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Effectiveness of two intracanal dressings in adult Portuguese patients: a qPCR and anaerobic culture assessment

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Abstract

Teles AM, Manso MC, Loureiro S, Silva R, Madeira IGC, Pina C, Cabeda JM. Effectiveness of two intracanal dressings in adult Portuguese patients: a qPCR and anaerobic culture assessment. *International Endodontic Journal*.

Aim To quantify bacterial equivalents before and after chemomechanical preparation using 3% sodium hypochlorite (NaOCl) and intracanal dressing with calcium hydroxide paste ($Ca(OH)_2$) or 2% Chlorhexidine digluconate gel (CHX) in necrotic pulps associated or not with apical periodontitis and to further compare this quantification with counts of anaerobic microorganisms.

Methodology Prospective clinical trial in 69 single-rooted adult teeth (strict inclusion criteria); CHX group: 34; $Ca(OH)_2$ group: 35. Bacteria samples were taken at baseline (S1), after chemomechanical preparation (S2) and after 14 days of intracanal dressing (S3). Bacterial equivalents were assessed by broad-range real-time polymerase chain reaction (qPCR), and live viable bacteria measured with conventional anaerobic culture (CFU/mL). Descriptive/inferential analysis was performed with SPSS vs. 20.0 ($\alpha = 0.05$) using the Kruskal–Wallis, Mann–Whitney and chi-squared tests and Spearman's correlation coefficients.

Results Both groups showed a significant decrease between S1 and S2 (Mann–Whitney *U*-test; $P < 0.001$) both in qPCR and in culture. In the $Ca(OH)_2$-group, no variation was observed between S2 and S3 by qPCR and culture. In contrast, the CHX group showed a significant increase from S2 to S3 by both techniques. The two groups were only significantly different in S3 (Mann–Whitney *U*-test; $P \leq 0.001$), with a worse performance in the CHX group. Again, these results were congruent by both approaches. Data from both approaches correlate reasonably ($r_S < 0.5$).

Conclusions Infected root canals contained a high bacterial load, and the chemomechanical root canal preparation reduced bacterial equivalents by 99.1% and anaerobic counts by 98.5%. Intracanal dressings were not efficient at reducing bacterial load, but the 14-day intracanal dressing with $Ca(OH)_2$ performed significantly better than CHX, particularly in cases with apical periodontitis.

Keywords: apical periodontitis, endodontic microbiology, intracanal dressing, qPCR, real-time PCR, root canal sampling.

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Introduction

Modern root canal treatment procedures aim at eliminating the infecting microorganisms during canal preparation. However, as it is usually impossible to assure complete bacterial eradication or due to lack of time to complete the treatment in one appointment, intracanal dressings between sessions have been advised to maximize disinfection. Calcium hydroxide paste ($Ca(OH)_2$) is the most commonly used medicament (Siqueira & Lopes 1999, Sathorn *et al.* 2005, Mohammadi *et al.* 2012), and its wide application is justified because of its antimicrobial activity and high

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pH (approximately 12.5–12.8). The lethal effects of the release of hydroxyl ions on bacterial cells are probably due to damage to the bacterial cytoplasmic membrane, denaturation of proteins or damage to the DNA (Mohammadi *et al.* 2012). In addition, it stimulates mineralization (induction of hard-tissue formation), dissolves organic material, produces a chemical and physical barrier (Vianna *et al.* 2007), stops inflammatory exudate (Tavares *et al.* 2011) and is currently the only clinically effective medicament for inactivation of endotoxins (Mohammadi & Dummer 2011).

Periapical cell infiltration initiates as soon as the inflammatory process starts in an attempt to eliminate the microorganisms and to prevent their dissemination into the fascial planes. During this response, a number of cell types release cytokines, chemokines, leukotrienes and prostaglandins into the area. These inflammatory mediators reinforce the recruitment of polymorphonuclear leucocytes (PMNs) and other leucocytes (Graves *et al.* 2011). In diseased periapical tissue, interleukin-1 (IL-1) plays a pivotal role stimulating osteoclastic bone resorption (da Silva *et al.* 2006), as well as contributing to inflammation by inducing IL-17 synthesis (McGeachy & Cua 2008). $Ca(OH)_2$ in root canals acts by denaturing immunoreactive IL-1, tumour necrosis factor-α (TNF) and calcitonin gene-related peptide as demonstrated by Khan *et al.* (2008) and confirmed recently by Tavares *et al.* (2011), who suggested the effectiveness of $Ca(OH)_2$ in dampening periapical inflammation after root canal cleaning procedures. It has also been reported that it is possible that $Ca(OH)_2$ diminishes TNF-α local concentrations, because it has been shown to denature proteins (Khan *et al.* 2008).

Despite all these advantages, $Ca(OH)_2$ has limitations (Trope *et al.* 1999, Shuping *et al.* 2000, Peters & Wesselink 2002, Sathorn *et al.* 2005, Waltimo *et al.* 2005, Figini *et al.* 2007) and, as an alternative, 2% chlorhexidine digluconate gel (CHX) has emerged (Barbosa *et al.* 1997, Manzur *et al.* 2007, Paquette *et al.* 2007, Wang *et al.* 2007), but is by far much less well studied.

Anaerobic bacteria, either strict or facultative, have been shown to be the most prevalent in primary root canal infections on several epidemiological studies (Baumgartner & Falkler 1991, Fouad *et al.* 2002, Munson *et al.* 2002, Abdullah *et al.* 2005, Ferrari *et al.* 2005, Blome *et al.* 2008, Özok *et al.* 2012). Most of these studies have relied on the labour-intensive and time-consuming bacterial culture. This is a well-established method for analysing endodontic microbiology, but because it shows low sensitivity and because many microorganisms remain uncultivable (Siqueira & Rôças 2009), it is thought to provide underestimated data.

Application of molecular techniques for the study of the polymicrobial nature of endodontic pathosis enables a more complete analysis. The speed of results, the detection of anaerobes even in samples not collected or transported under special conditions, the possibility to study samples from antibiotic-treated subjects, the possibility to batch study large number of stored samples are major advantages of these techniques.

One of the most interesting molecular approaches for the detection/quantification of bacteria is the broad-range quantitative PCR amplification of the 16S rRNA gene (16S rDNA). This can be accomplished by real-time PCR (qPCR) using primers targeting conserved regions of the 16S rDNA. This approach returns 16S rDNA quantifications, independently of the nature of the DNA (live/dead or viable/nonviable bacteria, or even free naked DNA) and is thus a complementary, independent approach to bacteria culture.

As the discrepancies of the published literature relating to the efficiency of intracanal dressings may be related to both geographical differences in the endodontic bacteria species (Baumgartner *et al.* 2004, Foschi *et al.* 2005, Rôças *et al.* 2008), and the fact that only a subset of the sampled bacteria will be detected by culture, in the present study, bacterial equivalents (detected by 16S rRNA qPCR) were measured in the root canal before and after treatment by chemomechanical preparation alone and after the intracanal use of $Ca(OH)_2$ or CHX as interappointment dressings.

Material and methods

Clinical material

The study adhered to the Helsinki Declaration (William, 2008). Before the beginning of the study, the patients understood and gave written informed consent. The study was approved by the Ethics Committee of the Health Sciences Faculty of Fernando Pessoa University.

For inclusion, teeth had to have a single canal and intact pulp chamber walls, necrotic pulps confirmed by negative response to sensitivity pulp tests, and in some cases, clinical and radiographic evidence of AP.

The sample included 18 central and 16 maxillary lateral incisors, 10 mandibular and 10 maxillary premolars, nine maxillary and six mandibular canines from 69 subjects (35 women/34 men; mean age 49.7 years). Of those, 26 had necrosis alone and 43 had apical periodontitis. After chemomechanical preparation, they were randomly divided into two groups for dressing with $Ca(OH)_2$ or CHX. The random order of assignment to both groups was previously generated using random numbers and was applied to participants as they entered the trial.

Aseptic techniques were used, and all clinical and sampling procedures were conducted by us as follows (Fig. 1).

First session

Based on Siqueira *et al.* (2007a), after rubber dam application, tooth disinfection was performed with 3% hydrogen peroxide, until no further bubbling occurred followed by a 3% sodium hypochlorite (NaOCl) rinse for 1 min. After access cavity preparation with sterile burs, the operative field was disinfected as described above followed by inactivation of NaOCl with 5% sodium thiosulfate ($Na_2S_2O_3$). A sterile cotton swab embedded in sterile saline solution were used for collection of the disinfection operative field control (Sx-fig. 1) and collected in tubes with 5 mL phosphate-buffered saline (PBS). For inclusion of the tooth in the study, these control samples had to be uniformly negative. S1 sample was acquired by placing three successive size 25 sterile paper points in the canal for 1 min each approximately 1 mm short of the canal terminus and used to soak up the fluid in the canal and transferred into vials containing 2 mL sterile reduced transport fluid (RTF). The working length (WL) was established with Root ZX apex locator (Morita, Kyoto, Japan) and confirmed by digital radiography. Canals were enlarged with ProTaper instruments (Dentsply Maillefer, Ballaigues, Switzerland) used in a crown-down manner with a brushing motion. Instruments were withdrawn when resistance was felt and changed for the next instrument. File sequences used were: Sx files were used until resistance was encountered; S1, S2, F1, F2 and F3 files were used at WL. Between consecutive files, irrigation with 2 mL 3% NaOCl (27G needle) 2 mm shorter than the WL with apico-coronal movements to improve irrigant flow rate and size 10 K-file 1 mm beyond the WL were used to prevent apical blockage according to findings of Ng (2008). In large canals, F4 and F5 were used at WL.

After smear layer removal (5 mL 10% Citric Acid, 5 mL 3% NaOCl and 5 mL 10% $Na_2S_2O_3$), the S2

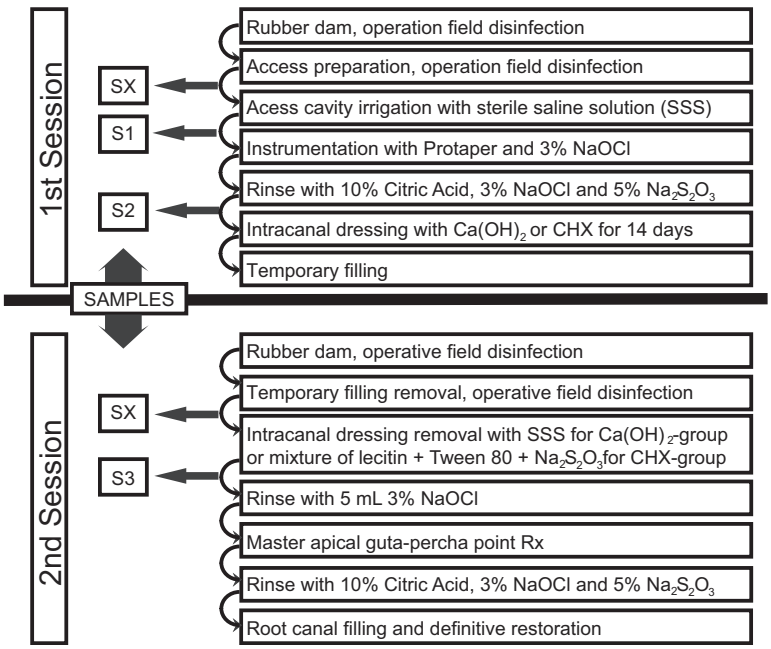

Figure 1 Study Protocol showing operative and sampling procedures.

sample was acquired the same way. After drying, the intracanal dressings were applied using lentulo spiral fillers with clockwise motion and packed with a sterile cotton pellet at the level of the canal orifice. Another sterile cotton pellet plug was placed, and access cavities were then filled with Coltosol (Coltène/Whaledent Inc, Cuyahoga Falls, OH, USA) for 14 days.

Second session

After similar field disinfection and restoration removal, a sterility control sample was again performed (Sx-Fig. 1), and the medicament was rinsed away with 5 mL of saline sterile solution for the $Ca(OH)_2$ group or mixture of lecithin, Tween 80 and $Na_2S_2O_3$ for the CHX group. Removal of intracanal dressings was confirmed visually with the help of an operating microscope (Carl Zeiss microscopy Gmbh, Munich, Germany). S3 samples were then acquired with three size 30 sterile paper points and stored as above. Subsequently, another rinse with 3% NaOCl was performed, the master apical files (size 35 or 50 K-file) were verified, and a radiograph with the correspondent gutta-percha point was exposed. Then, the canals were irrigated with 10% citric acid, followed by 3% NaOCl and finally rinsed with 70% alcohol. After drying with sterile paper points, canals were filled with gutta-percha points (principal and accessories) and TopSeal (Dentsply Maillefer) using the lateral compaction technique, sealed with Synergy D6 Flow (Coltène/Whaledent) and the access cavity temporized with Coltosol (Coltène/Whaledent).

Paper points used for canal sampling (S1, S2, S3) were transferred into vials containing RTF and processed in the laboratory within 2 h.

Microbiological procedures

Anaerobic culture

Samples in RTF vials were managed as described (Siqueira *et al.* 2007a). Aliquots of 100 μL of undiluted and highest dilution (10^{-3}) were spread onto BHI (Liofilchem, Roseto degli Abruzzi, Italy), and Anaerobe Basal (Oxoid, Basingstoke, UK) agar plates supplemented with 5% defibrinated horse blood (Oxoid, Basingstoke, UK). Plates were incubated within jars, at 37 °C, anaerobically for 14 days. Colony-forming units (CFUs) were then counted.

After this, each sample vial was immediately frozen at −20 °C until further analysis by molecular techniques.

DNA extraction

Bacterial DNA was extracted using the Qiagen DNA mini Kit (Qiagen, Hamburg, Germany) according to the manufacturer's instructions for Gram-positive bacterial DNA extraction (i.e. including a lysozyme bacterial wall lysis). DNA was eluted in 200 μL of the elution buffer included in the kit.

DNA was stored at −20 °C until used for qPCR.

Bacterial 16S rDNA quantification calibrator

Pure *Escherichia coli* colonies were harvested from agar culture plates, suspended in PBS and quantified by light densitometry using a Densimat instrument (Biomerieux, Parma, Italy). The MacFarland scale was then converted to bacterial cell concentration using the manufacturer's conversion table. From this, sample DNA was extracted as above, and the resulting DNA solution was used to create a 10-fold dilution series for qPCR results calibration.

16S rDNA quantitative real-time PCR

Bacterial Ribosomal 16S rRNA gene was quantitatively amplified using the 347F (5′-GGA GGC AGC AGT RRG GAA T-3′) and 803R (5′-CTA CCR GGG TAT CTA ATC C-3′) degenerate primers designed by others (Nossa *et al.* 2010). Quantitative real-time PCR (qPCR) was performed on a Lightcycler 1.2 Instrument (Roche, Penzberg, Germany), using the iTaq Universal SybrGreen Supermix (Biorad, Foster City, CA, USA). Briefly, 15 μL total reactions consisted of 1× supermix (Biorad), primers (0.3 μ mol/L each) and 5 μL of extracted DNA sample. All PCR reactions included serial dilutions of the calibrator DNA, and a negative control. Temperature cycles included an initial enzyme activation and DNA denaturation step at 95 °C for 2 min, followed by 30 rounds of amplification including a denaturation step for 10 s at 94 °C, 52 °C annealing for 15 s and amplification during 30 s at 72 °C.

After amplification, product specificity was checked by melting temperature analysis. Results were analysed by crossing point (*C*p) determination with calibration by serial dilution of a calibrator sample. All samples were tested in duplicate. Linear regressions observed for the calibration curves were evaluated by R^2, and found to be above 90%. All single quantification points were used for statistical analysis.

Statistical analysis

Statistical analysis was performed using IBM© SPSS© Statistics vs.20.0 (IBM Corporation, New York, NY, USA) at a significance level of 0.05.

Differences in bacterial counts between S1, S2 and S3 and amongst medication groups, and initial diagnosis, were first analysed with the Kruskal–Wallis test as non-normal distributions were always found (Shapiro–Wilk test). Upon detection of significant differences, these were further investigated with the Mann–Whitney *U*-test. Comparison of intracanal dressing effect at S3 was assessed using the Mann–Whitney *U*-test (Table 1). Correlation between quantification methods were assessed using Spearman's correlation coefficients.

Results

Randomization of intracanal dressing proved independent of diagnosis ($P = 0.687$), tooth position ($P = 0.436$) and 'master apical file' (I = 0.233) (chi-squared test, data not shown).

Quantitative microbiological analysis

Between S1 and S2, for both experimental groups and by the two approaches (16S rRNA gene qPCR and Anaerobic Culture), a consistent significant decrease on bacterial equivalents and bacterial load ($P < 0.001$) was found (Table 1).

The same was not true for S2–S3 comparison: CHX-treated samples had a significant ($P < 0.05$) increase in bacterial equivalents and CFU's. On the contrary, no difference was observed for $Ca(OH)_2$ both by culture and molecular techniques (Table 1).

Analysis of the putative influence of the different initial diagnosis (necrosis or apical periodontitis), and the performance of the intracanal dressing tested is shown in Table 1. In teeth presenting only necrotic pulps, the increases of bacterial equivalents between S2–S3, for both $Ca(OH)_2$- and CHX group were not significant, unlike the CFU/mL with which a maintenance was observed. Within teeth with apical periodontitis, significantly lower values in S3 were observed within $Ca(OH)_2$- than in CHX-treated patients (Mann–Whitney *U*-test, $P \leq 0.001$; Table 1) by both techniques.

The analysis of the correlation (Spearman's correlation coefficients) between the two approaches always showed a positive association, although not significant for all combinations under scrutiny. Correlation was significant for S1, independently of the initial diagnosis ($r_s = 0.410$; $P < 0.001$), although more evident for apical periodontitis cases ($r_s = 0.462$; $P = 0.002$). Again, in S2, both technologies showed correlating results ($r_s = 0.296$; $P = 0.013$), but this correlation was more evident in necrotic pulps ($r_s = 0.429$; $P = 0.026$). Comparing medicaments (S3 samples), in the $Ca(OH)_2$ group, a significant correlation was observed independently of the initial diagnosis ($r_s = 0.372$; $P = 0.030$), particularly with necrotic pulps ($r_s = 0.609$; $P = 0.021$).

Discussion

In the design of the experiment, the major concern was on the relevance of samples to the clinical situation. In this regard, the last paper point can be regarded as the most important because it absorbs the liquid from the apical region. In narrow root canals, the paper points were difficult to handle; so, it was challenging to collect valid samples of the apical regions in those teeth. To overcome this problem, sterilized small files (sizes 008, 010, 015) were used without handles, and these were included with the paper points in the vials of the samples.

Studies on the dynamics of root canal infections have shown that the relative proportions of anaerobic microorganisms and bacterial cells increase with time and that the facultative anaerobic bacteria are outnumbered when the canals have been infected for 3 months or more (Fabricius *et al.* 1982). The consumption of oxygen and production of carbon dioxide and hydrogen along with the development of a low reduction–oxidation potential by the pioneer species favour the growth of anaerobic bacteria (Figdor & Sundqvist 2007) leading to pulpal necrosis followed by apical periodontitis. The anaerobic atmosphere was therefore chosen for the culture approach as the selective endodontic milieu supports the development of anaerobic microorganisms.

Molecular techniques can overcome the shortcomings of culture methods because they exhibit increased sensitivity and specificity as well as the ability to reliably detect culture-difficult and even as-yet uncultivated bacteria (Siqueira & Rôças 2009). Thereby, it seems logical to use them in investigations such as the present one.

Broad-range PCR and culture results reflect different albeit related realities. Therefore, the temptation to perform direct comparisons should be avoided. In

Table 1 Comparison of the Classical Microbiology Culture CFU quantification results with qPCR bacteria equivalents quantification

		Molecular Biology (bacterial equivalent/sample)					Culture – CFU/mL (anaerobic)				
Diagnosis	Statistics	S1	S2	S3 CHX	S3 $Ca(OH)_2$	P^a	S1	S2	S3 CHX	S3 $Ca(OH)_2$	P^a
ALL	*n*	138	138	68	70		69	69	34	35	
	Av. (±StDev)	6.1×10^8 ($\pm 6.5 \times 10^9$)	5.3×10^6 ($\pm 7.4 \times 10^6$)	4.3×10^7 ($\pm 2.0 \times 10^8$)	7.1×10^6 ($\pm 2.2 \times 10^7$)		1.8×10^4 ($\pm 4.9 \times 10^4$)	2.7×10^2 ($\pm 1.7 \times 10^3$)	6.2×10^3 ($\pm 1.0 \times 10^4$)	2.0×10^3 ($\pm 6.9 \times 10^3$)	
	P25–P75	3.9×10^6 – 3.2×10^7	0–7.4×10^7	2.8×10^7 – 1.9×10^8	0–5.0×10^7		0–6.3×10^4	0–0	0–9.1×10^4	0–3.8×10^1	
	Median	7.6×10^6 a	2.7×10^6 b	9.1×10^6 aA	2.2×10^5 bB	<0.001	510 a	0 b	345 aA	0 Bb	<0.001
				P<0.001					*P*<0.001		
NEC	*n*	54	54	24	28		27	27	12	14	
	Av. (±StDev)	1.8×10^7 ($\pm 6.1 \times 10^7$)	4.6×10^6 ($\pm 7.4 \times 10^6$)	8.6×10^6 ($\pm 1.1 \times 10^7$)	6.0×10^6 ($\pm 1.2 \times 10^7$)		8.5×10^3 ($\pm 2.9 \times 10^4$)	5.5×10^2 ($\pm 2.7 \times 10^3$)	1.2×10^3 ($\pm 3.8 \times 10^3$)	4.6×10^3 ($\pm 1.0 \times 10^4$)	
	P25–P75	8.2×10^4 – 9.3×10^7	0–6.5×10^7	2.3×10^1 – 1.4×10^8	0–6.6×10^7		0–3.6×10^4	0–0	0–7.5×10^1	0–1.5×10^4	
	Median	2.7×10^6	3.7×10^4	4.7×10^6	1.9×10^6	0.052	1.9×10^2 a	0 b	0 b	0 b	<0.001
				P = 0.277					*P* = 0.972		
AP	*n*	83	84	44	42		42	42	22	21	
	Av. (±StDev)	9.9×10^8 ($\pm 8.4 \times 10^9$)	5.6×10^6 ($\pm 7.4 \times 10^6$)	6.1×10^7 ($\pm 2.5 \times 10^8$)	7.8×10^6 ($\pm 2.7 \times 10^7$)		2.4×10^4 ($\pm 5.8 \times 10^4$)	8.9×10^1 ($\pm 4.0 \times 10^2$)	9.0×10^3 ($\pm 1.2 \times 10^4$)	1.3×10^2 ($\pm 3.2 \times 10^2$)	
	P25–P75	3.4×10^7 – 3.8×10^8	2.0×10^1 – 7.6×10^7	4.1×10^7 – 3.4×10^8	0–4.3×10^7		1.7×10^2 – $^1 3.3 \times 10^4$	0–0	2.2×10^2 – 1.9×10^4	0–1.1×10^2	
	Median	1.6×10^7 a	3.9×10^6 b	1.0×10^7 aA	9.2×10^4 bB	<0.001	1.0×10^3 a	0 b	1.6×10^3 aA	0 bB	<0.001
				P<0.001					*P*<0.001		

[a]Kruskal–Wallis test.

a,b,c – different letters stand for significant differences between sampling moments, according to the Mann-Whitney U test; A,B – different letters stand for significant differences between intracanal dressing, according to the Mann-Whitney U test; *P* – significance level; Av., average; StDev, standard deviation; P25–P75 25th and 75th percentiles; CFU, colony-forming units.

particular, comparisons of positive/negative results between techniques must be avoided, as they indicate completely different realities. Thus, a direct analysis (sample by sample) of the two methodologies does not reflect concordance or disagreement, but rather the different physical entities being measured. It was interesting to observe the correlations of results from the two methodologies were always positive (thus with similar trends), indicating that the study measured different but related physical entities. In fact, data from microbiologic root canal sampling and culture counts may be an underestimation of the total *in vivo* bacterial load, as reported previously due to lack of detection of: (i) nonviable bacteria due to sampling and transportation problems (Siqueira & Lopes 1999, Sathorn *et al.* 2007); (ii) noncultivable bacteria due to specific culture requirements (i.e. fastidious bacteria) (Conrads *et al.* 1997, Munson *et al.* 2002, Foschi *et al.* 2005, Gomes *et al.* 2006). On the other hand, broad-range PCR measures bacterial DNA load, irrespective of its nature: live and dead, viable and unviable bacteria or even naked DNA, all contribute to the total DNA being quantified. Thus, qPCR results cannot be treated as bacteria counts, but rather as bacteria equivalents, a unit reflecting the total amount of 16S rRNA gene present in the sample and that once was part of a living bacteria cell. It is thus the sum of all bacteria detectable by culture plus a large array of dead or nonviable or noncultivable bacteria plus all naked DNA resulting from nonphagocytic bacteria lyses. Even if bacteria are present in phagocytic cells, it may be expected to be detectable by qPCR as the DNA extraction method should be able to lyse all phagocytic cells and expose all of its content. Although this may seem at first as a disadvantage, it may in fact reflect the total dynamics of bacterial presence in the root canal, as it is completely insensitive to the limitations of culture techniques.

Using both strategies, the aim was to compare treatment performance, disregarding the technical limitations of the bacteria detection technique being used. Thus, the aim was to analyse bacteria detection dynamics with both techniques as surrogate markers to evaluate the effect of disinfection with chemomechanical preparation alone and with the two intracanal dressings tested.

The expected further disinfection of intracanal dressings failed as reported in previous studies (Trope *et al.* 1999, Shuping *et al.* 2000, Peters & Wesselink 2002, Sathorn *et al.* 2005, Waltimo *et al.* 2005, Figini *et al.* 2007, Paquette *et al.* 2007). In addition to the technical limitations described above, bacterial dynamics in the root canal, particularly between S2 and S3 samples may also be affected by lingering bacteria that are, most often, located and able to survive in inaccessible areas (Peters *et al.* 2001) to instrumentation and sampling. Thus, culture reversals or increased bacterial equivalents from S2 to S3 may reflect a possible flaw during S2 sample collection (Siqueira *et al.* 2007b) or the growth of bacteria from their original hidden anatomical location to the main canal. Another explanation could be, that the use of an intracanal medicament, may favour contamination of the sample calling attention to the risk of false-negative and false-positive culture results (Reit *et al.* 1999, Sathorn *et al.* 2007). Finally, it could be due to the presence of bacteria intrinsically resistant to the irrigant or to the intracanal dressing, possibly embedded in tissue remnants or arranged in biofilm structures, and thus protected from the lethal effects of medicaments (Siqueira *et al.* 2007b). Finally, inactivation or decreased activity of the intracanal dressings tested induced by dentine constituents, inflammatory exudate seeping into the canal, bacterial products, and components of the necrotic tissue, particularly in the subsurface layers of the root canal walls, might have been the main factors behind the reduced antibacterial effect (Portenier *et al.* 2001, Manzur *et al.* 2007, Mohammadi *et al.* 2012). It is possible that deeper in dentine (outside the main root canal), $Ca(OH)_2$ is present as a saturated solution or at concentrations even below that level (Haapasalo *et al.* 2000).

Application of a 14-day intracanal dressing with $Ca(OH)_2$ significantly increased the number of negative cultures compared with CHX, but not compared with S2 cultures.

In a laboratory study (Tavares *et al.* 2011), $Ca(OH)_2$, placed for 15 days, maintained throughout the experimental period, the numbers of all cytokines, proinflammatory or regulatory and the chemokine CCL-2/MCP-1 assessed. Thus, the benefits of $Ca(OH)_2$ become evident; if it does not reduce cytokine basal expression observed at day 0, it impedes the increase of all cytokines during the experimental time. However, whether these effects are due to its anti-inflammatory, antibacterial or physical barrier properties is a matter of debate (Tavares *et al.* 2011).

Despite the antibacterial effect of CHX, its activity is pH dependent (McDonnell & Russell 1999). In endodontic applications, the antibacterial efficacy of CHX has compared favourably with that of NaOCl (Vianna *et al.* 2006), and substantive antimicrobial activity has been reported when CHX was used as an intracanal

medicament (Basrani *et al.* 2002). Nevertheless, *in vivo* studies in which CHX was used for intracanal medication have reported, only a moderate bacterial reduction below threshold levels: in 78% of canals using a 0.12% liquid solution (Barbosa *et al.* 1997), in 46% of canals using a 2% liquid solution (Paquette *et al.* 2007) and in 55% of canals using a 2% gel (Manzur *et al.* 2007).

Conclusions

Within the limits of the present study, it is suggested that for teeth with necrotic roots associated or not with apical periodontitis, a thorough chemomechanical preparation using 3% NaOCl is able to significantly reduce the bacterial load of root canals to levels capable of inducing the desirable successful outcome. A further significant reduction was not accomplished by inclusion of intracanal medicaments for 14 days. Thus, the benefit of intracanal dressing to improve the overall treatment outcome is questionable. Nevertheless, the long-term clinical impact of the protocols using either of these two medicaments deserves further investigation.

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