Evaluation of in vitro biofilm elimination of Enterococcus faecalis using a continuous ultrasonic irrigation device

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Abstract: This study sought to evaluate biofilm elimination using the HBW Ultrasonic Ring based on continuous ultrasonic irrigation. Forty-five premolars and molars with complex curvatures were included. An Enterococcus faecalis biofilm was established for 30 days on the extracted teeth. The teeth were then stratified into three experimental groups for instrumentation and irrigation (i.e., HBW Ultrasonic Ring, conventional irrigation, and passive ultrasonic irrigation). Pre- and post-instrumentation samples were collected, and reductions of bacterial load were evaluated by McFarland’s scale, counting of colony-forming units, and scanning electronic microscopy. The HBW Ultrasonic Ring promoted a higher reduction in bacterial load relative to conventional irrigation (P < 0.05) and a similar reduction compared with passive ultrasonic irrigation (P > 0.05). These results suggest the HBW Ultrasonic Ring is a promising alternative modality for simultaneous instrumentation and irrigation during root canal treatment, achieving an appropriate level of bacterial reduction and allowing the passage of the irrigating solution throughout the entire working length.

Keywords: biofilm, Enterococcus faecalis, HBW Ultrasonic Ring, manual conventional irrigation, passive ultrasonic irrigation

Introduction

Anaerobic microorganisms are the principal causal agents of pulp tissue infections, and their complete elimination from root canals is almost impossible, due to the complex anatomy of root canals, thus constituting a clinical challenge for endodontists. As the infection progresses through enamel, dentin, and pulp, the environment becomes increasingly anaerobic, with great bacterial diversity [1-4]. The typical microbiota in this context includes organisms from genera such as Prevotella, Porphyromonas, Fusobacterium, Actinomyces, Streptococcus, and Enterococcus. The microorganism Enterococcus faecalis (E. faecalis) is most frequently reported in persistent infections associated with endodontic failure; it is a facultative, Gram-positive coccus that can be isolated from up to 35% of infected root canals [5]. Commonly, these microorganisms form biofilms inside root canals, complicating their complete elimination [6].

The formation of biofilm inside root canals occurs in several ways, beginning immediately after the first invasion of oral microorganisms into the pulp chamber. For this process, bacteria need to stay in a fluid medium beginning immediately after the first invasion of oral microorganisms into infected root canals [5]. Commonly, these microorganisms form biofilms facultative, Gram-positive coccus that can be isolated from up to 35%. Anaerobic microorganisms are the principal causal agents of pulp tissue infections, and their complete elimination from root canals is almost impossible, due to the complex anatomy of root canals, thus constituting a clinical challenge for endodontists. As the infection progresses through enamel, dentin, and pulp, the environment becomes increasingly anaerobic, with great bacterial diversity [1-4]. The typical microbiota in this context includes organisms from genera such as Prevotella, Porphyromonas, Fusobacterium, Actinomyces, Streptococcus, and Enterococcus. The microorganism Enterococcus faecalis (E. faecalis) is most frequently reported in persistent infections associated with endodontic failure; it is a facultative, Gram-positive coccus that can be isolated from up to 35% of infected root canals [5]. Commonly, these microorganisms form biofilms inside root canals, complicating their complete elimination [6].

is fundamental during root canal treatment; the ideal irrigation solution should remove the microbial load, lubricate the walls of the root canal, and dissolve the organic tissue. Sodium hypochlorite (NaOCl) is the chemical irrigator most frequently adopted in endodontics given its properties [11].

There are diverse techniques available for supplying the irrigant solution, such as conventional irrigation and passive ultrasonic irrigation. The latter involves the use of an ultrasound-activated instrument placed inside the root canals filled with the irrigant solution, which induces an acoustic current in the solution around the tip. It has been demonstrated that ultrasonically-activated NaOCl can eliminate more microorganisms, pulp, and debris tissue than conventionally applied NaOCl [12].

The HBW Ultrasonic Ring (US patent 9,839,492 B2) is a device that can perform instrumentation and irrigation simultaneously (Fig. 1A-C). It is an ultrasonic tip made of stainless steel 316 that activates endodontic instruments, transforming them into potential ultrasonic instruments during the root canal treatment. The HBW Ultrasonic Ring, with its dual function, has the advantage of displaying an excellent capacity for file cutting, while, at the same time, the irrigant is activated. The central concern with this device, however, is the effect of its innovation in instrumentation and ultrasonic irrigation on normal or complicated root canals with narrow canals or major curvatures [13]. Other benefits of the HBW Ultrasonic Ring include: more conservative access since it eliminates the need for a straight line of access to the canal; creation of a safe and reliable glide path, where the initial file follows the canal anatomy even in narrow or calcified canals; and the provision of adequate irrigation at the full working length, generating disinfection without the use of needles or other additives inside the root canals and, thus, eliminating the risk of irrigant extrusion [13].

This study sought to evaluate the root canal cleaning and disinfection performance observable when using the HBW Ultrasonic Ring in comparison with passive ultrasonic irrigation and conventional irrigation.

Materials and Methods

For the present study, extracted permanent teeth of patients from the Oral Surgery Clinic of the Faculty of Dentistry (San Luis Potosí University) were collected. All involved patients provided a thorough clinical history and signed an informed consent form. The Faculty of Dentistry’s Ethical and Investigation Committee approved this study (CEIFE-031018). The study included 45 molars and premolars with dental caries and/or periodontal disease. Periapical X-rays were taken of all teeth (Timex 70 E, X-Ray digital; Gnatus Medical-Dental Equipments, Ribeirao Preto, São Paulo, Brazil), which were analyzed with AutoCAD 2015 for Mac (Autodesk, Inc., San Rafael, CA, USA), while the curvatures were measured using Schneider’s method [14]. Molars and premolars with roots that had curvatures greater than 30° and at least three walls of the clinical crown, mature apex, and complete root were included. Teeth showing cracks or resorptions and calcified canals were excluded from further analysis. Access and patency were made with #10 K-files. All teeth were disinfected carefully with 5.25% NaOCl, 17% ethylenediaminetetraacetic acid, disodium salt, and sterile distilled water during 4 min each solution in an ultrasonic bath (BioSonic UC50; Whaledent Inc, Mahwah, NJ, USA). Later, the teeth were sterilized in an autoclave at 121°C with 15 pounds of pressure for 15 min.
Separately, *E. faecalis* samples were isolated from the root canals of patients who came to the Endodontics Clinic with secondary endodontic infections. Microbiological specimens were collected with sterile paper tips, placed in thioglycolate tubes, and incubated in an anaerobic chamber (COY Laboratory Products, Grass Lake, MI, USA) at 37°C for 48 to 72 h. Then, the microbiological samples were spread in anaerobic agar with 5% sheep blood (BD BBL; Cuautitlán Izcalli, Estado de México, México). The microorganisms were identified through biochemical testing using API 20 Strep (bioMérieux, Marcy I’Etoile, Lyon, France) according to the manufacturer’s instructions.

In vitro biofilm formation was subsequently performed in a Purifier Logic+ class II biosafety cabinet (Labconco Delta Series, Kansas City, MO, USA). The microorganisms were cultured on a blood agar plate (BD BBL; Cuautitlán Izcalli, Estado de México, México) and placed in a bacteriological stove (Felisa, Guadalajara, Jalisco, México) at 37°C for 24 h. Microscopic characteristics were evaluated by Gram staining (Hycel de México, Reactivos Químicos, México D.F., México) to verify the developed strain’s purity. *E. faecalis* was cultured in an Erlenmeyer flask with 150 mL of brain heart infusion (BHI) culture medium, and the roots were inoculated and incubated in the bacteriological stove at 37°C. During this stage, the culture medium was changed every 72 h for 30 days [15].

After the aforementioned 30 days, following operative field disinfection with 30% hydrogen peroxide (H₂O₂), 5.25% NaOCl and 10% sodium thiosulfate (Na₂S₂O₃) for one minute each, the teeth were placed in a metal device with a plastic base, which was covered with a heavy silicone material by condensation (Coltène/Whaledent AG, Altstätten, Switzerland). Each tooth was isolated completely with a rubber dam and Young’s arch. A microbiological sample of the operative field was taken with a sterile cotton tip, spread in trypticase soy agar, and incubated at 37°C for 48 h to confirm operative field disinfection. Preinstrumentation samples were collected by placing three #15 sterile paper tips for one minute each inside the root canal, facilitating microorganism sampling from the root canal; the paper tips were then placed in BHI. For assessments of instrumentation and irrigation, the teeth were divided into three experimental groups that were treated with the following protocols: continuous ultrasonic irrigation (CUI) employing the HBW Ultrasonic Ring (n = 16) (group 1), positive apical pressure (PAP) employing conventional irrigation (n = 12) (group 2), and passive ultrasonic irrigation (PUI) (n = 12) (group 3). The instrumentation of group 1’s teeth was carried out with #8, #15, and #25 K-files and activated with the HBW Ultrasonic Ring alongside simultaneous irrigation with 2 mL of 5.25% NaOCl. The files were instrumented for one minute, avoiding prolonged exposure to ultrasound. The final irrigation protocol was performed using 2 mL of 17% ethylenediaminetetraacetic acid for 1 minute to promote organic material degradation and allow for good dentinal permeability. The protocol was inactivated with 2 mL of 10% sodium thiosulfate applied for 1 minute. In group 2, the manual instrumentation was carried out with #8, #10, #15, #20, #25, and #30 K-files; after each file irrigation was performed, 5.25% NaOCl (2 mL) was applied for 1 minute with a needle and Endo-Eze syringe (Ultradent Products, Inc., South Jordan, UT, USA). In group 3, manual instrumentation was carried out with #8, #10, #15, #20, #25, and #30 K-files with subsequent irrigation with 5.25% NaOCl (2 mL) for 1 minute after each file was applied. The last irrigation was activated with ultrasound and the #25 file for 1 minute, with replacements of 5.25% NaOCl introduced every 20 s. In all groups, post-instrumentation samples were taken with three sterile paper tips first inserted inside the root canals for one minute each and then placed in a sterile BHI culture medium. All pre- and post-instrumentation samples were incubated at 37°C for 24 h. Negative controls without biofilm formation and positive controls showing biofilm formation without instrumentation were also employed (both n = 5) (Fig. 2).

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**Fig. 1** Design and use of the HBW Ultrasonic Ring. This device is used in dentistry for activating endodontic instruments, as described in the Introduction section. A) The top view, view below, lateral view, and detailed overview of the HBW Ultrasonic Ring. B) Design of the HBW Ultrasonic Ring. C) Clinical use of the HBW Ultrasonic Ring

**Fig. 2** Flow diagram that shows the steps and the different conditions affiliated with the application of each instrumentation system
After 24 h of incubation, the sample bacterial concentration was evaluated using McFarland’s scale with a densitometer (Densimat; bioMérieux, Marcy l’Etoile, Lyon, France). Serial dilutions of the samples were made and inoculated on BHI agar plates (BD BBL; Cuautitlán Izcalli, Estado de México, México) and incubated at 37°C during 24 h. Later, the counting of colony-forming units (CFUs) was performed in a semi-automatic counter (Felisa, Guadalajara, Jalisco, México). To calculate the CFUs per milliliter, the following formula was adopted: \( \text{CFU/mL} = \frac{\text{(number of CFUs)} \times \text{(serial dilution)}}{\text{dilution factor in mL}} \).

The degree of biofilm elimination in each tooth was evaluated by scanning electron microscopy (SEM) (Jeol JSM-6610LV; Jeol Ltd., Tokyo, Japan). The roots were separated and longitudinally sectioned, then placed in a mix of 2% glutaraldehyde and 1% alcian blue stain for fixation over 24 h; later, a gradual dehydration protocol was enacted using 20%, 40%, 60%, 80%, 90%, and absolute ethyl-alcohol by placing the roots for 5 min in each solution. For critical point drying (Leica EM CPD030; Leica Microsystems, Wetzlar, Germany), the samples were submerged in acetone and placed inside the critical point drying chamber for seven cycles of washing with liquid carbon dioxide. Finally, the pieces were covered with gold in an automatic fine coater (JEOL JFC-1100; Jeol Ltd., Tokyo, Japan) to increase the electronic density. The samples were then observed by SEM at different magnifications.

Data are expressed as means and standard deviations or as medians and interquartile ranges (IQR), according to their distribution. The normality of data was calculated using the Kolmogorov-Smirnov test. According to the results of this test, comparisons among groups were performed using either the Student’s t-test or nonparametric Mann-Whitney U-test for the comparison of two independent groups. For comparisons among the three groups, the Kruskal-Wallis test or one-way analysis of variance was performed, depending on the data distribution, followed by the use of post hoc tests with Dunn’s multiple comparison method. The analysis was carried out using the GraphPad Prism version 5.0 software program (GraphPad Software Inc., San Diego, CA, USA). \( P \)-values lower than 0.05 were considered statistically significant.

**Results**

The bacterial load reduction in *E. faecalis* biofilm was evaluated according to both McFarland’s scale and the CFU count. There was a statistically significant post-instrumentation reduction compared with preinstrumentation in group 1 [median (IQR): 0.5 (0.42-0.67) vs. 7.3 (6.32-7.5); \( P < 0.0001 \)] (Fig 3A). In group 2, there was also a significant reduction observed in the post-instrumentation bacterial load relative to preinstrumentation [median (IQR): 7.05 (5.7-7.37) vs. 7.5 (7.4-7.5); \( P < 0.0001 \)] (Fig 3B). Finally, in group 3, a significant reduction was also noted in the post-instrumentation bacterial load compared with preinstrumentation [median (IQR): 0.6 (0.5-2.3) vs. 7.5 (7.4-7.5); \( P < 0.0001 \)] (Fig 3C). Similar results were found considering the reduction in bacterial load as determined by measuring CFU count between the pre- and post-instrumentation samples of the three different evaluated irrigation systems: group 1 [median (IQR): 1.65 × 10^9](Fig. 4A)
The evaluation of the bacterial load reduction between pre- and post-instrumentation using McFarland’s scale revealed a significant difference in favor of group 1 in comparison with group 2 [median (IQR): 0.5 (0.42-0.67) vs. 7.05 (5.7-7.37); P = 0.0006], and group 3 [median (IQR): 8.79 × 10^11 (7.9 × 10^11-1.02 × 10^12) vs. 0 (0.0-4.2 × 10^11); P = 0.0001], and (Fig. 3D-F). Similar results were also observed regarding bacterial load reduction when comparing CFU counts, where group 1, compared with group 2, showed a significant difference (mean ± standard deviation: 0.12 ± 0.34 vs. 3.34 × 10^10 ± 3.52 × 10^10; P = 0.0002) (Fig. 4B). Also, bacterial load reduction in the E. faecalis biofilm was evaluated by observation of the root canals by SEM. A biofilm diminution inside the root canals had occurred, with areas of totally permeable dentinal tubules noted, when the HBW Ultrasonic Ring (group 1) was used in comparison with group 2 and the positive controls (Fig. 5A-C). In addition, the reduction of bacterial load of group 1 in comparison to group 3 was similar, with areas of totally permeable dentinal tubules. Table 1 presents the compiled results.

### Discussion

This study aimed to evaluate the disinfection capacity by assessing the biofilm elimination of the novel device HBW Ultrasonic Ring compared with other instrumentation techniques, such as PUI and PAP. The disinfection of the root canal system and the elimination of microorganisms, debris, and microbial components are crucial steps by which to achieve endodontic treatment success. This objective is accomplished with chemomechanical debridement of the canal system, where the mechanical systems are paired with irrigation chemical solutions [16,17]. There are several irrigation techniques for root canal treatment. However, some of these techniques do not allow irrigating solutions to penetrate all areas of the root canals, since root canal anatomy is usually complex and whimsical, which represents a common problem in endodontics [18].

In the present study, the disinfection capacity of the HBW Ultrasonic Ring, an innovative CUI device used in endodontics for the instrumentation and irrigation of root canals, was assessed [13]. This novel technique

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**Table 1** Pre- and post-instrumentation bacterial loads according to the instrumentation system used

| Instrumentation system used | Continuous ultrasonic irrigation employing the HBW Ultrasonic Ring | Positive apical pressure employing conventional irrigation | Passive ultrasonic irrigation |
|-----------------------------|---------------------------------------------------------------|----------------------------------------------------------|-------------------------------|
| McFarland’s scale           |                                                               |                                                          |                               |
| Preinstrumentation          | 7.3 (6.32-7.5)                                                | 7.5 (7.4-7.5)                                            | 7.5 (7.4-7.5)                 |
| Postinstrumentation         | 0.5 (0.42-0.67)                                              | 7.05 (5.7-7.37)                                          | 0.6 (0.5-2.3)                |
| CFU count preinstrumentation| 1.65 × 10^10 (6.2 × 10^9-9.03 × 10^9)                        | 4.4 × 10^11 (3.8 × 10^10-6.64 × 10^10)                  | 8.79 × 10^11 (7.9 × 10^11-1.02 × 10^12) |
| Postinstrumentation         | 0.0 (NA)                                                     | 1.5 × 10^10 (2.92 × 10^9-7.33 × 10^9)                   | 0 (0.0-4.2 × 10^9)           |

Results are expressed as medians (IQRs). IQR, interquartile range; CFU, colony-forming units; NA, not applicable.
combines instrumentation and irrigation processes simultaneously, achieving superior canal disinfection, according to the findings of the present study, compared with other techniques. Additionally, this device attained favorable results in the biofilm elimination of *E. faecalis* when compared with conventional irrigation techniques. Similarly, the root canal cleaning efficacy was assessed by SEM, and it was found that the canals treated with the HBW Ultrasonic Ring exhibited improved biofilm removal in comparison with those in the other two treatment groups. This outcome was especially noticeable in the apical third, which is the most critical intracanal region to observe in this context due to its anatomical localization and challenging access. Other advantages of the HBW Ultrasonic Ring include its improved removal of pulp tissue debris in isthmus, calcified, or lateral canals and other anatomical variations. Also, it reduces the risks of damage associated with endodontic instrumentation such as fractures files, perforations, transports, and blockages significantly. Further, fewer files are needed during instrumentation, instrument separation, apical closing, and organic material removal, as debris [13].

*E. faecalis* has been commonly used as a test microorganism in the endodontic area for *in vitro* biofilm formation since it is one of the microorganisms most frequently found in endodontic secondary infections [19-21]. The technique of intracanal *in vitro* biofilm formation may vary depending on the period of formation. In the present study, biofilm formation for 30 days produced a robust biofilm, resembling the actual clinical environment. However, other studies have allotted only three days since preliminary studies have suggested that, after just 72 h, the biofilm is uniformly present on the root canal surface [22].

One of the objectives of irrigation and instrumentation, regardless of the technique employed, is the elimination of microorganisms inside the root canal system. The use of adequate irrigating solutions is essential for achieving this objective. Traditionally, NaOCl is the most commonly used irrigant in endodontics; nevertheless, the ideal concentration to employ remains under debate by different authors [23,24]. The NaOCl concentration used in this study was 5.25% since it is the most reported choice for use in the treatment of necrotic root canals. Radcliffe et al. [25] evaluated *in vitro* the effectiveness of NaOCl at 0.5%, 1.0%, 2.5%, and 5.25% with different contact times, concluding that *E. faecalis* proved to be significantly more resistant to NaOCl than the other solutions tested. These authors ultimately recommended a concentration of 0.5%, with an exposure time of 30 min, to reduce viable *E. faecalis* counts close to zero, while 10 min was needed for the 1.0% concentration, 5 min was required for the 2.5% concentration, and 2 min were needed for the 5.25% concentration [26]. Thus, the present study is consistent with these authors regarding the resistance of *E. faecalis*, the microorganism of choice for the formation of biofilm. Also, this technique is effective against this and other microorganisms with a similar resistance capacity. Using NaOCl concentrations above 2.5% is justified to ensure successful treatment without the risk of recurrence [25,26]. However, more studies about this topic would confirm the HBW Ultrasonic Ring’s usefulness in endodontics, such as testing it with other irrigants and different contact periods.

Castelo-Baz et al. [27] published a study comparing the working length of root canals in extracted teeth using the CUI, PUI, and PAP approaches. These authors did not observe any significant difference between the CUI and PUI techniques; however, the total penetration of the lateral canals was 0% in the PAP, 30% in the PUI, and 67% in the CUI groups. These authors concluded that CUI improves the disinfection process in both lateral and accessory canals, due to its deeper penetration capacity, which enhances the chance of success following the root canal treatment. This study has some important limitations since the HBW Ultrasonic Ring is a novel device that has not been assessed previously in experimental assays, and the number of samples employed was small. It would be interesting for future studies to include larger size samples of extracted teeth with different types of curvatures or assess the use of the HBW Ultrasonic Ring directly on patients in the clinical setting.

To conclude, the described experiments supported the evaluation of the properties of the HBW Ultrasonic Ring, a device that functions based on the principle of CUI. The HBW Ultrasonic Ring exhibited an increased disinfection capacity and deeper penetration in the interior of the root canal. Further, its ramifications about the other studied irrigating systems are of interest in that it showed a more extensive cleaning range as compared with both PUI and conventional irrigation. Also, this dual device supports the recuperation of fractured instruments, so it would be interesting to evaluate this property in future studies.

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**Conflict of interest**

The authors deny any conflict of interest exist related to this study.

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