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Original research article

Antimicrobial effect of the hydrodynamic action of 1 and 2.5% sodium hypochlorite in infected root canals

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Abstract

Introduction: The mouth has various microorganisms that can live in the free form, called planktonic, or in organized groups, called biofilm. **Objective:** To determine the antimicrobial effect of the hydrodynamic action of sodium hypochlorite (NaOCl) on the biofilm of *Enterococcus faecalis* with different of maturity biofilms. **Material and methods:** Sixty bovine incisors were decoronated, prepared, inoculated with *E. faecalis*, and examined after 7, 14, 28, and 60 days. Eppendorf tubes were connected to the coronal portion of the root. Urethane hoses were attached to the tubes and to the entrance of a peristaltic pump. The test irrigating was 1 and 2.5% NaOCl solutions that circulated at a constant flow of 50 mL min^{-1} for 20 min. Samples from the root canals were collected before and after the use of NaOCl solutions, and the bacterial growth was analyzed using turbidity of culture medium followed by ultraviolet spectrophotometry. **Results:** All protocols showed a significant reduction of the optical density of the culture medium ($p < 0,05$). However, none of them promoted the complete elimination of *E. faecalis*. **Conclusion:** The

hydrodynamic action of 1 and 2.5% NaOCl solutions reduced the microorganisms of the biofilm, but it was not sufficient to inactivate *E. faecalis* in different of maturity biofilms.

Introduction

The mouth has various microorganisms that can live in the free form, called planktonic, or in organized groups, called biofilm [9]. The formation of a biofilm involves events with sequential steps at the molecular level [35]. At first, there is the retention of substances (organic and inorganic) attracted to a solid surface [9, 24]. Then, it is verified the adhesion of microorganisms to this film through surface bacterial structures (fimbrias, flagella, and glycocalyx) until the observation of multiple layers of microorganisms [9, 24]. Finally, the formation of a structured solid layer occurs with exchanges of genetic material between cells through cell-cell communication [6]. It has been demonstrated that the microorganisms established in a biofilm form enjoy greater security and protection due to the difficulty of penetration of antimicrobial substances [26].

Enterococcus faecalis is a Gram-positive coccus, a facultative anaerobe commonly isolated from teeth with endodontic treatment and with failure [29, 32, 33]. It presents several virulence factors (aggregation substance, enterococcal surface proteins, gelatinase, cytolysin toxin, extracellular superoxide production, capsular polysaccharides, antibiotic resistance determinant) that can facilitate the adherence to the host cells and extracellular matrix, tissue invasions, besides interfering with immunomodulation and causing toxin-mediated damage [29]. Ørstavik and Haapasalo [27] observed that *E. faecalis* could invade the dentinal tubules, colonize the root canal and survive without the presence of other bacteria. Duggan and Sedgley [10] evaluated the ability of *E. faecalis* to form biofilm in endodontically treated teeth. The authors verified that this microorganism could live with low oxygen level, alkaline pH, variable temperature (10–45ºC), and in environments with nutritional scarcity.

Once the infection is established, root canal instrumentation is crucial for controlling microorganisms [13]. However, it has been shown that some areas of the root canal remain untouched during the instrumentation step [36, 37]. These areas can maintain bacterial biofilm intact and serve as a potential cause of persistent infection [33]. Irrigating solutions are antimicrobial agents also used during the sanitization process [11-13]. Several substances have been developed and proposed, but sodium hypochlorite (NaOCl) remains the most suitable solution for treating endodontic infection [11, 12, 15, 21]. Estrela *et al.* [15] analyzed the antimicrobial effect of 2% NaOCl by agar diffusion test and by direct exposure test on *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Candida albicans*, and one mixture of these microorganisms. The authors observed that the best performance of antimicrobial effectiveness of NaOCl occurred in the direct exposure test. Spratt *et al.* [31] evaluated the antimicrobial effect of different irrigating solutions on *E. faecalis* biofilm in membrane discs and found that NaOCl was the most effective solution.

Although the antimicrobial effect of the NaOCl on *E. faecalis* biofilm has been demonstrated in a large variety of methods [15, 19, 31], more evidence is required for the analysis performed on the dentin substrate [12, 16]. Thus, the purpose of this study was to determine the antimicrobial effect of the hydrodynamic action of 1 and 2.5% NaOCl on the biofilm of *E. faecalis* with different degrees of maturity.

Material and methods

Tooth preparation

Sixty freshly extracted bovine incisors with roots anatomically similar in size and shape and fully developed apices were selected for this study. The crowns were removed, and tooth length was standardized to 16 mm (from root apex to coronal border). The coronal root canal third was enlarged using sizes 2 and 3 Gates Glidden drills (Dentsply Maillefer, Ballaigues, Switzerland). The roots were prepared up to a size 40 K-file (Dentsply Maillefer) 1 mm short of the apical foramen, using a crown-down preparation technique. During instrumentation, the root canals were irrigated with 3 mL of 2.5% NaOCl (Longevità, Goiânia, GO, Brazil) at each file change. Root canals were dried and filled with 17% ethylenediamine tetraacetic acid (EDTA) (pH 7.2) (Biodinâmica, Ibiporã, PR, Brazil) for 5 min for smear layer removal. Next, the teeth were autoclaved at 120ºC for 30 min.

Test organisms

A reference strain of Gram-positive facultative anaerobic coccus (*E. faecalis*) obtained from the American Type Culture Collection was used (ATCC 29212). The bacterial strain was inoculated in 7 mL of brain heart infusion (BHI) (Difco Laboratories, Detroit, MI, United States) and incubated at 37ºC for 24 h. The experimental suspensions were prepared by cultivating the biological marker on the surface of brain heart infusion agar (BHIA) (Difco Laboratories), following the same incubation conditions. Bacterial cells were resuspended in saline to achieve the final concentration of about 3×10^8 cells mL^{-1}, adjusted to #1 MacFarland turbidity standard [16].

Experimental design

A split platform was used during the period of inoculation with the biological marker. The coronal portion of the root canal of each root was connected to the cut end of a 1.5-mL polypropylene Eppendorf tube (Cral, São Paulo, SP, Brazil) using a cyanoacrylate adhesive (Super Bonder, Itapevi, SP, Brazil) and epoxy resin (Durepoxi, São Paulo, SP, Brazil) to prevent leakage at the connection. The tooth-tube connections were entirely coated with two nail polish layers (Max Factor, Cosmetics and Fragrances, London, United Kingdom). The roots coupled to the polypropylene tubes were sterilized in 5% NaOCl (Longevità) for 30 min and then rinsed with sterile water for 30 min. The specimens were placed into the culture medium (BHI), and, to ensure sterilization, the test apparatus was incubated at 37ºC for 24 h. No growth was observed after this period.

Five mL of sterile BHI broth were mixed with 5 mL of the bacterial inoculum containing *E. faecalis* and inoculated using sterilized syringes of sufficient volume to fill the root canal. This procedure was repeated every 72 h during the 7, 14, 28, and 60-day period, always using 24-hour pure culture prepared and adjusted to #1 MacFarland turbidity standard. The teeth were maintained in a humid environment at 37ºC.

After the biofilm formation period, the root canals were dried and filled with distilled water. Sterile paper tips #40 (Tanari, Tanariman Industrial, Manacaru, AM, Brazil) were introduced into the root canal and maintained there for 3 min for microbiological collection. Each sample was collected using three paper tips, which were subsequently immersed in 5 mL of BHI added with neutralizing Tween 80 and sodium thiosulfate (P. A., Art Laboratories, Campinas, SP, Brazil) at appropriate concentrations, followed by incubation at 37ºC for 48 h. Microbial growth was analyzed by turbidity of the culture medium. An inoculum of 0.1 mL obtained from the medium was transferred to 5 mL of BHI and subsequently incubated at 37ºC for 48 h. After that, the bacterial growth was analyzed by the turbidity of the culture medium, being determined the presence or absence of bacteria, and using ultraviolet (UV) spectrophotometer (Nova 1600 UV, Piracicaba, SP, Brazil) (initial measurement). Next, the specimens were randomly divided into eight experimental groups (n = 5) and two control groups (n = 10) (Table I). The negative control was used to test sterility, and the positive control, to check bacterial viability throughout the experiment.

Table I – Distribution of experimental and control groups

Groups	Protocols	n
1	7-day biofilm + 1% NaOCl	5
2	7-day biofilm + 2.5% NaOCl	5
3	14-day biofilm + 1% NaOCl	5
4	14-day biofilm + 2.5% NaOCl	5
5	28-day biofilm + 1% NaOCl	5
6	28-day biofilm + 2.5% NaOCl	5
7	60-day biofilm + 1% NaOCl	5
8	60-day biofilm + 2.5% NaOCl	5
9	Positive control	10
10	Negative control	10

NaOCl: sodium hypochlorite

To evaluate the antimicrobial efficacy of the 1 and 2.5%-NaOCl solutions, a sterile urethane hose was connected to the polypropylene Eppendorf tube attached to the teeth and to the entrance of a peristaltic pump (Sarlo 90, São Paulo, SP, Brazil) [12]. The entrance of this apparatus was the urethane hose connected to the polypropylene tube, and its exit corresponded to the apical portion of the root canals. The irrigants circulated within the apparatus at a constant flow of 50 mL min^{-1} for 20 min.

At 20-min intervals, each tooth was removed from its apparatus under aseptic conditions, and further irrigation with 5 mL of sterile distilled water with a syringe was undertaken. The root canals were dried and refilled with sterile distilled water. After that, sterile paper points #40 (Tanari) were introduced into the canals and maintained there for 3 min for sample collection. The points were

transported and immersed in 5 mL of Letheen Broth (LB) (Difco Laboratories), a medium containing or added with neutralizers [Tween 80 and sodium thiosulfate (P. A., Art Laboratories)] in appropriate concentrations, followed by incubation at 37ºC for 48 h. Microbial growth was analyzed by turbidity of the culture medium. An inoculum of 0.1 mL obtained from the medium was transferred to 5 mL of BHI and subsequently incubated at 37ºC for 48 h. After this period, the bacterial growth was analyzed by the turbidity of the culture medium, being determined the presence or absence of bacteria, and using UV spectrophotometer (Nova 1600 UV) (final measurement).

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences software, version 20 (IBM, Chicago, IL, United States). The mean and standard deviation of the initial and final measurements were obtained. The Kolmogorov-Smirnov test assessed the data normality. The statistical difference between the initial and final measurements was assessed by the *t*-test for paired samples or by the Wilcoxon test. Values of $p < 0.05$ were considered significant.

Results

The results of the initial and final measurements of the biofilms with different periods of formation are shown in Table II. All protocols showed a significant reduction of the optical density of the culture medium ($p < 0,05$). However, none of them promoted the complete elimination of *E. faecalis*.

Table II – The mean and standard deviation of the biofilm's initial and final measurements in 7, 14, 28, and 60 days

Biofilm	Irrigation solution	Mean/SD optical density (nm)		
		Initial	Final	P-value
7-day	1% NaOCl	1.118 ± 0.090	0.744 ± 0.043	0.043**
	2.5% NaOCl	1.013 ± 0.111	0.665 ± 0.375	0.043**
14-day	1% NaOCl	1.012 ± 0.053	0.884 ± 0.076	0.001*
	2.5% NaOCl	0.942 ± 0.056	0.833 ± 0.068	0.043**
28-day	1% NaOCl	1.082 ± 0.160	0.752 ± 0.423	0.043**
	2.5% NaOCl	0.981 ± 0.026	0.691 ± 0.387	0.043**
60-day	1% NaOCl	1.014 ± 0.029	0.854 ± 0.088	0.004*
	2.5% NaOCl	0.931 ± 0.047	0.637 ± 0.316	0.043**
Positive control		1.064 ± 0.037	–	–
Negative control		0.004 ± 0.003	–	–

SD: standard deviation; NaOCl: sodium hypochlorite; nm: nanometers; **t*-test for paired sample; **Wilcoxon test

Discussion

The present study evaluated the antimicrobial effect of the hydrodynamic action of different NaOCl concentrations on bovine dentin infected with *E. faecalis*. The influence of the degree of maturity of the bacterial biofilm on this action was also evaluated. However, the results suggested a reduction, not an elimination, regardless of the time of contamination or the concentration of the solution.

Numerous biofilm models have been described to study intracanal medicaments [14, 20, 27, 30]. The experimental models involved infected human teeth *in vivo* [25], infected human teeth *ex vivo* [12, 14, 30], infected dog's teeth *in vivo* [22], infected bovine teeth *ex vivo* [20, 27], and biofilm model in membrane filters [19, 31]. The differences in

methodology amongst these methods are likely to lead to results that cannot be compared, and that extrapolation of the results to clinical conditions must be done with caution. A new model system to study antimicrobial strategies in endodontic biofilms was recently developed [16]. This model served as the basis for this study since it allowed for a satisfactory colonization time of the selected bacterial species with virulence and adherence properties. Bovine teeth were used in the present study due to their easy acquisition and similarity to human teeth [17].

Enterococcus faecalis was chosen as a biological marker for this study, considering its importance in root canal infections, mainly in teeth with endodontic treatment and failure [29, 32]. It is a Gram-positive coccus, a facultative anaerobe that tolerates low nutrient and oxygen conditions and high pH [13, 16, 18].

It has been shown that current instrumentation techniques by themselves cannot make root canals free of microorganisms [36, 37]. This highlights the need to use an auxiliary solution that neutralizes the microbial content and its by-products. Several irrigating solutions have been advocated to reduce endodontic infection [11-13]. Sodium hypochlorite (NaOCl) is a solution widely used in the endodontic sanitization process, as it presents fundamental properties such as a broad spectrum of action, antimicrobial effect, and the dissolution capacity of organic tissue that is directly influenced by the increase of its concentration [7, 11, 39]. Studies have shown that NaOCl is effective on *E. faecalis* in the biofilm or planktonic form [1, 12], which again justifies analyzing its action when kept in circulation in the root canal through a peristaltic pump.

It is important to notice that some aspects can compromise the quality of the NaOCl and must be considered, such as the pH of the solution, the chlorine content, the temperature, and the storage conditions [11, 15, 28]. Pécora *et al*. [28] evaluated the lifetime of the NaOCl solution as a function of storage conditions (temperature and light) and found that, the greater the incidence of sunlight and the higher the temperature, the greater the loss of the chlorine content of the solution. Another critical factor is the pH of the solution. This substance has greater stability at high pH, between 11 and 12, with the chlorine release being slower [11, 15].

An essential factor for the excellent performance of the irrigating solution is its antimicrobial potential. Estrela *et al*. [11] described the mechanism of action of NaOCl. This substance, in the presence of organic matter and fat, solubilizes them, transforming them into salts of fatty acids and glycerol in a saponification reaction. It also neutralizes amino acids leading to the formation of water and salt, an amino acid neutralization reaction, and, finally, the chloramination reaction occurs with hydrolysis and degradation of amino acids. In summary, the effectiveness of NaOCl is due to hydroxyl ions on the cytoplasmic membrane of bacteria. At the same time, high pH interferes with the integrity of this membrane, promoting biosynthetic changes. Also, the formation of chloramines interferes with cell metabolism [11].

Wang *et al*. [38] demonstrated that the use of 6%-NaOCl for 3 min on a 24-h biofilm eliminated more than 60% of *E. faecalis*, while fewer bacterial cells were killed when using this same substance during a similar period in a three-week biofilm. Du *et al*. [8] evaluated the antimicrobial activity of 2- and 6%-NaOCl and 2%-chlorhexidine on dentin contaminated with *E. faecalis* for 24 h and three weeks. The authors found that the microorganism's death depended on the type, concentration, and time of exposure to the irrigating solution and the biofilm's degree of maturity. For all analyzed substances, it was found that after 10 min of exposure to the irrigating solution, there was a more significant elimination of the microorganism. The NaOCl at 6% showed an elimination rate of the microorganism ranging from 53 to 88%, while for NaOCl at 2% this rate varied from 26 to 71%, and for chlorhexidine, the variation occurred between 24 to 68%. Considering the biofilm's degree of maturity, the authors observed a percentage of dead cells ranging from 34 to 88% in the young biofilm. In contrast, for the three-week biofilm, the data varied from 24 to 78%. Georgova *et al*. [18] evaluated the antimicrobial effect of different disinfection methods on root canal biofilms. *Enterococcus faecalis* and other Gram-positive bacteria proved to be more sensitive than Gram-negative microorganisms, and that NaOCl was the solution that demonstrated the most favorable results in reducing intraradicular biofilm. Bukhary and Balto [2] studied the antimicrobial potential of different irrigating solutions on *E. faecalis* biofilm in dentin discs and observed that NaOCl was the substance that showed the best result. Hong *et al*. [23] verified that NaOCl and calcium hydroxide affect the immune stimulatory power of lipoteichoic acid, reducing the inflammatory response in infections caused by *E. faecalis*.

The results found in the present study agree with those previously described regarding the

antimicrobial action of the NaOCl in a microbial reduction in young biofilm [8, 38]. It was possible to verify a reduction for both analyzed substances, above 60%, as described by Wang *et al.* [38]. However, it should also be considered that NaOCl has a surface tension equals to 75 dynes/cm, which is a high and undesirable value, considering that high surface tension prevents contact between the liquid and the surface, and, consequently, it makes it difficult for the irrigating solution to act on the microbiota and root canal walls [12]. There was a microbial reduction of more than 70% for more mature biofilms, regardless of the irrigation solution analyzed. This fact differs from other analyses, but it can be explained by checking out the methodology used in this study. This study did not consider only the irrigating solution's antimicrobial action, but mainly the mechanical action created by the constant flow of irrigating solution of 50 mL min^{-1} against the walls of the root canal, enabling a possible displacement of bacterial cells from the root canal. Estrela *et al.* [12] studied the antimicrobial effect of gaseous ozone, ozonated water, NaOCl, and chlorhexidine in human root canals infected with *E. faecalis*, using the hydrodynamic action generated from a peristaltic pump. The authors observed microbial reduction, but not elimination. Cachovan *et al.* [5] evaluated the antibacterial effect of a hydrodynamic system on extracted and contaminated teeth and found that the hydrodynamic system, that works with the agitation of the irrigating solution inside the root canal using pressure-suction technology, with a flow rate of 6.2 mL/min, presented better results than manual irrigation and passive ultrasonic irrigation in bacterial reduction. Moreover, the authors reported that the results might be also a consequence of the greater penetration of the irrigating solution into the dentinal tubules due to pressure, facilitating the action of the solution in regions of difficult access to the endodontic instrument.

Given the variability of studies associated with the potential for the antimicrobial action of NaOCl, the antibacterial strategy has come to occupy a prominent position. The hydrodynamic and physical activity with the irrigant flow acting as a disintegrating jet was intentional conduct, testing the possibility of disaggregating the biofilm in the different periods analyzed. However, even with a high flow of irrigant against the contaminated walls, there was no elimination of bacteria present in the form of aggregates. The action time for 20 min is justified in simulating an approximate time of activity of the irrigator acting during an endodontic treatment [12].

It is worth mentioning that the root canal treatment occurs through the mechanical action of the endodontic instrument in a complex root canal system and by the antimicrobial action of the irrigating solution and intracanal dressing [3, 4, 34]. Further studies are needed to continuously search for new alternatives related to irrigation solutions and irrigation techniques, to eliminate the root canal microbiota.

Conclusion

The hydrodynamic action of 1- and 2.5%-NaOCl solutions reduced the microorganisms of the biofilm, but it was not sufficient to inactivate *E. faecalis*.

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