Effect of endodontic irrigation, with and without the use of ultrasound, on removal of smear layer and biofilm

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ABSTRACT

Introduction: This study investigated the effect of different endodontic irrigation protocols, with or without ultrasonic activation, on cleaning and decontamination of the three thirds of the root canal. **Methods:** Teeth were inoculated with *E. faecalis* and remained in culture for 50 days for biofilm formation. The teeth were divided into eight groups according to the endodontic irrigant used and the use of ultrasonic activation: G1 = 2.5% NaOCl + ultrasound; G2 = 2% chlorhexidine solution + ultrasound; G3 = 2% chlorhexidine gel + ultrasound; G4 = H_2O + ultrasound; G5 = 2.5% NaOCl; G6 = 2% chlorhexidine solution; G7 = 2% chlorhexidine gel and G8 = H_2O. The roots were divided into two slices and analyzed by scanning electron microscopy (SEM). The images were classified according to the level of cleanness (presence of smear layer, 2000x) and decontamination (presence of bacteria, 10000x) on the coronal, middle and apical thirds. **Results:** Ultrasound improved the cleaning and decontamination ability of all endodontic irrigants tested, mainly of sodium hypochlorite and chlorhexidine solution. Chlorhexidine gel without ultrasound had the lowest values of cleaning; however, when combined with ultrasound, it provided a cleaning ability similar to 2.5% NaOCl. As for decontamination in the apical third, chlorhexidine solution without ultrasound presented better decontamination ability than chlorhexidine gel with ultrasound. **Conclusion:** It was concluded that ultrasound improved the cleaning ability on the three root canal thirds by bringing the endodontic irrigant in contact with microorganisms and dentin debris within the canal system, thus optimizing their removal.

Keywords: Endodontics. *Enterococcus faecalis*. Chlorhexidine. Scanning electron microscopy. Sodium hypochlorite.

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Introduction

Due to the complex anatomy of root canal systems, which include additional canals, oval extensions, isthmuses and apical deltas,[1,2,3] it is a challenge to shape and clean the root canal completely. Irrigation is an essential part of root canal treatment of which aim is to remove pulp tissue and/or microorganisms (planktonic microbiota or biofilm) from the root canal system.[4] Irrigation should also remove smear layer and dentin debris following instrumentation.[5]

Because of the small diameter of the canal and its ramifications, it becomes difficult for the irrigant to reach the whole apical region. *In vitro* studies report the use of ultrasonics devices to enhance the action of the irrigant.[6,7,8] Ultrasonic irrigation of the root canal can be performed with or without simultaneous ultrasonic instrumentation. Passive Ultrasonic Irrigation (PUI) consists of ultrasonic activation of an endodontic irrigant. Although a recent study has suggested an alteration to the term PUI[9] when canal shaping is not undertaken, the term PUI is still commonly used in the literature to describe this technique.[7] PUI can be performed with a small file or a nylon thread oscillating freely in the root canal to induce powerful acoustic microstreaming.[7]

The literature suggests that PUI may be an important supplement for cleaning and disinfecting the root canal system, compared with traditional syringe irrigation. Irrigation with ultrasound could remove more organic tissue, planktonic bacteria and dentine debris from the root canal.[7,8,10] On the other hand, a recent study claimed that although PUI can be an aid in cleaning/decontaminating the root canal, the main role played in bacteria elimination is assigned to the endodontic irrigant.[11]

Despite the findings available, studies designed to investigate the effect of ultrasonic activation of endodontic irrigants taking into consideration the differentiation between removal of smear layer and removal of microorganism are scarce. Therefore, with the goal of improving our understanding regarding the role of ultrasonic activation during irrigation procedures, this *in vitro* study was conducted to assess, by scanning electron microscopy, smear layer and microorganism removal from root canal coronal, middle and apical thirds.

Material and Methods

Sample preparation

This study protocol was approved by the Ethics Committee of the institution where the experiment was conducted. Sixty human mandibular premolars, extracted for clinical reasons, with one canal and complete root formation were selected.

Tooth crowns were removed, with the remaining root length between 15 and 18 mm in all samples. Coronal enlargement by LA Axxess burs (Sybron Endo, USA) was performed, and the working length (WL) was determined at 1 mm from the apical foramen. The canals were prepared with stainless-steel K-files #35 (Dentsply Maileffer, Ballaigues, Switzerland) by means of the crown-down technique. Irrigation was performed with 2% NaOCl (2% VirexPlus; Johnson Diversey Brasil Ltda, São Paulo, SP, Brazil). Each tooth was secured to a plastic microtube (Axygen Inc, Union City, CA, USA) with cyanoacrylate (SuperBonder Plastic Glue, Loctite, SP, Brazil), so that it remained in upright position, with the coronal portion facing upward. A hole was opened on the side of the plastic microtube to remove the culture medium. The teeth were divided into eight groups: n = 10 for the groups with ultrasound and n = 5 for the groups without ultrasound. Each group was placed in a polypropylene box (Heathrow Scientific, Vernon Hills, IL, USA) sterilized at 121 °C with a pressure of 118 KPa in autoclave (Dabi Atlante, Ribeirão Preto, SP, Brazil) for a period of 30 minutes.

Sterilization control

One additional tooth from each group was subject to sterilization control. After sterilization of each polypropylene box containing the teeth, a sterile paper cone was introduced into the root canal of one of the teeth in the box, and this cone was immediately inoculated in a tube containing sterile saline solution at 0.85%. The material was homogenized, and after 5 minutes, an aliquot of 100 µL saline solution was spread on blood agar, in duplicate, and incubated for 18 to 24 hours at 37 °C; presenting no bacterial growth. The tooth used for sterilization control was removed from the box and discarded; the other 60 teeth remained in each box.

Inoculum cultivation and preparation

E. faecalis (ATCC 29212) strain was cultivated in Brain Heart Infusion (BHI) broth for 18 to 24 hours,

at 37 °C, in a bacteriological incubator. The number of colony forming units (CFU/mL) of the inoculum was determined by counting the colonies on blood agar. To this end, the culture of *E. faecalis* was diluted serially up to 10^{-8} in saline solution at 0.85%; and 100 µL of the dilutions 10^{-6}, 10^{-7}, and 10^{-8} were spread on blood agar with the aid of a Drigalski handle, in duplicate. The plates were incubated at 37 °C for 24 hours, and after that period, the CFU/mL of the plates that grew from 15 to 150 colonies were counted.

The CFU/mL varied from 2.4 x10^{7} to 8.0 x 10^{7}. However, in each one of the 60 samples previously sterilized, 100 µL of *E. faecalis* were inoculated inside the root canal. After this procedure, the sterile BHI was added into the microtube, so that it was completely filled with the culture medium. The culture of *E. faecalis* was maintained for 50 days for the formation of biofilm, with one third of BHI being renewed every two days. The teeth were manipulated under aseptic conditions in a laminar flow hood. Once a week, an aliquot of BHI from the teeth was submitted to Gram staining and cultured on blood agar, followed by catalase and esculin tests to verify the absence of contamination.

Classification of groups

The roots were mounted on a base of utility wax (Wilson Polidental, Cotia, SP, Brazil) in order to avoid irrigant overflow. Groups were distributed as follows:

Group 1 = ultrasound + 2.5% sodium hypochlorite (NaOCl+US): the root canal was filled with 2.5% sodium hypochlorite, agitated with a digital file for 15 seconds. The auxiliary chemical was agitated with a K-file #15 (Dentsply Maillefer, Ballaigues, Switzerland) for 15 seconds. Irrigation was performed with a disposable sterile syringe (BD Brasil, São Paulo, SP, Brazil). Manual agitation was performed with a K-file #30 (Dentsply Maillefer, Ballaigues, Switzerland) at the working length for 15 seconds. Subsequently, passive ultrasonic irrigation (PUI) was performed with Nac Plus ultrasound (Adiel, Ribeirão Preto, SP, Brazil), using the scale power 2 for Endodontics. A K-file #30 was coupled to the ultrasound via an adapter (QuickEnd-Holder, Adiel). Four irrigation cycles were performed.

Group 2 = ultrasound + 2% chlorhexidine solution (CHX+US): the ultrasonic activation protocol was performed following the same specifications already described for Group 1. A 5 mL disposable syringe (BD Brazil, São Paulo, SP, Brazil) was used for insertion of the chlorhexidine liquid.

Group 3 = ultrasound + 2% chlorhexidine gel (CHXg+US): the ultrasonic activation protocol was performed following the same specifications already described for Group 1. A 3 mL disposable syringe (BD Brazil, São Paulo, SP, Brazil) with a hypodermic needle was used for insertion of chlorhexidine gel. Chlorhexidine gel removal was performed by 0.9% saline solution and a 5 mL disposable syringe (BD Brazil, São Paulo, SP, Brazil) with a 25 x 0.6 hypodermic needle (Injex Indústria Cirúrgica, Ourinhos, SP, Brazil).

Group 4 = ultrasound + distilled water (H_2O+US): the ultrasonic activation protocol was performed following the same specifications already described for Group 1.

Group 5 = 2.5% sodium hypochlorite (NaOCl): the use of NaOCl was performed following the same specifications already described for Group 1. The irrigation procedure was repeated four times, without the use of ultrasound.

Group 6 = 2% chlorhexidine solution (CHX): the use of CHX liquid was performed following the same specifications already described for Group 2, without the use of ultrasound.

Group 7 = 2% chlorhexidine gel (CHXg): the use of CHXg was performed following the same specifications already described for Group 3, without the use of ultrasound.

Group 8 = distilled water (H_2O): the use of H_2O was performed following the same specifications already described for Group 4, without the use of ultrasound.

Preparation for scanning electron microscopy

Scanning electron microscopy (SEM) was performed at the Center for Electron Microscopy and Microanalysis at the university where the experiment was conducted.

The roots were fixed for seven days in 2.5% glutaraldehyde and then washed three times for 30 minutes in 0.2 mol/L phosphate buffer and distilled water solution in a ratio of 1:1. Thereafter, the samples were dehydrated by immersion in 30%, 50%, 70%, 90%, and 100% acetone. Longitudinal grooves were carved on the free surfaces of the roots with a diamond saw (Dhpro; Rhadartrade, Paranaguá, PR, Brazil), taking care not to invade the inner part of the root canal. Complete

fracture was made with a chisel and a hammer, providing two halves of each sample. The best half of each tooth was chosen and placed on stubs with the portion of the root canal facing upward.

Subsequently, the 60 samples (40 from the groups with ultrasound and 20 from the groups without ultrasound) were coated with gold-palladium for conduction of electrons.

Evaluation was carried out in the three thirds of each half, with a scanning electron microscope (XL 30; Philips, Eindhoven, Netherlands). Magnification of 2000x was selected to verify root canal cleanness (smear layer); whereas 10000x was selected to differentiate smear layer from bacterial biofilm.

Statistical analysis

Data were analyzed with the aid of SPSS v.17.0 (SPSS, Chicago, IL, USA). To detect a difference of at least 1.5 standard deviation units between the mean scores observed in the groups, reaching a statistical power of 90% with a significance level of 5%, a need for ten experimental units per group was estimated.

One evaluator, unaware of the experimental groups, analyzed all SEM images twice (180 images under 2000x and 180 images under 10000x). Each image was attached to an individual slide using Microsoft Power Point™, and subsequently organized according to the level of cleanness (presence of smear layer, 2000x), that is: number 1 was the cleanest while number 60 was the least clean. The same protocol was used for analysis of contamination (presence of bacteria, 10000x). This classification by rank was performed for each third (coronal, middle, and apical). One-way analysis of variance (ANOVA) was applied on these data, followed by Tukey's *post hoc* test. The level of significance was set at $\alpha = 0.05$.

Results

Both analyses (cleaning and decontamination) showed adequate inter-examiner agreement rates (intraclass correlation coefficient $p > 0.05$).

Figure 1 illustrates a SEM image revealing of cleaning traits (2000x) for Group 7 (CHXg) and Group 1 (NaOCl+US); and decontamination (10000x) for Group 8 (H_2O) and Group 1 (NaOCl+US).

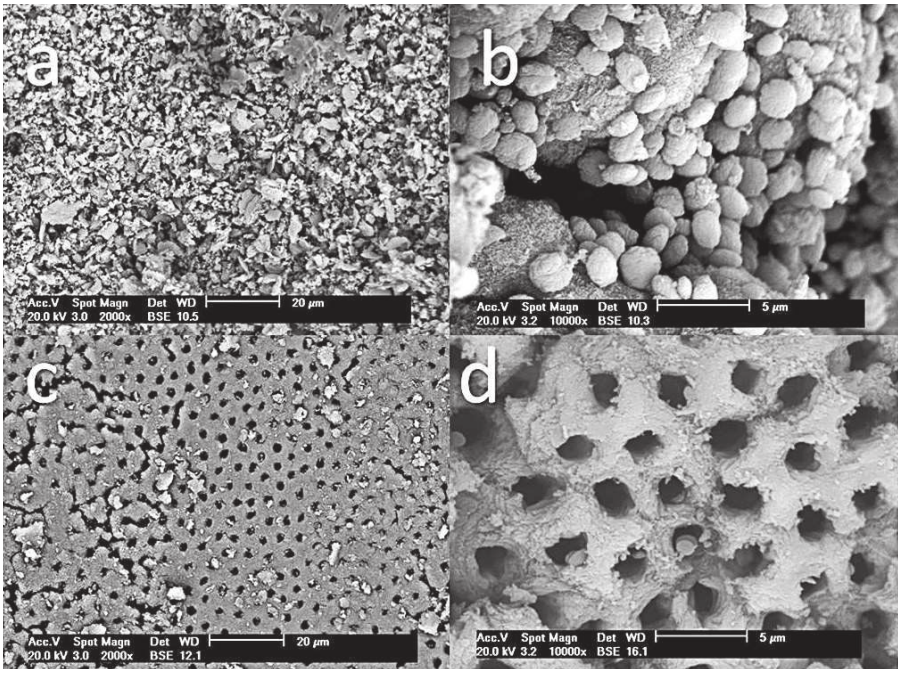

Figure 1. SEM images of groups displaying cleaning (2000x) and decontamination (10000x) features: **A**) 2% chlorhexidine gel (2000x); **B**) Distilled water (10000x); **C**) 2.5% sodium hypochlorite + ultrasound (2000x); **D**) 2.5% sodium hypochlorite + ultrasound (10000x).

Table 1 shows the cleaning and decontamination ability of different irrigation protocols in relation to the root thirds. Overall, the results can be summarized as follows:

» Under 2000x, all groups with ultrasound presented very similar results in relation to cleaning, showing no statistical significant differences when compared in terms of the root thirds. Groups 1 (NaOCl+US) and 2 (CHX+US) had the highest values of cleaning on the three thirds and did not show statistical differences between them. In the apical third, Groups 1 (NaOCl+US), 2 (CHX+US) and 4 (H_2O+US) had higher values of cleaning when compared to Group 5 (NaOCl), as well as when compared to the control, i.e., Group 8 (H_2O). Unlike the other groups with ultrasound, Group 3 (CHXg+US) did not present statistical differences when compared to Group 5 (NaOCl). Group 7 (CHXg) had the lowest values of cleaning in all root thirds, with statistical differences in relation to all groups with ultrasound (except for Group 3 [CHX+US] in the apical third).

» Under 10000x, all groups with ultrasound showed higher values of decontamination when compared to the groups without ultrasound. The groups with the best decontamination results were Groups 1 (NaOCl+US) and Group 2 (CHX+US). Among the groups with ultrasound, the only statistical difference was found on the apical third of Groups 2 (CHX+US) and 3 (CHXg+US), with the latter presenting the lowest values of decontamination. Group 6 (CHX) had higher decontamination values when compared to Group 3 (CHXg+US) in all thirds, although these two groups did not show statistical differences on the apical third. The group with the worst value of decontamination in the apical third was Group 8 (H2O). The group with the worst value of decontamination in the middle and coronal third was Group 7 (CHXg).

Discussion

Cleaning and decontamination of root canal systems in infected teeth are key procedures to achieve the success of endodontic treatment.[8,12] However, the complexity of root canal anatomy makes it very difficult to efficiently remove smear layer, as well as decontaminate and seal all ramifications of the root canal system.[13] The literature shows different irrigation devices and techniques proposed to improve root canal cleaning and disinfection.[8,11,14,15] In the '80s, ultrasonic activation of endodontic instruments was suggested as a means to improve canal debridement.[16] The procedure has been reported in the literature as an aid to irrigate and disinfect root canal systems.[6] Therefore,

Table 1. Comparison of contamination levels between different cleaning treatments applied to human root canal.

Variable	NaOCl+US (n = 10)	CHX+US (n = 10)	CHXg+US (n = 10)	H_2O+US (n = 10)	NaOCl (n = 5)	CHX (n = 5)	CHXg (n = 5)	H_2O (n = 5)	*p*
MEV 2.000x									
Apical third	42.2±12.9^{c}	42.4±11.0^{c}	26.2±13.8$^{a.b.c}$	41.1±13.9$^{b.c}$	19.8±15.1^{a}	18.0±13.1^{a}	5.2±6.1^{a}	19.2±14.9$^{a.b}$	<0.001
Medium third	47.0±13.1^{c}	38.4±14.1$^{b.c}$	35.5±11.6$^{b.c}$	34.6±11.2$^{b.c}$	20.0±13.8$^{a.b}$	9.8±4.2^{a}	4.2±3.1^{a}	21.0±16.2$^{a.b}$	<0.001
Coronal third	39.9±17.2$^{c.d}$	39.0±16.3$^{c.d}$	27.0±11.2$^{b.c.d}$	43.1±9.7^{d}	31.4±15.4$^{b.c.d}$	20.2±11.3$^{a.b.c}$	4.0±2.7^{a}	12.4±9.9$^{a.b}$	<0.001
MEV 10.000x									
Apical third	49.8±6.3^{e}	45.9±15.1$^{d.e}$	26.8±10.4$^{a.b.c}$	28.2±11.7$^{b.c.d}$	11.2±6.9$^{a.b}$	30.4±10.1$^{c.d}$	14.4±10.8$^{a.b.c}$	8.6±10.4^{a}	<0.001
Medium third	47.3±10.8^{c}	42.8±10.5^{c}	31.3±13.0$^{b.c}$	33.8±13.4$^{b.c}$	18.8±9.4$^{a.b}$	9.4±6.5^{a}	8.4±2.7^{a}	19.0±20.9$^{a.b}$	<0.001
Coronal third	43.9±11.8^{d}	43.3±12.0^{d}	38.4±15.1$^{c.d}$	32.5±9.7$^{b.c.d}$	18.6±14.9$^{a.b.c}$	11.0±8.8^{a}	5.8±4.7^{a}	14.4±5.9$^{a.b}$	<0.001

Data are presented as mean ranks ± standard deviation within thirds. H_2O: Distilled water, H_2O+US: Ultrasound and distilled water, NaOCl: Sodium hypochlorite, US+NaOCl: Ultrasound and sodium hypochlorite, CHXg: Chlorexidine gel, CHXg +US: Chlorexidine gel and ultrasound, CHX: Chlorexidine, CHX+US: Chlorexidine and Ultrasound. *P*: significance using ANOVA on ranks. Different index letters represent statistical significant difference at the *post hoc* procedure (Tukey's test).

every effort made towards irrigation protocols aiming at improving clinical results are welcome.

The present study was motivated by the challenge to remove smear layer (cleaning) and microorganisms (decontamination) from the root canal system, and intended to add evidence to existing knowledge by contributing to enhance our understanding regarding the effect of ultrasonic irrigation as an aid to different chemical substances.

Our main findings can be summarized as follows: 1) ultrasound improved the cleaning and decontamination ability of all irrigants tested, mainly of sodium hypochlorite and chlorhexidine solution; 2) as regards cleaning: i) chlorhexidine gel without ultrasound provided the worst cleaning ability, ii) chlorhexidine gel with ultrasound had a cleaning ability similar to sodium hypochlorite at 2.5%; and 3) as regards decontamination, in the apical third, chlorhexidine solution without ultrasound presented better decontamination ability than chlorhexidine gel with ultrasound.

Although recent clinical studies have not found good results for irrigation techniques using ultrasound, particularly regarding disinfection and periapical healing,[17] evidence from *in vitro* studies has already shown that ultrasonic activation of irrigating solutions during root canal treatment improves cleaning and disinfection.[7,8,10,15] Similarly, our findings are also in agreement with the literature review published by Van der Sluis et al.[7] In that study, the authors concluded that PUI appears to be an adjunctive treatment for cleaning/decontaminating the root canal system and that PUI is more effective than syringe irrigation (without ultrasound).

An efficient dentin debris removal from the root canal system will allow adequate cleaning. The majority of published studies has provided evidence that the SEM technique is an important resource to investigate the presence of smear layer.[6,13,14,18,19] However, many different variables in these studies (irrigating solutions, activation method for these solutions, SEM magnification, evaluation methods, etc.) do not allow direct comparison among results. Overall, studies show increased smear layer removal primarily from the coronal portion of the root canal, rather than the apical portion. In this regard, it is interesting to note that our findings showed that ultrasound improved the cleaning ability of irrigating solutions towards the apical third (Table 1). This pattern was also found by Mancini et al[14] who evaluated the effectiveness of different irrigating methods in removing the smear layer 1, 3, 5, and 8 mm from the apex of endodontic canals. Although these authors showed that PUI had decreased the ability to remove smear layer along endodontic walls from the apex to the crown, they found that the Endo Vac system increased the flow and distribution of irrigating solutions within the root canal system, especially at the apical third, showing the highest degree of cleanliness 1 mm from the apex.

Since chlorhexidine gel was introduced as an endodontic irrigant, the literature has shown its advantages, such as: biocompatibility, water-solubility and viscosity; thereby facilitating instrumentation.[20,21] In other words, chlorhexidine gel acts like a lubricant. It is interesting to note that our findings showed that chlorhexidine gel without ultrasound had the worst cleaning ability. This result is in disagreement with some authors who claimed that chlorhexidine gel reduces smear layer formation and maintains dentinal tubules open due to its viscosity, thus keeping the debris in suspension and reducing smear layer formation.[21,22] One hypothesis to explain the poor cleaning ability of chlorhexidine gel might be its higher surface tension when compared to chlorhexidine liquid. The higher surface tension of gel might have limited the ability of chlorhexidine gel to reach the apical third and penetrate into the dentinal tubules, thereby hindering dentin debris removal. There are reports claiming that reduced surface tension could improve the intimate contact of irrigants with the dentinal walls of the root canal system.[24] In this study, ultrasonic activation of chlorhexidine gel improved its cleaning ability. Therefore, it can be concluded that ultrasonic activation of chlorhexidine gel used in mandibular premolars *in vitro* appears to be an adjunctive treatment for cleaning the root canal, when the choice for irrigation is chlorhexidine gel.

The literature has recently reported that antimicrobial effectiveness is the most important property required for an irrigant solution to be used during treatment of teeth with apical periodontitis.[12] Regarding decontamination, evidence shows that PUI is efficient at removing planktonic bacteria through the flushing effect. The physical mechanisms describing the effect of ultrasonic irrigation on biofilm in the root canal are unknown, although the mechanism of cavitation, which has been recently studied,[25] is able to remove or even destroy biofilm.[7]

Our findings are different from the study by Grundling et al[11] who did not find significant differences between conventional irrigation and irrigation with ultrasound in any of the root thirds evaluated. Although our methodology (infection model) was similar to their study, we used human extracted teeth instead of bovine teeth. Bovine teeth have dentinal tubules with larger diameters, which favors endodontic irrigant flow (even without ultrasound). Such a factor might be one of the reasons why they did not find differences between conventional irrigation and ultrasonic-activated. Our methodology may reproduce the clinical scenario, thus denoting differences amongst cleaning protocols. In other words, the challenge for endodontic irrigant to reach the root canal system and dentinal tubules of human teeth is a large one Thus, based on the results yielded by this study, we concluded that irrigation with ultrasound resulted in higher decontamination when compared with the irrigation without ultrasound.

Therefore, within the limitations inherent to the methodology of our study, the present findings suggest that ultrasound improves the ability to bring the endodontic irrigant in contact with microorganisms and dentin debris within the root canal system, thus improving their removal.

As regards smear layer removal, sodium hypochlorite and chlorhexidine solution, when combined with ultrasonic activation, had the highest ability to clean the root canal on the three thirds. These two combinations were effective at cleaning the apical third, better than sodium hypochlorite alone. Chlorhexidine gel had the lowest cleaning values in all thirds. Therefore, liquid irrigation, using either sodium hypochlorite or chlorhexidine, and ultrasonic agitation, are suggested to reduce the presence of smear layer.

As regards microbial control, sodium hypochlorite and chlorhexidine solution, when combined with ultrasonic activation, showed the best decontamination ability amongst groups. Chlorhexidine solution was more effective than chlorhexidine gel, even when the latter was combined with ultrasound. Based on the above, and regarding the two chlorhexidine formulations, it is preferable to use liquid solution to decontaminate the root canal system with or without ultrasound.

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