An *in vitro* evaluation of the ability of ozone to kill a strain of *Enterococcus faecalis*

R. S. Hems, K. Gulabivala, Y.-L. Ng, D. Ready & D. A. Spratt
Unit of Endodontology, Eastman Dental Institute for Oral Health Care Sciences, University College London, London, UK

Abstract

Hems RS, Gulabivala K, Ng Y-L, Ready D, Spratt DA. An *in vitro* evaluation of the ability of ozone to kill a strain of *Enterococcus faecalis*. *International Endodontic Journal*, **38**, 22–29, 2005.

Aim To evaluate the potential of ozone as an antibacterial agent using *Enterococcus faecalis* as the test species.

Methodology Ozone was produced by a custom-made bench top generator and its solubility in water determined by ultraviolet (258 nm) spectrophotometric analysis of solutions through which ozone was sparged for various time-periods. The antibacterial efficacy of ozone was tested against both broth and biofilm cultures. Ozone was sparged for 30, 60, 120 and 240 s, through overnight broth cultures of a strain of *E. faecalis* (E78.2) and compared with those that were centrifuged, washed and resuspended in water. *Enterococcus faecalis* (E78.2) biofilms were grown on cellulose nitrate membrane filters for 48 h and suspended in water through which ozone gas was sparged with stirring for 60, 120 and 240 s in a standard fashion. In a separate test, biofilms were also exposed to gaseous ozone. Sodium hypochlorite (NaOCl) was used as a positive control. All experiments were repeated four times.

Results There were significant ($P < 0.05$) reductions of bacteria in the unwashed (2 $\log_{10}$ reductions) and washed (5 $\log_{10}$ reductions) broth cultures following 240 s applications. Biofilms incubated for 240 s with ozonated water showed no significant reduction in cell viability attributable to ozone alone, whereas with NaOCl no viable cells were detected over the same time. Gaseous ozone applied for 300 s had no effect on these biofilms.

Conclusions Ozone had an antibacterial effect on planktonic *E. faecalis* cells and those suspended in fluid, but little effect when embedded in biofilms. Its antibacterial efficacy was not comparable with that of NaOCl under the test conditions used.

Keywords: antibacterial, *Enterococcus faecalis*, ozone.

Received 13 May 2004; accepted 13 September 2004

Introduction

The treatment of apical periodontitis involves elimination of root canal infection by a combination of mechanical and chemical means. Mechanical instrumentation alone may only reduce the numbers of bacteria from the root canal system by 50% (Byström & Sundqvist 1981). This is at least partly because even in maxillary anterior teeth, only a proportion of the root canal surface is planed by the action of files (Mannan *et al.* 2001). As a result, antibacterial irrigants have to be relied upon to penetrate to the noninstrumented surfaces (Byström & Sundqvist 1983).

Sodium hypochlorite (NaOCl) is the current irrigant of choice due to its antibacterial (Byström & Sundqvist 1983, 1985) and tissue-dissolving effects (Moorer & Wesselink 1982). Although NaOCl is a potent antibacterial agent, it is toxic at high concentrations (Spångberg *et al.* 1973). It also weakens dentine by reducing its flexural strength and resilience, therefore rendering the tooth more susceptible to deformation (Grigoratos *et al.* 2001, Sim *et al.* 2001) and possibly

Correspondence: K. Gulabivala, Unit of Endodontology, Eastman Dental Institute for Oral Health Care Sciences, University College London, 256 Gray's Inn Road, London WC1X 8LD, UK (Tel.: 020 7915 1033; fax: 020 7915 1273; e-mail: k.gulabivala@eastman.ucl.ac.uk).

fractures. Furthermore, the principles of current root canal treatment protocols, which have existed for almost a century, have a failure rate of 16% (Lewsey *et al.* 2001). There is therefore a need to develop new and more effective treatment strategies.

A number of different approaches to eliminating infection from root canal systems have been proposed including; the noninstrumentation technique (Lussi *et al.* 1995), laser technology (Kimura *et al.* 2000, Seal *et al.* 2002), irrigation with electrochemically activated water (Marais & Williams 2001, Gulabivala *et al.* 2004) and application of ozone (Deltour *et al.* 1970, Chahverdiani & Thadj-Bakhche 1976).

Ozone (O_3) is a powerful oxidizing agent (Broadwater *et al.* 1973) and has been used in the water industry for many years to kill bacteria. It has also been tested in medicine to decontaminate hospital side rooms (Dyas *et al.* 1983), rooms contaminated with methicillin-resistant *Staphylococcus aureus* (Berrington & Pedlar 1998) and in auto-haemotherapy (Bocci 1992). In the dental field ozone has been advocated for treatment of gum infections, during surgery, for failed implant cases (Sandhaus 1969), root caries (Baysan *et al.* 2000), root canal treatment (Deltour *et al.* 1970, Chahverdiani & Thadj-Bakhche 1976) and for its potential application in reducing bacterial counts in dental unit water delivery systems (Filippi 1997).

The aim of this study was to investigate the antibacterial effectiveness of ozone using a single strain of *Enterococcus faecalis* as the test organism in laboratory planktonic and biofilm test models, in order to establish its potential as a root canal disinfectant.

Materials and methods

Generation and characterization of ozone

An ozone-generating device (Purezone®; Ipswich, UK) was used in this study to deliver ozone in air at the rate of 5.8 $cm^3\ s^{-1}$ (at atmospheric pressure and 21 °C) through a nozzle connected to the device. Ozonated water was produced by sparging de-ionized water (Milli-Q Water Purification System; Millipore, Bedford, MA, USA) with ozone using a closed-ended silicone tube perforated by numerous small holes and attached to the ozone delivery nozzle.

Preparation of all glassware used throughout this study was carried out by thoroughly rinsing with ozonated water, autoclaving at 121 °C for 15 min and finally drying. All experiments with ozone were carried out in a fume cupboard. Ozone demand-free water was used throughout this study and prepared according to Kim & Yousef (2000).

The maximum concentration of aqueous ozone was determined spectrophotometrically according to Bader & Hoigné (1981). Briefly, ozone was sparged through 10 mL of water ($n = 4$) in a glass container for various time-periods (5, 10, 20, 30 and 60 s), pipetted into a quartz cuvette and the UV absorbance measured (Ultra spec 2000; Pharmacia Biotech, Cambridge, UK) at 258 nm. Ozone demand-free water was used as a reference. The optimum concentration of ozone was 0.68 $mg\ L^{-1}$ and was produced after sparging for 30 s.

Preparation of cultures

A root canal isolate previously identified as *E. faecalis* strain E78.2 (by comparative 16S rRNA gene sequence analysis) was used in this study. Bacterial cells were resuspended in 10 mL of nutrient broth (Oxoid Ltd, Basingstoke, UK) in a universal glass container, and incubated for 18 h in a CO_2 incubator at 37 °C. Prior to testing, an aliquot (100 μL) was removed from all cultures to provide a baseline count. These were serially diluted in reduced transport fluid (Syed & Loesche 1972) plated onto blood agar, incubated in a CO_2 incubator at 37 °C for 24 h and enumerated.

Enterococcus faecalis broth cultures ($n = 4$) were sparged with ozone for various time-periods (30, 60, 120 and 240 s). Immediately following sparging, 1 mL of broth was transferred to 9 mL of neutralizing broth (Difco Ltd, East Moseley, UK) and vortexed for 10 s. A positive control of sodium hypochlorite was used and consisted of 200 μL of 2.5% v/v NaOCl (Thin Bleach; Tesco plc, London, UK) added to 10 mL of *E. faecalis* broth resulting in a 0.05% solution. A negative control was performed by sparging atmospheric air through *E. faecalis* broths for 240 s and at equivalent flow rates to that of the ozone generator. Serial dilution and plate counts were performed on all samples and CFU mL^{-1} calculated as previously described.

A modification of the above protocol was also developed to investigate the potential neutralizing effects of the nutrient broth. *Enterococcus faecalis* broth cultures were prepared as previously described. Cultures were centrifuged (Centura 2; Fisher Scientific UK Ltd, Loughborough, UK) at 1780 ***g*** for 20 min. The supernatant was removed and the cells resuspended in ozone demand-free water. A baseline enumeration and sparging with ozone and controls were carried out as previously described.

The effect of ozone on *E. faecalis* cells grown as a biofilm was investigated using the simple biofilm model described by Spratt *et al.* (2001). Briefly, biofilms were grown on sterile nitrocellulose discs on blood agar plates containing 5% defribrinated horse blood. Biofilms were aseptically transferred to glass universal bottles containing 10 mL of water or NaOCl (2.5% v/v). Ozone was sparged through the water containing the biofilms for 60, 120 and 240 s ($n = 4$ for each time-period). This was carried out either with stirring at 200 rpm or without. Following removal, the membrane filters were carefully transferred to neutralizing broth and vortexed for 1 min to resuspend the bacteria. This was followed by culture and enumeration. Additionally, upon removal of the biofilm an aliquot of the 'incubating' water was removed and cells enumerated after all experiments.

The effect of gaseous ozone on biofilms was investigated. The biofilm disc was suspended on a piece of wire in a conical glass container and ozone was passed around the disc at the rate of 5.83 $cm^3\ s^{-1}$ at 21 °C for 300 s. The disc was then placed in neutralizing broth and cells enumerated as previously described. A control of no addition of ozone for 300 s was also performed.

Statistical analysis of the data

The mean CFUs per unit determined for test cultures before and after treatment for each experimental group were calculated and compared using paired *t*-tests. The antimicrobial effect of ozone on *E. faecalis* biofilm was analysed using the independent *t*-tests.

Results

Antibacterial effects of ozone on planktonic *E. faecalis* cells

The viability of bacterial cells recovered before and after ozone treatment on *E. faecalis* cells suspended in broth are presented in Table 1a. Reduction in bacterial cell numbers (1–2 $\log_{10}$) was observed for all time-points but significant reduction ($P = 0.015$) could only be detected for the 240 s time-point. No reduction in bacterial cell viability was observed when air was sparged through the broth culture (data not shown).

The effect of sparging ozone through *E. faecalis* resuspended in ozone demand-free water (pH 7) is given in Table 1b. There was significant reduction (5 $\log_{10}$, $P = 0.034$) of bacterial cell viability after 240 s treatment. No reduction in cell viability was observed when air was sparged through the suspended culture (data not shown).

There were no significant differences in reduction of bacterial cell viability between broth culture and ozone demand-free water at 240 s treatment.

Antibacterial effects of NaOCl (0.05%) on planktonic *E. faecalis* cells

NaOCl (0.05%) treatment of planktonic *E. faecalis* cells in broth culture (Table 1c) resulted in a significant ($P < 0.05$) reduction of recovered bacterial cells compared with baseline controls at all sampling points and no viable cells were detected at 240 s. Additionally, no viable cells were detected after NaOCl treatment of planktonic *E. faecalis* cells resuspended in ozone demand-free water after 30 s (Table 1d).

Antibacterial effects of ozone on *E. faecalis* cells grown as biofilms

The numbers of bacterial cells recovered from the biofilms after sparging with ozone are presented in Table 2a, along with the effect of treatment on the bacteria dislodged from the biofilm during the treatment (Table 2b). There were significantly less viable bacterial cells recovered from the biofilm following stirring (1–3 $\log_{10}$ reduction, $P = 0.001$) or ozone sparging together with stirring (2–4 $\log_{10}$ reduction, $P = 0.001$) compared with the baseline controls (Table 2a). However, there was no significant difference in the numbers of viable cells recovered from biofilms that had been stirred only and those that had been stirred and treated with ozone. There was also significant reduction in the numbers of viable cells recovered from the biofilms after sparging air past the biofilms (with stirring for 240 s) compared with the baseline control (3 $\log_{10}$ reduction, $P = 0.030$) (Table 2a). In contrast, using 2.5% (v/v) NaOCl (with stirring) no viable cells were detected at any of the time-points (Table 2a).

The effect of ozone on *E. faecalis* cells which became dislodged from the biofilm following the above treatments was also assessed. There was no significant difference in numbers of viable bacterial cells dislodged from the biofilm before and after stirring action alone (Table 2b). However, when ozone was sparged through whilst stirring, a 4 $\log_{10}$ reduction ($P = 0.020$) in viable bacterial cells was achieved after 60 s and no viable cells were detected after 120 s. There was no

Table 1 Effect of ozone and NaOCl (0.05%) on *Enterococcus faecalis* cells suspended in broth culture or ozone demand-free water

Duration of treatment (s)	Mean (SD) CFU mL^{-1} Before treatment	After treatment	Mean difference (SD)	95% Confidence interval of differences	*P*-value
(a) Sparging of ozone on *E. faecalis* cells in broth culture ($n = 4$)					
30	8.8×10^{10} (1.01×10^{11})	5.25×10^{9} (1.8×10^{9})	8.28×10^{10} (9.98×10^{10})	-7.6×10^{10}–2.42×10^{11}	0.196
60	2.53×10^{10} (1.83×10^{10})	8.75×10^{9} (6.9×10^{9})	1.65×10^{10} (2.13×10^{10})	-1.74×10^{10}–5.04×10^{10}	0.220
120	1.78×10^{11} (1.35×10^{11})	1.34×10^{9} (6.6×10^{8})	1.70×10^{11} (1.41×10^{11})	-5.60×10^{10}–3.95×10^{11}	0.097
240	3.35×10^{11} (1.3×10^{10})	2.97×10^{9} (3.3×10^{9})	3.32×10^{10} (1.30×10^{10})	1.24×10^{10}–5.40×10^{10}	0.015[a]
(b) Sparging of ozone on *E. faecalis* cells resuspended in ozone demand-free water ($n = 5$)					
30	4.16×10^{9} (4.6×10^{9})	5.33×10^{9} (6.2×10^{9})	-1.17×10^{9} (8.01×10^{9})	-1.11×10^{10}–8.78×10^{9}	0.761
60	2.24×10^{10} (1.8×10^{10})	1.14×10^{7} (2.1×10^{7})	2.24×10^{10} (1.80×10^{10})	-1.86×10^{7}–4.49×10^{10}	0.050
120	2.52×10^{10} (2.0×10^{10})	5.92×10^{5} (3.5×10^{5})	2.52×10^{10} (2.03×10^{10})	-1.76×10^{7}–5.04×10^{10}	0.050
240	1.31×10^{11} (9.2×10^{10})	2.14×10^{6} (2.4×10^{6})	1.31×10^{11} (9.26×10^{10})	1.56×10^{10}–2.46×10^{11}	0.034[a]
(c) NaOCl (0.05%) treatment of *E. faecalis* cells in nutrient broth ($n = 4$)					
30	2.93×10^{11} (8.1×10^{10})	1.04×10^{10} (1.2×10^{10})	2.82×10^{11}	1.50×10^{11}–4.15×10^{11}	0.007[a]
60	2.93×10^{11} (8.1×10^{10})	2.61×10^{8} (1.5×10^{8})	2.91×10^{11}	1.62×10^{11}–4.22×10^{11}	0.006[a]
120	2.93×10^{11} (8.1×10^{10})	2.1×10^{9} (3.4×10^{9})	2.90×10^{11}	1.57×10^{11}–4.23×10^{11}	0.006[a]
240	2.93×10^{11} (8.1×10^{10})	0	–[b]	–[b]	–[b]
(d) NaOCl (0.05%) treatment of *E. faecalis* cells in ozone demand-free water ($n = 4$)					
30	7.31×10^{10} (1.37×10^{11})	0	–[b]	–[b]	–[b]
60	7.31×10^{10} (1.37×10^{11})	0	–[b]	–[b]	–[b]
120	7.31×10^{10} (1.37×10^{11})	0	–[b]	–[b]	–[b]
240	7.31×10^{10} (1.37×10^{11})	0	–[b]	–[b]	–[b]

[a]Significant at 5% level.
[b]No statistical analyses were carried out due to 100% kill.

Table 2 Effect of stirring, ozone and air sparging on *E. faecalis* biofilms and the dislodged cells suspended in ozone demand-free water

Treatment time (s)	Stirring only ($n = 4$)	Stirring and ozone sparging ($n = 4$)	Stirring and air sparging ($n = 4$)	2.5% NaOCl ($n = 4$)	Stirring and 2.5% NaOCl ($n = 4$)
(a) Mean (SD) CFU/disc of bacterial cells recovered from biofilms					
0 (Baseline count with no stirring)	4.99×10^{9} (3.5×10^{9})				
60	8.45×10^{7} (9.2×10^{7})[a]	3.95×10^{7} (1.1×10^{7})[a]	–[b]	–[b]	0
120	8.45×10^{8} (1.9×10^{8})[a]	2.65×10^{6} (2.3×10^{5})[a]	–[b]	0	0
240	3.2×10^{6} (1.04×10^{6})[a]	2.35×10^{5} (1.7×10^{5})[a]	5.2×10^{6} (5.24×10^{6})[a]	–[b]	0
(b) Mean (SD) CFU mL^{-1} of dislodged bacterial cells recovered from liquid suspending the biofilms					
0 (Baseline count with no stirring)	3.85×10^{8} (1.7×10^{8})				
60	1.48×10^{9} (1.3×10^{9})	3.95×10^{4} (3.9×10^{4})[a]	–[b]	–[b]	–[b]
120	5.3×10^{8} (2.8×10^{8})	0	–[b]	–[b]	–[b]
240	9.35×10^{9} (2.7×10^{9})	0	1.29×10^{10} (1.7×10^{10})	–[b]	–[b]

[a]Significant difference between baseline count and bacteria recovered after treatment at 5% level.
[b]Not tested.

significant difference between the numbers of viable cells recovered after sparging air past the biofilms (with stirring for 240 s) compared with the baseline control (Table 2b).

The effect of gaseous ozone on biofilms was also assessed and the data for a 300 s exposure are presented in Table 3. Gaseous ozone had no significant effect on *E. faecalis* viability in biofilms.

Discussion

Ozone is a selective oxidant and affects only certain compounds but when it dissolves in water, it becomes highly unstable and rapidly decomposes through a complex series of chain reactions (Hoigné & Bader 1976, Shin *et al.* 1999). As a result, hydroxyl ($OH^{\cdot}$) radicals are generated, which are amongst the most

Table 3 The effect of a 5-min treatment with gaseous ozone on *E. faecalis* biofilms ($n = 4$)

Mean (SD) CFU/disc				
Without ozone treatment	After ozone treatment	Mean (SD) difference	95% Confidence interval of differences	*P*-value
2.62×10^{10} (2.7×10^{10})	5.75×10^{9} (2.38×10^{9})	2.04×10^{10} (1.39×10^{10})	-1.38×10^{1}–5.46×10^{10}	0.194

reactive oxidizing species. Ozone reacts with various chemical compounds in aqueous systems in two different and coexisting modes; one involving direct reactions of molecular ozone and the other a free radical-mediated reaction (Staehelin & Hoigné 1985). Both these mechanisms may be involved in the destruction of bacteria by ozone. *Escherichia coli* cells however, have been found to be inactivated primarily by molecular ozone (Hunt & Mariñas 1997).

The absorbance of ozone in the water increased almost linearly with time, from 5 to approximately 60 s (data not shown). The stability of the ozone in the water was low and the ozone dissipated very quickly in ozone demand-free water at room temperature over 5 min, in agreement with Shechter (1973).

Enterococcus faecalis, a Gram-positive facultative anaerobe, was chosen as the test microorganism because it has significant implication in treatment resistant cases (Molander *et al.* 1998, Sundqvist *et al.* 1998), and is difficult to kill (Ørstavik & Haapasalo 1990). The hardy nature of this bacterium is such that it can grow and survive as a monoculture under diverse conditions including; in nutrition depleted root canal systems, the gastrointestinal tract and the genitourinary tract (Felmingham *et al.* 1992).

The organic matter in the nutrient broth for culturing *E. faecalis* may protect the cells by providing a large number of suitable targets for the ozone to react with, thereby shielding the bacteria from the ozone (Restaino *et al.* 1995). Therefore, ozone demand-free water was also used as the medium for testing and as a comparison.

Neutralizing broth was used prior to determination of the number of viable bacteria as it contains sodium thiosulphate that instantly reduces the dissolved ozone and inactivates it (Farooq & Akhlaque 1983).

The antibacterial nature of ozone was tested on *E. faecalis* in planktonic as well as biofilm forms. The use of planktonic cultures alone would not represent a clinically relevant test (Shih *et al.* 1970, D'Arcangelo *et al.* 1999) as bacteria also exist as biofilms on the walls of root canals (Nair 1987). Moreover, the susceptibility of bacterial phenotypes in biofilms is different to those of the planktonic phenotype (Wilson 1996).

The most realistic *in vitro* model would be to grow bacterial biofilms on the root canal surfaces of extracted teeth but the test results may be confounded by the variation in root canal anatomy. The natural variation in anatomy results in both differential growth and variable exposure to the antimicrobial agent (Shih *et al.* 1970, Siqueira *et al.* 1997), consequently resulting in a greater statistical spread of results and the need for a much larger sample size to demonstrate significant differences (Gulabivala *et al.* 2004). The use of a bacterial biofilm grown on a simple membrane eliminates the variable of root canal anatomy and reduces the variations in quantity of growth and contact of the biofilm with the antimicrobial agent. It is therefore a useful and convenient method for rapid preliminary testing of new antimicrobial agents (Spratt *et al.* 2001).

The biofilm was stirred to enhance ozone contact with its entire surface. In order to rule out the possible antibacterial effect of the physical nature of sparging alone, *E. faecalis* cells suspended in broth or ozone demand-free water were treated with air sparging as a control.

The bactericidal effects of 0.05% NaOCl on planktonic bacteria were used as the positive control and compared with the effects of ozone. Although 0.5–5% NaOCl are commonly used for chemomechanical cleaning of root canals (Byström & Sundqvist 1985), a much lower concentration (0.05%) was used in the studies on planktonic bacteria based on the inhibitory concentration reported for NaOCl when used against planktonic *E. faecalis* (Ghori *et al.* 2001). It was impossible to make direct comparisons between ozone and NaOCl because the concentration of ozone changes constantly during continuous sparging. In contrast, the higher concentration of 2.5% NaOCl was used as a positive control for the studies on *E. faecalis* biofilms based on a previous study (Spratt *et al.* 2001), although no previous data were found on biofilm inhibitory concentration.

When *E. faecalis* cells were treated with ozone over the time-periods 30–240 s using nutrient broth as the medium, there was a 1–2 $\log_{10}$ reduction of bacterial counts. A significant reduction could only be detected after 240 s of treatment. The duration of action was

therefore an important consideration in its antibacterial effect.

When using ozone demand-free water as a suspension medium for testing the antibacterial effect of ozone (in place of nutrient broth), a significant reduction of bacterial counts was again achieved at 240 s. There were no significant differences in bacterial kills when either nutrient broth or ozone demand-free water were used as the suspension medium at any of the time-points of treatment. This may be in contrast to previous studies where the presence of organic matter has been shown to deplete ozone (Broadwater *et al.* 1973, Restaino *et al.* 1995). The antimicrobial effect of NaOCl is also reduced in the presence of organic matter in the test medium (Moorer & Wesselink 1982). Therefore, NaOCl (0.05%) was less effective at killing *E. faecalis* cells in nutrient broth (no viable cells detected after 240 s) compared with ozone demand-free water (no viable cells detected after 30 s).

The results from the biofilm study show that stirring alone and stirring together with air sparging, both reduced the number of bacteria on the biofilm. This was probably because the shear generated during these processes removed the cells from the biofilm and into the surrounding medium. Stirring and ozone (together) did not increase the loss of viable *E. faecalis* cells from the biofilm compared with the controls (stirring alone, stirring with air sparging); implying that ozone had no effect on *E. faecalis* cells in the biofilm. In contrast, the *E. faecalis* cells displaced into the surrounding medium by stirring were killed by sparging with ozone (together with stirring) (Table 2). The inference appears to be that the biofilm phenotype does not have an inherent resistance to ozone. The resistance of the bacterial cells in the biofilm must be attributed to the depletion of ozone as it diffuses into the biofilm by virtue of its organic composition. The biofilm extracellular polysaccharide matrix therefore probably protects against ozone.

When 2.5% NaOCl was tested on the biofilm, no viable cells were detected after 120 s without stirring (Table 2), confirming its efficacy as a root canal irrigant against *E. faecalis*. The concentration of reactive molecules in the 2.5% NaOCl is far higher than that in the ozonated water and is therefore a biased comparison; the reactive molecules cannot approach such concentrations in ozonated water, as far as it is known. Nevertheless, a direct comparison is still valid from a clinical perspective.

Gaseous ozone had no significant antibacterial effect on the biofilms. This was predictable considering that the effectiveness of ozone is highest in solution and given the previous results with biofilms, gaseous ozone would not be expected to exert any great antibacterial effect. At the termination of the experiments, it was noted that the biofilms were desiccated yet the bacterial count was similar to the controls that did not appear desiccated. This observation reiterates the resistance of *E. faecalis* to adverse conditions.

Ozone was not significantly less effective in killing planktonic *E. faecalis* when organic material was present. However, given the resistance of bacterial cells embedded in a biofilm matrix to ozone, there would appear be a limited role for ozonated water as a root canal irrigant. The view is further supported by the need for fresh generation of the solution because of its rapid dissipation and safety considerations. The much higher effectiveness of NaOCl on both planktonic and biofilm cultures of *E. faecalis*, substantiates the view further.

When considering new techniques, safety is an important issue. Ozone has limitations as it is irritating to the respiratory system (Hazucha *et al.* 1989). Very low concentrations (0.2–0.5 ppm) may cause headache, and irritation or dryness of the nose, throat and eyes (McDonnell *et al.* 1983). Higher concentrations (1–10 ppm over a few hours) may cause lung congestion, oedema, haemorrhage, changes to the blood and loss of vital lung capacity. It is irritating to the eyes and can cause redness, pain and blurred vision. These effects are noticeable at about 0.2 ppm. Epidemiological studies (Cody *et al.* 1992) suggest a possible link between ozone pollution and allergic airway disease, however, the data is limited (Krishna *et al.* 1995).

Conclusions

Within the limitations of this study, the following conclusions may be drawn about ozone's ability to kill *E. faecalis*:

- Ozone in solution was antibacterial against planktonic *E. faecalis* after 240 s.
- It was not effective against *E. faecalis* cells in a biofilm unless they were displaced into the surrounding medium by agitation.
- The biofilm phenotype was not more resistant to killing by ozone than the planktonic phenotype.
- Gaseous ozone had no effect on the *E. faecalis* biofilm.
- NaOCl (0.05%) was more effective in planktonic tests in the absence of organic matter in the suspension medium.

• NaOCl (2.5%) was more effective on the *E. faecalis* biofilm with stirring than without.

Acknowledgements

The authors gratefully acknowledge the loan of the Purezone® ozone generating machine from Mr P Nichols, Purezone, Ipswich, UK and Mr W Chambers, Tex Engineering, Ipswich, UK for the duration of this study.

They also acknowledge the statistical advice provided by Dr D Moles and thank Proctor and Gamble and the Oral and Dental Research Trust for part funding this study.

References

Bader H, Hoigné J (1981) Determination of ozone in water by the indigo method. *Water Research* **15**, 449–56.

Baysan A, Whiley RA, Lynch E (2000) Antimicrobial effect of a novel ozone-generating device on microorganisms associated with primary root carious lesion in vitro. *Caries Research* **34**, 498–501.

Berrington AW, Pedlar SJ (1998) Investigation of gaseous ozone for MRSA decontamination of hospital side-rooms. *Journal of Hospital Infection* **40**, 61–5.

Bocci V (1992) Ozonation of blood for the therapy of viral diseases and immunodeficiencies. A hypothesis. *Medical Hypotheses* **39**, 30–4.

Broadwater WT, Hoehn RC, King PH (1973) Sensitivity of three selected bacterial species to ozone. *Applied Microbiology* **26**, 391–3.

Byström A, Sundqvist G (1981) Bacteriologic evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. *Scandinavian Journal of Dental Research* **89**, 321–8.

Byström A, Sundqvist G (1983) Bacteriologic evaluation of the effect of 0.5% sodium hypochlorite in endodontic therapy. *Oral Surgery, Oral Medicine, Oral Pathology* **55**, 307–12.

Byström A, Sundqvist G (1985) The antibacterial action of sodium hypochlorite and EDTA in 60 cases of endodontic therapy. *International Endodontic Journal* **18**, 35–40.

Chahverdiani B, Thadj-Bakhche A (1976) L'Ozonethérapie en traitement radiculaire. *Acta Medical Iranica* **XIX**, 192–200.

Cody RP, Weisel CP, Beinbaum G, Lioy PJ (1992) The effect of ozone associated with summer-time photochemical smog on the frequency of asthma visits to hospital emergency departments. *Environmental Research* **58**, 184–94.

D'Arcangelo C, Varvara G, De Fazio P (1999) An evaluation of the action of different root canal irrigants on facultative aerobic – anaerobic, obligate anaerobic and microaerophilic bacteria. *Journal of Endodontics* **25**, 351–3.

Deltour MM, Vincent J, Lartigau G (1970) Effet Lethal de L'ozone sur certaines souches de bacteries aerobies dan un modele de chamber pulpaire. *Revue d'odonto-stomatologie du midi de la France* **15**, 278–84.

Dyas A, Boughton BJ, Das BC (1983) Ozone killing action against bacterial and fungal species; microbiological testing of a domestic ozone generator. *Journal of Clinical Pathology* **36**, 1102–4.

Farooq S, Akhlaque S (1983) Comparative response of mixed cultures of bacteria and virus to ozonation. *Water Research* **17**, 809–12.

Felmingham D, Wilson APR, Quintana AI, Gruneberg RN (1992) *Enterococcus* species in urinary tract infections. *Clinical Infectious Diseases* **15**, 295–301.

Filippi A (1997) Ozone in oral surgery – current status and prospects. *Ozone Science and Engineering* **19**, 387–93.

Ghori S, Gulabivala K, Spratt DA (2001) Assessment of the antimicrobial nature of electrochemically activated water. *Journal of Dental Research (Special Edition)* **80**, 695 (Abstract 1355).

Grigoratos D, Knowles J, Ng Y-L, Gulabivala K (2001) Effect of exposing dentine to sodium hypochlorite and calcium hydroxide on its flexural strength and elastic modulus. *International Endodontic Journal* **34**, 113–9.

Gulabivala K, Stock CJR, Lewsey JD, Ghori S, Ng Y-L, Spratt DA (2004) The efficacy of electrochemically activated water as an irrigant in an infected tooth model. *International Endodontic Journal* **37**, 624–31.

Hazucha MJ, Bates DV, Bromberg PA (1989) Mechanism of action of ozone on the human lung. *Journal of Applied Physiology* **67**, 1535–41.

Hoigné J, Bader H (1976) The role of hydroxyl radical reactions in ozonation processes in aqueous solutions. *Water Research* **10**, 377–86.

Hunt NK, Mariñas BJ (1997) Kinetics of *Escherichia coli* inactivation with ozone. *Water Research* **31**, 1355–62.

Kim J-G, Yousef AE (2000) Inactivation kinetics of food-borne spoilage and pathogenic bacteria by ozone. *Food Microbiology and Safety* **65**, 521–8.

Kimura Y, Wilder-Smith P, Matsumoto K (2000) Lasers in endodontics: a review. *International Endodontic Journal* **33**, 173–85.

Krishna MT, Mudway I, Kelly FJ, Frew AJ, Holgate ST (1995) Ozone, airways and allergic airways disease. *Clinical and Experimental Allergy* **25**, 1150–8.

Lewsey JD, Gilthorpe MS, Gulabivala K (2001) An introduction to meta-analysis within the framework of multi-level modelling using the probability of success of root canal treatment as an illustration. *Community Dental Health* **18**, 131–7.

Lussi A, Messerli L, Hotz P, Grosrey J (1995) A new non-instrumental technique for cleaning and root filling canals. *International Endodontic Journal* **28**, 1–6.

Mannan G, Smallwood E, Gulabivala K (2001) Effect of access cavity location and design on degree and distribution of intstrumented root canal surface in maxillary anterior teeth. *International Endodontic Journal* **34**, 176–83.

Marais JT, Williams WP (2001) Antimicrobial effectiveness of electro-chemically activated water as an endodontic irrigation solution. *International Endodontic Journal* **34**, 237–43.

McDonnell WF, Horstman DH, Hazucha MJ et al. (1983) Pulmonary effects of ozone exposure during exercise: dose-response characteristics. *Journal of Applied Physiology* **54**, 1345–51.

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

Moorer WR, Wesselink PR (1982) Factors promoting the tissue dissolving capability of sodium hypochlorite. *International Endodontic Journal* **15**, 187–96.

Nair PRN (1987) Light and electron microscope studies of root canal flora and periapical lesions. *Journal of Endodontics* **13**, 29–39.

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

Restaino L, Frampton EW, Hemphill JB, Palnikar P (1995) Efficacy of ozonated water against various food-related microorganisms. *Applied and Environmental Microbiology* **61**, 3471–5.

Sandhaus S (1969) Ozonanwendung in der chirugischen und klinischen Zahn-, Mund- und Kieferheilkunde. *Zahnärzliche Praxis* **24**, 1–5.

Seal GJ, Ng Y-L, Spratt DA, Bhatti M, Gulabivala K (2002) An *in vitro* comparison of the bactericidal efficacy of lethal photosensitisation or sodium hypochlorite irrigation on *Streptococcus intermedius* biofilms in root canals. *International Endodontic Journal* **35**, 268–74.

Shechter H (1973) Spectrophotometric method of determination of ozone in aqueous solutions. *Water Research* **7**, 729–39.

Shih M, Marshall FJ, Rosen S (1970) The bactericidal efficiency of sodium hypochlorite as an endodontic irrigant. *Oral Surgery, Oral Medicine and Oral Pathology* **29**, 613–9.

Shin W-T, Mirmiran A, Yiacoumi S, Tsouris C (1999) Ozonation using micro-bubbles formed by electric fields. *Separation and Purification Technology* **15**, 271–82.

Sim TPC, Knowles JC, Ng Y-L, Shelton J, Gulabivala K (2001) Effect of sodium hypochlorite on mechanical properties of dentine and tooth surface strain. *International Endodontic Journal* **34**, 120–32.

Siqueira JF, Machado AG, Silveira RM, Lopes HP, De Uzeda M (1997) Evaluation of the effectiveness of sodium hypochlorite used with three irrigation methods in the elimination of *Enterococcus faecalis* from the root canal *in vitro*. *International Endodontic Journal* **30**, 279–82.

Spångberg L, Engström B, Langeland K (1973) Biologic effects of dental materials. *Oral Surgery, Oral Medicine and Oral Pathology* **36**, 856–69.

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.

Staehelin J, Hoigné J (1985) Decomposition of ozone in water in the presence of organic solutes acting as promoters and inhibitors of radical chain reactions. *Environmental Science Technology* **19**, 1206–13.

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

Syed SA, Loesche W (1972) Survival of human dental plaque flora in various transport media. *Applied Microbiology* **24**, 638–44.

Wilson M (1996) Susceptibility of oral bacterial biofilms to antimicrobial agents. *Journal of Medical Microbiology* **44**, 79–87.