Resumo

Introdução: A eliminação de microorganismos do sistema de canais radiculares infectados é uma etapa crucial que envolve o completo debridamento mecânico-químico e a utilização de uma medicação intracanal. O hidróxido de cálcio vem sendo indicado nestes casos por ser estável por longos períodos, por possuir boa biocompatibilidade, ser bactericida em uma área limitada, além de induzir a formação de tecido mineralizado e ser efetivo contra a formação de exsudatos inflamatórios. A Clorexidina (CHX) é uma molécula hidrofóbica e lipofílica positivamente carregada, que vem sendo utilizada como solução irrigadora e medicação intracanal. Isto se deve a sua ação antimicrobiana contra microorganismos gram-positivos e gram-negativos, assim como leveduras, anaeróbios facultativos e aeróbios. Vem sendo indicada em casos de infecções persistentes e falhas no tratamento endodôntico.

Objetivo: Avaliar a influência do hidróxido de cálcio e da CHX na resposta imune periapical em humanos.

Métodos: Os níveis de expressão do mRNA das citocinas IFN-γ, TNF-α, IL-1β, IL-17A e IL-10, assim como o da quimiocina MCP-1 foram avaliados através do real-time PCR, imediatamente após a limpeza e modelagem dos canais e após 15 dias.

Resultados: Os níveis das citocinas IL-1β, IFN-γ, IL-10 e da quimiocina CCL2/MCP-1 aumentaram nos dentes que não receberam a medicação intracanal. Entretanto, a utilização do hidróxido de cálcio e da clorexidina promoveu uma mudança significativa na expressão do mRNA das citocinas.

Conclusões: A análise das citocinas e da quimiocina CCL-2/MCP-1 demonstraram os benefícios do hidróxido de cálcio e da clorexidina quando utilizados como medicação intracanal por impedirem o aumento da expressão dos mediadores durante o período de avaliação.

Palavras-chave: citocinas; clorexidina; hidróxido de cálcio; infecção endodôntica.

Abstract

Introduction: Root canal treatment typically involves cleaning and shaping procedures followed by treatment with antibacterial endodontic dressing between appointments and, ultimately, three-dimensional, hermetic filling. The use of calcium hydroxide is an effective step in killing bacteria that remain after cleaning and shaping procedures. It also induces hard tissue formation and is effective for stopping inflammatory exudates. Chlorhexidine (CHX) is effective as an irrigation solution and is utilized as an endodontic dressing.

Objective: The aim of this study was to assay the influence of calcium hydroxide and CHX on periapical interstitial fluid from human root canals.

Methods: The mRNA expression levels of the cytokines IFN-γ, TNF-α, IL-1β, IL-17A, and IL-10 as well as the chemokine MCP-1 were assayed by real-time PCR, immediately after root canal cleaning and 15 days later.

Results: Levels of IL-1β, IFN-γ, IL-10, and the chemokine CCL2/MCP-1 were increased in teeth without endodontic dressings. However, with calcium hydroxide or CHX interappointment dressings, statistically significant changes were observed in cytokine mRNA expression.

Conclusions: Analyses of cytokines and the chemokine CCL-2/MCP-1 demonstrated the benefits of calcium hydroxide and CHX as root canal dressings because it impedes the increase of all mediators during the experimental time.

Keywords: calcium hydroxide; chlorhexidine ; cytokine expression; root canal infection

1- Introdução e Relevância

O desenvolvimento de patologias pulpares e periapicais estão intimamente relacionados à presença de microorganismos e seus subprodutos.

Uma vez instalados nos SCR, os microrganismos induzem uma resposta de defesa nos arredores do ápice radicular (Stashenko, 1990). A resposta inflamatória, que aí se processa, recruta células imunocompetentes para conter e impedir a disseminação dessa infecção para outros sítios, culminando com a formação de uma lesão crônica e a 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 (Stashenko *et al.,* 1998). Daí o grande interesse em se conhecer esses mediadores e seus efeitos sobre as células imunocompetentes (Silva *et al.*, 2005).

Detecta-se uma grande variedade de células nas lesões perirradiculares humanas, dentre elas citam-se: os linfócitos $TCD4^+$ e $TCD8^+$, macrófagos, células plasmáticas, mastócitos, eosinófilos**.** As células T, entretanto, são as mais numerosas nessas lesões (Colic *et al.*, 2009b).

Os linfócitos $TCD4^+$ e $CD8^+$, após seu contato com antígenos ou serem estimulados por outras células inflamatórias, podem produzir uma grande variedade de citocinas (Marton & Kiss, 2000). As células $TCD4^+$ atualmente

subdividem-se 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 e destruição óssea perirradicular (Stashenko *et al.*, 1998, Colic *et al.*, 2009b). A resposta Th2 induz a síntese e atividade das 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 considerada ponte entre esta e a resposta adaptativa. (Yu & Gaffen, 2008). 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).

As quimiocinas participam do processo inflamatório ao promoverem a ativação de selectinas que, por sua vez, estão envolvidas na adesão de células às paredes endoteliais. Sua expressão localizada nos tecidos gera gradientes quimiotáticos, que são responsáveis pela migração guiada e a manutenção de células inflamatórias nesses locais (Mantovani *et al*., 1998, Silva *et al*., 2005). A Proteína Quimiotática para Monócitos (MCP-1) tem sido detectada em granulomas periapicais estando associada à modulação das lesões periapicais humanas (Kabashima *et al*., 2001).

Durante o tratamento endodôntico, a eliminação de microorganismos do sistema de canais radiculares (SCR) infectados é uma etapa crucial que envolve o completo debridamento mecânico-químico. Devido à complexidade

anatômica do SCR, sua completa descontaminação não pode ser assegurada unicamente por este procedimento (Bystrom & Sundqvist, 1981). Apesar de promover uma redução significativa da contagem de microorganismos (Sakamoto *et al*., 2007), estudos *ex vivo* e evidências clínicas demonstram que mesmo após a instrumentação do SCR com limas de aço inoxidável ou de NiTi, paredes dentinárias podem permanecer intocadas por estes instrumentos (Peters *et al.,* 2001).

Remanescentes necróticos do tecido pulpar podem servir de fonte nutricional para os microorganismos. Desta forma, faz-se necessário o auxílio de um protocolo de irrigação química eficiente, juntamente com a utilização de uma medicação intracanal. Esta última tem o intuito de eliminar ou reduzir substancialmente a contaminação persistente no interior de túbulos dentinários, fissuras, reentrâncias e ramificações do SCR, além de prevenir sua proliferação entre sessões (Siqueira *et al*., 1998, Law *et al*., 2004).

O hidróxido de cálcio é a medicação intracanal mais utilizada na prática endodôntica (Estrela *et al*., 2001). Vem sendo indicado como medicação intracanal por ser estável por longos períodos, possuir boa biocompatibilidade, ser bactericida em uma área limitada, além de induzir a formação de tecido mineralizado e ser efetivo contra a formação de exsudatos inflamatórios Bystrom *et al*., 1985). Sua ação antimicrobiana está relacionada à dissociação iônica que ocorre em presença de umidade (Estrela *et al*., 2001). Os íons hidroxila liberados em tais circunstâncias são radicais livres altamente oxidantes que demonstram elevada reatividade com várias biomoléculas (Siqueira & Lopes 1999). Os efeitos letais dos íons hidroxila sobre as células

bacterianas operam-se por meio dos seguintes mecanismos de ação (Siqueira & Lopes1999):

• dano à membrana citoplasmática bacteriana;

• desnaturação proteica;

• dano ao DNA.

A Clorexidina (CHX) é uma molécula hidrofóbica e lipofílica positivamente carregada que vem sendo utilizada como solução de irrigação e medicação intracanal. Isto se deve a sua ação antimicrobiana contra microorganismos gram-positivos e gram-negativos, assim como leveduras, anaeróbios facultativos e aeróbios (Gomes *et al*., 2003, Ercan *et al*., 2007). Vem sendo indicada em casos de infecções persistentes e falhas no tratamento endodôntico (Stuart *et al*., 2006, Cook *et al*., 2007). Sua eficácia está relacionada à interação da molécula positivamente carregada com o carregamento negativo dos fosfolípedes e lipopolissacarídeos presentes na membrana celular bacteriana. Tal interação aumenta a permeabilidade celular, o que permite a entrada da molécula da CHX na célula bacteriana e a alteração do seu equilíbrio osmótico (Gomes *et al*., 2003a,b, Athanassiadis *et al*., 2007).

A CHX, assim como as tetraciclinas, possui uma característica única, que está relacionada à substantividade antimicrobiana adquirida pelo tecido dentinário após o seu uso (Khademi *et al*., 2006). Os íons carregados positivamente liberados pela CHX podem ser adsorvidos à dentina, prevenindo a colonização bacteriana à superfície dentinária por um tempo maior que o período de aplicação do medicamento (Athanassiadis *et al*., 2007).

Apesar de vários estudos *in vivo* terem investigado as propriedades antimicrobianas das medicações intracanais, o efeito destes medicamentos

sobre a resposta imune periapical não foi explorado. Este estudo analisou a expressão gênica de citocinas nos tecidos perirradiculares expostos à terapia endodôntica à base do hidróxido de cálcio e da clorexidina em gel a 2%.

2- Objetivos

- Identificar a expressão gênica de citocinas e quimiocinas nos tecidos perirradiculares de indivíduos portadores de infecções endodônticas submetidos à terapia endodôntica de rotina, na presença ou ausência de medicação intracanal.

2.1- Objetivos específicos

- Caracterizar, por PCR em tempo real, a expressão gênica das citocinas IFN-γ, TNF-α, IL-1-β, IL-17A, IL-10 e da quimiocina MCP-1 no tecido periapical de dentes infectados submetidos à terapia endodôntica.

- Avaliar o efeito da utilização da pasta de hidróxido de cálcio sobre a expressão daqueles mediadores, quando utilizada como medicação intracanal.

- Avaliar o efeito da utilização da clorexidina em gel a 2% sobre a expressão gênica das citocinas e quimiocina acima descritas, quando utilizada como medicação intracanal.

Trabalhos científicos:

Artigo 1- The effects of calcium hydroxide on cytokine expression in endodontic infections

Artigo aceito para publicação no periódico *Journal of Endodontics* (*In press*)

Artigo 2- The impact of chlorhexidine-based endodontic treatment on periapical cytokine expression in teeth.

The effects of calcium hydroxide on cytokine expression in endodontic infections

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Abstract

Introduction: The use of calcium hydroxide is an effective step in killing bacteria that remain after cleaning and shaping procedures. It also induces hard tissue formation and is effective for stopping inflammatory exudates.

Methods: The aim of this study was to assay and to compare the influence of calcium hydroxide on periapical interstitial fluid from human root canals. The mRNA expression levels of the cytokines IFN-γ, TNF-α, IL-1β, IL-17A, and IL-10 as well as the chemokine MCP-1 were assayed by real-time PCR, immediately after root canal cleaning and 15 days later.

Results: Levels of IL-1β, IFN-γ, IL-10, and the chemokine CCL2/MCP-1 were increased in teeth without endodontic dressings. With calcium hydroxide interappointment dressings, no statistically significant changes were observed in cytokine mRNA expression. However, when comparing teeth that received the medication with those that did not, expression levels of IL-1β, IFN-γ and IL-10 were statistically lower in those teeth that received calcium hydroxide.

Conclusions: Analyses of cytokines and the chemokine CCL-2/MCP-1 demonstrated the benefits of calcium hydroxide as a root canal dressing because it impedes the increase of all mediators during the experimental time.

Introduction

The goals of endodontic treatment are removal of bacteria and their byproducts from infected root canals and the complete seal of the disinfected root canal space (1-4). Currently, although cleaning and shaping may be assumed to be of greater importance, endodontic dressing remains an effective step in killing the remaining bacteria. Calcium hydroxide has been determined as suitable for use as an intracanal medicament because it is stable for long periods and bactericidal in a limited area (5). Its antimicrobial activity is related to the release of hydroxyl ions in an aqueous environment (6-8). It also induces hard tissue formation and is effective for stopping inflammatory exudates (5,9). Despite the wide use of calcium hydroxide as an intracanal medicament, systematic reviews have questioned its efficacy (10,11).

Although a number of *in vivo* studies have investigated the antibacterial property of calcium hydroxide, its effects on immune periapical response have not been explored. The aim of this study was to assay and compare the influence of calcium hydroxide on periapical tissues from human root canal infections when it is used in interappointment dressings. To achieve this, the mRNA expression levels of the cytokines IFN-γ, TNF-α, IL-1β, IL-17A, and IL-10, as well as the chemokine CCL2/MCP-1, was assayed by real-time PCR, immediately after root canal cleaning and fifteen days later.

Methods

Human subjects

The subject pool consisted of 20 patients presenting teeth with pulp necrosis. Subjects were drawn from patients with indications for endodontic treatment that were referred to the School of Dentistry at the Universidade Federal de Minas Gerais (Belo Horizonte, MG, Brazil). Patients were excluded from this study if they had taken antibiotics in the three months prior to the initiation of endodontic therapy. In addition to necrotic root canal, patients presented periapical lesions. All participants signed the Free Agreement Formulary. This study was approved by the Ethics Committee of the Universidade Federal de Minas Gerais (ETIC 0011.0.215.203-10).

Sample collection

Clinical samples were taken from teeth with pulp necrosis which were diagnosed based on clinical and radiographic analyses in addition to pulp sensibility tests. All selected patients did not present acute periapical symptoms at the appointment time. Teeth were isolated using a rubber dam followed by a complete asepsis. Cleaning and shaping of the root canals were completed using ProTaper NiTi files (Dentsply Maillefer) in conjunction with 5.2% sodium hypochlorite, as previously described (12). The samples were collected immediately after root canal cleaning to characterize the cytokine/chemokine expression profile. After cleaning and drying, three paper points (20) 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. With this procedure, RNA was extracted from the periapical interstitial fluid. Two groups were designed: one that received endodontic dressings based on calcium hydroxide (experimental group) and another where no endodontic

dressing was inserted into the root canals (control group). In the experimental group, a powder/liquid (saline) mixture of calcium hydroxide was inserted into root canals using K files and endodontic condensers (13). The coronal accesses of the teeth were restored with eugenol-based cement. Fifteen days later, the teeth were opened and sampled as described above. 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 the root canals were sealed with vertically compacted thermoplasticized obturation (14).

Sample Preparation

Total RNA was extracted from each sample with TRIzol reagent (GIBCO/BRL Laboratories, Grand Island, NY) as previously described (15). Briefly, chloroform was added, and the mixture was centrifuged at 12,000*g* at 4°C for 15 minutes; the aqueous phase was collected, and RNA was precipitated by isopropanol. Samples were centrifuged at 12,000*g* at 4°C for 10 minutes. The RNA precipitate was washed once with 75% cold ethanol, 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 PCR

Primer sequences 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 Step One Real-time PCR Systems (Applied Biosystems). Complementary DNA was synthesized using 1 µg of RNA through a reverse transcription reaction as described by Silva *et al*. (16). PCR was performed under standard

conditions as follows: a holding stage at 95°C (10 minutes); a cycling stage of 40 cycles at 95°C (15 seconds) followed by 60°C (1 minute); and a melt curve stage at 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-17A, IL-10 and CCL2/MCP-1 mRNA expression are shown in Table I.

An SYBR-Green detection system (Applied Biosystems) was used to assay primer amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization, performed at each reaction. All samples were run in duplicate. Reactions were performed in a volume of 25 µL and contained 1 µg of cDNA. Sequence Detection Software version v 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 $2^{-\Delta\Delta CT}$ method (17, 18). The values were calculated as the mean value of the duplicates for each patient, and the expression levels of mRNA 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. Because the samples did not present a normal distribution, the Wilcoxon test was used to determine significant differences in samples from the same groups ($p < 0.05$). The Mann-Whitney test was used to compare the differences between the groups ($p < 0.05$).

Results

mRNA expression of IL-1β ($p = 0.034$), IFN-γ ($p = 0.003$), IL-10 ($p = 0.009$) and the chemokine CCL2/MCP-1 ($p = 0.010$) was increased in the control group (without endodontic dressing) 15 days after cleaning and shaping procedures . TNF-α and IL-17A mRNA expression levels were similar at both times ($p > 0.05$). In the experimental group (with endodontic dressing), no statistically significant changes were observed in cytokine mRNA expression when comparing both times of sampling ($p > 0.05$). However, when comparing control and experimental groups at day 15, mRNA expression of IL-1β ($p = 0.032$), IFN-γ ($p = 0.004$) and IL-10 ($p = 0.032$) was statistically lower in cases where calcium hydroxide was placed inside the root canal.

Discussion

Calcium hydroxide is the most commonly used intracanal medicament due to its well-known and recognized antimicrobial activity. This activity is influenced by the speed of the dissociation of hydroxyl ions, which create a high pH environment that inhibits almost all microorganisms which remain in infected root canals after cleaning and shaping procedures (19, 20). Additionally, it inactivates endotoxins, stimulates mineralization, dissolves organic material, and produces a chemical and physical barrier (21). However, its effect on periapical immune response, specifically cytokine expression, is unknown.

In this study, the analyses of the effects of calcium hydroxide on periapical tissues were performed at two different periods, immediately after cleaning and shaping procedures (Day 0) and later (Day 15) in teeth that had or had not

received endodontic dressings. The well-tested sampling collection was performed as described elsewhere (15, 18).

IL-1 plays a pivotal role in periapical diseases, stimulating osteoclastic bone resorption (22, 23), as well as contributing to inflammation by inducing IL-17 synthesis (24). In this study, the expression of IL-1β m-RNA was significantly lower at day 15 when calcium hydroxide had already been long-acting in root canals when compared with control cases. This effect was recently confirmed by *in vitro* data that showed calcium hydroxide acting on denaturing immunoreactive IL-1α, TNF-α, and CGRP (25). Hence, this result suggests the effectiveness of calcium hydroxide in dampening periapical inflammation after root canal cleaning procedures.

As a potent immunologic mediator of acute and chronic inflammatory responses, TNF-α has the capability to increase bone resorption (26,27). However, in this study significant differences in its mRNA expression were not observed in control or experimental groups. However, it is possible calcium hydroxide diminishes TNF-α local concentrations, since it has been shown to denature proteins, including TNF-α (25). In accordance with our findings, Brito *et al.* (18) have shown that TNF-α and IL-17A mRNA expression levels were similar at both periods analyzed (0 and 7 days) in teeth that had not received endodontic dressings, although high TNF- α mRNA and protein levels have been reported in human chronic periapical exudates (15, 26, 28, 29).

Calcium hydroxide interappointment dressing lowers IFN-γ mRNA expression when compared to its expression in the control group. Interestingly, our group has reported that when bacterial loads of the infected root canal were diminished, the levels of mRNA expression of IFN-γ, IL-1β and RANKL

significantly decreased at 7 days (18). Conversely, in this study, we found that, at day 15, the mRNA expression of IFN-γ and IL-1β increased in teeth of control group (without any dressing) but remained at its basal expression in those teeth that received calcium hydroxide. Together, these results are more likely to be related to the reinfection of root canals that might occur in teeth that do not receive a physical barrier or an antibacterial therapy. Additionally, the maintenance of IFN-γ mRNA expression was parallel to IL-17 mRNA expression in the experimental group, in accordance to previous studies which have demonstrated that as many as half of all IL-17$^+$ cells are also IFN-γ$^+$ in humans (30-32).

Chemokines act as vital initiators and promulgators of inflammatory reactions, and an increased expression of CCL2/MCP-1 has been associated with increased recruitment of cells to inflammatory sites (33-35). Here, we observed that in teeth which did not receive an endodontic dressing, the mRNA expression of CCL2/MCP-1 was increased at day 15 in relation to day 0, while it was not observed in teeth from the experimental group. This reinforces our suggestion that the absence of an endodontic dressing is detrimental to cytokine expression throughout the recovery period. Accordingly, the increased expression of CCL2/MCP-1 was shown in chronic periapical lesions when compared with healthy teeth (15, 35).

Immunosuppressive mechanisms mediated by transforming growth factor-β (TGF- β) and IL-10 are responsible for healing processes and the restriction of the inflammatory/immune mechanisms (36-38). Here, we detected increased IL-10 mRNA expression at day 15 when root canals did not receive an endodontic dressing. Previously, analyzing IL-10 mRNA expression 7 days after

root canal cleaning procedures, we found that the increased mRNA expression of the mediator IL-10 was associated with the reduced expression of proinflammatory cytokines (18). In this study, with the same root canal conditions, but 15 days later, we observed a statistically significant increase in the expression of pro-inflammatory cytokines, while IL-10 mRNA expression still remained high. Interestingly, message for all cytokines, pro-inflammatory or regulatory, and the chemokine CCL-2/MCP-1, were kept constant throughout the experimental period in those teeth for which a calcium hydroxide dressing was used. Then, the benefits of calcium hydroxide become evident: if it does not reduce cytokine basal expression observed at day 0, it impedes the increase of all cytokines during the experimental time. However, whether these effects are due to its anti-inflammatory, antibacterial or physical barrier properties is a matter of debate.

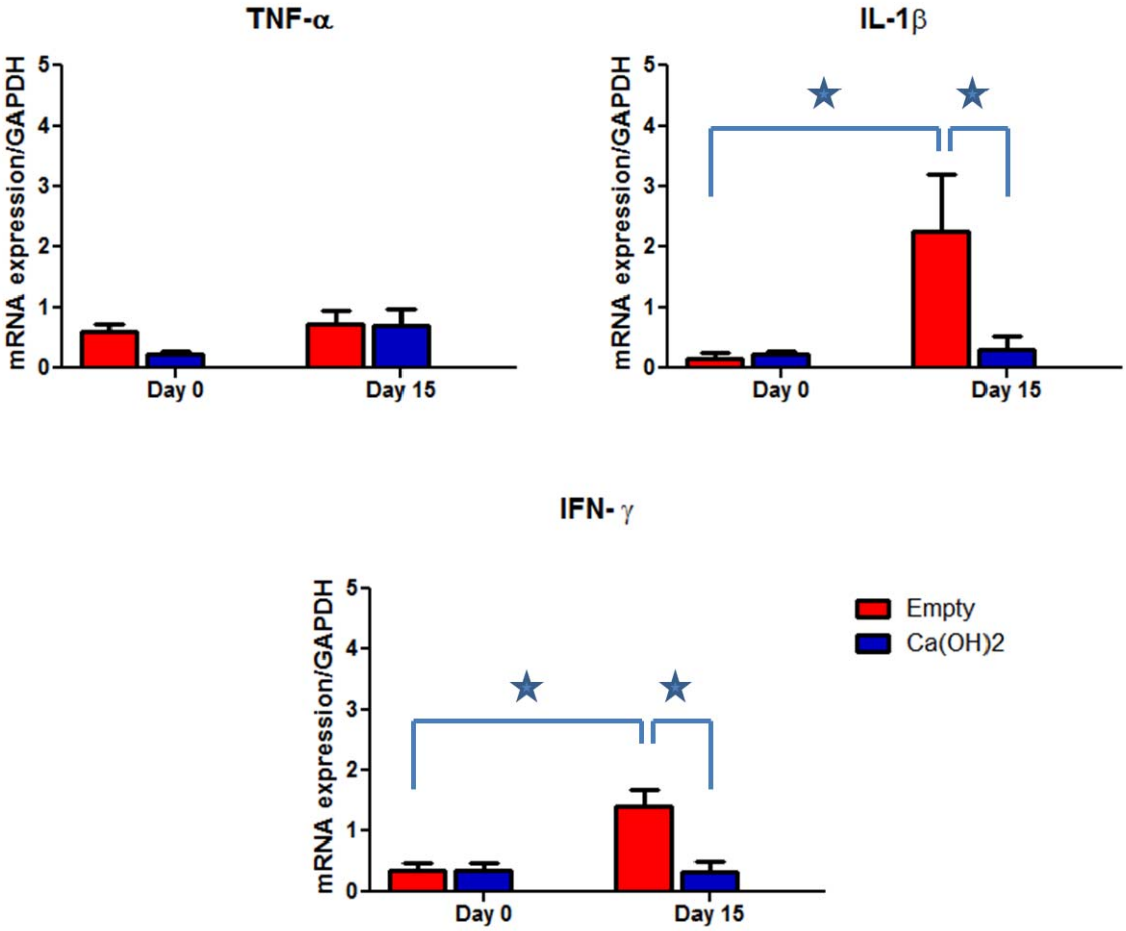

Figure 1. Expression of TNF-α, IL-1β and IFN-γ genes in perirradicular tissues of humans with root canal infections. Levels of expression were determined by real-time PCR and quantified by comparison with the internal control (GAPDH). Bars represent the mean values of samples recovered from teeth that did or did not receive endodontic dressings based on calcium hydroxide; lines represent the standard error of the mean. * Indicates $P < .05$ by the Wilcoxon or Mann-Whitney tests.

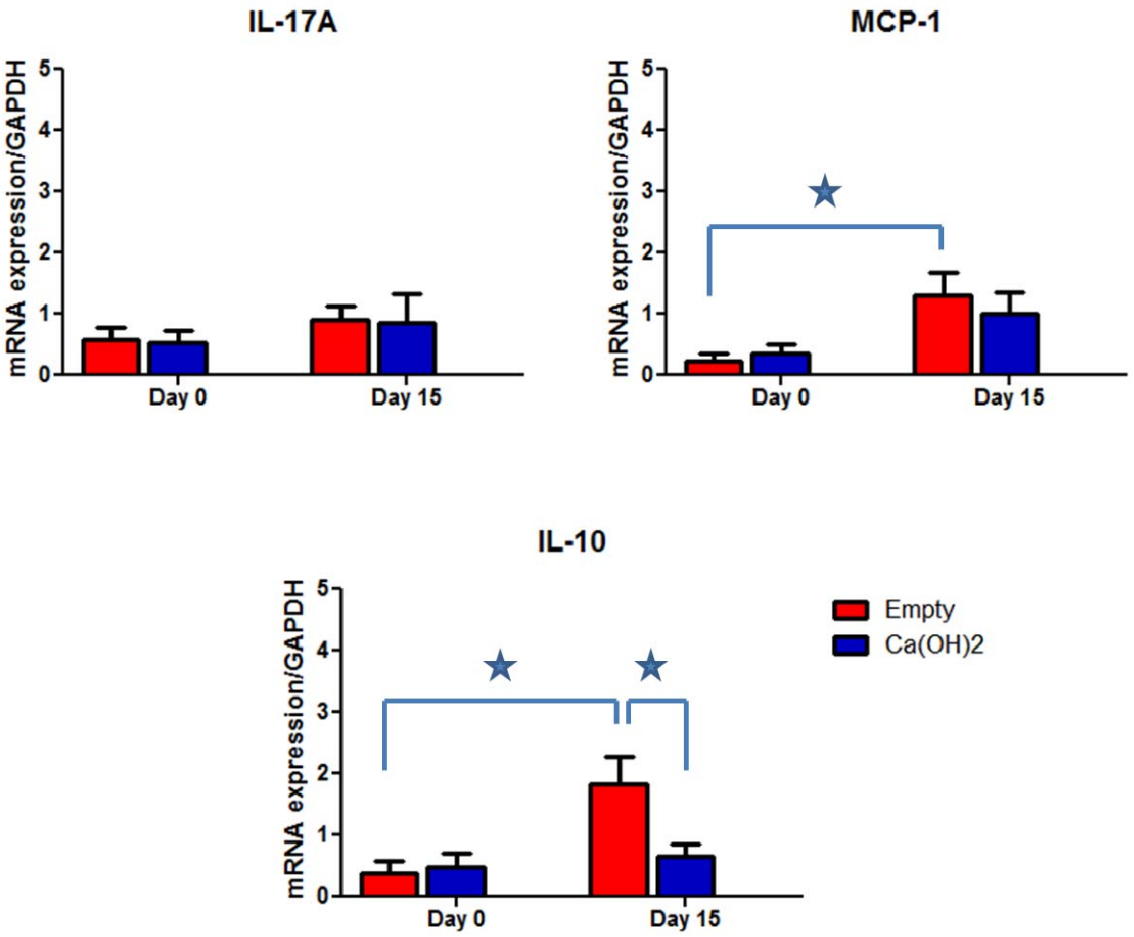

Figure 2. Expression of IL-17A, IL-10 and CCL2/MCP-1 genes in perirradicular tissues of humans with root canal infections. Levels of expression were determined by real-time PCR and quantified by comparison with the internal control (GAPDH). Bars represent the mean values of samples recovered from teeth that did or did not receive endodontic dressings based on calcium hydroxide; lines represent the standard error of the mean. * Indicates *P* < .05 by the Wilcoxon or Mann-Whitney tests.

Table 1. Primer sequences

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-17A	5’-CAA TGACCT GGA ATT ACC CAA- 3’	70	52
	5’-TGA AGG CAT GTG AAA TCG AGA- 3’		
IFN-γ	5’-GAA CTG TCG CCA GCA GCT AAA- 3’	80	95
	5’-TGC AGG CAG GAC AAC CAT TA- 3’		
CCL2/	5’-AAG ACC ATT GTG GCC AAG GA- 3’	80	93
MCP-1	5’-CGG AGT TTG GGT TTG CTT GT- 3’		
	5’-GGT TGC CAA GCC TTG TCT GA- 3’	81	107
IL-10	5’-TCC CCC AGG GAG TTC ACA T- 3’		

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

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The impact of chlorhexidine-based endodontic treatment on periapical cytokine expression in teeth.

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Abstract

Introduction: Root canal treatment typically involves cleaning and shaping procedures followed by treatment with antibacterial endodontic dressing between appointments and, ultimately, three-dimensional, hermetic filling. Chlorhexidine (CHX) is effective as an irrigation solution and is utilized as an endodontic dressing. The aim of this study was to examine the influence of CHX on periapical cytokine expression.

Methods: Expression levels of the cytokines IFN-γ, TNF-α, IL-1β, IL-17A, IL-10 and the chemokine CCL2/MCP-1 were assayed by real-time PCR immediately after root canal cleaning and 15 days later.

Results: mRNA expression of IL-1β, IFN-γ, IL-10 and CCL2/MCP-1 was increased at day 15 in teeth without endodontic dressing. No statistical change was observed in the mRNA expression of cytokines when comparing sampling times for teeth that received endodontic dressing. The mRNA expression of IL-17, CCL2/MCP-1, IFN-γ and IL-10 was statistically lower in those teeth receiving intracanal inter-appointment dressings than in those not receiving them.

Conclusion: The results demonstrate that CHX application between appointments correlated with lower proinflammatory and immunoregulatory cytokines 15 days after the dental procedure.

Introduction

The eradication of bacteria and their byproducts from infected root canals and the complete sealing of the disinfected root canal space are the major objectives in root canal treatment (Ricucci 1998, Ricucci & Langeland 1998, Fabricius *et al.* 2006, Siqueira & Roças 2008). These goals may be accomplished using mechanical instrumentation and chemical irrigation, which can be used in conjunction with inter-appointment endodontic dressing (Bystrom *et al.* 1985, Mohammadi & Abbott 2009).

Chlorhexidine (CHX) is a positively charged hydrophobic and lipophilic molecule that enters cells through active or passive transport mechanisms and interacts with phospholipids and lipopolysaccharides on the cell membranes of bacteria (Athanassiadis *et al.* 2007). Its effectiveness as an irrigation solution is related to the interaction of the positive charges of the molecule with the negatively charged phosphate groups on microbial cell walls (Gomes *et al.* 2003a,b), which alters the cells' osmotic equilibrium. Besides being used as an irrigation solution, CHX is also utilized as an endodontic dressing (Mohammadi & Abbott 2009).

In spite of several endodontic studies have focused on CHX antibacterial effect, its role in the periapical immune response remains a gap in knowledge. The aim of this study was to compare the periapical tissues of human root canal infections that received inter-appointment CHX dressing to those that did not. To achieve this, the mRNA expression levels of the cytokines IFN-γ, TNF-α, IL-

1β, IL-17A and IL-10, as well as the chemokine CCL2/MCP-1, were assayed by real-time PCR immediately after root canal cleaning and 15 days later.

Methods

Twenty patients presenting with dental pulp necrosis who were referred to the School of Dentistry at the Universidade Federal de Minas Gerais (Belo Horizonte, MG, Brazil) were selected for this study. None of them had taken antibiotics in the three months prior to the root canal treatment. This study was approved by the Ethics Committee of the Universidade Federal de Minas Gerais (ETIC 0011.0.215.203-10).

Pulp sensitivity tests and radiographic analyses were performed to diagnose teeth as necrotic. Those teeth that presented with acute symptoms were excluded. Clinical and sampling procedures were performed as described elsewhere (Henriques *et al.* 2011, Brito *et al.* 2012, Tavares *et al. in press*). Briefly, cleaning and shaping of the root canals was completed using ProTaper NiTi files (Dentsply Mailleffer) in conjunction with 5.2% sodium hypochlorite. The samples were collected immediately after root canal cleaning (day 0) and 15 days later (day 15). In the experimental group, 2% CHX gel was placed into the root canals with an Ultradent Capillary Tip (Ultradent, South Jordan, UT, USA), while in the control group, no endodontic dressing was inserted into the root canals. Three paper points (#20) were introduced into the root canal, passing through the root apex (2 mm) for 1 minute. After withdrawal, the paper points were cut 4 mm from the tip and dropped into a microcentrifuge tube. The samples were stored at -70^{o}C. In teeth with multiple canals, the first and second samples were collected from the same canal. At this stage of the treatment,

none of the teeth presented with clinical signs or symptoms, and root canals were sealed with vertically compacted thermoplasticized obturation (Bowman & Baumgartner 2002).

Total RNA was extracted from each sample with TRIzol reagent (GIBCO/BRL Laboratories, Grand Island, NY, USA), as described previously (Henriques *et al.* 2011, Brito *et al.* 2012, Tavares *et al. in press*). Complementary DNA (cDNA) was synthesized using 1 μg of RNA (Silva *et al.* 2008). Polymerase chain reaction (PCR) was carried out under standard conditions as follows: a holding stage at 95^{o}C (10 minutes); a cycling stage of 40 cycles at 95^{o}C (15 seconds) followed by 1 minute at 60^{o}C; and a melting stage at 95^{o}C (15 seconds), 60^{o}C (1 minute) and 95^{o}C (15 seconds). The primer sequences for IFN-γ, TNF-α, IL-1β, IL-17A, IL-10 and CCL2/MCP-1 were designed using PRIMEREXPRESS software (Applied Biosystems, Foster City, CA, USA) based on nucleotide sequences available in the GenBank database. Primer sequences are shown in Table I. The real-time PCR assay was performed using Step One Real-time PCR Systems (Applied Biosystems). A SYBR-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. Reactions were carried out in a volume of 25 μL and contained 1 μg of cDNA. Sequence Detection Software version 2.0 (Applied Biosystems) was used to analyze data after amplification. Results were obtained as threshold cycle (Ct) values, and expression levels were calculated using the comparative $2^{-\Delta\Delta CT}$ method (Schmittgen & Livak 2008, Brito *et al.* 2012). The values were calculated as the mean value of the duplicates for each patient, and the

expression levels of mRNA in all samples were defined as the ratio of each specific primer to GAPDH expression. Finally, 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. Because the samples did not present a normal distribution, the Wilcoxon test was used to determine statistical differences in samples from the same groups ($p < 0.05$). The Mann-Whitney test was used to compare the differences between the groups ($p < 0.05$).

Results

In the group where no endodontic dressing was inserted into the root canals (control group), the mRNA expression of IL-1β, IFN-γ, IL-10 and the chemokine CCL2/MCP-1 was elevated at day 15 ($p<0.05$). The TNF-α and IL-17A mRNA expression was similar at the two time points ($p>0.05$). In contrast, no statistical change ($p>0.05$) in cytokine mRNA was observed between time points for the experimental group. Moreover, when comparing control and experimental groups at day 15, the mRNA expression of IL-17, CCL2/MCP-1, IFN-γ and IL-10 was statistically lower in the experimental group where CHX was used as a root canal dressing ($p<0.05$).

Discussion

Intracanal medications are mainly used to reduce or eliminate bacteria located inside the root canal system and to prevent their proliferation between appointments (Siqueira & Uzeda 1998, Law & Messer 2004). It has been demonstrated that such treatment improves the patient outcome (Bystrom *et al.*

1985, De Rossi *et al.* 2005). Due to its strong antibacterial activity against gram-positive and gram-negative microorganisms as well as against yeast, facultative anaerobes and aerobic bacteria (Gomes *et al.* 2003a, Ercan *et al.* 2006), CHX has been recommended not only as an irrigation solution but also as an intracanal medication (Mohammadi & Abbott 2009, Kontakiotis *et al*. 2008). CHX, as well as tetracycline, are unique in that dentine medicated with them acquire long-term antimicrobial properties (Khademi *et al.* 2006) because the positively charged ions released by CHX can be adsorbed into the dentine, preventing microbial colonization of the dentine surface (Athanassiadis *et al*. 2007).

Despite its well-known antibacterial properties, the effect of CHX on the periapical immune response, specifically on cytokine expression, remains unclear. To explore its effect, we used a well-established sampling collection methodology (Henriques *et al*. 2011, Brito *et al*. 2012, Tavares *et al. in press*), comparing samples from immediately after cleaning and shaping procedures (Day 0) to those collected later (Day 15) in teeth that had received or not received 2% CHX gel as a root canal dressing.

Proinflammatory and immunoregulatory cytokines are important for the pathogenesis of periapical lesions (Colic *et al.* 2009). A type 1 immune response, characterized by the production of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), is involved in the progression, bone destruction and remodeling of periapical lesions (Takeichi *et al.* 1996), while immunosuppressive mechanisms mediated by Treg- or Th2-derived cytokines are responsible for restricting the inflammatory immune mechanisms (Kawashima & Stashenko 1999, Fukada *et al*. 2009). IL-10, initially described as

a Th2 cytokine, and transforming growth factor-β (TGF-β) exhibit strong anti-inflammatory properties. The Th17 cell subset participates in the exacerbation of inflammation, yielding potent effects on various cells of the innate immune system (Kolls & Linden 2004, Colic *et al.* 2007). Recently, we analyzed T-lymphocyte and cytokine expression in periapical tissues and showed that distinct root canal treatments might play regulatory roles in controlling local immune/inflammatory processes (Brito *et al*. 2012).

In this study, a statistical increase in the type 1 cytokine IL-1β mRNA expression was observed in the control group between 0 and 15 days after root canal cleaning procedures. However, teeth in which 2% CHX gel was placed inside root canals exhibited no statistical changes in cytokine or chemokine levels during this same time interval. Similarly, a previous study showed that when calcium hydroxide was used as the endodontic dressing, IL-1β mRNA expression was maintained at a low level between 0 and 15 days (Tavares *et al*. *in press*).IL-1β is involved in the progression of bone destruction (Takeichi *et al*. 1996), and anti–IL-1α antisera were able to significantly neutralize bone resorption activity in periapical lesions (Wang & Stashenko 1993). The maintenance of basal expression levels of IL-1β in those teeth that received 2% CHX gel suggests that this dressing probably acts by dampening the bone destruction induced by IL-1β.

TNF-α significantly stimulates local bone resorption by inducing the production of essential osteoclast differentiation factors (Prso *et al.* 2007, Menezes *et al.* 2008). High TNF-α levels have been reported in human chronic periapical exudates (Safavi & Rossomando 1991, Artese *et al.* 1991, Ataoglu *et al.* 2002, Henriques *et al.* 2011). In our study, TNF-α mRNA expression was

similar at both times analyzed for both control and experimental groups ($p>0.05$). Previously, using calcium hydroxide as a root canal medication, we observed very similar results (Tavares *et al.* 2012). Brito *et al.* (2012) also reported that TNF-α remained at basal levels 7 days after root canal cleaning in teeth that did not receive any endodontic dressing. Taken together, these results suggest that the maintenance of basal TNF-α mRNA expression is related to the restriction of root canal infection rather than the presence of endodontic dressing.

The mRNA expression of IFN-γ was statistically increased in the control group, but it was maintained at basal levels in the experimental, CHX-treated group. This is in keeping with a similar study examining the effect of calcium hydroxide treatment (Tavares *et al. in press*). It is interesting to note that the mRNA expression of IFN-γ and IL-1β increased in teeth without any dressing at day 15 but decreased at day 7 (Brito *et al.* 2012), implying that bacterial recolonization may occur around day 7 in untreated teeth but not in teeth that had received 2% CHX gel. Whether these results are the consequence of CHX antibacterial or physical properties is unknown, but they indicate that 2% CHX gel favors a periapical cytokine response.

Similar to IFN-γ, IL-17 is a cytokine that acts in delayed-type reactions by increasing chemokine production in various tissues so as to recruit monocytes and neutrophils to the site of inflammation (Kuby *et al.* 2007, Tesmer *et al.* 2008). In this study, the maintenance of IFN-γ mRNA expression was paralleled by IL-17 mRNA expression in the experimental group, and this is in keeping

with the observation that as many as half of all IL-17$^+$ cells are also IFN-γ^+ in humans (Acosta Rodriguez *et al.* 2007, Wilson *et al.* 2007, Colic *et al.* 2009).

Several studies have shown that increased expression of the chemokine MCP-1 was associated with greater recruitment of cells to inflammatory sites (Silva *et al.* 2005, De Rossi *et al.* 2008, Marçal *et al*. 2010). In this study, we found that MCP-1 mRNA expression increased in the periapical tissues of teeth that did not receive an endodontic dressing but not in teeth where 2% CHX gel was applied to the root canals. In agreement with a previous study (Tavares *et al. in press*) and with the results concerning the other cytokines analyzed in this study, the absence of an endodontic dressing may be detrimental to the inflammatory response in the periapical area.

The healing processes and the restriction of inflammatory/immune mechanisms are mediated by the immunosuppressive mechanisms of transforming growth factor-β (TGF- β) and IL-10 (Kawashima & Stashenko 1999, Sasaki *et al*. 2000, Colic *et al*. 2010). In this study, we detected an increase in IL-10 mRNA expression concurrent with an increase of type 1 cytokines at day 15 when the root canals did not receive an endodontic dressing. In contrast, when root canals were dressed with CHX, a reduction in IL-10 mRNA expression and type 1 cytokine expression was observed. Cross-regulation, by which Th1 cells inhibit the Th2 response and vice versa, has been proposed (Stashenko *et al.* 1998). It was supported by a recent study that showed increased IL-10 mRNA expression in association with a reduced expression of proinflammatory cytokines at day 7 after chemo-mechanical procedures in teeth that did not receive root canal dressing (Brito *et al*. 2012). Taken together, the results of this study demonstrate that after CHX had

already been used as a dressing, both proinflammatory and immunoregulatory cytokine expression remained lower when compared to the control group. The critical effect of CHX on the periapical immune response is open for debate. Moreover, comprehensive clinical studies of multiple samples quantitatively examined for cytokine expression in periapical tissues after different treatments are necessary to better understand the effects of root canal dressing and to design the best target therapy.

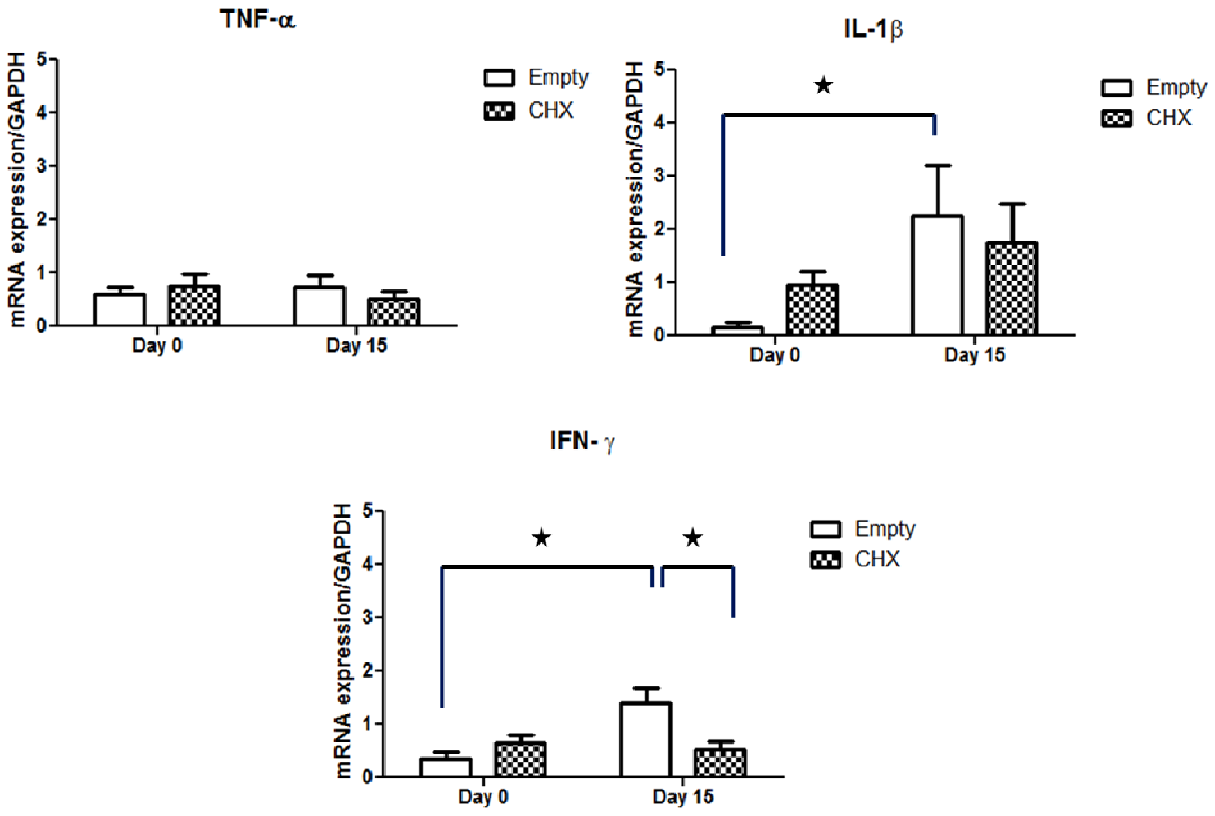

Figure 1. mRNA expression of TNF-α, IL-1β and IFN-γ in the periradicular tissues of humans presenting with root canal infections. Levels of expression were determined by real-time PCR and quantified by comparison with the internal control (GAPDH). Bars represent the mean values of samples

recovered from teeth that did or did not receive 2% chlorhexidine gel as endodontic dressing; lines represent the standard error of the mean. * Indicates *P* < .05 by the Wilcoxon or Mann-Whitney tests.

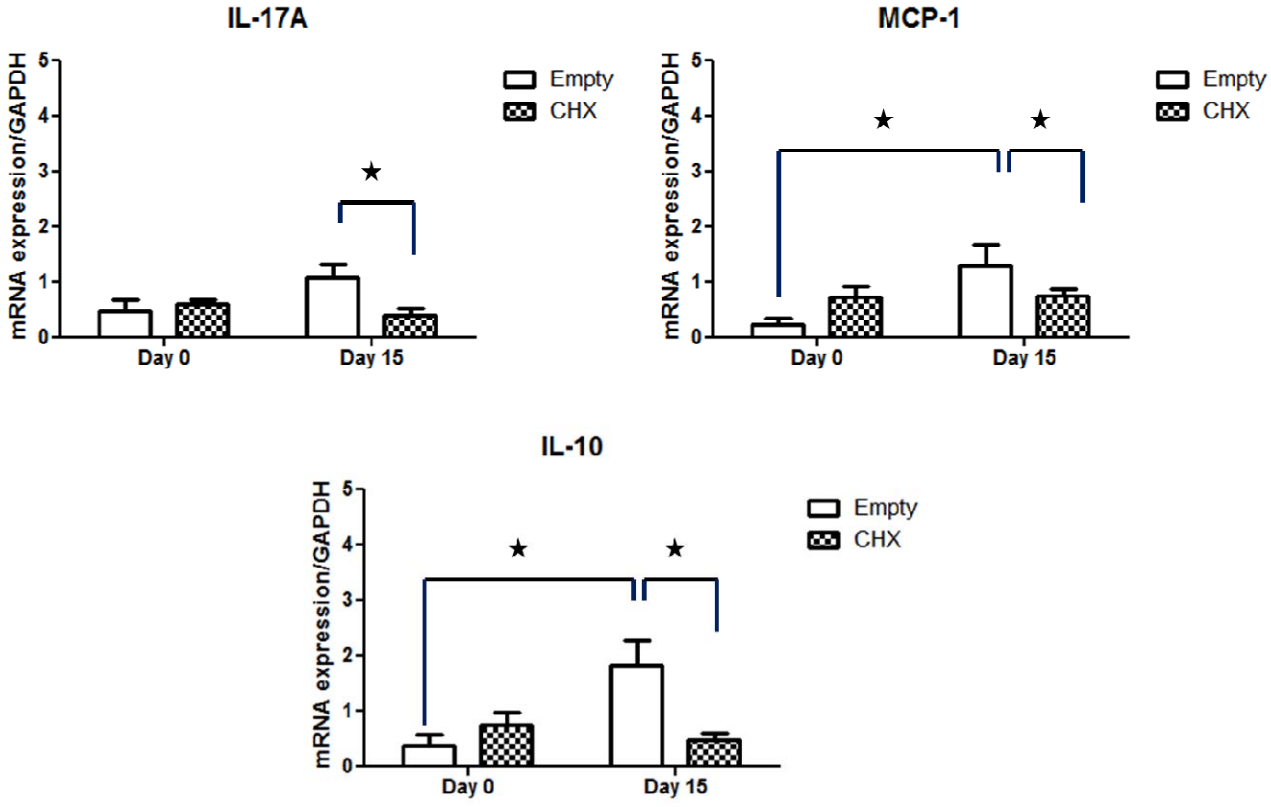

Figure 2. mRNA expression of IL-17A, IL-10 and CCL2/MCP-1 in periradicular tissues of humans presenting with root canal infections. Levels of expression were determined by real-time PCR and quantified by comparison with the internal control (GAPDH). Bars represent the mean values of samples recovered from teeth that did or did not receive 2% chlorhexidine gel as endodontic dressing; lines represent the standard error of the mean. * Indicates *P* < .05 by the Wilcoxon or Mann-Whitney tests.

Table 1. Primer sequences

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-17A	5’-CAA TGACCT GGA ATT ACC CAA- 3’	70	52
	5’-TGA AGG CAT GTG AAA TCG AGA- 3’		
IFN-γ	5’-GAA CTG TCG CCA GCA GCT AAA- 3’	80	95
	5’-TGC AGG CAG GAC AAC CAT TA- 3’		
CCL2/	5’-AAG ACC ATT GTG GCC AAG GA- 3’	80	93
MCP-1	5’-CGG AGT TTG GGT TTG CTT GT- 3’		
	5’-GGT TGC CAA GCC TTG TCT GA- 3’	81	107
IL-10	5’-TCC CCC AGG GAG TTC ACA T- 3’		

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

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Considerações finais

O sucesso do tratamento endodôntico depende de vários fatores: seleção do caso clínico, correto diagnóstico e criteriosa execução da técnica de preparo mecânico-químico e obturação tridimensional do SCR. Contudo, falhas no tratamento endodôntico podem ocorrer em casos em que há a persistência ou a reintrodução de microrganismos no sistema de canais radiculares. Neste contexto, a utilização da medicação intracanal insere-se como adjuvante na eliminação do processo infeccioso e reparo dos tecidos periapicais (Farhad & Mohammadi 2005).

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, Henriques *et al.,* 2011, Brito *et al*., 2012). 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.

Até recentemente, os estudos sobre as respostas imunoperiapicais em humanos utilizavam a remoção cirúrgica do granuloma ou cisto, quando então, realizavam-se as análises dos espécimes clínicos. Tais procedimentos, devido à sua natureza invasiva, inviabilizavam estudos sobre o efeito imunológico da terapia endodôntica em humanos. Utilizando-se uma metodologia que se mostrou eficaz (Henriques *et al.,* 2011, Brito *et al*., 2012), nos foi permitido coletar o fluido intersticial perirradicular de forma conservadora, evitando-se os

procedimentos cirúrgicos. Avaliamos, no presente estudo, os efeitos de medicações intracanais utilizadas na clínica endodôntica de rotina.

Os efeitos do hidróxido de cálcio e da clorexidina sobre os tecidos perirradiculares foram analisados em dois tempos distintos, imediatamente e 15 dias após o preparo mecânico-químico. As amostras foram coletadas de dentes que receberam (grupo teste) ou não (grupo controle) a medicação intracanal.

Vários trabalhos reportaram a efetividade do hidróxido de cálcio como medicação intracanal. Sua ação antimicrobiana foi atribuída a sua capacidade de gerar uma barreira física no interior dos canais com a liberação gradual de íons cálcio e hidroxila (Bystrom *et al*.,1985). Por outro lado, alguns estudos questionavam sua eficácia em eliminar algumas espécies microbianas associadas ao insucesso no tratamento endodôntico, sabidamente o *E. faecalis*, o que levou-os a sugerir a utilização da clorexidina como medicação intracanal (Ballal *et al.,* 2007, Krithikadatta *et al.,*2007, Lee *et al.*, 2008).

Interessantemente, em recente estudo da microbiota de dentes com lesões refratárias ao tratamento endodôntico, nosso grupo de pesquisa demonstrou que o papel do *E.faecalis* nessas infecções é questionável e deve ser revisto (Henriques *et al*., 2011b), assim como sugeriram outros autores (Rôças *et al.,* 2004, Sakamoto *et al*., 2008). Também demonstramos, que as respostas imuno-periapicais à infecção presente naqueles dentes com lesões refratárias ao tratamento endodôntico apresentam um perfil pró-inflamatório (Henriques *et al*., 2011).

Observou-se, de maneira interessante, no presente estudo que a expressão gênica dos mediadores IFN-γ e IL-1β aumentaram no 15º dia, naqueles dentes que não receberam a medicação intracanal. Contudo, sob as

mesmas condições, Brito *et al.* (2012) observaram a redução da expressão destes mediadores, após 7 dias. Tais achados parecem estar relacionados à reinfecção tardia que deve acontecer nos canais radiculares daqueles dentes que não receberam a medicação intracanal, reforçando a ideia de que a sua presença auxilia a resposta imune periapical.

Neste contexto, todas as citocinas, próinflamatórias ou regulatórias, assim como a quimiocina CCL-2/MCP-1, mantiveram os mesmos níveis de expressão no grupo experimental diferentemente do observado no grupo controle. O benefício da medicação intracanal se torna evidente: se por um lado a medicação não reduz a expressão basal de citocinas observadas logo após o preparo mecânico-químico, por outro ela impede o aumento dessa expressão 15 dias após a sua utilização. Se este efeito está relacionado à substantividade antibacteriana, à ação antiinflamatória ou à formação de uma barreira física, esta ainda é uma questão a ser esclarecida.

Todavia, para que se tenha uma análise mais acurada dos resultados obtidos no presente trabalho, os mesmos serão futuramente confrontados aos achados microbiológicos dos mesmos sítios, analisando-se as amostras coletadas no mesmo período daquelas coletadas para a etapa imunológica. A associação desses achados trará, certamente, uma compreensão mais bem fundamentada dos mecanismos que operam nos tecidos perirradiculares nas diferentes etapas do tratamento endodôntico.

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Anexo – Decisão do comitê de Ética da UFMG

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM PESQUISA - COEP

Parecer nº. ETIC 0359.0.203.000-10

Interessado(a): Prof. Antonio Paulino Ribeiro Sobrinho
Departamento de Odontologia Restauradora
Faculdade de Odontologia - UFMG

DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP aprovou, no dia 27 de outubro de 2010, após atendidas as solicitações de diligência, o projeto de pesquisa intitulado **"Análise microbiológica e imunológica de canais radiculares infectados e os efeitos da terapia endodôntica sobre estes parâmetros"** bem como o Termo de Consentimento Livre e Esclarecido.

O relatório final ou parcial deverá ser encaminhado ao COEP um ano após o início do projeto.

Profa. Maria Teresa Marques Amaral
Coordenadora do COEP-UFMG

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