

BACTERIOLOGICAL EVALUATION OF ROOT CANAL CLEANING AFTER CONSERVATIVE VERSUS TRADITIONAL ENDODONTIC ACCESS CAVITY (AN IN VITRO STUDY)

Michael E. Girgis^{1*} BDS, Sybel M. Moussa² Ph.D., Samya S. Omar³ PhD

ABSTRACT

INTRODUCTION: Effective irrigant delivery and agitation protocols with recent advances in magnification, endodontic instruments, and obturation systems can lead to more conservative access opening designs.

OBJECTIVES: The purpose of this in vitro study was to compare the cleaning of the root canal system with 2 different designs of access opening, traditional versus conservative.

METHADODOLOGY: 100 extracted upper premolars were divided into four groups according to the access opening performed: Gp I and Gp III Conservative endodontic access, GpII, and IV traditional access opening. Then all teeth were autoclaved, inoculated with *Enterococcus faecalis*, and incubated for 30 days. Gp I and Gp II were then prepared with Wave One Gold and irrigated with Passive ultrasonic irrigation while Gp III and Gp IV were not instrumented and served as negative control. Finally, all samples were decalcified and submitted to staining by Brown and Brenn stain.

RESULTS: The traditional access opening design showed better cleaning than conservative access. The obtained results showed statistical significance between the two access opening designs regarding the chemical cleaning.

CONCLUSIONS: Conservative and conventional access cavities exhibited different cleaning outcomes. Endodontic microscope assisted conservative access could not be done as a valuable alternative for conventional access without hindering cleaning.

KEYWORDS: access cavity, traditional, conventional, *E.faecalis*, conservative.

-
1. Instructor of Endodontics, Conservative Dentistry Department, Faculty of Dentistry, Alexandria University, Alexandria, Egypt.
 2. Professor of Endodontics, Conservative Dentistry Department, Faculty of Dentistry, Alexandria University, Alexandria, Egypt.
 3. Professor of Oral Biology, Oral biology Department, Faculty of Dentistry, Alexandria University, Alexandria, Egypt.

* Corresponding author:
E-mail: Michaelelabib@gmail.com

INTRODUCTION

It has been well established that the objective of the coronal access preparation is to identify the root canal entrances to provide a smooth free-flowing tapered channel from the orifice to the apex. This will allow instruments, irrigants, and medicaments to attempt shaping and cleaning of the entire length and circumference of the canal while preventing procedural errors, with as minimal loss of structural integrity to the tooth as possible. (1)

Although traditional designs of access cavity preparation (Traditional Endodontic Cavity (TEC)) have not changed for many years, excessive removal of tooth structure especially at pulp chamber walls and around the canal orifices may decrease the tooth resistance to fracture during function. (2) This will lead to subsequent tooth extraction and loss and thus decreased belief in root canal treatment as a strong treatment alternative.

Recently; because of the evolution of radiography and endodontic microscopy as well as the great increase of flexibility and fracture resistance of endodontic instruments and recent advances in obturation systems; clinicians have the potential to become more conservative without any need to compromise tooth structure which is convenient with new trends in modern dentistry.

A new conservative endodontic cavity (CEC) preparation design was suggested by Clark and Khademi in 2014 (3). They attributed the success of endodontic treatment to dimensions of coronal and radicular preparation. This is believed to make achieving the maximum possible strength and longevity possible. But, it may compromise shaping and cleaning of canals and may also increase stresses overused instruments. (4)

The effect of the new suggested access opening design on the efficacy of the instrumentation as well as the tooth resistance to fracture were evaluated by Rajesh

Krishan et al (5). They concluded that the CEC while was associated with the risk of compromised canal shaping due to increased area of untouched canal walls; it conserved coronal dentin and conveyed a benefit of increased fracture resistance.

A traditional access opening could be described as the complete removal of the pulp chamber roof in order to create a tapered channel from the cavity outline on the outer crown surface to the canal orifice. (6) A conservative access cavity preparation is accomplished by reaching the canal orifices without the need for complete removal of the pulp chamber roof and trying to keep as much of the coronal dentine as possible. (7)

Recent instrumentation studies and techniques focus on the importance of chemo-mechanical preparation of the root canal system. (8) This means that the Cleaning protocol is as important as or even more important than the shaping phase because irrigation acts as a flush to remove organic and inorganic debris as well as a bactericidal agent, tissue solvent, and lubricant. (9)

Cleaning is mainly achieved by several irrigation protocols which vary according to materials used as well as delivery and activation systems while keeping in mind that apical areas can only be disinfected if reached by the irrigant. (10,11) Passive ultrasonic irrigation is used to describe an irrigation method where there was no contact of the canal walls with an endodontic file but energy is transmitted to the irrigant in the root canal by means of ultrasonic waves from an oscillating file or a smooth wire. This induces acoustic streaming and cavitation of the irrigant. (12, 13)

One of the reliable methods to evaluate the cleaning ability is to detect the presence or absence of bacteria in histological sections stained by Brown and Brenn method to check for the existence of pulp tissues and either they are infected or not. (14)

The research question is: Does conservative endodontic access in conjunction with passive ultrasonic irrigation provide the same cleaning ability as the traditional access opening when evaluated in tissue sections stained by Brown and Brenn?

The null hypothesis is that no difference will be found between traditional versus conservative endodontic access cavities.

MATERIALS AND METHODS

A) Materials

Study Samples size calculation

A total sample size of 100 teeth i.e. 25 per group (number of groups = four). Is the enough required sample to detect > 0.7 Cohen kappa for inter-rater agreement (reliability) leading to kappa of 0.85. One hundred extracted human first maxillary premolars collected from patients ranging from 15 to 25 years old who extracted their teeth due to orthodontic reasons from Oral Surgery and maxillofacial Department at Faculty of Dentistry Alexandria University, Clinics, and hospitals in Alexandria Governate following appropriate informed consent. The study was accepted by the Research Ethics Committee of the Faculty of Dentistry, Alexandria Dental Journal. Volume 45 Issue 3.

Alexandria University (IBR NO: 00010556-IORG0008839).

Study design

The study was conducted as a parallel, controlled, experimental study in which four groups were examined.

Teeth selection criteria

Sound maxillary premolar teeth (type I according to Vertucci's classification) with completely formed roots and closed apices were included. Normal depth of the pulp chamber was checked on preoperative x-ray (the level of the pulp chamber roof shouldn't be below the cemento-enamel junction) (20).

Teeth with root caries, internal resorption or calcified canals detected on radiographs, and previously endodontically treated teeth were excluded.

Randomization technique

A computer-generated list of random numbers was used to randomly assign 100 eligible teeth into one of the four study groups. An equal allocation ratio was adopted, with twenty-five teeth in each group. The teeth allocation was done using Research Randomizer* online software.

Chemicals used in this study: Storage medium: sterile saline + 0.1% thymol, 5.25% NaOCl, 17% EDTA solution

Instruments used in this study: Wave One Gold Rotary Files, Mani Stainless steel k-files, microsurgical ultrasonic tips BS6-Satelec.

B) METHODS.

A. Preparation of teeth

One hundred human maxillary premolars were collected from patients ranging from 15 to 25 years old who extracted their teeth due to orthodontic reasons from Oral Surgery and maxillofacial Department at Faculty of Dentistry Alexandria University, Clinics, and hospitals in Alexandria Governate following appropriate informed consent. Then they were cleaned from any remaining hard or soft tissues remnants on their external surface. Teeth were preserved in normal saline and Thymol 0.1% to prevent candida infection. (15)

The apical foramina of all teeth were sealed by cyanoacrylate. The tooth length was measured, then each tooth was separately mounted in a mold of condensation silicone putty to facilitate sample handling. The study was performed in Conservative Dentistry department and Oral Biology department in the faculty of dentistry, Alexandria University.

Finally, each tooth was radiographed in both mesiodistal and buccolingual views in order to measure the pulp chamber dimensions and pulp horns height.

B. Grouping

The (100) premolars were also randomly distributed into four groups each of (n= 25) according to the type of access opening performed

Group I: Conservative access opening was used with canal preparation.

Group II: Traditional access opening was used with canal preparation.

Group III: Conservative access performed without canal preparation.

Group IV: Traditional access performed without canal preparation.

C. Access Opening Preparation

Using a 4x magnification an access opening preparation was made in each tooth according to its group.

Group I and III (Conservative access cavity groups)

A rose head bur (tip size 0.5mm) (Horico – Germany) was used to reach the dentine then a 169 bur (SS White, USA) was directed to the center of the occlusal table and moved in a buccolingual direction in a depth of seven mm followed by a needle-shaped stone (Horico – Germany) which was used to finalize the access by increasing the depth till exposure of the buccal pulp horn occurs, then extending the access towards the lingual pulp horn without complete deroofting.

Group II and IV (Traditional access cavity groups)

A rose head bur (tip size 1.6 mm) (Horico – Germany) was used to penetrate the dentine then a tapered fissure bur (SS White, USA) was directed to the center of the occlusal table in order to expose the pulp chamber followed by safe ended stone (Horico – Germany) which was used to finalize the access by extending it to include both the buccal and palatal pulp horns in order to remove the pulp chamber roof completely. (Figure 1)

D. Teeth sterilization and bacterial inoculation (16)

The samples were sterilized at a temperature of 121^o C with a pressure of 118 Kappa in class B autoclave for a period of 30 minutes.

Then one tooth from each group was randomly selected and submitted to the sterilization control. A sterile paper point was placed in contact with the root canal walls for 15 seconds and individually transported to a plastic micro tube containing 1mL 0.9% saline solution. The material was homogenized and was cultivated on blood agar after 5 minutes. The samples were then be incubated at 37^o C for 48 hours in order to verify sterilization.

E. feacalis (ATCC 29212) was sub-cultured on blood agar plates for 24 hours before inoculation, and then a suspension of the bacterial cells was prepared in sterile saline solution and vortexing was performed. The bacterial inoculums were then compared to match 0.5 on the McFarland scale to have standardized bacterial suspension. The beakers used to contain the specimen were first sterilized in heal force[®] biology safety cabinet and then teeth were added each in a corresponding beaker and covered with parafilm. Each received a patch with the sample number on its external surface.

Beakers were then opened in the presence of a flame, and sterile pipettes were used to add 15 mL of the bacterial inoculums to each one. They were then closed and kept at 37^oC for 30 days, with the replacement of half the inoculums broth with fresh sterile medium every 2 days to avoid medium saturation (Figure 10). The turbidity

of the medium during the incubation indicated bacterial growth.

E. Canal preparation

For group I the pulp chamber was first flooded with one ml of 5.25% NaOCl solution and then Microsurgical US tip BS6 (Acteon Satelec- France) was used to debride the pulp under the pulp horns which was then flushed with 2 ml 5.25% of the same solution. The P5 neutron ultrasonic device (Acteon Satelec – France) was set to power seven for a duration of one minute.

The tooth length was measured for each tooth separately before putting them in the silicone mold. This anatomical tooth length was used to provide canal patency with Kfile #10 (Mani – Japan) which was used to negotiate canals down to the actual working length which was decided to be 0.5 mm shorter. A glide path was secured using Kfile #15 to the full working length, then canals were prepared using Wave One Gold (Dentsply-Sirona, Ballaigues, Switzerland) single file system mounted to X-smart plus (Dentsply-Sirona, Ballaigues, Switzerland) endodontic motor in a reciprocating motion. An iso-standard apical size of 25 and 6% taper was achieved.

Shaping was finished in two steps in each canal, first, the occlusal two-thirds were shaped and irrigation with 5 ml of NaOCl was performed, then canal patency was rechecked and another 5 ml of the same solution was used to irrigate the canal. The remaining part of the canal was prepared to the full working length followed by irrigation with 5 ml of the previously used NaOCl. The irrigation needle was placed 2mm short from the working length. In the end, canal patency was checked using k file #10 and a final 5 ml irrigation of NaOCl was performed. Using a side vented 30-G needles, the total volume of irrigation was 25 ml of NaOCl solution for each tooth.

At the end of the preparation, passive ultrasonic activation was performed with k15 tip from Satelec while the canal was flooded with 2mL 17% EDTA solution (Prevest - India). The ultrasonic tip was put inside the canal two mm shorter than the working length for one min. It was mounted to P5 Neutron device set to power six.

This last step was repeated with the same specifications but this time substituting the EDTA solution with two mL 5.25% sodium hypochlorite solution. A sterile saline rinse was performed between the two irrigation materials in order to prevent any material precipitation and canal blockage.

For group II the same protocol of chemo-mechanical preparation was followed as described before for the other group, the only difference is that there was no ultrasonic curettage for the pulp chamber prior to canals shaping and irrigation as the whole roof was completely removed.

F. Decalcification, sectioning and Brown and Brenn staining (14)

All teeth were post fixed in 10% neutral buffered formalin for two days, and then were washed in running water for four hours and decalcified in 10% Trichloro-Acetic acid. After complete decalcification, teeth were processed to obtain five microns thick longitudinal paraffin sections, which were stained with Brown and Brenn for evaluation

of the presence of gram +ve and gram -ve bacteria. When examining the slides different colors appeared indicating Gram-positive bacteria which was stained with blue and purple, Gram-negative bacteria was stained by red stain on a yellow background.

Penetration of bacteria into dentinal tubules was studied by a light microscope at 400x magnification. The extent of invasion was referred as the percent of infected area compared to the total area of the slide. (17)

A round area of two mm in diameter in each slide was evaluated in order to calculate the bacterial load by E.faecalis bacteria to compare its diminution between the different groups. (Figure 2)

Data management and Statistical analysis:

Normality was checked using descriptive statistics, plots (histogram and boxplots) and Kolmogorov–Smirnov test of normality. All variables showed non-normal distribution, so mean, Standard Deviation (SD), median and interquartile range (IQR) were calculated. Comparing the four study groups was done using Brown-Forsythe test followed by a post hoc test for multiple comparisons using Games-Howell adjustment between the groups. Significance was set at P <0.05. Data were analyzed using IBM SPSS statistical software (version 25).

Figure (2): Modified Brown and Brenn stain, 100X Compound light micrograph showing: (A) group IV: formation of a heavy bacterial biofilm on the pulpal surface

(B) Group III: residual pulp tissue in the pulp space adjacent to Bacterial penetration in the root dentin (C) Group II two patches of Gram+ bacteria close to the outer surface of cementum, but not reaching it (insets 1 and 2) (D) Group I showing the slightly larger area of DTs bacterial involvement than in the previous group

RESULTS

The Mean ± Std. Deviation of the Percentage of the infected area of the four experimental groups, as well as a 95% confidence interval of the mean, are shown in table (table 1).

The percentage of infected area score in negative control was considered the baseline of bacterial infection before preparation to which the infected area reduction was compared with the study groups.

Presence of bacterial growth was seen in the negative control groups with a mean of: for group III 32.69 % and for group IV is 43.41%.

The two study groups' slides showed less bacterial presence with a mean value of 13.69% in group I and 5.92% in group II (Figure 3)

Statistical significance differences are shown in (table 2) between the four experimental groups by Post-hoc Multiple comparisons Games-Howell method. It was found that group II had the least percent of the infected area followed by group I, while group IV showed the highest percentage of infected area.

A statistically significant difference in the bacterial percentage load was found among the four experimental groups (p=0.000).

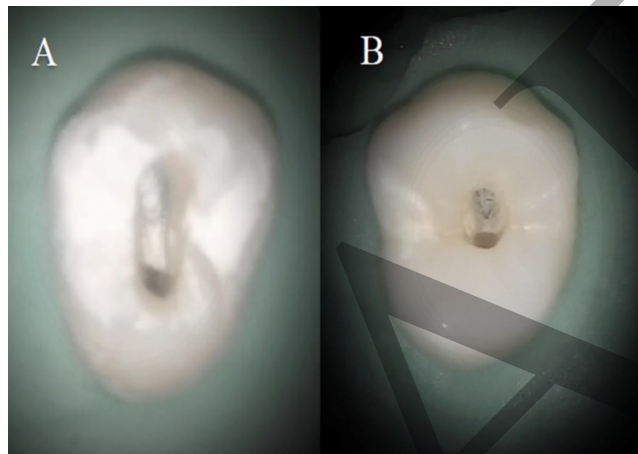


Figure (1): showing an occlusal view of the two access opening designs (a) Conventional access opening (b) Conservative access opening

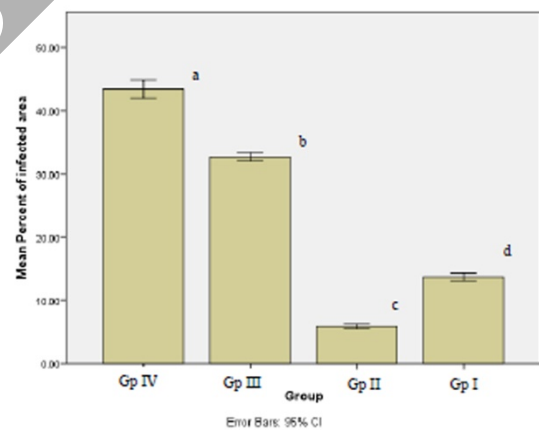
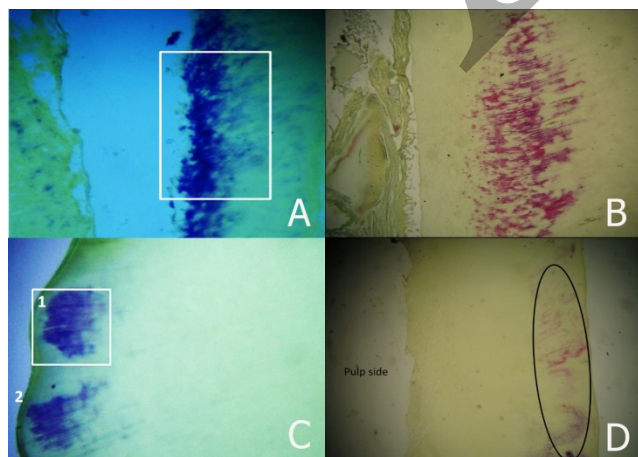


Figure (3) Bar chart with 95% CI error bars of mean percent of the infected area (%) in the studied groups (different superscript letters indicate pairwise statistical significant difference using Games-Howell method).

Table (1): Showing Percent of the infected area among the 4 Study Groups.

	Control 1	Control 2	Study 1	Study 2
• n	25	25	25	25
	37.51-	31.07-	4.06-7.15	11.09-

• Min-Max	48.89	35.09	5.92±0.8	16.19
• Mean ± Std. Deviation	43.41±3.09	32.69±1.3	1	13.69±1.2
• 95% CI for mean	41.9677-44.8615	5	5.5415-6.3002	5
		32.0578-33.3248		13.1058-14.2803
Test of significance	$F_{(BF)(df=3)}=1733.663$ $p=0.000^*$			

n : Number of samples
 Min-Max: Minimum - Maximum
 CI: Confidence interval
 BF: Brown-Forsythe test
 * : Statistically significant (p<0.05)

Table(2): Post-hoc Multiple comparisons using the Games-Howell method

	Control 1	Control 2	Study 1	Study 2
Control 1		$p=0.000^*$	$p=0.000^*$	$p=0.000^*$
Control 2			$p=0.000^*$	$p=0.000^*$
Study 1				$p=0.000^*$
Study 2				

* Statistically significant at p < 0.05

DISCUSSION

Proper disinfection and complete eradication of all strains of bacteria especially persisting types in the root canal system is a major challenge in today’s treatment regimens. Although the chemo-mechanical preparation is crucial for the long term success and preservation of the endodontically treated tooth, we should not ignore the effect of final tooth restoration and its effect on the long term service in vivo.

Each clinician should keep as much of the sound tooth structure intact as possible, without the need to remove sound dentine in order to reach better results from the endodontic point of view regardless of the prosthetic needs.

So, we should rather change our techniques and modify the way we are gaining access to the root canal system, than removing sound tooth structure.

The use of magnification and proper illumination, highly flexible instruments, as well as, ultrasonic energy to passively activate intracanal irrigants may allow us to reach these goals.

However, no enough evidence-based work was accomplished to discuss the variations happening with the change in the geometry of the access cavity from the microbiological point of view, and the actual effect on bacterial eradication; which is actually the most important factor, and hence, raised the idea for this study.

Enterococcus faecalis was chosen because it accounts for 64-78% of cases with persistent or secondary

infection. They are gram-positive cocci that can occur singly, in pairs or as short chains. They are facultative anaerobes, possessing the ability to grow in the presence or absence of oxygen. In addition, they have various inherited virulence factors and mechanisms that enable it to survive the effects of root canal treatment and persist as a pathogen in the root canals and dentinal tubules of teeth. (18) Starved *E. faecalis* was found to form biofilms through a harsh environment, and this may contribute to its role in persistent intra-radicular infections. Also, it has the ability to invade dentinal tubules and adhere to collagen in the presence of human serum and can penetrate the dentinal tubules of root canal walls up to 800-1000µm deep. (19) The time taken for *E. faecalis* colonization in the root canals varies among studies. However, a longer incubation period yields a more mature biofilm, that’s why it is increased in recent studies. Therefore in the current study, root canals were inoculated and incubated for a period of one month, in accordance with Mehrvarzfar et al (2011) (20) and Balic et al (2016) (21), as they revealed by scanning electron microscopy heavy colonization of *E. faecalis* and a biofilm-like structure on the canal surface after only 15 days of inoculation and incubation.

Wave One Gold® files on a reciprocating endodontic motor were chosen in this study. Wave One Gold® is a gold treated Ni-Ti alloy. Heat treatment of conventional NiTi wires that are in the austenite phase transforms them into a rhombohedral crystal structure called as an intermediate R-phase between austenite and martensite. The R-phase shows good super elasticity and shape memory effects; its Young’s modulus is typically lower than that of austenite. Thus, an instrument made out of R-phase wire would be more flexible. This file is considered from the Stress-induced Martensite (SM Alloy). It’s also a unique SM-wire because it’s subjected to high temperatures twice; it is subjected to special heat treatment under constant strain in a range of 3-15 kg in a temperature range of about 410° to 440°C. After machining the working portion of the file, the finished instrument is heat-treated a second time in a range of 120°C to 260°C. It was believed it’s a file system that can comply with canal morphology without breaking due to overstress caused by incomplete deroofing. (22)

In this study, a modified Brown and Brenn method was used in accordance with Ricucci in 2010 (23) and Adalberto in 2012 (24). This method was believed to be more significant because of the practical field of view in the specimen. With this method we were able to show the actual presence of bacteria on a larger scale as well as the amount bacterial penetration in the dentinal tubules. (Figure 2)

The Brown and Brenn staining method uses a light transmission microscope in order to detect the bacterial colonies in tissue sections. The scanning electron microscope (SEM) may be a strong challenger to the light microscope.

Golding in 2016, stated that the use of (SEM) to detect pathogens, although being a reliable method, requires an adequate concentration of bacterial cells. And that why

over the years (SEM) yielded low test sensitivity for many types of microbiological investigations. (25)

But (SEM) still has some benefits over the traditional histopathological stain, which is revealing morphological features of isolated organisms as well as for diagnosis of the organism itself, but difficulty with specimen preparation methods has limited the use of SEM for routine microbiology.

Another limitation of the SEM is that it allows exclusively for the observation of the canal wall surface. (26) Therefore, only the external wall surface can be analyzed. While *E.Feacalis* were found to form deep inside the canal walls.

On comparing the bacterial count reduction among the four experimental groups when stained by Brown and Brenn stain, which shows the reflection of the two access opening designs on the antibacterial effect of the same chemo-mechanical preparation protocol, it was found that percent of infected area score in group II was less than group I with statistical significance ($p < 0.001$).

Differences in percent of the infected area between group I and II; and Group III and IV respectively were statistically significant ($p < 0.001$).

Although the irrigation protocol could have yielded proper chemical cleaning; it is believed that mechanical shaping has been hindered by the smaller access opening cavity. (27)

The irrigation protocol in this study, was made in accordance with Souza et al, who in 2019 evaluated the efficacy of passive ultrasonic irrigation, continuous ultrasonic irrigation versus irrigation with reciprocating activation device in penetration into main and simulated lateral canals (28); also with accordance with Koçak et al who in 2017 studied the influence of passive ultrasonic irrigation on the efficiency of various irrigation solutions in removing smear layer (29).

These findings were found opposite with the results of the study done by Prasanna Neelakantan et al, in 2017, when they evaluated the remaining pulp tissue after canal instrumentation with the two different access opening design. They did this evaluation histologically by H&E stain. They found that there was no statistical significance in the remaining pulp tissue in all of the root canal system and isthms area. (30)

Our findings were found to be consonant with the work done in 2014 by Krishan et al, who evaluated the untouched canal walls in canals instrumented with Wave One rotary file with different access opening designs. They found that the proportion of untouched canal walls differed significantly in apical areas of the root canal system. (27)

The limitation of the present study was the difficulty to directly compare the results of the current study with that of the previous studies due to different methodologies, different incubation periods, and different methods of testing. Another limitation may be related to the absence of a Scanning electron microscope group in which the behavior and maturity of bacterial biofilm could be studied.

The hypothesis of the present study could not be completely rejected as Conservative endodontic access opening showed significant results compared to the Traditional access group in the coronal and middle root canal system thirds. While in apical third, Traditional access lead to superior antibacterial effects.

CONCLUSION

Within the limitations of this study, the following conclusions can be addressed:

1. Although conservative access design is believed to increase in post-operative tooth strength, it yielded less disinfection when compared to the traditional design. Hence, it can lead to less service time for treated teeth especially in necrotic cases.
2. The conservative endodontic access is not an easily REPRODUCIBLE design and thus is not easily teachable.

Conflict of interest:

The authors declare that they have no conflicts of interest.

Acknowledgments:

The authors would like to acknowledge Prof. Dr. Abdelfatah Hamouda Abdelfattah (Professor of microbiology) for his help with the microbiology work.

REFERENCES

1. Patel S and Rhodes J. A Practical Guide to Endodontic Access Cavity Preparation in Molar Teeth. *Br Dent J* 2007; 203:133–40.
2. Kishen A. Mechanisms and Risk Factors for Fracture Predilection in Endodontically Treated Teeth. *Endod Top* 2006; 13:57–83.
3. Clark D and Khademi J. Modern Molar Endodontic Access and Directed Dentin Conservation. *Dent Clin North Am* 2010; 54:249–73.
4. Parashos P, Messer MHH. Rotary NiTi Instrument Fracture and its Consequences. *JEndod* 2006; 32:1031–43
5. Krishan R, Frank P, Arezou O, Anil K, Thuan D and Shimon F. Impacts of Conservative Endodontic Cavity on Root Canal Instrumentation Efficacy and Resistance to Fracture Assessed in Incisors, Premolars, and Molars. *J Endod* 2014 ; 40 : 1160 - 6
6. Ingle J I, Bakland L K and Baumgartner J C. *Ingle's endodontics* 6 (2008). Hamilton, Ontario, BC Decker.
7. Clark D and Khademi J. Case Studies in Modern Molar Endodontic Access and Directed Dentin Conservation. *Dent Clin North Am* 2010; 54:275–89.
8. Byström A, Sundqvist G, Byström A SG. Bacteriologic evaluation of the effects of percent sodium hypochlorite in endodontic therapy. *Scand.J. Dent. Res.* 1981: 89: 321-8.
9. Jeffrey LH, Johanna KD, James DJ and Nestor C. Antimicrobial Efficacy of Two Irrigation Techniques in Tapered and Non-tapered Canal Preparations: An in Vitro Study. *J Endod* 2008; 34: 1374-7

10. Johnson WT, Noblett WC, Torabinejad M, Walton RE. Cleaning and shaping in Endodontics: Principles and Practice. 4th ed. St.Louis, MO: Saunders, 2009:258–86.
11. Lisha G, Jong R, Junqi L, Kyung K, David H and Franklin R. Review of Contemporary Irrigant Agitation Techniques and Devices. *J Endod* 2009; 35:791- 804
12. Vinoth K, Kavitha S, Lakshminarayanan L, Gomathi NS, and Kumar V. Influence of Irrigating Needle-tip Designs in Removing Bacteria Inoculated into Instrumented Root Canals Measured Using Single-tube luminometer. *J Endod* 2007; 33:746–8.
13. Roberto E, Antonio J, Oliver V, Francisco T, Giampiero R and Rafael C. Effect of Passive Ultrasonic Activation on Organic Tissue Dissolution from Simulated Grooves in Root Canals Using Sodium Hypochlorite with or without Surfactants and EDTA. *J Endod* 2017; 43: 1161–5.
14. Ricucci D. and Bergenholtz G. Bacterial status in root-filled teeth exposed to the oral environment by loss of restoration and fracture or caries – a histobacteriological study of treated cases. *Int Endod J.* 2003; 36: 787-802.
15. Anna M, Ilkay E, Maria D, Ramona B, Arianna D, Seyed F, et al. Antibacterial and antifungal activities of thymol: A brief review of the literature. *JFoodChem* 2016;210:402-14
16. Ana P, Matheus A, Daniela C, Yuri D, Douglas C and AnaPaula F. Comparative Evaluation of Calcium Hypochlorite and Sodium Hypochlorite Associated with Passive Ultrasonic Irrigation on Antimicrobial Activity of a Root Canal System Infected with *Enterococcus faecalis*: An In Vitro Study. *J Endod* 2014 ;40 :1953 - 7
17. Elie N, Plancoulaine B, Signolle JP, Herlin P. A simple way of quantifying immunostained cell nuclei on the whole histologic section. *Cytometry A.* 2003; 56: 37-45.
18. Prada I, MicóMuñoz P, GinerLluesma T, MicóMartínez P, ColladoCastellano N, Manzano SA. Influence of microbiology on endodontic failure. Literature review. *Med Oral Patol Oral Cir Bucal.* 2019; 24: 364–72.
19. Ran S, Wang J, Jiang W, Zhu C, Liang J. Assessment of dentinal tubule invasion capacity of *Enterococcus faecalis* under stress conditions ex vivo. *International Endodontic Journal* 2015; 48: 362– 72.
20. Mehrvarzfar P, Saghiri MA, Asatourian A, Fekrazad R, Karamifar K, Eslami G, et al. Additive effect of a diode laser on the antibacterial activity of 2.5% NaOCl, 2% CHX and MTAD against *Enterococcus faecalis* contaminating root canals: an in vitro study. *J Oral Sci.* 2011; 53: 355–60.
21. Balić M, Lucić R, Mehadžić K, et al. The efficacy of photon-initiated photoacoustic streaming and sonic-activated irrigation combined with QMiX solution or sodium hypochlorite against intracanal *E. faecalis* biofilm Lasers. *Med Sci* 2016; 31: 335.
22. Srivastava S. Current Strategies in Metallurgical Advances of Rotary NiTi Instruments: A Review. *J Dent Health Oral Disord Ther* 2016; 9: 03-33.
23. Ricucci D, Siqueira JF. Biofilms and apical periodontitis: Study of prevalence and association with clinical and histopathologic findings. *J Endodon* 2010; 36:1177-88.
24. Adalberto R, Siqueira JF, Ricucci D, Lopes W. Dentinal Tubule Infection as the Cause of Recurrent Disease and Late Endodontic Treatment Failure: A Case Report. *J Endodon* 2012; 38:250-4.
25. Golding CG, Lamboo LL, Beniac DR, Booth TF. The scanning electron microscope in microbiology and diagnosis of infectious disease. *Sci Rep.* 2016; 6: 265-76.
26. Ricucci D, Pascon EA, Siqueira JF. The Complexity of the Apical Anatomy. In: Versiani M, Basrani B, Sousa-Neto M, eds. *The Root Canal Anatomy in Permanent Dentition.* Springer, Cham: E-Publishing; 2018: 241-54.
27. Krishan R, Paqué F, Ossareh A, Kishen A, Et al. Impacts of Conservative Endodontic Cavity on Root Canal Instrumentation Efficacy and Resistance to Fracture Assessed in Incisors, Premolars, and Molars. *J Endodon* 2014; 40:1160-6
28. Souza CC, Bueno CE, Kato AS, Limoeiro AG, Fontana CE, Pelegri RA. Efficacy of passive ultrasonic irrigation, continuous ultrasonic irrigation versus irrigation with reciprocating activation device in penetration into main and simulated lateral canals. *J Conserv Dent* 2019;22:155-9
29. Koçak S, Bağcı N, Çiçek E, Türker A, CanSağlam B, and Koçak MM. Influence of passive ultrasonic irrigation on the efficiency of various irrigation solutions in removing smear layer: a scanning electron microscope study. *Microsc Res Tech.* 2017; 80: 537–42
30. Prasanna N, Khadija K, Geoffrey P, Chui Y, et al. Does the Orifice-directed Dentin Conservation Access Design Debride Pulp Chamber and Mesial Root Canal Systems of Mandibular Molars Similar to a Traditional Access Design? *JEndodon* 201