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Comparative Evaluation of Mechanical Biofilm Removal by Different Root Canal Instrumentation Systems Using Confocal Laser Microscopy

Evaluación Comparativa de la Eliminación Mecánica de Biopelícula Mediante Diferentes Sistemas de Instrumentación de Conductos Radiculares Utilizando Microscopía Láser Confocal

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ABSTRACT: The aim of this study was to evaluate the capacity of three different file systems to mechanically remove a mature multispecies anaerobic biofilm. Roots of fifteen extracted molars were used to develop a multispecies anaerobic biofilm. Root canals were prepared at working length using the following instrumentation systems: Reciproc® blue, WaveOne® Gold, XP-endo® Shaper and Readysteel™ K-Flexofile. Three samples were left without any preparation (control group). Bacterial viability was determined using a LIVE/DEAD® Kit. Two- and three-dimensional images of the apical third were obtained using confocal laser microscopy and further analyzed with the IMARIS software. The relative biofilm removal after instrumentation was determined. Data were analyzed with a Kruskal-Wallis non-parametric test and pairwise comparisons ($p < 0.05$). The 2D analysis revealed a significantly higher bacterial reduction for Reciproc® blue treated roots compared to manual instrumentation. However, no differences were observed between mechanized systems. Manual instrumentation (Readysteel™ K-Flexofile) displayed the lowest bacterial reduction among all systems. Although none of the instrumentation systems used were able to completely remove the bacterial biofilm from the apical third of the root canals, the results tend to point that the Reciproc® blue system allows a greater reduction of residual bacteria from the root canal surface.

KEY WORDS: Biofilms, Endodontics, root canal preparation, confocal microscopy.

INTRODUCTION

The goal of root canal treatment is therapeutic success, regardless of clinical conditions. The execution of the endodontic triad, which consists of chemomechanical preparation, microbial control, and a proper filling of the root canal system, is the foundation for this success (Estrela *et al*., 2008; Tanalp & Güngör, 2014). One of the most important steps in treatment is chemomechanical preparation. In addition to shaping the root canal, this procedure also involves the removal of vital and necrotic tissues, as well as infected root dentine (Pérez *et al*,. 2020).

Bacteria and their products are considered the main etiologic agents of pulp necrosis and apical lesions (Siqueira *et al*. 2018). The main cause of failure in the root canal treatment is the persistence of microorganisms after endodontic therapy or reinfection of the root canal system due to incorrect coronary sealing (Wu *et al*., 2006; Schirrmeister *et al*., 2009), microleakage, failure in chemomechanical preparation, and failure in the limit and quality of the root filling (Gulabivala & Ng, 2023; Siqueira & Roccas, 2008). Studies investigating the intraradicular microbiota associated with treatment failures have reportedthe presence of microorganisms in 35% -100% of cases (Schirrmeister *et al*., 2009) and there is a high interindividual variability in the apical microbiome composition (Siqueira *et al*., 2024).

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The advancement of technology has led to the manufacture of mechanized nickel-titanium instruments for root canal preparation. Modifications such as inactive tips, different cross sections and taper, improve work safety, reduce instrument fractures, reduce working time, and create a better taper of the root canal (Fernandes *et al*., 2020). Although these objectives have been demonstrated in several studies, few have evaluated their ability to reduce the number of microorganisms within the root canals in an *ex vivo* multispecies model (Nakamura *et al*., 2013; de Oliveira *et al*., 2015; Zavattini *et al*., 2020). Most studies of endodontic biofilms have been performed using models with monospecies bacterial cultures, grown on membranes, glass or plastic, with continuous or frequent nutrient supply, ranging from a few hours to a few days of life (Oz *et al*., 2023). These models do not adequately reflect the complexity of the root canal system anatomy, nor do they simulate the clinical situation. When reviewing the literature, there are no reports that have compared the effects of mechanized instrumentation systems between Reciproc® blue (RB), WaveOne® Gold (WOG) and XP-endo® Shaper (XP-S) in reducing bacterial populations present in infected root canals. Therefore, it is important to develop ex vivo models of multispecies anaerobic endodontic biofilms that resemble *in vivo* endodontic biofilms to properly study the cleaning and disinfection of the root canal system.

Confocal Laser Scanning Microscopy (CLSM) has become a very effective method to study the structure of biofilms (Sánchez-Sanhueza *et al*., 2018; Jerez-Olate *et al*., 2022). The use of fluorescent markers allows for the observation of specific cells or even certain components of the extracellular matrix. Live and dead bacteria can be visualized, and biofilm thickness can be quantified by its size or fluorescence intensity using a three-dimensional biomass reconstruction (Neelakantan *et al*., 2017).

The aim of this study was to evaluate the capacity of three different file systems to mechanically remove a mature multispecies anaerobic biofilm. Roots of fifteen extracted molars were used to develop a multispecies anaerobic biofilm.

MATERIAL AND METHOD

The manuscript of this ex-vivo study has been written according to Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines (Nagendrababu *et al*., 2021). Protocol authorized by the Bioethics Committee C.I.Y.B N°04/18 that incorporates the informed consent signed by the patient for the use of his extracted tooth in this study.

Sample size. Eighteen human molars extracted were collected, maintained in 0.9 % sterile saline solution since extraction. Roots were used, palatal roots of upper molars and distal roots of lower molars were specifically selected. Only roots with straight or moderate curvatures were chosen, which were determined according to Schneider's classification criteria (Schneider, 1971); for that, periapical radiographs were taken with plates of activated phosphorus size 2, processed in the VistaScan software (Dürr Dental®, Stutgart, Germany) and analysed with DBSWIN software. Any morphological anomality, calcified canals, clinical impermeable, and/or roots with incomplete apical closure were excluded for the study.

Sample preparation. The selected roots were standardized to a length of 9 mm. The working length (WL) was established by subtracting 1 mm from the actual root length, determined by visualizing the tip of a size #10 K-file at the apical foramen. The samples were randomly divided into five groups (n = 3). Group 1: root canal preparation was performed using 40R Reciproc® blue (VDW GmbH, Munich, Germany). Group 2: root canal preparation was performed using WaveOne® Gold medium (Dentsply Sirona, Ballaigues, Switzerland). Group 3: root canal preparation was performed using XP-endo® Shaper (FKG Dentaire, La Chaux-de- Fonds, Switzerland). Group 4, positive control, was manually operated with Readysteel™ K-Flexofile n° 40 (Dentsply Sirona, Ballaigues, Switzerland) files. Group 5, negative control, 3 specimens were inoculated with the strains, but were not instrumented. Three specimens as sterilize control were used to standardize the analysis of the samples, to be able to measure the natural fluorescence emitted by the dentin and thus be able to eliminate it from the final analysis of the images, thus avoiding obtaining distorted results.

Sample assembly. The roots were placed on eppendorf tubes with transparent self-curing acrylic, obtaining a representative disc 1 mm thick from the middle area of the apical third. The discs were obtained using a manual cutting system with immobilized ends. After sectioning, the samples underwent two cycles of 10 % EDTA ultrasonic

washing for 5 min (Transsonic 460 Ultrasonic Cleaning Units) to remove dentin debris from the main canal. The samples were then rinsed in 0.9 % sterile saline for 10 min. Finally, the sectioned and disassembled samples were autoclaved at 121°C for 30 min.

Creation of the multi-species anaerobic artificial biofilm model and sample inoculation. The study used strains of *Porhyromonas gingivalis* ATCC 33277, *Prevotella melaninogenica* ATCC 25845, *Enterococcus faecalis* ATCC 29212, *Streptococcus mutans* ATCC 18556, plus *Propionibacterium acnes* (clinical strain UC A1), *Enterococcus faecalis* (clinical strain UC C1) and *Streptococcus constellatus* (clinical strain UC D2).

Each bacterial strain was independently cultured on Fastidious Anaerobe agar plates (Laboratory M, Bury, United Kingdom) with 5 % defibrinated sheep blood, containing 1 ml/L of hemin and 1 ml/L of vitamin K1, in anaerobic chamber at 37 °C for 8 days. A representative colony of each strain was inoculated into 5 mL of Fastidious Anaerobe broth (Laboratory M, Bury, United Kingdom) and incubated in an anaerobic chamber at 37 °C for 8 days. The turbidity of each strain was adjusted using an Oxoid turbidimeter (Fisher Scientific Company, Ottawa, Canada) to reach a concentration equivalent to 0.5 McFarland (1.5 X 10^8 CFU/mL). Subsequently, 5 mL of each strain was combined in an Erlenmeyer flask, which was constantly stirred using a Vortex Super Mixer shaker (Lab-line instruments INC, Melrose Park, USA), to achieve homogeneity. The sectioned and disassembled samples were placed in a 24-well plate. Each well was filled with 1 ml of multispecies bacterial inoculum and 1 ml of Fastidious Anaerobe broth (Laboratory M, Bury, United Kingdom) to completely cover the sample. The samples were incubated in an anaerobic chamber for 21 days until a mature biofilm formed. The culture medium was refreshed at 7 and 14 days. At 21 days, the samples were gently immersed in 0.9 % sterile saline to remove loosely adhered bacteria before being reassembled in the Eppendorf tube.

Sample instrumentation. Mechanized and manual instrumentation was performed by an expert operator. During the instrumentation process, a total of 9 mL of 0.9 % sterile saline was used as an irrigant, delivered with monoject syringes (Kendall, USA.) equipped with a 27 G needle. The negative control group was only rinsed with 0.9 % sterile saline without any instrumentation being performed.

Analysis of the sample with Confocal Microscopy. To evaluate the effectiveness of the different mechanized instrumentation systems, the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen, Carlsbad, CA) was prepared according to the manufacturer's instructions. Immediately after canal preparation, the samples were stained with 500 µl of the reagent and incubated in the dark for 10 minutes using aluminum foil (Alusa Foil, Aluhome, Chile). The samples were then observed with a confocal laser scanning microscope (LSM 780, ZEISS, Germany), and the images obtained were analysed with IMARIS software, version 7.5.2, Measurement Pro module (Bitplane, USA). An argon laser was used for illumination, with an excitation wavelength of 488 nm and an acquisition spectrum adjusted to the following parameters: green (490-560 nm) and red (560-639 nm). Biofilm thickness was quantified by the volume of total biomass (μm^3) and by the intensity of residual fluorescence post-instrumentation.

Statistical analysis. Statistical analysis was performed using InfoStat statistical software. The analysis began with descriptive statistics, using bar graphs to display means of the percentages. Subsequently, a Kruskal Wallis Non-parametric test was used to compare the central tendencies of the percentage of residual bacterial volume per system, followed by pairwise comparisons. Differences were considered significant when $p < 0.05$.

RESULTS

Two- and three-dimensional images of the apical third were obtained using confocal laser microscopy (CLSM). For the 2D analysis, a vertical central axis was selected, and a maximum intensity projection of the 3D image was calculated from that perspective. The 360 degrees of the image were pixelated every 0.25 degrees, resulting in a total of 1440 pixels on the X-axis (Fig.1). This analysis provides a proportional value of the total tooth surface area (μ^2) that is positive for fluorescence (pixels), with pixels/μ^2 as the unit of measurement. The percentage (%) indicates the amount of fluorescence per surface area, where fluorescence in this study, from a biological perspective, corresponds to the quantity of residual bacteria visible under CLSM.

Table I summarizes the central tendency measures obtained from the 2D analysis, describing the proportional value of the total tooth surface area that is positive for fluorescence (pixels/μ^2). This value

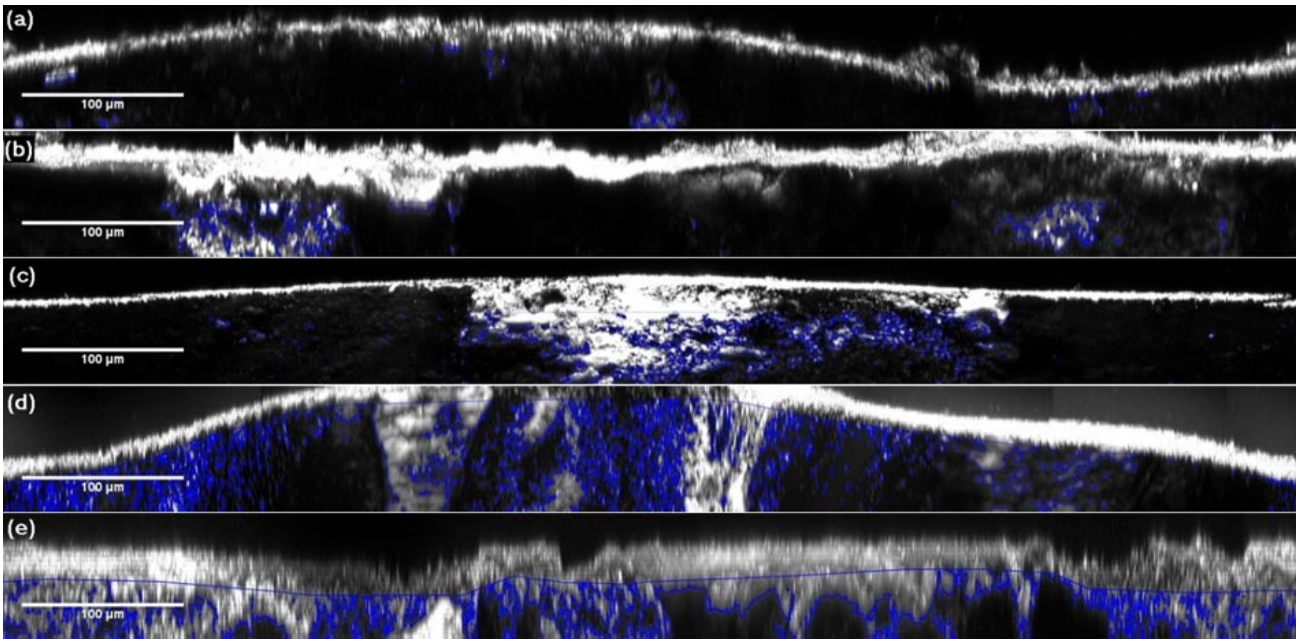

Fig. 1. Mean percentage of residual bacteria on the root canal surface after instrumentation with different file systems, based on the 2D analysis.

Table I. Descriptive statistics from the 2D analysis. Data represent the proportion of the root canal surface positive for fluorescence (pixels/µm²), corresponding to the percentage of residual bacteria remaining after instrumentation with different file systems (N = 15).

File Systems	N	*Media (%)*	*Mín.*	*Máx.*
Reciproc Blue	3	3 %	2 %	7 %
Wave One Gold	3	9 %	7 %	11 %
XP-endo Shaper	3	16 %	13 %	20 %
Manual	3	29 %	24 %	34 %
Negative Control	3	57 %	55 %	62 %

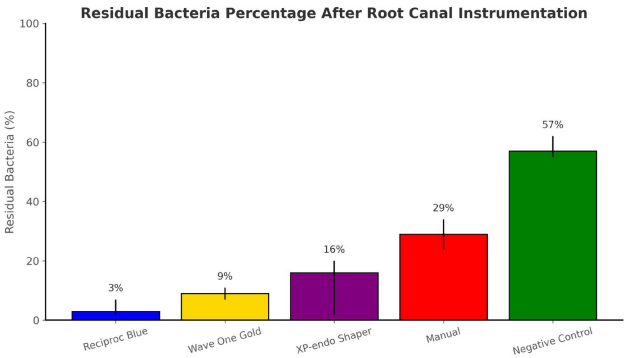

Fig. 2. Representative images from the 2D analysis, showing the proportion of the root canal surface positive for fluorescence (pixels/µm²), corresponding to residual bacterial presence after instrumentation with: (a) Reciproc® Blue, (b) WaveOne® Gold, (c) XP-endo® Shaper, (d) manual instrumentation, and (e) negative control.

translates into a percentage (%) of residual bacteria on the root canal surface after instrumentation with the different file systems. When comparing the percentage of post-instrumentation residual bacteria, it is evident that the Reciproc® blue system left the lowest number of bacterial residues (3 %) in the measured area (Fig. 2), while manual instrumentation resulted in a higher percentage of residual bacteria (29 %) in the measured area (Fig. 2). The trend suggests that the system with the greatest bacterial reduction was Reciproc® blue.

DISCUSSION

The cleaning and preparation of the apical region of the root canal are critical steps, with the last millimeters being especially crucial. At this level, the anatomy of root canals varies significantly due to the presence of curvatures, intercanal connections, and apical deltas (Mjör *et al*., 2001; Mamat & Nik Abdul Ghani, 2023). Among the different root canal configurations, the oval shape presents considerable challenges in cleaning. The extremities of these canals often cannot be included in the rounded preparation created by instrument rotation, sometimes leading to failures in endodontic treatment due to the inability to fully clean these areas (Bortoluzzi *et al*., 2015).

Palatal roots of upper molars and distal roots of lower molars were used in this study, which vary in apical diameter. According to Wu MK *et al*, who reported on median (range) root canal diameters, the palatal root has a buccolingual diameter of 0.29 mm and a mesiodistal diameter of 0.33 mm at 1 mm from the apex. At 5 mm from the apex, the buccolingual diameter increases to 0.55 mm, and its mesiodistal diameter to 0.74 mm, suggesting that the apical morphology tends to be oval. The distal root shows a buccolingual diameter of 0.46 mm and a mesiodistal diameter of 0.35 mm at 1 mm from the apex, and at 5 mm, its buccolingual diameter is 1.07 mm and the mesiodistal diameter is 0.59 mm, reflecting a pattern similar to that of the palatal root (Wu *et al*., 2000). There is significant variability in the morphology of the apical root canal system, which can include numerous accessory canals, intra-canal calcifications, and varying amounts of irregular secondary dentin (Mjör *et al*., 2001).

In this study, a saline solution was used as the irrigant because it does not affect bacterial viability nor does it disrupt the biofilm (Machareonsap *et al*., 2024). Although the literature suggests that sodium hypochlorite dissolves organic matter within the root canals, aiding in both instrumentation and disinfection (Maezono *et al*., 2024), it was not used in this study to ensure that only the mechanical action of the instruments was evaluated, without the influence of any antimicrobial chemicals, which could act as a confounding factor in the analysis of results .

Based on the analysis of two- and three-dimensional images, the observed trend indicates that Reciproc® blue is the instrumentation system that allows for the greatest reduction of residual bacteria from the root canal surface, compared to WaveOne® Gold, XP-endo® Shaper, and manual instrumentation. These results could be related to changes in root canal volume after chemomechanical preparation, where previous studies have shown that Reciproc-Blue showed higher increase in root canal volume, followed by WaveOne-Gold, while XP-EndoShaper did not significantly increase root canal volume during preparation (Caviedes-Bucheli *et al*., 2021). A previous study evaluated the effectiveness of different instrumentation systems in reducing bacterial load within oval-shaped root canals. The results indicated that Reciproc Blue was more effective than XP-Endo Shaper when only 0.9 % saline solution was used. But with the use of NaOCl, XP-Endo Shaper and Reciproc Blue presented similar effectiveness (Carvalho *et al*., 2019).

One study compared the cleaning, shaping, and disinfection capabilities of two instrument systems (Reciproc® blue and XP-endo® Shaper) using micro-computed tomography and a histobacteriological approach (Pérez *et al*., 2020). They found no significant differences between the two instruments in terms of the percentage of bacteria-free specimens in the apical section (56%). The differences with our study are likely due to methodological variations and the sample size. In our study, Reciproc® blue left 3 % residual bacteria, while XP-endo® Shaper left 16 %.

When comparing the WaveOne Gold system with the XP-endo Shaper, the WaveOne Gold system demonstrated greater bacterial reduction. These results differ from those reported by Ureyen *et al*., where instrumentation in straight and round canals of premolar teeth with the Hyflex EDM and XP-endo Shaper resulted in significantly greater bacterial reduction than WaveOne Gold (Üreyen Kaya *et al*., 2019). The differences between the results of that study and ours may be attributed to variations in the methodologies used and the canal morphologies analyzed.

Although the trend in this study indicates that XP-endo® Shaper leaves 16 % residual bacteria, this is not a discouraging result. According to the study by Versiani *et al*., XP-endo® Shaper was as effective as iRace and EdgeFile in preparing oval canals of mandibular incisors (Versiani *et al*., 2018). However, despite its extreme flexibility and its ability to contract and expand within the root canal, XP-endo® Shaper could not reach the areas that other instruments left untreated, contrary to what is suggested by the manufacturer.

A randomized clinical study compared the *in vivo* antibacterial efficacy of Reciproc Blue, XP-endo Shaper, and XP-endo Shaper associated with XP-endo Finisher systems in infected oval-shaped root canals with primary apical periodontitis. The findings indicated that there was no statistically significant difference between RB and XP-S instrumentation in terms of bacterial reduction. However, a marked bacterial reduction was observed after the use of the XP-endo Finisher instrument (Amaral *et al*., 2020).

In this study, the samples used as bacterial controls (without instrumentation) showed 57% residual bacteria in the measured area, rather than 100% as might have been expected, considering that these samples were not instrumented. This could be due to the elimination of planktonic bacteria in the root canal

through the 0.9% saline wash to which all samples were subjected after being inoculated with the multispecies bacterial biofilm. Thus, this 57% would represent the bacteria that remained post-irrigation with saline solution.

Additionally, a study of 3D images was conducted, but the results did not entirely align with those found in the 2D image analysis. This discrepancy may be primarily due to differences in the morphology of the canals at the apical level, as observed in different cross-sections. Files may struggle to reach the extremities of canals with oval or tapered shapes. In the case of XP-endo® Shaper, its high rotation speed (800 rpm) could result in a greater impact of bacterial residues and dentinal debris on the canal walls. The development of this research faced certain limitations. The sample size (n = 3) was directly affected by a lack of resources, limiting our ability to discuss the statistical trends of the data. Further studies are needed to enhance the generalizability of these findings. Additionally, it may be worth considering avoiding oval canals in future studies, although it is important to note that the apical 3 mm, which tend to be more rounded, were used in all canals.

DECLARATION OF INTERESTS.The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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RESUMEN: El objetivo de este estudio fue evaluar la capacidad de remover mecánicamente una biopelícula anaerobia madura multiespecie de tres sistemas de limas mecanizadas. Las raíces de 15 molares extraídos fueron usadas para el desarrollo de una biopelícula anaeróbica multiespecie. Los conductos radiculares fueron preparados a longitud de trabajo usando los siguientes sistemas de instrumentación: Reciproc® blue, WaveOne® Gold, XP-endo® Shaper y Readysteel™ K-Flexofile. 3 muestras se dejaron sin ninguna preparación (grupo control). La viabilidad bacteriana fue determinada usando el kit LIVE/DEAD®. Se obtuvieron imágenes en 2 y 3 dimensiones del tercio apical usando microscopía confocal láser y posteriormente analizadas con el software IMARIS. Se determinó la eliminación relativa de la biopelícula después de la instrumentación. Los datos se analizaron con la prueba no paramétrica de Kruskal-Wallis y comparaciones por pares ($p < 0,05$). El análisis en 2D reveló una reducción bacteriana significativamente mayor para las raíces intrumentadas con Reciproc® blue en comparación con la instrumentación manual. Sin embargo, no se observaron diferencias entre los sistemas mecanizados. La instrumentación manual (Readysteel™ K-Flexofile) evidenció la reducción bacteriana más baja de todos los sistemas. A pesar de que ninguno de los sistemas utilizados fue capaz de remover completamente la biopelícula bacteriana en el tercio apical de los conductos radiculares, los resultados tienden a mostrar que el sistema Reciproc® blue permite una mayor reducción de bacterias residuales de la superficie de los conductos radiculares.

PALABRAS CLAVE: Biofilms, endodoncia, preparación del canal radicular, microscopio confocal.

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