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 interferon (IFN)-γ, tumor necrosis factor-α, interleukin (IL)-1β, IL-17A, and IL-10 as well as the chemokine MCP-1 were assayed by real-time polymerase chain reaction 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. *(J Endod 2012;38:1368–1371)*

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Key Words

Calcium hydroxide, cytokine expression, root canal infection

The goals of endodontic treatment are removal of bacteria and their by-products 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 interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-17A, and IL-10, as well as the chemokine CCL2/MCP-1, were assayed by real-time polymerase chain reaction (PCR) immediately after root canal cleaning and 15 days later.

Methods

Human Subjects

The subject pool consisted of 20 patients with teeth with pulp necrosis. Subjects were drawn from patients with indications for endodontic treatment who 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 during the 3 months before the initiation of endodontic therapy. In addition to necrotic root canal, patients had 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 that were diagnosed on the basis of 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 by using a rubber dam, followed by complete asepsis. 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, as previously described (12). The samples were collected immediately after root canal cleaning to characterize the cytokine/chemokine expression profile. After cleaning and drying, 3 paper points (13) 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, 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 mixture of calcium hydroxide was inserted into root canals by using K-files and endodontic condensers (14). 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 had clinical signs or symptoms, and the root canals were sealed with vertically compacted thermoplasticized obturation (15).

Sample Preparation

Total RNA was extracted from each sample with TRIzol reagent (GIBCO/BRL Laboratories, Grand Island, NY) as previously described (16). 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 by using PRIMEREXPRESS software (Applied Biosystems, Foster City, CA) on the basis of nucleotide sequences available in the GenBank database. The real-time PCR assay was performed by using Step One Real-time PCR Systems (Applied Biosystems). Complementary DNA was synthesized by using 1 μg of RNA through a reverse transcription reaction as described by Silva et al (17). 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 1.

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 2.0 (Applied Biosystems) was used to analyze data after amplification. The results were obtained as threshold cycle (Ct) values. Expression levels were calculated by using the comparative $2^{-\Delta\Delta CT}$ method (18, 19). 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 by using SPSS for Windows (version 15.0; SPSS Inc, Chicago, IL). Data were subjected to the Shapiro–Wilks 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 ($P < .05$). The Mann–Whitney test was used to compare the differences between the groups ($P < .05$).

Results

The mRNA expression of IL-1β ($P = .034$), IFN-γ ($P = .003$), IL-10 ($P = .009$), and the chemokine CCL2/MCP-1 ($P = .010$) was increased in the control group (without endodontic dressing) 15 days after cleaning and shaping procedures. The TNF-α and IL-17A mRNA expression levels were similar at both times ($P > .05$). In the experimental group (with endodontic dressing), no statistically significant changes were observed in cytokine mRNA expression when comparing both times of sampling ($P > .05$). However, when comparing control and experimental groups at day 15, mRNA expression of IL-1β ($P = .032$), IFN-γ ($P = .004$), and IL-10 ($P = .032$) was statistically lower in cases where calcium hydroxide was placed inside the root canal (Figs. 1 and 2).

Discussion

Calcium hydroxide is the most commonly used intracanal medicament because of 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 that remain in infected root canals after cleaning and shaping procedures (13, 20). In addition, 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.

TABLE 1. Primer Sequences

Gene	Sense and antisense	Mt (°C)	bp
GAPDH	5′-GCA CCA CCA ACT GCT TAG CA- 3′ 5′-TGG CAG TGA TGG CAT GGA GGA- 3′	80	96
TNF-α	5′-TTC TGG CTC AAA AAG AGA ATT G- 3′ 5′-TGG TGG TCT TGT TGC TTA AGG- 3′	76	73
IL-1β	5′-TGG CAG AAA GGG AAC AGA A- 3′ 5′-ACA ACA GGA AAG TCC AGG CTA- 3′	73	59
IL-17A	5′-CAA TGACCT GGA ATT ACC CAA- 3′ 5′-TGA AGG CAT GTG AAA TCG AGA- 3′	70	52
IFN-γ	5′-GAA CTG TCG CCA GCA GCT AAA- 3′ 5′-TGC AGG CAG GAC AAC CAT TA- 3′	80	95
CCL2/ MCP-1	5′-AAG ACC ATT GTG GCC AAG GA- 3′ 5′-CGG AGT TTG GGT TTG CTT GT- 3′	80	93
IL-10	5′-GGT TGC CAA GCC TTG TCT GA- 3′ 5′-TCC CCC AGG GAG TTC ACA T- 3′	81	107

bp, base pairs of amplicon size; Mt, melting temperature.