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Effect of ultrasonic activation on the reduction of bacteria and endotoxins in root canals: a randomized clinical trial

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Abstract

Nakamura VC, Pinheiro ET, Prado LC, Silveira AC, Carvalho APL, Mayer MPA, Gavini G. Effect of ultrasonic activation on the reduction of bacteria and endotoxins in root canals: a randomized clinical trial. *International Endodontic Journal*.

Aim This randomized clinical trial aimed to compare the effectiveness of ultrasonic activation with that of nonactivated irrigation on the removal of bacteria and endotoxin from root canals.

Methodology Fifty patients with necrotic pulps and asymptomatic apical periodontitis were randomly allocated into two groups according to the final irrigation protocol after root canal preparation: Group UI – ultrasonic irrigation ($n = 25$) and Group NI – needle irrigation ($n = 25$). The root canals were medicated with calcium hydroxide for 14 days. Microbiological sampling was performed before (S1) and after the root canal preparation (S2), after the irrigation protocols (S3) and after the removal of the intracanal medication (S4). Total bacteria counts were determined by qPCR and the endotoxin levels by the limulus amebocyte lysate assay. Intragroup analyses were performed using the Wilcoxon test for related samples, whereas intergroup analyses were performed using the Mann–Whitney *U*-test ($P < 0.05$).

Results All S1 samples were positive for bacteria, with median numbers of 1.49×10^6 and 8.55×10^5 bacterial cells for the UI and NI groups, respectively. This number significantly decreased in S2 samples (UI: 1.41×10^4; NI: 3.53×10^4; both with $P < 0.001$). After final irrigation protocols, there was a significant decrease in bacterial load from S2 to S3 samples in both groups (UI: 4.29×10^3; NI: 1.08×10^4; $P < 0.01$). Intergroup analysis revealed a significant difference between irrigation methods regarding bacterial counts in S3 samples ($P < 0.05$). In contrast, no significant differences were observed between groups for endotoxin levels ($P > 0.05$).

Conclusions Ultrasonic activation was more effective than nonactivated irrigation for reducing the number of bacteria but not the endotoxin levels in root canals of teeth with apical periodontitis.

Keywords: apical periodontitis, bacterial infection, endotoxin, root canal treatment, ultrasonic activation.

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Introduction

Bacteria are the main aetiological agents of pulpitis and apical periodontitis. Intraradicular bacteria have virulence factors such as lipopolysaccharide (LPS), or endotoxin, which can activate the inflammatory process in apical tissues. LPS is the major structural component of the outer cell wall of gram-negative bacteria, and the release of endotoxin is involved in the development of apical periodontitis (Hong *et al.* 2004). Therefore, the aim of root canal treatment in infected root canals of teeth with apical periodontitis is to provide maximal reduction of bacteria and their by-products to promote apical healing.

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Molecular-based clinical studies have shown that although chemo-mechanical preparation significantly reduced the bacterial load in root canals, many cases remain infected after root canal preparation (Vianna *et al.* 2006, Rôças & Siqueira 2011a,b, Rôças *et al.* 2013, 2014, Neves *et al.* 2014, 2016). Similarly, studies evaluating the effect of chemo-mechanical procedures on endotoxin levels have indicated that the use of rotary nickel–titanium files and irrigant significantly reduced endotoxin content but was not able to eliminate it from root canals (Vianna *et al.* 2007, Martinho & Gomes 2008, Gomes *et al.* 2009, Martinho *et al.* 2010, Xavier *et al.* 2013, Herrera *et al.* 2015, 2016). Therefore, alternative approaches have been proposed to supplement the antimicrobial effect of chemo-mechanical preparation in infected root canals, including a final irrigation protocol using additional ultrasonic activation of the irrigant (Siqueira & Rôças 2011).

Ultrasonic activation is based on the transmission of acoustic energy through the irrigant by a stainless steel wire or endodontic file (Van der Sluis *et al.* 2007). This energy is dissipated through the irrigant by ultrasonic waves leading to cavitation (Roy *et al.* 1994) and acoustic streaming (Ahmad *et al.* 1987). The latter is the biophysical force most commonly associated with endodontic files during ultrasonic activation (Lea *et al.* 2010). On the other hand, the appearance of cavitation inside root canals *in vivo* is debated, especially at energy levels recommended for endodontic procedures (Lea *et al.* 2010). Although the mechanism of action for ultrasonic activation in root canal irrigation is not clear, laboratory studies have shown that ultrasonic activation of antimicrobial solutions promotes the removal of dentine debris (Rödig *et al.* 2010, Amato *et al.* 2011) and bacterial biofilm from infected root canals (Townsend & Maki 2009, Harrison *et al.* 2010, Cachovan *et al.* 2013, Ordinola-Zapata *et al.* 2014, Mohmmed *et al.* 2016). However, the clinical effectiveness of ultrasonic activation in improving root canal disinfection after chemo-mechanical preparation has yet to be proven (Siqueira & Rôças 2011, Paiva *et al.* 2012, 2013).

Randomized clinical trials (RCT) are considered the 'gold standard' for assessing intervention effects (Koletsi *et al.* 2012). To date, only one RCT evaluating the antimicrobial effect of ultrasonic activation has been reported (Beus *et al.* 2012). The authors evaluated the effect of ultrasonic activation on the bacterial culture status of teeth when compared to a nonactivated irrigation and found no significant difference between them. In the latter study, each protocol resulted in a high frequency of negative cultures, which may be related to the constraints of culture methods, including low sensitivity and inability to detect uncultivated bacterial species (Siqueira & Rôças 2009). One strategy to overcome these problems is the use of culture-independent molecular approaches, which have been considered the method of choice to assess the antimicrobial effectiveness of endodontic procedures (Siqueira & Rôças 2009). There are no RCTs comparing the antibacterial effectiveness of ultrasonic and conventional irrigation using molecular-based methods. Moreover, there are no RCTs assessing the effectiveness of ultrasonic activation in reducing endotoxin from infected root canals with apical periodontitis. It is possible that mechanical agitation of the irrigant could improve bacterial DNA and endotoxin reduction after root canal preparation.

Therefore, the main goal of this RCT was to evaluate the effect of ultrasonic activation on the reduction of bacterial and endotoxin levels in root canals when compared to a nonactivated irrigation protocol using quantitative polymerase chain reaction (qPCR) and the limulus amebocyte lysate (LAL) assays. The null hypothesis is that the irrigation protocol used after the chemo-mechanical preparation does not influence the reduction of bacteria and endotoxin within the root canal system. Additionally, the antibacterial effect of each step of the endodontic therapy was evaluated, including chemo-mechanical preparation, final irrigation protocols and calcium hydroxide intracanal medicament.

Materials and methods

Study design, sample size and participants

This single-blinded, 2-arm, randomized controlled clinical trial compared an ultrasonic activation as the test protocol and a nonactivated irrigation protocol as the control regarding their effectiveness in reducing bacteria and endotoxin from root canals after chemo-mechanical preparation. Clinical procedures were performed by two endodontic specialists (VCN, LCP) who had limited their work to endodontics for at least 4 years. The study was not operator blinded because of the various devices used during the irrigation protocols. However, data analysis was performed by a researcher (ETP) who was blind to the treatment group.

Patients reporting to the postgraduate endodontic clinic from January 2014 to July 2016, within the age group of 18–65 years, were enrolled. Criteria for inclusion were: patients who had asymptomatic teeth with necrotic pulps confirmed by a negative response to sensitivity pulp tests; radiographic evidence of apical periodontitis in single rooted teeth or in one root with a single canal from multirooted teeth. The following exclusion criteria were applied: patients who had received antibiotics during the previous 3 months or had any general disease; teeth that could not be properly isolated with rubber dam; nonrestored teeth; periodontal pockets depths greater than 4 mm; and radiographic evidence of previous endodontic treatment, open apex, crown/root fracture, root resorption or calcifications. Moreover, teeth with root canal curvatures greater than 20° (Schneider 1971) and roots shorter than 15 mm or longer than 25 mm were excluded. This study was conducted in accordance with the Helsinki declaration and was approved by the Institutional Ethical Committee (#428.730). Patients signed a written term of free and informed consent prior to study commencement.

The sample size was estimated based on the means and standard deviations of the pilot study using BioEstat 5.3 software (Mamiraua, AM, Brazil). Calculation of statistical significance was estimated using the Wilcoxon test (power of 80% and significance level of 5%). A sample size of 20 patients per group was calculated based on the results of the pilot study. Considering the dropouts, a final sample size of 25 patients per group was decided. Randomization was performed using a block randomization method generated by an electronic online randomizer (Research Randomizer, www.randomizer.org). Based on this computer randomization list, the participants were assigned to one of two groups: '1' for ultrasonic activation and '2' for a nonactivated irrigation protocol. The number list was kept in a sealed envelope by 1 of the authors (VCN). The operators (VCN, LCP) were not aware of the irrigation technique until the time of irrigation. After chemo-mechanical preparation, the number list was removed from an envelope to determine which irrigation protocol would be performed.

Interventions

All materials, except for the thermo-sensitive ones, were heat sterilized at 180 °C for 4 h to become free from endotoxins (Hagman 2012). The thermo-sensitive materials, such as irrigation/aspiration needles, were treated with Cobalt 60 gamma radiation 20 kGy for 6 h (Xavier *et al.* 2013). The methods used for the microbiologic samples have been described previously (Gomes *et al.* 2004, Martinho *et al.* 2010). Aseptic techniques were used throughout treatment and sample collection. Teeth were isolated with rubber dams, and the operative field was disinfected with 30% H_2O_2 (v/v) and 2.5% NaOCl for 30 s each, followed by 5% sodium thiosulphate to inactivate the disinfectant agents. Access cavities were started with sterile high-speed diamond burs irrigated with sterile saline to remove caries and restorations. Before entering the pulp chamber, the access cavity was disinfected again, and a bacteriologic sample was taken with sterile paper points, as a control sample, to check the sterility of the disinfected surface. Then, the access cavity was completed using new sterile diamond burs. Control samples were placed in cryotubes and frozen at −20 °C for further DNA extraction. The absence of bacteria in the control sample was verified by polymerase chain reaction (PCR) using universal primers for the bacteria domain.

After access cavity preparation, the root canal was filled with sterile saline solution, and the working length was established 1.0 mm short of the apical foramen. This was established with an electronic apex locator (J. Morita Brazil, São Paulo, SP, Brazil). A size 15 H-file was pushed against the root canal walls to suspend bacteria and endotoxins into the solution. Five sterile paper points were placed individually inside the root canal for 1 min each to collect the initial content of bacteria and endotoxins (S1). The first paper point was placed in a pyrogen-free glass tube and frozen at −20 °C for further endotoxin analysis. The remaining paper points and the H-file, devoid of the handle, were transferred to cryotubes containing 300 μL of RNAlater solution (Life Technologies, Carlsbad, CA, USA) and frozen at −80 °C for bacterial analysis. In each case, a single root canal was sampled to confine the microbial evaluation to a single ecological environment.

Root canal preparation was performed with R40 or R50 Reciproc instruments (VDW GmbH, Munich, Germany), depending on the initial diameter of the root canal. The selection of instruments to be used followed the manufacturer's instructions. All instruments were used only once. Each canal received an initial flush with 10 mL of 2.5% NaOCl delivered by a disposable syringe and 30G side-vented endodontic needles (EndoEZE, Ultradent Products Inc., South

Jordan, UT, USA); then, the Reciproc instrument was inserted into the cervical third with an in-and-out pecking motion. After a cycle of three in-and-out movements, as more pressure was needed to advance the instrument further into the canal, the file was removed, and its flutes were cleaned. The root canal was irrigated again with 10 mL of 2.5% NaOCl, and a new cycle of three in-and-out movements was performed in the middle third followed by new irrigation. The instrument was inserted up to the working length with a brushing motion against the root canal walls. At the end of the preparation, the canal was irrigated with 10 mL of 2.5% NaOCl, completing a total volume of 40 mL. The canal was dried using paper points and flushed with 5 mL of 5% sodium thiosulphate for 1 min. The root canal was then filled with sterile saline, and a post-instrumentation sample (S2) was taken as described above.

Teeth were divided into two groups according to the final irrigation protocol used after chemo-mechanical preparation. In the ultrasonic irrigation (UI) group, the irrigant was activated by a smooth wire with 0.2 mm diameter and .01 taper (Irrisonic – Helse, Ribeirão Preto, SP, Brazil), driven by an piezoelectric ultrasonic device (CVDentus, São José dos Campos, SP, Brazil) set at 10% power following the manufacturer's recommendations. The tip of the insert was positioned at 2 mm from the working length, avoiding touching the root canal walls. First, the root canal was filled with 2 mL of 2.5% NaOCl delivered by disposable syringe and 30G side-vented endodontic needles, inserted up to 2 mm short of the working length and then activated for 30 s. The canals were refilled with fresh 2 mL of 2.5% NaOCl and activated for an additional 30 s. Then, the remaining fluid was aspirated, and 2 mL of 17% EDTA was added inside the root canal and activated for 30 s. The root canal was refilled with 17% EDTA and activated for another 30 s. The remaining fluid was aspirated, and the root canal was filled again with 2 mL of 2.5% NaOCl and activated for 30 s. The 2.5% NaOCl was renewed (2 mL), and a final ultrasonic activation was performed for 30 s. In the needle irrigation (NI) group, the volume of the irrigants, their sequence, the depth and time of irrigation were similar to the UI group but with no-ultrasonic activation. The flow rate was approximately 2 mL min^{-1}. In both groups, 2.5% NaOCl was inactivated again using 5% sodium thiosulphate, and a new sample was taken after the final irrigation protocol (S3).

After S3 samples were taken, the root canals were dried using paper points and filled with Calen paste (S.S. White, Rio de Janeiro, RJ, Brazil), which comprises $Ca(OH)_2$, zinc oxide, colophony (pine resin) and polyethylene glycol 400. The paste was inserted in the canals using an ML endodontic syringe (S.S. White) attached to a Septojet XL needle (Septodont Brasil Ltd, Barueri, SP, Brazil). Radiographs confirmed the proper root canal filling with the intracanal medication. The access cavities were filled with 2 mm of temporary restorative material (Dentalvile, Joinville, SC, Brazil) and glass–ionomer cement (Vidrion R, S.S. White).

After 14 days, the tooth was isolated, the temporary restoration was removed and disinfection procedures of the operative field were performed following the same protocol used in the first visit. A new control sample of the dental crown and surrounding dentine of the pulp chamber was taken. The intracanal medicament was removed with 10 mL of 17% EDTA and agitation with 15 K files. Then, a fourth sample (S4) was taken following the same procedures performed in previous collections. Completion of the root canal treatment proceeded with the root filling using lateral condensation of gutta-percha and AH Plus sealer (Dentsply DeTrey). The access cavities were restored with temporary endodontic cement and composite resin (Z350, 3M Corporation, Saint Paul, MN, USA), and a final radiograph was taken.

Primary outcome measures: bacteria quantification by qPCR

DNA extraction was performed with the MasterPure purification kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions. Briefly, after centrifugation at 10,000 × ***g*** for 10 min, supernatants were discarded, and pellets were resuspended in a solution containing 300 μL of tissue and cell lysis solution and 2 μL of 50 μg μL^{-1} proteinase K. After incubation for 15 min at 65 °C, mixtures were cooled on ice for 5 min and added to 200 μL of MPC protein precipitation reagent. After centrifugation at 10,000 × ***g*** for 10 min, supernatants were collected and subjected to isopropanol precipitation. Total nucleic acid samples were resuspended in 35 μL of TE buffer. The concentration and purity of the DNA were analysed by spectrophotometry (NanoDrop 1000 – Thermo Fisher Scientific, Wilmington, DE, USA).

Universal primers (Life Technologies Corporation, Carlsbad, CA, USA) were designed for the conserved

regions of the 16S rRNA gene of the bacteria domain according to Shelburne *et al.* (2000): Forward: 5′-CC A TGA AGT CGG AAT CGC TAG-3′ and reverse: 5′-G CT TGA CGG GCG GTG T-3′.

Real-time PCR reactions were performed in 96-well plates with a total volume of 20 μL per reaction containing: 10 μL of Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 2 μL of sample DNA and 100 nmol L^{-1} of each primer. Deionised water served as negative control. Cycling conditions for qPCR reactions were as follows: 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, and extension at 95 °C for 15 s. The reactions were performed with a Step One Plus thermocycler (Applied Biosystems, Foster City, CA, USA), and results were analysed using Step One Plus software v2.3 (Applied Biosystems). The standard curve was built using recombinant plasmids containing the 1500 fragments encoding 16S rRNA gene of *E. faecalis* (Teixeira *et al.* 2009). Plasmid standard dilutions (from 10^7 to 10 DNA copies) were run in triplicate, and the limit of quantification was 10^2 DNA copies. Correlation coefficient (r^2), amplification efficiency (E) and y-intercept values of the standard curve for the qPCR using universal primers were 0.989, 97.8% and 34.5, respectively. DNAs were also run in triplicate, and mean values for DNA measurements were used to calculate the total number of DNA copies per root canal sample.

Secondary outcome measures: quantification of endotoxin by LAL assay

The endotoxin levels of the samples were analysed by the quantitative kinetic turbidimetric LAL assay (Pyrogent 5000, Lonza Group Ltd, Walkersville, MD, USA) following the manufacturer's instructions. The root canal samples were suspended in 1 mL of pyrogen-free water, agitated in vortex for 1 min and serially diluted to 10^{-2}. To perform the analysis, 100 μL of the root canal samples was added in quadruplicates in 96-well plates, which were then incubated at 37 °C for 10 min. After incubation, 100 μL of reconstituted LAL reagent (Pyrogent 5000 reagent + reconstitution buffer, Lonza Group Ltd) was added to each well. The absorbance for each well was measured with an enzyme-linked immunosorbent assay plate reader (ELX808LBS Absorbance Plate Reader, Lonza Group Ltd) at 340 nm. A standard curve was generated using 10-fold serial dilutions (0.01, 0.1, 1 and 10 EU mL^{-1}) of an *Escherichia coli* endotoxin solution (100 EU mL^{-1}) provided by the manufacturer. Pyrogen-free water (Lonza Group Ltd) served as negative control. The mean endotoxin levels were estimated by comparison with the standard curve values, using WinKQLC Endotoxin Detection and Analysis Software (Lonza Group Ltd).

Statistical analysis

Data were summarized and statistically analysed with SPSS Statistics Desktop software (IBM Corporation, Armonk, NY, USA). The adherence analysis was performed using the Kolmogorov–Smirnov test that demonstrated non-normal distribution across the analysed groups. Intragroup analyses were performed using the Wilcoxon test for related samples. Comparisons between groups were performed using the Mann–Whitney test for quantitative analysis of bacteria and endotoxins and chi-squared tests for qualitative analysis (incidence of positive samples for bacterial DNA and endotoxin). Multiple linear regression was used to examine the effect of independent variables (age/gender of the patient, tooth type and Reciproc file size) on the dependent variables (bacteria and endotoxin levels). All analyses were performed with a significance level of 5%.

Results

A CONSORT flow diagram illustrating subject flow during the clinical trial is presented in Fig. 1. The recruitment period was from January 2014 to July 2016, and data analyses were completed in December 2016. The 50 participants consisted of 37 females and 13 males, with a mean age of 39 years ranging from 18 to 63 years. Clinical characteristics of the teeth and their distribution in treatment groups are shown in Table 1. Multiple linear regression was performed on the independent variables including age/gender of the patient, tooth type and Reciproc file size. None of the investigated variables had a significant effect on bacteria and endotoxin levels at any point in time.

All control samples were PCR negative, which indicated no bacterial contamination. Therefore, all cases were included for bacterial analysis. Quantitative data for bacteria are summarized in Table 2. In the UI group, the median number of bacterial cells in S1 samples decreased significantly in S2 ($P < 0.001$) and S3 samples ($P < 0.01$). In the NI group, intragroup

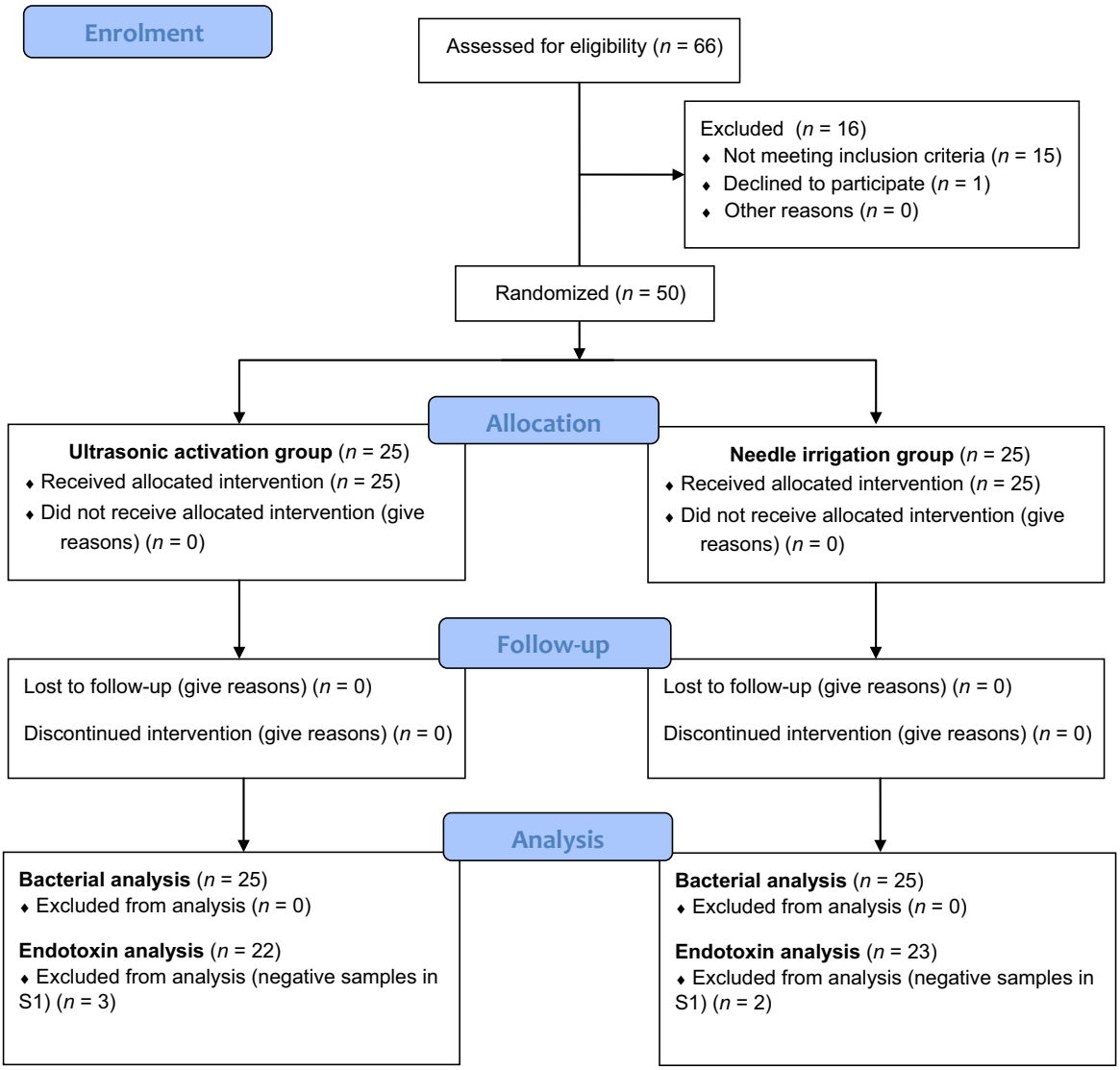

Figure 1 CONSORT flow diagram.

analysis also revealed a significant difference for bacterial levels after each step of the treatment. Comparisons between groups revealed that the UI group had lower bacteria levels than NI in S3 samples ($P < 0.05$), whereas no significant differences were found at baseline (S1) and after root canal preparation (S2). After the use of calcium hydroxide medication, the bacterial counts in S4 became similar in both groups.

Five patients were excluded for endotoxin analysis because S1 samples yielded negative results (three patients from UI group and two from NI group). Table 3 provides an overview of the median values of endotoxin levels at the various sampling times. A significant difference was observed between each step of the treatment for endotoxin levels in both groups. No significant difference was observed regarding endotoxin levels in the intergroup analysis in any step of the treatment ($P > 0.05$).

The qualitative analysis for bacteria and endotoxin is summarized in Tables 4 and 5, respectively. No significant difference was observed between groups for bacteria or endotoxin detection in S3 samples.

Discussion

This clinical study aimed to compare the antimicrobial efficacy of ultrasonic activation and nonactivated

Table 1 Distribution of 50 cases according to gender, tooth type and root canal preparation file in the treatment groups: ultrasonic irrigation (UI) and needle irrigation (NI)

	Treatment groups		Bacterial counts (median)			
			S1		S2	
	UI (n = 25)	NI (n = 25)	UI	NI	UI	NI
Gender						
Female	17	20	1.49×10^6	6.56×10^5	1.34×10^4	3.53×10^4
Male	8	5	3.76×10^6	1.45×10^6	6.01×10^4	5.25×10^4
Tooth type						
Anterior and premolar	21	22	1.68×10^6	1.15×10^6	1.38×10^4	4.83×10^4
Molar	4	3	1.88×10^6	2.01×10^6	4.57×10^4	3.33×10^4
Root canal preparation						
R40 (0.40 mm, Taper 0.6)	12	11	1.87×10^6	2.01×10^6	1.34×10^4	1.66×10^4
R50 (0.50 mm, Taper 0.5)	13	14	1.69×10^6	1.15×10^6	3.61×10^4	7.62×10^4

Table 2 Median values (range) of bacteria in root canal samples of teeth with primary endodontic infections taken before (S1) and after the root canal preparation (S2), after the irrigation protocols (S3) and after calcium hydroxide medication (S4)

	Treatment groups			
Samples	Ultrasonic irrigation	Needle irrigation	P value	95% confidence interval for difference of means
S1	1.49×10^6 (2.66×10^3–3.29×10^7)	8.55×10^5 (1.91×10^2–4.66×10^7)	0.3879	-4.34×10^6 to 5.2×10^6
S2	1.41×10^4 (0–5.67×10^5)	3.53×10^4 (0–3.05×10^6)	0.9768	-1.68×10^5 to 3.46×10^6
S3	4.29×10^3 (0–2.22×10^4)	1.08×10^4 (0–3.38×10^5)	0.0426*	3.7×10^3 to 6.23×10^4
S4	2.39×10^3 (0–3.82×10^4)	4.56×10^3 (0–1.4×10^5)	0.1229	-4.39×10^2 to 2.66×10^4

*There was a significant difference between the median values of ultrasonic irrigation and needle irrigation groups for total bacteria counts in S3 samples (Mann–Whitney U-test, $P < 0.05$).

Table 3 Median (range) values of endotoxin levels (EU mL^{-1}) in root canal samples of teeth with primary endodontic infections taken before (S1) and after the root canal preparation (S2), after the irrigation protocols (S3) and after calcium hydroxide medication (S4)

	Treatment groups			
Samples	Ultrasonic irrigation (n = 22)	Needle irrigation (n = 23)	P value	95% confidence interval for difference of means
S1	57.45 (10–309)	64.95 (10–195)	0.36	−26.72 to 49.32
S2	12.7 (0–36.1)	11.1 (0–53.9)	0.4047	−6.93 to 8.32
S3	6.53 (0–19.4)	5.75 (0–14.8)	0.9439	−2.74 to 3.89
S4	0.46 (0–9.3)	1.2 (0–11.6)	0.7336	−1.44 to 2.66

irrigation protocols. In addition to the comparison between final irrigation protocols, the effects of root canal treatment procedures on the reduction of bacteria and endotoxin levels were also evaluated. In both groups, chemo-mechanical procedures using reciprocating instruments and 2.5% NaOCl irrigation promoted a substantial reduction in the bacterial load and endotoxin levels in root canals. The drastic reduction in the amount of bacteria and endotoxin from S1 to S2 samples can be explained by the mechanical action of the reciprocating instrument along with the chemical properties and the flow of the 2.5% NaOCl solution (Martinho *et al.* 2014). These data are in accordance with previous clinical studies evaluating the effectiveness of chemo-mechanical procedures using 2.5% NaOCl irrigation in reducing the number of bacteria (Vianna *et al.* 2006, Rôças & Siqueira 2011b, Paiva *et al.* 2012, 2013,

Table 4 Number (%) of teeth with positive qPCR results before (S1) and after the root canal preparation (S2), after the irrigation protocols (S3) and after calcium hydroxide medication (S4)

	Treatment groups		
Samples	Ultrasonic irrigation	Needle irrigation	*P* value
S1	25 (100%)	25 (100%)	1
S2	19 (76%)	20 (80%)	0.7328
S3	17 (68%)	18 (72%)	0.7576
S4	16 (64%)	15 (60%)	0.7708

Table 5 Number (%) of teeth with positive LAL results before (S1) and after the root canal preparation (S2), after the irrigation protocols (S3) and after calcium hydroxide medication (S4)

	Treatment groups		
Samples	Ultrasonic irrigation	Needle irrigation	*P* value
S1	22 (100%)	23 (100%)	1
S2	20 (90.9%)	16 (69.5%)	0.2077
S3	17 (77.3%)	15 (65.2%)	0.5557
S4	10 (45.45%)	12 (52.17%)	0.5688

Rôças *et al.* 2013, 2014, Neves *et al.* 2014, 2016) and endotoxin in root canals (Martinho *et al.* 2010, 2014, Herrera *et al.* 2015, 2016).

Both final irrigation protocols significantly reduced the bacterial and endotoxin levels when compared to post-instrumentation samples. Moreover, the number of cases with positive results for bacteria and endotoxin did not differ between the final irrigation protocols. This finding may be related to the great volume and depth of irrigation, as previously reported by Beus *et al.* (2012). Moreover, the anatomical characteristics of the studied teeth may have influenced the results. In this investigation, most teeth were anterior/premolar and were prepared with R40 or R50 Reciproc instruments (VDW GmbH). Therefore, the introduction of fine irrigation needles in wide canals may have improved irrigant effectiveness in the syringe irrigation group.

Although both irrigation methods were effective in reducing bacterial levels, the ultrasonic activation provided greater bacterial reduction than the nonactivated irrigation protocol. The comparison of quantitative data showed significant difference in median bacterial levels between groups. The higher microbial reduction after ultrasonic activation may be related to acoustic streaming and/or warming of the irrigant (Ahmad *et al.* 1987, Van der Sluis *et al.* 2007, Lea *et al.* 2010), which may have improved the effectiveness of the NaOCl in removing planktonic bacteria or disrupted biofilms after root canal instrumentation. On the other hand, placing the side-vented needles 2 mm short of the working length may have limited the cleaning efficacy in the nonactivated group as the irrigant replacement may have been restricted to 1–1.5 mm apically to the needle tip (Boutsioukis *et al.* 2010, Chen *et al.* 2014).

The findings of this study indicate ultrasonic activation is more effective than needle irrigation in removing bacteria from root canals in a clinical setting, as predicted by previous laboratory studies comparing various irrigation techniques (Townsend & Maki 2009, Cachovan *et al.* 2013). However, it is important to note that the results of molecular studies have not yet been correlated with treatment outcomes. Therefore, the difference in median bacterial levels between ultrasonic activation and needle irrigation groups found in the present molecular study might not be clinically relevant as previously demonstrated by Liang *et al.* (2013). The latter trial failed to correlate the efficacy of ultrasonic activation with improved treatment outcome of single-rooted teeth.

With respect to endotoxin reduction, ultrasonic activation did not significantly decrease the remaining LPS levels when compared to the needle irrigation group. This finding is in contrast with a previous clinical study that evaluated the efficacy of ultrasonic activation of EDTA in reducing endotoxin levels in infected root canals (Herrera *et al.* 2016). The divergences between studies may be due to differences in irrigation protocols, especially to the volume of irrigants. The greater volume of irrigation used in the present study may have contributed to endotoxin reduction after root canal preparation, irrespective of the use of ultrasonic activation. It is also important to note that the inclusion of many single-rooted teeth in this study may have contributed to the efficacy of manual syringe irrigation in endotoxin reduction. Considering that ultrasonic activation would have a better performance inside complex root canal systems, such as areas of flattening or isthmus, it would be expected to be more beneficial in a multirooted tooth than a single canal. Further clinical studies including more multirooted teeth would help elucidate this issue.

After the final irrigation protocols, all root canals were medicated with calcium hydroxide for 14 days.

In both groups, the use of calcium hydroxide as an interappointment medication promoted additional reduction of bacterial and endotoxin levels, which is supported by previous molecular studies (Marinho *et al.* 2012, Oliveira *et al.* 2012, Paiva *et al.* 2013, Xavier *et al.* 2013, Martinho *et al.* 2014). The antimicrobial effects of $Ca(OH)_2$ are probably due to protein denaturation and damage to DNA and cytoplasmic membranes (Mohammadi & Dummer 2011), whereas its anti-endotoxin effect occurs by hydrolysis of lipid A in fatty sugars and nontoxic amino acids (Safavi & Nichols 1993). However, even after $Ca(OH)_2$ use, many cases had still detectable levels of DNA and endotoxin. These findings are in accordance with previous *in vivo* studies (Rôças & Siqueira 2011a,b, Paiva *et al.* 2013).

Although the microbial load from root canals may be drastically reduced after current root canal treatment procedures, high levels of bacteria and endotoxins may still be detected in the main root canal. In this context, it is important to highlight that qPCR/LAL methods used in the present study have allowed the quantification of bacteria/endotoxin that were removed from the root canal, but they do not give an indication of the effect of the irrigation procedures on the removal of biofilm from the root canal wall. Considering that bacteria organized as intraradicular biofilms are the main cause of apical periodontitis, it is possible that the remaining biofilm may affect the outcome of the treatment. The clinical implication of residual infection after endodontic procedures should be further evaluated in prospective clinical studies. Moreover, the bacterial diversity of residual infections should also be evaluated.

Conclusions

The results of this clinical study, using quantitative molecular methods, revealed that ultrasonic activation was more effective than the nonactivated irrigation protocol in reducing bacteria from root canals with primary endodontic infections. It was, however, as effective as the nonactivated irrigation protocol in reducing intracanal endotoxin levels. Although each step of the root canal treatment contributed to enhancing disinfection, bacterial DNA and endotoxins were detected in a significant number of cases even after using intracanal medication. The consequence of residual infection should be further evaluated in prospective clinical studies.

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Conflict of interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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