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# Effectiveness of intracanal dressing protocols on *Enterococcus faecalis* biofilm in a bovine teeth model – an *in vitro* study

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## Abstract

**Aim:** The purpose of study was to evaluate, *in vitro*, the effectiveness of intracanal dressing protocols in root canals infected with *E.faecalis*.

**Methods:** Eighty eight bovine incisors were contaminated with *E. faecalis*, remaining in culture for 30 days for biofilm formation. The teeth were divided into ten groups according to presence of disinfectant penetration (DP), intracanal dressing and medication placement site: G1 (CHX gel) – 2% chlorhexidine (CHX) gel (cervical third), G2 (CHX liq) – 2% CHX liquid (cervical third), G3 (TC) – tricresol formalin (canal entrance). In these groups (n=10), DP was not performed. G4 (DP+CHX gel) – 2% CHX gel (all thirds), G5 (DP+CHX liq) – 2% CHX liquid (all thirds), G6 (DP+TC) – tricresol formalin (canal entrance), G7 (DP+$Ca(OH)_2$) – calcium hydroxide paste (all thirds). In these groups (n=10) DP with 2% NaOCl was performed. Groups G8 (DP NaOCl) – DP with 2% NaOCl, G9 (DP $H_2O$) – DP with distilled water, and G10 – (no treatment) were considered controls (n=6). Microbiological test (CFUs counting) and scanning electron microscopy (SEM) were performed to evaluate and illustrate respectively the effectiveness of proposed treatments.

**Results:** Microbiological test demonstrated that groups G4 (DP+CHX gel), G5 (DP+CHX liq), G6 (DP+TC) and G7 (DP+$Ca(OH)_2$) showed no bacterial growth, being statistically different from all other groups ($p<0.05$).

**Conclusion:** 2% chlorhexidine gel, 2% chlorhexidine liquid and calcium hydroxide paste in all root canal thirds, as well as tricresol formalin on root canal entrance, are effective intracanal dressings against *E. faecalis*, when associated to previous DP with 2% NaOCl.

**Key words:** Biofilm; *Enterococcus faecalis*; Bovine teeth; Intracanal dressing

## Eficácia de protocolos de medicação intracanal sobre o biofilme de *Enterococcus faecalis* em um modelo de dentes bovinos – estudo *in vitro*

### Resumo

**Objetivo:** O propósito deste estudo foi avaliar, *in vitro*, a eficácia de diferentes protocolos de medicação intracanal em canais radiculares infectados com *Enterococcus faecalis*.

**Métodos:** Oitenta e oito incisivos bovinos foram contaminados com *Enterococcus faecalis*, permanecendo em cultura por 30 dias para a formação do biofilme. Os dentes foram divididos em dez grupos de acordo com a presença de penetração desinfetante, medicação intracanal utilizada e o local de colocação desta medicação: G1(CHX gel) – clorexidina gel 2% (terço cervical), G2(CHX liq) – clorexidina líquida 2% (terço cervical), G3(TC) – tricresol formalina (entrada do canal); nestes grupos (n=10) não foi realizada penetração desinfetante com hipoclorito de sódio 2%. Seguindo, G4(DP+CHX gel) – clorexidina gel 2% (todos os terços), G5(DP+CHX liq) – clorexidina líquida 2% (todos os terços), G6(DP+TC) – tricresol formalina (entrada do canal), G7(DP+Ca (OH)2) – pasta de hidróxido de cálcio (todos os terços); nestes grupos (n=10) foi realizada penetração desinfetante com hipoclorito de sódio 2%. Seguindo, G8(DP NaOCl) – penetração desinfetante com hipoclorito de sódio 2%, G9(DP H2O) – penetração desinfetante com água destilada, G10(sem tratamento); estes grupos (n=6) foram considerados controles. Teste microbiológico (contagem de UFCs) e microscopia eletrônica de varredura (MEV) foram realizados para avaliar e ilustrar respectivamente a eficácia dos tratamentos propostos. Análise estatística foi realizada através de ANOVA, seguido pelo *post-hoc* de Tukey ($\alpha=0.05$).

**Resultados:** O teste microbiológico demonstrou que os grupos G4(DP+CHX gel), G5(DP+CHX liq), G6(DP+TC) e G7(DP+Ca(OH)2) não apresentaram crescimento bacteriano, sendo estatisticamente diferentes dos demais grupos ($p<0,05$).

**Conclusão:** Clorexidina gel 2%, clorexidina líquida 2% e pasta de hidróxido de cálcio em todos os terços do canal radicular, bem como tricresol formalina na entrada do canal radicular, podem ser consideradas efetivas medicações intracanais contra *Enterococcus faecalis*, quando associadas à penetração desinfetante prévia com hipoclorito de sódio 2%.

**Palavras-chave:** Biofilme; *Enterococcus faecalis*; Dentes bovinos; Medicação intracanal

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## Introduction

Most pathological changes affecting pulp and periradicular tissues have microbial etiology. Bacteria and their products play essential role in the pathogenesis and progression of such conditions [1,2].

Studies have reported that bacteria found in endodontic microbiota can be removed using sodium hypochlorite in chemomechanical preparation [3,4]. However, some bacteria such as *Enterococcus faecalis* possess resistance to endodontic treatment and remain viable into dentinal tubules even after root canal preparation [5].

On this way, intracanal dressing is advocated to prevent multiplication of microorganisms remaining even after careful cleaning and shaping of root canal [6].

Calcium hydroxide has been recommended as intracanal dressing due to some properties such as antibacterial activity, endotoxin neutralization and inducement of hard tissue formation [7,8]. However, microorganisms such as *Enterococcus faecalis* may persist [9] and its antimicrobial activity may vary depending on the location into root canal [10].

Tricresol formalin has been used as an alternative to intracanal dressing, especially in situations which the root canal is not enlarged enough to allow calcium hydroxide placement. It is considered a strong disinfectant and effective bactericide [11].

Chlorhexidine gluconate has been used because of its broad antimicrobial spectrum [12-14] and substantivity [15]. However, the actual impact of these substances on teeth with a biofilm along the canal space and dentinal tubules needs further investigations.

The purpose of this study was to evaluate, *in vitro*, the effectiveness of intracanal dressing protocols in root canals of bovine teeth infected with *Enterococcus faecalis*. For that, the influence of previous disinfectant penetration with 2% sodium hypochlorite and the site of medication placement were assessed.

## Materials and Methods

This study was submitted to the Science and Ethics Commission of the School of Dental Medicine of Pontifical Catholic University of Rio Grande do Sul – PUCRS.

### Sample obtaining and preparation

Eighty eight bovine incisors were extracted from animals killed for commercial reasons. The dental crowns were sectioned so that all the roots remained with 18 mm in length. The pulp tissue was removed by irrigation with 2% sodium hypochlorite (NaOCl) (Virex Plus – JohnsonDiversey, São Paulo, Brazil) and instrumentation with #60 k-file (Dentsply-Maillefer, Ballaigues, Switzerland) calibrated in 17 mm. Then, a final rinse with 17% EDTA (Iodontosul, Porto Alegre, Brazil) was performed for *smear layer* removal.

Each root was fixed in a plastic micro-tube (GenuineAxygenQuality, CA, USA), so that it remained upright with the cervical portion facing upward. A hole was opened in the side of micro-tube for culture medium exchange. The samples were randomly divided into seven experimental groups (n=10) and three control groups (n=6). The samples were sterilized in autoclave (Dabi Atlante – Ribeirão Preto, SP, Brazil) for a period of 30 minutes.

### Culture and inoculum preparation

The culture and inoculum preparation were performed according to previous study [16]. The reference strain used was *Enterococcus faecalis* (ATCC 19433). The bacteria were cultivated in BHI (Brain Heart Infusion) broth for 18 to 24 hours, at 37°C, in bacteriological incubator.

100 µL of *Enterococcus faecalis* culture were inoculated inside the root canal of the 88 samples previously sterilized. Following, the sterile BHI was added into the micro-tube so that it was completely filled with the culture medium. The culture of *Enterococcus faecalis* was maintained for 30 days in order to obtain the biofilm formation, with the renewal of one third of the BHI every 2 days. Once a week, an aliquot of BHI from the teeth was submitted to Gram staining and cultured on blood agar followed by catalase and esculin tests to verify the absence of contamination with other microorganisms.

### Classification of the groups

The roots were mounted on utility wax basis (Wilson, Cotia, Brazil) to avoid substance extravasation. The group distribution is demonstrated in Table 1, according to presence of disinfectant penetration, intracanal dressing and medication placement site.

Firstly, the root canal was filled with 2% NaOCl in all groups, except 9 (DP $H_2O$) and 10 (no treatment). A size 2 LA Axxess (Sybron-Endo,Orange,USA) was used to prepare the cervical third, followed by irrigation with 2 ml of 2% NaOCl and concomitant aspiration. The same procedure was performed in group 9 (DP $H_2O$), replacing the chemical substance with distilled water.

The disinfectant penetration (DP) was simulated through root canal filling with 2% NaOCl or distilled water according to Table 1, and agitation of the solution with a K #25 file (Dentsply-Maillefer,Ballaigues,Switzerland) for 60 seconds.

Irrigation with 2 ml of distilled water followed by aspiration was performed before the intracanal dressing placement, in order to neutralize de NaOCl.

Both formulations of 2% chlorhexidine (CHX) (Essencial Pharma, Itapetininga, Brazil) were introduced into root canal using a disposable sterile syringe (Descarpack, São Paulo, Brazil) and Ultradent needle (Ultradent, Indaiatuba, Brazil). Tricresol formalin (TC) (Essencial Pharma, Itapetininga, Brazil) was impregnated in a sterilized cotton pellet which was positioned in the root canal entrance. The calcium hydroxide paste ($Ca(OH)_2$) (Calen – SS White, Rio de Janeiro, Brazil) was introduced into root canal with ML endodontic syringe (SS White, Rio de Janeiro, Brazil) attached to a Septojet XL needle (Septodont, Barueri, Brazil)

**Table 1.** group distribution according to presence of disinfectant penetration, intracanal dressing and medication placement site.

| Group | N | Disinfectant penetration (substance) | Intracanal Dressing | Medication placement site |
|---|---|---|---|---|
| 1. CHX gel | 10 | No | 2% Chlorhexidine gel | Cervical third |
| 2. CHX liq | 10 | No | 2% Chlorhexidine liquid | Cervical third |
| 3. TC | 10 | No | Tricresol formalin | Canal entrance |
| 4. DP+CHX gel | 10 | Yes (2% NaOCl) | 2% Chlorhexidine gel | All thirds |
| 5. DP+CHX liq | 10 | Yes (2% NaOCl) | 2% Chlorhexidine liquid | All thirds |
| 6. DP+TC | 10 | Yes (2% NaOCl) | Tricresol formalin | Canal entrance |
| 7. DP+$Ca(OH)_2$ | 10 | Yes (2% NaOCl) | Calcium hydroxide paste | All thirds |
| 8. DP NaOCl | 6 | Yes (2% NaOCl) | No | – |
| 9. DP $H_2O$ | 6 | Yes ($H_2O$) | No | – |
| 10. no treatment. | 6 | No | No | – |

CHX gel: chlorhexidine gel;
CHX liq: chlorhexidine liquid;
TC: tricresol formalin;
DP: disinfectant penetration;
$Ca(OH)_2$: calcium hydroxide;
NaOCl: sodium hypochlorite;
$H_2O$: distilled water.

The roots were sealed with sterilized cotton pellet and Cavit (3M, Sumaré, Brazil). In the group 10, no procedure was performed.

The samples were stored in bacteriological incubator at 37ºC for 7 days.

### Microbiological analysis

After storage period, the intracanal dressing was removed by irrigation with 5 ml of distilled water. Then, 5 teeth in the experimental groups and 3 teeth in the control groups were immediately immersed in the fixation solution and were used for analysis in scanning electron microscopy (SEM). The remaining teeth in each group were used for microbiological test. Following treatment, the canal was immediately filled with sterile saline solution, which was stirred with a file number 60 (Dentsply, Maillefer - Ballaigues, Switzerland) for 15 seconds. An aliquot of 50 µL of the solution was removed from the canal and transferred to a tube containing 450 µL of sterile saline solution at 0.85%. The material was homogenized and diluted to $10^{-3}$. Aliquots of 100 µL of the solution and the dilutions were cultivated on the surface of the blood agar, in duplicate, with the aid of a Drigalsky handle, being incubated for 18 to 24 hours at 37°C. After the incubation period, the counting of number of colony-forming units of the plates was performed.

### SEM preparation and analysis

The roots were fixed for 7 days in 2% glutaraldehyde and washed three times for 30 minutes in a 1:1 ratio of 0.2M phosphate buffer and distilled water. After dehydration, the roots were longitudinally sectioned, providing two halves of each sample. The samples were coated with gold and the image acquisition was made under SEM (Philips XL 30, Eindhoven, Netherlands), using the backscattering resource (BSE). The image records were made at 5000x in the canal wall, into all thirds of root canal, in order to illustrate the effectiveness of proposed treatments.

### Data analysis

One-way ANOVA was applied in the microbiologic evaluation, followed by Tukey´s post hoc procedure, at 5% of significance level. Descriptive analysis was performed over SEM illustrations.

## Results

The results are expressed in Figure 1. Groups 4 (DP+CHX gel), 5 (DP+CHX liq), 6 (DP+TC) and 7 (DP+$Ca(OH)_2$) showed no bacterial growth, being statistically different from all other groups ($p<0.05$). Group 2 (CHX liq) showed a lower median of CFU/ml than groups 1 (CHX gel), 3 (TC), 8 (DP NaOCl), 9 (DP $H_2O$) and 10 (no treatment), being statistically different from them ($p<0.05$). Groups 1 (CHX gel), 3 (TC), 8 (DP NaOCl) showed a lower median of CFU/ml than groups 9 (DP $H_2O$) and 10 (no treatment), being statistically different ($p<0.05$). However, there were no significant differences between groups 1 (CHX gel), 3 (TC) and 8 (DP NaOCl).

SEM revealed that root canal walls of samples from group 10 (no treatment) were densely colonized by *Enterococcus faecalis* (Fig 2 – A and B). In several areas, cells were organized in biofilm and were seen penetrating the dentinal tubules. At the same time, root canal walls of samples from groups 4 (DP+CHX gel), 5 (DP+CHX liq), 6 (DP+TC) and 7 (DP+$Ca(OH)_2$) (Fig 2 – C, D, E, F, G, H, I and J) showed absence of bacteria.

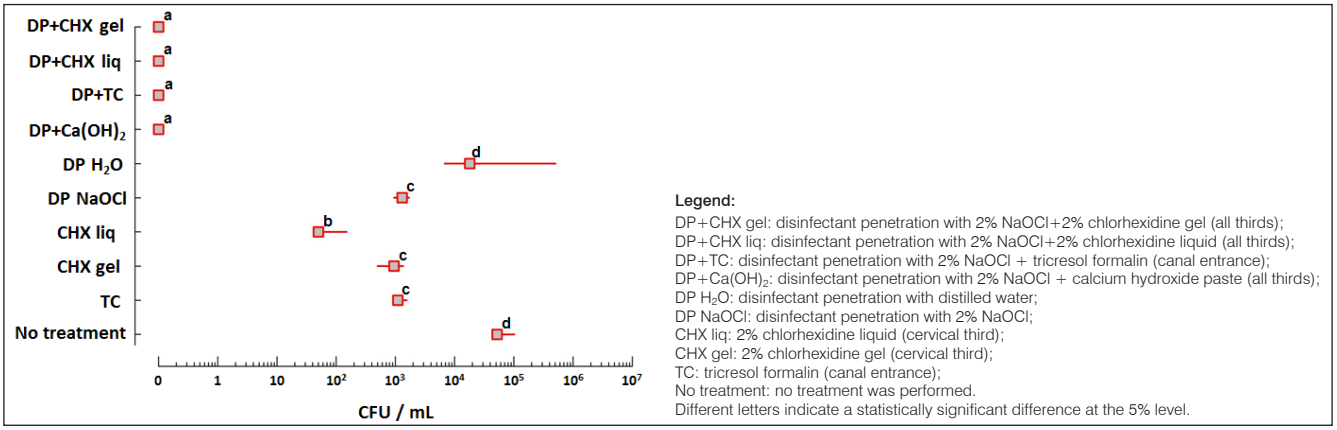


Fig. 1. Graph of microbiological test results, depicting the median of CFU/ml observed for all groups

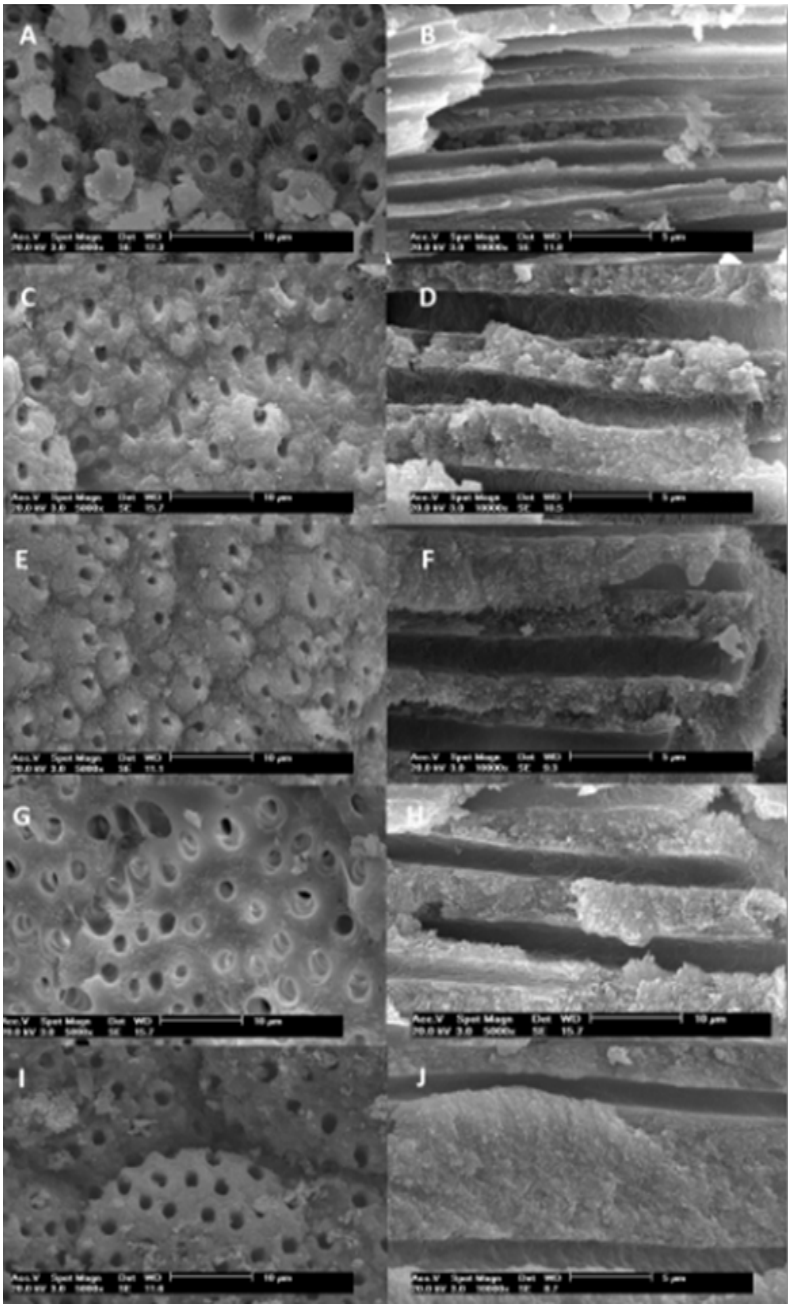


**Fig. 2.** SEM illustrating the pattern of colonization by *Enterococcus faecalis* and the effectiveness of proposed treatments.
**A** and **B**: G10 (no treatment) in the canal wall and exposed tubule area respectively;
**C** and **D**: G4( DP+CHX gel) in the canal wall and exposed tubule area respectively;
**E** and **F**: G5 (DP+CHX liq) in the canal wall and exposed tubule area respectively;
**G** and **H**: G6 (DP+TC) in the canal wall and exposed tubule area respectively;
**I** and **J**: G7 (DP+$Ca(OH)_2$) in the canal wall and exposed tubule area respectively.

## Discussion

One of the most important factors of endodontic success is an effective decontamination of root canal system. Chemical substances and intracanal dressings are available to perform this role concurrently with mechanical action of endodontic instruments.

The model of biofilm formation used in this study simulates the clinical conditions which are found in infected root canals. *Enterococcus faecalis* was chosen because of its ability to successfully colonize the root canal system in the biofilm form [5,17].

However, there is no consensus in literature about time of biofilm formation, varying from 24 hours [18] to 21 [19] and 50 days [16]. In the present study, 30 days of biofilm formation was adopted, believing that the biofilm would be better structured and mimicking the clinical situation. Then, decontamination protocols were effectively tested.

Bovine teeth were used to perform the model of biofilm formation in the present study, as in previous studies [11,20]. These teeth are used because of the anatomical and physical similarities with human teeth, plus the ease of obtaining [21].

The counting of colony forming units (CFUs) was used to evaluate the effect of the proposed treatments in the present study. This method was chosen based in previous studies [11,20] and because it allows bacteria quantification per milligram of dentin [22].

The groups where DP with 2% NaOCl was performed, previously to intracanal dressing placement in the descripted regimens, showed better results in the *Enterococcus faecalis* elimination when compared to groups where intracanal dressing was used alone. These findings testify that previous neutralization of microbial content is necessary to promote an appropriate cleaning of root canal system, as well as the antimicrobial activity of NaOCl showed in previous studies [23,24].

According to present study, 2% CHX liquid, when used only in the cervical third, showed a lower median in the counting of CFUs when compared to gel formulation in the same regimen and concentration. It can be explained by the lower superficial tension of liquid formulation, which provides a higher diffusion into root canal system and dentinal tubules.

Studies have suggested that CHX gluconate is an effective intracanal medication due its antimicrobial activity [11-13], which is in agreement of the findings of the present study. The present results showed that both CHX formulations, when placed in all extension of root canal, after previous DP with NaOCl, promoted complete elimination of *Enterococcus faecalis*. CHX efficacy is explained by interaction between positive charge of the molecule and negatively charged phosphate groups on microbial cell walls, altering the cells' osmotic equilibrium. This increases the permeability of cell wall, which allows the CHX molecule penetration, resulting in bacteria cell death [11].

The procedures performed in groups 6 (DP+TC) and 7 (DP+$Ca(OH)_2$) promoted complete elimination of *Enterococcus faecalis*. Both regimens were associated to previous DP with 2% NaOCl. These findings are in accordance with previous studies which showed the antimicrobial activity of tricresol formalin [15] and calcium hydroxide [25, 26]. The tricresol formalin antimicrobial activity occurs from formaldehyde action over microorganism components, making penetration and inducing injury in bacterial cell [27]. In other hand, the mechanism of action of calcium hydroxide is dependent on dissociation of the calcium and hydroxyl ions, followed by its diffusion through the dentinal tubules and ramifications of the root canal [8]. Furthermore, it promotes the inhibition of bacterial LPS [28].

Groups 4 (DP+CHX gel), 5 (DP+CHX liq), 6 (DP+TC) and 7 (DP+$Ca(OH)_2$) have not showed statistical difference between them. These findings suggest that 2% CHX gel, 2% CHX liquid, tricresol formalin and calcium hydroxide paste can be used as intracanal dressing, helping with the elimination of *Enterococcus faecalis*, when associated with previous DP with 2% NaOCl. However, components of tricresol formalin, especially formaldehyde, may cause mutagenic effects [29], being considered potentially carcinogenic [30].

The present study suggests the use of CHX gluconate as an alternative of intracanal dressing, from the moment that is effective against *Enterococcus faecalis* and doesn't have the disadvantages of the other tested substances. In addition, chlorhexidine promotes substantivity, ensuring its activity for long period of time in dentin [14], absence of citotoxicity [31] and beneficial effects in the bond strength between restorative material and dentin [32,33].

Under the limitation of this study, it can be concluded that 2% CHX gel, 2% CHX liquid and calcium hydroxide paste in all root canal thirds, as well as tricresol formalin on root canal entrance, are effective intracanal dressings against *Enterococcus faecalis*, when associated to previous DP with 2% NaOCl.

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