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Antibiofilm Effect of Different Irrigation Solutions Activated with KTP Laser

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Research Article

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History

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ABSTRACT

Objectives: *The aim of this study was to evaluate the antimicrobial efficacy of Potassium Titanium Phosphate (KTP) laser-activated irrigation solutions on intraradicular Enterococcus faecalis biofilms in in-vitro conditions by using a scanning electron microscope.*

Materials and Methods: *120 single-root, single canal permanent mandibular premolar human teeth were used. Sterilization and disinfection of the teeth were performed. Enterococcus faecalis biofilms were obtained after 4 weeks by re-inoculation procedures. Each group was divided into 6 groups consisting of 20 roots and root canal disinfection protocols were applied using irrigation solutions with 5.25% NaOCl, saline, super-oxidized water solution, 8 ppm ozonated water, 2% CHX, 17% EDTA, all activated by KTP laser. It was smear-planted to a solid medium which split as before and after the disinfection applications for the aim of Counting Microorganism colonies from root canals and data were evaluated statistically. In this statistical evaluation one way ANOVA and Tukey tests were used. Before and after the irrigation procedures for the presence and elimination of biofilm the root canals were processed for scanning electron microscopy and biofilm was examined on the standard images.*

Results*: As a result of the statistical comparison performed among all groups, while NaOCl ensures the highest amount of elimination as a positive control group, the lowest amount of bacterial elimination was detected in the saline group that applied as the negative control group ($p<0.05$). None of the experimental groups achieved the whole elimination of Enterococcus faecalis biofilm. While there was no statistically significant difference between super-oxidized water and aqueous ozone groups that indicated the strongest antibiofilm effect ($p>0.05$), EDTA showed the lowest antibiofilm effect ($p<0.05$).*

Conclusions: *The use of 5.25% NaOCl solution activated by KTP laser, which shows the highest antibiofilm efficiency among the study groups, in clinical applications is very effective in terms of biofilm elimination in root canal treatments and is especially promising in the success of long-follow-up treatments. However, the KTP laser activation procedures of super-oxidized water solution and 8 ppm ozonated water may be insufficient as a safe disinfection method.*

Keywords: *Biofilm, laser-activated irrigation, Enterococcus faecalis, KTP laser.*

KTP Lazer ile Aktive Edilen Farklı İrrigasyon Solüsyonlarının Antibiyofilm Etkisi

Bilgi
 # Bu çalışma 22-24 Kasım 2022 tarihleri arasında düzenlenen 'Sivas Cumhuriyet Üniversitesi 2. Uluslararası Diş Hekimliği Kongresi'nde sözlü bildiri olarak sunulmuştur.

Süreç

Geliş: 16/11/2022
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ÖZ

Amaç: *Bu çalışmanın amacı, Potasyum Titanyum Fosfat (KTP) lazer ile aktive edilen irrigasyon solüsyonlarının intraradiküler Enterococcus faecalis biyofilmleri üzerindeki antimikrobiyal etkinliğinin taramalı elektron mikroskobu kullanarak in vitro koşullarda değerlendirilmesidir.*

Gereç ve Yöntemler: *Bu çalışmada 120 tek kök tek kanallı daimi mandibular küçük azı insan dişleri kullanıldı. Dişlerin sterilizasyon ve dezenfeksiyon işlemleri yapıldı. Enterococcus faecalis biyofilmleri re-inokülasyon prosedürleri ile elde edildi. Her grup 20 kökten oluşan 6 gruba ayrıldı ve tamamı KTP lazer ile aktive edilen %5,25'lik NaOCl, serum fizyolojik, süper okside su solüsyonu, 8 ppm ozonlu su, %2'lik CHX, %17'lik EDTA ile irrigasyon protokolleri uygulandı. Kök kanallarından Mikroorganizma kolonilerinin sayımı amacıyla dezenfeksiyon uygulamaları öncesi ve sonrası olarak ayrılan katı besiyerine smear ekilmiş ve veriler istatistiksel olarak değerlendirilmiştir. Bu istatistiksel değerlendirmede tek yönlü ANOVA ve Tukey testleri kullanılmıştır. Biyofilm varlığı ve ortadan kaldırılması için irrigasyon işlemlerinden önce ve sonra kök kanalları taramalı elektron mikroskobu için işlendi ve standart görüntüler üzerinde biyofilm incelendi.*

Bulgular: *Tüm gruplar arasında yapılan istatistiksel karşılaştırmalar sonucunda NaOCl pozitif kontrol grubu olarak en yüksek miktarda eliminasyonu sağlarken, en düşük bakteri eliminasyonu negatif kontrol grubu olarak uygulanan serum fizyolojik grubunda tespit edilmiştir ($p<0,05$). Deney gruplarının hiçbiri, Enterococcus faecalis biyofilminin tamamen ortadan kaldırılmasını sağlamadı. Antibiyofilm etkinliği açısından en güçlü etkiyi gösteren süper okside su ile ozonlu su grupları arasında istatistiksel olarak anlamlı bir fark bulunmaz iken ($p>0,05$); en düşük antibiofilm etkiyi EDTA grubu göstermiştir ($p<0,05$).*

Sonuçlar: *Çalışma grupları arasında en yüksek antibiyofilm etkinliği gösteren KTP lazer ile aktive ettiğimiz % 5,25' lik NaOCl solüsyonunun klinik uygulamalarda kullanılmasının kök kanal tedavilerinde biyofilm eliminasyonu açısından oldukça etkili ve özellikle uzun takipli tedavilerin başarısında umut vericidir. Ancak süper oksitlenmiş su solüsyonu ve 8 ppm ozonlu suyun KTP lazer aktivasyon prosedürleri güvenli bir dezenfeksiyon yöntemi olarak yetersiz kalabilir.*

Anahtar Kelimeler: *Biyofilm, Lazer ile Aktive Edilen İrrigasyon, Enterococcus faecalis, KTP Lazer.*

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Introduction

The elimination of resistant microorganisms in deep areas of the root canal system is the main problem in today's treatment protocols. One of the main goals of endodontic treatment is the elimination of microorganisms that cause periapical periodontitis from the infected root canal system.[1] The most common bacterial species in resistant and secondary infections is *Enterococcus faecalis* (*E. faecalis*). The fact that *E. faecalis* has never been isolated or has been isolated at low levels in teeth that have not previously applied root canal treatment proves that this bacterial species is one of the main bacterial species that cause root canal treatment failures.[2,3] In the successful implementation of the shaping and cleaning of the root canals; It has been stated that the complete removal of the vital and necrotic pulp tissue, affected dentin tissue and other residues in the root canal and disinfection of the root canal cavity play a very important role.[4]

The use of lasers in endodontics increases the success rate of root canal treatment. Laser beams are used to remove debris and smear layer in endodontics, to penetrate the dentin tissue more and to reach the areas that cannot be reached in the complex structure of the root canal system more than traditional methods, and to eliminate the disadvantages of the chemomechanical preparation.[5,6] It is claimed that lasers can reach the inaccessible areas of the root dentin tubules and progress to a depth of more than 1000 µm, showing a specific antibacterial effect and eliminating these negative factors by ensuring that all microorganisms in the deep layers can be eliminated.[7]

In light of all this information, this study aims to evaluate the antimicrobial activities of the irrigation activation procedure with potassium titanium phosphate (KTP) laser activation on the *E. faecalis* biofilm layer formed in the root canal by scanning electron microscopy in vitro.

Materials and Methods

The study was initiated after the ethics committee approval dated 14.07.2015 and numbered 2015-07/08 was obtained from Cumhuriyet University Clinical Research Ethics Committee. The present study was carried out by Sivas Cumhuriyet University Faculty of Dentistry, Department of Endodontics clinic, and Sivas Cumhuriyet University Faculty of Pharmacy, Department of Pharmaceutical Microbiology clinic.

In the present study, 120 permanent human mandibular premolar teeth, which were extracted for orthodontic or periodontal reasons, without caries, and without restoration, were used. The pulp tissue in the root canals was removed using tirnerf (Vereinigte Dentalwerke GmbH & Co. KG, Munich, Germany) and the canal path was determined by entering the root canals with #15 K-File (Mani Inc., Tochigi, Japan) hand instruments. The length of the canal tool was measured by advancing until the tip of the canal tool was visible in the apical opening, and the working length was determined for each root by retreating 1 mm from this measured length. Root canals were shaped using ProTaper Next rotary files (Dentsply Maillefer, Ballaigues, Switzerland) using X1, X2, and X3 files respectively, and the apical third of all canals were standardized with X3. During the preparation, the canals were irrigated with 1 ml of 5.25% NaOCl solution after each file use. To remove the smear layer formed during preparation, the root canals are then were irrigated using sequentially 17% EDTA (AppliChem GmbH, Germany), 5.25% NaOCl, and distilled water and were dried with a paper point. Before microbiological applications, the glass bottles in which we will place the teeth were packed in groups of 10 and sterilized at 121 °C for 20 minutes (min) by placing them in an autoclave to ensure sterilization (Melag, Euroclave 23V-S, Germany). Then, randomly selected teeth were sent to Ethylene Oxide (EtO) sterilization, 10 in each package. As a result, to use in the present study, first of all, all teeth were root canal preparations, disinfection, and sterilization processes were completed and placed in sterile glass bottles with rubber caps. Following these processes, 120 teeth, which were purified from microorganisms and placed in the bottles, were obtained. The teeth placed in the bottles were divided into 6 groups for *E. faecalis* inoculation, with 20 teeth in each group.

Contamination with *E. faecalis* Biofilm

E. faecalis (ATCC 29212) strains were cultured on blood agar (Brain-heart infusion agar, Acumedia Manufactures, Inc., Lansing, Michigan, USA) and were incubated at 37 °C for 24 hours (h). Before each experiment, 0.5 McFarland turbidity was set with a kristalspecTM device. Then was subcultured on Trypticase soy broth (Detroit, Michigan, USA) and incubated aerobically at 37 °C for 24 h. The turbidity of *E. faecalis* culture was adjusted to No. 0.5 Mc Farland Standard. The value of 10 µl of bacterial suspension (Final concentration of about 1.5 X 10^8) was transferred to the mechanically expanded lumen of the root canal using a sterile micropipette except for 10 canals which were preferred as negative control and then kept at 37 °C for 24 h. The entrance of root canals was sealed with temporary filling material (Cavit; 3M ESPE, USA). All samples were stored at 37 °C for 10 days in a humidity atmosphere and the reinoculation procedure was repeated every 72 h with fresh culture on the first, fourth, seventh, and tenth days. The biofilm's scanning electron microscopy (SEM) micrograph was examined at 10,000x magnification, as shown in Figure 1.

Experimental and Control Groups with Activation Procedures

Samples in which *E. faecalis* biofilm was obtained in root canals were randomly selected to have 20 teeth in each group. Then, root canals were disinfected with six types of irrigation solutions each activated by the 2 Watt (W) KTP laser applications. The same KTP laser activation procedure was applied to irrigation solutions groups formed as 5.25% NaOCl, physiological saline, super-oxidized water solution, 8 ppm ozonated water, 2% CHX, 17% EDTA, and this standard application is as follows;

Root canals of 20 teeth infected with *E. faecalis* are irrigated with these irrigation solutions with a flow rate of 2.5 ml/min for 15 seconds (s), followed by laser activation with a 200 µm diameter fiber optic tip of the 2W KTP laser for 15 s with continuous circular movements from the apex to the coronal done. This cycle was repeated 6 times. The total volume of the irrigant was 7.5 ml. The total time of the protocol was determined as 3 min and then the remaining bacteria in the root canal were counted.

Microbiological Count and SEM Examination

After the irrigation activation procedures were applied to the root canals of the teeth contaminated with *E. faecalis*, the samples were taken into glass tubes with 5 ml BHI, after waiting for 5 min, sterile paper cones numbered 40 were placed in the root canals moist with the irrigated solution. After the Eppendorf tubes were vortexed for 5 min, samples were taken with sterile plastic specials and the second half of the bloody medium was inoculated. The inoculated petri dishes were kept in an oven at 37 ºC for 24 h and then counted according to the Colony Forming Units (CFU) classification. The antibacterial activity indicators of the study groups were recorded by calculating the bacterial counts obtained as a result of the first and last bacterial sowing. Two of the roots in the experimental and control groups to be used as imaging samples were randomly divided into two vertically with sterile separators, and the used parts were fixed in 4% glutaraldehyde for 3 h at 4 °C before imaging. Then, it was exposed to dehydration by keeping it in ethanol solutions at increasing concentrations (40%, 50%, 70%, 80%, 90%, 100%). After drying the dehydrated samples at room temperature, they were coated with gold-palladium and examined by SEM.

Statistical Analysis

The data of our study were evaluated by uploading them to the SPSS (Ver:22.00) program. While evaluating the data, one-way ANOVA and Tukey test was applied.

Results

The logarithms of the counting results obtained after the application were taken and the minimum, maximum, mean and standard deviation, and median (median) values are shown in Table 1. In the present study, statistically significant differences were found between the NaOCl and saline groups used as the control groups and the other 4 experimental groups ($p<0.05$). While the ozonated water and superoxide water groups did not show a statistically significant difference ($p>0.05$), the differences between these groups and the other experimental groups were found to be statistically significant ($p<0.05$). Among the experimental groups, super oxidized water and ozonated water showed the strongest antibacterial effect statistically in infected root canals ($p<0.05$).

Discussion

This study aims to evaluate the antimicrobial activities of irrigation with KTP laser activation on *E. faecalis* biofilms formed in root canals by scanning electron microscopy in vitro.

In our study, *E. faecalis* biofilms were formed in vitro for 1 month as a monoculture on the root dentin walls of an extracted single root, single canal teeth. The presence of *E. faecalis* biofilms formed in the first step and the penetration of *E. faecalis* into the dentinal tubules were determined by scanning electron microscopy. In the second stage, the biofilm elimination efficiency of irrigation solutions activated by the KTP laser on *E. faecalis* biofilms formed in the canal was determined. The purpose of using the *E. faecalis* biofilm is that this bacterium is highly resistant to chemical and mechanical processes and is one of the main factors of treatment failures. *E. faecalis* is a facultative anaerobic gram (+) test microorganism that causes resistant apical inflammation and is found in monocultures.[8] *E. faecalis* is the bacterium that is the main factor in failures after root canal treatment.[9] Because of the low nutritional conditions and resistance to drugs used during root canal treatment, it can maintain its vitality in difficult conditions.[10]

Pinheiro et al.[8] isolated *E. faecalis* in 52.94% of unsuccessful root canal treatments. They explained these rates by the fact that the vital and virulence factors of *E. faecalis* are high, and that they consume the nutrients in the environment in a way that does not allow other microorganisms to live by showing more intense invasion into the dentinal tubules compared to other microorganisms.

In studies on biofilm, the time required for biofilm formation varies. Biofilms were formed for periods ranging from 24 h to 6 weeks, and the efficacy of antimicrobial agents was evaluated.[11,12] However, no standardization could be determined regarding the biofilm formation time in the studies. In our study, fresh *E. faecalis* suspension was injected into the roots every other day for 4 weeks to form a mature biofilm.

Within the scope of our study, the SEM technique was used to view our biofilm samples formed in dental tissue. Scanning electron microscopy has been frequently used in the literature to observe biofilm formation within the tooth.[13, 14] Yañez *et al.*[15] reported that the use of SEM techniques in imaging provides excellent depth of field and is quite suitable for describing morphology. Laser dentistry is mainly used in surgery, periodontal procedures, and operative procedures. However, the use of lasers in the field of endodontics has a very high potential.

Our study, it was aimed to activate different irrigation solutions used in endodontic treatments with the KTP laser, which has a halved wavelength Nd:YAG laser system, and thus to evaluate the antibiofilm efficacy of this disinfection procedure in root canals.

There are not enough studies about KTP laser in the literature. Therefore, since the KTP laser is a halved

Nd:YAG laser system; Some studies in the literature related to the Nd:YAG laser system are included in our discussion.[16, 17]

Nd:YAG lasers are frequently preferred devices for removing the smear layer, removing debris, cleaning, shaping, disinfecting the root canal system, and covering the apical region after apicectomy. It has been found that Nd:YAG laser reduces periapical inflammation, accelerates the drying of the root canal lumen, and has a bactericidal effect while removing dentin.[16, 17] Gutknecht *et al.*[18] determined that the Nd:YAG laser applied at a pulse frequency of 15 Hz at an energy level of 1.5 W produced an antibacterial effect of 97.12-99.91%.

In a study, it was reported that Nd:YAG laser beams showed a high antimicrobial effect on gram-positive and gram-negative microorganisms in the dentin distant from the canal. It has been stated that microorganisms such as *E. faecalis* are very sensitive to laser beams[19]. Schoop et al.

[20] When the KTP laser was applied to *E. faecalis* with 1 W, there was a significant decrease in the number of microorganisms, while the effectiveness was higher when they applied 1.5 W, so in the present study, it was thought that it could make irrigation more effective and the KTP laser was applied at 2W power. To provide a more homogeneous effect on the dentin surface, the laser beam must be applied from the lateral parts of the fiber optic cable, not only from the end. Therefore, in our study, the fiber optic tip was applied with circular movements from apical to coronal.

The cleaning efficiency of the laser-activated irrigation technique; depends on the dynamics of the steam bubble formed as a result of pulse beats. While each pulse of the laser accelerates the fluid flow, a constant flow rate is observed in conventional irrigation. Therefore, laser activation is more successful than conventional irrigation. For this reason, we activated different irrigation solutions with the KTP laser in our study.

In the efficiency of the irrigation solution; concentration, application volume, application time, temperature, and pH level have been reported to be important.[21] All of the irrigation solutions tested in our study were used at room temperature, in the same volume, and at the most effective concentrations known.

According to the results of our study, 5.25% NaOCl, which we activated with a 2 W KTP laser, which we used as the positive control group, was found to be the most effective agent in eliminating *E. faecalis* biofilm in both culture methods and SEM examinations.

Retamozo et al.[22] used 450 dentin samples obtained from bovine incisors in their study to determine the sufficient NaOCl concentration and irrigation time to disinfect dentin samples infected with *E. faecalis*. After infecting these samples with *E. faecalis*, they applied NaOCl solution at concentrations of 1.3%, 2.5%, and 5.25% for 5, 10, 15, 20, 25, 30, 35, and 40 min. At the end of their study, they reported that they obtained the most effective result with the application of 5.25% NaOCl solution for 40 min, and the application of 1.3% and 2.5% NaOCl at the same time was insufficient to eliminate *E. faecalis*. They emphasized that a high concentration of NaOCl and a long application time are needed to ensure the complete elimination of *E. faecalis*-contaminated dentin. In our study, NaOCl was used at a rate of 5.25%.

In a study comparing the antibacterial activity of 980 nm wavelength diode laser and 5.25% NaOCl against *E. faecalis*; Compared with the diode laser, NaOCl was reported to be successful in eliminating 99.87% of *E. faecalis*.[23]

In a study evaluating the antimicrobial activities of NaOCl, MTAD, and Tetraclean against *E. faecalis* biofilm, the only solution that could destroy *E. faecalis* biofilm within five min was 5.25% NaOCl, and MTAD and Tetraclean were totally eliminated. It has been stated that they need a longer period.[24]

In another study evaluating the effectiveness of various irrigation solutions against E. facealis, which is in planktonic and biofilm form; It has been reported that NaOCl, used at a rate of 3%, is the most effective agent and eliminates *E. faecalis* in both forms in 2 min.[25]

In light of the results of these studies, the antimicrobial activity of 5.25% NaOCl on the *E. faecalis* biofilm was examined as the positive control group, and the results of our study were similar to the results of the present study mentioned above.[26- 28]

Although the saline solution, which we used as the negative control group in our study, was found to be the most ineffective agent among all groups, it caused a decrease in the number of bacteria in parallel with the literature studies.

Although it is said that EDTA is a chemical agent that does not have an active antimicrobial effect, it is known that it causes a decrease in the number of bacteria in the canal by removing the inorganic component of the smear layer formed on the root canal walls.[29]

In a study examining the antibacterial activities of 17% EDTA, 2% CHX, 0.2% cetrimide, MTAD, and QMix against *E. faecalis* biofilm formation on dentin blocks for 2 min, the antibacterial activity of 17% EDTA against *E. faecalis* biofilm formation was higher. found low.[30]

In the study, in which the antimicrobial activities of 5.25% NaOCl, 10% citric acid, 17% EDTA, 3% H2O2, 0.2% cetrimide and saline solution as the control group, against *E. faecalis* and E. coli in root canals were evaluated, % in both groups. 17% EDTA solution has been reported to have the lowest antimicrobial activity.[31] Parallel results were also obtained in our study. The fact that the antibiofilm effect is less than the other experimental groups can be attributed to its low antimicrobial property.

In a study examining the effects of various irrigation solutions against *E. faecalis* biofilm in root canals, no significant difference was found between the application time of 2% CHX solution used for 1 and 5 min, and the rate of destruction of *E. faecalis* biofilm by 2% CHX was found 60.49%.[32] In a study evaluating the effects of NaOCl, EDTA, citric acid, phosphoric acid, and 2% CHX against *E. faecalis* biofilm, NaOCl was found to be the most effective agent; followed by 2% CHX; EDTA, citric acid, and phosphoric acid were found to be ineffective against *E. faecalis* biofilm.[33]

In parallel with the literature information mentioned above, the 2% CHX solution, which we used in our study, was found to be more successful in eliminating *E. faecalis* biofilm than the 17% EDTA solution, while it was found to be less successful compared to the other experimental groups.

There are also studies in the literature that do not show parallelism with the results of our study. *E. faecalis* was inoculated into the canals of single-rooted maxillary teeth for 60 days, and then, in a study examining the antibacterial activities of ozonated water, gaseous ozone, 2.5% NaOCl, and 2% CHX washing solutions, none of the washing solutions used in 20 min. It has been reported that it has no antimicrobial effect against *E. faecalis* during the contact period. In our study, however, the 5.25% NaOCl solution that we used completely eliminated the *E. faecalis* biofilm, while the 2% CHX solution caused a decrease in the biofilm layer. We think that the different findings are due to the different incubation times and the method of obtaining the bacteria.[34]

Super oxidized water is a solution that has been widely used in recent years because it is non-toxic, biocompatible, safe for patients and the environment, and inexpensive.[35] However, there are few studies in the literature evaluating the antimicrobial effect of super-oxidized water.

In a study investigating the in vitro efficacy of super oxidized water against various microorganisms including *E. faecalis* 29212 strain at different concentrations, Medilox super oxidized water was found to be effective in all standard and clinical strains at 1/1 dilution for 1 min and at all other test times.[36]

There are significant limitations to the application of lasers within the root canal system. Laser energy from the optical fiber tip or laser guiding tip travels directly through the root canal before it has a chance to reach the lateral canals. Therefore, it is not always possible to maintain a uniform contact area along the canal surface using a laser. Many researchers have shown that during the interaction between the laser and the tooth structure, photon energy is converted into heat energy. This heating effect must be carefully controlled to avoid damage to the vital cells of the surrounding tissues.[37] Successful endodontic treatment depends on the elimination of all microorganisms. Continuity of infection is the most important cause of failure in endodontic treatment. Fabricius et al.[38] showed that bacteria can survive for many years in treated root canals and stated a significant relationship between nonhealing apical periodontitis and the presence of bacteria. For these reasons, the major role of the disinfection procedure applied during root canal treatment in the success of the treatment should always be considered.

Conclusion

Activation of ozonated water and super-oxidized water irrigation solutions with KTP laser showed strong antibacterial activity. However, it could not completely eliminate the bacteria in the canal. Considering the logarithmic growth of bacteria, it is obvious that bacteria can reach their maximum numbers again in a suitable environment in a very short time as a result of not being able to be completely eliminated. It is known that the prognosis for recovery will be successful if the bacteria can be completely eliminated during root canal treatment. Therefore, the activation of these irrigations with a 2 W KTP laser for disinfection in root canals will not provide the expected success. For this reason, we think that laser-activated disinfection systems can only be used as supportive treatment in root canal treatment.

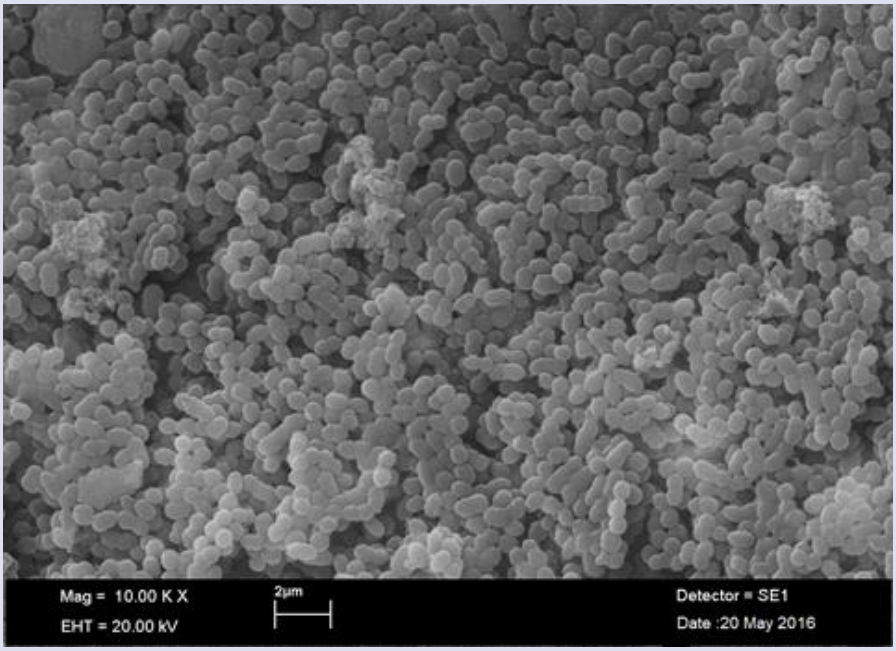

Figure 1. SEM images of Enterococcus faecalis biofilms obtained from the root canal at 10,000x magnification after four weeks

Table 1. The minimum, maximum, mean and standard deviation, median values with statistical comparisons between groups with log CFU count values obtained after activation procedures

Groups		Minimum	Maximum	Mean±standard deviation (Log CFU mL^{-1})	Median
Group 1	Saline	4.000	7.000	6.36±1.03	7.000
Group 2	NaOCl	0.000	0.000	0.00±0.00	0.000
Group 3	Super-Oxidized Water	0.700	1.100	0.86±0.13[a]	0.800
Group 4	Ozonated Water	0.600	1.500	1.10±0.27[a]	1.000
Group 5	CHX	1.400	2.600	1.77±0.35	1.800
Group 6	EDTA	2.200	3.300	2.71±0.35	2.600

Superscripts with the same letters ([a]) indicate no sign of the difference between groups. ($p<0.05$)

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