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EFFECT OF DIFFERENT IRRIGATION REGIMENS ON ENTEROCOCCUS FAECALIS ELIMINATION FROM INFECTED ROOT CANALS (AN IN-VITRO COMPARATIVE STUDY)

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EFFECT OF DIFFERENT IRRIGATION REGIMENS ON ENTEROCOCCUS FAECALIS ELIMINATION FROM INFECTED ROOT CANALS (AN IN-VITRO COMPARATIVE STUDY)

Abstract

Microorganisms harbored in the complexities of root canal systems might lead to endodontic failures and development of apical periodontitis. Enterococcus faecalis is the most common isolated bacteria in these cases. Aim: This study was conducted to compare the efficiency of four irrigation regimens on the elimination of an inoculated strain of E. faecalis. Materials and Methods: Forty single rooted extracted premolars were inoculated with a standard strain of Enterococcus faecalis (ATCC 29212) and incubated at 37˚C for two weeks to allow infection of the dentinal tubules. The bacterial suspension was replaced with a new one every 48 hours. The teeth were then divided into four groups of n=10 each, to apply irrigation regimens as follows: Group i: irrigation with normal saline, Group ii: syringe irrigation (NaOCl 2.625%), Group iii: NaOCl 2.625%+EndoActivator and Group iv: NaOCl 2.625%+diode laser. Samples were collected at baseline and after irrigation. Colony-forming unit counts were performed. For baseline count of E. faecalis, differences in means between the groups were tested using the one way ANOVA F test. For the count of E. faecalis after the irrigation, differences in means was assessed using the Kruskal Wallis test. Results: The mean values of % of E. faecalis killed were respectively 99.2 ± 2.53% in group iv, 98.3± 5.34% in group iii, 29.0 ± 5.46% in group ii and 4.77 ± 0.78%) in group i. Conclusion: Maximum removal of E. faecalis strains was achieved by activating 2.625% sodium hypochlorite with the diode laser.

Keywords

Bacterial Eradication, Enterococcus faecalis, Syringe irrigation, EndoActivator, Diode laser.

1. INTRODUCTION

One of the main objectives of root canal treatment is a complete debridement of the root canal system, eliminating the microorganisms and their products as well as organic and inorganic substances from the root canal space (Wu *et al.,* 2006). In this context, chemomechanical preparation of the root canal system is an essential step for endodontic success. However, the complexity of the root canal system and the numerous anatomical variations such as lateral, accessory and secondary canals, apical deltas, and curvatures have an essential impact on the success of endodontic therapy (Khademi *et al.,* 2017). In addition to the influence of the root canal anatomy, it was proven that 35–50% of the root canal walls of the main canal are left untouched with mechanical instrumentation. Therefore, microorganisms persist in these areas (Peters *et al*., 2001). Although various mechanical methods have been developed, an optimal disinfection of the root canal system remains impossible and additional chemical irrigations are needed for a better eradication of the intracanal biofilm (Haapasalo *et al.,* 2014). The mechanical preparation of the root canal system should create an environment facilitating the flow of the irrigants (Li *et al*., 2020). To enhance irrigant dispersal and its activation, many irrigation techniques and devices have been tested and developed. In this context, improved irrigation techniques such as sonic and ultrasonic irrigation, as well as laser-activated irrigation, appear to be more effective than classical syringe needle irrigation (De Moor *et al.,* 2010). Many studies have shown that *Enterococcus faecalis* is the most common bacteria isolated in cases of endodontic failures (Sedgley *et al.,* 2006; Molander *et al*., 2007). *E. faecalis* is a gram-positive facultative anaerobic bacterium which tolerate and adapt to the ecologically demanding conditions in the filled root canals (Ran *et al.* 2015). According to their study, *E. faecalis* resists starvation, survives in environments with deficiency of nutrients and has the ability to flourish again when the nutrient source is reestablished. The aim of this study was to evaluate and compare the effectiveness of four modes of irrigation: saline irrigation, syringe irrigation with 2.625% sodium hypochlorite, sonic agitation with EndoActivator and diode laser activation of the 2.625% sodium hypochlorite on the elimination of the inoculated microbial strains of *Enterococcus faecalis* ATCC 29212 (American Type Collection Culture).

2. MATERIALS AND METHODS

2.1 Teeth Selection

Forty freshly extracted single-rooted (type I root canal) human mandibular first and second premolars of approximate similar size were selected for use in this study. All teeth had completely developed roots and were extracted for periodontal or orthodontic reasons. Approval was obtained by the Institutional Review Board at Beirut Arab University (I.R.B.-B.A.U.) under the number 2014-H-001-D-P-0010. All premolars demonstrating fractured or immature apices were excluded from this study. The presence of a single root canal was determined by preliminary radiographs taken in both mesio-distal and bucco-lingual directions. After taking the periapical radiographs, teeth with external and internal root resorption, calcification, caries, visible cracks or fractures, having more than one canal and/or previous root canal treatments were excluded. The teeth were cleaned to remove blood, plaque, calculus or attached periodontal tissues using prophylaxis brush. Then, they were placed in a disinfectant solution 2.625% NaOCl (Clorox, Vernon, CA, USA) for 30 minutes and stored in distilled water at room temperature. The teeth were then decoronated by using a double-sided sintered diamond disk (Kerr, CA, USA) mounted on a high-speed contra-angle with water coolant. The tooth length was standardized to 15 mm from the root apex to the coronal border. The teeth were then air-dried and steam autoclaved at 121°C for 30 minutes in a Class B autoclave (Mocom, Italy). To get sure of sterilization, specimens were incubated at 37°C for 24 hours and samples from canals of five specimens were obtained using # 35 Hedström file (Mani, Japan) and cultured. No bacterial growth was observed. The apex and the external surface of each root were hermetically sealed with epoxy- resin material (Dutch boy, USA) in order to prevent bacterial leakage. To make handling, instrumentation and identification easier, the roots were mounted vertically in plaster blocks.

2.2 Preparation of the Root Canal

After preparation of the access cavity, the patency of each root canal was established using a #10 K file (DentsplyMaillefer, Ballaigues, Switzerland). Cleaning and shaping of samples were performed using the Protaper Universal rotary nickel titanium system (DentsplyMaillefer, Ballaigues, Switzerland) mounted on an X-SMART Motor (DentsplyMaillefer, Ballaigues, Switzerland) according to the manufacturer's instructions with crown-down technique at a rotational speed of 300 rpm up to F4 (40 / 0.06) reaching the working length (WL). Working length was established at one mm shorter than the apex. After each instrument, the root canal was irrigated with one ml of 2.625% NaOCl solution (Clorox, Vernon, CA, USA) using a disposable three ml luer-lock syringe and 30-gauge needle (Max-i-Probe (DentsplyMaillefer, Ballaigues, Switzerland). Following preparation, all canals received a final irrigation sequence of five ml of 2.625% sodium hypochlorite (0.05 mL/sec) and five ml of sterile water (Baxter sterile water, USA) (Saber and El- Hady, 2012). Each root canal was dried with sterile paper points. Then the teeth were randomly grouped into four groups each one consisting of 10 teeth according to the irrigation modality to be used. Each tooth has been labeled according to its group number.

2.3 Inoculation of the Canal with *E. Faecalis*

Each group of teeth was transmitted into a 500 ml flask each of them containing 100 ml of Tryptic Soy Broth media (Oxoid, England). Bacterial suspension was prepared by growing standard strain of *E. faecalis* bacteria ATCC 29212 (American Type Culture Collection) in brain heart infusion broth (BHI) (Becton, France) at 37˚ C for 24 hours. The number of bacteria was adjusted at around 9 x 10^7 cfu/ml using normal saline. An inoculum from this bacterial suspension was added to each flask and incubated at 37˚C for two weeks to allow infection of the dentinal tubules. During this period, canals were replenished with fresh bacterial suspension every 48 hours in order to preserve a fresh medium and to ensure maintenance of the culture viability (Sohrabi *et al*., 2016).

2.4 Grouping of Teeth.

After the incubation period, root canals were sampled for bacterial counting before applying any irrigation regimen (Type A samples). The canals were divided randomly (simple randomization method) into four groups (i, ii, iii and iv of n=10 each). Subsequently, the samples were submitted to the irrigation modalities as follows:

Group i: Canals were irrigated with normal saline only using a three ml luer-lock syringe and a 30-gauge lateral side Max-i-Probe needle (DentsplyMaillefer, Ballaigues, Switzerland), for two minutes.

Group ii: Canals were irrigated with a final rinse of three ml of 2.625% NaOCl solution (Clorox, Vernon, CA, USA) using a three ml luer-lock syringe and a 30-gauge lateral side Max-i-Probe needle (Dentsply Maillefer, Ballaigues, Switzerland), (0.05 ml/sec) for two minutes. The needle was applied without pressure in a vertical movement of three mm, at 1 mm from the apical end of the preparation.

Group iii: Canals were irrigated with a final rinse of three ml of 2.625%NaOCl solution (Clorox, Vernon, CA, USA) for two minutes. The EndoActivator system (Dentsply Tulsa Dental Specialties, USA) was used to agitate the irrigating solution for 60 seconds in short vertical strokes up and down at 10,000 cycles per minute and 167 Hz with a 25/04 polymer tip (Dentsply Tulsa Dental Specialties, USA). The procedure was repeated three times

Group iv: Canals were irrigated with a final rinse of three ml of 2.625% NaOCl solution (Clorox, Vernon, CA, USA) for two minutes. The diode laser (SIROLaser Xtend, Dentsply Sirona, USA) was applied in the irrigating solution. Root canal decontamination was performed with an optical fiber of 200μm diameter inserted two mm shorter than the working length. Laser irradiation was performed with the irrigant in the canal for a period of 20 seconds, repeated three times at intervals of 10 seconds between each one. Emission and withdrawal of the tip were done with a helicoidal and circumferential motion with a speed of 2mm / second

in order to permit maximum irradiation of the walls. The fiber emitted from its distal end (the beam diverged 15-22° upon exciting the tip). The practitioner was wearing safety goggles (Dentsply Sirona, USA).

2.5 Bacterial Sampling And Microbiological Analysis

All samples were transported and processed in the microbiology laboratory at the faculty of Pharmacy, Beirut Arab University (BAU) within one hour of sample collection. The viable bacterial count in cfu/ml was determined using pour plate method. The root canals were sampled for bacterial counting before and after using the irrigation regimens. Microbial samples (type A) were collected before applying any irrigation modality. Then, each of the teeth in groups i, ii, iii and iv was subjected to an irrigation modality as described before. At the end of the four disinfection protocols, new samples (type B) were harvested. Microbial samples (type A & B) were collected as follows: For each tooth a sterile K 35 file (DentsplyMaillefer, Ballaigues, Switzerland) was inserted in the root canal and the canal walls were slightly touched with an in-out motion, moving the file once around the canal wall. Three successive sterile paper points (size 35) (DentsplyMaillefer, Ballaigues, Switzerland) placed to the full working length for two minutes, were immediately transferred to an Eppendorf tube containing 500 µl of sterile saline solution and glass beads, then vortexed. After serial 10-fold dilutions, 100 µl of the diluted saline were spread on Tryptone Soya Agar (TSA) plates (Oxoid, England) and incubated at 37˚C for 24 hours. The Eppendorf tube was labeled with the tooth number, time and date. The number of colonies was counted and the absolute number of bacteria was calculated based on the Eq. (1)

Eq. (1) $$\text{Viable count (cfu / ml)} = \frac{\textit{Number of colonies} \times \textit{(dilution factor)}}{\textit{Volume taken to plate surface}}$$

2.6 Statistical Analysis

Sample size was determined based on expected large effect size after treatment. An eta square of 0.58 was selected, with 4 groups compared using a one-way Anova, with a pre-specified alpha of 0.05 and a desired power of 80%. The sample size was calculated to be 40: 10 per group (Cohen J., 1988). The values of pre- and post-irrigation were recorded in excel spreadsheet and the statistical analyses were performed using Statistical Package for the Social Sciences (IBM-SPSS V25) software. Means and standard deviations were used to summarize numerical data. Frequency and percentages were used to summarize categorical data. *E. faecalis* count was tested for normality of distribution at baseline and after irrigation using the Shapiro Wilk test. Since normality could be assumed at baseline, difference in *E. faecalis* among treatment groups was tested using the one-way Anova F test. However normality could not be assumed after irrigation and hence difference in *E. faecalis* count after irrigation was tested using the Kruskal Wallis test, the nonparametric equivalent of the ANOVA F test. Pairwise comparisons were carried out once the omnibus *p*-value was significant. The Bonferroni correction was utilized to prevent type I error inflation. Change in *E. faecalis* count from baseline to after irrigation was tested using paired t-test for group i and group ii since normality could be assumed. Wilxocon matched pairs test were used for group iii and group iv since assumption of normality could not be met. Difference in percentages of teeth with persistence *E. faecalis* status after treatment was tested using the Fisher exact test due to the small number of expect cell counts. All analyses were carried out at the 0.05 significance level.

3. RESULTS

3.1 Pre- Irrigation Normal Distribution at Baseline

The four treatment groups had similar count of *E. Faecalis* at baseline. The counts were as follows: 96.6 $x10^6$ for group i, 96.42 $x10^6$ for group ii, 95.5 $x10^6$ for group iii, and 94.8 $x10^6$ for group iv. Pairwise comparison revealed that with the exception of group i versus group ii on one hand (*p*-value=0.105), and group iii versus group iv on the other hand (*p*-value=0.173), all other comparisons were statistically significant (*p*-values <0.001). Nonetheless the differences were minimal.

3.2 Post–Irrigation *E. Faecalis* Status among All Groups

As indicated in Table 1 and as shown as Fig.1, all teeth in group i and group ii retained presence of *E. faecalis* after irrigation. Whereas only one tooth in group iii and one tooth in group iv had a positive status of *E. faecalis* post to irrigation (*p*-value was < 0.001).

Table 1: Percentage of *E. faecalis* persistence status after treatment in the four groups.
Reference: Done by the Authors

Group / Frequency of *E. faecalis*	Group i		Group ii		Group iii		Group iv		*p*-value
	N	%	N	%	N	%	N	%	
Negative	0	0.0%	0	0.0%	9	90.0%	9	90.0%	
Positive	10	100.0%	10	100.0%	1	10.0%	1	10.0%	<.001
	10		10		10		10		

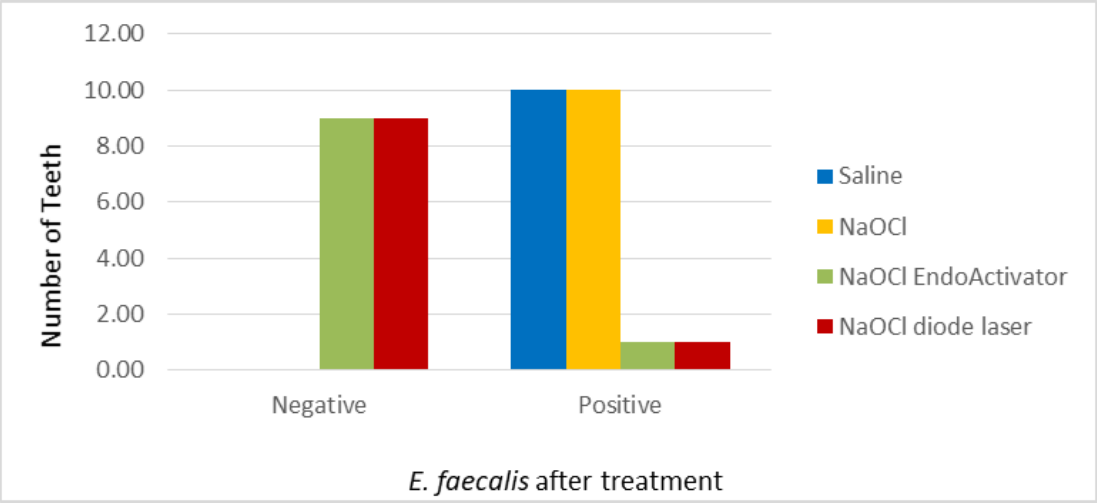

Fig.1: Number of positive and negative status of *E. faecalis* in the four groups.
Reference: Done by the Authors

3.3 Comparison between Pre and Post-Operative *E. Faecalis* Count In Same Group

The drop in the *E. faecalis* average count after treatment in each of the four groups is as indicated in Table 2. The drop in the count was tested for statistical significance using the paired t test for groups i and ii and returned a (*p*-value <.001). For group iii and iv, the Wilcoxon match paired test returned a *p*-value =0.005 indicating a statistically significant change in the mean count of *E. faecalis.*

Table 2: Paired t-test comparing *E. faecalis* average count in the same group before and after treatment.
Reference: Done by the Authors

Mean ± SD of *E. faecalis* count in 10 Million ($x10^7$)	Before treatment	After treatment	Paired t test, *df*=9	*p*-value
Group i	9.66 ± 0.083	9.2 ± 0.128	19.919	<0.001
Group ii	9.742± 0.048	6.918 ± 0.541	16.928	<0.001
			Wilcoxon Matched pairs test	
Group iii	9.555± 0.097	0.159± 0.503	2.805	0.005
Group iv	9.48 ± 0.056	0.076 ±0. 24	2.805	0.005

* Wilcoxon match paired test was used since normality assumption could not be assumed.

The overall comparison of the group means was tested using the Kruskal Wallis test. The *p*-value was <0.001 indicating that there was a statistical significance among the four groups' means as shown as Fig. 2.

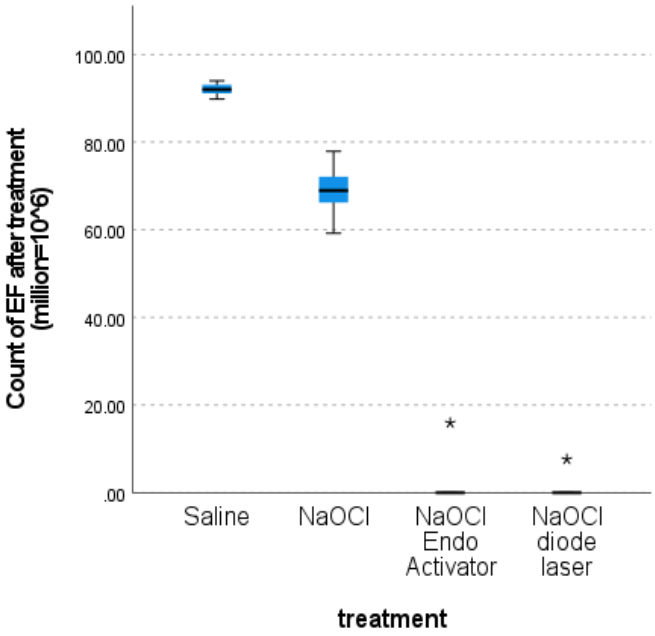

Fig.2: Overall comparison of the groups' results after treatment, using the Kruskal Wallis test
Reference: Done by the Authors

3.4 Pair-Wise Comparisons of Treatment Modalities

Kurskal Wallis pair-wise comparisons of groups' results after treatment were as indicated in Table 3. Each row is testing the null hypothesis that both groups distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .050. Significant values have been adjusted by the Bonferroni correction for multiple tests.

Table 3: Kurskal Wallis pair-wise comparisons of groups with Bonferroni adjusted *p*-values
Reference: Done by the Authors

Compared Groups	Test Statistic	Std. Error	Std. Test Statistic	*p*-value	Bonferroni Adj. *p*-value
Group iv- Group iii	.100	4.985	.020	.984	1.000
Group iv- Group ii	15.050	4.985	3.019	.003	.015
Group iv- Group i	25.050	4.985	5.025	<.001	<.001
Group iii- Group ii	14.950	4.985	2.999	.003	.016
Group iii- Group i	24.950	4.985	5.005	<.001	<.001
Group ii- Group i	10.000	4.985	2.006	.045	.269

3.5 Comparison of the Groups Ii, Iii and Iv Versus Group I After Irrigation

The difference between the three active treatment modalities (groups ii, iii and iv) and the saline group (group i) was tested using the Kruskal Wallis multiple comparison correction techniques. The *p*-values are as indicated in Table 3. The mean count of group ii showed no statistical significance when compared to that of group i .When comparing the mean count of group iii to group i, *p*-value indicates a statistical difference. When comparing the mean count of group iv, to group i, *p*-value indicates a statistically significant difference.

3.6 Comparison of Groups Iii and Iv Versus Group Ii After Irrigation

As indicated in Table 3, the Kruskal Wallis multiple comparison correction test gave *p*-value indicating a statistical difference between groups ii and iii. Similarly, a statistical significance between groups ii and iv was revealed by the *p* value obtained,

3.7 Comparison between Groups Iii versus Group Iv After Irrigation

As indicated in Table 3, the results of Kruskal Wallis multiple comparison correction test for the mean count of *E. faecalis* between groups iii and iv showed a (*p*-value =.999) indicating that there was no statistical significance between groups iii and iv.

4. DISCUSSION

In the current *in-vitro* study, the choice of *E. faecalis* as a unique bacterium, was based on its behavior in infected root canals. It endures a prolonged period of nutritional deprivation, binds to dentin and invades dentinal tubules. According to Swimberghe *et al.* in 2019, our set-up is one of the most common biofilm model systems. However, incubation times of *E. faecalis* differed considerably, ranging from one to seventy days. In our study, we tried to simulate the typical clinical scenario of the canal surinfection as it is well known that when a tooth is infected, the pathology can easily amplify if the cause is not treated. In the present *in-vitro* study, regarding the incubation time of *E. faecalis*, the inoculum from the *E. faecalis* bacterial suspension was incubated for two weeks to allow infection of the dentinal tubules and to permit the formation of biofilms. During this period, the bacterial suspension was replaced with a new one every 48 hours in order to preserve a fresh medium (Sohrabi *et al.*, 2016). A lot of studies were carried out in this field. Gergova *et al*. in 2016, showed that 48 hours were enough for the *E. faecalis* bacterial strains to build well-formed biofilms.

The aim of our *in-vitro* study was to assess the efficacy of four irrigation modalities in removing the inoculated bacterial population of *E. faecalis* from the root canal system. The present study was based on mandibular human premolars with a single root canal. The used methodology allowed the standardization of samples in order to reduce the bias. This was confirmed by the homogeneity of the initial samples, two weeks after bacterial inoculation with a standard strain of *E. faecalis* ATCC 29212 (American Type Culture Collection). After implementation of the four investigated irrigation protocols, sampling was performed. Our results revealed that the agitation of 2.625% sodium hypochlorite by the EndoActivator in group iii, and the activation by the diode laser in group iv significantly lowered the cfu of *E. faecalis* compared to the initial ratio.

In the present *in-vitro* study, intragroup analyses were performed using the paired t-test for related samples. The drop in the *E. faecalis* count after treatment in each of the four groups returned a *p*-value < .001) indicating a statistically significant change in the mean count of *E. faecalis* in each of the four groups postoperatively. Intergroup analyses were performed using Kruskal Wallis test and the following results were obtained.

Regarding the postoperative mean count of *E. faecalis*:

No statistical significance between normal saline (group i) and 2.625% NaOCl syringe irrigation (group ii). These results are in accordance with those of Afkhami *et al.* in 2017. Their findings did not reveal differences between conventional NaOCl irrigation and saline irrigation.

When comparing each of the EndoActivator group (group iii) and the diode laser group (group iv) to the saline group (group i), the postoperative mean count of *E. faecalis* showed a reduction indicating a significant statistical difference between group iii versus group i and between group iv versus group i. Our results are in agreement with those obtained by Brito *et al.* in 2009 who found a significant reduction of intracanal *E. faecalis* count when comparing agitation with the EndoActivator to saline irrigation. The results of the current *in- vitro* study are also in accordance with those of Sun & Zhu in 2017 who concluded that specimens treated with diode laser radiation showed great effect of elimination against biofilm of *Enterococcus faecalis* compared with saline irrigation.

A significant statistical difference was reported between 2.625% NaOCl syringe irrigation and the EndoActivator groups. Our results are also in accordance with those of the *in-vitro* study conducted by Al- Obaida *et al.* in 2019. A significant statistical difference was also reported between 2.625% NaOCl syringe irrigation group and diode laser group. This result is in accordance with the results of Shaktawat *et al.* in 2018 who found that diode laser irradiation along with irrigation protocols was more efficient against *E. faecalis* when compared with the groups subjected to irrigation protocol alone.

In the current *in-vitro* study, there was no statistical significant difference regarding the decrease of *E. faecalis* colony forming units when 2.625% NaOCl was activated by either the EndoActivator or the diode laser although the clinical findings showed a better reduction of the bacteria in the diode laser group. Our results are in agreement with those obtained by Neelakantan *et al.* in 2015, who found that diode laser was the most effective in reducing *E. faecalis* biofilms.

From the activation systems that we used in our study, diode laser activation and sonic agitation contributed positively to both the mechanical and the chemical aspects of the irrigation procedure. Their contribution improved the outcome of endodontic treatments. Biofilm research in endodontics is still an open field that should contribute into a better understanding of mechanisms of infections.

5. CONCLUSION

Within the parameters of this study:

A- Regarding the removal of *E. faecalis* strains from root canals, there was no statistical significant difference between the EndoActivator and diode laser groups.

6. ACKNOWLEDGEMENT

The Authors declare that there are no conflicts of interest in this study.

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