Antimicrobial efficacy of endodontic irrigation solutions against planktonic microorganisms and dual-species biofilm

A.M. Darrag
Faculty of Dentistry, Tanta University, Egypt

Abstract

Objective: To compare the antimicrobial effect of different irrigation solutions against Enterococcus faecalis (E. faecalis) and Streptococcus mutans (S. mutans) planktonic microorganisms and dual-species biofilm within the root canal.

Materials and methods: Forty three extracted human single-rooted teeth (18–25 years old) were selected and decoronated. The root canals were prepared to a master apical size 40 using hand K-files with distilled water irrigation. Roots were sterilized, microbial suspension of mixed culture of the tested microorganisms were inoculated into canals and incubated for 48 h. Three randomly chosen teeth were used to check up root infection, the remaining forty infected root canals were divided equally into 4 groups according to the irrigant solutions used. Group A: sterile distilled water; Group B: 5.25% Sodium Hypochlorite (NaOCl); Group C: 2% Chlorhexidine (CHX); Group D: 200 mg/mL N-Acetylcysteine (NAC) solution. Finally, the canals were washed with saline. The first microbiological sampling for planktonic bacteria was taken from root canal lumen and the number of CFU of tested organisms was counted. Each root canal was then split longitudinally, and a 4-mm-long mid-part of one half was cut, sterilized and inoculated with the mixed microbial suspension for 48 h. Specimens of each group were immersed in 5 ml of tested irrigation solution for 5 min. The proportions of dead bacterial cells in the biofilm were assessed by CLSM.

Results: Both planktonic bacteria were more sensitive to NAC solution recording significantly the lowest mean CFU values. S. mutans was statistically more sensitive to NaOCl irrigation compared to E. faecalis. CLSM showed significantly more proportion of dead cells in dual-species biofilm when NAC irrigation solution was used.

Conclusion: NAC solution can be considered as a potential alternative irrigation solution in root canal infections because of its antimicrobial efficacy against both S. mutans and E. faecalis planktonic bacteria and dual-species biofilm.

Keywords: Antimicrobial effect; Biofilm; Dual-species; Enterococcus faecalis; Irrigation solution; Streptococcus mutans

1. Introduction

The success of endodontic treatment depends on the eradication of micro-organisms from the root canal system and prevention of re-infection [1]. A bacteria-free root canal system is difficult to be obtained due to the anatomical complexities of many root canals, organic residues and unreachable bacteria located deep in the dentinal tubules [2]. Bacteria in the root canal
are present either as free-floating planktonic single cells or attached to each other or to the root canal walls to form a biofilm [3]. Biofilms, by definition, are protected structured community of bacterial cells embedded in a self-produced polymeric matrix adherent to each other and/or to surfaces or interfaces [4]. Thus various irrigating solutions are essential to be used during and immediately after root canal preparation to remove debris and necrotic pulp tissue and to ensure microorganisms’ minimization [5].

It was known that, Enterococcus faecalis is a versatile pathogenic microorganism that plays a major role in the etiology of persistent endodontic infections after initial treatment and commonly found in high numbers of root canal failures [6]. Several traits of E. faecalis are related to its persistence in root canal infections, these include extensive genetic polymorphisms, invasion into dentinal tubules, and rapid adaptation to adverse conditions. It can adhere to the root canal walls, accumulate, and form communities organized in biofilm [7].

In vitro studies have shown the ability of E. faecalis biofilm to calcify as it undergoes “maturation” within the root canal [8]. So far, most studies on E. faecalis biofilm characteristics have been performed on monoculture biofilm. Although E. faecalis has sometimes been isolated as the sole infectious organism in root canals, the notion that endodontic infections are typically polymicrobial has become more and more favorable [7,9]. E. faecalis was reported to coexist with several other taxa in root canal—treated teeth [10].

The presence of Streptococcus mutans species leads to the microbe—microbe interactions which increase total biofilm formation [7] and change the characteristics of the biofilm, thus alter the resistance pattern to antimicrobials [11]. These possess outstanding abilities to penetrate dentinal tubules both individually and in co-aggregation with other organisms and form a strong biofilm [12]. A variety of streptococcal species have not been isolated only at significant rates in samples from primary infected teeth, but also during root canal treatment, after instrumentation and application of intracanal dressings, and in 47% of retreatment cases [13].

The predominance of Streptococci together with other Gram-positive organisms suggests resilience and ability to survive under difficult nutritional conditions [14].

The bacterial population inside the root canal was found to be significantly reduced during irrigation regardless the type of irrigant solution used due to the mechanical effect generated by the flow and back flow of the solution [15]. The most popular irrigating solution is NaOCl which is an effective antimicrobial agent, good lubricant and an excellent organic solvent [16,17]. However, it is highly irritating to the periapical tissues, especially at high concentrations [18].

In addition, it was found that CHX digluconate is a bisguanide disinfectant that has broad antimicrobial substantive activity against some resistant bacteria such as E. faecalis [19] because it has the ability to be adsorbed and released gradually from the hydroxyapatite surfaces. It is also effective mainly against Gram +ve and also Gram —ve bacteria as well as yeasts [20]. Thus it has been widely used as an auxiliary canal irrigant or a canal soaking agent against E. faecalis [2]. Furthermore, it was found to have antibacterial action against S. mutans [21–24].

NAC is a derivative of the amino acid L-cysteine [25] which is a well-established antioxidant mucolytic agent that is considered a non-antibiotic drug with antibacterial properties which decreases biofilm formation by a variety of bacteria [26]. This solution disrupts disulphide bonds in mucus and reduces the viscosity of secretions [27]. Furthermore it exerts anti-inflammatory activity [28] through its ability to inhibit the expression and release of a variety of pro-inflammatory cytokines [29]. In addition, Ehsani et al. [25] had proved that NAC can be a substitute for ibuprofen for post-endodontic pain. It was indicated that NAC solution is an effective chemoprotectant that can safely be used to protect the pulp and the surrounding periradicular tissues from adverse toxic effects of resin-based and methacrylate dental restorative and endodontic materials [30–32].

However, the possible effectiveness of NAC against oral bacteria biofilm has not been extensively explored. Recently, it was published that NAC inhibits growth and eradicates biofilm of E. faecalis [33] although there are limited reports of NAC against the main oral bacteria like S. mutans. Thus it would be interesting to test the incorporation of NAC as a root canal irrigant solution to overcome the inadequacy of most root canal irrigants to kill root canal microorganisms. This study was thus conducted as a randomized clinical trial to evaluate and compare the antimicrobial effect of 5.25% NaOCl, 2% CHX and 200 mg/mL NAC solutions against E. faecalis and S. mutans planktonic microorganisms and dual-species biofilm in root canals.

2. Materials and methods

2.1. Specimen selection and preparation

Forty three intact, unrestored non-curious, mature human single-rooted freshly extracted teeth for orthodontic or periodontal reasons from adults (18–25 years
old) were selected. Teeth were cleaned and stored in normal saline solution at room temperature and used within 3 months after extraction. Each tooth was examined radiographically to ensure the presence of a single canal. Teeth were de-coronated using a diamond disc at a low speed straight hand piece under water cooling to standardize roots of all the teeth to approximately 14 ± 1 mm length and a minor apical foramen of size 20 [34]. Then, working length (WL) for each root was recorded radiographically using a size 10 K-file up to 1 mm short of the apical foramen. Apical enlargement of all samples was done up to size 40 and the root canals were prepared with step back technique using hand K-files. During the process of instrumentation, all canals were irrigated with 5 ml of distilled water [15] using 26 gauge stainless steel needles with disposable syringe.

2.2. Sterility and asepsis control

Following root canal preparation, the apical foramina were sealed with acrylic resin\(^3\) to prevent bacterial leakage. Subsequently, each root specimen was autoclaved\(^4\) to be sterilized within a glass tube containing 3 ml of Brain Heart Infusion (BHI) broth\(^5\) for 20 min at 121 °C and incubated\(^6\) for 48 h at 37 °C. The BHI broth was inspected daily to ensure the absence of any signs of turbidity to control the sterility [35,36]. These procedures were performed inside a laminar airflow cabinet\(^7\) (Fig. 1) at Microbiology and Immunology Department, Faculty of Pharmacy, Tanta University.

2.3. Root canal inoculation and group assignment

The bacterial strains used in this study were \textit{E. faecalis} (ATCC 29212) and \textit{S. mutans} (ATCC 25175)\(^8\). Both bacterial strains were grown anaerobically on BHI agar at 37 °C for 24 h 4 ml of mixed microbial suspension was collected in a sterile glass tube from equal volumes of \textit{E. faecalis} and \textit{S. mutans} suspensions, which had its optical density adjusted to approximately 1.5 × 10\(^8\) colony forming units CFU/mL by comparing its turbidity to a 0.5 McFarland standard spectrophotometrically.\(^9\)

The glass tubes containing specimens were opened and 2 ml of sterile BHI was removed and replaced with 2 ml of the bacterial suspension using a sterile pipette. The bottles were closed and incubated at 37 °C for 48 h [15]. To check root canal infection, samples were taken from 3 randomly selected canals using sterile paper points at the end of the inoculation period and inoculated onto an agar dish for 24 h at 37 °C [37]. After incubation, the remaining forty roots were removed from the test tubes, rinsed thoroughly with sterile physiological saline and were randomly divided into four experimental groups with 10 roots in each group according to the solutions used for irrigation. Group A: sterile distilled water (control group); Group B: 5.25% NaOCl\(^10\) solution; Group C: 2% CHX\(^11\) solution; Group D: NAC solution at a concentration of 200 mg/mL which was freshly prepared by dissolving 0.2 g in 1 mL of sterile distilled water according to Quah et al. [33]. A volume of 5 ml of the tested irrigant solution was used for each sample and was allowed to remain in the canal for 5 min. Then a final flush was performed using 4 ml of sterile distilled water in each sample using 26 gauge stainless steel needles with a disposable syringe [15].

2.4. Microbial sampling and bacterial counting

Excess moisture from the canal was removed using a sterile paper point, then the first microbiological sampling for planktonic bacteria was taken from each root canal lumen using a dry sterile standardized paper point size 40 that was kept in the canal to the full WL for 10 s to absorb the fluid, then transferred to a sterile Eppendorf containing 1 ml of sterile saline solution and vortexed for 30 s. After obtaining 1:10 serial dilution from each sample, aliquots were plated out on

\footnotesize{
1 Eastwind Diamond Abrasives, Windsor, Canada.
2 Dentsply Maillefer, Ballaigues, Switzerland.
3 Acrostone, Egypt.
4 Systec D-Series, Germany.
5 Difco, Detroit, USA.
6 Kottermann 2736, Germany.
7 MicroFlow LFC013, Bioquell UK Ltd.
8 Sigma–Aldrich, St. Louis, France.
9 CrystalSpec, Sparks, MD21152, USA.
10 Clorox Co, 10th of Ramadan, Egypt.
11 Septodent Healthcare India Pvt, Ltd, Maharashta, India.
}
BHI agar plates and spread using sterilized glass rod L-spreaders loop and the plates were incubated anaerobically at 37 °C for 2 days. The numbers of bacterial colonies for both *E. faecalis* and *S. mutans* were counted and expressed as CFUs using a colony counter.12

2.5. Biofilm evaluation

The effect of tested endodontic irrigating solution on the biofilm bacteria was evaluated using confocal laser scanning microscopy (CLSM).13 Each root was split longitudinally, the most patent half was selected and the dentin surface was flattened using a 600 grit sandpaper.14 The coronal and apical parts of the root halves were cut with a high-speed diamond disk15 and discarded, resulting in a 4-mm long mid-part of the root sample. The prepared dentin specimens, with the canal side up, were embedded in acrylic resin16 blocks to facilitate handling. Root dentin blocks were washed with distilled water, sterilized, inoculated with a freshly prepared mixed microbial suspension of *E. faecalis* and *S. mutans* and incubated for 48 h as previously described. After 2 days of biofilm growth, specimens of each group were immersed in 5 ml of sterile distilled water, 5.25% NaOCl, 2% CHX and NAC solutions for groups A, B, C and D respectively for 5 min.18

The mounted specimens were examined to observe biofilms and determine dead and viable bacterial counts by using CLSM with three-dimensional images. Specimens for CLSM were rinsed with sterile physiologic solution for 2 min to remove loosely adherent planktonic bacteria. Then they were stained with SYTO-9 and Propidium Iodide LIVE/DEAD BacLight Bacterial Viability Stain17. The staining solution was prepared by adding 1 μL of SYTO-9 and 1 μL of Propidium Iodide stains to 1 mL of sterile distilled water following the manufacturer’s instructions. Each sample was incubated for 20 min at room temperature protected from light in the stain, then gently washed in sterile distilled water and immediately mounted on a coverslip for examination under CLSM.

The mounted specimens were observed by using a 10× lens. Confocal LIVE/DEAD images were analyzed and quantitated by using Olympus FV10-ASM 1.7 software. The volume ratio of red fluorescence (dead cells) to green-and-red fluorescence (live and dead cells) indicated the proportion of dead cells in the biofilms where a high ratio indicates the positive antimicrobial effect of the studied therapeutic agent.

2.6. Statistical analysis

The CFU values were transformed to their log 10. The mean values of log 10 CFU of target bacteria, *E. faecalis* and *S. mutans*, and the mean proportion of dead cells on the biofilm with the standard deviation (SD) were calculated. The results were statistically analyzed and summarized in Table 1 using univariate One-way ANOVA and Tukey-HSD post hoc analysis, when necessary, for mean comparison between groups at a significance level of *P* ≤ 0.05 using SPSS version 11.5. Student’s *t*-test was used to assess significance between *E. faecalis* and *S. mutans* mean values of each group at 95% level of confidence.

3. Results

The present data demonstrated that, none of the tested root canal irrigant solution completely eliminated the tested planktonic microorganisms in root canal lumen and biofilm.

Considering planktonic microorganisms, the antimicrobial effect of different irrigation solutions on each tested microbiota was analyzed using ANOVA. Regarding *E. faecalis*, the mean CFU counts showed an ascending rank for NAC, 2% CHX, 5.25% NaOCl and control groups with a high statistical significant difference among the tested groups which was recorded between control group and all the other tested groups, in addition to group B and D.

Comparing the effect of the different tested irrigating solutions on *S. mutans*, the lowest mean value of CFU was noticed using NAC solution followed by NaOCl and CHX solutions, while the highest mean value was found with control group. It was obvious that, the control group was responsible for the recorded statistical significant difference since these were obtained between control group and all the other tested groups.

Regarding the effect of each root canal irrigation solution on both tested organisms, Student’s *t*-test revealed that *S. mutans* was statistically more sensitive to NaOCl irrigation compared to *E. faecalis*. On the other hand there were no statistical significant differences recorded between the mean log 10 of CFU values of both *E. faecalis* and *S. mutans* when distilled
Table 1
Planktonic bacterial count (log CFU) in root canal lumen and proportion of dead cell in biofilm on root canal surface exposed to different irrigation solutions.

| Planktonic organism | Irrigation          | Distilled water (group A) mean ± SD | 5.25% NaOCl (group B) mean ± SD | 2% CHX (group C) mean ± SD | 200 mg/mL NAC (group D) mean ± SD | ANOVA P-Value |
|---------------------|---------------------|------------------------------------|---------------------------------|--------------------------|----------------------------------|----------------|
| E. Faecalis         |                     | 12.34 ± 1.80                       | 6.09 ± 0.18 a                   | 5.81 ± 0.37 ab           | 3.60 ± 0.02 b                    | 0.001*         |
| S. Mutans           |                     | 14.52 ± 1.25                       | 3.91 ± 0.12 b                   | 4.75 ± 0.92 b            | 3.08 ± 0.87 b                    | 0.003*         |

Proportion of dead cell in biofilm

Tukey HSD test: groups with similar letters ‘a’ or ‘b’ (italicized) are not significant.
* Significant at 5% level.

In addition, using one way ANOVA and Tukey HSD test at 95% level of confidence, the results of biofilm assessment using CLSM images revealed that the mean value of the proportion of dead bacterial cells was significantly dependent on the type of root canal irrigation solution currently used. Compared to control group (Fig. 2a), viable bacterial cells were scarcely detectable in biofilms on dentin surfaces exposed to NAC solution (Fig. 2b) recording significantly higher proportion of dead cells (0.94 ± 0.17), followed by groups B and C (0.69 ± 0.09 and 0.23 ± 0.07 respectively) as obviously shown in Fig. 2c and d. However no significant difference was detected between control and 2% CHX groups. Both killed fewer bacteria than 5.25% NaOCl and NAC groups in the dual-species biofilm as shown in Table 1.

4. Discussion

The eradication of bacteria by endodontic treatment from the root canal is difficult [40] because of biofilm formation on the dentin walls of the main root canal [41]. So, one of the major goals in the field of modern clinical microbiology is to attempt to develop new strategies capable of reducing the incidence of biofilm infections and of effectively curing chronic conditions related to the establishment of these difficult-to-eradicate bacterial structures [42].

Most of Earlier literature about endodontic disinfection has focused on killing of planktonic microorganisms which can be easily eliminated by a variety of different methods [39]. Increased awareness of the role of biofilms in infections of the human body where a bacterial biofilm remain a major challenge has caused a shift in study design, so that biofilms rather than planktonic microorganisms are now used in studies of the efficacy of various disinfecting methods and agents [43–45]. Accordingly, the rationale of this study stems from the antimicrobial properties of tested irrigation solutions, 5.25% NaOCl, 2% CHX and 200 mg/mL NAC and abilities to eradicate both planktonic and dual-species biofilm of *E. faecalis* and *S. mutans* organisms.

These types of bacteria were chosen because *E. faecalis* was reported as therapy resistant bacteria in root canals either in primary endodontic infections or in root canal treated teeth [46]. In addition, *Streptococcus* comprise a relatively high proportion of the microorganisms recovered from the canals of teeth with post-treatment disease [47]. In addition, Jiang et al. [4] indicated that the presence of preformed *S. mutans* biofilms resulted not only in increased biofilm formation of *E. faecalis* but also increased microbial resistant against antimicrobial agents due to the presence of the dual-species biofilm.

The first step in biofilm formation is surface attachment [39]. The mechanisms whereby oral bacteria adhere to solid surfaces are influenced by the properties of the outer hard surface [48], so root dentin blocks were used currently trying to simulate the clinical conditions where previous studies have shown that dentin has an inhibitory effect on the antibacterial effectiveness of disinfecting agents [49]. Bacterial adhesion has been suggested to occur in 2 main phases. Phase 1 is a physicochemical process which occurs within seconds to minutes, whereas phase 2 is considered as a biologic cellular-molecular process of biofilm maturation, occurring in a time frame of few hours to two days [50]. Therefore in the present study dentin samples were inoculated with bacteria for the maximum required period 48 h to allow maturation of the biofilm structure on dentin.

In biofilm research viability testing, using CFU counts is frequently applied although it is laborious,
time consuming, and not suitable for high-throughput screening. Moreover, biofilms may be removed from the surface and dispersed before plating during microbial sampling, thus the efficacy of this process is often in question [4]. The development of CLSM and the improvement of direct viable staining methods have stimulated the study of bacteria’s viability in the last years, especially in the biofilm area. Potential advantages of using CLSM for the study of dentin infection currently includes: observation of live bacteria on root canal walls [51], in addition to avoiding biofilm detachment/dispersed steps and allowing 3D visualization of structural organization in biofilms [52].

Regarding the tested irrigating solutions, 5.25% concentration of NaOCl was used because Berber et al. [53] found no differences among various concentrations of NaOCl in reducing E. faecalis within root canal lumen but only at the higher concentrations, it was able to disinfect the dentinal tubules. On the other hand, 2% Chlorhexidine was chosen because it has a bacteriostatic action at lower concentrations of 0.2% and bactericidal effect at higher concentrations [34]. While Cindy et al. [54] found that, in the case of NAC solution, the most important effect was observed at 200 mg/mL as antimicrobial agents against oral bacteria, so this concentration was chosen. Sterile distilled water was used as an irrigant in control group because it is an inert solution without any antimicrobial effect.

Following the analysis of data, the current results revealed that NAC was able to kill both planktonic and biofilm forms of E. faecalis and S. mutans most effectively. This could be attributed to the mechanism of antibacterial effects of NAC that is most likely due to its thiol group (–SH), which is the active moiety of NAC. This group plays an important role in free radical scavenging and destruction of disulfide bonds of bacterial protein. The reaction of thiol group with the disulfide bonds of bacterial proteins leads to the irreversible damage of bacterial proteins that are essential for bacterial growth and metabolism thus reduced sessile cell viability [55,56].
Regarding the recorded significant role of NAC solution on disruption of biofilm bacteria, it was hypothesized that NAC is an antioxidant that can have indirect effects on bacterial cell metabolism and exo-polysaccharides production [33] that may reduce cell viability. This reduction of bacterial exo-polysaccharides which are the main component of the biofilm will affect the trapping and concentrating essential minerals and nutrients from the surrounding environment thus promoting the disruption of mature films and inhibiting bacterial adhesion and viability [57].

Previous studies reported that NAC inhibited the growth and biofilm formation of several microbes including the oral bacteria E. faecalis [33] as shown in the current results. In addition, earlier studies have shown that NAC decrease biofilm formation and promote the disruption of mature biofilms [55,56].

In comparison to the current antimicrobial effect of NAC solution, it was found that, NaOCl or CHX were less effective on E. faecalis. It has been suggested that the buffering capacity of dentin against some antimicrobials [58] or tissue debris on the dentin surface might reduce the efficacy of NaOCl or CHX on the smear layer and thereby limit the effect on bacterial attachment. Quah et al. [33] added that the antimicrobial effects of NAC on E. faecalis were not affected by the presence of dentin. In addition, a significant reduction in dead cell proportion with 2% CHX irrigation compared to NAC found in this study corroborates with the finding of Cindy et al. [54] who revealed that the efficacy of NAC solution in inhibiting S. mutans growth was better than CHX solution.

From the present results, 2% CHX showed less antimicrobial efficacy compared to 5.25% NaOCl on both S. mutans species and biofilm. This was consistent with [59,60] who demonstrated that the better antibacterial efficacy of NaOCl observed in comparison to CHX could be attributed to the antibiofilm effects of NaOCl. The NaOCl acts as organic tissue solvent thus eliminating the bacterial attachment to dentin and other organisms. In contrary, Ercan et al. [18] and Mittal et al. [35] observed that CHX gluconate was significantly more effective than NaOCl. This controversy can be explained by the fact that the present investigation was carried on both planktonic bacteria and dual-species biofilm, whereas their studies were performed on planktonic bacteria only. Regarding the effect of CHX and NaOCl on E. faecalis, the present results found that their effects were comparable as supported by Menezes et al. [37]. In contrary, Bansal et al. [34] found that NaOCl had better antibacterial efficacy than CHX, this difference may be related to different methodology where they used the irrigation solution throughout instrumentation that may have an important role in the reduction of bacterial count.

The present finding revealed that S. mutans was significantly sensitive to NaOCl than E. faecalis in planktonic condition where the proportion of dead cells associated with NaOCl treatment confirmed this finding. This was supported by Jiang et al. [4] who demonstrated that the presence of dual-species biofilm containing S. mutans increased NaOCl resistance of E. faecalis. Deng et al., 2009 [7] showed that the presence of other microorganisms had a marked effect on E. faecalis biofilm mode of growth. This finding is similar to what was found in other studies [11,61].

Additionally, whenever CHX was tested, it was found that it is more effective on S. mutans than E. faecalis. Its action is to combine with phospholipids membrane of S. mutans and cause its disruption since it is a membrane active agent. Additionally at high concentration it inhibits membrane bound Adenine Tri-Phosphatase (ATP-ase) enzyme to bacterial cell membrane which is essential for hydrolysis of ATP and liberation of energy required for survival of S. mutans [62]. In addition, several studies agreed with the current results concluding that CHX did not possess sufficient inhibitory activity to eliminate E. faecalis from infected root canals and dentinal tubules. Also, other authors [63] confirmed the current findings indicating that E. faecalis resisted CHX.

Under the limitation of this study which is using two bacterial strains only however the endodontic infection has a polymicrobial nature, the conclusion can be summarized that 200 mg/mL NAC solution might be considered as a potential alternative irrigation solution in root canal infections because of its antimicrobial efficacy against both S. mutans and E. faecalis planktonic bacteria and dual-species biofilm compared to commonly use irrigating agents, NaOCl or CHX. Further studies are required to focus on other aspects of an ideal root canal irrigant as smear layer removal and its effect on adhesive root canal filling seal and bond strength to establish the use of NAC solution as a new strategy in root canal irrigation and disinfection.

Acknowledgment

I would like to thank Dr. Aml M. Saeed, associate professor of Microbiology and Immunology, Faculty of Pharmacy, Beni-Suif University, for providing the S.
mutans and E. faecalis strains and her help and effort in microbiological technical assistance.

References

[1] Haapasalo M, Shen Y, Qian W, Gao Y. Irrigation in endodontics. Dent Clin North Am 2010;54:291–312.
[2] Mohammadi Z, Abbott PV. The properties and applications of chlorhexidine in endodontic: review. Int Endod J 2009; 42:288–302.
[3] Rucucci D, Siqueira Jr JF. Biofilms and apical periodontitis: study of prevalence and association with clinical and histopathologic findings. J Endod 2010;36:1277–88.
[4] Jiang LM, Hoogenkamp MA, van der Sluis LWM, Wesselink PR, Crielard W, Deng DM. Resazurin metabolism assay for root canal disinfectant evaluation on dual-species biofilms. J Endod 2011;37:31–5.
[5] Ferraz CC, Gomes BP, Zaia AA, Teixeira FB, Souza d’Almeida against Decurcio DA. Efficacy of sodium hypochlorite and chlorhexidine in endodontic: review. Int Endod J 2009;42:288–302.
[6] Stuart CH, Schwartz SA, Beeson TJ, Owatz CB. Enterococcus faecalis: its role in root canal treatment failure and current concepts in retreatment. J Endod 2006;32:93–8.
[7] Deng DM, Hoogenkamp MA, Exterkate RAM, Jiang LM, van der Sluis LWM, Cate JMT, et al. Influence of Streptococcus mutans on Enterococcus faecalis biofilm formation. J Endod 2009;35:1249–52.
[8] Kishen A, George S, Kumar R. Enterococcus faecalis-mediated bimineralized biofilm formation on root canal dentine in vitro. J Biomed Mater Res 2006;77:406–15.
[9] Love RM. Invasion of dentinal tubules by root canal bacteria. Endod Top 2004;9:52–65.
[10] Rocas IN, Hulsmann M, Siqueira Jr JF. Microorganisms in root canal-treated teeth from a German population. J Endod 2008; 34:926–31.
[11] Ozok AR, Wu MK, Luppens SB, Wesselink PR. Comparison of growth and susceptibility to sodium hypochlorite of mono- and dual-species biofilms of Fusobacterium nucleatum and Peptostreptococcus (micromonas) micros. J Endod 2007;33:819–22.
[12] da Silva LA, Nelson-Filho P, Faria G, de Souza-Gugelmin MC, Ito YJ. Bacterial profile in primary teeth with necrotic pulp and periapical lesions. Braz Dent J 2006;17:144–8.
[13] Chávez de Paz L. Gram-positive organisms in endodontic infections. Endod Top 2004;9:79–96.
[14] Chávez de Paz L, Svensäter G, Dahlén G, Bergenholtz G. Streptococci from root canals in teeth with apical periodontitis receiving endodontic treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2005;100:232–41.
[15] Vijaykumar S, Gunasekhar M, Himagiri S. In vitro effectiveness of different endodontic irrigants on the reduction of Enterococcus faecalis in root canals. J Clin Exp Dent 2010;2:e169–72.
[16] Estrela C, Silva JA, Gonçalves De Alencar AH, Leles CR, Decurcio DA. Efficacy of sodium hypochlorite and chlorhexidine against Enterococcus faecalis—a systematic review. J Appl Oral Sci 2008;16:364–8.
[17] Vianna ME, Gomes BP, Berber VB, Zaia AA, Ferraz CC, Jose’ de Souza-Filho F. In vitro evaluation of the antimicrobial activity of chlorhexidine and sodium hypochlorite. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004;97:79–84.
[18] Erkan E, Ozekinci T, Atakul F, Kadri G. Antibacterial activity of 2% chlorhexidine gluconate and 5.25% sodium hypochlorite in infected root canal: In-vivo study. J Endod 2004;30:84–7.
[19] Baca P, Junco P, Arias-Moliz MT, Castillo F, Rodriguez-Archipila A, Ferrer-Lusque CM. Antimicrobial substantivity over time of chlorhexidine and cetrimide. J Endod 2012;38:927–30.
[20] Athanassiadis B, Abbott PV, Walsh LJ. The use of calcium hydroxide, antibiotics and biocides as antimicrobial medicaments in endodontics. Aust Dent J 2007;52:64–82.
[21] Kandaswamy D, Venkateshbabu N, Gogulnath D, Kindo AJ. Dentine tubule disinfection with 2% chlorhexidine gel, propolis, morinda citrifolia juice, 2% povidone iodine, and calcium hydroxide. Int Endod J 2010;43:419–23.
[22] Valera MC, da Rosa JA, Maekawa LE, Ozok AR. Action of propolis and medications against Escherichia coli and endoxin in root canals. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010;110:70–4.
[23] Signoretti FG, Gomes BP, Montagner F, Barrichello Tosello F, Jacinto RC. Influence of 2% chlorhexidine gel on calcium hydroxide: the activity of reducing endotoxin. Oral Surg Oral Med Oral Pathol Radiol Endod 2011;111:653–8.
[24] Tüzünler T, Ulusoy AC, Baygın O, Yahyaoglu G, Yalcin I, Buruk K, et al. Direct and transdental (indirect) antibacterial activity of commercially available dental gel formulations against Streptococcus mutans. Med Princ Pract 2013;7:1–5.
[25] Ehsani M, Moghadamnia A, Zahedpasha S, Ghobaran Maliji G, Haghanifar S, Aghajanpour SM, et al. The role of prophylactic ibuprofen and N-acetylcysteine on the level of cytokines in periapical exudates and the post-treatment pain. J Pharm Sci 2012;20:30–5.
[26] Schwandt LQ. Prevention of biofilm formation by dietary products and N-acetylcysteine on voice protheses in an artificial throat. Acta Otolaryngol 2004;124:726–31.
[27] Livingstone CR, Andrews MA, Jenkins SM, Marriott C. Model systems for the evaluation of mucolytic drugs: acetylcysteine and S-caboxymethylcysteine. J Pharmacol 1990;42:73–8.
[28] Sadowska AM, Manuel YK, De Backer WA. Antioxidant and anti-inflammatory activity of NAC in the treatment of COPD: discordant in vitro and in vivo dose-effects: a review. Pulm Pharmacol Ther 2007;20:9–22.
[29] Lappas M, Permezel M, Rice GE. N-Acetyl-cysteine inhibits phospholipid metabolism, proinflammatory cytokine release, protease activity, and nuclear factor-kappaB deoxyribonucleic acid-binding activity in human fetal membranes in vitro. J Clin Endocrinol Metab 2003;88:1723–9.
[30] Paranjpe A, Cacalan NA, Hume WR, Jewett A. Mechanisms of N-acetyl Cysteine—mediated protection from 2-Hydroxyethyl Methacrylate—induced apoptosis. J Endod 2008;34:1191–7.
[31] Kim NR, Park HC, Kim I, Lim BS, Yang HC. Anti-inflammatory efficacy of NAC in the treatment of COPD: discordant in vitro and in vivo dose-effects: a review. Pulm Pharmacol Ther 2007;20:9–22.
[32] Minamikawa H, Yamada M, Deyama Y, Suzuki K, Kaga M, Yawaka Y, et al. Effect of N-acetylcysteine on Rat dental pulp cells cultured on mineral trioxide aggregate. J Endod 2011;37:637–41.
[33] Quah SY, Wu S, Lui JN, Sum CB, Tan KS. N-acetylcysteine inhibits growth and eradicates biofilm of Enterococcus faecalis. J Endod 2012;38:81–5.
[34] Bansal R, Jain T, Mittal S, Kumar T, Jindal N, Kaur D. A comparison of the antibacterial efficiency of MTAD (mixture of tetracycline, citric acid and detergent), 2.5% sodium hypochlorite and 2% chlorhexidine root canal irrigants against
Enterococcus Faecalis in root canals of single rooted mandibular premolars—an in vitro study. J Dent Med Sci 2013;5:47–53.

[50] Kishen A, Sum CP, Mathew S, Lim CT. Influence of irrigation regimens on the adherence of Enterococcus faecalis to root canal dentin. J Endod 2008;34:850–4.

[51] Ordinola-Zapata R, Bramante CM, Gomes de Moraes I, Bernardinelli N, Porto C, Campanelli AP, et al. The use of confocal laser scanning microscopy for the study of dentin infection. Microsc Sci Technol Appl Educ 2010;583–9.

[52] Shen Y, Qian W, Chung C, Olsen I, Haapasalo M. Evaluation of the effect of two chlorhexidine preparations on biofilm bacteria in vitro: a three-dimensional quantitative analysis. J Endod 2009;35:981–5.

[53] Berber VB, Gomes BP, Sena NT, Vianna ME, Ferraz CC, Zaia AA, et al. Efficacy of various concentrations of NaOCl and instrumentation techniques in reducing Enterococcus faecalis within root canals and dental tubules. Int Endod J 2006;39:10–7.

[54] Cindy HR, Rene HD, Sergio GR, Diana RP, Claudio CR. Rifampicin and N-acetylcysteine inhibit oral bacterial growth and biofilm formation. The Pharmaceut Innovat J 2013;2:16–23.

[55] Marchese A, Bozzolasco M, Gualco L, Debbia EA, Schito GC, Schito AM. Effect of fosfomycin alone and in combination with N-acetylcycteine on E. coli biofilms. Int J Antimicrob Agents 2003;22:S95–100.

[56] Olofsson AC, Hermansson M, Elwing M. N-acetylcysteine affects growth, extracellular polysaccharide production, and bacterial biofilm formation on solid surfaces. Appl Environ Microbiol 2003;69:4814–22.

[57] Zhao T, Liu Y. N-acetylcysteine inhibit biofilms produced by Pseudomonas aeruginosa. BMC Microbiol 2010;10:140–7.

[58] Haapasalo M, Qian W, Portenier I, Waltimo T. Effects of dentin on the antimicrobial properties of endodontic medicaments. J Endod 2009;35:917–25.

[59] Clegg MS, Vertucci FJ, Walker C, Belanger M, Britto LR. The effect of exposure to irrigant solutions on apical dentin biofilms in vitro. J Endod 2006;32:434–7.

[60] Arias-Moliz MT, Ferrer-Luque CM, Espigares-García M, Baca P. Enterococcus faecalis biofilms eradication by root canal irrigants. J Endod 2009;35:711–4.

[61] Kara D, Luppens SB, Cate JM. Differences between single- and dual-species biofilms of Streptococcus mutans and Veillonella parvula in growth, acidogenicity and susceptibility to chlorhexidine. Eur J Oral Sci 2006;114:58–63.

[62] Hugo WB, Russell AD. Chemical disinfectants, antiseptics, and preservatives. In: Pharmaceutical microbiology. 6th ed. Blackwell Science Ltd; 2000.

[63] Srikumar GPV, Varma KR, Shetty KHK. Comparison of the antibacterial efficiency of MTAD, 2.5% sodium hypochlorite and 2%chlorhexidine against Enterococcus faecalis-an ex vivo study. Endodontontology 2012;24:41–7.