

Antibacterial Effect of Different Concentration of Boric acid against *Enterococcus Faecalis* Biofilms in Root Canal

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Abstract

The aim of this study was to investigate the antibacterial effects of different concentration of boric acid in root canals that were infected with *Enterococcus faecalis* biofilms. 104 single-canal premolar teeth were prepared and then disinfected and sterilized. *E.faecalis* were inoculated into the root canals and kept at 37°C for 24 hours. The re-inoculation procedure was repeated on the first, fourth, seventh and tenth days with fresh culture. The infected root canals were divided into one negative (saline), one positive (sodium hypochlorite) control group, and three experimental groups (boric acid; 2%, 4% and 6%) (n=20). Paper points were placed in root canals before and after irrigation to control and evaluate the biofilm formation. Biofilms were counted on blood agar plates, and the data were evaluated and statistically analyzed using one-way ANOVA and Tukey's test. There were statistically significant differences among three experimental group (boric acid; 2%, 4% and 6%). Although 6% concentration of boric acid indicated the highest antibacterial effect, it could not exhibit the same antibacterial effect as NaOCl. As a new product boric acid indicated considerable bactericidal effect against *E.faecalis* biofilms. However, the use of boric acid can be advisable with high concentrations and long irrigation time for root canals disinfection.

Keywords: Boric acid, root canal, microorganisms

Introduction

In endodontics, various root canal chemo-mechanical approaches have been investigated with different irrigation solution, since the researcher emphasised the deeper penetration of several resistant bacteria into dentin tubules (1). One of the common root canals microorganisms that mostly isolated is *Enterococcus faecalis* (2,3).

In recent times, one of the most important approaches in modern microbiology is biofilm. Especially, biofilm formation of *E. faecalis* that was commonly and mostly isolated from root canal, began to be used more frequently in recent microbiological investigations (4-7). The biofilm formation is a complementary portion of the prokaryotic life cycle. Recent researches demonstrated some properties of biofilms; they are structurally complex, dynamic systems with attributes of primordial multicellular organisms and multifaceted ecosystems. Moreover, the crucial properties of these survival and propagation mechanisms in the natural environment and infectious diseases are based on four mechanisms; the physical barrier properties of the extracellular polysaccharide matrix (8), slow growth of bacterial cells residing within a biofilm (9), deep locations of cells that stay alive under decreased oxygen tension (10) and highly resistant phenotypic states (11).

Another issue that needs to be given importance for successful endodontic treatment is effective root canal irrigation. In this direction, several clinicians and researchers have mostly used sodium hypochlorite that is the common irrigant (NaOCl)(6,12,13) It has many advantageous properties such as its broad antimicrobial activity, the capacity to prevent the formation of and dissolve the smear layer, in association with chelating agents, and its ability to dissolve tissue remnants (14). On the other hand, NaOCl has a cytotoxic effect on vital tissue and can therefore elicit inflammatory reactions if it reaches the periapex (15).

Recently, new disinfectant agents have been searched for endodontic treatments. One of these new disinfectant agents is Boric acid that is a non-volatile mineral and shows low-toxicity, fungicidal effect and herbicidal properties (16). Boric acid and its salts, borates, have been used in medicine as a bactericide, a fungicide, and an antiseptic since the 1860s. It is used as a wettable powder, liquid (applied as a spray or aerosol), emulsifiable

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concentrate, granule or dusts. It has been considered as a safer alternative material to highly volatile, synthetic chemical pesticides (16). Moreover, it has been researched as an irrigation solution for periodontal therapy in patients with chronic periodontitis (17) and has been recommended systemic boric acid for osteoblastic activity (18). It has some disadvantages that has been linked to adverse health effects such as respiratory irritation that can occur from chronic inhalation of airborne boric acid or borates. Researchers showed eye irritation, dryness of the mouth, nose, or throat, sore throat, and cough at mean exposures of 4.1 mg/m³ (19). Furthermore, boric acid may caused inhibition of bone loss (20).

None of researchers have emphasized the antimicrobial efficacy of boric acid in root canals. In light of this information, the present study aimed to evaluate the antibacterial effect of different concentration of boric acid on *E. faecalis* biofilms in human root canals.

Materials and Methods

2.1. Selection of teeth

104 single-root single-canal human mandibular premolar teeth that were freshly extracted for orthodontic or periodontal reasons, were used in the present study. Digital radiographs of teeth were taken in the buccal and approximal directions to determine the number and morphology of the canals. Informed consent was obtained from the patients before the study, and the study was approved by the Local Ethics Committee on Human Research of Cumhuriyet University (2013-09-03).

2.2. Preparation of samples

After having been cleaned of residues, the freshly-extracted human teeth were kept at +4°C in a 0.9% saline solution until the study was applied. Below the level of the enamel-cementum junction, the coronal portions of the teeth were cut using sterile diamond discs under cooling water to obtain a 14-16 mm length for each root. Then, the root canals were entered with #15 K-File (Mani Inc., Tochigi, Japan) hand tools, and the paths of the canals were determined. The tip of the file was transmitted to measure the length of each canal until it became visible in the apical foramen. It was then withdrawn 1 mm from the measured length. The root canals were shaped with ProTaper (Dentsply, Tulsa Endodontics, OK, USA) rotary Ni-Ti instruments using the crown-down method with an the electric motor (Denta Port DP-ZX, J. Morita MFG, CORP, Kyoto, Japan). Firstly, the coronal third of the roots were expanded with SX files. The median third of roots were then reached with S1 and S2 files. The F1, F2, and F3 files were applied, respectively, to shape the apical third of the canals. The canals were irrigated with 1 ml of 5.25% NaOCl solution after the variation of each file.

The roots were irrigated with 17% EDTA, 5.25% NaOCl, and distilled water for 5 min each to remove the smear layer, which was formed during the root canal preparation and then dried with paper point. Bottles were placed in an autoclave to ensure sterilization for 20 min each at 121°C (Melag, Euroklav 23V-S, Germany). Then, a 3-fold nail polish (L'Oreal Jet-Set Diamond,

Paris, France) was applied to the entire root surface of each tooth, including the root tips. Finally, the rubber caps that were embedded in the teeth, were sterilized by EtO. Then, these caps were placed in bottles.

2.3. Biofilms formation

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 h. Prior to each experiment, 0.5 McFarland turbidity was set with a kristalspec™ device. Then, the strains were subcultured in a Trypticase soy broth (Detroit, Michigan, USA) and incubated aerobically at 37°C for 24h. The turbidity of the *E. faecalis* cultures was adjusted to No. 0.5 Mc Farland standard. The value of 10 µl of the bacterial suspension (final concentration of about 1.5 X 10⁸) was transferred to the mechanically expanded lumen of the root canals using a sterile micropipette, except for 10 canals, which were used as negative controls. They were then kept at 37°C for 24 h. The entrances of the root canals were sealed with Cavit™ temporary filling material (3M ESPE, Dental Products, USA). All samples were stored at 37°C for 10 days in a humid atmosphere, and the re-inoculation procedure was repeated every 72h with fresh cultures on the first, fourth, seventh and tenth days. The resulting stereomicroscopic image was examined to identify the biofilm formation at the end of the tenth day (Fig. 1).

2.4. Scanning Electron Microscopy Analysis (SEM)

SEM analysis was performed to identify the biofilm formation at the end of the tenth day (Fig. 1). Moreover, the remaining bacteria obtained from the inner surface of root canal following the 6% Boric acid irrigation (Group 4) were shown on SEM (Fig. 2). The root canals were immersed in a fixative solution containing 4% buffered paraformaldehyde for 24h. The root canals were dehydrated in ascending degrees of ethanol series, following their separation with a diamond disc (Horico, Hopf Ringleb & Co.,Gmbh & Cie, Germany) longitudinally. The samples were air-dried at room temperature for 24h, after sputter-coating with 25 to 30 µm of gold layer (Hummer VII, Analect, USA). The specimens were examined at × 20.000 magnification with a scanning electron microscope (SEM) (Jeol JSM 6400, Noran Instruments, Tokyo, Japan), and digital images were taken at the center of the coronal (9 mm from apex), middle (6 mm from apex), and apical (3 mm from apex) thirds of each canal in both root segments.

2.5. Experimental and Control Groups

Group 1 Saline (negative control): Root canals infected by *E. faecalis* biofilm were irrigated with 0.9% saline solution at a 10 mL/min flow rate for 2 min.

Group 2 Boric acid 2%: Boric acid was diluted with distilled water to a concentration of 2% in beaker on a magnetic stirrer (RH Basic 2 IKAMAG, IKA® Werke Staufen/ GERMANY). Root canals infected by *E. faecalis* biofilm were irrigated with 2% Boric acid at a 10 mL/min flow rate for 2 min.

Group 3 Boric acid 4%: Boric acid was diluted with distilled water to a concentration of 4% in beaker on a magnetic stirrer. Root

Figure 1. A scanning electron microscopic image of developed *E. faecalis* biofilm in a root canal.

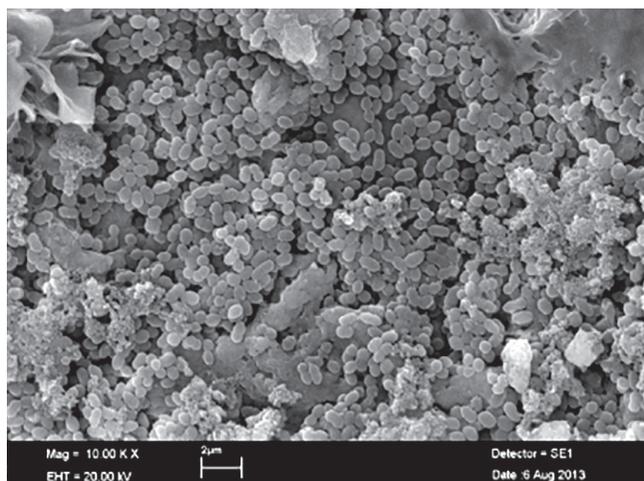
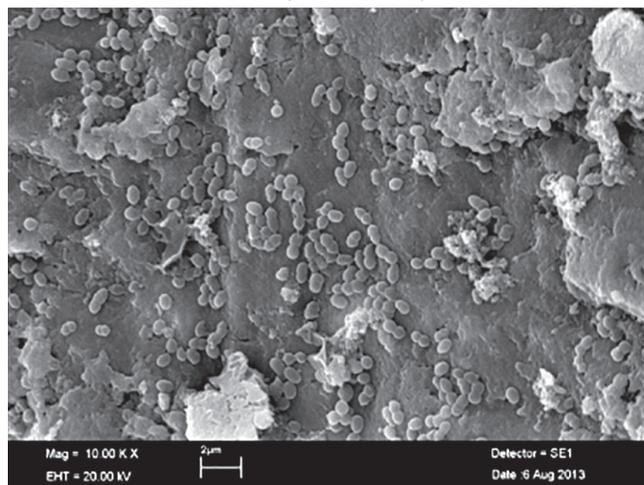


Figure 2. Stereomicroscopic images of remaining bacteria in Group 4.



canals infected by *E. faecalis* biofilm were irrigated with 4% Boric acid at a 10 mL/min flow rate for 2 min.

Group 4 Boric acid 6%: Boric acid was diluted with distilled water to a concentration of 6% in beaker on a magnetic stirrer. Root canals infected by *E. faecalis* biofilm were irrigated with 6% Boric acid at a 10 mL/min flow rate for 2 min.

Group 5 NaOCl (positive control): Root canals infected by *E. faecalis* biofilm were irrigated with 5.25% NaOCl at a 10 mL/min flow rate for 2 min. Then the remaining bacteria of all experimental samples were counted in root canals.

2.6. Bacterial evaluation

Paper points were placed in the root canals before and after irrigation to control and evaluate the biofilms' formation. Biofilm counting ensured standardization; examples with CFU values under 1.5×10^8 CFU/ml were excluded. After irrigation, CFU

Table 1. Mean (Standard Deviation) together with their statistical comparisons, median, and bacterial reduction (%) values that were obtained from all groups.

Groups	Mean (SD) (Log CFU mL ⁻¹)	Median (Log CFU mL ⁻¹)	Bacterial Reduction (%)
Group 1 Saline (Negative control)	7.000 (0.000) ^{a,b,c}	7.000	14 %
Group 2 Boric acid 2%	6.700 (0.470) ^{d,e,f}	7.000	18 %
Group 3 Boric acid 4%	4.200 (0.410) ^{a,d,g,h}	4.000	49 %
Group 4 Boric acid 6%	2.500 (0.513) ^{b,e,g,i}	2.500	69 %
Group 5 NaOCl (Positive control)	0.000 (0.000) ^{c,f,h,i}	0.000	100%

CFU, colony-forming units; By the one way ANOVA, $F= 1323.411$; $P= 0.000$ ($P<0.001$).

Values with same superscript letter are statistically different at $p<0.001$ by Tukey's Test.

counts of the breeding colonies of microorganisms were performed in blood agar plates. Then, the CFUs were calculated.

2.7. Statistical analysis

The variation data for the irrigation solutions were analyzed using SPSS statistical software (Version 14.0, SPSS Inc., Chicago, USA). The data were subjected to statistical analysis among the five different groups using one-way ANOVA. Tukey's test was applied when significant differences appeared, in order to examine pairwise differences at a significance level of 0.05.

Results

Mean (SD) with their statistical comparisons, median, and bacterial reduction (%) values that were obtained from all groups are given in Table 1. When the antibacterial effects of all disinfection agents were compared with each other;

Although there was no statistically significant difference between Group 1 (negative control) and Group 2 ($P>0.05$), statistically significant differences were found in Group 1 when compared with other all groups ($P<0.05$). Group 5 (positive control) showed statistically significant differences among other all groups ($P<0.05$).

The pairwise comparison indicated that; there were statistically significant differences among each experimental group (Group 2, 3 and 4). Moreover, while 6% concentration of boric acid showed the highest antibacterial effect, 2% boric acid demonstrated the lowest bactericidal effect among experimental groups.

The resulting stereomicroscopic image was examined to identify the biofilm formation at the end of the tenth day (Fig. 1). The SEM

image showed very dense bacterial colonization on the inner surface of the root canal. The SEM image of Group 4 demonstrated sparsely clustered bacteria on the inner surface of root canal (Fig. 2).

Discussion

In endodontics, microbiological studies have gained more importance due to the formation of intrinsic resistance microorganisms, such as biofilms. A commonly used definition of a biofilm is a “microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other.” In a biofilm, cell densities are significantly higher than in a planktonic culture (21). Therefore, unlike planktonic bacteria, most biofilm cells have higher levels of waste products, secondary metabolites, and secreted factors, as well as lower nutrient and oxygen limitations (22,23). Moreover, biofilms are known to express genes differently than planktonic cells (24,25). Furthermore, biofilms are much more metabolically active than planktonic cells in the stationary phase (26). The result of these special properties show that; biofilms are hard to treat and eliminate from root canals so that they may simulate the conditions in vivo. For these reasons, we used biofilms like those used in the recent studies (4,5).

In recent times, researchers have investigated several irrigation solutions for root canal systems. However, none of them achieved complete elimination except from NaOCl. For this reason, endodontics studies have tried alternative irrigants like boric acid that have not been emphasized enough in endodontic literature. NaOCl has been used in several investigations in which biofilms were formed in root canals. The results of these recent studies showed that; although 1% NaOCl was found to be highly effective, it could not destroy all biofilms within 2 min (6,12). Moreover, Alves et al.(13) investigated the antibiofilm and antibacterial effects of 2.5% NaOCl on *E. faecalis* biofilms in root canals, and found that, it eliminated most of the biofilms. On the other hand, in another study, 5% NaOCl showed 100% biofilm reduction (27). In the light of the afore-mentioned studies, we preferred to use 5.25% NaOCl against *E. faecalis* biofilms in root canals. Consequently, the results of the present study demonstrated complete elimination. The present study showed parallel results with Subbiya's (27) study.

Boric acid has been searched in various studies by clinicians in the field of medicine (28-31). These investigations introduced some properties about benefits of boric acid. For example, boric acid found beneficial in preventing necrotizing enterocolitis rat model (28), exhibits potent hepatoprotective effects (29), increase in antioxidant-defense system activity (29), may provide bacterial reduction in bacterial vaginosis patients (30), may exhibit antimicrobial properties against the gram-negative bacterium, the gram-positive bacterium and the fungi (31).

In dentistry, a few researchers have investigated the effects of boric acid. For instance, in a previous study, Saglam et al.(17) the effects of boric acid irrigation were evaluated as an adjunct to scaling and root planing on clinical and microbiologic parameters and compared this method with chlorhexidine irrigation in patients with chronic periodontitis. Boric acid and chlorhexidine gluconate

indicated the similar reductions in deep pockets, plaque index and gingival index scores. That study emphasized that 0.75% concentration of boric acid may be an alternative to the chlorhexidine for chronic periodontitis patients. In other investigation, the effects of systemic boric acid were evaluated on the levels of expression of RANKL and osteoprotegerin (OPG) in a rat periodontitis model. As a result, boric acid was found effective in reducing bone loss by affecting the RANKL/OPG balance in periodontal disease (20) The results of these studies emphasized the bactericidal efficacies of boric acid (17,20). However, these effects of boric acid have never been investigated by endodontists in human root canals. This lack of knowledge has created the need to study boric acid in literature. For this purpose, the present study aimed to evaluate the antibacterial effect of different concentration of boric acid on *E. faecalis* biofilms in human root canals. Based on the results of present study, the antibacterial effect and increased concentrations of boric acid showed directly proportional relation. The SEM image showed very dense bacterial colonization on the inner surface of the root canal. The SEM image of 6% boric acid demonstrated sparsely clustered bacteria on the inner surface of root canal (Fig. 2). The highest concentration of boric acid (6%) demonstrated the most effective bactericidal properties but it could not reach the same effect of NaOCl. Consequently, boric acid has a remarkable bactericidal effect. In our opinion the antimicrobial efficacy of boric acid should be researched in further studies as a root canal irrigant.

Conclusions

As a new product boric acid, considerable bactericidal effect has been obtained against *E. faecalis* biofilms. However, the use of boric acid can be advisable only with high concentrations and long irrigation time for root canals disinfection.

Conflict of Interests

All authors have nothing to disclose and have no commercial or financial interests in the products described in this paper.

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