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Antimicrobial efficacy of ozonated water, gaseous ozone, sodium hypochlorite and chlorhexidine in infected human root canals

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Abstract

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Aim To determine the antimicrobial efficacy of ozonated water, gaseous ozone, sodium hypochlorite and chlorhexidine in human root canals infected by *Enterococcus faecalis*.

Methodology Thirty human maxillary anterior teeth were prepared and inoculated with *E. faecalis* for 60 days. Eppendorf tubes were connected to the coronal portion of the teeth. Urethane hoses were attached to the tubes and to the entrance of a peristaltic pump. The exit of the apparatus corresponded to the apical portion of the root canals. The test irrigating solutions were ozonated water, gaseous ozone, 2.5% sodium hypochlorite (NaOCl), 2% chlorhexidine that circulated at a constant flow of 50 mL min^{-1} for 20 min. Samples from the root canals were collected and immersed in 7 mL Letheen Broth (LB), followed by incubation at 37 °C for 48 h. Bacterial growth was analysed by turbidity of the culture medium and subculture on a specific nutrient broth. A 0.1 mL inoculum obtained from LB was transferred to 7 mL of brain heart infusion and incubated at 37 °C for 48 h. Bacterial growth was checked by turbidity of the culture medium carried out in triplicate.

Results No solution used as an irrigant over a 20-min contact time demonstrated an antimicrobial effect against *E. faecalis*.

Conclusion The irrigation of infected human root canals with ozonated water, 2.5% NaOCl, 2% chlorhexidine and the application of gaseous ozone for 20 min was not sufficient to inactivate *E. faecalis*.

Keywords: chlorhexidine, intracanal dressing, ozonated water, ozone, sodium hypochlorite.

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Introduction

The outcome of root canal treatment in the presence of apical periodontitis is directly influenced by the use of acceptable clinical procedures under strictly aseptic conditions, in addition to the immunological response of the host (Estrela *et al.* 2004, Nair *et al.* 2005). Selective pressures related to oxidation–reduction potential, nutrient supply and microbial interactions are related to the maintenance of endodontic infections (Sundqvist & Fidgor 2003).

Several reports have discussed factors related to the aetiology of post-treatment disease in endodontics: (i) microbial aetiologic factors (intraradicular and extraradicular infection – bacteria, fungi); (ii) nonmicrobial aetiologic factors (endogenous – true cysts; exogenous – foreign-body reaction) (Nair *et al.* 1990a,b, 1993, Nair 2003).

Enterococcus faecalis has been identified frequently in cases with post-treatment disease (Siren *et al.* 1997, Molander *et al.* 1998, Sundqvist *et al.* 1998, Portenier *et al.* 2003). Siren *et al.* (1997) investigated

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the relationship between clinical treatment procedures and the occurrence of facultative enteric bacteria in root canal infections. *E. faecalis* was the common finding in the enteric bacteria group. Sundqvist *et al.* (1998) observed that the microbial flora of teeth with persistent apical periodontitis was mainly simple species of predominantly Gram-positive organisms. *E. faecalis* (38%) was the species most commonly recovered, and the overall healing rate of re-treatment was 74%. Portenier *et al.* (2003) investigated *E. faecalis* survival in root filled teeth with apical periodontitis. *Enterococcus faecalis* was the dominant microorganism in root filled teeth presenting post-treatment apical periodontitis and was often isolated from the root canal in pure culture. However, it was also found together with other bacteria and yeasts. In mixed infections, *E. faecalis* typically is the dominant isolate. Although the pathogenicity of *E. faecalis* in endodontic infections is well documented, this microorganism has been rarely associated with acute infections and flare-ups.

The aim of root canal preparation is to eliminate microorganisms from the root canal system using biomechanical procedures (cleaning, enlarging, shaping) combined with the use of antimicrobial therapies. Calcium hydroxide, sodium hypochlorite (NaOCl) and chlorhexidine are antimicrobial agents often used in the treatment of endodontic infection. These medicaments have distinct characteristics and the literature contains numerous reports on their antimicrobial efficacy (Byström *et al.* 1985, Safavi *et al.* 1990, Estrela *et al.* 1999, Haapasalo *et al.* 2000, Gomes *et al.* 2001, Holland *et al.* 2003, Estrela *et al.* 2004, Kvist *et al.* 2004, Nair *et al.* 2005).

A primary root canal infection is associated with an endodontic microbiota generally composed of Gram-negative anaerobic bacteria. In root filled teeth, the microorganisms can persist and maintain the apical periodontitis (Sundqvist *et al.* 1998). On the other hand, the microbiota in cases with post-treatment disease is largely composed of Gram-positive organisms, particularly *E. faecalis* (Sundqvist *et al.* 1998, Sundqvist & Fidgor 2003).

Ideally, an intracanal medicament should be able to neutralize the virulence of microorganisms and pathogenic factors (such as proteins, enzymes, toxins, aggregation substances) and induce a host response that favours periapical tissue healing. However, the continuous presence of positive microbial cultures after root canal shaping, disinfection and use of calcium hydroxide as interappointment intracanal dressing justifies the investigation of other antimicrobial substances.

The use of ozonated water for treatment of endodontic infections has been suggested (Nagayoshi *et al.* 2004, Hems *et al.* 2005). Ozone has also been used in the water industry to eliminate bacteria (Lezcano *et al.* 1999, 2001) and its properties could be useful in dentistry (Baysan & Lynch 2005). Ozone is a blue gas, containing three oxygen atoms, it is irritant, toxic and unstable; it is also very reactive. Studies have reported interesting results when ozone-treated water was used in the dental unit (Lezcano *et al.* 1999, 2001, Filippi 2002, Murakami *et al.* 2002, Baysan & Lynch 2005).

Nagayoshi *et al.* (2004) observed that ozonated water had nearly the same antimicrobial activity as 2.5% NaOCl during irrigation, especially when combined with ultrasonication; they also reported a low level of toxicity against cultured cells. However, Hems *et al.* (2005), evaluating the ability of ozone to kill an *E. faecalis* strain verified that its antibacterial efficacy was not comparable to that of NaOCl.

Modern concepts of microbial control have been directed towards the use of intracanal medicaments that act against different types of respiratory bacteria (aerobic, anaerobic and microaerophiles), have the ability to affect cell wall synthesis or alter the cytoplasmic membrane permeability and interfere with protein synthesis or chromosomal replication (Estrela & Holland 2003). In primary endodontic infections there is a predominance of Gram-negative anaerobic bacteria and it is conceivable that oxygen toxicity could be able to inactive all anaerobic bacteria. However, it is not known whether oxygen behaves in the same way in cases of secondary infection, in which facultative Gram-positive bacteria are found predominantly.

Several questions about the effect of ozone on endodontic microbiota remain unclear, for example, the ideal ozone concentration, its depth of action in dentinal tubules, and the ideal time to reach full antimicrobial efficacy.

The purpose of this study was to determine the antimicrobial efficacy of ozone water, gaseous ozone, 2.5% NaOCl and 2% chlorhexidine in human root canals infected by *E. faecalis*.

Materials and methods

Test organisms

A reference strain of Gram-positive facultative anaerobic coccus (*E. faecalis*; ATCC 29212) obtained from the

American Type Culture Collection was used. The bacterial strain was inoculated in 7 mL of brain heart infusion (BHI; Difco Laboratories, Detroit, MI, USA) and incubated at 37 °C 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 give a final concentration of about 3×10^8 cells mL^{-1}, adjusted to No. 1 MacFarland turbidity standard.

Tooth preparation

Thirty extracted human maxillary anterior teeth with intact cementum obtained from the tooth bank of the Brazilian Dentistry Research and Learning Center (CEPOBRAS) were selected for this study. The teeth were removed from storage in 0.2% thymol solution and were immersed in 5% sodium hypochlorite (Fitofarma, Lt. 20442, Goiânia, GO, Brazil) for 30 min to remove organic tissues.

After initial radiographs were taken, standard access cavities were prepared and the cervical third of the canals was enlarged with sizes 3 and 4 Gates-Glidden drills (Dentsply Maillefer, Ballaigues, Switzerland). The teeth were prepared up to a size 50 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 1% NaOCl at each change of file.

Thereafter, the crowns were removed and tooth length was standardized to 16 mm (from root apex to coronal border). Under continuous water/air spray, the apical 1 mm of each root was removed at 90° to the long axis of the tooth, with a fissure bur in a high-speed handpiece. Root canals were dried and filled with 17% EDTA (pH 7.2) for 3 min for smear layer removal. After cleaning and shaping of the root canal system, the teeth were autoclaved for 30 min at 120 °C.

Experimental design

In the experimental model, a split platform was used during the period of inoculation with the biological marker (Fig. 1a). The coronal portion of the root canal of each tooth 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 layers of nail polish (Max Factor, Cosmetics and Fragrances, London, UK). The specimens (teeth coupled to the polypropylene tubes) were sterilized in 5% NaOCl 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 37 °C for 24 h. No growth was observed after this period.

The teeth were randomly assigned to four experimental and two control groups ($n = 5$), according to the tested irrigants, as follows. Group 1: ozonated water; group 2: gaseous ozone; group 3: 2.5% NaOCl (Fitofarma); group 4: 2% chlorhexidine digluconate (Fitofarma); group 5: positive control; group 6: negative control.

For groups 1 and 2, ozone was produced by electric discharge through oxygen current (PXZ3507; Eaglesat Tecnologia em Sistemas Ltda., São José dos Campos, SP, Brazil) and bubbled into 1 L sterile distilled water at 7 g h^{-1} ozone flow rate (1.2%). For group 2, only the gaseous ozone was used, under the same conditions. The negative control was used to test sterility and the positive control was used to check bacterial viability throughout the experiment. Therefore, during the 60-day period of contamination of the root canals, five noninoculated teeth were kept incubated at 37 °C, as an aseptic control, and five teeth were inoculated with *E. faecalis*, incubated and analysed under identical conditions.

Five millilitres of sterile BHI were mixed with 5 mL of the bacterial inoculum, and the experimental groups were inoculated with *E. faecalis* for 60 days, using sterilized syringes of sufficient volume to fill the root canal. This procedure was repeated every 72 h, always using 24-h pure cultures prepared and adjusted to No. 1 MacFarland turbidity standard. The teeth were maintained in a humid environment at 37 °C.

To evaluate the antimicrobial efficacy of the irrigating 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). 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 (Fig. 1b–d). 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

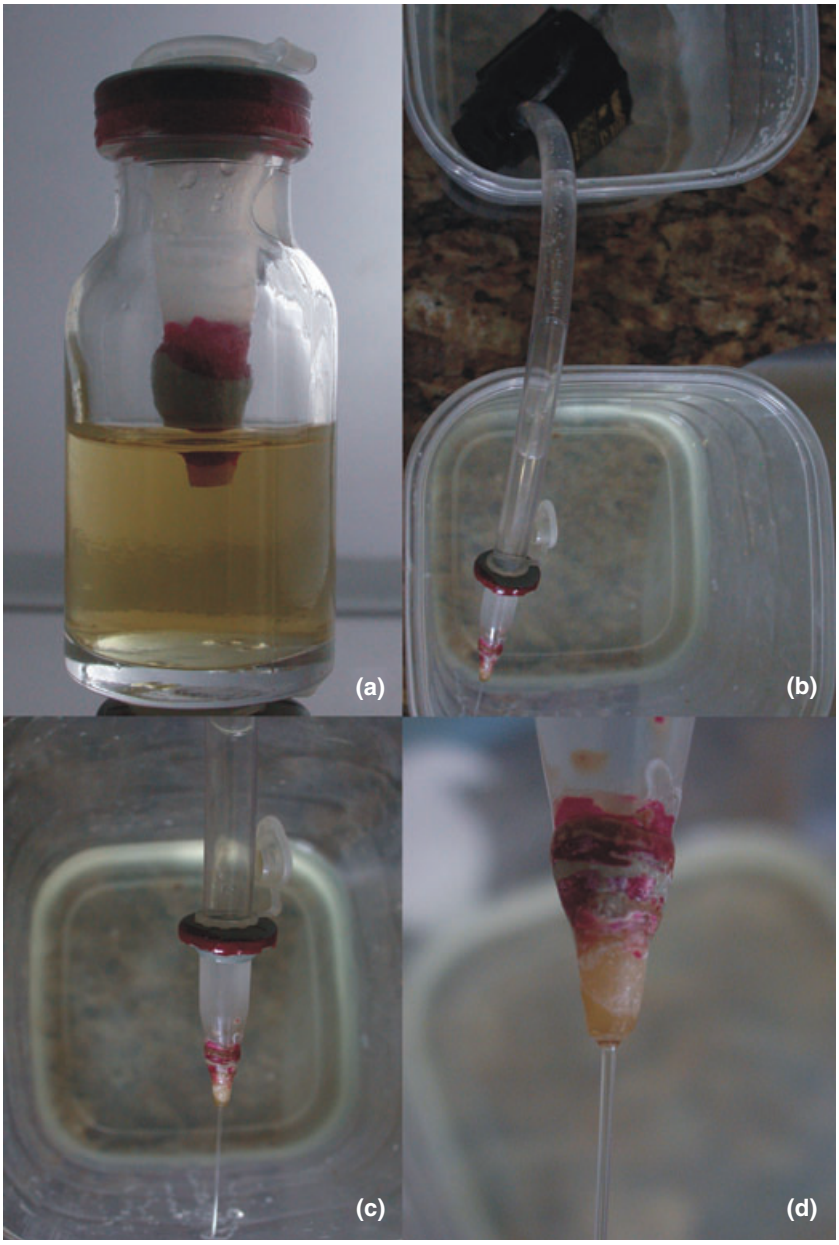

Figure 1 Overall view of the study model used to assess the antimicrobial efficacy of the irrigants in infected human root canals. (a) Platform used for inoculation with the biological indicator during 60 days; (b) irrigating system with peristaltic pump; (c) irrigants circulating at a constant flow of 50 mL min^{-1}; (d) closer view of the previous image.

irrigation with 5 mL of sterile distilled water with sterile syringe was undertaken. The root canals were dried and refilled with sterile distilled water. Thereafter, sterile paper points (Tanari, Tanariman Indústria, Ltda., Manacaru, AM, Brazil; size 45) were introduced into the canals and maintained for 3 min for sample collection. Each sample was collected by using three paper points. The points were individually transported and immersed in 7 mL of Letheen Broth (LB; Difco Laboratories), a medium containing or added with neutralizers [Lecithin, Tween 80 and sodium thiosulfate (P.A., Art Laboratories, Campinas, SP, Brazil)] in appropriate concentrations, followed by incubation at 37 °C for 48 h in a reduced oxygen atmosphere. After

72 h of the first collection, a new collection was done as described above.

Microbial growth was analysed by turbidity of the culture medium. Thus, after assessing LB changes, an inoculum of 0.1 mL obtained from the medium was transferred to 7 mL of BHI, and subsequently incubated at 37 °C for 48 h. Microbial growth was also checked by turbidity of the culture medium and, in some cases, by Gram staining. All collections were carried out in triplicate under aseptic conditions.

Results

The antimicrobial efficacy of the solutions as irrigants is shown in Table 1.

In both sample collection intervals (immediately and after 72 h), ozonated water, gaseous ozone, 2.5% NaOCl and 2% chlorhexidine used as irrigants had no antimicrobial effect against *E. faecalis* over a 20-min contact time in infected root canals. Bacteria were viable in the positive control group, whilst the negative control group was free of microorganisms under the experimental conditions.

Discussion

The positive culture of microorganisms following the application of the irrigating solutions (ozonated water, gaseous ozone, 2.5% NaOCl, 2% chlorhexidine) that circulated at a constant 50 mL min^{-1} flow for 20 min confirmed their inability to sterilize an infected human root canal.

Several studies have investigated the antimicrobial efficacy of intracanal medicaments using different experimental models (Byström *et al.* 1985, Haapasalo & Ørstavik 1987, Safavi *et al.* 1990, Ørstavik & Haapasalo 1990, Sundqvist *et al.* 1998, Estrela *et al.* 1999, 2004, Holland *et al.* 2003, Nair *et al.* 2005). Some authors evaluated intracanal medicaments in infected human teeth *in vivo* (Byström *et al.* 1985, Nair *et al.* 2005), infected dog's teeth *in vivo* (Holland *et al.* 2003, Estrela *et al.* 2004), infected human teeth *ex vivo* (Akpata & Blechman 1982, Perez *et al.* 1993, Estrela *et al.* 1999, Buck *et al.* 2001, Love 2001, Peters *et al.* 2001, Vivacqua-Gomes *et al.* 2005) and infected bovine teeth *ex vivo* (Ørstavik & Haapasalo 1990, Peters *et al.* 2000, Gomes *et al.* 2003, Saleh *et al.* 2004). In these studies, intracanal medicaments were investigated on microbial samples obtained from natural human root canal infections and from culture collection to yield artificial infection of human or bovine root canal. Unfortunately, the differences in methodology amongst these methods is likely to lead to results that cannot be compared and also that extrapolation of the results to clinical conditions must be done with caution.

Table 1 Antimicrobial efficacy of medications in human root canals infected by *E. faecalis*

Medications	20 min	72 h
Ozonated water		
Sample 1	+ + +	+ + +
Sample 2	+ + +	+ + +
Sample 3	+ + +	+ + +
Sample 4	+ + +	+ + +
Sample 5	+ + +	+ + +
Gaseous ozone		
Sample 1	+ + +	+ + +
Sample 2	+ + +	+ + +
Sample 3	+ + +	+ + +
Sample 4	+ + +	+ + +
Sample 5	+ + +	+ + +
2.5% Sodium hypochlorite		
Sample 1	+ + +	+ + +
Sample 2	+ + +	+ + +
Sample 3	+ + +	+ + +
Sample 4	+ + +	+ + +
Sample 5	+ + +	+ + +
2% Chlorhexidine digluconate		
Sample 1	+ + +	+ + +
Sample 2	+ + +	+ + +
Sample 3	+ + +	+ + +
Sample 4	+ + +	+ + +
Sample 5	+ + +	+ + +
Positive control		
Sample 1	+ + +	+ + +
Sample 2	+ + +	+ + +
Sample 3	+ + +	+ + +
Sample 4	+ + +	+ + +
Sample 5	+ + +	+ + +
Negative control		
Sample 1	− − −	− − −
Sample 2	− − −	− − −
Sample 3	− − −	− − −
Sample 4	− − −	− − −
Sample 5	− − −	− − −

Result of each repeat experiment: (+++) Positive result = growth presence/inefficacy; (−−−) Negative result = growth absence/efficacy.

Spratt *et al.* (2001) studied the bactericidal effect of 2.25% NaOCl, 0.2% chlorhexidine, 10% iodine or phosphate-buffered saline on single species of biofilms (*Prevotella intermedia*, *Peptostreptococcus micros*, *Streptococcus intermedius*, *Fusobacterium nucleatum* and *Enterococcus faecalis*) derived from a range of root canal isolates. They concluded that the efficacy of a particular agent was dependent on the nature of the organism in the biofilm and on the contact time. NaOCl was

generally the most effective agent tested followed by iodine. However, the clinical effectiveness of these agents must be regarded in the light of the complexity of root canal anatomy and polymicrobial nature of root canal infections. Abdullah *et al.* (2005) evaluated and compared the efficacy of 3% NaOCl, 10% povidone iodine, 0.2% chlorhexidine, 17% EDTA and calcium hydroxide on a clinical isolate of *E. faecalis* grown as biofilm or planktonic suspension phenotype. The difference in gradients of bacterial killing amongst the biofilm, planktonic suspension or pellet presentation was significant and dependent upon the agent, except for NaOCl and calcium hydroxide, in which no difference could be detected. NaOCl was the most effective agent and achieved 100% kill for all presentations of *E. faecalis* after a 2-min contact time.

Although the present study analysed only human root canal infection *ex vivo* from pure culture collection, it appears that a 60-day period was enough for *E. faecalis* to invade root dentinal tubules and show resistance to ozonated water, ozone, 2.5% NaOCl, 2% chlorhexidine. Different findings have been reported, depending on the incubation time, model of study, nutrient supply, root third and bacterial invasion of root dentinal tubules (Olgart *et al.* 1974, Akpata & Blechman 1982, Haapasalo & Ørstavik 1987, Safavi *et al.* 1990, Ørstavik & Haapasalo 1990, Perez *et al.* 1993, Peters *et al.* 1995, 2000, 2001, Gomes *et al.* 2003, Abdullah *et al.* 2005).

Enterococcus faecalis has been the focus of attention as a recognized pathogen, isolated both in mixed microbiota and in monocultures. Several virulence factors (aggregation substance, enterococcal surface proteins (Esp), gelatinase, cytolysin toxin, extracellular superoxide production, capsular polysaccharides, antibiotic resistance determinant) can facilitate the adherence to host cells and extracellular matrix, tissue invasions, immunomodulation effect and cause toxin-mediated damage (Portenier *et al.* 2003). Love (2001) investigated a possible mechanism that would explain how *E. faecalis* could survive and grow within dentinal tubules and reinfect filled canals. The author postulated that a virulence factor of *E. faecalis* in root filled teeth with post-treatment disease may be related to the fact that *E. faecalis* cells maintain the capability to invade dentinal tubules and adhere to collagen in the presence of human serum. Evans *et al.* (2002) reported the mechanisms involved in *E. faecalis* resistance to calcium hydroxide. *Enterococcus faecalis* was resistant to calcium hydroxide at pH 11.1, but not at pH 11.5. Pretreatment with calcium hydroxide (pH 10.3) induced no tolerance to further exposure at pH 11.5. Survival of *E. faecalis* in calcium hydroxide seemed to be unrelated to stress-induced protein synthesis, but a functioning proton pump was critical for *E. faecalis* survival at high pH.

Based on these factors, it is inferred that when a medicament does not reach the target microorganism, its killing potential cannot be realized. Therefore, it cannot be stated whether the microbial strains were resistant to one or other medication. In this case, it is likely that the microorganisms were able to survive, adapt and tolerate the critical ecological conditions.

NaOCl and chlorhexidine are antimicrobial agents frequently used in the treatment of endodontic and periodontal infections. Studies have shown that the magnitude of the antimicrobial efficacy of a medicament can be influenced by the methodology, microbial characteristics in the biofilm, exposure time and concentration of the substance tested (Spratt *et al.* 2001, Estrela *et al.* 2003). The antimicrobial effect of NaOCl by direct contact on *E. faecalis* occurred after 2 min (Estrela *et al.* 2003, Abdullah *et al.* 2005).

The findings of this investigation are consistent with those of previous studies that showed bacterial persistence after use of potent irrigants in endodontic infections (Byström *et al.* 1985, Sjögren *et al.* 1991, Estrela *et al.* 2004, Nair *et al.* 2005). Therefore, it is important to take into consideration that root canal infection is not a random event, as observed by Sundqvist & Fidgor (2003) in a recent study about the survival of endodontic pathogens. The type and combination of microbial microbiota are developed in response to the surrounding environment. Factors that influence whether species shall die or survive include the particular ecological niche, nutrition, anaerobiosis, pH and competition with other microorganisms.

The use of ozone is justified as a new option of irrigating agent with antimicrobial action. The antimicrobial effect of ozone results from oxidation of microbial cellular components. Ozone is a highly reactive form of oxygen that is generated by passing oxygen through high-voltage (Tortora *et al.* 1998). Oxidation is the removal of electrons from an atom or molecule, a reaction that often produces energy. Several biological oxidations involve the loss of hydrogen atoms (dehydrogenation reactions). Oxygen is essential for the survival of cells that follow aerobic metabolism, although it has a dramatically toxic effect on microaerophiles and anaerobic bacteria. Aerobic respiration involves ATP generation at specific sites in the electron transport chain via oxidative phosporyla-

tion (the final electron acceptors include oxygen). Although aerobic bacteria contain a variety of enzymes that protect them from oxygen toxicity, microaerophilic and anaerobic bacteria are devoid of these protective mechanisms. The final electron acceptors in anaerobic respiration include inorganic substrate – sulphate ions, nitrate ions and carbonate ions. Oxygen is not used in fermentation process; anaerobic bacteria use organic compounds as final oxygen acceptor during its energetic metabolism (Maiden *et al.* 1992, Tortora *et al.* 1998).

Nagayoshi *et al.* (2004) examined *ex vivo* the effect of ozonated water against *E. faecalis* and *S. mutans* infections in bovine dentine and compared the cytotoxicity against L-929 mouse fibroblasts between ozonated water and NaOCl. In conclusion, ozonated water had nearly the same antimicrobial activity as 2.5% NaOCl during irrigation, especially when combined with sonication (flow rate: 30 mL min^{-1}), and showed a low toxicity level against cultured cells. In this experiment, root canals were irrigated by flushing for 10 min (flow rate, 30 mL min^{-1}) with the following solutions: 4 mg L^{-1} of ozonated water (O3aq), 4 mg L^{-1} of O3aq plus ultrasonication, distilled water (DW) and DW with plus ultrasonication. One specimen was not irrigated and acted as a positive control, whilst another specimen was flushed with 2.5% NaOCl for 2 min (flow rate, 30 mL min^{-1}) and acted as a negative control.

The present study used human root canal dentine and the irrigants were allowed to circulate at a constant flow of 50 mL min^{-1} for 20 min. Ozone was produced and bubbled into 1 L sterilized distilled water at a high flow rate (7 g h^{-1} ozone – 1.2%). Within the conditions of concentration (2.5% NaOCl, 2% chlorhexidine, ozonated water – 7 g h^{-1} ozone, and gaseous ozone) and contact time (20 min) set for this investigation, the irrigants tested did not show antimicrobial efficacy.

These outcomes differ from those of a recent study (Nagayoshi *et al.* 2004), which may possibly be ascribed to differences in the methodologies. On the other hand, the irrigant concentration, the contact time and the study model notwithstanding, the results of the present investigation are in accordance with those of previous studies (Buck *et al.* 2001, Hems *et al.* 2005). Hems *et al.* (2005) evaluated ozone potential as an antibacterial agent using *E. faecalis* as the target species. Ozone was produced by a custom-made bench top generator and its solubility in water was determined by ultraviolet (258 nm) spectrophotometric analysis of solutions through which ozone was sparged for different periods. Ozone demand-free water was used as a reference. Ozone optimal concentration was 0.68 mg L^{-1} and it was produced after sparging for 30 s. The results showed that biofilms incubated for 240 s with ozonated water showed no significant reduction in cell viability attributable to ozone alone, whereas no viable cells were detected with NaOCl over the same time. Gaseous ozone applied for 300 s had no effect on these biofilms. Ozone had an antibacterial effect on planktonic *E. faecalis* cells and those suspended in fluid, but little effect was observed when the microorganism was embedded in biofilms. Its antibacterial efficacy was not comparable to that of NaOCl under the tested conditions.

Therefore, root canal preparation with careful disinfection and use of an intracanal medicament that has good antimicrobial efficacy, tissue dissolution capacity, and acceptable biocompatibility, will definitely improve the prognosis of treatment of apical periodontitis.

Further research is essential to offer new guidelines for the treatment protocol of endodontic infections. It should be kept in mind that the major factor contributing to the healing or maintenance of an infection involves the host immunological response. Studies should be carried out to investigate the applicability of ozone in different clinical situations.

Conclusions

Under the tested conditions and within the limitations of this study, it may be concluded that irrigation of infected human root canals with ozonated water, 2.5% sodium hypochlorite, 2% chlorhexidine and the application of gaseous ozone for 20 min was not sufficient to inactivate *E. faecalis*.

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References

Abdullah M, Ng Y-L, Gulabivala K, Moles D, Spratt DA (2005) Susceptibilities of two *Enterococcus faecalis* phenotypes to root canal medications. *Journal of Endodontics* **31**, 30–6.

Akpata ES, Blechman H (1982) Bacterial invasion on pulpal dentin wall in vitro. *Journal of Dental Research* **61**, 435–8.

Baysan A, Lynch E (2005) The use of ozone in dentistry and medicine. *Primary Dental Care* **12**, 47–52.

Buck RA, Eleazer PD, Staat RH, Scheetz JP (2001) Effectiveness of three endodontic irrigants at various tubular depths in human dentin. *Journal of Endodontics* **27**, 206–8.

Byström A, Claesson R, Sundqvist G (1985) The antibacterial effect of camphorated paramonochlorophenol, camphorated phenol and calcium hydroxide in the treatment of infected root canals. *Endodontics Dental Traumatology* **1**, 170–5.

Estrela C, Holland R (2003) Calcium hydroxide: study based on scientific evidences. *Journal of Applied Oral Science* **11**, 269–82.

Estrela C, Pimenta FC, Ito IY, Bammann LL (1999) Antimicrobial evaluation of calcium hydroxide in infected dentinal tubules. *Journal of Endodontics* **26**, 416–8.

Estrela C, Ribeiro RG, Estrela CRA, Pécora JD, Souza-Neto MD (2003) Antimicrobial effect of 2% sodium hypochlorite and 2% chlorhexidine tested by different methods. *Brazilian Dental Journal* **14**, 58–62.

Estrela C, Holland R, Bernabé PFE, Souza V, Estrela CRA (2004) Antimicrobial potential of medicaments used in healing process in dog's teeth with apical periodontitis. *Brazilian Dental Journal* **15**, 181–5.

Evans M, Davies JK, Sundqvist G, Figdor D (2002) Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. *International Endodontic Journal* **35**, 221–8.

Filippi A (2002) Water disinfection of dental units using ozone – microbiological results after 11 years and technical problems. *Ozone Science & Engineering* **24**, 479–83.

Gomes BPFA, Ferraz CCR, Vianna ME, Berber VB, Teixeira FB, Souza-Filho FJ (2001) In vitro antimicrobial activity of several concentrations of sodium hypochlorite and chlorhexidine gluconate in the elimination of *Enterococcus faecalis*. *International Endodontic Journal* **34**, 424–8.

Gomes BPFA, Souza SFC, Ferraz CCR et al. (2003) Effectiveness of 2% chlorhexidine gel and calcium hydroxide against *Enterococcus faecalis* in bovine root dentine in vitro. *International Endodontic Journal* **36**, 267–75.

Haapasalo MPP, Ørstavik D (1987) In vitro infection and disinfection of dentinal tubules. *Journal of Dental Research* **66**, 1375–9.

Haapasalo HK, Sirén EK, Waltimo TMT, Ørstavik D, Haapasalo MPP (2000) Inactivation of local root canal medicaments by dentine: an in vitro study. *International Endodontic Journal* **33**, 126–31.

Hems RS, Gulabivala K, Ng Y-L, Ready D, Spratt DA (2005) An in vitro evaluation of the ability of ozone to kill a strain of *Enterococcus faecalis*. *International Endodontic Journal* **38**, 22–9.

Holland R, Otoboni-Filho JA, Souza V, Nery MJ, Bernabé PFE, Dezan E Jr (2003) A comparison of one versus two appointment endodontic therapy in dogs' teeth with apical periodontitis. *Journal of Endodontics* **29**, 121–5.

Kvist T, Molander A, Dahlén G, Reit C (2004) Microbiological evaluation of one- and two-visit endodontic treatment of teeth with apical periodontitis: a randomized, clinical trial. *Journal of Endodontics* **30**, 572–6.

Lezcano I, Rey RP, Gutiérrez MS, Baluja C, Sánchez E (1999) Ozone inactivation of *Pseudomonas aeruginosa*, *Escherichia coli*, *Shigella sonnei* and *Salmonella typhimurium* in water. *Ozone Science & Engineering* **21**, 293–300.

Lezcano I, Rey RP, Gutiérrez MS, Baluja C, Sánchez E (2001) Ozone inactivation of microorganisms in water. Gram positive Bacteria and yeast. *Ozone Science & Engineering* **23**, 183–7.

Love RM (2001) *Enterococcus faecalis* – a mechanism for its role in endodontic failure. *International Endodontic Journal* **34**, 399–405.

Maiden MFJ, Lai CH, Tanner A (1992) Characteristics of oral Gram-positive bacteria. In: Slots J, Taubman MA, eds. *Contemporary Oral Microbiology and Immunology*. St Louis, MO: Mosby, pp. 342–72.

Molander A, Reit C, Dahlen G, Kvist T (1998) Microbiological status of root-filled teeth with apical periodontitis. *International Endodontic Journal* **31**, 1–7.

Murakami H, Mizuguchi M, Hattori M, Ito Y, Kawai T, Hasegawa J (2002) Effect of denture cleaner using ozone against methicillin-resistant *Staphylococcus aureus* and *E. coli* T1 phage. *Dental Materials* **21**, 53–60.

Nagayoshi M, Kitamura C, Fukuizumi T, Nishihara T, Terashita M (2004) Antimicrobial effect of ozonated water on bacteria invading dentinal tubules. *Journal of Endodontics* **30**, 778–81.

Nair PNR (2003) Non-microbial etiology: foreign body reaction maintaining post-treatment apical periodontitis. *Endodontic Topics* **6**, 96–113.

Nair PNR, Sjögren U, Krey G, Kahnberg KE, Sundqvist G (1990a) Intraradicular bacteria and fungi in rootfilled, asymptomatic human teeth with therapy-resistant periapical lesions: a long-term light and electron microscopic follow-up study. *Journal of Endodontics* **16**, 580–8.

Nair PNR, Sjögren U, Krey G, Sundqvist G (1990b) Therapy-resistant foreign body giant cell granuloma at the periapex of a root-filled human tooth. *Journal of Endodontics* **16**, 589–95.

Nair PNR, Sjögren U, Schumacher E, Sundqvist G (1993) Radicular cyst affecting a root-filled human tooth: a long-term post-treatment follow-up. *International Endodontic Journal* **26**, 225–33.

Nair PNR, Henry S, Cano V, Vera J (2005) Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after 'one-visit' endodontic treatment. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **99**, 231–52.

Olgart L, Brannstron M, Johnson G (1974) Invasion of bacteria into dentinal tubules. *Acta Odontologica Scandinavica* **32**, 61–70.

Ørstavik D, Haapasalo MPP (1990) Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. *Endodontics Dental Traumatology* **6**, 142–9.

Perez F, Rochdt T, Lodter JP, Calas P, Michel G (1993) In vitro study of the penetration of three bacterial strains into root dentine. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **76**, 97–103.

Peters LB, Wesselink PR, Moorer WR (1995) The fate and role of bacteria left in root dentinal tubules. *International Endodontic Journal* **28**, 95–9.

Peters LB, Wesselink PR, Moorer WR (2000) Penetration of bacteria in bovine root dentine in vitro. *International Endodontic Journal* **33**, 28–36.

Peters LB, Wesselink PR, Buijs JF, van Winkelhoff AJ (2001) Viable bacteria in root dentinal tubules of teeth with apical periodontitis. *Journal of Endodontics* **27**, 76–81.

Portenier I, Waltimo TMT, Haapasalo M (2003) *Enterococcus faecalis* – the root canal survivor and 'star' in post-treatment disease. *Endodontic Topics* **6**, 135–59.

Safavi KE, Spångberg LSW, Langeland K (1990) Root canal dentinal tubule desinfection. *Journal of Endodontics* **16**, 207–10.

Saleh IM, Ryuter IE, Haapasalo MPP, Ørstavik D (2004) Survival of *Enterococcus faecalis* in infected dentinal tubules after root canal filling with different root canal sealer in vitro. *International Endodontic Journal* **37**, 193–8.

Siren EK, Haapasalo PP, Ranta K, Salmi P, Kerosuo ENJ (1997) Microbiological findings and clinical treatment procedures in endodontic cases selected for microbiological investigation. *International Endodontic Journal* **30**, 91–5.

Sjögren V, Fidgor D, Spångberg L, Sundqvist G (1991) The antimicrobial effect of calcium hydroxide as a short-term intracanal dressing. *International Endodontic Journal* **24**, 119–25.

Spratt DA, Pratten J, Wilson M, Gulabivala K (2001) An in vitro evaluation of the antimicrobial efficacy of irrigants on biofilms of root canal isolates. *International Endodontic Journal* **34**, 300–7.

Sundqvist G, Fidgor D (2003) Life as an endodontic pathogen. Ecological differences between the untreated and the root-filled root canals. *Endodontic Topics* **6**, 3–28.

Sundqvist G, Figdor D, Persson S, Sjögren U (1998) Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative retreatment. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **85**, 86–93.

Tortora GJ, Funke BR, Case CL (1998) *Microbiology – An Introduction*, 6th edn. Menlo Park, CA: Benjamin/Cummings Publ. Comp.

Vivacqua-Gomes N, Gurgel-Filho ED, Gomes BPFA, Ferraz CCR, Zaia AA, Souza-Filho FJ (2005) Recovery of *Enterococcus faecalis* after single- or multiple visit root canal treatments carried out in infected teeth ex vivo. *International Endodontic Journal* **38**, 697–704.