In vitro assessment of the immediate and prolonged antimicrobial action of chlorhexidine gel as an endodontic irrigant against *Enterococcus faecalis*

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Objective. The aim of this study was to assess in vitro the antimicrobial activity of 2% chlorhexidine gel against *Enterococcus faecalis*, comparing it to other endodontic irrigants (2% chlorhexidine liquid and 5.25% sodium hypochlorite).
Study design. Eighty roots of human mandibular premolars were prepared by serial instrumentation technique, autoclaved, and contaminated for 7 days with *E faecalis* monocultures. The roots were then divided into 5 groups according to the irrigant substance used during the standardized biomechanical preparation. In order to evaluate the antimicrobial action of the irrigant substances, 3 microbial samples were taken: initial (before the biomechanical preparation); post-treatment (immediately after the biomechanical preparation), and final (7 days after the biomechanical preparation). The microbiological samples were plated to count the colony-forming units (CFU).
Results. The 2% chlorhexidine gel and 2% chlorhexidine liquid significantly reduced the *E faecalis* CFU in the post-treatment and final microbiological samples. The 5.25% sodium hypochlorite also reduced the *E faecalis* CFU immediately after the root canal instrumentation, but it was not able to keep the root canal free of detectable *E faecalis* in the final sample.
Conclusions. The 2% chlorhexidine gluconate (gel and liquid) antimicrobial ability was more effective than 5.25% sodium hypochlorite in keeping low CFU of *E faecalis* for 7 days after the biomechanical preparation.
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Bacteria are the main causative factors in pulpal and periapical pathosis.[1] Although obligate anaerobic bacteria dominated the root canal microbiota, some facultative strains, eg, *Enterococcus faecalis*, have been involved in persistent infections influencing the prognosis of the root canal treatment.[2]

Due to the complex anatomy of the root canal system, an effective disinfecting in endodontics is only achieved by augmenting mechanical preparation with antimicrobial irrigants.[3]

Therefore, various substances have been used during and immediately after root canal preparation to remove debris and necrotic pulp tissue and to help eliminate microorganisms that cannot be reached by mechanical instrumentation.[4] It is highly desirable that the chemical agents selected as endodontic irrigants possess 4 major properties: antimicrobial activity, dissolution of organic tissues, aid in debridement of the canal system, and nontoxicity to periapical tissues.[5]

A concentrated solution of sodium hypochlorite (NaOCl) has been recommended for its greater effectiveness than diluted solutions. Other properties attributed to NaOCl, such as organic tissue dissolution[6] and low surface tension[7] are also important in the cleaning of root canals. However, NaOCl is known to be highly irritant to the periapical tissues,[8] mainly at high concentrations. NaOCl is incapable of removing inorganic material. Consequently, the combination of NaOCl and EDTA has been recommended for root canal therapy.[9] For this reason the search for another irrigant with a lower potential to induce adverse effects is desirable.

Chlorhexidine digluconate has also been recommended for root canal irrigation[10] of infected teeth because of its antimicrobial action and its adsorption to dental hard tissues with gradual and prolonged release at therapeutic levels, frequently called residual effect or substantivity.[11,12] These substances have been used during chemomechanical preparation and are usually in liquid form. However, the inability of chlorhexidine liquid to dissolve pulp has been a problem. The chlorhexidine in gel form has already been suggested for root canal dressing[13] and for its use to irrigate the root canal.[14] The gel base used in the present study was the natrosol gel

(hydroxyethyl cellulose) that is a nonionic, highly, inert and water-soluble agent.[15] Vivacqua Gomes et al[16] showed that chlorhexidine gel (viscous form) does not interfere with the sealing ability of the obturation cement, being soluble and removable with a final flush of 5 mL distilled water. It maintains the dentin walls almost clean of smear layer due to its mechanical cleansing ability, which compensates for its inability to dissolve organic tissue.[14]

Thus, the objective of this study was to assess in vitro the antimicrobial action and residual effect of 2.0% chlorhexidine gluconate based on natrosol gel as an endodontic irrigant, comparing it with 2.0% chlorhexidine liquid and 5.25% NaOCl.

MATERIAL AND METHODS
Specimen preparation

Eighty freshly extracted, straight, single-root teeth with complete apex formation were used. The crowns of the teeth were removed at the cementoenamel junction using a rotary diamond saw and tap water irrigation. The diameter of the root canals was standardized in the apex using K file size 25.

All teeth were submitted to an ultrasonic bath for 10 min in 17% EDTA followed by 10 min in a 5.25% NaOCl bath, according to Perez et al[17] to eliminate the smear layer produced during the initial preparation. Their apical foramens were then sealed with epoxy resin to prevent bacterial leakage.

Specimen sterilization

The teeth were individually placed in bijoux bottles containing 5 mL of Brain Heart Infusion Broth (BHI, Oxoid; Basingstoke, UK) medium and autoclaved at 121°C, 1 atm, for 20 min. They were then kept in an incubator at 37°C for 24 h to check the efficacy of the sterilization treatment.

Contamination with *Enterococcus faecalis*

Isolated 24-h colonies of pure cultures of *E faecalis* (ATCC 29212) grown on 5% defibrinated sheep blood + BHI agar plates were suspended in 5.0 mL of BHI. The cell suspension was adjusted spectrophotometrically to match the turbidity of 3.0×10^8 CFU/mL (equivalent to ≈ 1.0 McFarland standard).

The bijoux bottles containing each specimen were opened under laminar flow. Sterile pipettes were used to remove 5.0 mL of sterile BHI and to replace it with 5.0 mL of the bacterial inoculum. The bottles were closed and kept at 37°C for 7 days, with the replacement of 2.0 mL of contaminated BHI for 2.0 mL of freshly prepared BHI every 2 days to avoid medium saturation. The turbidity of the medium during the incubation period indicated bacterial growth. The purity of the cultures was confirmed by Gram staining, catalase production,

colony morphology on BHI agar + blood, and using a biochemical identification kit (API 20 Strep, bioMérieux; Marcy-l'Etoile, France).

Specimen division

The contaminated teeth were divided into 3 study groups of 20 teeth each and 2 control groups of 10 teeth each according to the irrigant used during root canal preparation as follows:

Group 1: 20 teeth irrigated with 2% chlorhexidine gluconate gel
Group 2: 20 teeth irrigated with 2% chlorhexidine gluconate liquid
Group 3: 20 teeth irrigated with 5.25% NaOCl
Control 1: 10 teeth irrigated with distilled water
Control 2: 10 teeth irrigated with natrosol gel

The same manufacturer (EssencialPharma, Laboratory of Manipulation; Itapetininga, Brazil) prepared all the irrigants.

Antibacterial assessment

Previous to the mechanical preparation, the canals were flushed with 3 mL of sterile saline, and, immediately after, the root canals were dried with sterile paper points that had been placed in flasks containing 1 mL of sterile BHI. This microbial sample was called "initial."

The root canals were instrumented and enlarged with K files (Dentsply Maillefer; Baillaigues, Switzerland) until a #35 file could be introduced 1 mm short of the apex. The canals were irrigated with 1 mL of the test irrigant between each file change.

At the end of the biomechanical preparation all root canals were flushed with 3 mL of the test irrigant and 3 mL of the appropriate neutralizer, followed by a final flush performed with 3 mL of sterile saline delivered in the same way. Neutralizer for NaOCl was 0.6% sodium thiosulfate, and 0.5% Tween 80 + 0.07% lecithin was used for chlorhexidine.[18]

The root canals were dried with sterile paper points that had been placed in flasks containing 1 mL of sterile BHI. This microbial sample was called "post-treatment" (immediately after the biomechanical preparation).

After this, the root canals were filled with 40 μL of sterile BHI. The access cavity was sealed with gutta-percha. The specimens were incubated aerobically for 7 days at 37°C. After an interval of 1 week the sealing was carefully removed and the canals were irrigated with 3 mL sterile saline and dried with sterile paper points that had been placed in flasks containing 1 mL of sterile BHI. This microbial sample was called "final" (7 days after the biomechanical preparation).

Table. CFU mean counts and rank averages in the initial, post-treatment, and final culture (7 days after instrumentation)

Group	Initial CFU mean count ($\pm$ SD)	Post-treatment CFU mean count ($\pm$ SD)	Post-treatment ranks average	Final CFU mean count ($\pm$ SD)	Ranks average final
G1—2% CG gel	433600 ($\pm$ 567384.2)	0 ($\pm$ 0.00)	34.50 a	0 ($\pm$ 0.00)	21.50 a
G2—2% CG liquid	423300 ($\pm$ 456823)	0 ($\pm$ 0.45)	36.22 a	0 ($\pm$ 0.00)	21.50 a
G3—2.5% NaOCl	153600 ($\pm$ 199306.8)	200 ($\pm$ 615.58)	38.10 a	107900 ($\pm$ 134869.1)	60.15 b
C1—Distilled water	32200 ($\pm$ 22537.8)	1600 ($\pm$ 3238.65)	46.75 ab	52400 ($\pm$ 95218.11)	60.20 b
C2—Natrosol gel	116600 ($\pm$ 174783.8)	15800 ($\pm$ 31992.36)	50.60 b	39400 ($\pm$ 5611.4)	57.50 b

SD, standard deviation; *CG*, chlorhexidine gluconate.
Averages followed by the same letter are not significantly different ($P \geq .05$).

The flasks, which contained microbial samples, were agitated in a mechanical mixer until the paper points disintegrated, and 10-fold serial dilutions to 10^{-2} were made in dilution blanks. The dilution 10^{-2} aliquots of 50 µL were inoculated onto triplicate blood agar plates (BHIA). The blood agar plates were incubated aerobically for 48 hours at 37°C.

The colony-forming units (CFU) were counted and the purity of the cultures was confirmed by Gram staining, catalase production, colony morphology on BHI agar + blood, and by the use of a biochemical identification kit (API 20 Strep, bioMérieux; Marcy-l'Etoile, France).

Statistical analysis

The data was statistically analyzed using the BioEstat 2.0 program (CNpQ, 2000, Brasilia, DF, Brazil). Due to the the the great standard deviation of the CFU means, a rank transformation was indicated for this study. It is a statistical tool that produces a table containing the ordinal rank of each value in a data set, in other words, rank transforms the dependent variable. In the present analysis, high rank averages indicate great CFU means (Table). The Kruskal-Wallis test was applied, with the level of significance established at 5% ($P < .05$).

RESULTS
Initial culture (before instrumentation)

The infection methodology adopted for the present investigation was adequate, because after 7 days of incubation of the contaminated teeth, it was possible to recover pure cultures of viable *E faecalis* from all teeth.

Post-treatment culture (immediately after instrumentation)

The mechanical instrumentation and irrigation with all tested substances significantly reduced the number of bacteria in the root canal. The specimens treated with distilled water or natrosol gel also demonstrated significant reduction in the number of bacteria in the root canal. A nonparametric analysis of variance (Table) revealed that there were no differences among groups 1, 2, and 3 ($P > .05$). However, the bacterial reduction observed in group 3 (5.25% sodium hypochlorite) was not statistically different ($P > .05$) compared to the control group irrigated with distilled water (Table).

Final culture (7 days after instrumentation)

There was a generalized increase in the CFU count of the specimens treated with distilled water and natrosol gel and 5.25% sodium hypochlorite 7 days after the instrumentation.

On the other hand, with the chlorhexidine gel and liquid treated teeth, a lower *E faecalis* CFU count was maintained. A nonparametric analysis of variance (Table) revealed that the differences between 2% chlorhexidine gel and 2% chlorhexidine liquid were not significant for both groups of teeth ($P > .05$). There were also no differences among the 5.25% sodium hypochlorite, the distilled water, and the natrosol gel groups ($P > .05$).

DISCUSSION

Several models have been proposed in literature for the study of dentin infection and most of them use *E faecalis* as the microorganism of choice.[14,18,19,20]

The infection methodology adopted for the present investigation was adequate, because after 7-day incubation of the contaminated teeth, it was possible to recover pure cultures of viable *E faecalis*.

Mechanical instrumentation−associated irrigation has been considered to play a key role to the success of endodontic treatment.[21] Sodium hypochlorite, owing to its powerful germicidal and bactericidal properties, is still the most frequently used root canal irrigant.[22] However, several investigators have suggested the use of chlorhexidine gluconate as an equally valuable root canal irrigant.[23,11] Chlorhexidine gluconate is a cationic bisguanide that combines to the cell wall of the microorganism and causes leakage of intracellular components.[24,25] At low concentrations of chlorhexidine, small-molecular-weight substances will

leak out, resulting in a bacteriostatic effect. At higher concentrations, chlorhexidine has a bactericidal effect owing to precipitation and/or coagulation of the cytoplasm, probably caused by protein cross-linking.[26]

Specific neutralizers were applied after the end root canal instrumentation to make sure that no irrigant vestige would be transferred to the culture medium, altering bacterial growth. Therefore the irrigant substances acted only during the instrumentation procedures.

Our results showed that all evaluated irrigants significantly reduce *Enterococcus faecalis* in vitro immediately after biomechanical instrumentation procedures. The specimens treated with distilled water and natrosol gel also demonstrated a decrease in the colony-forming-unit (CFU) count. However, there was a CFU increase in the specimens treated with distilled water and natrosol gel 7 days after biomechanical instrumentation procedures. These results are in accordance with those of Bystrom & Sundqvist,[21] who demonstrated the immediate reduction of bacteria after root canal preparations but in half the cases bacteria remained in the canals after 4 mechanical instrumentations, and it was observed that remaining bacteria were able to recontaminate the whole root canal.

On the other hand, with the NaOCl-treated specimen, the results in this study indicate that in spite of the 5.25% NaOCl solution application, all bacteria were not exterminated in the infected root canals. Therefore, there was an absolute increase in microorganisms for 100% of the specimens 7 days after the biomechanical instrumentation. Our findings are in agreement with the results of other investigators,[3,27,28] who have shown the presence of living microorganisms 2 to 7 days after root canal instrumentation with NaOCl.

Our results also showed that 2% chlorhexidine, gel and liquid, was absorbed and released from dentin and enamel even after 7 days of root instrumentation. The outcome was a maintained lower *E faecalis* CFU count. Our findings are in agreement with the results of other investigators, who have shown the bactericidal property and substantivity of chlorhexidine when used as a root canal irrigant.[11,29,30]

These results and other studies[12,14,16] indicate that chlorhexidine, mainly in gel form due to its cleansing ability, should be considered as an endodontic irrigant. On initial exposure to chlorhexidine, antimicrobial activity is at least as effective as with NaOCl. And as revealed in this study, its substantive antimicrobial activity offers potential protection of the canal tissues for as many as 7 days after instrumentation. Although NaOCl may be equally effective on initial exposure, it is not a substantive antimicrobial agent.

Thus, this study shows that the mechanical effects of instrumentation, coupled with the substantive antimi-

crobial activity of 2% chlorhexidine, gel and liquid, are more effective than 5.25% NaOCl in keeping a low *Enterococcus faecalis* CFU count 7 days after biomechanical instrumentation. It was concluded that chlorhexidine gluconate, in gel and liquid forms, has potential for use as an auxiliary antimicrobial agent during the biomechanical procedures.

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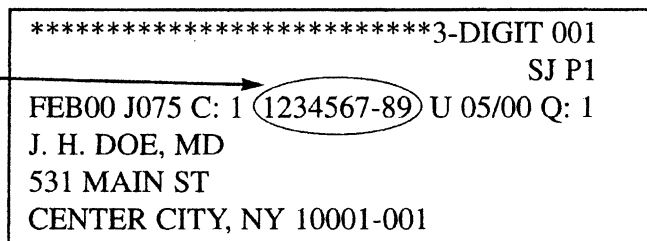
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