

IN VITRO ANTIBACTERIAL EFFECT OF DIFFERENT IRRIGATING SOLUTIONS ON *ENTEROCOCCUS FAECALIS*

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ABSTRACT

Was evaluated the minimum inhibitory concentration (MIC) and the antibacterial effect (AE) of 2.5% NaOCl, 0.2% chlorhexidine gluconate (CHX) and 17% EDTA on *Enterococcus faecalis*.

The antibacterial capacity was assessed by diffusion in agar. The AE was evaluated on contaminated root dentin, employing apical and middle portions of human roots, sterilized and contaminated with *Enterococcus faecalis*, immersed in the irrigation solutions and incubated at 37°C. Viable cells were counted at 0, 4, 8 and 24 hours.

MIC: NaOCl and CHX: 0.2%, EDTA below 5%. Diffusion in agar: NaOCl 2.5%= 21 mm. CHX 0.2%= 14mm. EDTA 17%= 20 mm.

Effect on root dentin: NaOCl 2.5%: *Enterococcus faecalis* was totally inhibited for 24 hours in the apical area, and for 8 hours in the middle area. CHX 0.2% elicited a reduction of more than 5 log CFU and EDTA 17% induced a reduction of more than 3 log CFU at all the time points examined in the apical and middle areas.

Key Words: Irrigation, *E. Faecalis*, Antibacterial effect.

EFFECTO ANTIBACTERIANO DE DIFERENTES SOLUCIONES DE IRRIGACIÓN SOBRE *ENTEROCOCCUS FAECALIS*

RESUMEN

Se evaluó el efecto antibacteriano (ea) y la concentración inhibitoria mínima (cim) sobre *Enterococcus faecalis* de NaOCl 2,5%, Gluconato de Clorhexidina 0,2% y EDTA 17%.

La capacidad antibacteriana fue valorada mediante el test de difusión en agar. El efecto antibacteriano se evaluó empleando trozos radiculares apicales y medios que fueron esterizados, contaminados con *Enterococcus faecalis*, sumergidos en las diferentes soluciones de irrigación e incubados a 37°C. El recuento de células viables se realizó a 4, 8 y 24 horas.

Cim: NaOCl y CHX: 0.2%, EDTA menor al 5%. Difusión en agar: NaOCl 2.5%= 21 mm. CHX 0.2%= 14mm. EDTA 17%= 20 mm.

Efecto sobre la dentina radicular: NaOCl 2.5%: *Enterococcus faecalis* fue totalmente inhibido hasta las 24 horas en el área apical y hasta las 8 horas en el área media. CHX 0.2% mostró una reducción de más de 5 log UFC y EDTA 17% provocó una reducción de más de 3 log UFC en todos los tiempos testeados en los tercios apical y medio.

Palabras Clave: Irrigación, *E. Faecalis*, Efecto antimicrobiano.

INTRODUCTION

The success of endodontic therapy depends mainly on the elimination of bacteria and irritants from the root canal before performing the obturation. Their persistence is the main cause of treatment failure (1). According to Evans et al. (2), the aim of chemical debridement is the removal of residual tissue and bacterial biofilm, mainly from the non-instrumented areas.

Once the root canal has been cleaned it must be obturated to eliminate surviving bacteria. Other-

wise, some bacteria such as *Enterococcus faecalis*, that are more resistant to treatment, will survive for upto 10 days in dentin tubules without a source of nutrients (3), even in the presence of calcium hydroxide applied topically (4).

The aim of the present study was to determine the antibacterial capacity of 2.5% sodium hypochlorite, 0.2% chlorhexidine gluconate and 17% EDTA on *Enterococcus faecalis* isolated from infected root canals by diffusion in agar and by assessing their effect on contaminated root dentin.

MATERIALS AND METHODS

The irrigation solutions employed were 2.5% sodium hypochlorite (NaOCl), 0.2% aqueous solution of chlorhexidine (CHX), 17% EDTA and distilled water as control.

The antibacterial action of the irrigation solutions under study was assessed by three methods, i.e. diffusion, dilution and evaluation of antibacterial action on contaminated root dentin.

Diffusion in agar

The stored bacterial strains were activated in brain-heart infusion broth in 3 successive steps.

The bacterial inoculum was prepared from the suspension of an 18-hour culture in Müller Hinton (Britania-Argentina) broth, adjusting turbidity to 0.5 of the scale of the Mc Farland nephelometer.

We prepared three Petri dishes with Müller-Hinton (Britania- Argentina) agar to a height of 4 mm. Two dishes were inoculated with 0.2 ml of bacterial suspension employing a Drigalsky spatula. Holes 4 mm in diameter were punched in one of the dishes and filled with 40 ml of each of the irrigation solutions under study. The remaining dish containing no irrigation solutions was inoculated with bacteria and used as a positive control. The third dish containing the different irrigating solutions but no bacteria was used to test the sterility of the culture medium.

The dishes were cultured, inverted, at 37°C for 24 hours. We then measured the diameter of the inhibition halos.

Evaluation of minimum inhibitory concentration (mic) by dilution in agar

We prepared a series of Petri dishes with Müller Hinton agar and added the following series of irrigation solutions:

NaOCl: 0.05% - 0.10% - 0.20% - 0.5% - 1.5% - 2.5%

CHX: 0.05% - 0.10% - 0.20% - 0.5% - 1% - 1.5%

EDTA: 5% - 10% - 15% - 17% - 20% - 25%

MIC was assessed on *Enterococcus faecalis* obtained from root canals, stored and activated in BHI broth in three successive steps. The following ATCC control strains were used for quality control: *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922,

Escherichia coli ATCC 35218, *Pseudomonas aeruginosa* ATCC 2785.

The dishes containing the series of dilutions of the different irrigating solutions were inoculated with 2 ml of each of the bacterial suspensions under study adjusted to a turbidity value of 0.5 on the Mc Farland nephelometer scale. They were then incubated at 37°C for 24 hours.

Bacterial growth or viability was tested employing a dish containing the bacteria but no irrigating solution. The sterility of the culture medium was assessed by evaluating a dish containing no bacteria.

The minimum inhibitory concentration corresponds to the minimum concentration of the irrigating solution that inhibits bacterial growth.

Evaluation of antibacterial effect of the irrigation solutions on contaminated root dentin *Preparation of the tooth specimens*

We employed 8 human, recently extracted teeth (upper incisors and canines) throughout. We eliminated the crown, excised the pulp tissue with a pulpotome, instrumented the teeth with the step-back technique, irrigating alternately with 2.5% sodium hypochlorite and 17% EDTA and performed a final wash in distilled water. Root cement was eliminated with Nr. 11 and 12 Gracey curettes. The future fracture lines were marked, i.e. longitudinally by buccal and lingual to obtain a mesial and a distal portion, and transversally along the whole circumference to obtain apical, middle and coronary fragments. The fragments were obtained employing a chisel and a hammer. A cylindrical, high speed, water-refrigerated N° 837 0,10 (3097 Sorensen Brazil) stone was used to cut the specimens.

The groups were formed according to the irrigation solution employed as follows:

- NaOCl 2.5% (n=8): 4 apical fragments and 4 middle fragments
- EDTA 17% (n=8): 4 apical fragments and 4 middle fragments
- CHX 0.2% (n=8): 4 apical fragments and 4 middle fragments
- Distilled water (n=8): 4 apical fragments and 4 middle fragments

In this way we obtained 32 tooth fragments in total, 16 corresponding to the apical third and 16



Fig 1: Apical area contaminated with *E. faecalis*.

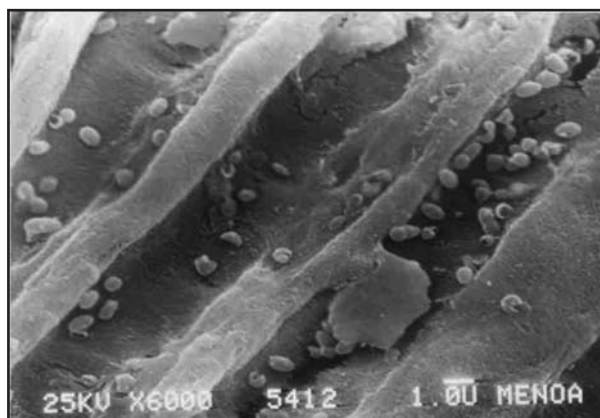


Fig 2: Middle area contaminated with *E. faecalis*.

corresponding to the middle third. They were divided such that the experimental and control groups contained 8 fragments each. As far as possible, the groups contained equal numbers of apical and middle fragments and the control and experimental groups contained fragments of the same specimens.

The fragments were rinsed sequentially for 3 minutes in EDTA 17%, 2.5% NaOCl and distilled water to make dentin permeable. The fragments were then sterilized for 20 minutes in autoclave at 120°C.

Each fragment was then placed individually in 2 ml of brain-heart infusion broth and inoculated with 0.2 ml of an 18 hour suspension of *Enterococcus faecalis* (10^8 colony forming units per ml), previously activated in three successive steps. The samples were incubated at 37°C for 96 hours to allow bacteria to penetrate the dentin tubules.

Each apical and middle fragment of contaminated root was immersed for 2 minutes in 2 ml of the corresponding irrigation solution. The fragments were then washed for 30 seconds in 5 ml of sterile distilled water. Each third was then placed in 2 ml of brain-heart infusion broth and incubated in an upright position at 37°C in 5% CO₂ for 24 hours in an incubator.

Colony forming units were counted in keeping with Clark et al. (5) at 0, 4, 8 and 24 hours after the irrigation of the root fragments with experimental and control solutions. At each time-point we performed the corresponding series of dilutions in brain-heart infusion broth 1:10. Samples of 0.1 ml were seeded in the selective culture medium for *Enterococcus faecalis*, i.e. blood agar with sodium azide (Britania-Argentina). The material was spread using a

Drigalsky spatula and incubated for 24 hours at 37°C in 5% CO₂.

When isolated colonies were observed, viable bacterial cells were counted. Inhibition was determined in terms of the ratio of viable cells in teeth irrigated with distilled water (control) to viable cells in teeth irrigated with each of the experimental solutions. The control sample included non contaminated apical and middle portions that served to determine tooth sterility.

The identity of the recovered bacteria was confirmed to be *Enterococcus faecalis* based on the appropriate biochemical features.

We included an apical fragment and a middle fragment in the control group to confirm the penetration of bacteria in the dentine tubules. We had previously made longitudinal grooves on the root surface to facilitate fracture. Once the period of contamination had finalized, the tooth fragments were fractured, processed and observed by Scanning Electron Microscopy (Figs. 1 and 2).

RESULTS

Diffusion in agar

We used this method in a pilot study employing each of the three experimental solutions under study. All three solutions exhibited an inhibitory effect, i.e. NaOCl 2.5%= 21 mm; EDTA 17%= 20 mm; CHX= 14mm.

Dilution in agar (MIC)

The minimum inhibitory concentration values of the irrigation solutions NaOCl and CHX for the 7 strains are presented in Figure 3. The MIC for EDTA and all the strains tested was less than 5%.

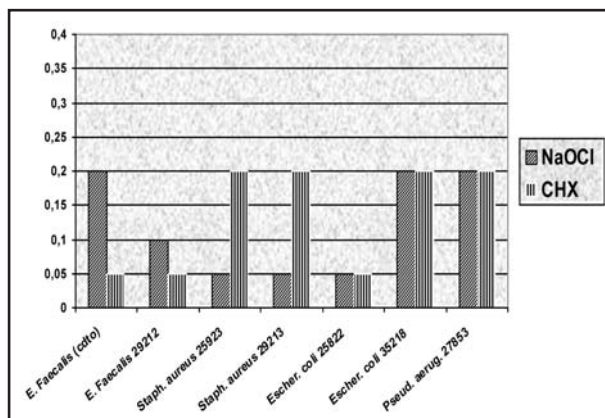


Fig 3: Minimum inhibitory concentration - NaOCl and CHX.

Antibacterial action of endodontic irrigation solutions on root dentin contaminated with *Enterococcus faecalis*

Figures 4 and 5 show the growth curves of *Enterococcus faecalis* in the apical and middle thirds up to the last experimental time-point, i.e. 24 hours.

NaOCL 2.5% fully inhibited *Enterococcus faecalis* in the apical area up to an incubation time of 24 hours. In the middle area, growth was inhibited up to 8 hours after treatment. However, growth was observed at 24 hours. At 24 hours, values were 12 log CFU lower than control values.

CHX 0.2% elicited a reduction in excess of 5 log CFU as compared to control for all the time-points assessed in the apical and middle thirds.

EDTA 17% induced a reduction in bacterial growth of more than 3 log CFU as compared to control in the apical and middle thirds at all the experimental time-points.

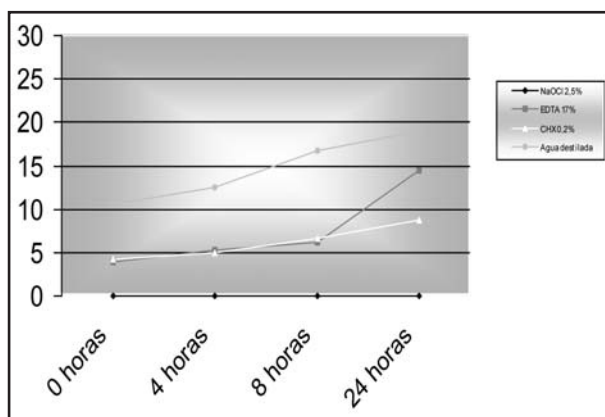


Fig 4: Apical third. Growth curve of *E. faecalis*.

The effect of all the irrigation solutions in the apical and middle thirds was compared for all the experimental time-points examined employing Tukey's test, setting statistical significance at $p=0.05$. NaOCl 2.5% was the most potent antibacterial solution, followed by 0.2% chlorhexidine gluconate and 17% EDTA at all the time-points evaluated, with exception of the 24 hours time-point at which no statistically significant differences were found between NaOCl 2.5% and CHX 0.2%.

Despite the fact that statistically significant differences were found between the antibacterial effect of the different irrigation solutions, they can all be considered effective according to the standards of the NCCLS (1999) that establish that an antibacterial effect involves a 3 log reduction in base 10 (99.99% death) in CFU in the time period under study.

DISCUSSION

Enterococcus faecalis was isolated from infected root canals of patients who attended the Endodontics Clinic of the Faculty of Dentistry, National University of Tucumán, Argentina.

The sodium hypochlorite solution has been recommended as an irrigating solution for chemo-mechanical cleaning of root canals and as a broad spectrum antibacterial agent (6). These capabilities are particularly relevant in the light of complex root canal systems and the need to remove residual organic matter from otherwise inaccessible sites (2).

The chlorhexidine solution is employed as an acetate salt or as gluconate and its value was initially explored in Periodontics. Its potential value in Endodontics as a liquid and as a gel in different con-

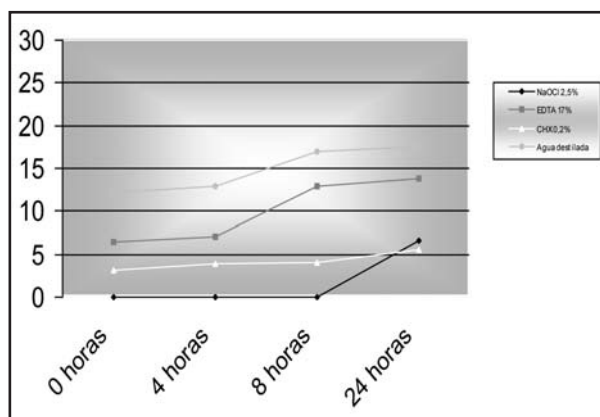


Fig 5: Middle third. Growth curve of *E. faecalis*.

centrations was examined later. Its antibacterial and substantivity properties were demonstrated (7).

In the present study we employed three different methods to evaluate the antibacterial action of irrigation solutions. We used the diffusion method in a preliminary assessment. This method is accepted as an adequate way of comparing the antibacterial effect of different dental materials and medicaments such as the different irrigation solutions examined herein. Extrapolation of the experimental data reported herein to a clinical scenario requires great caution (8).

Siqueira et al. (8) employed the diffusion method to assess antibacterial action on *Enterococcus faecalis* and reported inhibition halos of 9 mm with NaOCl 2.5% and CHX 0.2%, whereas a 7 mm halo was described for EDTA 17%. Admittedly, the absolute values of inhibition differ from those reported herein. However, the relative antibacterial efficacy of the different solutions are in agreement. The inhibition halo described herein for 2.5% sodium hypochlorite on *E. faecalis* is in keeping with the data reported by Siqueira et al. (9).

Heling et al. (10) reported a minimum inhibitory concentration value of 0.157% for sodium hypochlorite on *Enterococcus faecalis*. These data are in close agreement with the values reported herein, i.e. 0.1% for *E. faecalis* ATCC 29212 and 0.2% for *E. faecalis* isolated from root canals.

We performed this technique on contaminated dental fragments, in keeping with Buck et al. (11) who also applied it successfully on middle third root fragments. We also examined apical fragments given their relevance in terms of providing contributory information on the contamination and antibacterial effect in an anatomically complex site. Buck et al. (11) contaminated the fragments by exposing only one surface to the bacteria. Unlike those authors, we contaminated the whole fragment and allowed the solutions to act on all the surfaces. In our opinion the technique used in the present study is simpler in terms of avoiding the invasion of the canal area. The uncertainty of canal invasion exists when impermeabilization of the remaining walls is necessary.

Our data differ from those of Ohara et al. (12) who found that 0.2% chlorhexidine was more effective than 5.25% sodium hypochlorite in direct contact with anaerobic bacteria for 1, 15, 30, and 60 minutes and one week. These discrepancies could be attributed to methodological differences between both studies. In the present study we assessed the

antibacterial activity on contaminated fragments whereas those authors analyzed the efficacy of the solutions in direct contact with the bacteria.

Heling and Chandler (13) failed to detect statistically significant differences between 1% sodium hypochlorite and 0.2% chlorhexidine. However, they found that 1% sodium hypochlorite inhibited *E. faecalis* in bovine dentin more effectively. The discrepancy with our data may be attributed to the fact that those authors used a lower sodium hypochlorite concentration.

Our results are in agreement with those of Marais and Williams (14). These authors found that 3.5% sodium hypochlorite was an effective antibacterial irrigation solution when tested on teeth contaminated with strict and facultative anaerobes such as *Enterococcus faecalis*. No colonies were found in samples taken immediately following irrigation or one week after sealing the non-medicated canals.

Our results are also keeping with Abdullah y col. (15) who compared the efficacy of 0.2% chlorhexidine gluconate, 17% EDTA and 3.0% NaOCl on *Enterococcus faecalis* biofilm. NaOCl was the most effective agent and achieved 100% kills of *E. faecalis* after a two minutes contact time.

Our data closely resembled the results of Ringel et al. (16), who compared, *in vivo*, the effect of 2.5% NaOCl and 0.2% chlorhexidine gluconate on teeth with necrotic pulps and reported that the NaOCl solution was more effective.

The capacity of the irrigating solution to penetrate dentin is of great importance. Vahadaty et al. (17) found that NaOCl 2% and CHX 2% were effective at a depth of 100 μ m. However, at greater depths these solutions were less effective. In the present study, at 24 hours we found bacteria in the culture medium corresponding to the middle third. This finding may be attributed to an incapacity of the irrigating solution to reach this area, thus allowing bacteria to reproduce and become detectable. The apical third failed to evidence growth upto 24 hours, probably because the dentin was more impermeable and less prone to bacterial invasion in depth during the experimental periods examined.

Shih et al. (18) failed to observe bacterial growth in recently extracted human teeth inoculated with *E. faecalis* immediately after irrigation with 5.25% NaOCl. Similar results were obtained herein in the apical and middle thirds employing NaOCl at 2.5%. Our data on 0.2% chlorhexidine gluconate are in keeping with Delany et al. (19) who found a signifi-

ificant reduction in the number of colony forming units following irrigation of infected canals of extracted teeth with this solution.

Lynne et al. (20) reported that 0.12% chlorhexidine was effective in reducing the number of colony forming units in bovine teeth previously contaminated with *Enterococcus faecalis*.

The 17% EDTA solution reduced by more than 3 log the number of colony forming units as compared to control. Although this solution can be considered effective, it is the least effective of the

solutions examined. Heling et al. (21) found that the EDTA solution, there was some elimination on *S. aureus* after incubation for 45 min.

Our findings are also in keeping with Heling and Chandler (13) who reported that the solution of 17% EDTA was significantly less effective than solutions of 1% sodium hypochlorite and 0.2% chlorhexidine. The direct extrapolations to clinical conditions must be exercised with caution, because the in vitro environment created in this experiment are different to the root canals conditions.

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