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ORIGINAL ARTICLE

Effect of Disinfection Protocols on Bacterial Reduction in Mandibular Molars

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ABSTRACT

Objective: This study aimed to evaluate the bacterial reduction effect of disinfection protocols used in mandibular molars infected by *Enterococcus faecalis*.

Methods: Eighty extracted mandibular molars were prepared and inoculated with *E. faecalis* for 2 months. The teeth were then divided into 2 control groups (n=4) and 4 experimental groups (n=18) according to the disinfection protocol utilized: G1. WaveOne Gold (WOG), passive ultrasonic irrigation (PUI) and Ultracal; G2. WOG, PUI and calcium hydroxide (CH); G3. WOG, XP-Endo Finisher (XPF) and Ultracal; G4. WOG, XPF and CH. Bacteriological samples were collected previously (S1), after preparation (S2), final agitation (S3) and intracanal dressing (S4). Microbial growth was assessed according to culture turbidity and UV spectrophotometry. Statistical analyses used the Friedman test for paired samples and Kruskal-Wallis test for non-paired data ($p<0.05$).

Results: No protocol eliminated *E. faecalis* effectively. The S2, S3 and S4 samples were statistically different from the S1 samples in G1, G2, G3 and G4 ($p<0.05$). Statistical differences were observed in bacterial reduction between G1 and G2 and G1 and G3 after the intracanal medicament (S4) ($p<0.05$).

Conclusion: The combination of WOG with PUI and CH disinfection protocol showed higher percentages of bacterial reduction.

Keywords: Calcium hydroxide, endodontic infection, *Enterococcus faecalis*, root canal instrumentation

HIGHLIGHTS

- The aim of this study was Evaluate the bacterial reduction effect of disinfection protocols used in mandibular molars infected by *Enterococcus faecalis*.
- The results of this study revealed significant differences in bacterial reduction between the successive samples (S2, S3 and S4) when compared with the initial sample (S1)
- This study concluded that the use of the WOG + PUI + CH disinfection protocol resulted in the greatest bacterial reduction.

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INTRODUCTION

The root canal system is disinfected using mechanical instrumentation and antimicrobial agents (1, 2). The instrumentation phase, essential to reduce the bacterial load, contributes to the healing of the periapical tissues (3). However, it may be difficult for an endodontic instrument to touch the walls of curved, oval-shaped and flattened canals (4, 5). Bacterial biofilm may survive in inaccessible areas of the root canals, thus compromising the treatment outcomes (1, 6).

The agitation of an irrigating solution has been recommended as one method to improve disin-

fection in areas of great anatomic complexity (1, 4). Two techniques developed for that purpose involve passive ultrasonic irrigation (PUI) and XP-Endo Finisher (XPF). Both methods improve irrigant dispersion and optimize disinfection (7–10).

The use of an intracanal medicament is usually an adjunct to the disinfection protocol of the root canal treatment procedure (11). The most commonly used intracanal medicament is calcium hydroxide (CH) (12–14). Various CH products are readily available, offering non-setting formulations. UltraCal XS (Ultradent, St. Louis, MO) is one example, which utilizes a syringe-delivery system containing 35% CH, 19% barium sulfate, 3% propylene glycol, and 2% methylcellulose (15, 16). The adequate choice of a vehicle, fundamental for an effective antimicrobial action, may contribute to the healing of periapical tissues (11–14).

Root canal disinfection should focus on the use of protocols that provide effective elimination of the root canal microorganisms (5, 7, 10). Studies that evaluated all three, type of instrument, final agitation of irrigating solution and intracanal medicament, are rare (3, 10, 17, 18). The amount of reduction should be assessed at all operative phases, so that an ideal disinfection model can be established (13). This study compared the microbial reduction effect of various disinfection protocols. The null hypothesis tested was that there would be no difference among the protocols in relation to bacterial reduction.

MATERIALS AND METHODS

The present investigation was conducted in accordance with Declaration of Helsinki and approved by the by Evangelical University of Goiás Research Ethics Committee.

Sample Size Calculation

The sample size was determined using G*Power 3.1 for Mac (Universitat, Düsseldorf, Germany). The Wilcoxon-Whitney test was selected for statistical analyses, with alpha set at 0.05, power at 0.95, and an n2:n1 ratio of 1. The software suggested that the ideal sample size should be 18 in each group.

Sample Selection

Eighty mandibular molars were selected for this study. All teeth were stored in a 0.2% thymol solution and subsequently inserted in 5% sodium hypochlorite (NaOCl; Asfer, Brazil) for 30 minutes as described in a previous study (19).

Periapical radiographic images were taken to ensure the absence of root obliterations, internal or external resorption, fractures, prior root canal treatment, and to verify the closure of the root apex.

The images were obtained using the paralleling technique and an intraoral Spectro X70 Electronic x-ray unit (Dabi Atlante, Brazil) along with a phosphor plate sensor and the Digora® Optime Classic (Soredex, Helsinque, Finland). Radiographic analysis was conducted using Digora for Windows 1.51 software on a workstation equipped with Windows 10 Professional (Microsoft Corp., Redmond, USA), Intel Intel i7 3.5 GHz – 6300 (Intel Corp, Santa Clara, CA), NVIDIA GeForce turbo cache graphics card (NVIDIA Corporation, Santa Clara, CA) and an EIZO – Flexscan S2000 monitor, with a resolution of 1600×1200 pixels (EIZO NANAO Corp., Japan).

Only mandibular molars with three root canals were eligible for inclusion. All teeth had length less than 22 mm, and their curvature was considered moderate (20).

Root Canal Preparation

Conventional access was prepared, using round diamond burs (#1013, KG Sorensen, Barueri, SP, Brazil) and canal patency was established with a K-Flexofile #15 instrument (Dentsply Maillefer, Switzerland). WaveOne® Gold (WOG) Primary #25/.07 (Dentsply Maillefer) instruments and an eletronic apex locator (X-Smart Plus; Dentsply Maillefer) were used following manufacturer's instructions. Subsequently, all specimens were sterilized by autoclaving for 30 min at 120˚C.

Biofilm Formation

The specimens were mounted on a platform (Fig. 1) to allow for the inoculation of the selected bacterial strain (4, 6). Next, the coronal area of each root canal was connected to a 5 mL plastic tube (FLEPA, São Paulo, Brazil). The tube cap was then removed to allow for adjustment to the coronal third of the root. Following the adjustment, two layers of cyanoacrylate adhesive (Super Bonder®, Henkel, Brazil) were applied to seal the connection. The tube-tooth set were kept at room temperature until the adhesive was dry. Then the specimens received a layer of epoxy resin (Durepóxi Loctite®, Henkel, Brazil) (Fig. 1a).

After mounting the test set, it was immersed in 5% NaOCl (Asfer, Indústria Química) for 30 min (Fig. 1b). The set was then rinsed with distilled water (Fig. 1c) and coupled to a 20 mL tube with a cap containing 10 mL of brain heart infusion (BHI) (Difco Laboratories, Detroit, MI) (Fig. 1d). During the entire contamination time, the apical portion of the tooth remained submerged. To ensure infection control, the test set was incubated at 37˚C for 24 hours. After the incubation time, no bacterial growth was found.

To form the biofilm, an *E. faecalis* reference strain (ATCC 29212) was acquired. The bacterial strain was inoculated in 7 mL of BHI (Difco Laboratories) and then incubated at 37˚C for 1 day. *E. faecalis* was cultured on the surface of BHI agar 1 day prior to inoculating the teeth, under the same incubation conditions. The bacterial inoculum was prepared by resuspending cells in saline at a final concentration of approximately 3×108 cells mL-1, adjusted to the #1 McFarland turbidity standard. To contaminate the samples, 5 mL of sterile Brain Heart Infusion (BHI) broth was mixed with 5 mL of the bacterial suspension Using sterile syringes, the samples in the experimental groups (n=18) and the positive control group (n=4) were inoculated with *E. faecalis* for 2 months. This inoculation was performed every 3 days using pure culture prepared 1 day prior and adjusted to the #1 McFarland turbidity standard (Fig. 1e). The specimens were maintained in a microbiology incubator at 37˚C. Microscopic analyzes (Gram stain) were performed on randomly selected samples from contaminated tubes to confirm that the present contamination consisted only of the biological indicators used in inoculation. Four uncontaminated samples were stored at 37°C for 2 months in a microbiological oven with aseptic control as part of the negative control protocol.

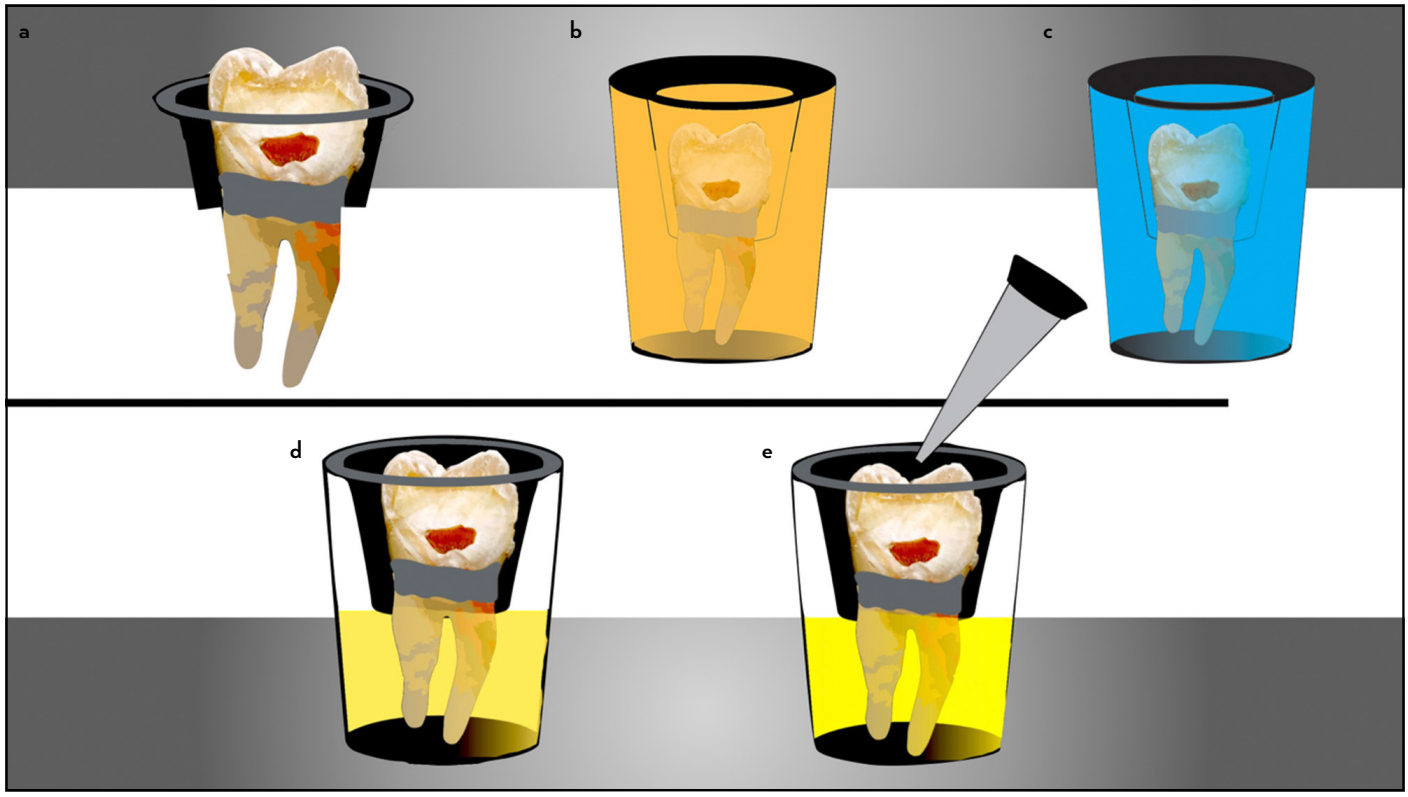

Figure 1. Biofilm formation: (a) the specimens received a layer of epoxy resin; (b) specimen immersed in NaOCl for 30 min; (c) The set was then rinsed with distilled water and (d) coupled to a 20 mL tube with a cap containing 10 mL of BHI; (e) inoculations performed every 3 days. Platform adapted from previous studies (4, 6)

NaOCl: Sodium hypochlorite, BHI: Brain Heart Infusion

Disinfection Protocols

After the formation of the biofilm, the tube-tooth set were allocated into the following groups (Fig. 2):

Group 1: WOG + PUI + Ultracal: The specimens underwent disinfection using a medium WOG file followed by final PUI using an E-1 Irrisonic device (Helse Dental Technology, Brazil) coupled with an EMS Piezon Master 200 ultrasound unit (EMS, São Bernardo do Campo, Brazil). The ultrasound unit was regulated to 10% power and operated in three 20-second cycles. Finally, CH paste applied in combination with methylcellulose (Ultracal XS®; Ultradent, St. Louis, MO) as an intracanal medicament.

Group 2: WOG + PUI + CH: In this group, the specimens underwent disinfection using a medium WOG file followed by final PUI with an E-1 Irrisonic device coupled with an ultrasound unit. The ultrasound which was regulated to 10% power and operated in three 20-second cycles. Subsequently, CH powder (Biodinâmica, Brazil) mixed with distilled water was used as an intracanal medicament.

Group 3: WOG + XPF + Ultracal: In this group, specimens were disinfected using a medium WOG file (Dentsply Maillefer, Switzerland). Final irrigant involved the agitation of the irrigant solution using XPF (FKG Dentaire, Swiss Dental Products, La Chaux de-Fonds, Switzerland) coupled to an electric motor (X-Smart Plus; Dentsply Maillefer), which was operated at 800 rpm and 1 N/cm torque in three 20-second cycles (10). Subsequently, a CH paste combined with methylcellulose was applied as an intracanal medicament.

Group 4: WOG + XPF + CH: In this group, the specimens were disinfected using a medium WOG file. Final irrigant involved agitation of the irrigant solution using XPF coupled to an electric motor operated according to previous specifications. Subsequently, CH powder (Biodinâmica, Ibiporã, Brazil) mixed with distilled water was used as an intracanal medicament.

All specimens were disconnected from the platform and instrumented in the same way, using a medium WOG reciprocating file coupled to an electric motor

(X-Smart Plus; Dentsply Maillefer) according to the manufacturer's instructions. During instrumentation, the canals were irrigated with 10 mL of 2.5% NaOCl solution (Asfer, Brazil). The 30-mm diameter irrigation tip (Navitip; Ultradent Products Inc., 505 West 10200 South, South Jordan, UT) of 5 mL syringe (Ultradent) was positioned 2 mm short of the root apex. One file was used for each 12 teeth.

After drying the root canals with an absorbing paper point #35/.06, the canals were filled with 3 mL of 17% EDTA and agitated for 20 seconds using an E-1 Irrisonic device. This operative phase was performed once in each specimen as an initial preparation of the canals. After that, the irrigating solution agitation protocol for each group was repeated using 2.5% NaOCl, for three 20-s cycles using a standardized volume of 10 mL of irrigant.

In the fourth phase of the protocol, the canals were filled with the intracanal medicament under test for each group, either Ultracal or CH, and kept for 21 days after sealing with a glass-ionomer

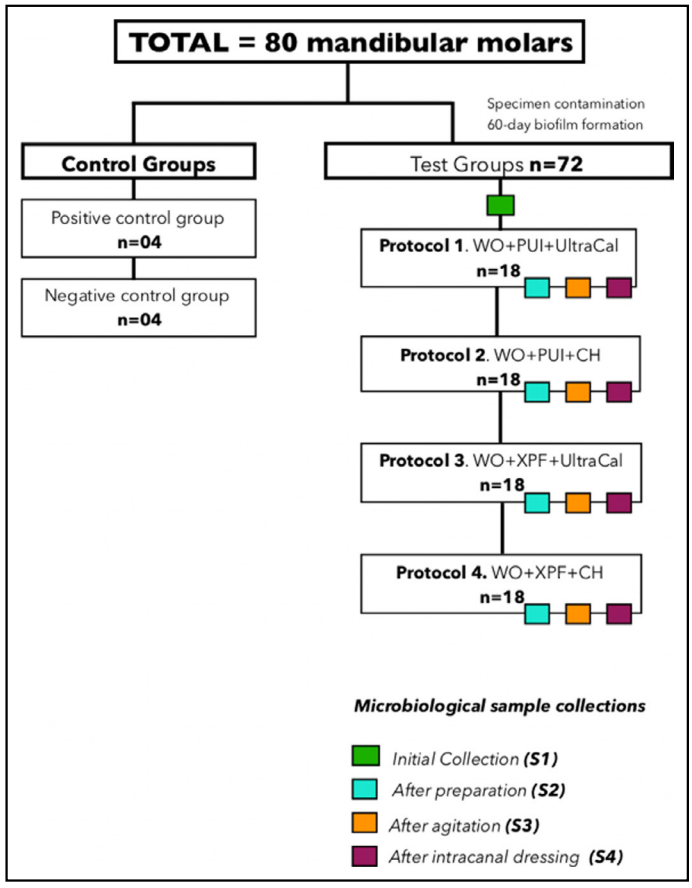

Figure 2. Distribution of test groups according to disinfection protocol, including preparation, final agitation technique and type of intracanal medicament

WOG: WaveONe Gold; PUI: Passive ultrasonic irrigation; CH: Calcium hydroxide; XPF: XP-Endo-Finisher

cement (Vidrion, SS, Rio de Janeiro, Brazil). In groups 1 and 3, the canals were filled with Ultracal following to the manufacturer's instructions. Specimens in G2 and G4 were filled with a CH paste (Biodinâmica, Ibiporã, Brazil) combined with distilled water. The paste was placed throughout the instrumented portion of the root canal using a #35 K-Flexofile (Dentsply Maillefer, Ballaigues, Switzerland) and #35 paper cones. Radiographs were taken to confirm the correct filling the intracanal medicament. All clinical phases were conducted by an experienced endodontist.

Microbiological Collection

Pre-instrumentation collection

Before instrumentation, the root canals were dried and filled with distilled water. Three #35 paper points were introduced into each root canal and left in place for three minutes to collect the first microbiological sample (S1). Subsequently, these paper points were placed in 7 mL of Letheen broth (LB; Difco Laboratories, Detroit, MI) containing Tween 80 and research-grade sodium thiosulfate (Laboratório Art, Brazil) both at 1% concentration, with an inoculum of 0.1mL, and then incubated at 37˚C for 48 hours. Microbial growth was assessed based on culture turbidity using a UV spectrophotometer (Spectrophotometer Model Nova 1600 UV, Brazil). All samples were collected under aseptic conditions.

Post-instrumentation collection

After root canal preparation, the second sample (S2) was collected in the identical way as described for S1.

Collection after agitation of irrigating solution

After agitation of the irrigating solution, the third sample was collected (S3). The collection was conducted under conditions similar to those of S1 and S2 collections.

Collection after application of intracanal medicament

After the intracanal medicament was kept in the roots (21 days), the dressing paste was removed, and another sample was collected (S4). The collection was conducted under conditions similar to those of the other three collections (S1, S2 and S3).

Calculation of Percentage of Bacterial Reduction

This percentage of variation was calculated after describing the group values by the median. This variable is: $\left(\frac{\text{median } Sx - \text{median } S1}{\text{median } S1}\right) * 100$, with x=2,3,4.

Statistical Analysis

The quantitative variables were described as medians and interquartile ranges (IQR 25–75%). The Shapiro-Wilk test was used to test the normal distribution of the results. The Friedman test was used for paired analysis, to compare optical density values in each disinfection protocol group between collection time points. The Kruskal Wallis test was used for non-paired analysis to compare optical density values between samples in the different disinfection protocols. Multiple comparisons were made using the Dwass-Steel-Critchlow-Fligner test. The significance level was set at $p<0.05$ for all tests. Data analyses were performed using The Jamovi 2.3 software (The Jamovi Project, 2023).

RESULTS

Table 1 presents the medians (nm) and interquartile ranges (IQR 25–75%) of the culture optical density for all microbiological samples (S1, S2, S3 and S4) and shows the bacterial reduction in the samples collected after initial contamination and under different protocols. Despite significant decreases of culture optical density in the samples of all groups after initial contamination ($p<0.05$), no protocol totally eliminated *E. faecalis*.

The analysis of optical density values for each test group revealed significant differences in microbial reduction between the consecutive samples (S2, S3 and S4) when compared with the initial sample (S1) for G1, G2, G3 and G4 ($p<0.05$). The comparison of the optical density values between samples in the various groups demonstrated differences in microbial reduction between G1 and G2 and G1 and G3 ($p<0.05$) in the samples collected after the intracanal medicament (S4). G2 had the greatest bacterial reduction (85%) of all groups.

DISCUSSION

The objective of the protocols tested in this study was to investigate the optimal method for reduction of bacterial contamination. Results revealed a substantial bacterial reduction

TABLE 1. Median (nm) and interquartile range (IQR 25–75%) values of culture optical densities (nm) in the four microbiological sample collections (S1, S2, S3 and S4)

Disinfection protocol	Initial collection (S1)		After preparation (S2)			After agitation (S3)			After intracanal dressing (S4)		
	Median	IQR 25–75%	Median	IQR 25–75%	(%) Bacterial reduction in relation to S1	Median	IQR 25–75%	(%) Bacterial reduction in relation to S1	Median	IQR 25–75%	(%) Bacterial reduction in relation to S1
WOG+PUI+Ultracal	0.293^{A,a}	0.224–0.347	0.192^{B,b}	0.112–0.251	34%	0.075^{C,c}	0.013–0.129	74%	0.057^{D,a}	0.052–0.088	80%
WOG+PUI+CH	0.281^{A,a}	0.229–0.396	0.174^{B,b}	0.119–0.217	38%	0.082^{C,c}	0.037–0.128	70%	0.040^{D,b}	0.036–0.053	85%
WOG+XPF+Ultracal	0.270^{A,a}	0.231–0.350	0.199^{B,b}	0.179–0.217	26%	0.140^{C,c}	0.070–0.191	48%	0.044^{D,b}	0.036–0.049	83%
WOG+XPF+CH	0.227^{A,a}	0.181–0.300	0.163^{B,b}	0.137–0.206	28%	0.091^{C,c}	0.048–0.140	59%	0.051^{D,a,b}	0.033–0.062	77%
Positive Control	0.350	0.255–0.606									

Different lowercase letters in the column indicate significant differences ($p<0.05$). Different uppercase letters in lines indicate significant differences ($p<0.05$). IOR: Interquartile range; WOG: WaveOne gold; PUI: Passive ultrasonic irrigation; CH: Calcium hydroxide; XPF: XP-endo finisher. Percentage of bacterial reduction calculated by the formula $\left(\frac{\text{median } Sx - \text{median } S1}{\text{median } S1}\right) * 100$, with x=2,3,4.

after the instrumentation, final irrigating solution agitation and intracanal medicament application. The significant differences in microbial reduction found between G1 and G2 and G1 and G3 in the samples obtained following intracanal medicament led to the rejection of the null hypothesis. These findings confirm that the type of disinfection protocol used affects the bacterial reduction.

A medium WOG reciprocating file was used for canal preparation in all test groups to reduce the number of variables under analysis. The final agitation techniques using PUI and XPF were compared with both intracanal medicaments used, Ultracal and CH.

Other studies found that the irrigating solution should be agitated after the instrumentation to potentiate the reduction of the microbial load in the root canals (7, 13, 21). Teves et al. (10) conducted an *in vitro* study using scanning electron microscopy (SEM) and 50 mandibular first premolars to evaluate the influence of XPF on the elimination of multispecies biofilm in comparison with passive ultrasonic irrigation and conventional irrigation (CI). Their data demonstrated that removal of multispecies biofilm in the XPF and PUI groups was higher than in the CI group ($p<0.05$). However, most studies did not include the application of intracanal dressing, and, therefore, did not produce a thorough understanding about the real impact of the sequence of all endodontic treatment phases on bacterial reduction (1, 7, 22).

The analysis of the preparation-agitation-dressing protocols in this study showed that Group 2 (WOG + PUI + CH) was the one with the greatest bacterial reduction (85%). This finding may be explained by the combined effects of PUI which generates acoustic streaming of the irrigant and improves the efficacy of NaOCl in removing bacterial biofilm disrupted by root canal instrumentation, and of the CH paste, which has a high content of calcium (Ca^{+}) and hydroxyl (OH^{-}) ions.

The type of delivery system used with the CH paste has a significant effect on ion penetration into the dentinal tubules, as well as on alkalinity (12, 14, 15, 23–25). Estrela & Pesce (15) evaluated the release of OH^{-} and Ca^{+} ions in CH pastes using delivery systems with different acid-base properties in the presence of subcutaneous connective tissue in dogs. The percentages of Ca^{+} and OH^{-} ions at 7, 30, 45 and 60 days indicated a greater ion release for pastes with aqueous water-soluble vehicles, such as saline and anesthetic solutions, when compared with the values of pastes using a viscous water-soluble vehicle, such as propylene glycol 400. The vehicle used is either water-soluble or else, with a certain viscosity. Together with acid-base properties and the level of dentine permeability, it may affect the speed of dissociation and diffusion of OH^{-} ions. The UltraCal paste has an aqueous methylcellulose base that helps in the diffusion of calcium hydroxide (CH) ions through the dentine. However, the effectiveness of the paste may not be optimal due to the presence of irregular particles and varying sizes. The particle size in UltraCal ranges from 0.5 to 2.5 µm, which may not be suitable for optimal penetration and diffusion through the dentinal

tubules. Ideally, particles smaller than the diameter of dentinal tubules, which are typically between 1 and 3 µm, are required for optimal penetration and diffusion (26).

Some vehicles may affect the pH of CH, resulting in lower rates of bacterial reduction. Evans et al. (22) evaluated the mechanism of resistance of *E. faecalis* to CH and found that these bacteria are resistant at a pH equal to or lower than 11.1.

Bacterial biofilm disruption and destruction in anatomically complex areas, such as isthmus, recesses and ramifications, remain a relevant issue in the field of endodontic research, as no disinfection technique has so far proven to eliminate all biofilm effectively (2, 4, 7, 8, 10). Although several *in vitro* studies using different methods have been conducted to achieve significant antimicrobial efficacy, findings remain contradictory or inconsistent. Many evaluations have been conducted using single-rooted teeth with an anatomy that is less complex than that of multirooted teeth (3, 8, 9, 14, 22, 25). This is one important parameter that was considered in the present investigation in which various disinfection protocols were assessed in mandibular molars infected by *E. faecalis*. The purpose of the 60-day biofilm in this study was to simulate a challenging clinical condition. According to Estrela et al. (4), shorter times of biofilm formation may complicate comparisons and the extrapolation of results to the formulation of better-defined clinical protocols that may add greater predictability to endodontic treatments.

The method used in this study included two analyses: qualitative, using the visual evaluation of culture turbidity; and quantitative, using UV spectrophotometry findings to eliminate the subjectivity of the visual method. This method has been widely used (4, 5). Others, such as molecular methods, that use a qPCR approach to count the number of bacteria, may not discriminate whether bacteria are viable or not, which may lead to errors in the interpretation of the effects of bacterial viability (6–8, 18, 22, 23, 25, 27, 28). As bacterial samples were obtained only from the main canals of mandibular molars using absorbent paper points, the study results should be interpreted with caution, because the deep regions of ramifications and dentinal tubules are not reached when this procedure (3, 9, 17, 23, 28).

The challenges to obtain root canal therapy success involve the understanding of the canal anatomy, the reduction of the microbial population and the patient's immune responses.

As no ideal set of procedures, including final irrigant agitation and type of intracanal medicament, has been defined so far, a perfect root canal system disinfection remains a challenge. Additional clinical essays should be developed to assess the influence of disinfection approaches on root canal infections.

CONCLUSION

Although bacterial reduction was found in all test groups, none of the techniques eliminated *E. faecalis* effectively. The use of WOG with PUI and CH as a combined disinfection protocol showed higher percentages of bacterial reduction.

Disclosures

Ethics Committee Approval: The study was approved by the Evangelical University of Goiás Research Ethics Committee (no: CAAE 81431717.1.0000.5076, date: 05/05/2020).

Authorship Contributions: Concept – H.F.O., O.A.G.; Design – H.F.O., L.C.G.T., I.F.S.J.; Supervision – H.F.O., D.A.D., O.A.G.; Funding – L.C.G.T.; Materials – H.F.O., L.C.G.T., I.F.S.J., F.F.C., A.R.G.; Data collection and/or processing – H.F.O., L.C.G.T., I.F.S.J., F.F.C., H.C.S., A.R.G.; Data analysis and/or interpretation – H.F.O., D.A.D., O.A.G.; Literature search – L.C.G.T., I.F.S.J., F.F.C., H.C.S., A.R.G.; Writing – H.F.O.; Critical review – D.A.D., O.A.G.

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REFERENCES

1. Estrela C, Holland R, Estrela CR, Alencar AH, Sousa-Neto MD, Pécora JD. Characterization of successful root canal treatment. Braz Dent J 2014; 25(1):3–11.
2. Nair PN, Henry S, Cano V, Vera J. Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after "one-visit" endodontic treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2005; 99(2):231–52. [CrossRef]
3. Siqueira Junior JF, Rôças IDN, Marceliano-Alves MF, Pérez AR, Ricucci D. Unprepared root canal surface areas: causes, clinical implications, and therapeutic strategies. Braz Oral Res 2018; 32(Suppl 1):e65. [CrossRef]
4. Estrela C, Sydney GB, Figueiredo JA, Estrela CR. A model system to study antimicrobial strategies in endodontic biofilms. J Appl Oral Sci 2009; 17(2):87–91. [CrossRef]
5. Estrela C, Sydney GB, Figueiredo JA, Estrela CR. Antibacterial efficacy of intracanal medicaments on bacterial biofilm: a critical review. J Appl Oral Sci 2009; 17(1):1–7. [CrossRef]
6. Sundqvist G, Figdor D, Persson S, Sjogren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998; 85(1):86–93. [CrossRef]
7. Decurcio DA, Rossi-Fedele G, Estrela C, Pulikkotil SJ, Nagendrababu V. Machine-assisted Agitation Reduces Postoperative Pain during Root Canal Treatment: A Systematic Review and Meta-analysis from Randomized Clinical Trials. J Endod 2019; 45(4):387–93.e2. [CrossRef]
8. Neves MA, Provenzano JC, Rôças IN, Siqueira JF, Jr. Clinical antibacterial effectiveness of root canal preparation with reciprocating single-instrument or continuously rotating multi-instrument systems. J Endod 2016; 42(1):25–9. [CrossRef]
9. Paiva SS, Siqueira JF, Jr., Rôças IN, Carmo FL, Ferreira DC, Curvelo JA, et al. Supplementing the antimicrobial effects of chemomechanical debridement with either passive ultrasonic irrigation or a final rinse with chlorhexidine: a clinical study. J Endod 2012; 38(9):1202–6. [CrossRef]
10. Teves A, Blanco D, Casaretto M, Torres J, Alvarado DE, Coaguila-Llerena H, et al. Multispecies biofilm removal by XP-endo Finisher and passive ultrasonic irrigation: A scanning electron microscopy study. Aust Endod J 2022; 48(1):91–7. [CrossRef]
11. Estrela C, Sydney GB, Bammann LL, Felippe Júnior O. Mechanism of action of calcium and hydroxyl ions of calcium hydroxide on tissue and bacteria. Braz Dent J 1995; 6(2):85–90.
12. Estrela C, Holland R. Calcium hydroxide: study based on scientific evidences. J Appl Oral Sci 2003; 11(4):269–82. [CrossRef]
13. Holland R, Gomes JEF, Cintra LTA, Queiroz Í OA, Estrela C. Factors affecting the periapical healing process of endodontically treated teeth. J Appl Oral Sci 2017; 25(5):465–76. [CrossRef]
14. Pereira TC, da Silva Munhoz Vasconcelos LR, Graeff MSZ, Ribeiro MCM, Duarte MAH, de Andrade FB. Intratubular decontamination ability and physicochemical properties of calcium hydroxide pastes. Clin Oral Investig 2019; 23(3):1253–62. [CrossRef]
15. Estrela C, Pesce HF. Chemical analysis of the liberation of calcium and hydroxyl ions from calcium hydroxide pastes in connective tissue in the dog--Part I. Braz Dent J 1996; 7(1):41–6.

16. Hawkins JJ, Torabinejad M, Li Y, Retamozo B. Effect of three calcium hydroxide formulations on fracture resistance of dentin over time. Dent Traumatol 2015;31:380–4. [CrossRef]
17. Alves FR, Rôças IN, Almeida BM, Neves MA, Zoffoli J, Siqueira JF, Jr. Quantitative molecular and culture analyses of bacterial elimination in oval-shaped root canals by a single-file instrumentation technique. Int Endod J 2012; 45:871–7. [CrossRef]
18. Virdee SS, Seymour DW, Farnell D, Bhamra G, Bhakta S. Efficacy of irrigant activation techniques in removing intracanal smear layer and debris from mature permanent teeth: a systematic review and meta-analysis. Int Endod J. 2018; 51(6):605–21. [CrossRef]
19. de Oliveira HF, da Silva Júnior IF, Teixeira LCG, Camilo NG, de Almeida Decurcio D, Guedes OA, et al. Influence of different agitation techniques on bacterial reduction in curved root canals. Aust Endod J 2023; 49(1):104–10.
20. Schneider SW. A comparison of canal preparations in straight and curved root canals. Oral Surg Oral Med Oral Pathol 1971; 32(2):271–5. [CrossRef]
21. Gu LS, Kim JR, Ling J, Choi KK, Pashley DH, Tay FR. Review of contemporary irrigant agitation techniques and devices. J Endod 2009; 35(6):791–804.
22. Evans M, Davies JK, Sundqvist G, Figdor D. Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. Int Endod J 2002; 35(3):221–8. [CrossRef]
23. Estrela C, Pimenta FC, Ito IY, Bammann LL. Antimicrobial evaluation of calcium hydroxide in infected dentinal tubules. J Endod 1999; 25(6):416–8. [CrossRef]
24. Haapasalo M, Qian W, Portenier I, Waltimo T. Effects of dentin on the antimicrobial properties of endodontic medicaments. J Endod 2007; 33(8):917–25. [CrossRef]
25. Louwakul P, Saelo A, Khemaleelakul S. Efficacy of calcium oxide and calcium hydroxide nanoparticles on the elimination of *Enterococcus faecalis* in human root dentin. Clin Oral Investig. 2017; 21(3):865–71. [CrossRef]
26. Peña-Bengoa F, Magasich MC, Bustamante D, Wastavino C, Niklander SE, Cáceres C. Effect of ultrasonic activation on dentinal tubule penetration of Bio-C temp and ultracal XS: A comparative CLSM assessment. Eur Endod J 2023; 8(4):268–73. [CrossRef]
27. Estrela C, Rabelo LE, de Souza JB, Alencar AH, Estrela CR, Sousa Neto MD, et al. Frequency of root canal isthmi in human permanent teeth determined by cone-beam computed tomography. J Endod 2015; 41(9):1535–9. [CrossRef]
28. Sirén EK, Kerosuo E, Lavonius E, Meurman JH, Haapasalo M. $Ca(OH)_2$ application modes: *in vitro* alkalinity and clinical effect on bacteria. Int Endod J 2014; 47(7):628–38. [CrossRef]