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Research Article

Evaluation of the Effect of Enlargement of the Apical One Third by Protaper Universal Rotary Files System on Elimination of *Enterococcus faecalis* and Surface Irregularities in Single Canaled Premolars (A Comparative *In-Vitro* Study)

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Abstract

Aim of the Work: The aim of the study was to evaluate the effect of enlargement of the apical one third of root canals; by using F4 in comparison to F3 Protaper files; on the amount of *Enterococcus Faecalis* and surface irregularities in the apical third.

Materials and Methods: 24 freshly extracted human single canalled premolars were obtained. The root lengths were standardized to 15 ± 1 mm. The root canals were instrumented up to #20 K-file till the apical constriction for standardization. Following root canal preparation and sterilization, all root canals were completely filled with 30µ *E. faecalis* suspension and incubated at 37°C for one week with refreshment every 48 hours. All teeth were sampled after bacterial injection and before instrumentation for examination of bacterial presence inside the canals. For the apical one third assessment, Scanning Electron Microscope "SEM" was used to examine the surface irregularities.

Results: The results concerning bacterial reduction revealed that Group II (F4), showed a significantly higher *E. faecalis* reduction than Group I (F3) (P = 0.025). However, none of the preparation sizes rendered a bacterial free root canal system. Topographic surface analysis revealed that group II (F4) had significantly lower surface irregularities in comparison to group I (F3) (P = 0.036).

Conclusion: Within the limitations of this study, it could be concluded that none of the root canal preparation sizes resulted in complete *E. faecalis* elimination. Bacterial reduction has a direct relation with the enlargement of the mechanical preparation. The larger the file size the more significant is the bacterial reduction. This result might be related to the significant reduction in surface irregularities

Keywords: *Enterococcus faecalis*; Surface Irregularities; Scanning Electron Microscope; Colony Forming Unit

Introduction

The main objective of endodontic treatment is to eliminate bacteria and pathologic debris from the root canal system and to prevent reinfection. One of the main causes of endodontic failure is the presence of bacteria inside the root canal system at inaccessible areas [1]. The total elimination of bacteria has not been achieved yet. The difficulty in the removal of the residual bacteria has been attributed to the presence of the ramifications, lateral canals and anastomoses which are inaccessible during mechanical preparation [2,3]. These anatomical factors were found to be more common at the apical one third. Surface irregularities such as grooves and concave areas are part of the anatomical morphology of the root canal commonly found at the apical one third after mechanical preparation [2,3]. Historically, ending the preparation to only

three sizes larger than the first binding file rule was still being used in modified forms. However, this technique does not ensure removal of the infected inner dentine layer from all apical canal walls. The first file to bind in the apical root canal system did not necessarily reflect the true canal diameter at the proposed working length because the apical anatomy is often irregularly shaped and not a round configuration [2-4]. The enlargement of the apical area could be an effective procedure to reduce the bacteria and hence decrease the postoperative pain. This is attributed to the elimination of infected dentin and ramifications which contain residual bacteria, and to allow better penetration of the irrigant. Recently, enlargement of the root canal preparation was found to be effective in *E. faecalis* reduction regardless of the irrigant type [2-4]. Despite studies which proved that there is a direct relation between enlargement of the apical third and bacterial reduction, there are controversial studies that support minimal canal enlargement. However, according to a systematic review, the optimum apical enlargement size remains a controversial topic [2]. Owing to the presence of controversy regarding the effect of final preparation size on the amount of remaining bacteria, in addition to the limited data that existed regarding the correlation of surface micro irregularities and the amount of remaining bacteria, this study was undertaken.

Materials and Methods

24 freshly extracted human single canalled premolars were obtained from the department of Surgery at the Faculty of Dentistry, Cairo University. The collected teeth were cleaned from any hard deposits by using an ultra-sonic scaler and were disinfected in 5.25% sodium hypochlorite for 30 minutes. Teeth were examined radiographically to exclude those with calcification, resorptive lesions, and multiple canals. In addition, teeth were examined under surgical microscope to exclude those with cracks and open apices. The teeth were then stored in saline solution until use. Patient's informed consent was obtained according to the recommendations of the ethics committee of Cairo University. Sample size calculation was achieved using PS: Power and Sample Size Calculation software Version 3.1.2 (Vanderbilt University, Nashville, Tennessee, USA).

Root canal disinfection, preparation and inoculation

All the teeth were decapitated at the level of the cemento-enamel junction by using a water-cooled diamond disc. The root lengths were standardized to 15 ± 1 mm. Apical patency was determined by inserting a size 10 K-file. Working length was established by measuring teeth length of all the teeth from the cemento-enamel junction till the root apex by subtracting 1 mm from the length of an inserted #10 K-file with its tip visualized at the apical foramen. Teeth with apices larger than #20 were excluded. The root canals were instrumented up to #20 K-file till the apical constriction for standardization and to allow better penetration of the bacteria into the canals. NaOCl (5.25%) was used for irrigation during root canal debridement and between each size file and the following one. The teeth were then washed thoroughly with sterile saline and then the canals were dried by using paper points. Following root canal preparation, the enlarged apical foramina were sealed to prevent bacterial leakage. All specimens were then placed inside Eppendorf tube, packed in with a piece of wet cotton to assure the presence of humidity then placed in sterilization pouches and autoclaved for 15minutes at 121°C. The teeth were then mounted vertically in blocks to make both handling and instrumentation easier.

Bacterial preparation

For Bile eusculine test: *E. faecalis* was identified by Eusculine hydrolysis test. Black discoloration (black colonies) which represent *E. faecalis* was shown on agar plate inoculated with the tested organism overnight. The cysteine lactose electrolyte-deficient agar (cled test): *E. faecalis* appeared as pin point colonies yellow in color due to formation of lactose. A gram stained film test: Application of methyl violet then Gram iodine, ethyl alcohol, all for1 minute then washed, and final staining with diluted carbol fuchsine followed by examination under the microscope showing that the bacteria is gram (+ve) (Figure). A suspension was prepared by adding 1 ml of a pure culture of *Enterococcus faecalis* (ATCC 29212), which was grown in brain-heart infusion broth (BHI) for 24 hours, to fresh BHI. All root canals were completely filled with 30µ *E. faecalis* suspension by using sterile, 1-ml insulin syringe.

Sterile #15 K-type files were used to carry the bacterial suspension to the entire root canal length. Blocks were then placed inside sterile box and sterile plastic bags and incubated at 37°C for one week with refreshment every 48 hours. All teeth were sampled after bacterial injection and before instrumentation for examination of bacterial presence inside the canals.

Root canals inculated with *E. faecalis* were randomly divided into two groups of 12 root canals each. First Group, root canals were prepared to size F3 (#30) ProTaper Universal file. Second Group, root canals were prepared one more size of the ProTaper Universal files up to size F4, (#40).

Mechanical preparation:

The contaminated teeth were prepared by using Protaper Universal rotary files according to the manufacturer's recommendation. A gear reduction hand piece of 20:1 ratio was used with a torque controlled ENDO-MATE electric motor DT set at 3 Ncm and a speed of 300 rpm. First Group (F3), preparation was done with Protaper files in sequence with S1, followed by Sx to coronal two-thirds for coronal flaring. A sequential apical instrumentation to the full working length was done with S2, F1, F2 and finally F3 as a final master file. Second Group (F4), was done using the same preparation up to size F4 Protaper file as a final master file. Sterile saline irrigation was used after finishing the preparation of each file size using disposable 30 gauge needle fit to 5 ml disposable plastic syringe which was introduced as far as possible in the canal space without binding. The canals were kept flooded with the saline throughout the instrumentation procedure.

Bacterial assessment

After preparation, samples for counting residual bacteria inside the canals were taken. Sampling was done by filling all canals with sterile 0.9% saline solution and each sample was taken by using three sterile Protaper paper points, placed at the working length and allowed to saturate for one minute. Paper points were then transferred to sterile tubes containing 1mL of sterile BHI broth and vortexed for 1 min (Figure 1). Sterile micropipettes with yellow tips were used to take 30µ from tubes and then smeared on the surface of the *Brain- heart infusion* agar plates by using sterile L-shaped glass rod, and then the plates were incubated at 37°C for 48 hours. Plates were examined for bacterial growth and count colonies (Figure 2). The *E. faecalis* was determined by visualization of individual white pinpoint colonies on the agar plates. Visible colonies of *E. faecalis* were counted on each plate and expressed as confirmed by colony forming unit (CFU) plate, and then the results of CFU/samples were calculated (Figure 1).

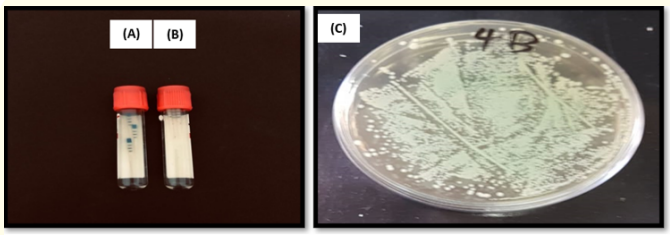

Figure 1: Bacterial assessment: (A) F3Paper points inside sterile tubes containing 1mL of sterile BHI broth, (B) F4 Paper points inside sterile tubes containing 1mL of sterile BHI broth, (C) Colony forming unit count (CFU) for the infected teeth to ensure *E. faecalis* biofilm growth.

Surface irregularities assessment

For the apical one third assessment, The teeth were then washed thoroughly with sterile saline and then the canals were dried by using paper points. EDTA solution (17%) was used as a final flush for smear layer removal. All the roots were sectioned longitudinally using a sharp thin tapered stone in a high speed hand piece under copious amount of cool water in order to cut a deep groove in the outer surface of the roots. This was followed by splitting the roots into two similar halves by means of a hammer and chisel inserted through the groove. Samples were coated with gold using (K550X sputter coater, England) and then they were mounted on a metallic disc before examination. Scanning Electron Microscope "SEM" was used to examine the surface irregularities at the apical third of the root canals of all the groups at various magnifications. The obtained multiple images of specimens were then taken at the apical 1-4 mm. The magnification started at 250X and increased up to 1500X. However, 1500X magnification was chosen to be the

standardized method for assessing the results. Scoring system suggested by Prati., *et al*. (2004) [5] for micro irregularities was used (Table 1).

Parameters	1	2	3	4
Surface irregularities	Absent	Isolated irregularities and grooves	Partially irregular with grooves	Irregular with grooves

Table 1: Scale of values assigned to the parameters evaluated.

Statistical analysis

Numerical data were explored for normality by checking the data distribution, calculating the mean and median values and using Kolmogorov-Smirnov and Shapiro-Wilk tests. Log transformation of bacterial count data was done to achieve normality. They were presented as mean and standard deviation values and were analyzed using independent t-test and paired t-test for inter and intragroup comparisons respectively. Ordinal data for irregularities and debris scale were presented as median and range values and were analyzed using Mann Whitney U test. The level of significance was set at P ≤0.05 within all tests. Statistical analysis was performed with IBM SPSS Statistics Version 25 for Windows.

Results

I-E faecalis count

Effect of instrumentation

Pre-instrumented *E. faecalis* bacterial count of F3 group was (2.55 ± 0.05) and F4 group was (2.56 ± 0.05). These value were significantly lowered after instrumentation to be (1.50 ± 0.35) and (1.00 ± 0.33), respectively (P < 0.001) (Table 2).

Effect of file size

F4 Group showed the highest percentage bacterial count reduction with a mean of (96.66 ± 2.10). Percentage of bacterial count reduction was lower in the F3 group with a mean of (88.63 ± 5.48). Comparison between the two groups revealed the difference to be statistically significant (P-value = 0.025) (Table 3).

Bacterial count (means ± SD)			P-value
Instrumentation	F3	F4	
Before	2.55 ± 0.05	2.56 ± 0.05	0.615ns
After	1.50 ± 0.35	1.00 ± 0.33	0.016*
P-value	<0.001*	<0.001*	

Table 2: Means ± standard deviations (SD) of log bacterial count (CFU) before and after instrumentation with different files.

*; significant (p ≤ 0.05) ns; non-significant (p > 0.05).

Percentage change of bacterial count (mean ± SD)		P-value
F3	F4	
88.63 ± 5.48	96.66 ± 2.10	0.025*

Table 3: Mean ± standard deviations (SD) of effect of file size on percentage change of bacterial count.

*; significant (p ≤ 0.05) ns; non-significant (p > 0.05).

Micro-irregularities presence

Effect of file size

Root canals prepared to size F3 showed mostly dentin surface with partial irregularities and multiple grooves with a significantly higher median irregularities score of 4 and a range of 2. While most root canals prepared up to size F4 revealed a lower median irregularities score of 2.5 and a range of 1 with most root canals showing only isolated irregularities with absence of grooves. Comparison between groups revealed a statistically significant difference (P-value = 0.036) (Table 4, Figure 2,3).

Irregularities [Median(Rang)]		P-value
F3	F4	
4.00(2.00)	2.50 (1.00)	0.036*

Table 4: Median and range values of irregularities score for different files.

*; significant (p ≤ 0.05) ns; non-significant (p > 0.05).

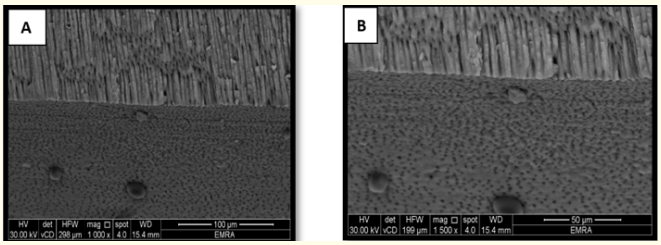

Figure 2: F4: SEM images (A and B) represents isolated irregularities (score. 2).

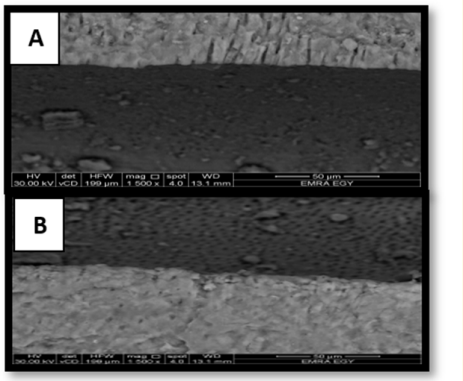

Figure 3: F3: SEM images (A and B) represent Partially irregular with grooves (score. 3).

Discussion

The role of micro-organisms inside the root canal system in developing apical periodontitis has been well established in the endodontic literature [6-10]. The main objective of endodontic treatment is to eradicate bacteria and their by-products from the root canal system, thus preventing the development of periapical diseases [6-10]. Previous studies have shown the correlation between the root canal preparation enlargement and the amount of remaining bacteria [2,3,11-16]. However, limited data existed regarding the correlation between surface micro-irregularities and the amount of remaining bacteria. The aim of this study was to evaluate the effect of enlarging the apical one third of root canals by using F4 compared to F3 Protaper files on the amount of *Enterococcus Faecalis* and surface irregularities in the apical third. Some studies have shown that these areas can reach up to 35% of surface of the main root canal [17-19]. This was more applicable for this study as it aimed to detect whether an increased root canal preparation size would affect the amount of untouched areas within the root canal and subsequently the amount of remaining bacteria and surface irregularities which in turn act as a niche for bacteria [16]. The root canals were mechanically prepared to size #20 stainless steel K- file to standardize the diameter of root canals and allow the introducing of *E. faecalis* [11,20]. Then, the canals were prepared using ProTaper Universal rotary system. Both shapers and finishers were chosen based on the evidence of previous studies which demonstrated the effectiveness of rotary system over hand technique [21]. Moreover, the Finisher files were invented to prepare only the apical one third [22]. So, unnecessary over-enlargement of the coronal third would be avoided [23]. F3 Protaper file was used as a control in this study because it corresponds to file size 30, which was found to have the suitable size for effective irrigant passage into the apical one third. Srikanth., *et al*. (2015) [24] determined the minimal apical enlargement for irrigant penetration into apical third of root canal system using (SEM). They reported that the minimal apical enlargement for penetration of irrigants to the apical third of root canal system was #30 size. F4 Protaper universal size was chosen because it allowed a specific enlargement of only the apical third of the canal with no change of the preparation size in the middle and coronal thirds. It has a tip diameter of 0.4mm with a progressive taper of 0.06mm from D1 to D3 which correspond to a canal preparation diameter of 0.46, 0.52, and 0.58 in D1, D2, and D3 respectively. On the other side, F3 provided a canal preparation diameter of 0.39, 0.48, and 0.57 in D1, D2, and D3 respectively. Accordingly F4 would not provide any additional enlargement to the canal preparation beyond the apical 3 mm [22]. Saline was used in this study as an irrigant to act as a lubricant and flush out the debris after each instrument exchange. A concentration of 0.9% was used as it has no antibacterial action [25], it was thus suitable for the study design as it allowed for adequate lubrication and flushing of debris without affecting the amount of remaining bacteria and subsequently affecting the results of the study. Therefore, the use of saline allowed for the assessment of mechanical instrumentation alone without acting as a variable [1,2,12]. A 30-gauge needle with a tip diameter of 0.31 mm was used, this tip diameter allowed

the needle tip to reach within 1-2 mm of tooth apex, thus providing proper flow of the irrigant throughout the apical third and the entire root canal space and subsequently proper flushing and cleaning of the debris resulting from the instrumentation. Sterilization of sample was done using autoclave after the chemo-mechanical preparation to obtain a totally sterile root canal system before the bacterial inoculation [11,26,27]. The roots were apically closed, double layered and placed in blocks to stimulate the in-vivo condition of a closed system, to confine the inoculated bacteria to the root canal system and to facilitate the handling [28,30]. *E. faecalis* was selected because of its ability to deeply invade the dentinal tubules [29], along with its ability to survive and re-colonize in these inaccessible areas [30]. Its inherent abilities made it more resistant to intracanal medicament or different types of irrigants [31]. It was also found to be the most frequently detected species in root-filled teeth with persistent lesions [31]. Multiple ranges of bacterial incubation period were suggested by variety of previous studies. Incubation periods used for *E. faecalis* have ranged from 1 day to 4 weeks. One week incubation period was chosen for this study of *E. faecalis* as it allowed for adequate invasion of the bacteria inside the dentinal tubules, the one week period was confirmed to be adequate in previous studies [13,16,20,27,32]. The brain heart infusion agar method was used to test the bacterial reduction, because it is the most widespread technique in the microbial activity assessment; as its efficacy was proved by many studies [1,26,33]. The apical region of the root canal was chosen because of the difficulty to disinfect. It is the most crucial area for the instrumentation process as the last few millimeters of the canal were found to be the region that most commonly harbors intra-radicular bacteria that can spread to periapical region and result in root canal treatment failure [16]. The larger preparation sizes have been shown to provide adequate irrigation that significantly decreasing the number of microorganisms [3,30,34]. Thus there appears to be a relationship between increasing the size of the apical preparation and bacterial reduction [1,2,5,12,16]. The present study showed that although significant bacterial reduction was achieved with both preparation sizes, none of the two groups resulted in the complete absence of *E. faecalis* when investigated using CFUs. This was in accordance to other studies which only investigated the effect of instrumentation without the additional use of chemical disinfection [27,35]. In terms of bacterial reduction, there was a statistically significant difference in the amount of bacterial reduction of the F4 group in comparison to the F3 group. F4 group showed a significantly higher percentage of bacterial reduction. The significant difference between (F4) group and (F3) group could be attributed to the root canal enlargement especially in the apical third, which could have resulted in more infected dentin removal and removal of surface irregularities and anastomosis and hence lower bacterial load.These results were in accordance with the results of previous studies by Rollison., *et al.* (2002) [122.0 × 107 colony-forming units], Marinho., *et al*. (2012) [11], Rodrigues., *et al*. (2017) [3] and Navabi., *et al*. (2018) [12] who demonstrated a significant bacterial reduction when the canal preparation was increased. However, the preparation was extended to file size 50 in Rollison., *et al*. study. While Marinho., *et al*. studied the effectiveness of the preparation against the bacterial endotoxins. On the contrary, our results were in contrast with those of Machado., *et al*. (2010) [32], Akhlaghi., *et al*. (2013) [27] and Moshari., *et al*. (2015) [36] who concluded that there was no significant increase in bacterial reduction when the canal preparation size was increased. The present study found that there is a significant reduction in root canal micro-irregularities between the groups which might have a role in the significant reduction of bacteria when the instrumentation size was increased.

Conclusion

Within the limitations of this study, it could be concluded that none of the root canal preparation sizes resulted in complete elimination of E. faecalis. Bacterial reduction has a direct relation with the enlargement of the mechanical preparation. The larger the file size the more significant is the bacterial reduction. This result might be related to the significant reduction in surface irregularities.

Conflict of Interest

The authors deny any conflicts of interest in this study.

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