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Effect of root canal procedures on endotoxins and endodontic pathogens

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Background/aims: The purpose of this study was to determine the amount of endotoxin (lipopolysaccharide) and cultivable bacteria in human necrotic root canals before (S1) and after chemo-mechanical preparation using chlorhexidine (CHX) gel as auxiliary chemical substance (S2), and after 7 days of intracanal dressing (S3) in order to evaluate the anti-endotoxin and antimicrobial effects of endodontic procedures.
Method: Twenty-four teeth were selected for the present study. Chemo-mechanical preparation was performed using 2% CHX gel and three different intracanal medicaments [$Ca(OH)_2$ paste; 2% CHX gel; and $Ca(OH)_2$ + 2% CHX gel]. A quantitative chromogenic *Limulus* amoebocyte lysate assay was used to measure the amount of endotoxin. Aerobic and anaerobic techniques were used to isolate and identify bacteria, and to determine the bacterial reduction by counting colony-forming units (CFU).
Results: Endotoxins and bacteria were present in 100% of the initial samples, with endotoxin concentration ranging from 62.93 to 214.56 UE/ml and CFU ranging from 4×10^5 to 2.6×10^6. After chemo-mechanical preparation a mean endotoxin reduction of 44.4% was found. Eight (33.3%) root canals were still positive by culture analysis with a mean reduction of bacteria (CFU) of 99.96%. After 7 days of intracanal dressing, endotoxin concentration decreased by only 1.4% compared with S2, and residual bacteria were recovered by culture analysis in 13 cases (54.1%). No significant difference was found among different intracanal medicaments.
Conclusion: Relatively high values of endotoxin were still present in the root canal after chemo-mechanical preparation although the majority of bacteria were eliminated. No improvement was achieved by 7 days of intracanal dressing.

Key words: calcium hydroxide; chlorhexidine; endotoxin; microbial reduction

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Numerous investigations have demonstrated that microorganisms and their products play the primary aetiological role in the development of necrotic pulps and periapical lesions (1, 20). Studies have shown that these infections are polymicrobial, with a prevalence of obligate anaerobic bacteria, including gram-negative species of the genera *Fusobacterium*, *Porphyromonas* and *Prevotella*, and also gram-positive species of the genus *Peptostreptococcus* (10, 11).

Gram-negative bacteria possess different virulence factors that are responsible for most of the products and sub-products that are toxic to the periapical area. One of the virulence factors of gram-negative bacteria is the presence of lipopolysaccharides (LPS), which are anchored in the outer cell membrane and which function as endotoxins in the host organism (33, 46).

LPS is released during multiplication or by bacterial death causing biological effects that leads to an inflammatory reaction and periapical bone resorption. Thus, root canal treatment of teeth with pulp necrosis and periapical lesions should not only sacrifice bacteria (30), but also remove the dead cells and/or promote the inactivation of the lipid A (the toxic portion of the endotoxin).

Cleaning, shaping and thoroughly irrigating the canal with an inert solution to remove debris, and the use of an auxiliary chemical substance with antimicrobial activity, greatly reduce the number of

bacteria (3, 4). Sodium hypochlorite (NaOCl) solution is, to date, the most commonly employed root canal auxiliary chemical substance. Recently, chlorhexidine (CHX) has been recommended as an alternative to NaOCl, especially in cases of open apex, because of its biocompatibility, or in cases of allergy related to bleaching solutions (43). It has been shown, however, that because of the anatomical complexity of the root canal system it is impossible to obtain complete disinfection in all cases, even after chemo-mechanical preparation (3, 4, 29). Therefore, an intracanal dressing with an antibacterial action is sometimes required to maximize disinfection of the root canal system (3). The need for intracanal dressing increases, especially in those cases where an infection is resistant to regular treatment and therapy cannot be successfully completed because of the presence of pain or constant exudation (41). For this reason, a wide variety of intracanal medicaments have been used, such as calcium hydroxide [$Ca(OH)_2$] pastes and CHX.

Calcium hydroxide is the most commonly used intracanal medicament, with its use being recommended for the neutralization of endotoxin (28, 36, 42) and of remaining organic tissue debris (16). Furthermore, it may be used to aid in the elimination of microbes (44), and may act as a barrier against microbial ingress and cut off nutrient supply (12). This intracanal medicament has been reported to be effective against several bacteria; however, studies have demonstrated that it does not effectively eliminate enterococci (13, 14, 37, 44). Other authors have proposed the combination of $Ca(OH)_2$ with different substances to enhance their antimicrobial properties (12, 44). One example is CHX, which has been suggested for use in combination with $Ca(OH)_2$ or alone as an intracanal medicament (12, 13).

The antimicrobial properties of intracanal medicaments have been investigated previously (30, 39, 44), as well as the anti-endotoxin effects of intracanal medicaments (2, 28, 34). Furthermore, the effectiveness of different substances has been evaluated histopathologically in canine root canals, with the finding that $Ca(OH)_2$ was able to neutralize endotoxin (36, 42). Since most of the studies applied NaOCl during chemo-mechanical preparation little is known concerning the *in vivo* effect of CHX gel on endotoxin and bacteria in combination with intracanal dressing.

The purpose of this study therefore was to determine the amount of endotoxin, and to identify and quantify bacteria in human necrotic root canals before (S1) and after chemo-mechanical preparation using 2% CHX gel as auxiliary chemical substance (S2), and after 7 days of intracanal dressing (S3). For intracanal dressing three different substances were tested: $Ca(OH)_2$, CHX and $Ca(OH)_2$ plus CHX, to see which of these intracanal medicaments would best complement CHX-based chemo-mechanical preparation.

Material and methods

Patient selection

Twenty-four patients, attending the Dental School of Piracicaba, SP, Brazil, for root canal treatment, were selected for this study. The age of the patients ranged from 18 to 65 years. The selected teeth were single-root, presented necrotic pulp tissues, and showed radiographic evidence of apical periodontitis. None of the patients showed signs and symptoms of endodontic origin during the endodontic procedures. A detailed medical and dental history was obtained from each patient. Patients who had received antibiotic treatment during the last 3 months or who had a general disease were excluded from the study. The Human Volunteers Research and Ethics Committee of the Dental School of Piracicaba approved a protocol describing the specimen collection for this investigation, and all patients signed their informed consent to participate in the study.

Microbiological sampling

The microbiological procedures performed in this study have been previously described (11, 45). Briefly, the teeth were isolated with a rubber dam. The crown and the surrounding rubber dam were disinfected with 30% H_2O_2 [volume/volume (V/V)] for 30 s followed by 2.5% NaOCl for an additional 30 s. Subsequently, 5% sodium thiosulphate was used to inactivate the disinfectant agents. An access cavity was prepared with sterile high-speed diamond burs under irrigation with sterile saline. Before entering the pulp chamber, the access cavity was disinfected following the protocol described above. The sterility was checked by taking a swab sample of the cavity surface and streaking on to blood agar plates with subsequent incubation at 37°C in both aerobic and anaerobic conditions. All subsequent procedures were performed aseptically. The pulp chamber was accessed with sterile burs refrigerated in saline. The samples were collected (S1) with five sterile pyrogen-free paper points, which were consecutively placed into the main root canal to a total length that was calculated from the preoperative radiograph. Afterwards, one paper point was placed in a pyrogen-free glass for the chromogenic *Limulus* amoebocyte lysate (LAL) test, and the others were pooled in a sterile tube containing 1 ml reduced transport fluid for culturing. The samples were transferred to the laboratory and processed for culture analysis within 15 min. The endotoxin samples were frozen at −20°C so that all samples could be processed at the same time. All samples were collected using the same technique.

Clinical procedures

After the first microbiological sampling, the pulp chamber was thoroughly cleaned with 2% CHX gel (Endogel, Itapetininga, SP, Brazil). The CHX gel consisted of a gel base (1% natrosol) and CHX gluconate at pH 7.0. Natrosol gel (hydroxyethyl cellulose) is a non-ionic, highly inert, water-soluble agent (45). The K-file (DYNA–FFDM, Bourges, France) size 10 or 15 was placed in the total length of the root canal as calculated from the preoperative radiograph. The coronal two-thirds of each canal were prepared initially using rotary files (GT® Rotary Files sizes .10/20 and .08/20; Malleifer-Dentsply, Bailagues, Switzerland) at 350 r.p.m. reaching 4 mm before the total length. Gates–Glidden drills, sizes 5, 4, 3 and 2 (DYNA–FFDM) were used until 2 mm before the length prepared with GT files was reached. The working length (1 mm from the radiographic apex) was checked with a radiograph after inserting an anatomical file in the canal to the estimated working length confirmed by apical locator (Forum Technologies, Rishon Le-Zion, Israel). The apical stop was established using K-files (DYNA–FFDM). The apical stop ended after the use of three files larger than the initial one. Step-back flaring of the canal was performed using larger files at intervals manipulated in a filing action. The file used to prepare the apical stop was used to recapitulate. Stepping back ended after the use of three files larger than the file that prepared the apical stop (45).

The 24 teeth were treated with 2% CHX gel as a disinfectant. The working time for the chemo-mechanical procedure was established at 20 min for all cases. Before using each file, 1 ml CHX gel was introduced into the root canal with a syringe (27-gauge needle), and immediately after instrumentation, 4 ml physiological saline solution was used to irrigate the canal. Before collecting the second (post-chemo-

mechanical) sample (S2), the inactivation of CHX was accomplished with a rinse of 5 ml pyrogenic-free solution containing 0.5% Tween-80 and 0.07% lecithin [weight/volume (W/V); chemical neutralizer of CHX] for 1 min. The second sample was then taken. The canals were then thoroughly washed with 4 ml of physiological saline solution and dried with sterile paper points, and the canals were randomly divided into three groups according to the intracanal medicament used: $Ca(OH)_2$ paste (M1), 2% CHX gel (M2), or the combination of $Ca(OH)_2$ and 2% CHX gel (M3) (13). The $Ca(OH)_2$ medicaments were plugged in the canal with Lentulo files (Malleifer-Dentsply) and with the blunt end of a sterile paper point. The access cavities were properly filled with two layers of Cavit (ESPE, Seefeld, Germany) and resin (Z-250, 3 M Dental Products, St Paul, MN).

Seven days after the placement of the intracanal medicament, the canal was aseptically accessed under rubber dam isolation using the protocol for disinfection described above. The medicaments were removed by rinsing the canals with 5 ml of their specific chemical neutralizers [Tween-80 plus 0.07% lecithin for CHX; 0.5% citric acid for $Ca(OH)_2$] and by carefully filing the canal with the master apical file. The third sample was then collected (S3) as described above. The root canal was fully washed with 4 ml physiological solution. The access cavities were then properly filled with two layers of Cavit (ESPE) and resin (Z-250, 3 M Dental Products).

Determination of endotoxin concentration

The quantitative chromogenic LAL assay – QCL-1000 LAL test kit (BioWhitaker, Inc., Walkersville, MD) was used to measure the endotoxin concentration in the root canals before and after endodontic procedures. This method utilizes a modified LAL and a synthetic colour-producing substrate to detect and quantify chromogenically the endotoxin of gram-negative bacteria. LAL has been used previously to measure endotoxin concentration in caries, pulp tissues (21, 22) and root canals containing necrotic pulp tissue (19), and in the bronchoalveolar lavage fluid of patients suffering from pneumonia (7, 17).

Standard curve

The endotoxin standard curve was generated following the manufacturer's instructions. A standard curve was plotted as a parameter for calculation of the amount of endotoxin in the sample using the endotoxins supplied in the kit (*Escherichia coli* 0111:B4) with a known concentration (25 EU/ml). After the LAL test procedures (described below), the absorbencies (ABS) of standard solutions in a series of endotoxin concentrations (0.1, 0.25, 0.5 and 1.0 EU/ml) were measured individually using an enzyme-linked immunosorbent assay plate-reader (Ultramark, Bio-Rad Laboratories, Inc., Hercules, CA) at 405 nm. The standard curve fulfilled the criteria for linearity ($r \geq 0.980$) as reported for the guideline on validation of LAL tests (25, 26).

Sample concentrations

Initially, six samples were tested to find the ideal concentration within the detection limits of the standard curves. For this procedure each sample was used pure and diluted (1/10, 1/100, 1/1000, 1/10,000). The tests were performed in a 96-well microplate (Corning Costar Corporation, Cambridge, MA) inside heating block equipment at 37°C throughout the assay. A standard curve was plotted, as described above, and four negative controls were added using water as a reagent (blanks). All reactions were performed in duplicate with the mean values used for calculations.

Spike concentration

The LAL test is a very sensitive test that may suffer from inhibition or enhancement. To avoid these factors, the addition of a known concentration of *E. coli* endotoxin (spiking procedure) is recommended. For all tests the spike recovery was 0.4 EU/ml. This activity was chosen because of its lower position on a logarithmic standard curve (its value was set as zero).

Chromogenic assay

Samples were run in duplicate using four blanks, and two standard curves from 0.1, 0.25, 0.5 and 1.0 EU/ml. After spiking the diluted samples (10^{-2}), standard and blank, 50 µl of each sample was placed in each well. Next, 50 µl LAL solution was added. The microplate containing the LAL, blank, standards and samples, was shaken for 1 min at 37°C. Ten minutes after the addition of LAL, 100 µl substrate solution (pre-warmed to 37°C) was added to each well. The plate was shaken for 1 min and incubated at 37°C for a further 5 min (total 6 min). Finally, 100 µl of a stop reagent (acetic acid, 25% V/V) was added and plates were read using a spectrophotometer (Ultramark, Bio-Rad Laboratories, Inc., Hercules, CA) at an absorbance of 405 nm.

The mean absorbance value of the blank was subtracted from the mean absorbance values of the standards and from the values of samples to calculate the mean absorbance. Since this absorbance value was directly proportional to the amount of endotoxin present, the endotoxic concentration (EU/ml) was determined from the standard curve (Fig. 1).

Culturing procedure

Inside the anaerobic chamber the transport media were shaken thoroughly in a mixer for 60 s (Vortex, Marconi, São Paulo, Brazil). Serial 10-fold dilutions were made up to 10^{-4} in tubes containing fastidious anaerobe broth (Lab M, Bury, UK). Fifty microlitres (undiluted and serial dilutions

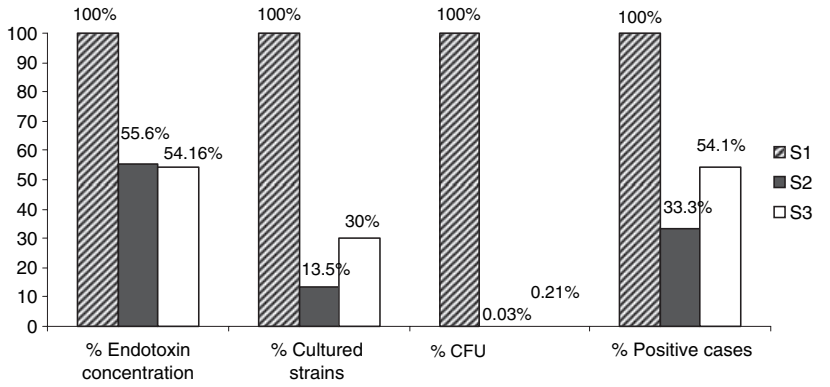

Fig. 1. Relative changes of endotoxin concentration, cultured bacteria, amount of cultivable bacteria and positive cases after chemo-mechanical preparation (S2) and after intracanal dressing (S3) expressed as percentage values compared with initial levels measured before the start of treatment (S1 = 100%).

10^{-2}, 10^{-3} and 10^{-4}) were plated using sterile plastic spreaders on to fastidious anaerobe agar (Lab M), supplemented with 5% defibrinated sheep blood, 5 mg/l haemin and 1 mg/l vitamin K1, to cultivate non-selectively obligate anaerobes and facultative anaerobes. Supplemented fastidious anaerobe agar was used as the selective culture medium by adding: (i) 0.001% (W/V) nalidixic acid and 0.5 mg/l vancomycin, to select gram-negative anaerobic bacteria; (ii) 0.0075% W/V kanamycin plus 0.5 mg/l vancomycin to select black-pigmented *Prevotella* and *Porphyromonas* species; (iii) 0.0075% W/V neomycin to select clostridia and other anaerobes; and (iv) 0.001% W/V nalidixic acid to select gram-positive anaerobes and *Actinomyces* spp. The plates were incubated at 37°C in an anaerobic atmosphere (80% N_2, 10% CO_2, 10% H_2) for up to 14 days to allow the detection of very slow-growing strains.

The same dilutions were plated onto brain–heart infusion agar plates (Oxoid, Basingstoke, UK), supplemented with 5% sheep blood, to allow the growth of aerobic (37°C, in air) or facultative anaerobic (37°C, in 10% CO_2) bacteria. Sabouraud–dextrose agar (Oxoid), supplemented with 100 μg/ml chloramphenicol (Medley, Campinas, SP, Brazil), was used to grow yeasts.

For biochemical identification, preliminary characterization of microbial species was based on the colony features (i.e. size, colour, shape, surface, texture, consistency, brightness and haemolysis) visualized under a stereoscopic lens (Lambda Let 2, Atto instruments CO, Hong Kong). Isolates were sub-cultured, gram-stained, tested for catalase-production, and their gaseous requirements were analysed by incubation for 2 days aerobically and anaerobically. Based on this information it was possible to select appropriate procedures for the identification of isolates by commercial biochemical test panels [Rapid ID 32 A, API Staph, Rapid ID 32Strep, API C Aux (BioMérieux SA, Marcy-l'Etoile, France) as well as the RapID ANA II System, RapID NH System (Innovative Diagnostic Systems Inc., Atlanta, GA)].

Statistical analysis

The data collected [colony-forming unit (CFU) counts, number of different species and endotoxin concentrations] were statistically analysed using SPSS for WINDOWS, version 12.0 (SPSS Inc, Chicago, IL). The comparison among the groups S1, S2 and S3 was performed using the Friedman test ($P < 0.05$), the Wilcoxon test was used subsequently. Comparison among the intracanal medicament groups was performed using the Kruskal–Wallis test ($P < 0.05$). Associations were tested by Fisher's Exact Test, followed by Odds ratio (OD), and confirmed by confidence interval (CI).

Results

Radiographs taken after application of the dressings showed that the teeth filled with $Ca(OH)_2$ (M1 and M3) were well compacted. Visualization of the CHX-filled canals (M2) was not possible because of the radiolucent character of CHX.

Overview of endotoxin and antimicrobial reduction

Table 1 provides an overview of the endotoxin concentrations, amount of cultivable bacteria (CFU/ml), number of culture-positive cases and the number of cultivable bacteria at the different steps S1, S2 and S3. Endotoxin and bacteria were present in all initial samples (S1, $n = 24$). The mean endotoxin concentration was 151.61 UE/ml, while the number of cultivable bacteria was 2.8×10^5 CFU/ml. The mean number of different species per root canal was 3.3. In total, 81 different bacterial strains were recovered by culture analysis, of which 69.1% were strict anaerobes (Table 1).

After chemo-mechanical preparation (S2) endotoxin was still present in all 24 cases, while cultivable bacteria were detected in only eight cases. The mean endotoxin concentration declined to 84.3 UE/ml, and the mean number of cultivable bacteria to 1×10^2 CFU/ml. A total of 11 bacterial strains was isolated, of which 72.7% were strict anaerobes.

After 7 days of intracanal dressing (S3) endotoxin was still present in all cases, and bacteria were detected by culture analysis in 13 cases. The mean endotoxin concentration had decreased slightly, to 82.12 UE/ml, while the mean number of cultivable bacteria had increased to 6×10^2 CFU/ml. Likewise, the total number of strains isolated increased to 30, of which 50% were strict anaerobes. Differences between S1 and S2 (both endotoxin concentration and CFU) were statistically significant ($P < 0.05$) but no significant differences were found between S2 and S3.

Relative changes

Figure 1 depicts the relative changes for the different steps S1, S2 and S3. The effect of the endodontic treatment procedures was most strongly pronounced after chemo-mechanical preparation (S2). The endotoxin concentration decreased to 55.6% of the initial value. Compared with S1, the number of positive cases declined to 33.3%, with the number of cultured strains declining to 13.5% and CFU declining to 0.03%. No significant further reduction of microbes or endotoxin concentration was achieved after 7 days of intracanal dressing. In fact, the number of positive cases increased to 54.1% compared with S1, as did the number of culture isolates (37% compared with S1), and the number of cultivable bacteria (0.21% compared with S1).

Comparison of different intracanal medicaments

The numbers of positive cases and of bacterial species were largely consistent among the three treatment groups in S3 [M1 = $Ca(OH)_2$ paste, M2 = 2% CHX gel, and M3 = $Ca(OH)_2$ with 2% CHX gel] (Table 1). However in M3, we observed a trend towards a lower bacterial load as well as a lower amount of endotoxin compared with M1 and M2 (mean values for M3: 9.7×10^1 CFU/ml and 53.4 UE/ml, respectively). In addition, the proportion of strict anaerobes was only 33% in M3, while the proportions in M1 and M2 were 60% and 54%, respectively. However, these differences were not statistically significant.

Species diversity

For all steps (S1, S2 and S3) gram-positive rods were the most frequently found morphotypes (Table 1). Table 2 shows the most frequently recovered bacteria by culture analysis. Before treatment (S1), the commonly found bacteria were *Propionibacterium acnes* (29%, seven of 24 positive samples), *Gemella morbillorum* (29%, seven of 24 positive samples), *Actinomyces naeslundii* (25%, six of 24 positive samples), *Eubacterium lentum* (25%, six of 24 positive samples), *Propionibacterium propionicum* (16%, four of 24 positive samples), *Fusobacterium nucleatum* (16%, four of 24 positive samples), *Prevotella intermedia* (12.5%, three of 24 positive samples), *Actinomyces viscosus* (12.5%, three of 24 positive samples), and *Eubacterium limosum* (12.5%, three of 24 positive samples). After chemo-mechanical preparation (S2), the most frequent bacteria found were gram-positive rods, and among them *P. acnes* (18%, two of eight

Table 1. Effect of endodontic procedures on endotoxin and bacteria

			After intracanal medicaments (different groups) (S3)			
	Initial samples (S1)	After chemo-mechanical preparation (S2)	$Ca(OH)_2$ paste	2% CHX gel	$Ca(OH)_2$ + 2% CHX gel	After intra-canal medicaments (all groups together)
Mean endotoxin concentration (UE/ml)	151.61 (±36.48) range: 62.93–214.56	84.30 (±42.27) range: 26.43–174.55	105.84 (±61.79) range: 35.58–192.26	87.12 (±52.23) range: 12.57–177.83	53.40 (±47.54) range: 7.5–173.29	82.12 (±58.36) range: 7.5–177.83
Mean amount of cultivable strains (CFU/ml)	2.8×10^5 ($\pm 5.6 \times 10^5$) range: 4×10^2–2.6×10^6	1×10^2 ($\pm 2.1 \times 10^2$) range: 0–7×10^2	2.0×10^2 ($\pm 2.8 \times 10^2$) range: 0–7.2×10^2	1.5×10^3 ($\pm 2.5 \times 10^3$) range: 0–8×10^3	9.7×10^1 ($\pm 1.3 \times 10^2$) range: 0–3.6×10^2	6×10^2 ($\pm 1.6 \times 10^3$) range: 0–8×10^3
Endodontic cases	24 (100%)	24 (100%)	8 (100%)	8 (100%)	8 (100%)	24 (100%)
Culture positive cases	24 (100%)	8 (33.3%)	5 (62.5%)	4 (50%)	4 (50%)	13 (54.1%)
Culture negative cases	0	16 (66.6%)	3 (37.5%)	4 (50%)	4 (50%)	11 (45.8%)
Bacterial species per positive sample[1]	mean: 3.3 (±1.5) median: 4 (range 1–5)	mean: 1.3 (±0.6) median: 1 (range 1–3)	mean: 2 (±0.8) median: 2 (range 1–3)	mean: 2.7 (±1.9) median: 2 (range 1–6)	mean: 2.2 (±0.8) median:2.5 (range 1–3)	mean: 2.3 (±1.3) median: 2 (range 1–6)
Total number of cultured bacteria	81 (100%)	11 (100%)	10 (100%)	11 (100%)	9 (100%)	30 (100%)
gram-positive cocci	12 (14.9%)	2 (18.2%)	1 (10%)	3 (27.3%)	2 (22.2%)	6 (20%)
gram-positive rods	43 (53.1%)	6 (54.6%)	7 (70%)	7 (63.6%)	4 (44.4%)	18 (60%)
gram-negative cocci	1 (1.2%)	0	1 (10%)	0 (%)	3 (33.3%)	4 (13.3%)
gram-negative- rods	25 (30.8%)	3 (27.2%)	1 (10%)	1 (9.1%)	0 (%)	2 (6.7%)
gram-positive bacteria	55 (67.9%)	8 (72.7%)	8 (80%)	10 (90.9%)	6 (66.6%)	24 (80%)
gram-negative bacteria	26 (32.1%)	3 (27.3%)	2 (20%)	1 (9.1%)	3 (33.3%)	6 (20%)
Facultative anaerobes	25 (30.9%)	3 (27.3%)	4 (40%)	5 (45.5%)	6 (66.6%)	15 (50%)
Strict anaerobes	56 (69.1%)	8 (72.7%)	6 (60%)	6 (54.5%)	3 (33.3%)	15 (50%)

The total number of cases was considered as 100% to calculate the percentage of positive and negative cases, as was the total number of bacteria to calculate the percentage of morphological/physiological microbial types.
[1]Only the positive samples were used to calculate the mean, standard deviation (±), median, and range.

positive samples) and *P. propionicum* (18%, two of eight positive samples) dominated. After 7 days of intracanal dressing (S3), the number of gram-positive rods had further increased. The most frequently found species were *P. acnes* (31%, four of 13 positive samples), *Clostridium argentinense* (23%, three of 13 positive samples), *Actinomyces meyeri* (15%, two of 13 positive samples), *A. naeslundii* (15%, two of 13 positive samples), and *Bifidobacterium* spp. (15%, two of 13 positive samples). In addition, the gram-positive coccus *G. morbillorum* (15%, four of 13 positive samples) was also frequently found.

Discussion

The purpose of the present study was to assess the reduction of endotoxin and bacteria after chemo-mechanical preparation of necrotic root canals with CHX and subsequent use of three different intracanal dressings for 7 days. Since bacteria are not only present in the main root canal but might hide and survive in the dentinal tubules, a sampling procedure covering the whole root canal system would be desirable. However, for *in vivo* studies sample collection is generally made from the main root canal. Thus it should be taken into consideration that endotoxin levels as well as the amount and diversity of endodontic bacteria might be underestimated. Nonetheless, even if the analysis is restricted to the main root canal, meaningful data can be obtained, especially for comparative purposes (i.e. evaluation of different treatment strategies).

Endotoxin and gram-negative bacteria in root canals

Our study focused on asymptomatic cases in which gram-negative bacteria are usually less dominant than in patients with endodontic symptoms (11, 18). For instance, the gram-negative species found in our study, namely *Fusobacterium*, *Prevotella*, *Porphyromonas*, *Capnocytophaga*, comprised only about one-third of the total bacteria cultured in the initial samples (S1). This proportion was maintained after chemo-mechanical preparation (S2) even though, of course, the overall number of viable cells was drastically reduced (Table 1). Irrespective of the number of cultivable gram-negative bacteria, the importance of endotoxin as a virulence factor in general and for the aetiology of periapical disease in particular is generally recognized (23). However, only relatively

Table 2. Bacteria found before and after endodontic procedures

	Initial samples	After chemo-mechanical preparation	After intracanal medicaments			
			M1	M2	M3	Total medicaments
Gram-positive cocci						
Aerococcus viridans			1		1	2
Gemella morbillorum	7	1		2	2	4
Micrococcus spp.	2					
Finegaldia magna	1					
Peptostreptococcus micros		1				
Peptostreptococcus prevotti	1			1		
Streptococcus mitis	1					1
Gram-negative cocci						
Neisseria spp.			1			1
Neisseria subflava					1	1
Veillonella spp.	1				1	1
Gram-negative rods						
Bacteroides gracilis	1					
Bacteroides ureolyticus		1				
Capnocytophaga spp.	2	1	1	1		2
Fusobacterium necrophorum	1					
Fusobacterium nucleatum	4					
Haemophilus aphrophillus	1					
Haemophilus haemolyticus	1					
Porphyromonas endodontalis	2					
Porphyromonas asaccharolytica	1					
Prevotella buccae	1					
Prevotella denticola	1					
Prevotella intermedia	4					
Prevotella loescheii	2					
Prevotella oralis	3					
Gram-positive rods						
Actinomyces israelli				1		1
Actinomyces meyeri	2		1		1	2
Actinomyces naeslundii	6			1	1	2
Actinomyces odontolyticus	2	1		1		1
Actinomyces viscosus	3		1			1
Bifidobacterium adolescentis	1					
Bifidobacterium spp.	1	1	1	1		2
Clostridium bifermentans	1					
Clostridium butyricum	2					
Clostridium sporogens	1					
Clostridium spp.	4					
Clostridium argentinense			1	2		3
Eubacterium lentum	6					
Eubacterium limosum	3					
Lactobacillus spp.					1	1
Lactobacillus lactis	1					
Lactobacilus acidophillus		1				
Propionibacterium propionicum	4	2	1			1
Propionibacterium acnes	7	2	2	1	1	4
Total	81	11	10	11	9	30

few studies have attempted to determine endotoxin levels in root canals, although such levels might have a predictive value for interpreting the success of root canal treatments (19, 21). Since endotoxins are released during death and lysis of gram-negative bacteria, a relative increase in endotoxin concentration can be expected after the instrumentation while the recovery of bacteria by culture analysis might be greatly reduced. This was observed in our study. After chemo-mechanical preparation (S2) the number of cultivable bacteria (as determined by CFU) was <1% compared with S1 while the amount of endotoxin in S2 was still 50% of the initial value in S1 (Fig. 1). This means that in S1 approximately 2.8×10^5 CFU/ml correspond 152 UE/ml endotoxin. In contrast, in S2 only about 100 CFU/ml corresponded to a value as high as 85 UE/ml (Table 1). This shift is striking because it indicates that efficient elimination of bacteria does not necessarily correspond to efficient elimination of endotoxin; the latter might still induce inflammatory reactions. This is particularly true because endotoxin concentration was measured using the chromogenic LAL test (QCL-1000), a method that is based on the enzymatic detection and quantification of biologically active endotoxin. The threshold levels of endotoxin required in the root canal to have damaging effects have still to be determined. However, with endotoxin values ranging from 10 to 200 UE/ml (before and after treatment) our data agree with the previously reported endotoxin levels to be present in the bronchoalveolar lavage fluid of patients suffering from pneumonia caused by gram-negative bacteria (7, 27). In contrast, endotoxin concentrations measured in dental caries and pulp tissues were about 100 times lower than in our study (21, 22). Although an exact comparison between studies is difficult because of differences in tissue samples, sampling technique and further methodological aspects, the endotoxin values found in our study suggest functional relevance and underline the principal importance of gram-negative bacteria even in asymptomatic endodontic cases.

As shown in Table 1 and Fig. 1 chemo-mechanical preparation (S2) was responsible for the major reduction of endotoxin. Such a reduction was probably the result of the mechanical removal of debris from the root canal during cleaning and shaping, aided by irrigation with physiological saline solution, rather than of the property of CHX to neutralize endotoxins. This is borne out by the fact that group M2 was treated with 2% CHX gel for 7 days (S3), but no significant reduction in endotoxin concentration was observed compared with S2. Endotoxin concentration was also not further reduced when $Ca(OH)_2$ was used as the intracanal dressing (M1), although this substance has been demonstrated to be effective in endotoxin reduction *in vitro* (2). Unfortunately, the proposed combination of both substances together for use as intracanal dressing (M3) did not show a synergistic effect against either endotoxins or microorganisms after 7 days of intracanal dressing.

Gram-positive bacteria in root canals

Gram-positive bacteria are also frequently recovered from necrotic pulps; however, only a few species from this group of organisms have been directly implicated in apical periodontitis (31, 40). Gram-positive bacteria do not contain LPS, and their role in the development of periapical disease is not clearly understood. It has been shown, however, that bacterial cell wall components other than LPS, namely lipoteichoic acid and murein (peptidoglycan), can also stimulate bone resorption (6, 17).

In the present study, gram-positive bacteria were frequently found in all samples (S1: 67.9%; S2: 72.7%; S3: 80%; Table 1),

which corresponds with the findings of Gomes et al. (9). Among those, the gram-positive rods constitute an important fragment of the remaining flora even after root canal filling (24) and were also frequently found in our samples (S1: 53.1%; S2: 54.6%; S3: 60%). This was especially evident after 7 days of intracanal dressing (18 out 30 were gram-positive rods) with *Actinomyces*, *Clostridium* and *Propionibacterium* being the genera most frequently recovered by culture analysis.

The mechanisms by which gram-positive rods remain viable in the root canals after instrumentation or filling may be determined by their interactions with other bacteria (35). Therefore, it may be of particular interest to explore the mechanisms by which these species ingress and prevail after root canal treatment and how they are pathogenically implicated in periapical lesions.

Gram-positive bacteria have the ability to establish themselves and survive in the periapical tissue outside the root canal. Such bacteria include *A. meyerii* and *P. propionicum* (15, 40). Alternatively, bacteria may develop an association with other bacteria (35). In the present study, positive associations were found with gram-positive bacteria: *Propionibacterium* spp. with *Clostridium* spp. ($P = 0.09$, OD = 0.81; CI = 0.81–177.4); *Propionibacterium* spp. with *Fusobacterium* spp. ($P = 0.03$, OD = 11.2; CI = 1.0–125.6); *E. lentum* with *F. nucleatum* ($P = 0.03$, OD = 17; CI = 1.3–223,1); and *Clostridium* spp. with *G. morbillorum* ($P = 0.03$, OD = 40; CI = 1.74–914.8).

Use of CHX as irrigating substance for chemo-mechanical preparation

The success of an antimicrobial endodontic treatment is usually rated according to the number of cases in which microbial growth can be observed after treatment, and according to the bacterial load that can be found in those positive cases (3, 4, 29, 39).

According to these criteria, chemo-mechanical preparation of the root canal system using 2% CHX gel appeared to be comparable with preparation using NaOCl, because in this study the total number of bacteria was significantly reduced to 0.03% of the original number (2.8×10^5 bacterial cells in the initial samples vs. 10^2 cells after chemo-mechanical preparation). These values are in accordance with a number of other studies (3, 4, 29, 30, 39) that reported 6.5×10^3 to 1×10^5 cells before and $0–10^3$ cells after treatment, depending on the pulp status and the presence of previous endodontic treatment. In addition, in our study, the proportion of bacteria-free root canals after chemo-mechanical preparation (S2) was 66.6% (16 of 24), which lies in the range of those reports using NaOCl as the irrigating substance. For instance, Sjögren et al. (38, 39) using 0.5% NaOCl to prepare the root canals found 50–60% culture-negative root canal samples after preparation, while Peters et al. (30) using 2% NaOCl found 76% culture-negative root canal samples, as did Vianna et al. (45) with 75% of root canals being negative cases using 2.5% NaOCl.

Reduction of species diversity

The number of different species was consistently reduced in all cases. The mean number of species in the initial samples (S1) was 3.3 (±1.5) or median 4 (range 1–7). This number dropped to a mean of 1.3 (±0.6) or median 1 (range 1–3) species after the chemo-mechanical preparation. Seven days after use of $Ca(OH)_2$ paste as intracanal dressing the mean was 2 (±0.8) or median 2 (range 1–3). Peters et al. (30) found slightly higher numbers of species using the same substance for intracanal dressing; however, they also found a higher range of species after chemo-mechanical preparation (range 2–6) and after 4 weeks of intracanal medicaments (range 2–6). In addition to species reduction per sample, the overall success of chemo-mechanical preparation also becomes evident when considering the total number of isolated strains. For instance, after chemo-mechanical preparation (S2) only 11 bacterial strains were cultured (vs. 81 strains at S1) (Table 2), and after 7 days of intracanal dressing (S3) 30 bacterial strains were recovered by culture analysis. This shows that the majority of species could be eliminated after chemo-mechanical preparation. Interestingly, in S2 and S3 species were found that were not detected in the initial samples (S1). For example, *Peptostreptococcus micros* and *Bacteroides ureolyticus* were found only after chemo-mechanical preparation (S2) but were not found in either the initial samples (S1) or after 7 days of intracanal dressing (S3) (Table 2). This shows that these organisms not only might have been overgrown on the agar plates in S1 by other bacteria but also that they resisted chemo-mechanical preparation but not 7 days of intracanal dressing. Conversely, *Aerococcus viridans*, *Neisseria subflava* and *Neisseria* spp., *Actinomyces israelli*, *Clostridium argentinense* and *Lactobacillus* spp. were only found after 7 days of intracanal dressing. Again these organisms might have been overgrown on the agar plates in S1 and S2 by other bacteria or these organisms had penetrated into the dentinal tubules and survived there, being able to re-colonize the main root canal during the 7 days of intracanal dressing. The latter explanation is especially plausible because the number of culture-positive cases was higher in S3 than in S2. This in turn makes the use of intracanal dressing for 7 days questionable, at least in our cases in which signs and symptoms of endodontic origin were absent.

Effect of 7 days of intracanal dressing

We chose a period of 7 days for evaluation because it is the usual period recommended between visits, when intracanal medicaments are employed (4, 8, 39, 44). However, the overall antimicrobial effect of the intracanal dressing was small regardless of the chemical compounds used [i.e. $Ca(OH)_2$ (M1), 2% CHX gel (M2) or $Ca(OH)_2$ + 2% CHX gel (M3)]. The failure of $Ca(OH)_2$, the most commonly used substance, is surprising but is in accordance with Peters et al. (30) who used NaOCl as irrigating solution and $Ca(OH)_2$ for 4 weeks as intracanal dressing and still found culture-positive cases with the bacterial load not significantly reduced after intracanal dressing. In fact, complete bacterial elimination has been reported in only a few studies. For instance Byström & Sundqvist (4) reported 100% of cultures to be negative after 4 weeks of intracanal dressing and Sjögren et al. (39) reported 100% bacteria-free samples after an intracanal dressing period as short as 1 week. On the other hand, Cvek (5) obtained only 90% culture-negative cases after 3 months, Reit & Dahlen (32) found 74% culture-negative cases after 2 weeks, and Ørstavik et al. (29) found 65% of their samples to be negative after 7 days of intracanal dressing. Since in all of these studies $Ca(OH)_2$ was used as the intracanal medicament, apparently treatment success (i.e. bacterial reduction) does not necessarily depend on the period of intracanal dressing.

Conclusion

In conclusion, endotoxin was only partially removed after chemo-mechanical preparation, although a large reduction of bacteria was achieved. A further significant reduction was not accomplished by inclusion of intracanal medicaments in the

root canal for 7 days. Thus, the benefit of intracanal dressing to improve the overall endodontic treatment success is questionable, at least in cases in which signs and symptoms of endodontic origin are absent.

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