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ORIGINAL ARTICLE

Periodontal and endodontic infectious/inflammatory profile in primary periodontal lesions with secondary endodontic involvement after a calcium hydroxide-based intracanal medication

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Abstract

Objective The aim of the present study was to investigate the effects of a calcium hydroxide-based intracanal medication (ICM) on periodontal and endodontic infectious/inflammatory contents and on periodontal clinical parameters in teeth with primary periodontal lesion and secondary endodontic involvement.

Materials and methods Ten patients with abnormal pulp test results and deep probing depth derived from primary periodontal disease with secondary endodontic involvement were included. Samples were collected from root canals (RC) and periodontal pockets (PP) in order to investigate the microbiological status, levels of endotoxin (LPS), cytokines, and matrix metalloproteinases (MMP), before and after ICM. PCR was used for microbiological assessment. The kinetic-chromogenic LAL assay was used for LPS quantification. Quantikine ELISA kits were used for measurement of IL-1 α, IL-1 β, TNF-α, PGE_2, MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13 levels. The statistical analyses were made using the Friedman and Wilcoxon tests ($p < 0.05$). *T* test was used to compare data on periodontal characteristics.

Results ICM did not reduce the number of microorganisms in PP and RC, except for *Fusobacterium nucleatum* in RC. There was a significant reduction in LPS, MMPs, IL-1 β, and TNF-α levels in PP after ICM. In RC, LPS, MMP13, PGE_2, and IL-1β levels remained unaltered ($p > 0.05$); however, the levels of the other MMPs and cytokines were reduced ($p < 0.05$). After 1 year of the root canal treatment, tooth mobility was significantly reduced ($p \leq 0.05$).

Conclusions The use of a calcium hydroxide-based ICM showed positive effects for periodontal treatment prognosis, as it reduced LPS, cytokine, and MMP levels in periodontal pockets.

Clinical significance Patients presenting deep probing depth and undergoing periodontal treatment for at least 6 months, with no positive response to periodontal therapy, might benefit with the endodontic treatment.

Keywords Endodontics · Periodontal disease · Calcium hydroxide · Microorganisms · LPS · Cytokines · Metalloproteinases · PCR

Introduction

Periodontal and pulp tissues are embryonically, anatomically, and functionally interrelated. Ectomesenchymal cell proliferation results in dental papilla and follicle formation, which are the embryonic precursors of the pulp and periodontium, respectively [1]. Anatomical features, such as the apical foramen, lateral canals, and dentinal tubules, are the main pathways of communication between pulp and periodontal tissues [2], allowing for the transmission of pathological irritants between them.

Accumulation of dental biofilm is the cause of both dental caries and periodontal disease. However, the effect of periodontal disease on the pulp is not so well established. Classic reports suggest that periodontal disease has no effect on pulp vitality as long as the main canal is not involved [3]. Nevertheless, with time, periodontal disease can have a degenerative effect on the pulp, including an increase in calcifications, fibrosis, and collagen resorption, as well as a direct inflammatory effect [3].

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Endodontic-periodontal lesions have been described as destructive lesions resulting from harmful stimuli found in varying degrees in both pulp and periodontal tissues [4]. Combined endodontic-periodontal lesions include pulp disease with secondary periodontal involvement (primary pulpal lesion), periodontal disease with secondary endodontic involvement (primary periodontal lesion), and true combined lesions [4].

Teeth with primary endodontic lesions presenting secondary periodontal involvement and teeth with true combined lesions have necrotic pulp tissues, reason why endodontic treatment is indicated [3]. Conversely, teeth with primary periodontal disease presenting secondary pulpal involvement frequently show pulp vitality and the endodontic treatment would not be clearly indicated. This is because, even if pathological changes occur in the pulp tissue influenced by a destructive periodontal disease, the pulp usually respond to the sensibility tests and does not undergo a degenerative process, as long as the main canal is not affected or the main blood supply is not compromised [3, 4].

The similar composition of microorganisms [5] and of the cell infiltrates [6] in both sites suggests the existence of some communication between pulp and periodontal tissues. Thus, possible cross-contamination between pulp and periodontal tissues may be inferred.

Accumulated evidence indicates that the tissue destruction in endodontic and periodontal infections is due to host-derived cytokines induced by lipopolysaccharides [7] (LPS/endotoxins), acting as the major components of the outer leaflet of the outer membrane of gram-negative bacteria [8]. It has been reported the influence of inflamed/infected periodontal/endodontic content in cytokines release [9]. These inflammatory mediators, such as interleukin (IL)-1β, tumor necrosis factor α (TNF-α), and prostaglandin E_2 (PGE_2), have been also detected in periapical tissues and root canal exudates [9]. Proinflammatory cytokines are able to stimulate, either directly or indirectly, the release of matrix metalloproteinases (MMPs) in the periapical/periodontal region, maintaining a persistent inflammatory process [10, 11]. MMPs are deeply involved in the pathogenesis of pulp, periodontal, and periapical tissue destruction [11].

The best and safest method to clean/decontaminate the root canal is through the careful removal of its content by means of instrumentation, irrigation, and aspiration [(chemo-mechanical preparation (CMP)] [5]. However, even though CMP can remove a large proportion of intracanal bacterial populations, bacteria may persist on unprepared dentinal walls and in isthmi, ramifications, recesses, and dentinal tubules. For this reason, application of an intracanal medicament (ICM) after CMP for a given period of time between appointments may be necessary to improve disinfection. The efficacy of the ICM is dependent on the antimicrobial activity of the agent used, the time it remains in the canal, its application in the entire extent of the prepared canal, and the susceptibility of the species involved in the root canal infection [12].

Calcium hydroxide is widely used as ICM due to its high pH, its ability to stimulate mineralization, its antibacterial properties, and its tissue-dissolving capability [2]. The use of an ICM could act not only inside the canal, but also in the external root surface, since the root canal space can be considered a reservoir for the local delivery of antimicrobials. This approach could be an adjuvant to the periodontal treatment [2].

Therefore, the aim of the present study was to investigate the effects of a calcium hydroxide-based ICM on periodontal and endodontic infectious/inflammatory contents and on periodontal clinical parameters in teeth with primary periodontal lesion and secondary endodontic involvement.

Materials and methods

The present study was approved by the Research Ethics Committee of the Piracicaba Dental School. All patients signed an informed consent form for their participation in the study. The research was conducted according to Gomes et al. [5].

Subject population

Ten subjects presenting primary periodontal lesion and secondary endodontic involvement, who were treated at the Periodontics Outpatient Clinic of the Piracicaba Dental School, State University of Campinas—UNICAMP, were included in this study.

Primary periodontal lesion with secondary endodontic involvement was defined as the presence of periodontal pockets ≥ 6 mm; abnormal pulp response to the thermal vitality tests, when compared to the surrounding/similar teeth (control); clinical and radiographic evidence of periodontal disease; and absence of apical periodontitis. Pulp status was assessed through thermal vitality tests and cavity test. The apical status was determined through the observation of clinical signs, such as tenderness to percussion and pain on palpation.

The inclusion criteria were as follows: (a) patients undergoing periodontal treatment, with follow-up of at least 6 months with no response to the treatment, and with at least one side of the tooth with periodontal pockets equal to or greater than 6 mm; (b) the teeth involved should show extensive bone loss on proximal surfaces; and (c) the response of the pulp to the thermal tests should be abnormal [13]. The exclusion criteria were as follows: (a) teeth with caries or restoration beyond the enamel level or indication of contamination of the pulp tissue through the crown pathway, (b) teeth with cracks or with any type of resorption, (c) patients who

had received antibiotic treatment in the preceding 3 months and reported systemic disease. Smoking was not a criterion, but all patients were non-smokers.

Sampling procedure and clinical data collection

For degradation of preexisting LPS, all the materials used in the experiment were sterilized with cobalt 60 gamma radiation (20 KGy for 6 h). Three different samples were collected from each site for investigation of the levels of LPS, microorganisms, and proinflammatory cytokines/MMPs.

Periodontal samples

Before sampling procedures, the area of collection was isolated with cotton rolls to avoid saliva contamination. The crown was carefully cleaned with sterile gauze to remove supragingival biofilm. Periodontal samples were collected from the deepest periodontal pocket of the involved tooth before root canal treatment and after 30 days of ICM, according to Gonçalves et al. [14] and Gomes et al. [5]. The endotoxin was sampled by introducing sterile/pyrogen-free paper points (size #15; Dentsply-Maillefer, Balaigues, Switzerland) into the full length of the periodontal pocket and retained in position during 60 s [9]. Immediately afterwards, the paper point was placed on a pyrogen-free glass and frozen at − 20 °C for future Limulus Amebocyte Lysate (LAL) assay.

In order to investigate microbial content, periodontal biofilm samples were also collected from the deepest periodontal pocket of the involved tooth, using three consecutive sterile paper points, which were kept in place for 60 s and then pooled in a sterile tube containing 1 mL of VMGA III transport medium, which was frozen at − 20 °C until processed for DNA extraction by molecular method (16S rDNA).

Later on, another paper point was also introduced into the full length of the periodontal pocket and retained in position during 60 s and frozen at − 80 °C for future proinflammatory cytokine and MMP measurements.

The following periodontal clinical parameters were assessed at baseline (prior to treatment), after ICM and after 1 year of the root canal treatment by a calibrated examiner: pocket depth, bleeding on probing, clinical attachment level, and plaque index. All measurements were performed using a periodontal probe at six sites per tooth and recorded. Periapical and interproximal radiographs were obtained from all patients at baseline for diagnostic purposes only. A second periodontal sample was taken in a similar way after 30 days of ICM.

Endodontic samples

The method used for disinfecting the operative field has been previously published [5]. Initially, the teeth were isolated with a rubber dam. The crown and surrounding structures were disinfected with 30% H_2O_2 (volume/volume [*V*/*V*]) for 30 s, followed by 2.5% sodium hypochlorite (NaOCl) for the same period of time and then inactivated with 5% sodium thiosulfate. The disinfection of the tooth surface was monitored by taking a swab sample from both external and internal surfaces of the crown and its surrounding structure area around and streaking it on blood agar plates. Plates were incubated aerobically and anaerobically [5].

For preparation of the access cavity, a sterile/LPS-free, high-speed diamond bur (KG-Sorensen, Barueri, SP, Brazil) was used in conjunction with manual irrigation with sterile saline. Before penetration into the pulp chamber, the access cavity was disinfected according to the protocol described above. A new sterile/LPS-free bur was used to access the canal. In each case, even in multi-rooted teeth, a single root canal was sampled in order to confine the microbial evaluation to a single ecological environment. The criterion used to choose the canal to be investigated in multi-rooted teeth was the canal related to the deepest periodontal pocket [5].

Root canal length was determined by preoperative radiograph and confirmed by an apex locator (Novapex; Forum Technologies, Rishon le-Zion, Israel) and then the first endotoxin sample was taken by introducing sterile/pyrogen-free paper points (size #15; Dentsply Maillefer, Balaigues, Switzerland) into the full length of the canal and retained in position during 60 s [5, 9, 10]. Immediately thereafter, the paper point was placed on a pyrogen-free glass and frozen at − 20 °C for future LAL assay. When tooth had a narrow root canal interfering with the penetration of the paper point, patency was accomplished through minimal instrumentation with a #10 file (Dentsply, Maillefer, Ballaigues, Switzerland). The file had its cable removed and was transferred to a pyrogen-free glass and frozen at − 20 °C for future LAL assay.

In order to detect microorganisms in the root canals, particularly Gram-negative bacterial species, and to support the presence of root canal LPS, microbial samples were collected by introducing sterile paper points (size #15; Dentsply Maillefer, Balaigues, Switzerland) into the full length of the canal and retained in position during 60 s and then pooled into a sterile tube containing 1 mL of VMGA III transport medium, which was frozen at − 20 °C until processed for DNA extraction by molecular method (16S rDNA). When a dry canal was identified, an additional sterile paper point moistened in sterile saline was used to ensure sample acquisition. When a tooth had a narrow root canal that hindered the penetration of the paper point, patency was obtained through minimal instrumentation with a #10 file (Dentsply Maillefer). The file had its cable removed and was transferred to the tube containing the transport medium and subsequently stored at − 20 °C until processed [5].

Later, another paper point was introduced 1 mm beyond the apex until it reached the periapical tissues and was maintained

in position for 60 s. Next, the paper point was placed in a sterile tube for enzyme-linked immunosorbent assay (ELISA) and then frozen at − 80 °C for further proinflammatory cytokine and MMP measurements.

Afterwards, the root canals were prepared with Mtwo instruments (VDW, Munich, Germany) using permanent rotation (300 rpm). Mtwo instruments (10/.04, 15/.05, 20/.06, 25/.06, 30/.05, 35/.04, and 40/.04) were used to the full length of the root canal using a single-length technique, with gentle in-and-out movements, gradually forcing them apically [15]. The use of each instrument was followed by irrigation.

The canals were irrigated between each file with 1 mL 2% chlorhexidine gel (Endogel, Itapetininga, SP, Brazil) and then immediately rinsed with 5 mL of sterile saline. The chlorhexidine (CHX) gel consisted of gel base (1% natrosol) and CHX gluconate at pH 7.0. Natrosol gel (hydroxyethyl cellulose) is a non-ionic, highly inert, and water-soluble agent [16]. All irrigation procedures were performed with a 5-mL syringe and a 27-gauge needle (Ultradent Products, South Jordan, UT, USA).

After root canal preparation, CHX was inactivated with 5 mL of a solution containing 5% Tween 80 and 0.07% (*v*/*v*) lecithin for 1 min, which was removed with 5 mL of saline solution. Removal of the smear layer was performed with 3 mL of 17% ethylenediaminetetraacetic acid (EDTA) solution for 3 min, followed by a final flush with 5 mL of saline solution.

Subsequently, the canals were filled with freshly prepared paste of $Ca(OH)_2$ associated with 2% chlorhexidine gel (1:1). The paste was inserted into the canals by using a sterile/LPS-free Lentulo bur (Dentsply Maillefer). Special attention was paid to completely filling the root canal with the medication. A cotton pellet was used to condense the paste at the canal orifice level, and a periapical radiograph was taken to ensure the quality of the root canal filling. Next, the access cavities were sealed with light-cured resin composite (Filtek Z250 XT, 3 M Dental Products, St Paul, MN, USA).

After 30 days of ICM, the canals were aseptically accessed under rubber dam isolation according to the disinfection protocol, as previously described. $Ca(OH)_2$ was removed with a K-file size 40, 0.02 taper against the lateral walls of the root canal, and copious irrigation with saline solution. To help in the removal of traces of $Ca(OH)_2$, an irrigation with 3 mL of 17% EDTA for 3 min, followed by a final flush with 5 mL of saline solution was performed [17]. The second sampling procedures were then undertaken.

Before root canal obturation, final instrumentation was performed using three files larger than the last one used for apical preparation. Immediately afterwards, the root canals were rinsed with neutralizing substance, followed by final irrigation with 5 mL of saline solution. The root canals were irrigated with 3 mL of 17% EDTA for 3 min, followed by a final flush with 10 mL of saline solution. Finally, the root canals were dried and then filled using gutta-percha and Endométhasone N sealer (Septodont, Maidstone, UK) with warm vertical condensation and backfill. The access cavities were restored with 2 mm of Cavit (3 M Dental Products) and resin composite in all cases. The same operator performed all procedures in a standard manner, and all the patients continued the periodontal treatment after endodontic procedures had been completed.

Microbiological assessment (qualitative polymerase chain reaction amplification of the 16S rDNA gene)

The method used for molecular assessment has been previously published [9]. The reference bacterial strains used in this study were purchased from the American Type Culture Collection (ATCC) and are listed as follows: *Fusobacterium nucleatum* (ATCC 25586), *Parvimonas micra* (ATCC 15794), *Prevotella intermedia* (ATCC 49046), *Porphyromonas gingivalis* (ATCC 33277), *Tannerella forsythia* (ATCC 43037), and *Treponema denticola* (ATCC 35405).

DNA extraction

Microbial DNA from periodontal and endodontic samples, as well as from ATCC bacteria, was extracted and purified with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The DNA concentration (absorbance at 260 nm) was determined using a spectrophotometer (Nanodrop 2000, Thermo Scientific, Wilmington, DE, USA).

PCR assay

The PCR (with amplification of the 16S rDNA gene) was performed in a thermocycler (My-Cycler; Bio-Rad, Hercules, CA,USA) in a total volume of 25 μL containing 2.5 μL of 10X Taq buffer (1X) (Invitrogen, Eugene, OR, USA), 0.5 μL of deoxyribonucleoside triphosphate mix (25 μmol/L of each deoxyribonucleoside triphosphate [dATP, dCTP, dGTP, and dTTP] (Invitrogen, Eugene, OR), 1.25 μL of 25 mmol/L MgCl2, 0.25 μL of forward and reverse universal primers (0.2 mmol/L; Invitrogen, Eugene, OR), 1.5 μL of sample DNA (1 μg/50 μL), 1.5 μL of Taq DNA polymerase (1 U, Invitrogen, Eugene, OR, USA), and 17.25 μL of nuclease-free water.

Forward and reverse primer sequences as well as PCR cycling parameters are listed in Table 1. Negative controls corresponded to the reaction mixture without DNA. The detection of either positive or negative target species was based on the presence of clear bands of expected molecular size.

Table 1 PCR primer pairs and cycling parameters used for the detection of target bacterial species in periodontal pockets and root canals

Target bacteria	Primer pairs (5′-3′)	Amplicon size	Cycles
Universal (16rDNA)	F: TCC TAC GGG AGG CAG CAG T R: GGA CTA CCA GGG TAT CTA ATC CTG TT	466 bp	Initial denaturation at 95 °C for 10 min and 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and a final extension step at 72 °C for 25 s.
T. forsythia	F: GCG TAT GTA ACC TGC CCG CA R: TGC TTC AGT GTC AGT TAT ACC T	641 bp	Initial denaturation at 95 °C for 1 min and 36 cycles of 95 °C for 30 s, 60 °C for 1 min, and a final step at 72 °C for 2 min.
T. denticola	F: TAA TAC CGA ATG TGC TCA TTT ACA T R: TCA AAG AAG CAT TCC CTC TTC TTC TTA	316 bp	Initial denaturation at 95 °C for 2 min and 36 cycles of 94 °C for 30 s, 60 °C for 1 min, 72 °C for 2 min and a final step at 72 °C for 10 min.
P. micra	F: AGA GTT TGA TCC TGG CTC AG R: ATA TCA TGC GAT TCT GTG GTC TC	207 bp	Initial denaturation at 95 °C for 2 min and 36 cycles of 94 °C for 30 s, 60 °C for 1 min, 72 °C for 2 min and a final step at 72 °C for 10 min.
F. nucleatum	F: AGT AGC ACA AGG GAG ATG TAT G R: CAA GAA CTA CAA TAG AAC CTG A	1000 bp	Initial denaturation at 95 °C for 5 min and 30 cycles of 94 °C for 30 s, 40 °C for 1 min, 72 °C for 2 min and a final step at 72 °C for 10 min.
P. gingivalis	F: AGG CAG CTT GCC ATA CTG CG R: ACT GTT AGC AAC TAC CGA TGT	404 bp	Initial denaturation at 95 °C for 2 min and 36 cycles of 94 °C for 30 s, 60 °C for 1 min, 72 °C for 2 min and a final step at 72 °C for 2 min.
P. intermedia	F: TTT GTT GGG GAG TAA AGC GGG R: TCA ACA TCT CTG TAT CCT GCG T	575 bp	Initial denaturation at 95 °C for 2 min and 36 cycles of 94 °C for 30 s, 58 °C for 1 min, 72 °C for 2 min and a final step at 72 °C for 2 min.

Determination of endotoxin concentration (kinetic chromogenic LAL assay)

The method used for measuring the endotoxin levels has been previously published [9, 10]. The turbidimetric test (BioWhitaker, Inc., Walkersville, MD, USA) was used to measure endotoxin concentrations in the root canals using the LAL assay. First, as a parameter for the calculation of the amount of LPS in root canal samples, a standard curve was plotted using endotoxins supplied in the kit with a known concentration (100 EU/mL) and its dilutions with the following final concentrations (i.e., 0.01, 0.10, 1, and 10 EU/mL) according to the manufacturer's instructions.

Test procedure

All reactions were performed in duplicate to validate the test. A 96-well microplate (Corning Costar, Cambridge, MA, USA) was used in a heating block at 37 °C and maintained at this temperature throughout the assay. First, the endotoxin samples were suspended in 1 mL of LAL water supplied with the kit and agitated in a vortex mixer for 60 s and serially diluted to 10^{-1}. Immediately thereafter, 100 μL of the blank followed the standard endotoxin solutions in concentrations (i.e., 0.01, 0.10, 1, and 10 EU/mL) and 100 μL of the samples was added in duplicate to the 96-well microplate. The test procedure was performed according to the manufacturer's instructions. The absorbance of endotoxin levels was measured individually using an enzyme-linked immunosorbent assay plate reader (Ultramark, Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 340 nm. Because the mean absorbance value of the standards was directly proportional to the concentration of LPS, the endotoxin concentration was determined from the standard curve.

Cytokine and matrix metalloproteinase measurements

The method used for measuring the cytokine and matrix metalloproteinase has been previously published [9]. Specific Quantikine ELISA kits (R&D Systems, Minneapolis, MD, USA) were used for measurement of cytokines [interleukins (IL-1α, IL-1β), tumor necrosis factor α (TNF-α), prostaglandin E_2 (PGE_2)], and matrix metalloproteinases (MMPs) (MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13).

A monoclonal antibody specific to each cytokine/MMP was pre-coated onto a 96-well microplate. Standards and sample solutions were added to the ELISA well plate, and any specific-kit cytokine/MMP present was bound by the immobilized antibody. After washing any unbound substances, an enzyme-linked polyclonal antibody, specific to each cytokine/MMP, was added to the wells. Following the wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed proportionally to the amount of specific cytokine/MMP bound in the initial step, following the manufacturer's instructions. Color development was interrupted, and color intensity was determined using a microplate reader at 540 nm. The

Table 2 Mean values and standard deviation of periodontal characteristics with statistical significance

	Initial	1-year follow-up
Mobility	2.10 (0.74)^{A}	1.10 (0.32)^{B}
Probing depth	8.60 (2.12)^{A}	6.60 (2.32)^{B}
Clinical attachment	10.60 (1.71)^{A}	8.80 (1.75)^{B}

Different letters in the same row indicate statistically significant values (*T* test; $p < 0.05$)

cytokine/MMP concentration in each sample was determined from the standard curve.

Statistical analysis

The data were analyzed using SPSS for Windows (SPSS Inc., Chicago, IL, USA) and checked for normal distribution using the Kolmogorov-Smirnov test. *T* test was used to compare data on periodontal characteristics [initial (baseline) vs 1-year follow-up]. PCR data, comparing the first (initial) and second (post-medication) samples, were evaluated using the Friedman test ($p < 0.05$). LPS, cytokines, and MMPs, before and after medication, were evaluated using the Wilcoxon signed-rank test ($p < 0.05$).

Results

Clinical features

Ten adult patients (5 males and 5 females, aged 31 to 53 years; mean age of 41.1 years) presented the characteristics needed for this study, named abnormal response to the thermal vitality tests, presence of periodontal pockets, loss of attachment, and radiographically visible bone loss. All teeth used in this study presented markedly narrow root canals as the effect of periodontal disease on the pulp.

All of the ten teeth examined had abnormal response to the thermal vitality tests, positive pulp cavity tests, and periodontal pockets equal ($n = 3$) to or greater ($n = 7$) than 6 mm. The dental groups consisted of incisors (5/10) and molars (5/10). Five maxillary and five mandibular teeth were included in this study.

Regarding the periodontal characteristics, moderate/severe mobility was recorded in all cases in the initial samplings. Probing depth and clinical attachment averaged 8.6 and 10.4 mm, respectively. No teeth presented bleeding on probing. One month later, before the removal of the ICM, no difference was noted for the periodontal features. After 1 year of the root canal treatment and with the maintenance of the periodontal treatment, tooth mobility was significantly reduced ($p < 0.05$). Probing depth and the clinical attachment were significantly reduced to 6.6 and 8.8 mm, respectively ($p < 0.05$). Table 2 shows information on the initial and 1-year follow-up periodontal characteristics.

Microbial analysis (PCR)

At least one of the target strains was detected in all periodontal pockets. In relation to the root canals, no bacteria were detected in three out of ten cases.

Figure 1 shows the number of cases in which microorganisms were detected in root canals and periodontal pockets before and after ICM. In periodontal samples, *P. micra*, *T. forsythia*, and *P. gingivalis* were the most detected microorganisms. ICM did not significantly reduce the detection of microorganisms in periodontal pockets.

P. intermedia was not detected in endodontic samples. *F. nucleatum* was the most prevalent microorganism, and its detection was significantly reduced after the use of the ICM. For the other bacterial strains, no significant reduction was observed after ICM.

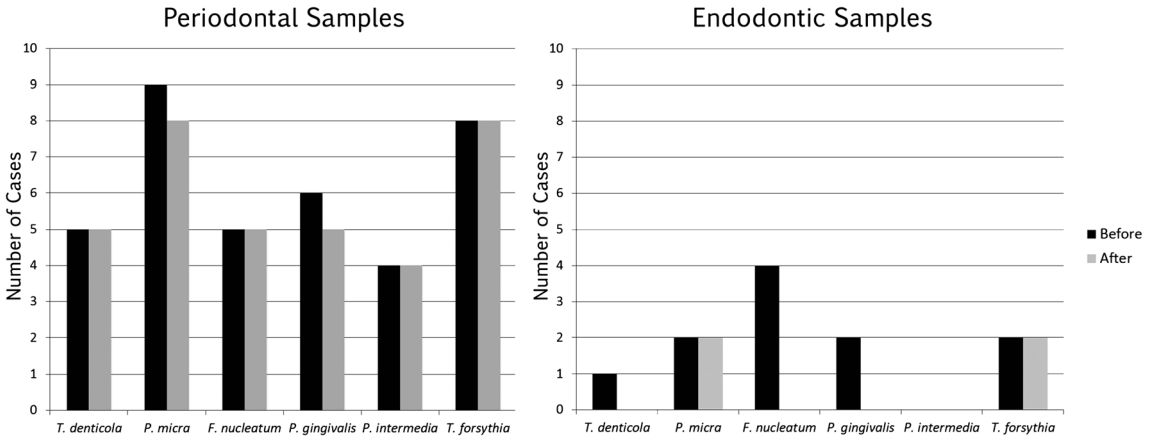

Fig. 1 PCR findings before and after medication for periodontal and endodontic samples

Endotoxin findings

For periodontal pockets, the median values of LPS before and after ICM were 223 and 40.35 EU/mL, respectively. The use of ICM significantly reduced LPS levels ($p < 0.05$).

In the root canals, differences were also observed in LPS levels before and after the use of ICM with the median values of 0.1 and 0.01 EU/mL, respectively ($p < 0.05$) (Table 3).

Cytokines findings

There was a significant reduction in IL-1 β and TNF-α levels in periodontal pockets after ICM. Regarding the endodontic samples, a significant reduction was observed in IL 1-α, IL 1-β, and TNF-α (Table 4).

MMPs findings

In relation to MMP levels, there was a significant reduction of all MMPs in periodontal samples after medication. For the endodontic samples, there was a significant reduction in MMPs -2, -3, -8, and -9. However, no significant difference was observed for MMP-13 after ICM (Table 5).

Discussion

The novelty of the present study lies in the fact that it has explored and delineated the infectious and inflammatory profiles of teeth with primary periodontal disease and secondary endodontic involvement. The limited literature on this theme and also the difficulties involving the treatment of advanced bone loss were encouraging factors for the development of this work.

There is a widespread consensus that primary periodontal lesions should be managed without devitalizing a tooth, only with periodontal therapy [4]. Endodontic therapy usually is not recommended if the pulp maintains its vitality and if there is no prosthetic indication [4]. However, studies have shown that teeth with chronic periodontitis led to changes in the histopathological features of the pulp, with progressive involvement, which could lead to a pulpal necrosis [3]. Untreated endodontic infection may influence the outcome of periodontal wound healing, increasing the risk of attachment loss [4]. Moreover, roots of periodontally compromised teeth can act as bacterial reservoirs from which recontamination of mechanically treated root surfaces can occur, as well as infection of the dental pulp [18].

Table 3 Median values of endotoxin levels (pg/mL) before and after medication for endodontic and periodontal samples

Samples	Before	After
Periodontal	$223^{A,a}$	$40.35^{B,a}$
Endodontic	0.1^{Ab}	0.01^{Bb}

Statistical analysis in the row, comparing before and after medication. Different capital letters in the same row and different lower case letters in the same column indicate statistically significant values (Wilcoxon signed-rank test; $p < 0.05$)

Clinical features

Endodontic treatment was indicated by periodontists for improvement of marginal healing and repair [4, 19]. Due to the difficulties in finding the patients who met the inclusion criteria, ten out of 140 patients were involved in this research. Pulp tissue with a degenerative aspect and an abnormal bleeding was observed during coronal access. This may be explained by the fact that this tissue was exposed to a long-lasting stimulus at a low intensity, which caused a slow and asymptomatic chronic degenerative reaction [4].

Tooth mobility, probing depth, and clinical attachment are the most commonly used and informative parameters to estimate the severity of soft tissue destruction and disease progression, as well as the response to periodontal treatment [20]. After 1 year of the root canal treatment, all the three periodontal characteristics evaluated in this study showed improvement.

Microbiological findings

It has long been accepted that as long as the pulp is vital, there is a host response mechanism in function that prevents the bacteria invading deeper into the pulp tissues. When exposed to caries or by trauma, vital pulps may have bacteria on the surface, but usually in low numbers and without a deep penetration into vital pulp tissues [21]. The presence of a long-standing periodontal disease causes degenerative changes in the pulp [3], and the partial necrosis of the pulp may result in a positive pulp test, especially in a multi-rooted tooth [4]. In our study, bacteria were detected in pulps with a positive pulp test, indicating that the vital pulp is not a sterile environment as previously believed.

Even considering the limitations of sampling biofilm using paper points, this approach is a well-established protocol based on that all "floating" species present in the main canal/periodontal pocket are or were members of the endodontic/periodontal biofilm.

In the present study, we used PCR to detect microorganisms commonly observed in endodontic-periodontal infections, including the red complex, which consists of three bacteria associated with severe forms of periodontal disease (*Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*) [22] and the other three bacteria equally related to endodontic infection (*Fusobacterium nucleatum*, *Prevotella intermedia*, and *Parvimonas micra* [8]. PCR is a highly sensitive method, able to detect very small quantities of

Table 4 Median values of Cytokines (pg/mL) before and after medication for endodontic and periodontal samples

Samples	PGE2		IL-1β		IL-1α		TNF-α	
	Before	After	Before	After	Before	After	Before	After
Periodontal	69.7 ^{A}	70.5 ^{A}	29.5 ^{A}	11.3 ^{B}	86.1 ^{A}	76.6 ^{A}	19.6 ^{A}	6.3 ^{B}
Endodontic	87.8 ^{A}	73.8 ^{A}	7.6 ^{A}	0.2 ^{B}	30.3 ^{A}	2.8 ^{B}	13.0 ^{A}	3.8 ^{B}

Statistical analysis in the row, comparing before and after medication. Different letters^{A,B} indicates values statistically significant (Wilcoxon signed-rank test; $p < 0.05$)

bacterial DNA, and also slow-growing and fastidious bacteria, such as the ones investigated here, proving to be effective in the present study.

At least one bacterial strain was detected in periodontal pockets in all cases. *P. micra*, *T. forsythia*, and *P. gingivalis* were the most prevalent microorganisms.

In endodontic samples, bacteria were detected in 7/10 root canals. *F. nucleatum* was the most prevalent microorganism in the root canals. The red complex was present in periodontal pockets before and after medication in three out of ten cases, but it was not found in the root canals.

T. forsythia was the most frequent bacterium belonging to the red complex detected in periodontal pockets (PP) and root canals (RC), before (8/10 PP; 2/10 RC) and after medication (8/10 PP; 2/10 RC). *T. forsythia* is an anaerobic gram-negative rod, belonging to the phylum *Bacteroidetes*, usually associated with various forms of the disease, including gingivitis and chronic and aggressive periodontitis [23]. Several studies have also related *T. forsythia* in the progression of clinical attachment loss associated with periodontitis [23]. Furthermore, they were found in combined endo-periodontal lesions [5].

P. gingivalis was detected in periodontal pockets before (6/10) and after medication (5/10). It was detected in the root canals (2/10) only before medication. It is a small, Gram-negative anaerobic rod-shaped bacterium, belonging to the phylum *Bacteroidetes*, that possesses a large number of putative virulence determinants. It is involved in the pathogenesis of periodontitis and is a member of more than 500 bacterial species that live in the oral cavity. It is highly detected in root canal infection and in endodontic abscesses [22].

T. denticola was detected in 5/10 periodontal pockets and in only one root canal, before medication, and in 5/10 and in 1/10 of the periodontal pockets and root canals, respectively, after medication. *Treponema* species are Gram-negative, motile, strictly anaerobic helically shaped bacteria, belonging to the phylum *Spirochaetae*. They are related to a variety of oral diseases, including root canal infections and periradicular abscess samples [22].

F. nucleatum was detected in 5/10 periodontal pockets before and after medication and in 4/10 root canals only before medication. *Fusobacterium* spp. are Gram-negative, non-motile, non-spore forming, obligate anaerobic rods belonging to the phylum *Fusobacteria*. *Fusobacterium nucleatum* and *Fusobacterium necrophorum* are the most common isolates in human infections, in different body sites such as in the skin or subcutaneous tissue, throat, female genital tract, and in the lungs. These species are twice as frequent found in symptomatic than in asymptomatic endodontic infections [24].

P. intermedia was detected in 4/10 periodontal pockets before and after medication, but it was not detected in the root canals. Our result is in line with Perez et al. [25], who showed that this microorganism does not have the potential to penetrate into the dentinal tubules. Bacterial penetration is related to the size, adhesive properties, and mobility of the bacteria [26]. *Prevotella* spp. are obligate anaerobic gram-negative rod-shaped bacteria, belonging to the phylum *Bacteroidetes*. *P. intermedia* is associated with periodontal disease, periapical periodontitis, and noma (an acute gangrenous disease) [27]. *P. intermedia* is also present at the diseased site of periapical periodontitis and shows a significantly higher detection ratio in symptomatic sites, respiratory tract, and can be associated with cystic fibrosis and chronic bronchitis [27].

P. micra was detected in periodontal pockets before (9/10) and after medication (8/10). It was detected in 2/10 root canals before and after medication. *P. micra* is a fastidious,

Table 5 Median values of MMP level (pg/mL) before and after medication for endodontic and periodontal samples

Samples	MMP-2		MMP-3		MMP-8		MMP-9		MMP-13	
	Before	After	Before	After	Before	After	Before	After	Before	After
Periodontal	732.4 ^{A}	176.0 ^{B}	351.5 ^{A}	67.0 ^{B}	192.5 ^{A}	16.3 ^{B}	498.2 ^{A}	221.8 ^{B}	62.3 ^{A}	5.6 ^{B}
Endodontic	0.2^{A}	0.1^{B}	0.2 ^{A}	0.1 ^{B}	0.3 ^{A}	0.1 ^{B}	0.2 ^{A}	0.1 ^{B}	0.1 ^{A}	0.1 ^{A}

Statistical analysis in the row, comparing before and after medication. Different letters^{A,B} indicates values statistically significant (Wilcoxon signed-rank test; $p < 0.05$)

anaerobic, Gram-positive coccus, belonging to the phylum *Firmicutes*, which is found in normal human oral and gastrointestinal microbiomes. It has been frequently isolated/detected in primary endodontic infections [28] and in found in combined endo-periodontal lesions, at the both sites [5]. A positive association was observed between *P. micra* and the presence of spontaneous pain [28].

LPS findings

Regarding the endotoxin findings, their levels were higher in periodontal pockets than in root canals. The detoxifying activity during CMP is attributed to the mechanical action of the instruments against dentin walls and the flow and back-flow of the irrigants, irrespective of which substance was used [5].

We selected chlorhexidine (CHX) as an auxiliary chemical substance to be used during CMP, not only due to its antimicrobial action and its adsorption to dental hard tissues, but also because instrumentation would be done in the full extension of the root canal, with foraminal patency and enlargement [5]. The foramen enlargement maneuver could increase the risk of irrigant extrusion through the apex, favoring the use of CHX, for being less irritating to the periapical tissues than NaOCl [29]. Furthermore, its lubrication and rheological properties are advantageous in the instrumentation of the narrow canals, commonly found in teeth with endodontic-periodontal lesions. The rheological property of the gel presentation keeps the debris in suspension, making their removal easier and decreasing the smear layer formation [29]. Immediately after each instrumentation, 5 mL of distilled water was used to irrigate the canal in order to remove traces of CHX [29].

In periodontics, CHX gel is also used as local delivery antimicrobial agent for treatment of pockets equal or greater than 5 mm, when the inflammation is still present, following conventional therapies, such as scaling and root planning [5].

ICM significantly reduced LPS levels from both sites. LPS level was low in the initial endodontic samples and even lower after the use of the ICM, agreeing with the findings of Sousa et al. [30], who used the same medication for 30 days. Previous studies from our group showed that $Ca(OH)_2$+ 2% CHX gel used for 7 [16] or 14 days [31] did not improve the disinfection achieved by CMP in vivo. The longer application of ICM (30 days) used in the present study and in Sousa's et al. [30] seemed to be important for improving the LPS detoxifying activity of $Ca(OH)_2$ in vivo. Moreover, a 30-day period may allow a significant contact time of the ICM not only inside the canal, but also in the external root surface [2]. The LPS reduction at both sites can be explained by the fact that a $Ca(OH)_2$-based medication can detoxify LPS [32].

Cytokines and MMP findings

The inflammatory mediators evaluated in this study have been detected in both periodontal and periapical tissues and play a critical role in the bone resorption process [19]. IL-1α plays a critical role in protecting the body from external invaders such as bacteria and viruses, and it is also involved in bone resorption [33]. IL-1β has been correlated with clinical signs/symptoms and greater bone resorption [14]. TNF-α stimulates the production of collagenase, PGE_2, and bone resorption-related factors [19]. PGE_2 has been reported in both painful and asymptomatic human dental pulps [34], varying in levels according to the degree of inflammation.

In the present study, PGE_2, IL-1 α, IL-1β, and TNF-α were expressed in both samples and the use of ICM significantly reduced IL-1β and TNF-α levels in periodontal samples and IL-1α, IL-1 β, and TNF-α levels in endodontic samples. PGE_2 levels were not reduced in both sites. This fact gives some insight into the state of the asymptomatic, chronically inflamed periodontal/pulp tissues [34].

IL-1α, IL-1β, and TNF-α are capable of inducing and regulating MMP expression in periodontal tissues [35]. MMPs are zinc- and calcium-requiring enzymes capable of degrading almost all extracellular matrix (ECM) and basement membrane components in normal tissue remodeling and especially in tissue-destructive diseases, including apical periodontitis [11]. Fibrillar collagens are the major components of periodontal ECM in pathological conditions. They are cleaved into smaller fragments by collagenases (MMPs − 1, − 8, and − 13) and further degraded by active gelatinases (MMPs − 2 and − 9) and other non-specific tissue proteinases [36].

In the present study, the initial levels of MMPs in periodontal samples were significantly higher than in endodontic samples, as expected, due to the presence of periodontal disease and absence of apical periodontitis. There was a significant reduction in all MMPs after ICM in periodontal samples.

In endodontic samples, there was a significant reduction of MMPs -2, -3, -8, and -9. On the other hand, no reduction in the MMP-13 levels was found, probably because the selected teeth did not present apical periodontitis. Levels of MMP-13 are intimately related to the intensity of the periapical inflammatory infiltrate [37].

During periodontal disease activity, there are periods of exacerbation or activity and periods of quiescence or inactivity. The first is characterized by loss of bone and connective tissue insertion, bleeding, gingival exudate, and presence of Gram-negative bacteria. The second is a period of remission, in which there is a proliferation of Gram-positive bacteria that stabilize the disease [38]. In the present study, the remission of periodontal disease did not occur due to the process of inactivity of periodontal disease. Clinically, during the 6 months prior to endodontic treatment, the pocket depth, attachment,

and bone loss progressively increased, characterizing a constant activity of periodontal disease at specific sites. In addition, Gram-negative bacteria were present in number and diversity. After endodontic treatment, there was a reduction of LPS, cytokines, and MMPs, probably close to physiological levels, which is very encouraging, since these factors are involved in periodontal disease. To confirm this information, further studies are required to include teeth with prosthetic indication for endodontic treatment and healthy periodontal tissues.

In conclusion, the preliminary findings in teeth with primary periodontal lesion with secondary endodontic involvement showed clinical alterations and presence of microorganisms in the root canals. The use of a calcium hydroxide-based ICM showed positive effects for periodontal treatment prognosis, as it reduced LPS, cytokine, and MMP levels in periodontal pockets.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The present study was approved by the Research Ethics Committee of the Piracicaba Dental School. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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