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Comparison of different final irrigant agitation techniques for the removal of *Enterococcus faecalis* biofilms from root canals: an in vitro study

Gunec, Gurkan; Haznedaroglu, Faruk; Kulekci, Guven; Topcuoglu, Nursen Bakir; Özcan, Mutlu

Abstract: Objective: The aim of this in vitro study was to compare the effectiveness of different final irrigant agitation techniques in the removal of *Enterococcus faecalis* biofilms from root canals. Material and Methods: In total, the root canals of 85 extracted single-rooted human maxillary incisors teeth were prepared using the Revo-S system to a 40/06 size. The apical foramen of each tooth was sealed by light-cured resin composite material to obstruct bacterial leakage. The specimens were sterilized in an autoclave at 121°C for 15 min and stored until further use. All teeth except five (negative control group) were inoculated with *Enterococcus faecalis* and incubated in a CO₂ chamber at 37°C for 7 days; the trypticase soy broth was changed every 2 days. For the determination of possible biofilm formation, five of the 80 teeth were randomly selected as a positive control group; one tooth of positive control group was analysed for biofilm development by scanning electron microscope (SEM) and these teeth received no final irrigant agitation procedure. Then, the remaining 75 teeth were randomly divided into five test groups (n=15 each) and were sequentially irrigated with 5% sodium hypochlorite (NaOCl), 17% ethylenediaminetetraacetic acid and 5% NaOCl. Following each irrigant application, different final irrigant agitation techniques were introduced for 60 s (3×20-s sessions). Group 1 received manual-dynamic agitation, group 2 received passive ultrasonic agitation (PUI), group 3 received EndoActivator agitation, group 4 received photon-initiated photoacoustic streaming (PIPS) with the Er:YAG laser and group 5 received conventional syringe irrigation. Colony-forming units (CFUs) were counted in samples from the positive control and test groups. Data were analysed using Kruskal-Wallis and post-hoc Mann-Whitney U multiple comparison tests. Results: *E. faecalis* elimination was significantly better in the experimental groups than in the positive control groups ($p < 0.001$). Manual-dynamic agitation and conventional syringe irrigation, with no significant differences between the two groups. Conclusion: Essentially, CFU reduction was significantly greater in the PUI, EndoActivator and PIPS groups than in the manual-dynamic agitation and conventional syringe irrigation groups ($p < 0.001$), with no significant differences among the former three groups.

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Comparison of different final irrigant agitation techniques for the removal of *Enterococcus faecalis* biofilms from root canals: an in vitro study

Comparaç o de diferentes t cnicas finais de agitaç o de irrigantes para a remoç o de biofilmes de *Enterococcus faecalis* de canais radiculares: estudo in vitro

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ABSTRACT

Objective: The aim of this in vitro study was to compare the effectiveness of different final irrigant agitation techniques in the removal of *Enterococcus faecalis* biofilms from root canals. **Material and Methods:** In total, the root canals of 85 extracted single-rooted human maxillary incisors teeth were prepared using the Revo-S system to a 40/06 size. The apical foramen of each tooth was sealed by photopolymerized resin composite material to obstruct bacterial leakage. The specimens were sterilized in an autoclave at 121°C for 15 min and stored until further use. All teeth except five (negative control group) were inoculated with *Enterococcus faecalis* and incubated in a CO₂ chamber at 37°C for 7 days; the trypticase soy broth was changed every 2 days. To determine of possible biofilm formation, five of the 80 teeth were randomly selected as a positive control group; one tooth of positive control group was analysed for biofilm development by scanning electron microscope (SEM) and these teeth received no final irrigant agitation procedure. Then, the remaining 75 teeth were randomly divided into five test groups (n=15 each) and were sequentially irrigated with 5% sodium hypochlorite (NaOCl), 17% ethylenediaminetetraacetic acid and 5% NaOCl. Following each irrigant application, different final irrigant agitation techniques were introduced for 60 s (3×20-s sessions). Group 1 received manual-dynamic agitation, group 2 received passive ultrasonic agitation (PUI), group 3 received EndoActivator agitation, group 4 received photon-

RESUMO

Objetivo: O objetivo deste estudo in vitro foi comparar a efic cia de diferentes t cnicas finais de agitaç o de irrigantes na remoç o de biofilmes de *Enterococcus faecalis* de canais radiculares. **Material e M todos:** No total, os canais radiculares de 85 dentes incisivos superiores unirradiculares humanos extra dos foram preparados usando o sistema Revo-S para um tamanho 40/06. O forame apical de cada dente foi selado por material comp sito de resina fotopolimeriz vel para obstruir o vazamento bacteriano. Os esp cimes foram esterilizados em autoclave a 121 ° C por 15 min e armazenados at  uso posterior. Todos os dentes, exceto cinco (grupo controle negativo), foram inoculados com *Enterococcus faecalis* e incubados em c mara de CO₂ a 37 ° C por 7 dias; o caldo de soja tripticase foi trocado a cada 2 dias. Para a determinaç o da poss vel formaç o de biofilme, cinco dos 80 dentes foram selecionados aleatoriamente como grupo controle positivo; um dos dentes do grupo controle positivo foi analisado para o desenvolvimento do biofilme por microscopia eletr nica de varredura (MEV) e estes dentes n o receberam nenhum procedimento final de agitaç o irrigante. Em seguida, os 75 dentes restantes foram aleatoriamente divididos em cinco grupos teste (n = 15 cada) e irrigados sequencialmente com hipoclorito de s dio a 5% (NaOCl),  cido etilenodiaminotetrac tico a 17% e NaOCl a 5%. Ap s cada aplicaç o de irrigantes, diferentes t cnicas finais de agitaç o foram introduzidas por 60 s (3 x 20 s sess es). Grupo 1 recebeu agitaç o manual-din mica, grupo 2 recebeu agitaç o ultra-s nica passiva (PUI),

initiated photoacoustic streaming (PIPS) with the Er:YAG laser and group 5 received conventional syringe irrigation. Colony-forming units (CFUs) were counted in samples from the positive control and test groups. Data were analysed using Kruskal–Wallis and post-hoc Mann–Whitney U multiple comparison tests. **Results:** *E. faecalis* elimination was significantly better in the experimental groups than in the positive control groups ($p < 0.001$). Manual–dynamic agitation and conventional syringe irrigation, showed no significant differences between the two groups. **Conclusion:** Essentially, CFU reduction was significantly greater in the PUI, EndoActivator and PIPS groups than in the manual–dynamic agitation and conventional syringe irrigation groups ($p < 0.001$), with no significant differences among the former three groups.

KEYWORDS

Enterococcus faecalis; Irrigant agitation; Irrigant solutions; Photon-initiated photoacoustic streaming; Passive ultrasonic irrigation.

grupo 3 recebeu agitação EndoActivator, grupo 4 recebeu fotoacústica iniciada por fóton (PIPS) com o laser Er: YAG e grupo 5 recebeu irrigação convencional com seringa. As unidades formadoras de colônia (CFUs) foram contadas em amostras dos grupos controle positivo e teste. Os dados foram analisados utilizando testes de comparação múltipla Kruskal-Wallis e post-hoc Mann-Whitney U. **Resultados:** A eliminação de *E. faecalis* foi significativamente melhor nos grupos experimentais do que nos grupos de controle positivo ($p < 0,001$). Agitação manual-dinâmica e irrigação com seringa convencional, sem diferenças significativas entre os dois grupos. **Conclusão:** Essencialmente, a redução de UFC foi significativamente maior nos grupos PUI, EndoActivator e PIPS do que nos grupos de agitação manual-dinâmica e de seringa convencional ($p < 0,001$), sem diferenças significativas entre os três grupos anteriores

PALAVRAS-CHAVE

Enterococcus faecalis; Agitação por irrigação; Soluções de irrigação; Fluxo fotoacústico iniciado por fótons; Irrigação ultrassônica passiva.

INTRODUCTION

Endodontal diseases are basically caused by microorganisms present in the root canals of affected teeth. [1] The success of endodontic treatment largely depends on the removal of these microorganisms and their by-products from infected root canals. [2] Cleaning and shaping procedures aid in the removal of these microorganisms and facilitate filling or obturation of the root canals, a procedure known as chemomechanical preparation. This procedure, in addition to mechanical cleaning using endodontic tools, involves the use of irrigation solutions that provide antibacterial and solvent effects. [3-5]

Previous studies demonstrated the presence of residual bacteria on root canal walls after cleaning and shaping, which was believed to be a result of a complex root canal anatomy that does not allow contact between cleaning tools and certain areas of the canal walls. [6,7]

Furthermore, several recent studies have shown that *Enterococcus faecalis*, a gram-positive facultative anaerobe, plays the most important role in secondary and persistent root canal and periapical infections. [8-11] One of the reasons is the ability of this bacterium to form a biofilm, which is a highly sheltered structure containing other microorganisms. The formation of bacterial clusters on the walls of infected root canals—in other words—biofilm formation, has been reported, although detailed descriptions were not provided. [11,12] It is therefore necessary to completely eliminate this bacterium from infected root canals for successful endodontic treatment, [13] and irrigation during chemomechanical preparation and mechanical cleaning has been reported to play an important role in this bacterial elimination procedure. [14,15]

Mechanical agitation has been found to increase the efficacy of irrigation solutions.

[16] Different techniques are used for irrigant agitation and are broadly classified as manual (syringe irrigation with needles or cannulae, Endobrush agitation and manual–dynamic agitation) and machine-assisted (rotary brush agitation, continuous irrigation during rotary instrumentation and sonic and ultrasonic agitation) techniques. [17] Among these, passive ultrasonic irrigation (PUI) is considered to be the most effective. [18-20]

Furthermore, many researchers reported that PUI enhances the organic tissue solvent and anti-bacterial properties of sodium hypochlorite (NaOCl) irrigation solution. [17-19]

Following its application in the field of medicine, laser technology has found widespread use in the field of dentistry. [21-23] Its main endodontic applications include root canal shaping, [24] root canal disinfection [25] and endodontic surgery. [26,27] The company Fotona recently introduced a photon-initiated photoacoustic streaming (PIPS) device and claim that this device particularly increases the efficacy of root canal irrigation when mounted on an Er:YAG laser. [28,29]

The aim of this in vitro study was to compare the effectiveness of PUI, EndoActivator agitation, PIPS and manual–dynamic agitation (gutta percha; GP) used during final irrigation with NaOCl and ethylenediaminetetraacetic acid (EDTA) in the removal of *E. Faecalis* biofilms from root canals.

METHODS AND MATERIALS

This in vitro study was approved by the institutional ethics committee of Istanbul University Faculty of Medicine, Turkey (Protocol number 03/366). Extracted for unknown reasons eighty-five single-rooted human maxillary incisors teeth were selected for this study. Immediately after extraction, adherent soft tissue was removed using a scalpel and any adherent hard tissue such as calculus was removed using an ultrasonic scaler. Then, the specimens were stored at 4°C distilled water until they were used.

The height of the teeth was standardized to 12 mm by cutting from the point below the cemento-enamel junction using a No. 0.012 diamond fissure bur (Maillefer, SA CH-1338, Ballaigues, Switzerland). The working length was calculated as the length of a #10 K-file (Maillefer, SA CH-1338, Ballaigues, Switzerland) inserted with its tip at the apical foramen, minus 1 mm.

The coronal 3 mm of all root canals was shaped using an endomotor (X-Smart, Dentsply Maillefer, USA) (1.5 Ncm and 300 rpm) attached with Endoflare (Micro-Mega, Besancon, France). Before the shaping procedure, the root canals were filled with 2.5% NaOCl (Wizard, Rehber Chemicals, Istanbul, Turkey) and prepared with 1–2-mm vertical strokes using Endoflare. Subsequently, the root canals were irrigated with 2 mL of 2.5% NaOCl using a 30-gauge needle (Endo-Eze, Ultradent, South Jordan, UT) with a tip having lateral holes. The same protocol was followed with every tool used after this step. After coronal shaping, the crown-down method was used for canal shaping with SC1 (25/06), SC2 (25/04), SU (25/06), AS30 (30/06), AS35 (35/06) and AS40 (40/06), which are files used with the standard Revo-S (Micro Mega, France) Ni-Ti rotary system.

Once canal shaping was complete, 5 mL of 17% EDTA (Wizard, Rehber Chemicals, Istanbul, Turkey) was injected into the canals for 1 min using a 30-gauge needle with a tip having lateral holes for smear layer removal. Then, EDTA was neutralized by irrigation with 5 mL of 2.5% NaOCl, 5 mL of distilled water and, finally, 5 mL of 10% sodium thiosulfate. The apical foramen was subsequently sealed using flowable composite resin (3M Esthete X Flow, Dentsply Maillefer, USA).

The prepared samples were embedded in acrylic resin (Imicryl 0-80, powder-liquid acrylic, Konya, Turkey) for an easy grip, with three-fourth of the root in the block and one-fourth outside. All prepared samples were sterilized in a B-type autoclave (Lina, WH, Austria) at 121°C and 1 atm pressure for 15 min.

Five of the total 85 teeth included in our study were randomly selected as a negative control group. Their access cavities were sealed with a temporary filling material, and the teeth were kept in an oven at 37°C for 7 days under aerobic conditions. For the determination of any growth in these control samples, the temporary filling material was removed under sterile conditions on the second, fourth and sixth days and fresh trypticase soy broth (TSB) was added to the root canals. No additional procedures were applied for these samples. The remaining 80 root canals were incubated with an *E. faecalis* (ATCC 29212) strain obtained from the American Type Culture Collection. The bacteria were first inoculated in trypticase soy agar (TSA) and incubated at 37°C for 24 h under aerobic conditions. Growing bacterial colonies were collected and inoculated in TSB medium, and 5 µL of the bacterial suspension (8×10^9 colony-forming unit [CFUs]/mL) was applied to the mechanically widened root canals using sterile micropipettes and spread using sterile ISO #30 K-files. The access cavities were closed with a temporary filling material (Coltosol®, Coltene, Whaleden). All samples were then placed in an oven at 37°C for 7 days in a humid environment under aerobic conditions. The temporary filling material was removed on the second, fourth and sixth days and fresh TSB was added to the root canals. In order to determine the possible biofilm formation, five of the 80 teeth were randomly selected as a positive control group and sent to the Oral Microbiology Laboratory at Istanbul University Faculty of Dentistry and the Nanobiotechnology Laboratory at Yeditepe University Faculty of Engineer and Architect. The colonies in TSA were counted and CFUs per millilitre were calculated (263.3×10^4 CFUs/mL) in Oral microbiology laboratory and one tooth of positive control group was analysed for biofilm development by scanning electron microscope (SEM) (Zeiss, Jena, Germany) in Nanobiotechnology laboratory.

The remaining 75 teeth were randomly divided into five experimental groups (n = 15 each). These teeth were irrigated with 5 mL of

5% NaOCl, 5 mL of 17% EDTA and, finally, 5 mL of 5% NaOCl using a 30-gauge needle with a tip having lateral holes. Different agitation techniques as described below were applied after each irrigant application, following which the root canals were irrigated with 5 mL of distilled water and 5 mL of 10% sodium thiosulfate. Any residual irrigant was removed.

Group 1 included 15 teeth that received manual–dynamic agitation with Gutta Percha (GP) (Gapadent, Tian Jin City, China). After using each solution, a #25/06 GP point was inserted up to 2 mm short of the working length and 100 1–2-mm vertical strokes from the apical to the coronal ends were applied for 60s.

Group 2 included 15 teeth that received PUI. In order to maintain PUI during irrigation, the irrigants were used in three volumes of 1 mL, 1 mL, and 3 mL. PUI was performed using an ultrasonic device (Electro Medical Systems, EMS, Switzerland) with a #30 probe mounted on its tip and adjusted to a power of 6/10. The irrigation probe was placed 2 mm short of the working length, activated and applied using apicocoronal movements. This procedure was applied for 20 s with each irrigant volume, with a 5-s interval between applications, leading to total activation duration of 60 s. The procedure was repeated three times.

Group 3 included 15 teeth that received EndoActivator (Dentsply Tulsa Dental, Tulsa, OK, USA) agitation. In order to maintain sonic agitation during irrigation, the irrigants were used in three volumes of 1 mL, 1 mL, and 3 mL. Sonic agitation was performed using an EndoActivator with a #30 probe mounted on its tip and adjusted to a power of 10000 cpm. The irrigation probe was inserted 2 mm short of the working length, activated and applied using apicocoronal movements. This procedure was applied for 20 s with each irrigant volume, with a 5-s interval between applications, leading to a total activation duration of 60 s. The procedure was repeated three times.

Group 4 included 15 teeth that received agitation with PIPS. In order to maintain laser

agitation during irrigation, the irrigants were used in three volumes of 1 mL, 1 mL, and 3 mL. Laser agitation was performed using an Er:YAG laser (Fidelis; Fotona, Slovenia) with a 12-mm-long conical probe having a 400- μm diameter mounted on its tip and adjusted to 15 Hz, 0.45 W, a 30-mJ power and a 50- μs pulse. The irrigation probe was placed 2 mm short of the working length, activated and applied using apicoronal movements. This procedure was applied for 20 s with each irrigant volume, with a 5-s interval between applications, leading to a total activation duration of 60 s. The procedure was repeated three times.

Group 5 included 15 teeth that received conventional syringe irrigation with no agitation. A 30-gauge needle tip (Endo-Eze, Ultradent, South Jordan, UT) having lateral holes was placed without pressure up to 2mm short of the apex and applied using apicoronal movements for 60 s.

Next, the root canal wall in each sample was cleaned using a #30 H-file (Maillefer, SA CH-1338, Ballaigues, Switzerland), following which sterile #30 paper points were inserted for 15 s. The samples thus obtained were assessed for the number of CFUs in TSA.

All statistical analyses were performed using SPSS Statistics software version 22.0 (IBM SPSS, Turkey). The Mann–Whitney U test was used for pair-wise comparisons of parameters between the experimental and positive control groups, while Kruskal–Wallis and post-hoc Mann–Whitney U multiple comparison tests were used for comparing the residual bacterial counts among the experimental groups. A p-value of <0.05 was considered statistically significant.

RESULTS

No growth was determined in the negative control samples. *E. faecalis* elimination was detected at various levels in all experimental groups and was significantly better than that in the positive control samples ($p < 0.01$) (Table 1).

All agitation techniques significantly decreased the number of bacterial cells in the infected root canals ($p < 0.001$). The bacterial count in group 1 was significantly higher than that in groups 2, 3 and 4 ($p < 0.01$), while that in group 5 was significantly higher than that in groups 2 ($p = 0.011$), 3 ($p = 0.006$) and 4 ($p = 0.011$) (Figure 1 and 2).

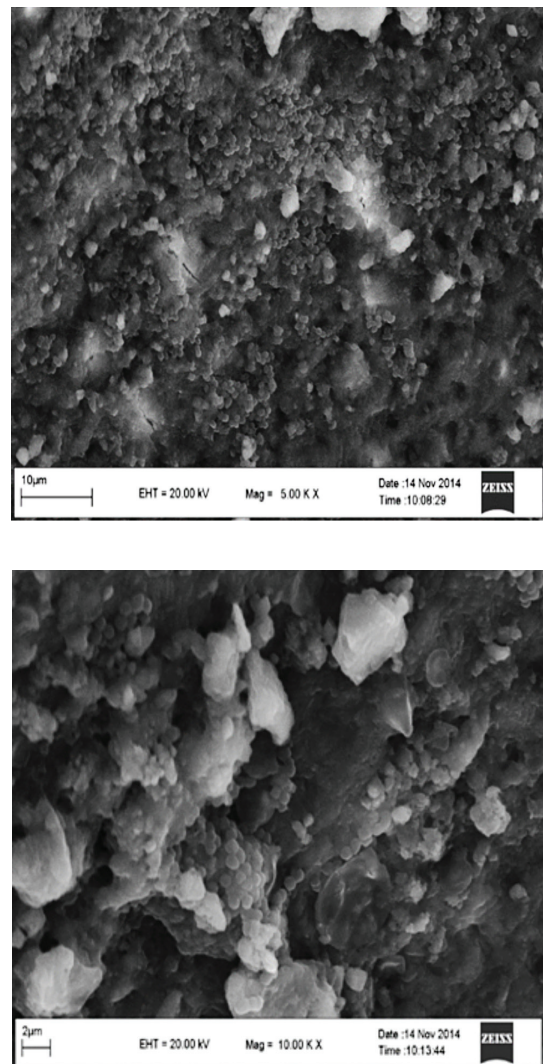


Figure 1 - The development *E. faecalis* biofilm of root canal walls in positive control group; original magnification, 5000x (A) and 10000x (B).

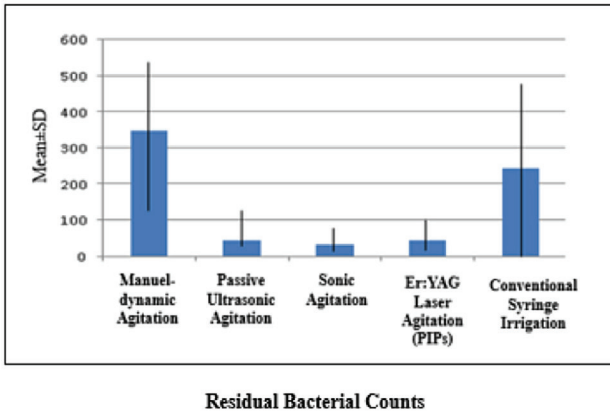


Figure 2 - Means and Standard Deviations of the residual bacterial count values after using the different final irrigation agitation technique.

Furthermore, there were no significant differences between groups 1 and 5 ($p = 0.108$), groups 2 and 3 ($p = 0.937$), groups 2 and 4 ($p = 0.476$) and groups 3 and 4 ($p > 0.05$).

Table 1 - Mean and Standard Deviation (SD) values and results of comparison between bacterial count scores for experimental groups

Experimental Groups	Mean±SD	Median	P value*
Positive Control Group	263333,33±198494,33	200000	
Manuel-dynamic Agitation	348.24±201	340	<0.001
Passive Ultrasonic Agitation	42.5±74,43	20	
Sonic Agitation	31.25±41,29	20	
Er:YAG Laser Agitation	44.71±54,10	20	
Conventional Syringe Irrigation	244±234	200	

Kruskal Wallis Test * $p < 0.01$

DISCUSSION

In this study, we compared the effectiveness of four different irrigant agitation techniques used with NaOCl and EDTA in the removal of *E. faecalis* biofilms from root canals: manual–dynamic agitation, PUI, EndoActivator agitation and PIPS. We found that all agitation techniques significantly decreased the number

of bacteria in the infected root canals, with manual–dynamic agitation and conventional syringe irrigation with no agitation showing the least favourable results.

Gram-positive facultative anaerobes are frequently observed in cultures of samples obtained from root canals with failed endodontic treatment. [8,30,31] We selected *E. faecalis* for our study because its elimination is extremely challenging, it penetrates deep into the dental tissues, it can survive even in negative conditions and it is resistant to intracanal medicaments. [11,32,33]

Irrigation performed with mechanical cleaning and shaping of root canals constitutes one of the most important stages of root canal treatment. [6] However, conventional chemomechanical preparation methods appear adequate for the total elimination of microorganisms from root canals. [34-36] The anti-bacterial effects of current irrigation solutions have been reported to be enhanced by increasing the concentration, temperature and amount of solution and by agitation. [37-39,40] Several clinicians reported that irrigant agitation increases the anti-bacterial efficacy and minimizes the rate of treatment failure and persistent infection. [29,41,43]

Previous studies reported that the vapour lock effect of irrigants did not allow the solution to reach the apical region of canals, inhibiting thorough chemomechanical preparation. [44] Manual-dynamic agitation with GP applied exclusively with conventional syringe irrigation was found to eliminate the vapour lock effect through hydrodynamic effects. [45] Furthermore, EndoActivator, which is a sonic device, was found to increase the efficacy of disinfection through oscillations and vibrations, [15,46] while PUIs exhibited similar effects through acoustic currents and cavitation formed by the ultrasonic waves in irrigants. [19,47] Recent years witnessed the introduction of a laser probe mounted on the tip of an Er:YAG laser that was specifically designed for root canal irrigation; this agitation procedure is known

as PIPS. [28,29,48,49] Different irrigation solutions can be agitated using PIPS, and several studies have shown that this method provides effective disinfection. [50,51] PIPS uses the conventional laser dose and energy at low levels and minimizes the toxic effects of lasers on healthy tissues. [29,51,52]

Various methods have been used for the evaluation of the different irrigant agitation methods, including scanning electron microscopy (SEM), [53,54] CFU counting [33,55] and molecular polymerase chain reaction (PCR). [56] SEM and PCR are very expensive techniques with average results; therefore, we used CFU counting in our study, which is also preferred by several researchers for its increased practicality and precision and easy implementation. [28,48,55]

Although the agitation techniques used in our study significantly decreased the residual bacterial counts in the root canals, none were able to eliminate the bacteria completely. This finding is consistent with those of previous studies, which demonstrated that root canals cannot be completely cleaned of microorganisms, regardless of the method of chemomechanical preparation. [5,35,57,58] In our study, there were no significant differences in bacterial counts among PUI, EndoActivator agitation and PIPS, whereas significant differences were observed between these methods and manual-dynamic agitation and conventional syringe irrigation. Nevertheless, the four agitation methods used in this study were $\geq 98\%$ successful in the elimination of *E. faecalis* biofilms from root canals, a finding compatible with that in previous studies reporting that a good irrigation technique can significantly decrease the bacterial count in root canals. [43,54,59-61]

CONCLUSIONS

Within the limitations of this in vitro study, the results suggest that PUI, EndoActivator and PIPS are considerably effective in the removal of *E. faecalis* biofilms from root canals. Furthermore, our results suggest that conventional irrigation

methods and the methods discussed in this study show nearly the same effectiveness in the elimination of bacterial biofilms from root canals, and we believe that the irrigation solution itself may be an important factor contributing to success. However, this study was based on an artificially induced biofilm under in vitro conditions, and biofilms formed in actual intraoral conditions may show different characteristics. Further similar studies conducted in vivo are necessary to clarify our findings and provide more realistic results.

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Clinical Relevance

Endodontic treatment failures are commonly microbiological problems and crown fractures. If we can remove the biofilms from root canals, the microbiological goals of endodontic treatment will be successful. Different final agitation techniques must clean the dirty canal walls perfectly so that clinical use of these four agitation techniques would practically, successfully and efficiently to remove the biofilms.

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