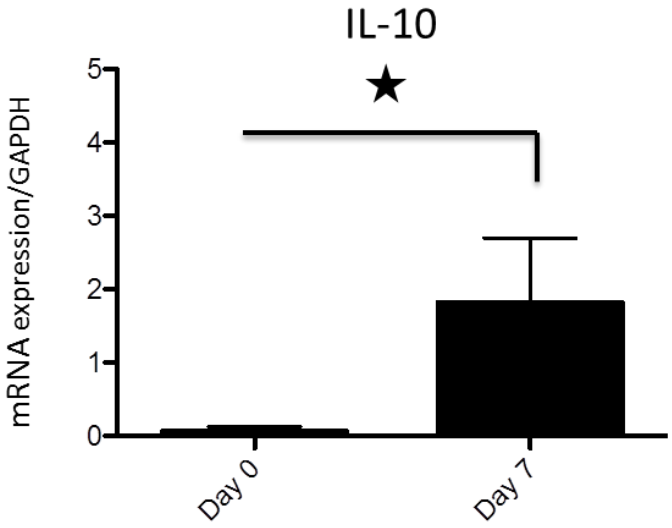
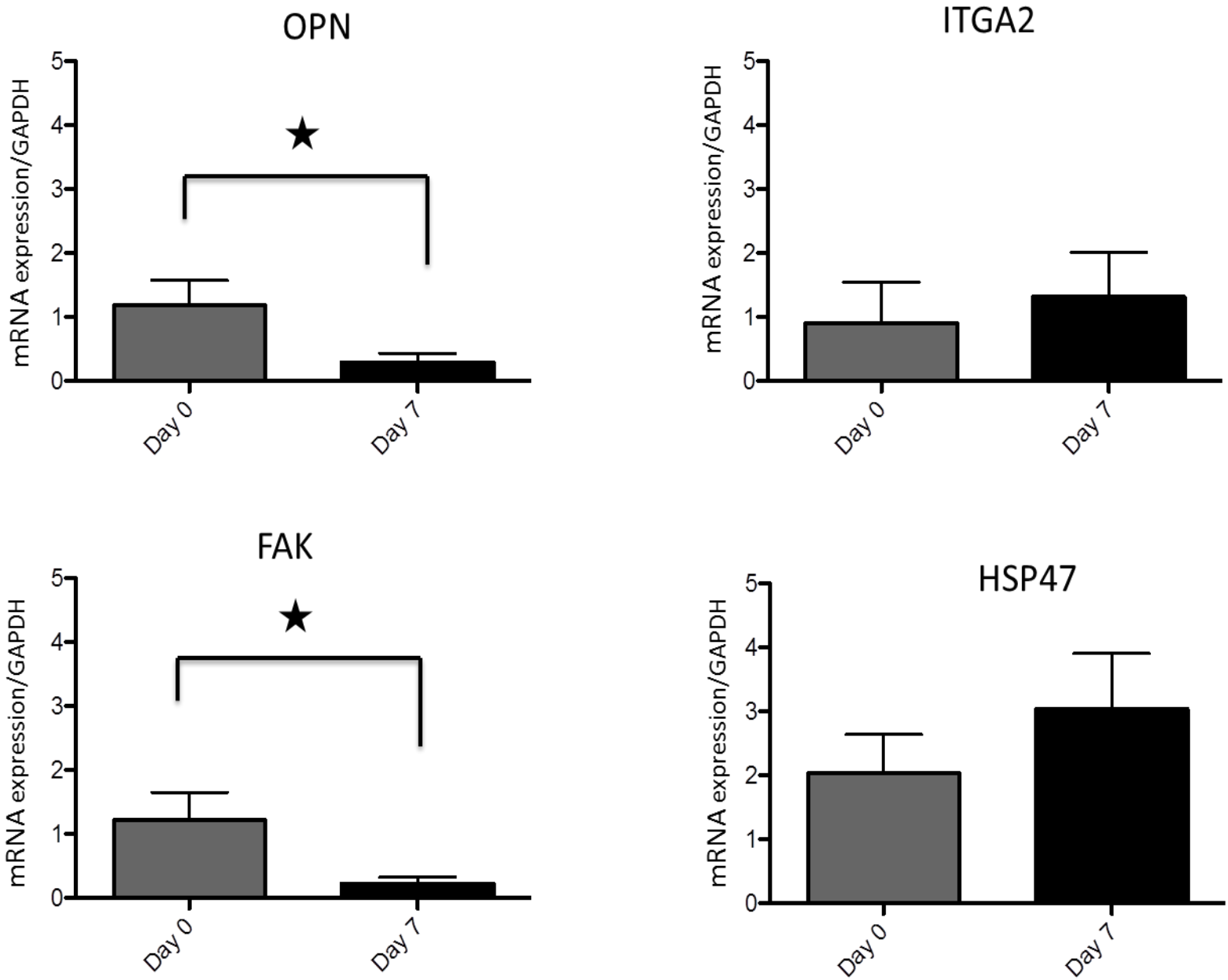

IL-10
★
mRNA expression/GAPDH
0
1
2
3
4
5
Day 0
Day 7

Figure 2. Expression of SSP1/OPN, ITGA2, FAK and Hsp47/SERPINH1 in infected root canal periradicular tissues. Expression was determined by real-time PCR and quantified by comparison with internal control (GAPDH). Bars represent mean values of samples recovered from 13 patients; lines represent standard error of mean.* indicates $p < 0.05$.



CONCLUSÃO

4 Conclusão

Conclui-se que a expressão de intregrina alfa2 (ITGA2), proteina do choque térmico 47 (Hsp47), das proteinas citosólicas OPN e FAK, bem como dos mediadores pro-inflamatórios IL-1β, IFN-γ, TNF-α e IL-17, das quimiocinas CCL2/MCP-1, CCL5 e IL-8, e da citocina reguladora IL-10 sofrem o efeito da presença ou da ausência de uma carga microbiana efetiva no interior do SCR. Ou seja, que ocorre um predomínio dos mediadores pró-inflamatórios na presença da infecção endodôntica e uma redução dos mesmos, em concomitância com o aumento da expressão da citocina IL-10, após a redução da carga bacteriana.

REFERÊNCIAS BIBLIOGRÁFICAS

Referências bibliográficas (Introdução)

1. Bleacher JC; Adolph VR; Dillon PW; Krummel TM.Fetal tissue repair and wound healing. Dermatol Clin 1993, v. 11(4), p. 677-83.
2. Brito LCN, Teles FR, Teles RP *et al*. Use of Multiple-Displacement Amplification and Checkerboard DNA-DNA Hybridization to Examine the Microbiota of Endodontic Infections. J Clin Microbiol 2007, v.45, p. 3039-49.
3. Caires NCM, Matos AM, Farias LM *et al*. Partial characterization of antagonistic substance produced by a *Clostridium butyricum* strain. Braz J Microbiol 2007, v.28, p. 265-9.
4. Canalli AA Conran N, Fattori A, Saad STO, Costa FF. Increased adhesive properties of eosinophils in sickle disease. Exp Hematol 2004, v.32(8), p. 728-34.
5. Chen L, Arbieva ZH, Guo S, et al. Positional differences in the wound transcriptome of skin and oral mucosa. BMC Genomics 2010, v.11, p.471.
6. Colic M, Gazivoda D, Vucevic D, Sasa V, Rudolf R, Lukic A. Proinflammatory and immunoregulatory mechanisms in periapical lesions. Mol Immunol 2009, v.47, p. 101-13.
7. de Brito LC, Teles FR, Teles RP, Totola AH, Vieira LQ, Sobrinho AP. T-Lymphocyte and Cytokine expression in human inflammatory Periapical lesions. J Endodon 2012, v.38, p.481-5.
8. Delzangles B, BoyLefevre ML, Forest N. Glycoproteins expression in apical pathologic tissues: Clinical incidences. J Endodon 1997, v.23 (9), p.565-568.
9. Diegelmann RF; Cohen IK; Kaplan AM. The role of macrophages in wound repair: a review. Plast Reconstr Surg 1981, v. 68(1), p. 107-13.

10. Fukada SY, Silva TA, Garlet GP,Rosa AL, da Silva JS, Cunha FQ. Factors involved in the T helper type 1 and type 2 cell commitment and osteoclastic regulation in inflammatory apical diseases. Oral Microbiol Immunol 2009, v.24, p.25-31.
11. Gomes BPFA, Pinheiro ET, Gadê-Neto CR *et al*. Microbiological examination of infected dental root canals. Oral microbial Immunol 2004, v.19, p.71-6.
12. Grenz H, Carbonetto S, Goodman SL. Alpha-3-beta-1 integrin is moved into focal contacts in kidney mesangial cell. J Cell Sci, 1993, v.105, p.739-751.
13. Hahn, C. L., Liewer, F. R. Innate immune responses of the dental pulp to caries. J Endodon, v. 33, n.6, p.643-651, 2007a.
14. Haubner R, Finsinger D, Kessler H. Stereoisomeric peptide libraries and peptidomimetics for designing selective inhibitors of the αvβ3 integrin for a new cancer therapy. Angew Chem Int Ed Eng 1997, v. 36, p.1374-1389.
15. Heder C, Peltonen M, Koening W, et al. Sytemic immune mediators and lifestyle canges in the prevention of type 2 diabetes: Results from the Finnish diabetes prevention study. Diabetes 2006, V.55,p.2340.
16. Hemler ME, Rutishauser U. Cell-to-cell contact extracellular matrix. Editorial overview. Curr Opin Cell Biol 2000, v. 12, p. 539-541.
17. Hynes RO. Integrins: Versatility, modulation, and signaling in cell adhesion. Cell 1992: 69; 11-25.
18. Ivarsson M, McWhirter A, Black CM, Rubin K. Impaired regulation of collagen pro-alpha 1(I) mRNA and change in pattern of collagen-binding integrins on scleroderma fibroblasts. J Invest Dermatol 1993, v. 101, p. 216-221.
19. Kawashima N, Stashenko P. Expression of bone resorptive and regulatory cytokines in murine periapical inflammation. Arc Oral Biol 1999, v.44, p. 55-66.

20. Kondo T, Ishida Y. Molecular pathology of wound healind. Forensic Sci Int 2010, v. 203, p. 93-8.
21. Lana MA, Ribeiro-Sobrinho AP, Stehling R *et al.* Microorganisms isolated from root canal presenting necrotic pulp and their drug susceptibility in vitro. Oral Microbiol Immunol 2001, v.16, p. 100-5.
22. Langholz O, Rockel D, Mauch C, Kozlowzka E, Bank I, Krieg T, Eckes B. Collagen and collagenase gene expression in three-dimensional collagen lattices are differentially regulated by α1β1 and α2β1 integrins. J Cell Biol 1995, v. 131, p.1903-1915.
23. Leibovich SJ; Ross R. The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. Am J Pathol 1975, v. 78, p. 71-100.
24. Majno G, Joris I. Cell, Tissues, and Disease.2nd ed.Oxford: Oxford University Press 2004, p.210-9.
25. McGeachy MJ, Cua DJ. Th17 cell differentiation: the long and winding road. Immunity 2008, v.4, p.445-53.
26. McKee MD, Nanci A. Postembedding colloidal-gold immunocytochemistry of noncollagenous extracellular matrix proteins in mineralized tissues. Microsc Res Tech 1995, v.31, p.44-62.
27. Miyamoto S, Teramoto K, Coso OA, Gutkind JS, Burbelo PD, Akiyama SK, Yamada KM. Integrin function – molecular hierarchies of cytoskeletal and signaling molecules. J Cell Biol 1995, v.131, p.791-805.
28. Mosmmam TR, Coffman RL. Heterogeneity of cytokine secretion patterns and function of helper T cells. Adv Immunol 1989, v.46, p.111-147.
29. Nagai N; Hosokawa M; Itohara S; Adachi E; Matsushita T; Hosokawa N; Nagata K .Embryonic lethality of molecular chaperone hsp47 knockout mice is associated with defects in collagen biosynthesis. J Cell Biol 2000, v. 150(6), p. 1499-506.

30. Nagata K. Expression and function of heat shock protein 47: A collagen-specific molecular chaperone in the endoplasmic reticulum. Matrix Biol 1998, v.16, p.379-386.
31. Narayanan LL, Vaishnavi C. Endodontic microbiology. J Conserv Dent 2010, v.13, p. 233-9.
32. Neville BW, Damm DD, Allen CM, Bouquot JE. Doenças da polpa e do periápice. Patologia oral e maxilofacial. Rio de Janeiro: Guanabara Koogan 2004, v.3, p.105-29.
33. Oliveira AAP, Farias LM, Nicoli JR, Costa E, Carvalho MAR. Bacteriocin production by Fusobacterium isolates recovered from the oral cavity of human subjects with and without periodontal disease and marmosets. Res Microbiol, 1998, v.149, p. 585-9.
34. Peters LB, Wesselink PR, van Winkelhoff AJ. Combinations of bacterial species in endodontic infections. Int Endod J 2002, v.35, p.698-702.
35. Renkl AC, Wussler J, Ahrens T *et al*. Osteopontin functionally activates dendritic cells and induces their differentiation toward a Th1-polarizing phenotype. Blood 2005, v.106, p.946-55.
36. Riikonen T, Westermarck J, Koivisto L, Broberg A, Kahari V-M, Heino J. Integrin α2β1 is a positive regulator of collagenase (MMP-1) and collagen α1 (I) gene expression. J Biol Chem 1995, v.270, p.13548-13552.
37. Rittling SR, Zetterberg C, Yagiz K, Skinner S, Suzuki N, Fujimura A, Sasaki H. Protective role of osteopontin in endodontic infection. Immunol 2009, v.129, p.105-114.
38. Romagnani S. Regulation of the T cell response.Clin Exp Allergy 2006, v.44, p.1357-66.
39. Ruoslahti E, Pierschbacher MD. New perspectives in cell adhesion: RGD and integrins. Science 1987, v. 238,p. 491-497.
40. Sato H, Seiki M. Regulatory mechanism of 92 kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells. Oncogene 1993, v.8, p.395-405.

41. Shinohara ML, Jansson M, Hwang ES, Werneck MB, Glimcher LH, Cantor H. T-bet-dependent expression of osteopontin contributes to T cell polarization. Proc Natl Acad Sci USA 2005, v.102, p. 17101-6.
42. Silva TA, Garlet GP, Fukada SY, Silva JS, Cunha FQ. Chemokines in oral inflammatory diseases: apical periodontites and periodontal disease. J Dent Res 2007, v.86, p.306-19.
43. Siqueira Jr JF, Rôças IN, Alves FRF, Silva MG. Bacteria in the apical root canal of teeth with primary apical periodontitis. Oral Surgery, Oral Med Oral Pathol Oral Radiol Endod 2009, v.107, p. 721-6.
44. Siqueira Jr JF, Rôças IN, Rosado AS. Investigation of bacterial communities associated with asymptomatic and symptomatic endodontic infections by denaturing gradient gel electrophoresis fingerprinting approach. Oral Microbiol Immunol 2004, v.19, p. 363-70.
45. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent Jr RL. Microbial complexes in subgingival plaque. J Clin Periodontol 1998, v.25, p. 134-44.
46. Socransky SS, Haffajje AD. Periodontal microbial ecology. Periodontol 2000 2005, v.38, p.135-87.
47. Sprinter TA, Wang JH. The three-dimensional structure of integrins and their ligants, and conformational regulation of cell adhesion. Adv Protein Chem 2004, v.68, p.29-63.
48. Sundqvist G. Associations between microbial species in dental root canal infections. Oral Microbiol and Immunol 1992, v.7, p. 257-62.
49. Taguchi T, Razzaque MS. The collagen-specific molecular chaperone Hsp47: is there a role in fibrosis? Trends Mol Med 2007, v. 13, p. 45-51.
50. Takahashi K. Microbiological, pathological, inflammatory, immunological and molecular biological aspects of periradicular disease. Int Endod J 1998, v.31, p.311-325.

51. Takeichi O, Saito I, Tsurumachi T, Moro I, Saito T. Expression of inflammatory cytokine genes in vivo by human alveolar boné-derived polymorphonuclear leukocytes isolated from chronically inflamed sites of bone resoorption. Calcif Tissue Int 1996, v.4, p.244-8.
52. Tavares WLF, Neves de Brito LC, Teles RP *et al.* Microbiota of deciduous endodontic infections analysed by MDA and Checkerboard DNA–DNA hybridization. Int Endod J 2011, v.44, p. 225-35.
53. Teixeira-Salum TB, Rodrigues DBR, Gervásio AM, Souza CJA, Rodriges Jr V, Loyola AM. Distinct Th1, Th2 and Treg cytokines balance in chronic periapical granulomas and radicular cysts. J Oral Pathol Med 2010, v.39, p. 250-6.
54. Thomas GJ, Jones J, Speight M. Integrins and oral cancer. Oral Oncol 1997, v. 33;, p.381-388.
55. Van der Flier A, Sonnenberg A. Structural and functional aspects of filamins. BBA - Mol Cell Research 2001, v.1538 (2), p.99-117.
56. Wang KX, Denhart DT. Osteopontin: role in immune regulation and stress responses. Cytokine Growth Factor Rev 2008, v.5-6, p.333-45.
57. Xiong H, Wei I, Peng B. Immunohistochemical localization of OL-17 in induced rat periapical lesions. J Endodon 2009, v.35, p.216-20.
58. Yang S, Zhu L, Xiao L, Shen Y, Wang L, Peng B, Haapasalo M. Imbalance of Interleukin-17+ T-cell and Fosp3+ Regulatory T-cell Dynamics in Rat Periapical Lesions. J Endodon 2014, v.1, p.56-62.
59. Yu JJ, Gaffen SL. Interleukin-17: a novel inflammatory cytokine that bridges innate and adaptive immunity. Front Biosci 2008, v. 13, p. 170-7.

60. Zou Z, Schmaier AA, Cheng L, Mericko P, Dickeson SK, Striker TP, Santoro SA, Kahn ML. Negative regulation of activated α2 integrins during thrombopoiesis. Blood 2009, v.113, p. 6428-6439.