Antimicrobial action, cytotoxicity, cleaning ability and erosive potential of Dentaqua solution compared with sodium hypochlorite

Matheus Albino Souza
Universidade de Passo Fundo

Liviu Steier
University of Pennsylvania

Gabriele Nichetti Vanin
Universidade de Passo Fundo

Mylena Lazaretti Zanella
Universidade de Passo Fundo

Camila Monteiro Pizzi
Universidade de Passo Fundo

Jordana da Silva Koch
Federal University of Rio Grande do Sul

Kellyn Rocca Souza
Federal University of Rio Grande do Sul

Eduarda Rizzon Ferreira
Universidade de Passo Fundo

Felipe Gomes Dallepiane
Universidade de Passo Fundo

Ubirajara Maciel da Costa
Universidade do Estado de Santa Catarina

Vanessa Valgas dos Santos
Universidade do Planalto Catarinense

Aleksandra Palatynska-Ulatowska
Medical University of Lodz

José Antonio Poli de Figueiredo
poli.figueiredo@outlook.com

Federal University of Rio Grande do Sul

Research Article
Abstract

Objectives: This study aimed to compare the antimicrobial action, cytotoxicity, cleaning ability, and erosion of dentine of hypochlorous acid (HClO) obtained from an electrolytic device at two different concentrations (Dentaqua) and three concentrations of sodium hypochlorite (NaOCl).

Methods: Microbiological test - The root canals of sixty single-rooted extracted human teeth were inoculated with Enterococcus faecalis and divided into 6 groups (n=10), according to decontamination protocol: DW (control); 1% NaOCl; 2.5% NaOCl; 5.25% NaOCl; 250 ppm HClO and 500 ppm HClO. The counting of colony-forming units evaluated the decontamination potential of each group, calculating the bacterial percentage reduction. Cytotoxicity test - Cytotoxicity was evaluated after inoculation of the same tested protocols in fibroblastic cells for 3 min, calculating the cell viability percentages. Specific statistical analysis was performed (α = 5%). Cleaning ability and erosion - Fifty-six single-rooted bovine lower incisors were divided into seven groups of 8 roots each, being the test groups 1% NaOCl; 2.5% NaOCl; 5,25% NaOCl; 250 ppm HClO and 500 ppm HClO, and a negative and positive control. Negative control was not contaminated, and the other groups were inoculated with Enterococcus faecalis. SEM images were ranked as from the cleanest to the least clean. Erosion was also assessed, being ranked from the least to the most eroded dentine.

Results: The highest bacterial reduction was observed in experimental groups, with no statistical differences between them (p > 0.05). The highest number of viable cells was observed in control group, followed by 250 ppm HClO and 500 ppm HClO groups, with statistical differences between them (p < 0.05). 1% NaOCl; 2.5% NaOCl; 5.25% NaOCl and 500 ppm HClO displayed the cleanest areas. All sodium hypochlorite groups displayed erosion with higher ranks with greater concentration, while hypochlorous acid did not display any erosion regardless the concentration.

Conclusions: It is possible to conclude that HClO obtained from an electrolytic device presented high antimicrobial activity and low cytotoxicity in both tested concentrations. 500 ppm HClO did not display erosion and showed great cleaning ability.

Clinical relevance: The use of 500 ppm hypochlorous acid may reduce unfavorable behavior of sodium hypochlorite whilst maintaining its antimicrobial action.

Introduction

The effective decontamination of the root canal system is essential during endodontic treatment, taking care to preserve adjacent tissues, since microorganisms represent the major reason for pulp and periapical pathologies (1), and chemical auxiliary substances induce inflammatory reactions when contacting connective tissue (2). Considering the anatomical complexity of the root canal system (3), the root canal instrumentation alone is not able to reach effective decontamination, providing a suitable environment for microbial survival and the development of persistent infection (4). Thus, chemical
auxiliary substances must be associated with mechanical instrumentation, aiming to provide effective antimicrobial activity with no damage to adjacent tissues, favoring apical healing.

Sodium hypochlorite (NaOCl) has been used as a chemical auxiliary substance during chemo mechanical preparation over the years on endodontics. The broad-spectrum antimicrobial activity (5) and ability to promote pulp tissue dissolution (6) are the main properties of NaOCl. In the other side, it induces significant modifications in organic components and mechanical properties of the root dentin (7) and its cytotoxicity induces inflammatory reactions on periapical tissue (2). It is also known that the properties of NaOCl are related to the elevation of its concentration. However, although using higher concentrations of NaOCl results in increased antimicrobial action (8) and tissue dissolution ability (9), it may also increase cytotoxicity (10) and damage to root dentin structure (11). Therefore, there is a constant search for new alternatives in the field of auxiliary chemical substances.

Zehnder [12] adds that the antimicrobial potential of NaOCl and its tissue dissolution capacity are directly related to the concentration, but so is its toxicity. The study also discusses the use of 5.25% hypochlorite by most American professionals. However, strong reactions were seen in the periapical tissues or mucosa of patients who underwent endodontic procedures, in which the solution, in this concentration, leaked through the rubber dam.

Marending et al. [13] elucidate that NaOCl is a non-specific oxidizing agent and can deproteinize hard tissues in biomedical applications. The authors also state that one of the side effects of this solution has not received its due importance within the endodontic literature: the impact on the dentin matrix.

According to Renovato et al. [14], of the 22% organic material in dentin, type I collagen is its main constituent. This collagen has a direct effect on the mechanical properties of dentin. It can suffer the solvent action of NaOCl, since this 5% solution can degrade around 14% of the dry weight of dentin samples in 24 hours..

Sodium hypochlorite can fragment long peptide chains and chlorinate terminal groups of proteins, affecting the mechanical properties of dentin by degrading its organic components. In this context, it was seen that a dentin surface exposed to 5% NaOCl has its peripheral dentin matrix severely altered [13]. Exposing dentin for two hours to a concentration of 3% significantly reduces its modulus of elasticity and its resistance to flexion compared to saline [15].

A 5.25% sodium hypochlorite solution significantly reduces the elastic modulus and flexural resistance of dentin, compared to a 0.5% NaOCl solution [12].

Pascon et al. [16] discuss changes in tooth hardness after the use of sodium hypochlorite during endodontic treatment. The use of irrigant would be a cumulative factor and could predispose the tooth to fractures due to overload during chewing. Regarding the decrease in flexural resistance, the result would be a reduced ability of the tooth to withstand deformations in the presence of some type of force. This way, after using NaOCl, less effort would be required for the cohesive bonds within the dentin to break.
These results were seen after irrigation with concentrations of 2.5%, 3%, 5%, 5.25% and 9% in a period between 24 minutes and 2 hours.

Renovato et al. [14] tested samples irrigated with 1%, 2.5% and 5% NaOCl. In all groups, it was possible to observe areas of erosion at different levels - moderate to severe, in all thirds of the root canal.

Dentaqua (Dentaqua, Conmel, Ireland) can produce hypochlorous acid (HClO) solution at different concentrations. It is obtained by the electrolysis of saline solution mixed with distilled water, demonstrating antimicrobial potential in the cleaning of dental unit waterlines [17], as well as low cytotoxicity when tested over epithelial tissues [18]. In addition, HClO has proinflammatory and anti-inflammatory properties, promotes healing by regulating cytokines and growth factors [19], and oxidizes organic matter present in wastewater [20]. Although the literature reveals a promising future for the use of HClO as chemical auxiliary substance, its antimicrobial activity in root canals and cytotoxicity potential over connective tissue cells need further investigations.

Thus, this study aimed to perform in vitro comparisons of antimicrobial action cytotoxicity and erosive potential of HClO obtained from an electrolytic device and several concentrations of NaOCl. The hypotheses to be tested in this study were that HClO would present similar antimicrobial activity and erosive potential but lower cytotoxicity, when compared to all tested concentrations of NaOCl.

**Materials and Methods**

This study was approved by the Research Ethical Committee of the University of Passo Fundo (protocol number 5.783.928).

**Antimicrobial evaluation**

Sixty single-rooted extracted human teeth were used in the present evaluation. All teeth were obtained from the Biobank of the School of Dentistry of the University of Passo Fundo (Passo Fundo, RS, Brazil). Dental crowns were sectioned with diamond disc so that all roots remained with 15 mm in length. All roots were prepared to remove pulp tissues and to standardize the canal diameter. Working length (WL) was established by introducing #10 K-file (Dentsply-Sirona, York, PA, USA) into the root canal until its tip was visualized at the apical foramen. From this measure, 1 mm was subtracted from WL. Roots were enlarged using manual K-files (Dentsply-Sirona) and serial instrumentation, up to a #35 file. Distilled water (DW) (Natupharma, Passo Fundo, RS, Brazil) was used as an irrigant solution and renewed at each instrument change. Subsequently, root canals were filled with 17% EDTA (Natupharma, Passo Fundo, RS, Brazil) for 1 minute to remove the smear layer, followed by irrigation with 5 mL of DW and drying with absorbent paper (Tanari, Manacapuru, AM, Brazil).

Roots were sterilized at 120°C in an autoclave (Kavo, Joinville, RS, Brazil) for 30 minutes. Five samples were randomly selected for sterilization control. The sterile paper point was placed in contact with the canal walls of each sample for 15 seconds and transported to a microtube containing 1 mL of 0.9%
saline solution (Basso, Caxias do Sul, RS, Brazil). The material was homogenized and a 100 μL aliquot was cultivated on blood agar after five minutes. Samples were incubated at 37°C for 48 hours and showed no sign of bacterial growth (16).

The reference strain was *Enterococcus faecalis* (*E. faecalis*) (ATCC 19433), which was cultivated in brain-heart infusion (BHI) broth (Acumedia–Neogen, Lansing, MI, USA) for 24 hours at 37°C in bacteriological incubator (Kavo, Joinville, SC, Brazil). The turbidity degree was adjusted to the McFarland's 1.0 scale, corresponding to 3.0 x 108 CFU/ml and optical density from 0.25 to 550 nm. A 100-μL culture aliquot was inoculated into the root canal of each sample, until extravasation to the root canal entrance. The culture was maintained for 14 days for biofilm formation, and the remaining volume was replaced every 48 hours with sterile BHI. Once a week, one BHI aliquot was collected from a randomly selected specimen of each group and submitted to Gram staining and cultured on blood agar, followed by catalase and esculin tests, to verify the absence of other microorganisms.

After contamination, the 60 samples were irrigated with 5 mL of DW and randomly distributed into six groups (n=10), according to test decontamination protocols: G1: DW (control); G2: 1% NaOCl; G3: 2.5% NaOCl; G4: 5.25% NaOCl; G5: 250 ppm HClO; G6: 500 ppm HClO. This number of samples was based on previous study that focused on antimicrobial strategies in endodontics (16).

DW and NaOCl solutions were obtained by manufacturing pharmacy (Natupharma, Passo Fundo, RS, Brazil). The 250 ppm and 500 ppm HClO solutions were obtained by specific Dentaqua device (Dentaqua, Conmel, Ireland) for each concentration. This device consists of a compartment on the top, a connector for the smaller bottle on the back, and a connector for the larger bottle on the front. Figure 1 illustrates the Dentaqua device. The electrochemical activation technology involves the generation of electrochemically activated solutions by passing a dilute NaCl solution through an electric field in a Flow-through Electrolytic Module (FEM), segregating the ions formed and producing two oppositely charged solutions with altered physical and chemical properties. The positively charged solution (anolyte) consists of a mixture of unstable mixed oxidants, such as HClO, in a physically excited state, which is capable of penetrating biofilms and is highly microbicidal. The negatively charged antioxidant solution (catholyte) predominantly consists of sodium hydroxide in an excited state.

The upper compartment is filled with sterile DW. The smaller bottle on the back is filled with brine solution, which is composed of sodium chloride. After filling, the smaller bottle was connected to the back of the device, while the larger bottle was connected to the front of the device. The Water Bottle button was pressed, performing a washing cycle of the device with DW for 3 minutes, discarding this solution in the larger bottle. The DW was then discarded, and the larger bottle was again connected to the device to produce HClO. The Ecasol button was pressed to produce HClO for a period of 10 minutes, which was obtained by the electrolysis of saline solution mixed with DW in the device, dispensing the HClO solution at 250 ppm and 500 ppm in the larger bottle, respectively, being ready to use.
In all groups, the root canals were completely filled with the tested solution, by using 5-mL syringe with 19-G needle, until extravasation to the root canal entrance. The tested solution remained in contact with root canal walls for 5 minutes. Subsequently, irrigation with 5 mL of tested solution was performed and the tested solution was renewed. Six 5-minute decontamination cycles were performed, totaling 30 minutes of the tested solution into root canals. Finally, irrigation with 5 mL of DW was performed and the root canals of all groups were dried using aspiration cannula and absorbent paper points (Tanari).

Microbiological analysis was performed in two stages: after contamination (S1) and after irrigation procedures (S2). Root canals were filled with sterile saline solution and #35 K-file promoted contact with root canal walls for 30 seconds, and #35 sterile absorbent paper points promoted the same intentional contact with root canal walls for 30 seconds. It was transferred to a tube containing 450 μL of 0.85% sterile saline solution, being subsequently homogenized and diluted to 10-3. Aliquots of 100 μL of each dilution were cultivated on blood agar in duplicate, being incubated for 24 h at 37°C. Subsequently, the number of CFUs was counted on plates. The antimicrobial activity was analyzed by the reduction percentage in S1 and S2 CFUs counts.

**Cytotoxicity evaluation**

In this evaluation, L929 gingival fibroblastic cells were used, which were provided by the Laboratory of Applied Virology of the Federal University of Santa Catarina (UFSC, Florianópolis, SC, Brazil). For cell maintenance, Minimum Essential Media (Sigma-Aldrich, Rio de Janeiro, RJ, Brazil) supplemented with 10% fetal bovine serum medium (Invitrogen, São Paulo, SP, Brazil) were used, being kept in 75 cm² culture flasks under humidity at 37°C and 5% CO2.

The assay was performed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide or MTT (Sigma-Aldrich), corresponding to a colorimetric test to evaluate cell viability. L929 cells were trypsinized, counted and distributed in 96-well plates at concentration of 1 x 105 cells per well. The Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Rio de Janeiro, RJ, Brazil) was used, enriched with 5% fetal bovine serum. After incubation for 24 hours at 37°C in oven with 5% CO2, cell confluence was observed under inverted microscope. Then, the DMEM medium was removed by aspiration and 100 µL of test chemical substances were added, being incubated for 3 minutes.

Wells were washed with 200 µL of sterile PBS and 50 mL of MTT solution (1 mg/mL in DMEM) were added for incubation for 4 hours. MTT was carefully removed avoiding damage to cells and 100 mL of dimethylsulfoxide (DMSO) were added to solubilize formazan crystals. DMSO was also added to empty wells for white colorimetric calculation. The plate was placed on mixer for 10 minutes and the absorbance was measured on a 490 nm filter. All experiments were performed in triplicate and cell viability percentages were calculated in relation to control group, according to the following formula:

\[ \text{viability (\%)} = \frac{\text{sample absorbance} - \text{mean blank absorbance}}{\text{control absorbance} - \text{mean blank absorbance}} \times 100 \]

**Cleaning ability and Erosion Potential Analysis (Scanning Electron Microscopy – SEM)**
Fifty-six single-rooted bovine lower incisors were selected after extraction with levers and forceps. They were stored in distilled water and 2.5% bleach (in a 50/50 ratio) until used. The criteria for the selected teeth to be included in the study were: predominantly straight canals that had similar and ideal diameters for carrying out the work and complete formation of the root apex. Exclusion criteria were deciduous teeth, teeth with incomplete development, any type of fracture or apical lacerations.

Tooth Cleaning and Sterilization: After selecting the samples, the external surface of the teeth was cleaned by root scaling. The remaining periodontal ligament attached to the roots was removed with a Gracey type nº 1/2, 3/4 and 5/6 periodontal curette (Neumar Instrumentos Cirúrgicos Ltda - Brazil) and a nº 12 scalpel blade. Then, samples were washed in running water for one minute and dried at room temperature.

The crown of the teeth was removed below the cementoenamel junction perpendicular to the long axis of the tooth with the aid of a diamond disc (KG Sorensen Indústria e Comércio Ltda., Barueri, São Paulo, Brazil) to standardize root length of 16mm (Figure 2 a). Grooves were made in the long axis of the buccal and lingual surfaces of the samples, with sufficient depth so that at the end of the experiment they could be cleaved more easily into two hemisections, but taking care to ensure that the interior of the root canal was not reached (Figure 2 b).

For root canal gauging, 21mm type K manual files (Dentsply Sirona Maillefer, Catanduva, São Paulo - SP) calibrated at 16mm were used. For larger-caliber canals, 20-gauge K-type files were selected, while for smaller-caliber canals, #10 K-type files were used.

After exploring the root canals, a small amount of New Max U utility wax (Technew Comércio e Indústria Ltda, Quintino Bocaiuva, RJ, Brazil) was fixed in the region corresponding to the apex, providing an apical seal to allow tooth irrigation protocol to be carried out (Figure 2 c). The samples were attached to a bench vise so that the grooved faces were perpendicularly attached to the vise, and below a container was placed to collect waste (water, solutions, organic waste).

All teeth received an adapted irrigation protocol for root canal disinfection: protocol by Grundling et al. [21]. Briefly, it consisted of an initial irrigation with distilled water (Asfer, São Caetano do Sul, São Paulo, São Paulo, Brazil) and activation for a time of three seconds. Afterwards, irrigation was performed with 2.5% sodium hypochlorite (Soda Chlorinated Asfer 2.5%, Dental Marc, Caxias do Sul, Rio Grande do Sul, Brazil) and activated again for three seconds. This was followed by using 17% trisodium EDTA (Iodontec Indústria e Comércio de Produtos Odontológicas Ltda, Porto Alegre, Rio Grande do Sul, Brazil) and activation for three seconds. The protocol was repeated three times. For the final wash, distilled water was used. Teeth were dried at room temperature. Helse E1 Irrisonic ultrasonic inserts (Helse, Santa Rosa de Viterbo, SP, Brazil) were used to clean the samples. The insert was positioned 3mm from the working length (16mm) and activated with power corresponding to one third of the equipment (Mini-Endo, SybronEndo, São Paulo, Brazil).
The decoronated teeth were positioned in 1.5ml microtubes (Kasvi, São José dos Pinhais, Paraná, Brazil) with the cervical portion upwards. A small hole was made in the center of the microtube lids with the help of an instrument heated over a flame. In this hole, the teeth were positioned and, subsequently, strips of Nexcare Micropore Tape (3M, Sumaré, São Paulo, Brazil) were used around the cap to adequately fix the tooth and reduce the chances of possible contamination. Next to the central hole, two smaller lateral holes were made with a disposable irrigation needle (Injex 21G, Ourinhos, São Paulo, Brazil); these holes were used to change the culture medium (Figure 2 d).

The microtube/tooth assembly was positioned in polypropylene boxes (Heathrow Scientific, Vernon Hills, Illinois, USA), which were individually packaged and sterilized in an autoclave (Figure 2 e). Sterilization time occurred in a 15-minute cycle at a temperature of 120°C.

Before inoculating the samples, they underwent sterility test using sterile paper cones (Absorbent paper tips 15-40 and 45-80, Diadent/TKD, Dental Web, Porto Alegre, Rio Grande do Sul, Brazil). These tips were introduced into the root canal and, shortly after, were transferred to a tube containing 3ml of BHI. These tubes were incubated at a temperature of 37°C for 24 hours. Sterility testing was performed on all samples. No type of bacterial growth was detected in the broth of the tubes after the sterility test, so the samples passed to the bacteria inoculation stage.

Culture Preparation: Enterococcus faecalis (ATCC 29212) was taken from -20 stock, cultivated in an aliquot of 200ul in 3ml of BHI broth and incubated for a period of 24 hours in the Immunology and Microbiology Laboratory of the School of Sciences of the Pontifical Catholic University of Rio Grande do Sul. Then, a 200 μL aliquot was removed and cultured again in a new 3ml BHI broth and incubated again for 24 hours.

After preparing the inoculum, its initial count was carried out by diluting it to -9. In the dilution box, 450 μL of saline solution was added to each of the 9 wells (each well corresponded to a dilution). In the first well, 50 μL of the initial inoculum was added. Then, serial dilution was carried out: 50 μL from the first well was passed to the second well, and so on, homogenizing the broth. After the serial dilution was completed, 10 μL of each dilution was inoculated using the drop technique, in triplicate.

E. faecalis strains were cultivated in a brain heart infusion (BHI) broth for 24 hours positioned in a shaker. Subsequently, a Gram stain test was performed to check whether there was contamination. After confirming the negative contamination test, the culture was kept in an oven at 37°C until use. E. faecalis were cultivated in BHI medium containing 1% trypticase (BBL, Cockeysville, MD), 1% proteosis peptone (Difco, Detroit, MI), 0.5% yeast extract (Difco, Detroit, MI), 0.5% sodium chloride, 5 mg/mg hemin, and 0.5% mg/ml vitamin K supplemented with 0.2% glucose at 37°C for 24 hours.

Inoculation of Samples: In the microtubes, the Enterococcus faecalis culture was added equally into the root canal of each sample (amount corresponding to 100 μL of the initial inoculum) through the central hole, for biofilm development. Furthermore, BHI broth was added through the side holes until the entire length of the tooth was submerged. In the negative control group, only BHI broth was introduced without
the presence of E. faecalis. The boxes with the microtubes inside were wrapped with plastic film and incubated in an oven at 37°C until the medium was changed again.

Every 48 hours, the culture medium was changed with the aid of disposable irrigation needles and 5ml luer lock disposable syringes (Injex, Ourinhos, São Paulo, Brazil). The largest amount of BHI broth possible (on average 2/3) was removed through the side holes made in the lids of the microtubes and discarded. A new culture medium was inserted at the same location. The samples were then taken to the oven at the same temperature of 37°C for another 48 hours, until the next exchange for a period of 7 days. The renewal of the medium was always carried out three times a week.

During each medium change, a drop of material from each group was collected from the inside of the microtube and placed on a blood agar plate. The 7 plates were placed in an oven at 37°C for a period of 48 hours, after which a visual assessment, collection and Gram analysis were carried out under a microscope (100x objective). This analysis aimed to verify whether there was staphylococcus-type bacteria contamination.

Classification of Treatment Groups

The samples were numbered from 1 to 56 and randomly divided into 7 groups, totaling 8 samples per group, as follows:

G1: NEGATIVE CONTROL - to prove the absence of erosive areas, changes in dentinal tubules, and bacterial growth, the samples were not inoculated and did not receive any type of treatment;

G2: E. faecalis + BHI + 1% NaOCl
G3: E. faecalis + BHI + 2.5% NaOCl
G4: E. faecalis + BHI + 5.25% NaOCl
G5: E. faecalis + BHI + 250 ppm HClO;
G6: E. faecalis + BHI + 500 ppm HClO;
G7: POSITIVE CONTROL - E. faecalis + BHI (no treatment but with bacterial growth)

All groups were, after carrying out the procedures, analyzed using Scanning Electron Microscopy (SEM). After the 7-day cultivation period, the samples were removed from the microtubes. Again, utility wax was fixed to the apex of each root (apical seal) and they were attached to the bench vice.

The irrigation protocol was carried out in the same way in samples from Dentaqua and Sodium Hypochlorite groups: 3 irrigation cycles with the solution belonging to each group; each cycle lasted 3 seconds. The irrigating needle was inserted 3mm short of the working length. During irrigation, a back-and-forth movement was carried out.
The 250 ppm and 500 ppm HClO solutions were obtained by specific Dentaqua device (Dentaqua, Conmel, Ireland) for each concentration, using the previously mentioned protocol.

Scanning Electron Microscopy (SEM): After teeth cleavage, samples remained for 7 days in a 2.5% glutaraldehyde solution (Glicolabor – Ribeirão Preto, São Paulo, Brazil). They were dehydrated and dried using a paper tip (Absorbent paper tips 15-40 and 45-80, Diadent/TKD, Burnaby, Canada), on a surface with the root canal portion facing upwards.

Samples were covered with 30nm gold/palladium alloy (Emitech K650 Sputter Coater, London, England), in a BAL-TEC SCD 005 Sputter Coater machine (BAL-TEC AG, Liechtenstein, Germany), at a vacuum level of approximately 5 x 10-2 mbar.

Observations using Scanning Electron Microscopy (XL 30; Philips, Eindhoven, Netherlands) were carried out at 5000x magnifications. Areas were standardized: between the cervical and middle third (cervical) and the middle and apical third (apical).

The most representative images of these regions were saved and analyzed on a ranking system, for two criteria (cleaning ability and erosion). Rank 1 was given for the image with the cleanest look, and the higher the rank, the worse was the cleaning ability. In the erosion analysis, rank 1 was given to the least eroded, and the higher the score, the greater the erosion profile. The positive control group could not be assessed because of the number of bacteria did not allow visualization of the dentine. For each area (cervical and apical) averages of each group were obtained. The objective was to verify the changes in the dentin structure after the applied treatment, making it possible to compare the cleaning ability, and the erosion magnitude in each group tested.

**Statistical analysis**

Data were analyzed using SPSS 11.0 software (SPSS, Chicago, IL, United States). Normal data distribution was confirmed by the Shapiro-Wilk test in antimicrobial and cytotoxicity evaluations. Data of antimicrobial and cytotoxicity evaluations were analyzed by one-way ANOVA followed by post-hoc Tukey’s test (p<0.05). SEM analyses of cleaning ability and Erosion were performed using the average with min-max, followed by Kruskal-Wallis and Mann-Whitney tests, with values adjusted for multiple comparisons by Benjamini-Hochberg procedure (p<0.05).

**Results**

Means (standard deviation) of bacterial reduction (%) in the evaluation of CFUs and viable cells (%) and cytotoxicity after tested decontamination protocols are presented in Table 1.
Table 1
Mean (standard deviation) of bacterial reduction (%) and viable cells (%) after treatment with tested irrigants.

<table>
<thead>
<tr>
<th>Group</th>
<th>Bacterial reduction (%)</th>
<th>Viable cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. DW</td>
<td>12.03 (4.37) a</td>
<td>98.88 (1.12) a</td>
</tr>
<tr>
<td>2. NaOCl 1%</td>
<td>97.59 (2.74) b</td>
<td>35.70 (0.10) b</td>
</tr>
<tr>
<td>3. NaOCl 2.5%</td>
<td>100.00 (0.00) b</td>
<td>35.66 (0.43) b</td>
</tr>
<tr>
<td>4. NaOCl 5.25%</td>
<td>100.00 (0.00) b</td>
<td>34.76 (0.98) b</td>
</tr>
<tr>
<td>5. HClO 250 ppm</td>
<td>97.18 (3.15) b</td>
<td>49.06 (1.99) c</td>
</tr>
<tr>
<td>6. HClO 500 ppm</td>
<td>99.94 (0.22) b</td>
<td>41.73 (1.15) d</td>
</tr>
</tbody>
</table>

* Different small letters, in the column, indicate significant differences between groups (p < 0.05).

** DW, distilled water; NaOCl, sodium hypochlorite; HClO, hypochlorous acid.

In the CFU evaluation, no statistically significant differences among groups 2 (1% NaOCl), 3 (2.5% NaOCl), 4 (5.25% NaOCl) 5 (250 ppm HClO), and 6 (500 ppm HClO) were observed (p > 0.05). All experimental groups were statistically different when compared to the control group (p < 0.05).

In the cytotoxicity evaluation, the highest percentage of viable cells was observed in the control group, followed by groups 5 (250 ppm HClO) and 6 (500 ppm HClO), with statistically significant differences from each other (p < 0.05). The lowest percentage of viable cells was observed in groups 4 (5.25% NaOCl), 3 (2.5% NaOCl) and 2 (1% NaOCl), with no statistically significant differences between them (p > 0.05).

Figures 3 and 4, display the SEM results on cleaning ability; Figs. 5 and 6 display SEM results for erosion. Tables 2 and 3 summarize the multiple comparisons on cleaning ability and erosion, respectively, pointing out significant differences among groups. Cleaning ability was best with sodium hypochlorite at different concentrations and with 500 ppm hypochlorous acid. The least to clean was 250 ppm hypochlorous acid. However, erosion occurred only within sodium hypochlorite groups, and the higher the concentration, the greater was the impact of erosion on dentine.
Table 2
Multiple comparisons on cleaning ability, with Benjamini-Hochberg adjusted p values

<table>
<thead>
<tr>
<th>p</th>
<th>p(BH)</th>
<th>p</th>
<th>p(BH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cervical cleaning 1 x 2 0,0030 0,0057 apical cleaning 1 x 2 0,0010 0,0019</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cervical cleaning 1 x 3 0,0120 0,0194 apical cleaning 1 x 3 0,0090 0,0135</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cervical cleaning 1 x 4 0,2480 0,2741 apical cleaning 1 x 4 0,0930 0,1028</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cervical cleaning 1 x 5 0,0010 0,0021 apical cleaning 1 x 5 0,0010 0,0019</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cervical cleaning 1 x 6 0,0270 0,0378 apical cleaning 1 x 6 0,0460 0,0604</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cervical cleaning 1 x 7 0,0010 0,0021 apical cleaning 1 x 7 0,0010 0,0019</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cervical cleaning 2 x 3 0,2940 0,3087 apical cleaning 2 x 3 0,0930 0,1028</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cervical cleaning 2 x 4 0,0160 0,0240 apical cleaning 2 x 4 0,0020 0,0035</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cervical cleaning 2 x 5 0,0120 0,0194 apical cleaning 2 x 5 0,0050 0,0081</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cervical cleaning 2 x 6 0,2480 0,2741 apical cleaning 2 x 6 0,0270 0,0378</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cervical cleaning 2 x 7 0,0010 0,0021 apical cleaning 2 x 7 0,0010 0,0019</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cervical cleaning 3 x 4 0,1720 0,2125 apical cleaning 3 x 4 0,0930 0,1028</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cervical cleaning 3 x 5 0,0010 0,0021 apical cleaning 3 x 5 0,0010 0,0019</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cervical cleaning 3 x 6 0,8340 0,8340 apical cleaning 3 x 6 0,4620 0,4620</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cervical cleaning 3 x 7 0,0010 0,0021 apical cleaning 3 x 7 0,0010 0,0019</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cervical cleaning 4 x 5 0,0010 0,0021 apical cleaning 4 x 5 0,0010 0,0019</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cervical cleaning 4 x 6 0,1720 0,2125 apical cleaning 4 x 6 0,4010 0,4211</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cervical cleaning 4 x 7 0,0010 0,0021 apical cleaning 4 x 7 0,0010 0,0019</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>p(BH)</td>
<td></td>
</tr>
<tr>
<td>----------------------</td>
<td>-----</td>
<td>-------</td>
<td>----------------------</td>
</tr>
<tr>
<td>cervical cleaning</td>
<td>5</td>
<td>6</td>
<td>0.0010</td>
</tr>
<tr>
<td>cervical cleaning</td>
<td>5</td>
<td>7</td>
<td>0.0010</td>
</tr>
<tr>
<td>cervical cleaning</td>
<td>6</td>
<td>7</td>
<td>0.0010</td>
</tr>
</tbody>
</table>
### Table 3
Multiple comparisons on erosion, with Benjamini-Hochberg adjusted p values

<table>
<thead>
<tr>
<th></th>
<th>p</th>
<th>p(BH)</th>
<th></th>
<th>p</th>
<th>p(BH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cervical erosion</td>
<td>1 x 2</td>
<td>0,0010</td>
<td>cervical erosion</td>
<td>1 x 2</td>
<td>0,0010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,0014</td>
<td></td>
<td></td>
<td>0,0014</td>
</tr>
<tr>
<td>cervical erosion</td>
<td>1 x 3</td>
<td>0,0010</td>
<td>cervical erosion</td>
<td>1 x 3</td>
<td>0,0010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,0014</td>
<td></td>
<td></td>
<td>0,0014</td>
</tr>
<tr>
<td>cervical erosion</td>
<td>1 x 4</td>
<td>0,0010</td>
<td>cervical erosion</td>
<td>1 x 4</td>
<td>0,0010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,0014</td>
<td></td>
<td></td>
<td>0,0014</td>
</tr>
<tr>
<td>cervical erosion</td>
<td>1 x 5</td>
<td>0,4010</td>
<td>cervical erosion</td>
<td>1 x 5</td>
<td>0,4010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,4296</td>
<td></td>
<td></td>
<td>0,4296</td>
</tr>
<tr>
<td>cervical erosion</td>
<td>1 x 6</td>
<td>0,6740</td>
<td>cervical erosion</td>
<td>1 x 6</td>
<td>0,9160</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,6740</td>
<td></td>
<td></td>
<td>0,9160</td>
</tr>
<tr>
<td>cervical erosion</td>
<td>2 x 3</td>
<td>0,0010</td>
<td>cervical erosion</td>
<td>2 x 3</td>
<td>0,0010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,0014</td>
<td></td>
<td></td>
<td>0,0014</td>
</tr>
<tr>
<td>cervical erosion</td>
<td>2 x 4</td>
<td>0,0010</td>
<td>cervical erosion</td>
<td>2 x 4</td>
<td>0,0010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,0014</td>
<td></td>
<td></td>
<td>0,0014</td>
</tr>
<tr>
<td>cervical erosion</td>
<td>2 x 5</td>
<td>0,0010</td>
<td>cervical erosion</td>
<td>2 x 5</td>
<td>0,0010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,0014</td>
<td></td>
<td></td>
<td>0,0014</td>
</tr>
<tr>
<td>cervical erosion</td>
<td>2 x 6</td>
<td>0,0010</td>
<td>cervical erosion</td>
<td>2 x 6</td>
<td>0,0010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,0014</td>
<td></td>
<td></td>
<td>0,0014</td>
</tr>
<tr>
<td>cervical erosion</td>
<td>3 x 4</td>
<td>0,0210</td>
<td>cervical erosion</td>
<td>3 x 4</td>
<td>0,0210</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,0263</td>
<td></td>
<td></td>
<td>0,0263</td>
</tr>
<tr>
<td>cervical erosion</td>
<td>3 x 5</td>
<td>0,0010</td>
<td>cervical erosion</td>
<td>3 x 5</td>
<td>0,0010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,0014</td>
<td></td>
<td></td>
<td>0,0014</td>
</tr>
<tr>
<td>cervical erosion</td>
<td>3 x 6</td>
<td>0,0010</td>
<td>cervical erosion</td>
<td>3 x 6</td>
<td>0,0010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,0014</td>
<td></td>
<td></td>
<td>0,0014</td>
</tr>
<tr>
<td>cervical erosion</td>
<td>4 x 5</td>
<td>0,0010</td>
<td>cervical erosion</td>
<td>4 x 5</td>
<td>0,0010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,0014</td>
<td></td>
<td></td>
<td>0,0014</td>
</tr>
<tr>
<td>cervical erosion</td>
<td>4 x 6</td>
<td>0,0010</td>
<td>cervical erosion</td>
<td>4 x 6</td>
<td>0,0010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,0014</td>
<td></td>
<td></td>
<td>0,0014</td>
</tr>
<tr>
<td>cervical erosion</td>
<td>5 x 6</td>
<td>0,1720</td>
<td>cervical erosion</td>
<td>5 x 6</td>
<td>0,1720</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,1985</td>
<td></td>
<td></td>
<td>0,1985</td>
</tr>
</tbody>
</table>

### Discussion

The main cause of endodontic failure is related to the permanence of bacteria into the root canal system, which survive after chemo-mechanical preparation [19]. Therefore, the use of auxiliary chemical substances with recognized antimicrobial activity is mandatory in the endodontic therapy. In the other
side, depending of chemical substance and concentration, the extrusion to periapical areas causes cell
damage, induces inflammatory reaction, impairs wound healing [2, 9], and the survival of stem cells,
which are required for successful tissue regeneration [22]. In this scenario, the present study evaluated
the use of HClO in different concentrations, which were obtained from an innovative electrolytic device,
regarding its antimicrobial activity and cytotoxicity. These properties are essential to enable the use of a
chemical substance in endodontic treatment and the comparison with conventional chemical substances
is necessary, to elucidate its potential in endodontics.

Despite the existence of a polymicrobial culture in cases of infection of the root canal system, a
monoculture of *E. faecalis* was used in the present study, to promote the infection of root canals.
*E. faecalis* is a highly-resistant anaerobic facultative gram + microorganism, which presents the ability to
penetrate dentinal tubules and colonize the root canal system in a biofilm format [23]. For these
characteristics, the effective antimicrobial action of the tested protocols over *E. faecalis* could indicate an
effective decontamination action over several microorganisms of the endodontic microbiota. Regarding
the required time to promote the biofilm formation, 14 days was used in the present study. It is enough
time to ensure the formation of a cohesive and well-structured bacterial biofilm [24]. Finally, the CFU
counting was used as an antimicrobial evaluation method in the present study. It is considered a
standard protocol that provides an effective bacterial quantification of the root canal space and it was
used in previous studies that focused on antimicrobial strategies against microbial biofilms [25, 26].

The fibroblastic cells are predominant in the connective tissue of the periapical area and periodontal
ligament [27]. These sites are affected by the extrusion of chemical substances during chemo-
mechanical preparation, resulting in the induction of inflammatory reactions that can bring post-operative
pain to the patient. Therefore, these cells were used in the present study, to approach the clinical reality
when a chemical substance meets adjacent tissues. In addition, the MTT assay was performed to assess
the cytotoxicity, with basis on changes in the number of viable fibroblastic cells, cell metabolism, and cell
morphology. This test presents higher sensitivity, the cell viability count is not affected by any
compounds released from samples, it is not influenced by any cellular metabolic action, making this
evaluation easier to perform when compared to other methods [28].

The rotary and reciprocating instrumentation provided advances in the shaping stage of root canal
treatment, making this instrumentation faster. However, the contact time of chemical auxiliary
substances with root canals was also reduced, decreasing the antimicrobial properties of chemical
substances [29, 30]. In the present study, the instrumentation was not performed during the
decontamination process, because present study aimed to evaluate the intrinsic antimicrobial potential of
the tested substances. Moreover, irrigation cycles were performed, with constant renewal of the tested
substances, simulating the clinical reality of the contact time of a chemical substance into the root canal
during endodontic treatment [31]. This is a critical step in the decontamination process. Otherwise, these
resistant bacteria adapt to the environmental conditions, acquire nutrients, and reach critical numbers to
exhibit virulence and pathogenicity, leading to the progression of periapical diseases in a clinical situation
[32].
The results of this study revealed that both concentrations of HClO obtained from the electrolytic device presented similar high antimicrobial activity against E. faecalis when compared to several tested concentrations of NaOCl. This finding confirms the first hypothesis. The antimicrobial effect of chlorinated substances, such as HClO, occurs due to the release of active chlorine. Through this mechanism, enzymatic inhibition and formation of chloramines are observed after reaction with bacterial cytoplasm components. Following, chlorine is a strong oxidizing agent, which promotes the irreversible oxidation of sulfhydryl groups of bacterial enzymes [33]. According to these mechanisms, bacterial cell metabolic reactions are interrupted, causing damage to cell DNA. In addition, the acidic pH and the high HClO concentrations also favor this effective antimicrobial activity [34], leading to high antimicrobial activity, which was observed in the present study.

The cytotoxicity evaluation showed that both concentrations of HClO obtained from the electrolytic device presented a higher percentage of viable fibroblastic cells, confirming the second hypothesis of the present study. In your turn, the several concentrations of NaOCl presented the lowest percentage of viable fibroblastic cells, being following some findings of the literature, where NaOCl revealed undesirable effects regarding cytotoxicity [2, 10]. The electrochemically activated solution produced by the Dentaqua device is composed of activated mixed oxidants formed in dynamic equilibrium, where HClO is the main oxidant. The previously described biological properties may also help to explain the present results. In addition, some findings in the literature revealed that HClO solution obtained from an electrolytic device induced low levels of adverse effects over connective tissue cells [11], in the same way as observed in the present study, even when used at high concentrations.

The chemical substances that act by releasing of active chlorine are influenced by their concentration, with higher biological effects when its concentration is increased. Furthermore, it is known that the concentration of solutions containing active chlorine decreases over time and storage [5]. However, according to results of present study, there was no higher antimicrobial activity by increasing the concentration of all tested chemical substances. Even at the lowest concentration of 250 ppm, the active chlorine content responsible for bacterial neutralization is already high, being enough to reach high antimicrobial action levels, when the HClO was tested. On the other hand, there was found a lower level of fibroblastic cell viability by increasing the concentration of HClO. The tested HClO solutions were placed in direct contact with fibroblastic cells and the active chlorine content was directly contacting these cell components. Thus, the higher HClO concentration may have caused such results. Even so, cytotoxicity was significantly lower when compared to several concentrations of NaOCl, which caused a significant reduction in fibroblastic cell viability, even when NaOCl was used at a low concentration of 1%.

The SEM analyses brought to light a problem that is not often observed in the literature. Despite being of universal endodontic use, sodium hypochlorite can cause erosion of dentinal tissues. The higher the concentration of NaOCl, the more erosion is caused. Wagner et al [36] found sodium hypochlorite to dissolve dentine collagen, and this is the probable cause of erosion. Authors constantly refer to EDTA or citric acid to cause erosion, but the use of sodium hypochlorite, considering that it occupies the root canal
space during most of preparation procedures, may cause more resorption than the acid solutions with limited time within the canal.

The use of HClO, prepared in the Dentaqua device, can be done in different concentrations. This study found that 250 ppm may not provide enough hypochlorous acid to help with root canal cleaning. On the other hand, 500 ppm allowed cleaning results at the same level of sodium hypochlorite. The raise in concentration did not affect the safety with regards to erosion, as only HClO groups and the negative control did not cause any erosion to the dentine.

The present study verified a device able to produce an electrochemically activated solution containing HClO at different concentrations as main component. In addition to producing a fresh substance at the desired concentration and ready for use, this study revealed promising findings regarding cleaning ability, low risk of causing erosion, together with antimicrobial activity and low cytotoxicity, even though the present study has some limitations. Microbiologic samples were only collected from the main canal. Therefore, it was not possible to assess the presence of bacteria in the depth of dentinal tubules or the viability of these bacteria. Regarding the cytotoxicity evaluation, the chemical auxiliary substances remain in contact with a reduced area of periapical tissues during endodontic treatment. In the present study, there is direct contact between the tested substances and fibroblastic cells. Thus, cytotoxicity may be lower in clinical reality, although significant differences were observed among the tested substances. Further studies are suggested to clarify these conditions, as well as to evaluate other variables involving the influence of HClO in the endodontic treatment.

Under the study limitations, it was possible to conclude that HClO obtained from an innovative electrolytic device presented high antimicrobial activity, low cytotoxicity, and no erosion of dentine in both tested concentrations, when compared to several concentrations of NaOCl. Cleaning ability was best with 500 ppm concentration.

Declarations

Conflict of Interest: Dr. Liviu Steier owns equities Dentaqua. The rest of the authors state that they have no financial affiliation (e.g., employment, direct payment, stock holdings, retainers, consultanthips, patent licensing arrangements, or honoraria) or involvement with any commercial organization with a direct financial interest in the subject or materials discussed in this manuscript, nor have any such arrangements existed in the past three years.

Ethical Approval: This study was approved by the Research Ethical Committee of the University of Passo Fundo (protocol No. 5.783.928).

Funding: This work has no financial support.

Informed consent: Informed consent is not applicable because samples were composed of extracted human teeth that were obtained from the Biobank.
Authors' contributions section

Matheus Albino Souza - supervising all experimental tests and writing the article.

Gabriele Vanin - performing microbiological and cytotoxicity tests.

Mylena Zanella - performing microbiological and cytotoxicity tests.

Camila Pizzi - performing microbiological and cytotoxicity tests.

Eduarda Ferreira - performing microbiological and cytotoxicity tests.

Felipe Dallepiane - performing microbiological and cytotoxicity tests.

Nathan Piccolo - performing microbiological and cytotoxicity tests.

Jordana da Silva Koch – performing SEM cleaning and erosion tests.

Kellyn Rocca Souza – performing SEM cleaning and erosion tests.

Aleksandra Palatynska-Ulatowska - performing SEM cleaning and erosion tests.

Ubirajara Maciel da Costa – supervising the cytotoxicity test.

Vanessa Valgas dos Santos – supervising the cytotoxicity test.

Liviu Steier – supervising all experimental tests and writing the article.

José Antonio Poli de Figueiredo - supervising all experimental tests and writing the article.

References


Figure 1

Representative image of the Dentaqua device. A – Water bottle button; B – Ecasol button; C – Upper compartment; D – Smaller bottle with brine solution; E – Larger bottle with HClO solution.
sequence of bovine tooth preparation for SEM analyses. a. crown sectioning; b. longitudinal grooves; c. wax apex closure; d. insertion of culture medium; e. propylene box with group samples.
Figure 3

Position ranks on cleaning after use of the test solutions. Left graph – cervical area; right graph – apical area. The lower the position rank, the best cleaning was obtained.

Figure 4

Position ranks on erosion of dentinal tubules after use of the test solutions. Left graph – cervical area; right graph – apical area. The lower the position rank, the least erosion occurs.
Figure 5

SEM images of dentinal tubules of cervical area from different groups. G1 - Negative control; G2 – 1% NaOCl; G3 – 2.5% NaOCl; G4 – 5.25% NaOCl; G5 – 250 ppm HOCl; G6 – 500 ppm HOCl; G7 – Positive control

Figure 6

SEM images of dentinal tubules of apical area from different groups. G1 - Negative control; G2 – 1% NaOCl; G3 – 2.5% NaOCl; G4 – 5.25% NaOCl; G5 – 250 ppm HOCl; G6 – 500 ppm HOCl; G7 – Positive control