Short-chain Fatty Acids in Infected Root Canals of Teeth with Apical Periodontitis before and after Treatment

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Abstract

Introduction: Short-chain fatty acids (SCFAs) are bacterial metabolic end products that may function as virulence factors. This study evaluated the occurrence of SCFAs in infected root canals before and after treatment. **Methods:** Samples were taken from root canals of teeth with apical periodontitis before (S1) and after (S2) chemomechanical preparation with either NaOCl or chlorhexidine as the irrigant and then after interappointment medication with calcium hydroxide (S3). High-performance liquid chromatography was used for detection of SCFAs. Selected bacterial taxa that are recognized producers of the target SCFAs were identified by real-time polymerase chain reaction. **Results:** Butyric acid was the most common fatty acid in S1, followed by propionic acid. Both molecules were also found in S2 and S3 from both NaOCl and chlorhexidine groups. Lactic acid was not present in detectable levels in S1, but it occurred in 1 postinstrumentation sample and in 9 samples taken after calcium hydroxide medication. Of the target taxa, *Fusobacterium nucleatum* was the most prevalent in S1 (76%), followed by members of the Actinobacteria phylum (71%), *Streptococcus* species (59%), and *Parvimonas micra* (53%). Gram-positive taxa, especially streptococci, were the most prevalent bacteria in S2 and S3. SCFA detection was matched with the respective potential producer species in most cases. **Conclusions:** This first report of SCFAs in infected root canals suggests that these molecules may play a role in the pathogenesis of apical periodontitis. Significance of persistence of SCFAs after treatment and its effects on the long-term outcome await elucidation. *(J Endod 2015;41:831–835)*

Key Words

Apical periodontitis, butyric acid, endodontic treatment, lactic acid, propionic acid, root canal infection, short-chain fatty acids

Bacteria infecting the root canals of teeth with apical periodontitis are usually organized in mixed biofilm communities (1). The pathogenic ability of multispecies biofilms is certainly related to the accumulation of virulence factors and antigens from the different component species. This bacterial "soup" may contact the periradicular tissues via apical foramen/foramina and evoke inflammation, with consequent formation of apical periodontitis (2). Virulence factors that are expected to concentrate in the biofilm include bacterial cellular constituents (eg, lipopolysaccharide, peptidoglycan, lipoteichoic acid, complex lipids, etc), and metabolic end products (3, 4).

Several end products of the bacterial metabolism are released to the extracellular environment and may be toxic to host cells, cause degradation of constituents of the extracellular matrix of the connective tissue, and interfere with host defense processes (5–7). Among them, short-chain fatty acids (SCFAs) are low-molecular-weight molecules that have been regarded as potential virulence factors involved in the pathogenesis of inflammatory conditions such as periodontal diseases (8, 9). Gingival inflammation has been directly and significantly correlated with SCFA concentrations in the gingival crevice (10). In addition, propionic and butyric acid concentrations in the crevicular fluid have been significantly associated with clinical measures of periodontal disease severity and inflammation and with the total microbial load (11). Concentrations of lactic, propionic, butyric, and isovaleric acids have been shown to decrease significantly in the gingival crevicular fluid of patients with marginal periodontitis after treatment, reaching levels comparable with the healthy control group (12). A role for SCFA in the pathogenesis of apical periodontitis, which is also an inflammatory disease of bacterial etiology, has been suggested (3).

SCFAs $[CH_3 - (CH_2)_x - COOH;\ X < 3$ thus $C \leq 5]$ include volatile acids (eg, propionic, butyric, acetic, and isovaleric) and non-volatile acids (eg, lactic and succinic acids) (13). Because endodontic pathogens are known to produce many SCFAs *in vitro*, these putative virulence factors are expected to accumulate in infected root canals. Nevertheless, no study has so far directly screened endodontic clinical samples for the presence of these bacterial metabolic end products. Detection of SCFAs in infected root canals is important in several aspects. First, it contributes to the knowledge of virulence factors potentially involved in the pathogenesis of apical periodontitis. Second, occurrence of these metabolic factors in pretreatment and post-treatment samples may permit inferences to be made as to how bacteria react to treatment procedures. Finally, because there is high interindividual variability in the bacterial diversity of endodontic infections and no specific species has been consistently associated with clinical symptoms or treatment outcome (14), there is a trend to look for bacterial products that may serve as biomarkers for certain clinical conditions (15, 16). This is because some redundancy in distinct communities is expected in terms of bacterial

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physiology and function, and consequently, the variability in bacterial products released in the environment is conceivably lower than the species composition. This is typical when one considers that several different species can produce the very same enzyme or metabolic product.

This study was undertaken to evaluate the presence and prevalence of several SCFAs in infected root canals before and after treatment with either sodium hypochlorite (NaOCl) or chlorhexidine (CHX) as the irrigant and interappointment dressing with a calcium hydroxide paste. Identification of selected bacterial taxa that are potential producers of the SCFAs under investigation was also performed.

Materials and Methods

Subjects and Sample Taking

Samples were taken from patients who had been referred for root canal treatment to the Department of Endodontics, Estácio de Sá University. Only single-rooted and single-canalled teeth from 18 adult patients (ages ranging from 20 to 39 years) with carious lesions, necrotic pulps confirmed by pulp tests, and clinical and radiographic evidence of asymptomatic apical periodontitis were included in this study. Exclusion criteria included teeth with gross carious lesions, teeth with root or crown fracture, teeth subjected to previous endodontic treatment, patients who received antibiotic therapy within the previous 3 months, symptomatic teeth, and patients with periodontal pockets deeper than 4 mm. The study was approved by the Ethics Committee at Estácio de Sá University, Rio de Janeiro, Brazil, and written informed consent was obtained from all the patients.

Samples were taken from the root canals as follows. After the tooth crown was cleansed with pumice, a rubber dam was placed, and the tooth and the surrounding field were decontaminated by a protocol that used 3% hydrogen peroxide followed by 2.5% NaOCl solution (17). Complete access preparations were made by using sterile burs without water spray. The operative field, including the pulp chamber, was again swabbed with 2.5% NaOCl, which was then inactivated with sterile 5% sodium thiosulfate. Sterility control samples were taken from the tooth surface with sterile paper points. All the teeth included in the study had sterility control samples uniformly negative after polymerase chain reaction (PCR) with universal bacterial primers.

The root canal was filled with 10 mmol/L Tris-HCl (pH 8.0), and a K-type file no. 15 was introduced up to approximately 1 mm short of the root apex, on the basis of radiographs, and used to gently file the canal walls. Afterwards, the fluid in the canal was aspirated by using a sterile disposable syringe and transferred to a cryotube. These sampling procedures were repeated several times until 100 μL sampled fluid was obtained. Sample was immediately frozen at -80°C. This initial root canal sample was called S1.

Each root canal was instrumented at the same visit by using BioRaCe instruments (FKG Dentaire, La Chaux-de-Fonds, Switzerland), with the working length established 1 mm short of the radiographic apex. Master apical files ranged from BR5 (40/.04) to BR7 (60/.04), depending on both the root anatomy and the preoperative root canal diameter. Patency of the apical foramen was confirmed with #20 K-type file throughout the procedures. Irrigation was performed with either 2.5% NaOCl (9 teeth) or 2% CHX (FGM, Joinvile, SC, Brazil) (9 teeth) by using disposable syringes and NaviTip needles (Ultradent, South Jordan, UT) inserted up to 4 mm short of the working length. Two milliliters of the irrigating solution (NaOCl or CHX) was used after each file size. After preparation, the root canal was dried and then flushed with 5 mL either 10% sodium thiosulfate solution or mixture of 0.07% lecithin, 0.5% Tween 80, and 5% sodium thiosulfate to neutralize any residual NaOCl or CHX, respectively. Postinstrumentation (S2) samples were taken from the root canals as described for S1 samples.

Smear layer was removed by using 17% EDTA for 3 minutes. The canal was dried with paper points, medicated with $Ca(OH)_2$ paste in camphorated paramonochlorophenol and glycerin, and placed in the canals by means of lentulo spiral fillers. A radiograph was taken to ensure proper placement of the paste in the canal, and the access cavity was filled with at least 4-mm thickness of temporary cement (Coltosol; Coltène/Whaledent Inc, Cuyahoga Falls, OH).

The second appointment was scheduled 1 week later. At this time, the tooth was isolated with a rubber dam, the operative field was disinfected before and after removal of the temporary cement, NaOCl was neutralized, and a sterility control sample was taken. All these procedures were carried out as outlined previously. The $Ca(OH)_2$ paste was rinsed out of the canal by using sterile saline solution and the master apical file. The root canal walls were gently filed, and a postmedication sample (S3) was taken from the canal as described for S1. The canal was filled with gutta-percha and Sealer 26 (Dentsply, Petrópolis, RJ, Brazil) by using cold lateral compaction, the tooth was temporized with glass ionomer cement, and a permanent restoration was planned. All clinical procedures were conducted by one experienced endodontist (J.C.P.).

High-performance Liquid Chromatography Analysis

Detection of SCFAs was performed directly in clinical samples. Fifty-microliter aliquots of each sample were diluted in 5 mmol/L H_2SO_4 at 1:2 ratio, followed by centrifugation at 10,000 rpm/10 min. Dilution of clinical samples was needed to reduce possible interferences related to the injection of complex samples in high-performance liquid chromatography (HPLC) and to keep samples at the detection limit, because they were highly concentrated and generated very intense peaks.

Analysis were conducted in HPLC equipped with DGU-20A5 degasser, LC-20AT liquid chromatograph, SIL-20A auto sampler, CTO-20A column oven, RID-10A refractive index detector, SPD-M20 A diode array detector, FRC-10A fraction collector, and CBM-20A communications bus module (Shimadzu, Kyoto, Japan). Aminex HPX-87H column (300×7, 8 mm; Bio-Rad Laboratories Ltd, Hercules, CA) was used with a mobile phase composed of 5 mmol/L H_2SO_4 and a flow rate of 0.6 mL/min. Total run time was 30 minutes, and the RID was used for the analysis. The column oven was set to 50°C. Commercial standards of the analyzed fatty acids (Sigma-Aldrich, São Paulo, SP, Brazil) were injected before the sample analysis. A total of 20 μL of each sample and standards was injected in the HPLC. Negative controls consisted of HPLC ultrapure water. Retention time and detection limits for each fatty acid targeted are depicted in Table 1. Experiments were run in duplicate.

Real-time PCR Analysis

DNA was extracted from a volume of 50 μL of each clinical sample by using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA), following the protocol recommended by the manufacturer. DNA extracts served as templates in a presence/absence real-time PCR assay for the detection of selected endodontic pathogens that are potential producers of the SCFAs targeted. The 16S rRNA gene-based PCR primers were specific for *Fusobacterium nucleatum*, *Parvimonas micra*, *Porphyromonas endodontalis*, *Pseudoramibacter alactolyticus*, members of the *Dialister* and *Streptococcus* genera, and the Actinobacteria phylum. Universal bacterial 16S rRNA gene-based primers were also used to serve as controls. PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on an ABI 7500 real-time