Final Endodontic Irrigation with 2% Peracetic Acid: Antimicrobial Activity and Cytotoxicity

Daniel de Oliveira Brandão-Neto[1] João Victor Zebende Mello[2] Marilia Fagury Videira Marceliano-Alves[3] Thais Machado de Carvalho Coutinho[3] Eduardo Fagury Videira Marceliano[4] Michelle Paiva Weydt Galhardi[5] Vívian Souza Tavares[2] Aline Peçanha Muzy Dias[2] Renata Ximenes Lins[1]

[1]Postgraduate Program in Dentistry, Health Institute of Nova Friburgo, Fluminense Federal University, Nova Friburgo, Rio de Janeiro, Brazil
[2]School of Dentistry, Health Institute of Nova Friburgo, Fluminense Federal University, Nova Friburgo, Rio de Janeiro, Brazil
[3]Post Graduation Program in Dentistry and Research, Iguaçu University, Nova Iguaçu, Rio de Janeiro, Brazil
[4]Dental Clinic Department, Brazilian Army General Hospital of Belem, Belem, Brazil
[5]Department of Dental Materials, Sao Jose University, Rio de Janeiro, Brazil

Address for correspondence Marilia Fagury Videira Marceliano-Alves, PhD in Endodontics, Avenue Abílio Augusto Távora, 2134 - Dom Rodrigo, Nova Iguaçu, Rio de Janeiro 26260-045, Brazil (e-mail: mmarceliano@hotmail.com).

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Abstract

Objective The aim of present study was to assess the cytotoxicity and antimicrobial efficacy of 2% peracetic acid (PAA) compared with 5.25% sodium hypochlorite (NaOCl) and 2% chlorhexidine (CHX).

Material and Methods For the cytotoxicity test, 100 µl of the tested solutions were added in 12 wells with ECV 304 endothelial cells in each group: NaOCl, CHX, and PAA, in addition to the control group. Each solution was evaluated after 24 hours of contact in four dilutions: 0.2, 0.1, 0.05 and 0.025 through mitochondrial function using MTT colorimetric assay. In the antimicrobial evaluation, 40 dentin blocks 5 mm in length and 0.2 g in weight were incubated with 400 µl of *Enterococcus faecalis* suspension for 21 days at 37°C. The contaminated samples were divided into three experimental groups within 5 minutes of contact: NaOCl group, CHX group, PAA group, as well as the positive control group. The specimens received treatment and were transferred to a tube with saline for serial dilution of the solution and seeding for isolation and colony forming unit (CFU) count.

Statistical Analysis The results obtained were expressed as mean (A570 nm) ± standard deviation (SD) and in a multiple linear regression model and multiple comparisons conducted.

Results The antimicrobial evaluation revealed that the NaOCl and CHX groups showed a statistically significant difference compared with the control group ($p < 0.001$), while the PAA reduced only the CFU growth. It can be concluded that, among the agents tested, PAA expressed greater cell viability, followed by CHX and NaOCl. However, it did not show greater antimicrobial activity *in vitro* in the mature biofilm of *Enterococcus faecalis*.

Keywords
- chlorhexidine
- disinfection
- enterococcus faecalis
- peracetic acid
- sodium hypochlorite

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Introduction

The success of the mechanical and chemical preparation of the root canal system is based on the effectiveness of endodontic instruments, irrigating solutions, and chelating agents that support the reliability and longevity of endodontic treatment.[1] The use of irrigating solutions during mechanical and chemical preparation favors the other stages of endodontic treatment.[2,3] Since the elimination of microorganisms from the root canal system presents a major challenge for the success of the treatment, significant chances of a favorable treatment require a maximum microbiological eradication of the infection before the filling step.[4-6]

Sodium hypochlorite (NaOCl) is the most used irrigating solution due to its excellent antimicrobial activity associated with the dissolution capacity of organic tissues.[7] However, it does not provide removal of the smear layer from the surface of the root dentin, requiring the additional use of demineralizing agents.[8] Besides, it is considered highly irritating to periapical tissues.[9,10]

During endodontic treatment, there is a possibility that the irrigating solution contacts the periapical tissues with debris that can be extruded through the apical foramen and cause deleterious effects on them.[11] Thus, it becomes relevant to evaluate the cytotoxic effects of an auxiliary chemical substance, so that cell viability of the periapical region is maintained, favoring the prognosis.[12]

A cleaning intervention with total removal of microorganisms adhered to the dentin surface of the root canal is not possible to be achieved, so several studies have demonstrated innovative techniques, with the objective of significantly improving disinfection, such as passive ultrasonic irrigation (PUI), photodynamic therapy (PDT), continuous irrigation techniques and apical positive and negative pressure irrigation methods.[13-15]

Peracetic acid (PAA) is a strong disinfectant with large antimicrobial spectrum against bacteria, viruses, fungi, and spores, even in low concentrations. This solution has activity even in the presence of organic residual material, with no residual toxic and /or mutagenic byproducts, low pH dependence and short contact time.[16,17] It is a balanced mixture of hydrogen peroxide, acetic acid, and stabilizing vehicle. Its main active agent is a stabilized combination of an active oxygen molecule, donated by hydrogen peroxide, and an acetic acid molecule.[18,19] The mechanism of action is similar to oxidizing agents such as hydrogen peroxide, which gives high oxidizing power and promotes oxidation of the SS and SH bonds of cellular components, acting on the cytoplasmic membrane and disabling physiological functions such as the osmotic barrier.[16,18,19] Its antibacterial effectiveness associated with its capacity for removing the smear layer has made PAA a possible alternative to be used as endodontic irrigant, which would simplify and speed up root canal preparation.

In this sense, the aim of this study was to evaluate the effectiveness of improving root canal disinfection with 2% peracetic acid, through antimicrobial activity against 21-day mature biofilm of *Enterococcus faecalis*, and its cytotoxic performance employed after contact with human ECV 304 cell line, compared with agents routinely used in endodontics, NaOCl (5.25%), and chlorhexidine (CHX, 2%).

Material and Methods

Cytotoxicity

For this evaluation, human endothelial cells of the ECV 304 cell line, obtained from spontaneously immortalized human umbilical cord veins, were used. The incubation parameters of ECV 304 endothelial cells were 37°C in an atmosphere of 95% oxygen, 5% carbon dioxide and 100% humidity for 7 days in F12 medium supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) along with 50 µg/mL of gentamicin sulfate (Sigma Chemical Co.; St Louis, MO, United States). The cells were collected by means of washing with serum-free α-MEM (Sigma Chemical Co.) after treatment with 5 mL of trypsin (0.1%)/1 mL of EDTA (0.01%) (Sigma Chemical Co) in phosphate-buffered saline (PBS) solution for 7 to 10 minutes. Fourth-pass cells were seeded on a 96-well plate at a density of 1.0×10^5 cells per well and allowed to fix for 24 hours in an α-MEM plus supplement. To assess cytotoxicity, a dose of 100 µL of the tested solutions was added to the wells of each group: 2% peracetic acid, 5.25% NaOCl, and 2% CHX. As a control group, only culture medium with supplements was used. Each solution was evaluated after 24 hours of contact and in four dilutions: 0.2, 0.1, 0.05, and 0.025. The tests were done in triplicate.

The evaluation of cytotoxicity in mitochondrial was assessed after incubation of viable cells; cell dehydrogenase enzymes from the MTT tetrazolium ring (pale yellow) were cleaved to convert the water-soluble tetrazolium salt to dark blue formazan crystals. A total of 0.5 mg/mL of MTT solution was added to each well to be solubilized with dimethyl sulfoxide (200 ¼), and the absorbance was determined in 570 nm using an ELISA plate reader (Thermomax Microplate Reader; Molecular Devices, Santa Monica, CA, USA).

For the statistical analysis, the absorption results obtained were expressed as mean (A570 nm) ± standard deviation (SD) and in a multiple linear regression model (SPSS/PC + Statistics 4.0 software: SPDD International BV, Gorinchem, Netherlands). Multiple Comparisons Test by Sidak were performed using the Graph Pad Prism 6.07 statistics program. Values of $p < 0.05$ were considered statistically significant.

Antimicrobial Activity

Forty lower incisors with prior indication of tooth extraction were used, according to the Treatment Plan of the Dental Clinic of the Faculty of Dentistry of the Health Institute of Nova Friburgo of Fluminense Federal University. There was no exclusion of any teeth obtained. The teeth were autoclaved and subsequently passed through a sample standardization process, received horizontal cuts with carbide sanding disks in the middle third of the root, which resulted in a dentin block with a dimension of 5 mm in length and 0.2 g in weight which guaranteed equivalence of dentin volume. The specimens were randomly distributed between the experimental and control groups.

The strain of *Enterococcus faecalis* (EFP38) was previously isolated from primary endodontic infection, identified by MALDI-TOF and PCR for 16S rRNA, and underwent the antimicrobial sensitivity test (AST). The strain was stored in Trypticase Soy Broth (TSB; Disco Laboratories, Detroit, MI, USA) with 20% glycerol at–20°C at the Department of Microbiology and Immunology of the State University of Rio de Janeiro.

From a microbial suspension in TSB with 1% glucose, biofilm formation was induced on the specimens. 400 µL aliquots of the bacterial suspension (1.0×10^8 colony forming unit [CFU]/mL) were transferred to sterile microtubes together with a specimen. The tubes were incubated at 37°C for 24 hours. Prior to the treatments to be tested, the specimens were washed in PBS and transferred to another sterile microtube, where the exposure to irrigating solutions was performed.

Experimental Groups

Manipulated agents were used for the experimental groups, 2% PAA (QUÍMICA SULFAL LIMITADA, Belo Horizonte - MG), NaOCl 5.25% (Mil Formulas Manipulation Pharmacy, Rio de Janeiro, Brazil), 2% CHX (Thousand Formulas, Manipulation Pharmacy, Rio de Janeiro, Brazil). Four groups were tested: NaOCl for 5 minutes, CHX) for 5 minutes, PAA for 5 minutes, and no treatment for control group. The specimens were removed from the microtubes and placed in microtubes containing PBS and glass beads to defrag the biofilm and release viable bacterial cells. This solution was serially diluted, plated in triplicate, and incubated at 37°C for 24 hours for later counting of CFU.

The viability of *Enterococcus faecalis* cells recovered after treatment with the agents was obtained by counting the CFU. From these data, the Mann–Whitney test was applied. Statistical significance was accepted for $p < 0.05$.

Results

Cytotoxicity

A viable cell concentrate was found in the control group after 24 hours. The 24-hour contact MTT assay showed a statistically significant difference ($p < 0.001$) in all dilutions (0.2/0.1/0.05/0.25) between the experimental and control groups, and between the experimental groups themselves.

The results showed that all agents affected cell viability. ►**Fig. 1** shows that among the experimental groups, 2% PAA expresses greater cell viability, followed by CHX 2%, and finally NaOCl 5.25%.

Antimicrobial Activity

The control group expressed a higher number of CFU per millimeter (CFU/mL). There was a statistically significant difference ($p < 0.001$) between the control group and both experimental groups NaOCl and CHX (►**Table 1**). The PAA group was the one that expressed bacterial growth, and showed a statistical difference compared with NaOCl and CHX groups ($p < 0.001$). There was no statistical difference between the PAA group and the control group ($p > 0.001$).

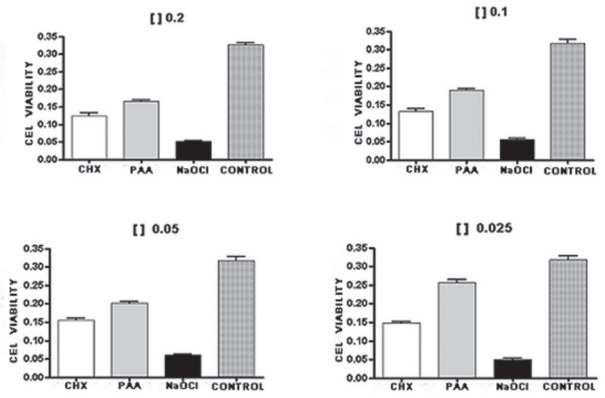

Fig. 1 Effect of cell viability after 24 hours with 2% chlorhexidine (CHX), 2% peracetic acid (PAA), 5.25% sodium hypochlorite (NaOCl) and Control Group in human endothelial cells (ECV 304) by MTT assay. Each bar represents average absorbance (A570 nm) ± standard deviation (SD). There is a statistically significant difference between all groups ($p < 0.01$).

Table 1 Means and standard deviation, and the CFU log number of bacterial cells remaining after antimicrobial activity test

Groups	*n*	Means *(log CFU mL–3)*	SD
2% PAA	10	6.38	0.2
2% CHX	10	0	0
5.25% NaOCl	10	0	0
Saline (control)	10	12.4	0.16

Abbreviations: CFU, colony forming unit; CHX, chlorhexidine; PAA, peracetic acid; SD, standard deviation; NaOCl, sodium hypochlorite.

The results showed that NaOCl 5.25% and CHX 2% had an effective antimicrobial effect against mature biofilm of *Enterococcus faecalis*. Under these conditions, PAA did not demonstrate a satisfactory antimicrobial effect.

Discussion

This *in vitro* study evaluated the cytotoxic effects of endodontic agents: 2% PAA, 5.25% NaOCl, and 2% CHX on human endothelial cells. The toxicity of these irrigating solutions is a relevant issue, since the deleterious effects on periapical tissues can delay healing.[20] Thus, an ideal solution needs, in addition to meeting antimicrobial requirements, to have biocompatibility with periapical tissues.[21]

The agents used were manipulated under prescription with ideal concentrations for disinfection in endodontic treatment, based on studies previously reported in the literature.[22-24]

The cytotoxicity of the irrigating solutions in this study was assessed by means of cell viability in an MTT colorimetric assay. This method is commonly used to assess cytotoxicity of dental materials[25] and has been used by several authors.[26,27] MTT is reduced through mitochondrial

dehydrogenase activity only in metabolically active cells, forming dark blue crystals[28] in which dead cells are distinguished from living cells.[25,29] It is a fast and accurate method of easy reproducibility, in addition to not requiring a washing step that can cause variations in the sample.[17,30] The ECV304 endothelial cell line was used in this study as well as in a previous study of cytotoxicity of dental materials.[31]

The cytotoxicity of PAA is still a conflicting issue. A previous study showed that 0.25% PAA did not significantly influence cytotoxicity when used to disinfect orthodontic ligatures, however Soto et al[30] showed that the use of 1 ppm of PAA to disinfect scaffolds for implants significantly affected the cell viability of keratinocytes, resulting in strong cytotoxic effects.[29]

About endodontic irrigating solutions, the cytotoxic effect of chemical auxiliary solutions has been increasingly studied.[29,31,32] This study showed that in the various dilutions, the cell viability of 2% PAA remained higher than the solutions of NaOCl 5.25% and CHX 2%, with a statistically significant difference between the groups ($p < 0.001$). Such results are like those found by other authors demonstrated that 1% PAA showed less cytotoxic effect than 2.5% NaOCl and 17% EDTA.[21] The evaluation was performed through cell proliferation previously exposed to three different dilutions of the agents: 0.01%, 0.05% and 0.1%, and at intervals of 1, 2, and 4 hours. On the other hand, it was showed that 1% PAA had a greater cytotoxic effect than 2.5% NaOCl, being the study performed with exposure to the agents in concentrations from 0.015% to 0.4% for a period of 10 minutes, and submitted to evaluation of cell metabolism, external morphology, ultrastructure, cytoskeleton and type of cell death.[8]

Considering the evaluated factors, it was found that 2% of PAA showed greater cell viability compared with the other irrigating solutions commonly used in endodontics, which indicates the potential of the agent for its clinical use in this context. Nevertheless, further studies are needed regarding its potential for dissolution of organic tissues, and other effects on root dentin, so that its use as an endodontic agent can be established.[32]

With regard to the evaluation of the antimicrobial action of endodontic agents, a strain of *Enterococcus faecalis* previously isolated from endodontic infection was used, identified by MALDI-TOF and PCR for 16S rRNA, and subjected to trypticase soy agar (TSA) having obtained the result of strain resistant to antibiotics.[33] Other authors reported a variation between 24 to 77% in the prevalence of *Enterococcus faecalis* in the root canals, and this bacterium could penetrate the dentinal tubules as well as the potential to be the infectious agent itself.[15,33,34] The formation of the biofilm for the study occurred for 21 days in human dentin blocks standardized in 5 mm in length, with similarity between the sample confirmed by the equivalence of the dentine volume weighing 0.2 g. Interestingly, this lifetime of 21 days, biofilm is more resistant to agents used in endodontic treatments.[35]

Arias-Moliz et al[36] found a minimal biofilm eradication of *Enterococcus faecalis* after exposure of 1, 5 and 10 minutes in different concentrations of NaOCl and CHX; the findings were that NaOCl was capable of minimal eradication of the biofilm after a period of one minute in concentrations below 0.1%, while CHX achieved this result after five minutes at 0.2%36. Bhasin et al. [22] also stated greater antimicrobial efficacy for 5.25% NaOCl, compared to 2% CHX, both against biofilm of *Enterococcus faecalis*.[36] The present study demonstrates antimicrobial efficacy after five minutes of contact for 5.25% NaOCl and 2% CHX. The 2% PAA did not show a significant microbiological decrease against the biofilm used in this study.

Guerreiro-Tanomaru et al[37] demonstrate that 1% PAA reduced the count of *Enterococcus faecalis* to 86% after 3 minutes, and eliminated it after 10 minutes, in contrast with 2% CHX and 2.5% NaOCl that eliminated the biofilm after 30 seconds of contact, so the authors concluded that PAA 1% is effective against this microorganism despite its lower action compared with 5.25% NaOCl and 2% CHX. In the present study, there is a great disparity in the results found between the experimental groups 5.25% NaOCl and 2% CHX in comparison to 2% PAA. Although PAA presents positive results for endodontic therapy, such as smear layer removal capacity,[32] and good cell viability, it also demonstrates a significantly lower bacterial decrease than NaOCl and CHX against *Enterococcus faecalis* biofilm.[38] Thus, its performance as a final irrigator would not improve the disinfection in the irrigation technique.

CHX demonstrated antimicrobial activity against the strain of *Enterococcus faecalis* and showed less cytotoxicity compared with NaOCl, which allows its use as an alternative to avoid the deleterious effects on periapical tissues.[31-37,39,40] Nevertheless, its antimicrobial action occurs on the cytoplasmic membranes of bacterial cells, which raises concerns about the resistance of these microorganisms.[39,40] That is the reason for NaOCl not being substituted, since its antimicrobial action occurs through unspecific destruction of the bacterial cell, which avoids the undesirable effect of microbiological resistance; with regard to the risks of damage to the periapical tissues, they can be controlled by a safe irrigation technique.[38]

This study demonstrated that the 2% PAA agent tested, even though presenting less cytotoxic effect, was not able to improve the disinfection, as it demonstrated ineffective antimicrobial activity against 21-day mature biofilm of *Enterococcus faecalis*. Therefore, the use of 2% PAA as a final endodontic agent is not applicable, with 5.25% NaOCl and 2% CHX being the agents that best demonstrated antimicrobial effect for disinfection.

Conclusion

This study demonstrated that although 2% PAA has less cytotoxic effect, as this agent has no antimicrobial efficacy against 21-day *Enterococcus faecalis* biofilm. Therefore, it does not offer applicability for root canal disinfection or as a final endodontic irrigating solution. Both tested agents, such as NaOCl and CHX, despite having an undesired toxic effect, seem to be the best options available for disinfecting the root canal.

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Conflict of Interest
None declared.

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