Brazilian Dental Journal (2018) 29(5): 459-464
http://dx.doi.org/10.1590/0103-6440201802172

ISSN 0103-6440

Antibacterial Efficacy of Synthetic and Natural-Derived Novel Endodontic Irrigant Solutions

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The aim of this study was to compare the efficacy of grape seed extract (GSE), calcium hypochlorite [$Ca(ClO)_2$], and sodium hypochlorite (NaOCl) irrigant solutions with rotary or reciprocating instrumentation for disinfection of root canals inoculated with *Enterococcus faecalis*. The mesiobuccal root canals of mandibular molars were prepared and inoculated with *Enterococcus faecalis* for 21 days. The roots were then randomly divided into the following eight experimental groups (n=11) according to the instrumentation technique and disinfection protocol: ProTaper Next or Reciproc R25 with sodium chloride (control group), 6% NaOCl, 6% $Ca(ClO)_2$, or 50% GSE used for irrigation during instrumentation. The antimicrobial activity was determined on the basis of a reduction in colony-forming units (CFUs) counted on bacterial samples collected before and after root canal instrumentation and expressed as a percentage of reduction. Data were evaluated by two-way ANOVA followed by Tukey HSD post-hoc tests ($p<0.05$). No significant differences were observed in bacterial reduction between the ProTaper Next and Reciproc R25 systems ($p>0.05$), regardless of the irrigant solution used. Furthermore, all active solutions (6% NaOCl, 50% GSE, and 6% $Ca(ClO)_2$) showed similar potential to reduce bacterial counts ($p>0.05$) and were significantly more effective than sodium chloride (control) ($p<0.05$). The results suggest that the GSE and $Ca(ClO)_2$ have potential clinical application as irrigant solutions in endodontic therapy since they present bactericidal efficacy against *Enterococcus faecalis*.

Key Words: calcium hypochlorite, grape seed extract, reciprocating motion, rotary instruments, sodium hypochlorite.

Introduction

Root canal preparation aims to eliminate bacteria from the contaminated canal system and yield a proper space for final obturation. During the cleaning and shaping stages, endodontic instruments and irrigant solutions are used to reduce bacterial contamination in the root canal (1). *Enterococcus faecalis* is a facultative anaerobic Gram positive bacterium isolated from 18% of primary endodontic infections and from 67% of the cases of endodontic failures (2). *E. faecalis* can survive in inappropriate nutritional situations and can stay viable as a single microorganism (3). It can enter the dentinal tubules and is capable of promoting biofilm formation (4), which creates an extracellular polymeric matrix that increases the resistance of microorganisms to irrigation protocols (5).

Nickel-titanium (NiTi) rotary files used in a continuous rotary motion and the Reciproc instrument, which is operated in a reciprocating motion, have become widely available and acceptable for root canal preparation (1,6-8). The ProTaper Next (PTN) (Dentsply Maillefer), which uses rotary movement, was developed with design features that include variable tapers and an off-centered rectangular cross-section. The set includes five shaping instruments: X1 (#17/.04), X2 (#25/.06), X3 (#30/.07), X4 (#40/.06), and X5 (#50/.06). It is expected that this entire file adapts through the root canal passively until the apical foramen is achieved (7,8). In contrast, another distinct concept proposes the use of a single-file system to shape the root canal completely from start to finish during endodontic therapy. The Reciproc system has an "S"-shaped cross-section file available in three sizes (R25, R40, and R50), and the appropriate size is selected according to the initial diameter of the root canal. The instrument has a variable taper along its shaft; in the last 3 mm from the tip, the R25, R40, and R50 instruments are 0.08-, 0.06-, and 0.05-mm/mm tapered, respectively (1,6,8). In reciprocating motion, the instrument rotates in one direction and then reverses the direction before completing a full rotary cycle. This motion has been shown to increase resistance to fatigue and thus extend the working life of the instrument (9).

Despite the good effectiveness of the current endodontic instruments, studies have shown that approximately 10%-50% of the main root canal surface area remains untouched by instruments during preparation of root canals (10). These areas may shelter microorganisms and consequently compromise the endodontic treatment outcome. Therefore, irrigant solutions play a major role during root canal cleaning and should disinfect the untouched areas. Sodium hypochlorite (NaOCl) is the most frequently used irrigant because of its broad antimicrobial spectrum and solvent

action on organic tissue (11). However, exposure of the apical foramen and periapical tissues to this solution can often cause caustic and toxic effects in vital tissues (11,12). Furthermore, NaOCl weakens the root dentin structure by reducing the mechanical integrity of the tissue (13,14). Considering the adverse effects of NaOCl, it is necessary to find alternative solutions that can be used as irrigants.

Calcium hypochlorite [$Ca(OCl)_2$] is commonly used for industrial sterilization and water purification. This agent has the ability to promote soft-tissue dissolution similar to NaOCl (15) and shows antibacterial activity against *E. faecalis* in straight root canals prepared with sequential hand instruments (16). However, there are no studies assessing the ability of $Ca(OCl)_2$ to eliminate *E. faecalis* in root canals using preparation techniques with reduced number of instruments such as PTN and Reciproc.

In addition to synthetic irrigant solutions, proanthocyanidin (PAC)-rich plant extracts like grape seed extract (GSE) have been studied as alternative natural irrigants (13). Plant-derived natural products represent an abundant source of antimicrobial compounds that can be applied in endodontics based on their potential to promote dentin biomodification, increase mechanical properties by mediating cross-links in collagen, and reduce dentin degradation via interaction with type I collagen and other components of the dentin organic matrix (17).

Therefore, the aim of this study was to compare the efficacy of GSE, $Ca(ClO)_2$, and NaOCl in combination with rotary or reciprocating instruments for disinfection of root canals inoculated with *E. faecalis*. The null hypotheses tested were as follows: (1) Reciproc and PTN have no ability to reduce *E. faecalis* contamination in the root canal. (2) In contrast to NaOCl, GSE and $Ca(ClO)_2$ have no antimicrobial activity when used as endodontic irrigants.

Material and Methods

Specimen Preparation

Approval for this study was obtained from the local IRB (protocol no. 1.472.852). The mesiobuccal canals of 96 extracted mandibular molars with curvatures of 10-20 degrees were used in this study according to the method described by Schneider (18). The teeth were stored in a 0.9% saline solution until use. Pre-operative radiographs were taken to ensure that the teeth had no fractures, canal bifurcation, calcifications, or prior root canal treatment. The roots were sectioned transversely at the coronal third using a water-cooled diamond disk to obtain root specimens with a length of 15 mm. Apical patency was determined by inserting a size-08 K-file (Dentsply Maillefer, Ballaigues, Switzerland) into the root canal until the tip was visible at the apical foramen. Only narrow canals with initial apical diameters no wider than a size-10 K-file (Dentsply Maillefer) were included. All specimens were instrumented with #10 and #15 files calibrated at 15 mm under irrigation with distilled water. The root canals were filled with 17% EDTA for 3 min to remove the smear layer and washed with 5 mL of distilled water.

The apical foramen was then sealed with resin composite (Z250; 3M ESPE, St. Paul, MN, USA), and the root surfaces were covered with two layers of a nail varnish. Then, all specimens were autoclaved for 30 min at 120 °C, and further contamination was performed as described below.

Contamination of Samples

The culture and inoculum preparations were performed according to previous studies (13,16). Before contamination, four teeth were randomly selected and subjected to sterilization control evaluation. A sterile paper point was placed in contact with the root canal walls for 15 s and homogenized with 1 mL of 0.9% saline solution. A 100-µL aliquot of the saline solution was cultivated on blood agar for 5 min, followed by incubation at 37 °C for 48 h to verify bacterial growth. Since no bacterial growth was observed, sterilization was confirmed and root specimens were subjected to the contamination protocol. For this protocol, *E. faecalis* (American Type Collection 19433) was cultivated on brain heart infusion (BHI) broth for 24 h at 37 °C, and 100 µL of the *E. faecalis* culture was inoculated into the root canal of the previously sterilized specimens. After this procedure, sterile BHI was added to the root canal to completely fill it with the culture medium. The *E. faecalis* culture was maintained for 21 days to promote bacterial growth, with the BHI being renewed every 48 h. All procedures were performed under aseptic conditions in a laminar flow hood. Once a week, a BHI aliquot from a randomly selected tooth from each group was subjected to Gram staining and cultured on blood agar followed by catalase and esculin tests to verify the absence of contamination with other microorganisms.

Root Canal Disinfection and Instrumentation

After contamination, specimens were randomly distributed into two groups (n=44) according to the instrumentation technique: ProTaper Next (PTN) or Reciproc R25. For PTN, files were used in accordance with the manufacturer's guidelines for root canal preparation. A 0.04-taper, tip size 17 instrument (X1) and a 0.06-taper, tip size 25 instrument (X2) were sequentially used. All ProTaper Next files were used in a brushing motion sequence to facilitate flute unloading and apical file progression until the working length was passively reached. Lastly, the X2 file was inserted up to the full extension of the root canal and a brushing motion was applied against the lateral walls of the root canal. For Reciproc R25 (tip size, 25; 0.08-taper)

(VDW), the file was gently inserted into the cervical third with an in-and-out pecking motion of low amplitude. After three in-and-out movements, when more pressure was needed to make the instrument advance further into the canal, the file was removed to clean the flutes. The canal was then copiously irrigated with the respective irrigant solution. The file was then reused in the same manner along the middle third followed by irrigation. This protocol was repeated until the R25 instrument reached the working length. Lastly, the file was inserted up to the full extension of the root canal with a brushing motion against the lateral walls of the root canal. Both files were used in an electric engine Reciproc Silver (VDW GMBH) using the settings predefined by the manufacturer.

During the instrumentation, the roots were then randomly divided into four experimental subgroups (n=11) according to the root canal irrigant: sodium chloride (control group), 6% NaOCl, 6% $Ca(ClO)_2$, or 50% GSE. NaOCl and $Ca(OCl)_2$ solutions (Farmaquímica SA Produtos Quımicos, Porto Alegre, Brazil) were prepared at a concentration of 6% in distilled water. A 50% GSE solution was prepared using oligomeric PACs from Vitis vinifera extract (Mega-Natural gold grape seed extract; Polyphenolics Madera, CA, USA) by diluting the extract in distilled water. All solutions were prepared immediately before their utilization. Irrigation was performed with a total of 5 mL of the respective solution using a syringe and a 30-gauge needle NaviTip (Ultradent Products, South Jordan, UT, USA), which was inserted into the root canal without binding, in an in-and-out motion for better flow. Finally, root canals were irrigated with 1 mL of 17% EDTA for one minute to remove the smear layer and the final irrigation was performed with 5 mL of sodium chloride in all groups.

Microbiological Analysis

Microbiological analysis was performed in samples obtained at two different stages: baseline or initial sample (S1), which was collected immediately after contamination with *E. faecalis* and before instrumentation procedures; and final sample (S2), which was collected after instrumentation using the irrigant solutions. For microbiological sample collection, the root canal of each specimen was filled with sterile saline solution and a sterile K-file #15 (Dentsply Maillefer) was placed into the root canal, promoting agitation of the solution for 30 s. A sterile absorbent paper point #15 was placed into the root canal and agitated in a circumferential manner to ensure contact with the walls for 30 s in the S1 and S2 collections. The absorbent paper point was then placed in a tube containing 450 µL of sterile saline solution to release the microbiological material, which was homogenized and diluted to 10–3. Aliquots of the saline solution and its dilutions containing the microbiological material were cultivated on blood agar in duplicate for 18-24 h at 37 °C. After this period, the number of colony-forming units (CFUs) was determined on the plates three times by two different trained observers and values were averaged. The efficiency of each instrumentation/decontamination protocol was measured by calculating the percentage reduction in the colony count (RCC) using the following equation: RCC = [(CFUs S1 - CFUs S2)/CFUs S1] x 100.

Statistical Analysis

The CFU counts were converted into $\log_{10}$ values to normalize the data and to perform statistical analysis. Normal distribution of bacterial reduction data was confirmed by the Kolmogorov-Smirnov test. Data were evaluated by two-way ANOVA followed by Tukey HSD post-hoc tests. The statistical significance level was established at $P<0.05$, and data were analyzed using StatPlus AnalystSoft Inc. version 6.0 (Vancouver, BC, Canada).

Results

Regardless of the instrumentation technique (PTN or Reciproc R25) and irrigant solution used, all protocols significantly reduced bacterial counts in comparison with the baseline (Table 1). No significant differences were observed between the Reciproc R25 and PTN methods ($p>0.05$). Furthermore, all active irrigant solutions [6% NaOCl, 50% GSE, and 6% $Ca(ClO)_2$] showed similar bacterial reduction ($p>0.05$) and were significantly more effective than sodium chloride (control group) ($p< 0.05$) (Table 1).

Discussion

The presence of remnant bacteria in the root canals after instrumentation is the main cause of endodontic therapy failure (19). The rotary and reciprocating instrumentation systems were proposed to enhance microbiological

Table 1. Percentage of CFU reduction in the root canals after chemomechanical instrumentation using different preparation techniques and irrigation regimens.

Groups	RCC (%) (Mean ± SD)	
	ProTaper X1 e X2	*Reciproc R25*
Sodium chloride	87.96% ± 9.34^{bA}	89.23% ± 5.77^{bA}
6% NaOCl	99.70% ± 0.49^{aA}	99.56% ± 0.50^{aA}
6% $Ca(ClO)_2$	98.02% ± 2.81^{aA}	99.65% ± 0.54^{aA}
50% GSE	96.97% ± 2.51^{aA}	96.72% ± 2.12^{aA}

Reduction in colony counts (RCC); grape seed extract (GSE); calcium hypochlorite [$Ca(ClO)_2$]; sodium hypochlorite (NaOCl). Means followed by different lowercase letters in the same column and uppercase letters in the same row are significantly different ($P<0.05$).

reduction in the root canal system and facilitate cleaning and shaping procedures when compared to manual instrumentation (20). In the present study, rotary (PTN) and reciprocating (Reciproc R25) systems were used with different irrigants to improve the decontamination of the root canals via chemomechanical actions. Both instrumentation techniques used herein presented similar ability to decontaminate the root canals when associated with the active irrigants, so our first null hypothesis was rejected. Moreover, all active irrigants showed similar results and promoted significant reduction of *E. faecalis* contamination in comparison with sodium chloride (control group). Therefore, the second null hypothesis was also rejected. In this study, to evaluate the effectiveness of the irrigants as antimicrobial agents, the control group was irrigated with sodium chloride only. In this group, there were no differences in bacterial reduction between the two instrumentation techniques. Although sodium chloride is not considered an active irrigant solution, significant reduction of microbial content was observed in the root canal, with PTN and sodium chloride showing 87.96% reduction and Reciproc R25 and sodium chloride showing 89.23% reduction of the microbial load. This reduction could be attributed to the mechanical activity induced by instrumentation and the flow of the irrigant inside the root canal.

The advantages of mechanical instrumentation include reduced working time for root canal preparation. Moreover, PTN has a rectangular cross-section design and an asymmetric rotary motion that has been described to improve the degree of canal-shaping (7,8). During rotation, the files create a mechanical wave of motion, which passes through the length of the working part of the instrument and reduces the contact between the instrument and dentin, resulting in enhanced debris removal and flexibility in the working part of the file (21). The Reciproc file works in a reciprocating motion that allows the instrument to rotate 360° in a sequence of repetitive and imbalanced back-and-forth rotational motion (1,6,8,9,22). This results in safe and easy instrumentation of the root canals with no previous hand filing needed (22) and/or curved root canal systems (8).

Although mechanical instrumentation has shown effective decontamination in previous studies (1,8), our results highlight the importance of chemomechanical preparation of root canals, since enhanced decontamination (>98%) was observed when mechanical root canal instrumentation was combined with an antimicrobial irrigant solution. NaOCl is a well-known solution for root canal preparation that ionizes into sodium (Na^+) and hypochlorite (OCl^-) ions in water and establishes an equilibrium with hypochlorous acid (HOCl), which has an effect on the vital functions of the microbial cell. HOCl exerts germicidal activity via oxidation of sulfhydryl groups of bacterial enzymes essential for metabolic reactions (11,23). However, NaOCl is a highly alkaline (pH 11-12.5) organic solvent that can induce dissolution of collagen by cleaving the bonds between carbon atoms and oxidizing the protein primary composition (24). It is also known to fragment long peptide chains and chlorinate protein terminal groups. Since the dentin organic matrix is constituted mainly of type I collagen, degradation of the organic elements of dentin by NaOCl affects the mechanical behavior of the tissue (13,14) and increases its susceptibility to vertical fractures, especially in teeth with thin root structure.

To overcome the problems associated with using NaOCl as an irrigant solution, $Ca(OCl)_2$ has been proposed as an alternative in endodontic therapy (14-16,25,26). It is very alkaline and tends to produce more chlorine than NaOCl (25), which partially explains its antimicrobial activity (16). However, in contrast to NaOCl, it has shown acceptable outcomes in terms of cell migration, viability, and level of inflammatory response (26). Moreover, as previously demonstrated by our group, $Ca(OCl)_2$ does not negatively affect the dentin mechanical behavior, unlike the effects of NaOCl (14). The low CFUs of *E. faecalis* reported in this study support previous evidence and confirm that $Ca(OCl)_2$ qualifies as a potential irrigant solution for endodontic procedures.

The antimicrobial potential of GSE when used as an irrigant during the root canal therapy has also been demonstrated by our group (13) and was confirmed in the present study. GSE is a plant-derived polyphenolic extract rich in oligomeric and polymeric forms of PACs (17). Polyphenols showed antimicrobial properties against Gram negative and Gram positive bacteria. Phenolic compounds are known for their ability to damage microbial cells by modifying the selective permeability of the plasma membrane, leading to leakage of vital intracellular substances (27). Furthermore, previous studies have shown that PACs can reinforce the dentin and enhance its mechanical properties to increase its stability against collagenase degradation (14,16), which may be beneficial when adhesive procedures like the use of fiber glass posts are performed in the root canal. It should be emphasized that a high concentration of GSE was used in the present study since it showed great antimicrobial activity comparable to that of NaOCl in a pilot study. A likely disadvantage of GSE for root canal preparation is the absence of tissue dissolution capacity, since it strengthens organic structures such as fibril collagen.

Therefore, the use of auxiliary irrigant solutions can significantly enhance the decontamination of root canals when associated with rotary or reciprocating instruments. GSE and $Ca(OCl)_2$ are promising irrigant solutions that promote significant bacterial reduction in the root canals when used with both rotary and reciprocating single-instrument systems. However, to establish protocols for their clinical application, further studies are necessary to evaluate their antimicrobial potential against other bacteria, to assess the possibility of dentin staining when using GSE, and to investigate the effect of $Ca(OCl)_2$ on dentin mechanical properties.

Resumo

O objetivo deste estudo foi comparar a eficácia do extrato de semente de uva (ESU), hipoclorito de cálcio [$Ca(ClO)_2$] e hipoclorito de sódio (NaOCl) como soluções irrigadores quando utilizadas com instrumentos reciprocantes e rotatórios para desinfecção de canais radiculares infectados com *Enterococcus faecalis*. Raízes mesio-vestibulares de molares inferiores foram preparados e inoculados com *E. faecalis* por 21 dias. As raízes foram aleatoriamente divididas em 8 grupos (n=11) de acordo com a técnica de instrumentação e protocolo de irrigação: ProTaper Next ou Reciproc R25 associados com soro fisiológico (grupo controle), $Ca(ClO)_2$ 6%, NaOCl 6% ou ESU 50%. A atividade antimicrobiana foi determinada pela redução do número de Unidades Formadoras de Colonias (UFCs) coletadas antes e após a instrumentação e expressas em porcentagens de redução. Os dados foram analisados estatisticamente pelos testes ANOVA seguido pelo teste complementar de Tukey HSD ($p<0,05$). Não foi encontrado diferença estatisticamente significante na redução bacteriana entre os sistemas ProTaper Next e Reciproc R25 ($p>0.05$), independente da solução irrigadora usada. Além disso, todas as soluções ativas (NaOCl, ESU e $Ca(ClO)_2$) mostraram similar potencial em reduzir a quantidade de bactérias ($p>0.05$) e foram significativamente mais efetivas que o soro fisiológico ($p<0.05$). Pode-se concluir que o ESU e o $Ca(ClO)_2$ apresentam potencial para aplicação clínica como irrigantes endodônticos uma vez que apresentaram efetividade antimicrobiana contra o *E. faecalis*.

Acknowledgments

The authors declare no financial affiliation (e.g., employment, direct payment, stock holdings, retainers, consultancies, patent licensing arrangements, or honoraria) or involvement with any commercial organization with direct financial interest in the subject or materials discussed in this manuscript, neither have any such arrangements existed in the past 3 years.

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Received January 20, 2018
Accepted June 21, 2018