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ORIGINAL ARTICLE

Comparing the Effect of Green Tea, Calcium Hydroxide and Chlorhexidine on *Enterococcus faecalis* biofim in Root Canal system (*Ex vivo*)

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ABSTRACT
Since removing microorganisms is necessary for treating the root canal, thus intracanal medicament are recommended between visits for reducing the bacteria in the root canal system. Enterococcus faecalis is a gram-positive bacterium that is usually separated from permanent infections of root canal. The purpose of this study was to evaluate the efficacy of Green tea and Calcium hydroxide and Chlorhexidine gel on the elimination of Enterococcus faecalis biofilm in root Canal system (Ex vivo). In this study 54 single-rooted and single-canal teeth that had the study conditions were prepared by the use of Protaper system. After autoclaving samples, the suspension of Enterococcus faecalis bacterium was inseminated inside the teeth. Samples were kept in 37 degree centigrade in orbital incubator for 2 weeks and for ensuring the formation of biofilm, 4 samples were surveyed by the use of SEM. After that the teeth were divided into three groups of experimental (n= 16) and control (n= 2) groups. In first experimental group calcium hydroxide, in second group chlorhexidine and in third group the green tea were put into the canal and samples were kept in incubator for 1 week. After one week, samples were washed with normal saline and then the sampling was conducted by the use of Hedstrom file and paper cone and the extracted fluid resulted from vortex of dentin were cultivated and CFU calculation was conducted 48 hours later, achieved data was surveyed by the use of Kruskal Wallis statistical test. Results showed that although there is a significant difference between three groups in terms of CFU amount, all three groups significantly decreased the percentage of Enterococcus faecalis. The current study findings showed that regarding the high sterilization of root canal by green tea and considering the benefits of this natural herbal extract such as fair price, low toxicity, anti-inflammatory properties and lack of side effects of calcium hydroxide and chlorhexidine, green tea could be used as an interacanal medicament in endodontics.
Keywords: *Green tea Extract, Calcium Hydroxide, Chlorhexidine, Biofilm of Enterococcus faecalis.*

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INTRODUCTION

Removing microorganisms existing in root canal is still a big problem for RCT [1, 2]. Although biomechanical preparation could effectively decrease microbiota, these procedures could not completely remove bacteria in the lateral and accessory root canals [3]. Since removing microorganisms is necessary for RCT, thus interacanal medicament during visits is recommended for more bacteria reduction in root canal system [2-4]. Researches show that *Enterococcus faecalis*, anaerobic gram-positive bacteria, is the most common type of bacteria found in teeth with root treatment failure [5]. Also researches show that compared to the planktonic form, when it is in the form of biofilm, the resistance of this bacterium to antibiotics increases to thousand times [6].

Nowadays different types of materials with antimicrobial properties are used as a intaracanal medicament and the most common materials are calcium hydroxide and chlorhexidine that have problems such as effect on dentine and changing the tooth color [7-11].
Calcium hydroxide was firstly introduced by Hermann in 1920 as a pulp caping material [12]. Calcium hydroxide is a soft white powder with no smell and chemical formula of Ca(OH)2 and in small amounts it is soluble in water and chemically speaking it is a strong base material and usually it owes its desirable properties to its alkaline PH [12, 13].
Bactericidal properties, liquidation of canal tissue remains, OH release and stimulating and mineralizing tissue are some of the important properties of this material [4, 14]. Fatal effects of hydroxyl ions on bacteria cells are perhaps due to the bacterial membrane damage, protein denaturation and damage to DNA [4]. Chlorhexidine is a wide range antibacterial material that due to its cationic nature is able to connect to bacteria with negative load and could damage the outer layers of cell and increase its permeability. This material has been studied both clinically and in laboratory as irrigant and intracanal medicament [15, 16]. Regarding their naturalness and fewer side effects, herbs have attracted the attention of many researchers in medical sciences. Green tea is produced from young leaves of Camellia Sinensis and due to its antioxidant, anticancer properties, cardiovascular effects, antibacterial effects and inflammatory effects, several researches have been conducted on it. Green tea includes antioxidant, polyphenol and also a wide range of vitamins and minerals [17, 18].
Some studies have suggested using natural extracts of plants for infections related to biofilm [19, 20].
In a research Murad *et al* (2012) compared the antibacterial activity of sodium hypochlorite, chlorhexidine and MTAD on biofilm of *Enterococcus faecalis* formed on dentin matrix (*in vitro*). 108 teeth were used in this research and after cutting the apical and coronal areas and creating a 5 mm length and doing instrumentation, they were longitudinally cut and 216 samples were created. The research results showed that there was no statistical difference between 2% chlorhexidine gel and sodium hypochlorite 2.5% and 5.25% in different times; but 2% chlorhexidine fluid and MTAD had less antimicrobial activity than sodium hypochlorite 2.25% and 5.25% chlorhexidine gel [21].
In a research Pujar *et al* (2011) evaluated the antimicrobial effect of Triphala, polyphenols of green tea (GTp) and sodium hypochlorite 3% on biofilm of *Enterococcus faecalis* formed on teeth (in vitro). In this research human extracted teeth with fully closed apex were used. Treatment results showed that hypochlorite 5% had the highest antibacterial effect against biofilm of *Enterococcus faecalis* and Triphala and green tea had the antibacterial properties that compared to the control group was statistically significant. As a result using herbal alternative as canal irrigator, considering the unpleasant properties of sodium hypochlorite, could be beneficial [6].
One of the research priorities is finding a substance that could have antibacterial features and fewer side effects at the same time. In the previous studies the antibacterial effect of green tea extract was proved in laboratory conditions but still its antibacterial effect as an interacanal medicament on biofilm of *Enterococcus faecalis* is not surveyed.
Based on the information gap related to the antibacterial effect of green tea as an interacanal medicament on biofilm of Enterococcus faecalis, the aim of this research is comparing the effect of green tea, calcium hydroxide and chlorhexidine on biofilm of *Enterococcus faecalis* in root canal system (*Ex vivo*).

MATERIALS AND METHODS

In this research the surveyed population includes single rooted extracted human premolar teeth without any internal or external resorption, calcification, cracks and decay at the surface of the root with developed apex and after preparing the canal they were out in autoclave and finally the pure *Enterococcus faecalis* suspension was inseminated in them. Regarding the number of samples in previous studies and since in this research the dependent variable is measured in terms of quantity, 54 samples were studied including: 16 samples in experimental group of green tea extract (group 1), 16 samples in experimental group of calcium hydroxide (group 2), 16 samples in experimental group of chlorhexidine (group 3), 2 samples in control group and 4 samples for watching by the use of SEM. The qualified samples were categorized in test groups by the use of simple randomization. This research is an empirical research with Ex Vivo lab conditions and its aim is comparing the effect of green tea, calcium hydroxide, chlrohexidine on biofilm of *Enterococcus faecalis* in root canal system.
Sample Preparation
54 teeth were collected from clinics in Tehran. Teeth were kept in normal saline until the last preparation stages. Firstly the rest of soft dental tissue from teeth and then by the use of Hand piece the crown was cut from CEJ area (Figure 1).

Fig. 1 Teeth samples separated from CEJ area

The qualified samples were prepared by the use of ProTaper (Mailolefer-Dentsply-Switzerland) rotary system after length determination up to F3.
Irrigation with 1% Sodium hypocholorite solution was performed throughout the instrumentation. To remove smear layer each canal was rinsed with EDTA 17% for 1 minute and 5% Sodium hypochlorite for 5 minutes and finally with normal saline.
Samples were put in micro-tubes with autoclave ability having a BHI (Brain-heart infusion) broth (nutritious environment for bacteria growth) (figure 2) and they were autoclaved under 121 degree centigrade and pressure of 15 Psi for 30 minutes. Then for ensuring the sterilization, samples were kept under 37 degree centigrade for 48 hours in incubator in order to repeat sterilization in case of seeing any darkness in BHI environment.

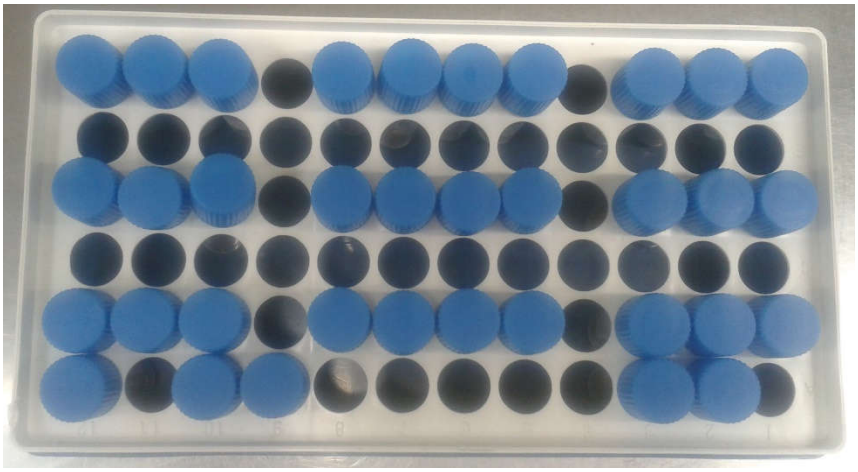

Figure 2- Placement of samples in micro-tube

Bacterial growth:
E. faecalis (American Type culture collection 29212) was cultured in brain heart infusion (BHI) broth. Bacterial concentration used in experiment was determined by MacFarlamd standard.
An inoculum of *E faecalis* (1.5_x 10^ CFU/ml) was injected into the canal using a sterile syring. All specimens incubated for 2 weeks at 37°C under shaking conditions in an orbital incubator.1 mL sterile BHI broth was refreshed every 2 days to ensure viability of the bacteria.
After gathering samples and after two weeks, 4 samples were cut for ensuring the formation of biofilm as longitudinal section. After that they were put in vacuum device for drying. Then samples were covered with gold and put under 30kv electron microscope (JSM 5510, JOEL, and Peabody. USA) with 400, 1000, 2000 and 4000 times magnification (Figure 3).

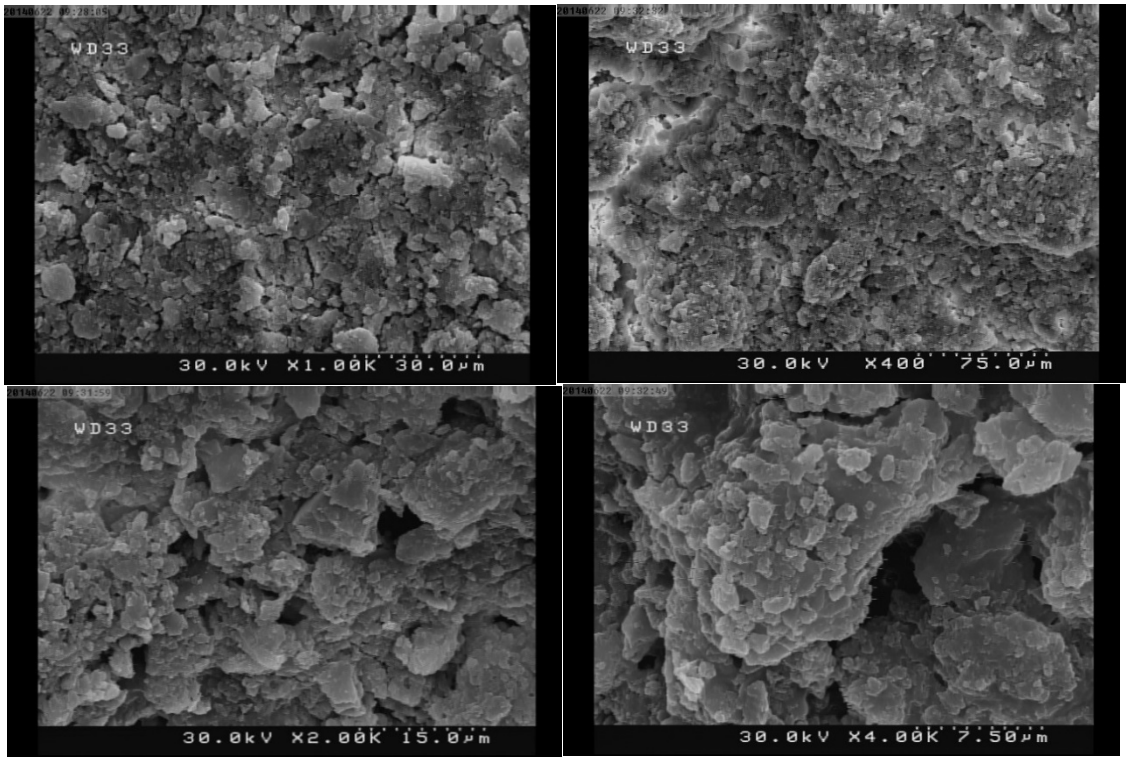

Figure 3- Images prepared by SEM

Samples were divided into 3 groups of 16 samples and control. Before inserting the interacanal medicament, teeth canals were extracted from liquid BHI environment inside canals by the use of sterile paper cone (GapaDent), no# 30.

A) In the first group with 16 teeth, green tea extract with creamy consistency was put into canal by the use of Lentulo no# 2.
B) In the second group with 16 teeth, calcium hydroxide (Merck -Germany Company) with creamy consistency was put into canal by the use of Lentulo no# 2.
C) In the third group with 16 teeth, chlorhexidine gel (Ultra Dent Company) was put in canal by the use of Lentulo no# 2.
- In the control group, after inseminating microorganism, the samples did not receive any treatment.

Then for all specimens, Coltosol (Colten, Switzerland) temporary dressing was used for closing the access cavity. Teeth were put into sterile micro tubes and samples were kept for one week under 37 degree centigrade and humidity of 100% and 5 to 10% CO2. After one week the temporary dressing was removed and samples were washed with normal saline. Then 5 cc of sterile normal saline was added by the use of 5cc sterile syringe in the canal and circumferential filing with hedstrome file was done for 20 seconds. File along with sterile paper cones used for collecting serum inside canal including dentin and living bacteria and they were transferred to test tube having 10cc sterile normal saline and then dilution was conducted in order to be able to count colonies.

Test tubes were Vortexed for 20 seconds and living bacteria were separated from file and paper cones.

At the next stage, sampling was conducted on vortexes solution and it was cultivated in BHI environment (figure 4). Also samples were taken and cultivated from control group.

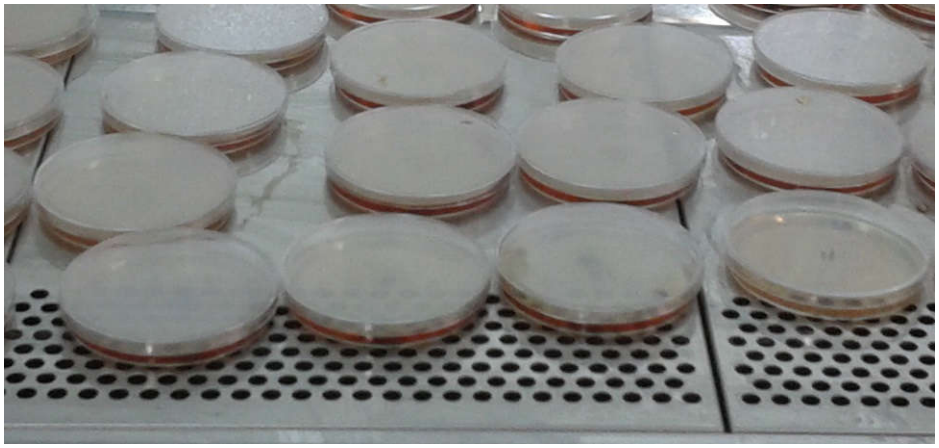

Fig. 4: Plates including cultivated samples.

Colonies were counted in the next 48 hours and CFU/ml was calculated. Data were surveyed by the use of Kruskal Wallis statistical test.

RESULT

According to Kruskal Wallis test, percentage of reduction of microorganisms did not have any significant difference in three groups of calcium hydroxide (99.8%), chlorhexidine (99.5%), and green tea (97%) (P>0.05) and this lack of difference is also clinically insignificant; however, there was a difference between three groups in terms of CFU (P<0.00001). Based on the achieved results, in all three groups the amount of remaining bacteria had a significant decrease and results are brought in tables 1 and 2.

Table 1. Distributing teeth samples based on number of colonies of bacteria divided based on treatment groups.

Kruskal Wallis (P value)	$\bar{X}$±SE	CFU/ml Groups
0.0001	138.73±108.64	Treatment by putting green tea extract as the interacanal medicament
	22.43±23.71	Treatment by putting chlorhexidine as the interacanal medicament
	5.28±13.21	Treatment by putting calcium hydroxide as the interacanal medicament

Table 2. Teeth samples based on percentage of reduction of micro-organisms divided based on medical groups.

Kruskal Wallis (P value)	$\bar{X}$±SE	Percentage of reduction of micro-organisms Groups
0.34	97.07±2.28730	Treatment by putting green tea extract as the interacanal medicament
	99.52±0.49917	Treatment by putting chlorhexidine as the interacanal medicament
	99.88±0.27826	Treatment by putting calcium hydroxide as the interacanal medicament

DISCUSSION AND CONCLUSION

Findings showed that although there was a significant difference between CFU calculated for three groups, it seems that reduction percentage of microorganisms in calcium hydroxide group (99.8%), chlorhexidine group (99.5%) and green tea group (97%) was insignificant and this insignificance is clinically acceptable as well.

Bacteria have an important role in pathogenesis and progress of pulp and periapical disease. The main aim of endodontics treatment is eliminating bacteria in root canal system as much as possible [1, 2]. *Enterococcus faecalis* is the most common type separated from persistent root canal infections [5]. Ability

of *Enterococcus faecalis* in formation of biofilm is the reason for resistance of this bacterium after root treatment. It has been shown that resistance of this bacterium to antibiotic becomes 1000 thousands more by the formation of biofilm (6); thus, one of the strengths of the current study is creating biofilm in samples and confirming them by SEM; whereas, in many studies only the canal is infected by *Enterococcus faecalis* [1, 22].

Lee *et al* (2015) surveyed the effect of EGCG on biofilm of *Enterococcus faecalis*. Results showed that EGCG is an effective antibacterial substance on both forms of biofilm and planktonic *Enterococcus faecalis* and it harnesses the bacteria growth and it suppresses the expression of specific genes related to formation of biofilm and virulence of this bacterium. This study result was compatible with our research results (green tea reduced the number of bacteria up to 97%) [23].

Delgado *et al* (2010) surveyed the antibacterial effect of calcium hydroxide and chlorhexidine on *Enterococcus faecalis* and results showed that chlorhexidine has more antibacterial effect compared to calcium hydroxide and this result and our research results are incompatible. Unlike our research, they had cut 4-5millimeters of the end of canal. As a result in our study chlorhexidine gel probably has not reached the 4-5mm end of canal due to being creamier; thus the possibility of bacteria in our study was more in chlorhexidine and this could justify the difference of results. Also their study was conducted on planktonic *Enterococcus faecalis*; whereas our study was conducted on biofilm of this bacterium [1].

Schafer *et al* (2004) surveyed the antibacterial properties of chlorhexidine and two calcium hydroxide formulas on *Enterococcus faecalis* and results showed that chlorhexidine has more antibacterial properties compared to calcium hydroxide [24]. This result is incompatible with our result and this could be justified. Firstly, in their study 2% chlorhexidine solution was used and due to being less creamy, chlorhexidine solution could penetrate all areas of canal compared to the gel we used; thus it could have a better effect. Also in this study biofilm of *Enterococcus faecalis* is not surveyed, unlike our research.

Our research was conducted on biofilm of *Enterococcus faecalis* which is one of the strengths of this research and confirming them by SEM [22].

Strength of this research is in sampling and also microbiological evaluation. Several methods are used in different researches for sampling. Prabhakar *et al* [2010] used sterile paper cone for taking samples from inside the canal [25]. Schafer *et al* [2005] achieved dentin by the use of Hedstrom file [24]. In our study we also used Hedstrom file and paper cone to prepare dentin filings from inside canal and this method let us have access to bacteria inside biofilm and dentin. At the next stage, number of living bacteria was calculated for microbiological evaluation. Due to calculating bacteria in quantitative manner, it has desirable accuracy [26, 27].

As it was earlier mentioned about the mechanisms of antibacterial effect of green tea extract, calcium hydroxide and chlorhexidine, perhaps the CFU difference is due to the difference of antibacterial mechanisms of this substance. As it was mentioned, green tea extract affects the cell membrane of micro-organisms; but mechanisms stated for alkaline calcium hydroxide and also cationic chlorhexidine and connection to negative surfaces of bacteria and damage to the cell walls of bacteria, this could make it harder for the anaerobic bacteria and these mechanisms are not stated for green tea. Thus this difference in antibacterial mechanisms could be due to the difference in CFU between these three substances.

CONCLUSION

In this research two important issues were surveyed. Firstly the application of green tea extract as an inter-canal medicine and secondly comparison of antibacterial properties of this substance and two other common substances of calcium hydroxide and chlorhexidine.

The current study findings showed that regarding the high ability of green tea to disinfect root canal and considering benefits of this substance, it is possible to use green tea extract instead of chlorhexidine and calcium hydroxide inside the canalin endodontics.

Regarding the findings achieved from this study and regarding the reduction percentage of micro-organisms for green tea (97%), calcium hydroxide (99.8%) and chlorhexidine (99.5%) and since the aim of treating endodontics is disinfecting root canal and not sterilizing it, thus this reduction amount is acceptable for disinfecting root canal and since one of our goals was reaching this substance with least side effects and easy access and regarding the side effects of calcium hydroxide on preapical tissues due to high PH and side effects of chlorhexidine such as change of color of tongue and teeth, change of sense of taste and allergy in some people, green tea extract could be a good alternative for two other substances.

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