

# An Evaluation of Antimicrobial Efficacy of Steralium, co+steralium, and 5% Sodium Hypochlorite against Enterococcus Faecalis Biofilm Formed on Tooth Substrate: (An in Vitro Study)

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## ABSTRACT

**Background:** Enterococcus faecalis is emerging as an important endodontic pathogen, which can persist in the environment for extended periods after treatment and may cause endodontic failure. It is known to produce biofilms, a community of bacteria enclosed within a protective polymeric matrix. This study aimed to establish whether the biofilm formation by Enterococcus faecalis can be inhibited with steralium, co+steralium, and 5% sodium hypochlorite in the root surface environment.

**Materials and Methods:** Extracted human teeth were biomechanically prepared, vertically sectioned, placed in the tissue culture wells exposing the root canal surface to E. faecalis to form a biofilm. At the end of the 3<sup>rd</sup> and 6<sup>th</sup> weeks, all groups were treated for 10 minutes with the previously mentioned tested solutions and control and were analyzed qualitatively and quantitatively.

**Results:** Qualitative assay with 3-weeks biofilm showed a complete inhibition of bacterial growth with co+steralium and NaOCl, except steralium and saline, which showed presence of bacterial growth. In quantitative analysis, steralium and saline- treated tooth samples have  $57.74 \pm 5.39^*$  CFU/ml.,  $140.71 \pm 8.49$  CFU/ml. respectively. Qualitative assay with 6-week biofilm showed growth when treated with steralium and saline treated tooth samples have  $346.0 \pm 78.88^*$  CFU/ml.,  $127.18 \pm 17.84$  CFU/ml. respectively whereas co+steralium and NaOCl has shown complete inhibition.

**Conclusions:** co+steralium and 5% sodium hypochlorite showed maximum antibacterial activity against E. Faecalis biofilm formed on tooth substrate. co+steralium showed statistically significant antibacterial activity. The use of co+steralium as a root canal irrigant might prove to be advantageous considering the several undesirable characteristics of NaOCl.

**Key words:** Biofilm, E. faecalis, steralium, co+steralium, NaOCl, root canal irrigant. (J Bagh Coll Dentistry 2016; 28(1):11-16).

## INTRODUCTION

Enterococcus faecalis is an important pathogen capable of causing root canal infections<sup>(1,2)</sup>, including main canal, lateral, accessories, and dentinal tubules<sup>(3,4)</sup> may cause endodontic treatment failure<sup>(5,6)</sup>. These organisms, which are ubiquitous within the environment, are generally found in mouth and other parts of GIT<sup>(7-10)</sup>. Enterococcus faecalis can survive for long period and resist many antimicrobials<sup>(2,11)</sup>. The resistance phenotype could be attributed to the ability of an Enterococcus faecalis clinical strains to form biofilms on abiotic surfaces<sup>11</sup>. The innate and emerging resistance of biofilm residing bacteria against antibiotics<sup>(12)</sup> in the current scenario consequently lead to the screening of various alternative sources to eradicate the bacterial pathogen<sup>(1)</sup>.

Sterillium® (PAUL HARTMANN AG 89522 Heidenheim Germany) Alcohol-based hand disinfection Active ingredients in 100 g: Propan-2-ol 45.0 g, propan-1-ol 30.0 g, mecetroniumetilsulfate (INN) 0.2 g<sup>(13)</sup>.

Bodedex® forte (PAUL HARTMANN AG 89522 Heidenheim Germany) is a modern, powerful instrument cleaner which is exceptionally suitable for cleaning of medicinal heat sensitive and heat-resistant instruments as well as laboratory apparatus. Bodedex® forte has a neutral pH-value and is therefore particularly gentle to materials<sup>(14)</sup>.

NaOCl was found to be significantly efficient in eliminating E. faecalis biofilms in vitro<sup>(15)</sup>. The main disadvantages of NaOCl are its unpleasant taste, high toxicity<sup>16</sup>, and its inability to remove the smear layer<sup>(17,18)</sup>. This study aims to: (i) assess the efficacy of sodium hypochlorite, steralium and, Bodedex® forte with steralium antimicrobial at removing Enterococcus faecalis from dentin surface; and (ii) investigate their effect on the formation of bacterial biofilms.

## MATERIALS AND METHODS

A pure culture of E. faecalis (American Type Culture Collection [ATCC] 29212) (central health lab., Baghdad, Iraq) was cultivated on Mueller-Hinton agar (Himedia, Mumbai, India), inoculated into Mueller-Hinton broth (Himedia, Mumbai, India), incubated at 37°C overnight and adjusted to an optical density (OD<sub>1\*107</sub>) of 1 with sterile Mueller-Hinton broth.

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The antibacterial activity of Sterillium® (PAUL HARTMANN AG 89522 Heidenheim Germany), Bodedex® forte (PAUL HARTMANN AG 89522 Heidenheim Germany) +steralium, and 5% sodium hypochlorite (Cholorex, Amman, Jordan) were initially tested on planktonic cells before evaluating them against *E. faecalis* biofilm formed on tooth substrate.

The antibacterial sensitivity test was performed by the disc diffusion method (National Committee for Clinical Laboratory Standard, 2000). Sterile blank discs (6-mm diameter; Himedia, Mumbai, India) were impregnated with **10 ml.** of test solutions (Sterillium®, Bodedex® forte +Sterillium®, and sodium hypochlorite). The broth culture of *E. faecalis* was swabbed on sterile Mueller-Hinton agar plates using sterile swabs. With the help of sterile forceps, the test solutions-incorporated discs were placed on the medium, and the plates were incubated at 37° C overnight. A standard Vancomycin disc (30 mg) was included for comparison.

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the test solutions were determined by the tube dilution method. Double dilution was made from a higher dilution 100 mg/ml to a lower dilution in a series of test tubes. Each tube was inoculated with bacterial suspensions and incubated at 37° C overnight. The MIC was regarded as the lowest concentration in the series of dilutions at which there was no visible growth of micro-organisms. The subcultures were made from the tubes, which did not yield any visible turbidity (growth) in the MIC assay on freshly prepared Mueller-Hinton agar plates. After 24 hours of incubation at 37° C, the MBC was regarded as the lowest concentration of the test solution that allowed less than 0.1% of the original inoculum to grow on the surface of the medium. In each experiment, test solutions were tested in triplicate.

### **Biofilm Formation on Tooth Substrate**

Sixty single-rooted human mandibular premolars with fully formed apices were used in this study. The teeth were cleaned of superficial debris, calculus, and tissue tags and stored in normal saline to prevent dehydration before use. Each tooth was radiographed to confirm the presence of a single patent canal. The tooth specimens were sectioned below the cemento-enamel junction with a diamond disc to obtain a standardized tooth length of 8 mm for uniform specimen.

The root canals were then instrumented using the crown-down technique and hand instruments (ProTaper, DENTSPLY Maillefer, Ballaigues, Switzerland). The canals were enlarged to an apical size F3. Two milliliters of 3% NaOCl was used between each instrument during the cleaning and shaping procedure. All the teeth were then vertically sectioned along the midsagittal plane into two halves. The concave tooth surface was minimally grounded by new diamond bur for each half to achieve a flat surface to enable placement in the tissue culture wells, exposing the root canal surface to *E. faecalis* to form a biofilm.

The sectioned samples were then divided into five experimental groups. Each group consisted of 30 samples each and assigned as group 1 (saline), group 2 (Sterillium®), group 3 (Bodedex® forte +steralium), and group 4 (5% sodium hypochlorite). Then, the samples were placed in the wells of tissue culture plates (Zellkultur Test Plates 24, Techno Plastic Products AG, Trasadingen, Switzerland) and sterilized by autoclave (Gemmy High Pressure Steam Autoclave Model TC-615).

The bacterium was cultured as described previously, and the wells containing tooth samples were inoculated with 2 ml. of bacterial solution and incubated at 37°C. The culture medium (Mueller-Hinton broth) was replaced every alternate day to avoid nutrient depletion and accumulation of toxic end products. The samples were taken from each well with a sterile paper point, inoculated onto Mueller-Hinton agar plates, and incubated at 37° C for 24 hours aerobically to check for cell viability and purity of culture.

At the end of the third week, all groups were treated for 10 minutes as follows: group 1, immersed in 3 ml. of sterile saline; group 2, immersed in 3 ml. of Sterillium®; group 3, immersed in 3 ml. of Bodedex® forte +steralium; and group 4: immersed in 3 ml. 5% NaOCl. Then, the biofilm on the root canal portion was scraped and inoculated on Mueller-Hinton agar plates and incubated for 24 hours at 37°C for qualitative analysis where  $n = 5$  for each group. The quantitative analysis was performed by vortexing the tooth samples with sterile saline for a few minutes followed by serial dilution method for all the groups where  $n = 10$  for each group. The same procedure was repeated for all groups once again at the end of the sixth week to analyze qualitatively and quantitatively.

### **Statistical Analysis**

Statistical analysis was performed by using one-way analysis of variance and compared by

the Student t test using SPSS software (student version 7.01; SPSS Inc, Chicago, IL). The criterion for statistical significance was defined as  $p < 0.05$ .

## RESULTS

Table 1 shows the zone of inhibition, MIC, and MBC of test solutions for *E. faecalis* (ATCC

29212). 5 % NaOCl test solutions showed a significant zone of inhibition in the disc diffusion assay when compared with Vancomycin and co+steralium ( $p < 0.05$ ). Maximum inhibition was observed by 5% NaOCl followed by steralium compared with co+steralium and Vancomycin. No statistical difference was observed between NaOCl and steralium

**Table 1: Susceptibility of *E. Faecalis* ATCC 29212 against the Test Solutions**

Test Solution	Zone of Inhibition	Minimal Inhibitory Concentration	Minimal Bactericidal Concentration
Steralium	24 mm	1.0 mg/ml.	2.0 mg/ml.
co+steralium	17mm*	2.0 mg/ml.	3.0 mg/ml.
5 % NaOCl	30 mm	0.50%	1.0%
Vancomycin	17 mm*	2 mg/ml.	3 mg/ml.

\* $P < 0.05$  with respect to NaOH

The qualitative assay with the 3-week biofilm on the canal portion showed complete inhibition of bacterial growth when treated with co+steralium and NaOCl, but the samples treated with steralium and saline showed the presence of

bacterial growth. In quantitative analysis, steralium and saline-treated tooth samples showed  $57.74 \pm 5.39^*$  CFU/ml. and  $140.71 \pm 8.49$  CFU/ml. (mean  $\pm$  standard deviation), respectively (Table 2)

**Table 2: Quantitative Analysis of Three-Week *E. Faecalis* Biofilm Formed on Tooth Substrate for Different Groups**

	Number of bacteria CFU/ml. (mean $\pm$ SD)
N.S.	$140.71 \pm 8.49$
steralium	$57.74 \pm 5.39^*$
co+steralium	0*
NaOCl	0*

\* $P < 0.05$  with respect to N.S. (one way ANOVA)

Qualitative assay with the 6-week biofilm on the canal portion showed co+steralium, and NaOCl has having complete inhibition.

Table 3 shows the bacterial population in the quantitative assay with the 6-week biofilm for steralium, co+steralium NaOCl and saline-treated tooth samples.

**Table 3: Quantitative Analysis of Six-Week *E. faecalis* Biofilm Formed on Tooth Substrate for different Groups**

	Number of bacteria CFU/ml. (mean $\pm$ SD)
N.S.	$346.0 \pm 78.88^*$
Steralium	$127.18 \pm 17.84$
co+steralium	0*
NaOCl	0*

\* $P < 0.05$  with respect to N.S. (one way ANOVA)

A significant reduction of bacterial population compared with the Control saline group, which showed  $346.0 \pm 78.88^*$  CFU/ ml.. In steralium group, has shown the bacterial count ( $127.18 \pm 17.84$ ), while co+steralium and NaOCl showed 100% eradication.

## DISCUSSION

*E. faecalis* is an important opportunistic pathogen, with the ability to colonize and persist in the root canal and on retreatment failure, and are resistant to traditional antibiotics<sup>(19,20)</sup>. It is hypothesized that its ability to persist in these environments, as well as its virulence, is a result of its capacity to form biofilms<sup>(21)</sup>. The antibiotic

resistance has been found to increase up to 1,500 times when compared with planktonic cells<sup>(22,23)</sup>. Therefore, testing the effect of an antibacterial irrigant on planktonic cells will not fulfill its effectiveness in in vivo conditions.

Bacteria-induced dissolution of the dentin surface and the ability of *E. faecalis* to form calcified biofilm on root canal dentin may be a factor that contributes to their persistence after endodontic treatment<sup>(24)</sup>. It is established that the biofilm-forming capacity and its structural organization are influenced by the chemical nature of the substrate. Biofilm experiments conducted on polycarbonate or glass substrate will not provide a true indication of the bacteria-substrate interaction<sup>(25)</sup>. Hence, *E. faecalis* biofilm was formed on a tooth substrate.

Some irrigants<sup>(20)</sup> are more effective against planktonic than biofilm cultures, Therefore, it is necessary to search for biofilm removal synergistic potential of biofilm removal in conjunction with steralium on *E. faecalis* planktonic cultures led us to study the antimicrobial activity of co+steralium used in combined form against *E. faecalis* biofilms. To enhance the efficacy of the irrigants, the action on biofilms should involve the elimination of the EPS matrix as well as the bacteria because this matrix could act as an additional source of nutrients and/or as a suitable surface for further cell growth<sup>(26)</sup>. Bearing in mind the methodology used, the results presented here suggest that in strategies that are not only active against microorganisms but also exert an effect on the EPS matrix. The lack of studies that closely examine biofilm susceptibility to antimicrobial associations and the action against *E. faecalis* biofilms; the associated use of co+steralium might provide better results than their applications as single agents.

Novel ways to combat dentin surface contamination of *E. faecalis* are required. Our experiment was conducted to evaluate the use new material and strategy against residual antimicrobial activity on root canals of human teeth cultured with *E. faecalis*. The uses of a biofilm removal agent with antimicrobial agents as the final irrigation regimen is proposed to biofilm removal eradicate *E. faecalis* biofilms allow the antimicrobial irrigant to have better access to the microorganism. Dissolution of biofilms by irrigant solutions is crucial because a significant area of the root canal system is inaccessible to endodontic instruments. One problem of root canal disinfection in vivo is the ability of organic compounds to inactivate the irrigant antimicrobial activity.

The current study is in accordance with the methodology done by Kishen et al<sup>(24)</sup>. All the groups were tested in direct contact with the biofilm formed on tooth substrate at different durations (3 weeks and 6 weeks).

In the initial antibacterial sensitivity test on planktonic cells, steralium its efficient against *E. faecalis*; steralium is a potent antimicrobial agent of proven substantivity, although it was not able to eradicate *E. faecalis* biofilms at any of the time periods we assayed. There is a need to use steralium together with another chemically compatible agent that enhances its efficacy against biofilms.

The pilot study showed that there is no antimicrobial Bodedex® forte effect against *E. faecalis* while The combined used of Bodedex® forte as co with steralium has been shown to exert less antimicrobial activity against *E. faecalis* planktonic than steralium alone but higher against biofilm cultures. Bodedex® forte is Non-ionic and amphoteric surfactants, removing biofilm agent that favors the action of irrigants, thus permitting more effective elimination of endodontic infection. Although in our case, the canals were refilled with BHI broth during the incubation period so that there would be an abundance of nutrients available for the bacteria. Both group 3 and 4 specimens were negative cultures on the three and six week that these agents eliminated the infected biofilm and the antibacterial strength of the agents used no regrowth after six week may indicates that eradication of the bacteria was complete.

NaOCl was effective at 0.5%, of the fact that sessile bacteria on surfaces or present within biofilm are much less readily inactivated than planktonic cells. A biocide gradient is produced throughout the biofilm, so that in thick biofilm there will be an “in-use” concentration as the biocide penetrates into the community<sup>25</sup>. The concentration of 60 mg/ml used in this study was found to be effective as an antibacterial against *E. faecalis*, and further reduction in concentration, when used in vivo, is still feasible because the bacterial count is expected to be much less than what we have used.

Five percent sodium hypochlorite is proven to be the best among all the groups in planktonic microbial inhibition, and exhibited excellent antibacterial activity both in 3-week and 6-week biofilm. Results from previous study reported that NaOCl was capable of eradicating *E. faecalis* biofilm after one minute at a concentration of 0.00625% that was grown in the Calgary biofilm forming device<sup>(27)</sup>. But the same concentration may not be effective on biofilm formed on tooth

substrate. co+steralium showed promising antibacterial efficacy on 3- and 6-week biofilm along and 5% sodium hypochlorite. While NaOCl is a very caustic, nonspecific agent whose action is not limited to necrotic tissue<sup>(28)</sup>, and it has deleterious effects on dentine that include reduction of the elastic modulus and the flexural strength<sup>(29,30)</sup>. The co+ steranium appear to be sufficient susceptible to disinfectants dentin from tested microorganisms with less limitation of NaOCl.

Our experiments suggest that, steranium and Bodedex® forte show a promise in this context. Further investigations need to be carried out to assess its use against a range of other pathogens and its potential to use Bodedex® forte with other potent antibiotic such as chlorhexidin.

Within the limitations of this study, 5% sodium hypochlorite showed maximum antibacterial activity against 3- and 6-week *E. faecalis* biofilm formed on tooth substrate. The use of co+steranium alternatives as a root canal irrigant might prove to be advantageous considering the several undesirable characteristics of NaOCl. Further research is needed to conclusively recommend co+steranium as a root canal irrigant.

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