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Ex vivo evaluation of the effects of several root canal preparation techniques and irrigation regimens on a mixed microbial infection

V. C. Nakamura[1], S. Cai[2], G. T. M. Candeiro[1], P. H. Ferrari[1], C. L. Caldeira[1] & G. Gavini[1]

[1]Department of Restorative Dentistry, Dental School; and [2]Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

Abstract

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Aim To assess the *ex vivo* effectiveness of the alternated use of 1% NaOCl and 15% citric acid in association with two instrumentation techniques for the disinfection of root canals infected with *Enterococcus faecalis* and *Candida albicans*.

Methodology Eighty human mandibular premolars with straight, oval root canals standardized to 15 mm in length were infected with a mixed culture of *E. faecalis* and *C. albicans* for 28 days. Five other teeth were used as controls and were neither contaminated nor instrumented. Specimens were divided into two groups ($n = 40$), according to whether the canal preparation technique used manual (K-type) or rotary (Protaper Universal) instruments. These groups were further divided into four subgroups ($n = 10$) according to the irrigation solution used: saline, 1% NaOCl, 1% NaOCl with alternated use of 15% citric acid and 5.25% NaOCl. Root canals were prepared with a crown-down technique until a size 50 K-file or with rotary preparation until an F5 instrument. Microbiological sampling was performed before (S1) and after (S2) the chemomechanical preparation, using sterile paper points. The specimens were split, and 0.02 g of dentine chips was collected from the root thirds to verify the presence of microorganisms in root canal walls.

Results Saline and 1% NaOCl were less effective in reducing microorganisms compared with 1% NaOCl with alternated use of 15% citric acid or 5.25% NaOCl alone ($P < 0.05$). Both manual and rotary preparations significantly reduced microorganisms regardless of the irrigation solution used ($P < 0.05$). However, there was no significant difference between the canal preparation techniques ($P > 0.05$).

Conclusions Irrigation with 5.25% NaOCl and 1% NaOCl alternated with 15% citric acid reduced microorganisms in infected root canals significantly more than saline and 1% NaOCl.

Keywords: chemomechanical preparation, endodontic irrigants, manual instrumentation, root canal disinfection, rotary instrumentation.

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Introduction

Bacteria and their by-products are the primary aetiological agents of pulp and periapical diseases. During root canal treatment, most of these microorganisms are eliminated by the chemomechanical preparation of the root canal, which is performed using endodontic instruments and irrigants (Rôças & Siqueira 2010). However, some microorganisms persist within the root canal system, making the outcome unpredictable.

Enterococcus faecalis is a Gram-positive anaerobic facultative coccus that has been widely studied in

Correspondence: Vitor C. Nakamura, Departamento de Dentística, Faculdade de Odontologia da USP, Av. Prof. Lineu Prestes, 2227, Cidade Universitária, São Paulo, SP, CEP. 05508-000 Brazil (e-mail: vcnakamura@usp.br).

endodontics because of its high prevalence in situations of persistent apical periodontitis (Stuart *et al.* 2006). This microorganism can form biofilms even in situations in which nutrients are scarce (George *et al.* 2005). *E. faecalis* is known to penetrate deeply into dentine, making its complete elimination difficult (Chivatxaranukul *et al.* 2008). In addition, it can survive in high-pH environments without interacting with other microorganisms (Stuart *et al.* 2006). Along with bacteria, yeasts can also be present in cases of persistent apical periodontitis (Siqueira *et al.* 2002). Despite being larger than bacteria, yeasts such as *Candida albicans* can colonize the dentinal tubules to depths of approximately 150 μm (Sen *et al. 1995*). In addition, the ability of this yeast to coexist with other microorganisms is a factor that can significantly increase their survival within the root canal (Ferrari *et al.* 2005).

Several techniques have been proposed to eliminate microorganisms from root canals. Rotary instrumentation has become widely adopted because its easy and rapid action promotes more uniform root preparations compared with manual files. Nevertheless, the use of irrigants is still required to aid in microbial elimination from the lumen and within dentinal tubules (Berber *et al.* 2006). In addition, the alternating use of decalcifying and antimicrobial irrigants, such as EDTA and NaOCl, are employed during instrumentation to achieve better disinfection (Soares *et al.* 2010). Citric acid is an option to remove the smear layer (Zehnder 2006, Prado *et al.* 2011) and shows appropriate cytotoxicity (Malheiros *et al.* 2005, Amaral *et al.* 2007) and antibacterial activity against *E. faecalis* (Krause *et al.* 2007, Arias-Moliz *et al.* 2008). However, there are no studies evaluating the use of 1% NaOCl with the alternating use of 15% citric acid on the disinfection of root canals.

Therefore, the aim of this study was to assess the disinfection provided by manual instrumentation using K-type files (Dentsply Maillefer, Ballaigues, Switzerland) and rotary instrumentation (Protaper Universal; Dentsply Maillefer) in root canals infected with *E. faecalis* and *C. albicans* in mixed culture. Additionally, the antimicrobial effectiveness of saline, 1% NaOCl, 1% NaOCl with alternated use of 15% citric acid and 5.25% NaOCl irrigation solutions was also examined. The null hypothesis was that the disinfection of oval root canals was not affected by the preparation techniques employed nor by the irrigants utilized.

Materials and methods

Specimen preparation

In total, 85 human mandibular premolars with a single root canal without internal resorption, calcifications or root dilacerations and with no other anatomical or pathological alterations were selected. The specimens were stored in saline for a minimum period of 7 days for hydration. The crowns were sectioned with diamond discs (KG Sorensen, São Paulo, SP, Brazil) to standardize the root length to 15 mm. Root canals were enlarged using a size 25 K-file (Dentsply Maillefer) and saline until the instrument tip was visualized at the apical foramen.

Transverse grooves that were 0.5 mm in depth were made 5.0 and 10.0 mm from the apex of each tooth using a 0.2-mm-thick diamond disc for subsequent specimen cleavage. Specimens were then immersed in an ultrasonic bath of 17% EDTA-T (Fórmula e Ação Pharmaceutical Manipulation Labs, São Paulo, SP, Brazil) for 3 min, followed by immersion in 5.25% NaOCl (Fórmula e Ação Laboratórios de Manipulação, São Paulo, SP, Brazil) for 5 min. Finally, specimens were immersed in distilled water for 3 min. The roots were dried and then covered with two layers of red nail polish, including the apical foramen.

After 24 h, the teeth were inserted individually into 1.5 mL polypropylene tubes (CRAL Ltda, São Paulo, SP, Brazil) and autoclaved at 121 °C for 20 min. The control group consisted of five teeth that were neither contaminated nor instrumented to demonstrate specimen sterility.

Microorganism inoculum preparation

The inoculum was prepared from 50 μL of *E. faecalis* strain (ATCC 29212) and a clinical isolate of *C. albicans*; each microorganism was inoculated into 50 mL of tryptic soy broth (TSB) (Difco Labs, Detroit, MI, USA) and cultured under aerobic conditions at 37 °C for 24 h. The density of each inoculum was calibrated until the turbidity reached level 4 on the McFarland scale, which corresponded to 1.2×10^9 colony-forming units (CFU) per mL. Inocula were then mixed in equal proportions and agitated.

Specimen contamination

The root canals were inoculated and incubated in aerobic conditions at 37 °C for 28 days. The culture

medium was renewed every 2 days. After the incubation period, the root canals were filled with sterile peptone water, and preoperative samples (S1) were collected using three size 20 sterile paper points (Dentsply Maillefer, Petrópolis, Brazil). Each paper point was held in the specimen at working length for 1 min and then stored in polypropylene tubes containing 1 mL of sterile peptone water. Next, 10-fold serial dilutions were performed. To confirm the contamination of the specimens, 50-μL aliquots were plated on Sabouraud dextrose agar and M-enterococcus agar (Difco Labs). After 48 h of incubation at 37 °C, microbial growth was recorded. The purity of the cultures was confirmed using Gram staining and colony morphology.

Experimental endodontic procedures

In total, 80 specimens were divided into two groups (n = 40) according to chemomechanical preparation technique: manual instrumentation using K-files (Dentsply Maillefer) or rotary instrumentation using Protaper Universal files (Dentsply Maillefer). Each group was further divided into four subgroups (n = 10) according to the irrigant used: saline, 1% NaOCl, 1% NaOCl with alternated use of 15% citric acid or 5.25% NaOCl. The working length was 14 mm for all specimens.

In canals that were prepared using the crown-down technique, the cervical and middle third were enlarged with size 3 and size 2 Gates-Glidden drills, whereas the apical preparations were performed with a filing motion at the working length, starting with size 30 K-files and working up to size 50 K-files. In teeth that were prepared using the rotary technique, Protaper Universal files (Dentsply Maillefer) were used at 350 rpm and 2N per cm of torque. First, a shaping file 1 (S1) was applied to the canal walls to enlarge the cervical and mid-thirds. Next, S2, F1, F2, F3, F4 and F5 instruments were manipulated in the same way throughout the working length. All instruments were autoclaved at 121 °C for 20 min prior to use.

In the subgroups rinsed with saline, 1% NaOCl and 5.25% NaOCl, 2 mL of the irrigation solution was renewed after use of each instrument. In these subgroups, a final rinse was performed using 10 mL of the respective solution after specimen preparation was complete. The total volume used in instrumentation was standardized to 22 mL. In subgroups irrigated with 1% NaOCl with alternated use of 15% citric acid, 1 mL of each irrigation solution was employed each time the instrument was replaced; for the final irrigation, 5 mL of each solution was used. To standardize the irrigation procedure, a peristaltic pump (VK Driller LTDA, São Paulo, Brazil) and a 30-G irrigation needle (Ultradent Products Inc., South Jordan, UT, USA) were used with a constant flow of 5 mL per min. At the end of each preparation, NaOCl solutions were buffered with 2 mL of a sterile solution of 5% sodium thiosulfate that was held within the root canal for 3 min. Subsequently, the root canals were filled again with sterile peptone water, and postoperative samples (S2) were collected with three size 20 sterile paper cones (Dentsply Maillefer); each paper cone was held inside the root canal for 1 min.

Dentine analysis

After postoperative canal sampling, the specimens were sectioned in thirds, and 0.02-g samples of dentine were collected from the apical, medium and coronal thirds of each root canal to test for the presence of microorganisms in the tubules. A sterile diamond No.3139 conical bur (Medical Burs Ind, São Paulo, Brazil) was used at 150 rpm without irrigation. Dentine chips were placed into polypropylene tubes containing 1 mL of sterile peptone water. A digital precision balance (Mettler-Toledo International Inc., São Paulo, Brazil) was used to standardize the weight of the dentine collected. Next, 10-fold serial dilutions were performed. To quantify the microbial growth, 100-μL aliquots were plated on M-enterococcus agar (Difco Labs) and Sabouraud dextrose agar in triplicate. The plates were incubated at 37 °C for 48 h; CFU counts were then performed.

Data were statistically assessed using the Mann–Whitney U-test at significance level of 5%; all analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA).

Results

In the dentine chip samples, saline and 1% NaOCl irrigation solutions were significantly less efficient in reducing the number of microorganisms (Table 2) compared with 1% NaOCl with alternated use of 15% citric acid and 5.25% NaOCl (P = 0.00002).

Regarding the root canal preparation techniques, both manual (K-files) and rotary (Protaper Universal files) instrumentation significantly reduced the numbers of *E. faecalis* and *C. albicans* organisms present in the root canal lumen regardless of the irrigation solution

Table 1 Means and standard deviations of CFU per mL for *Enterococcus faecalis* and *Candida albicans* in the root canal lumen before and after chemomechanical preparation

		Before instrumentation (CFU per mL)	After instrumentation (CFU per mL)	Percent reduction
Group	*N*	Mean ± SD	Mean ± SD	Mean ± SD
***E. faecalis* reduction before and after instrumentation**				
Manual (K File)				
Saline	10	$3.68 \times 10^8 \pm 1.19 \times 10^8$	$4.97 \times 10^3 \pm 4.13 \times 10^3$	99.99
NaOCl 1%	10	$3.36 \times 10^8 \pm 1.29 \times 10^8$	$1.8 \times 10^2 \pm 1.73 \times 10^2$	99.99 ± 0.01
NaOCl 1% + CA 15%	10	$3.18 \times 10^8 \pm 1.45 \times 10^8$	$1.1 \times 10^2 \pm 98.51$	99.99 ± 0.01
NaOCl 5.25%	10	$3.36 \times 10^8 \pm 1.42 \times 10^8$	0	100
Rotary (ProTaper)				
Saline	10	$3.06 \times 10^8 \pm 1.27 \times 10^8$	$5.4 \times 10^3 \pm 4.07 \times 10^3$	99.99
NaOCl 1%	10	$3.52 \times 10^8 \pm 1.56 \times 10^8$	$1.67 \times 10^2 \pm 2.24 \times 10^2$	99.99 ± 0.01
NaOCl 1% + CA 15%	10	$3.42 \times 10^8 \pm 1.2 \times 10^8$	$1.02 \times 10^2 \pm 1.05 \times 10^2$	99.99 ± 0.01
NaOCl 5.25%	10	$3.59 \times 10^8 \pm 1.07 \times 10^8$	0	100
***C. albicans* reduction before and after instrumentation**				
Manual (K File)				
Saline	10	$7.48 \times 10^4 \pm 1.04 \times 10^4$	$7.6 \times 10^2 \pm 1.5 \times 10^2$	98.97 ± 0.21
NaOCl 1%	10	$6.6 \times 10^4 \pm 1.8 \times 10^4$	$1 \times 10^2 \pm 1.15 \times 10^2$	99.87 ± 0.14
NaOCl 1% + CA 15%	10	$6.65 \times 10^4 \pm 2.24 \times 10^4$	51.67 ± 65	99.94 ± 0.08
NaOCl 5.25%	10	$6.4 \times 10^4 \pm 2.19 \times 10^4$	0	100
Rotary (ProTaper)				
Saline	10	$6.58 \times 10^4 \pm 2.1 \times 10^4$	$7.47 \times 10^2 \pm 1.71 \times 10^2$	98.72 ± 0.72
NaOCl 1%	10	$6.71 \times 10^4 \pm 2.67 \times 10^4$	99 ± 99	99.89 ± 0.11
NaOCl 1% + CA 15%	10	$6.87 \times 10^4 \pm 1.98 \times 10^4$	45 ± 57.76	99.95 ± 0.06
NaOCl 5.25%	10	$6.72 \times 10^4 \pm 1.91 \times 10^4$	3.33 ± 7.03	100 ± 0.01

CA, citric acid.

used (Table 1). There was no significant difference between these techniques ($P > 0.05$). For the dentine chip samples, the Mann–Whitney *U*-test revealed no statistically significant differences ($P > 0.05$) between the preparation techniques when samples from the same root third that were treated with the same irrigation solution were compared (Table 2).

The sterile condition of the root canal system prior to contamination with *E. faecalis* and *C. albicans* was verified, as no microbial growth was observed in the control group.

Discussion

The aim of root canal treatment is to restore or maintain the healthy condition of periapical tissues. This goal is achieved through the reduction or elimination of microorganisms within the root canal system. Chemomechanical canal preparation utilizes several instruments, techniques and chemical substances that attempt to promote the ideal conditions to fill the root canal space. Rotary instrumentation represents a viable option to perform canal preparation because it is faster and the final shape is more uniform compared with manual instrumentation. Additionally, several studies have focused on the effectiveness of disinfection utilizing this method (Berber *et al.* 2006, Singla *et al.* 2010). In endodontic practice, NaOCl is the most common chemical substance employed because of its antimicrobial properties and ability to dissolve organic matter (Krause *et al.* 2007, Siqueira *et al.* 2007, Retamozo *et al.* 2010). Thus, the present study assessed the effectiveness of disinfection through manual and rotary instrumentation within the root canal lumen and dentinal tubules contaminated with *E. faecalis* and *C. albicans* using saline, 5.25% NaOCl, 1% NaOCl and 1% NaOCl with alternated use of 15% citric acid as irrigation solutions.

Regardless of the preparation technique utilized and the irrigation solution employed, microbial populations were reduced by more than 98%. Nevertheless, dentine chip analysis revealed that microorganisms were still present in the dentinal tubules in all groups, consistent with a report from Câmara *et al.* (2009). These findings emphasize the difficulty of disinfecting within dentine. The target microorganisms in this study were selected based on their levels of virulence and resistance to antimicrobial agents; additionally, their high prevalence in cases of failed root canal treatments was taken into consideration (Siqueira & Sen 2004, Stuart *et al.* 2006).

Table 2 Means and standard deviations of CFU per mL for *Enterococcus faecalis* and *Candida albicans* in dentine ships, according to radicular third and preparation technique

Radicular third	Saline	1% NaOCl	1% NaOCl + 15% AC	5.25% NaOCl
***E. faecalis* collected from dentine ships**				
Cervical				
Manual	$2.2 \times 10^3 \pm 1.2 \times 10^3$ a	156.67 ± 110.33 b	90.00 ± 111.44 c	11.67 ± 22.30 c
Rotary	$2.5 \times 10^3 \pm 1.3 \times 10^3$ a	241.67 ± 227.34 b	63.33 ± 85.27 c	11.83 ± 22.34 c
Medium				
Manual	$1.8 \times 10^3 \pm 7.4 \times 10^2$ a	170.00 ± 128.33 b	48.33 ± 58.50 c	6.67 ± 11.65 c
Rotary	$2.2 \times 10^3 \pm 1.2 \times 10^3$ a	263.33 ± 206.50 b	31.67 ± 44.06 c	10.00 ± 14.05 c
Apical				
Manual	$1.3 \times 10^3 \pm 4.2 \times 10^2$ a	51.67 ± 46.11 b	13.33 ± 42.16 c	0.00 ± 0.00 c
Rotary	$1.6 \times 10^3 \pm 6.5 \times 10^2$ a	188.33 ± 112.28 b	11.67 ± 17.65 c	0.00 ± 0.00 c
***C. albicans* collected from dentine ships**				
Cervical				
Manual	595.00 ± 132.65 a	103.34 ± 80.43 b	25.00 ± 25.15 c	10.00 ± 14.05 c
Rotary	536.67 ± 114.61 a	140.00 ± 79.43 b	25.00 ± 26.35 c	5.00 ± 8.05 c
Medium				
Manual	571.67 ± 220.56 a	91.67 ± 80.60 b	16.67 ± 19.24 c	6.67 ± 11.65 c
Rotary	525.00 ± 116.07 a	100.00 ± 46.48 b	18.33 ± 24.15 c	5.00 ± 8.05 c
Apical				
Manual	200.00 ± 156.35 a	40.00 ± 53.98 b	10.00 ± 17.92 c	0.00 ± 0.00 c
Rotary	171.67 ± 92.65 a	43.33 ± 60.96 b	10.00 ± 14.05 c	0.00 ± 0.00 c

CA, citric acid.
Similar letters (from a to c) indicate absence of statistical difference in horizontal direction (Mann–Whitney $P > 0.05$).

Enterococcus faecalis has the ability to colonize dentinal tubules and can penetrate into dentinal tubules to a depth of 1000 μm in 21 days (Haapasalo & Ørstavik 1987). In addition to penetrating into the root canal walls, collagen-binding proteins strengthen the binding of *E. faecalis* to dentine (Kayaoglu *et al.* 2008). Further, this microorganism can form biofilms within the root canal system that make the complete disinfection of the root canal system difficult (Liu *et al.* 2010). The other microorganism used in this study was *C. albicans*, a yeast isolated from persistent lesions that has the ability to penetrate dentinal tubules, adhere to dentine (Sen *et al.* 1995) and form biofilms (Turk *et al.* 2008). As a dimorphic fungus, *C. albicans* can easily adapt to environmental variations through hyphal-to-yeast or yeast-to-hyphal transitions whenever needed (Siqueira & Sen 2004). Additionally, *C. albicans* can co-aggregate with several endodontic pathogens (Ferrari *et al.* 2005). In the present study, both species were viable throughout the entire culture period; this result was also observed by Cardoso *et al.* (2008). Thus, the results obtained in the present study showed that both root canal preparation techniques resulted in similar disinfection conditions when the groups using the same irrigation protocols were compared. These results are consistent with previous findings (Berber *et al.* 2006).

Both *E. faecalis* and *C. albicans* are susceptible to both concentrations of sodium hypochlorite. Amongst the irrigation protocols examined, 1% NaOCl was found to be more effective than only saline solution for eliminating both microorganisms. At a 5.25% concentration, sodium hypochlorite was more effective, resulting in negative cultures from the root canal lumen and the dentine chip samples.

Specimens that were irrigated using 1% NaOCl with alternated use of 15% citric acid exhibited significantly lower numbers of viable microorganisms compared with the groups that used 1% NaOCl only. Citric acid is an organic acid with low cytotoxicity (Amaral *et al.* 2007) and a demineralizing action that can remove smear layer efficiently (Zehnder 2006, Prado *et al.* 2011). Bystrom & Sundqvist (1985) and Soares *et al.* (2010) suggested the alternated use of 5.25% NaOCl and 17% EDTA for preparing root canals. The authors stated that such an association resulted in cleaner dentinal tubules with greater permeability. Similarly, in this study, the increase in dentinal permeability produced during root canal preparation may have allowed the 1% NaOCl to penetrate deeper into the dentinal tubules, thus reaching more bacteria and yeast. Further studies should be conducted to validate this hypothesis. However, Zehnder *et al.* (2005) demonstrated that associating

sodium hypochlorite with citric acid or EDTA decreases the antimicrobial capacity of sodium hypochlorite significantly. This discrepancy may be related to the fact that these authors mixed the substances rather than alternating their use, as proposed in this study. In fact, citric acid alone can exhibit antimicrobial action against strict and facultative anaerobic microorganisms (Krause *et al.* 2007, Arias-Moliz *et al.* 2008). Soares *et al.* (2010) stated that an association between sodium hypochlorite and a demineralizing chemical solution might promote a more powerful bacterial effect or interfere with the recolonization process. Georgopoulou *et al.* (1994) observed that citric acid was ineffective in reducing the number of microorganisms attached to biofilms. Therefore, it is more likely that the bacterial reduction observed in the groups that used 1% NaOCl alternately with 15% citric acid occurred as a result of increased dentine permeability rather than the antimicrobial efficacy of the acid itself.

The results obtained in this study demonstrate the greater efficacy of 5.25% NaOCl in eliminating *E. faecalis* and *C. albicans* compared with other irrigation protocols, regardless of the instrumentation technique used. However, the choice of the most adequate substance for endodontic therapy also depends on others factors that were not considered here, such as biocompatibility. It is well known that increasing the chloride concentration of sodium hypochlorite solutions also increases their cytotoxicity (Amaral *et al.* 2007). Several case reports on accidents involving this substance have been described in the literature, mainly when NaOCl was used at concentrations ranging from 2.5% to 6.15% (Kleier *et al.* 2008, Motta *et al.* 2009).

There were no significant differences between the CFU counts of the two different instrumentation techniques analysed. These data are consistent with other studies previously reported in the literature (Berber *et al.* 2006, Chuste-Guillot *et al.* 2006). It should be noted, however, that according to Paqué *et al.* (2010), only 20.1% to 40.4% of the total volume of oval-shaped root canals are touched by endodontic instruments during the chemomechanical preparation. Thus, the disinfection of untreated surfaces is performed only through the action of irrigants, which may explain the lack of difference between the results.

Similar to the results from previous studies, a higher CFU count was found in the cervical third, followed by the middle and apical thirds. These results are mainly because of the greater amount of dentinal tubules in these portions of the root canal system (Souza *et al.* 2008). Additionally, the CFU count in the apical third after chemomechanical preparation was low. This result might be explained by all of the root canals being enlarged to file size 50, which might increase the efficacy of irrigation (Brunson *et al.* 2010) and promote better disinfection (Mickel *et al.* 2007).

Microorganisms were collected with sterile paper points from the root canal system of extracted premolars. This sampling method has limitations because the paper points are only able to detect planktonic bacteria. Moreover, the paper points cannot access irregularities and other regions of the root canal system. Consequently, this approach might fail to harvest viable bacteria in biofilms and in some areas of the root canal system (Siqueira *et al.* 2010). Thus, diamond conical burs were used at low speed to remove dentine from the root canal walls and dentinal tubules, allowing for a more predictable sampling. Another critical factor to be considered is the limitation of the culture method because only viable and culturable microorganisms could be counted. Additionally, some cells of *E. faecalis* in biofilms can enter a stationary phase, which makes them undetectable with conventional culture methods (Liu *et al.* 2010). Thus, the data obtained from CFU counts must be interpreted with caution.

Conclusion

Both instrumentation techniques employed for chemomechanical preparation substantially reduced the number of viable microbial cells in the root canal lumen and in the inner walls of the dentine. Furthermore, the alternated use of 15% citric acid and 1% NaOCl during chemomechanical preparation enhanced disinfection, yielding results similar to 5.25% sodium hypochlorite.

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