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

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Abstract


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 log10 reductions) and washed (5 log10 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.

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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
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 & Hoigne (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 2000), root canal isolate previously identified as E. faecalis culture (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).

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% defibrinated 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

<table>
<thead>
<tr>
<th>Duration of treatment (s)</th>
<th>Mean (SD) CFU mL⁻¹</th>
<th>95% Confidence interval of differences</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before treatment</td>
<td>After treatment</td>
<td>Mean difference (SD)</td>
<td></td>
</tr>
<tr>
<td>(a) Sparging of ozone on E. faecalis cells in broth culture (n = 4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>8.8 × 10⁷ (1.01 × 10⁷)</td>
<td>8.28 × 10⁸ (9.98 × 10⁹)</td>
<td>-7.6 × 10⁻⁸ to -2.42 × 10¹¹</td>
</tr>
<tr>
<td>60</td>
<td>2.54 × 10⁹ (1.83 × 10⁹)</td>
<td>8.75 × 10¹⁰ (6.9 × 10¹⁰)</td>
<td>-1.74 × 10⁹ to -5.04 × 10¹⁰</td>
</tr>
<tr>
<td>120</td>
<td>1.78 × 10¹¹ (1.35 × 10¹¹)</td>
<td>1.34 × 10¹⁰ (6.6 × 10¹⁰)</td>
<td>-5.60 × 10⁻⁸ to -3.95 × 10¹¹</td>
</tr>
<tr>
<td>240</td>
<td>3.25 × 10¹¹ (1.33 × 10¹¹)</td>
<td>2.97 × 10¹⁰ (3.3 × 10¹⁰)</td>
<td>1.24 × 10⁹ to 5.40 × 10¹⁰</td>
</tr>
<tr>
<td>(b) Sparging of ozone on E. faecalis cells resuspended in ozone demand-free water (n = 5)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>30</td>
<td>4.16 × 10⁴ (4.6 × 10⁴)</td>
<td>5.33 × 10⁶ (6.2 × 10⁶)</td>
<td>-1.17 × 10⁶ (8.0ym 10⁶)</td>
</tr>
<tr>
<td>60</td>
<td>2.24 × 10⁵ (1.8 × 10⁵)</td>
<td>1.14 × 10⁶ (2.1 × 10⁶)</td>
<td>2.24 × 10⁵ (1.80 × 10⁵)</td>
</tr>
<tr>
<td>120</td>
<td>2.52 × 10⁵ (2.0 × 10⁵)</td>
<td>5.92 × 10⁶ (3.5 × 10⁶)</td>
<td>-2.52 × 10⁵ (2.03 × 10⁵)</td>
</tr>
<tr>
<td>240</td>
<td>1.31 × 10⁷ (9.2 × 10⁵)</td>
<td>2.14 × 10⁶ (2.4 × 10⁶)</td>
<td>1.31 × 10⁷ (9.26 × 10⁵)</td>
</tr>
<tr>
<td>(c) NaOCl (0.05%) treatment of E. faecalis cells in nutrient broth (n = 4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>2.93 × 10¹¹ (8.1 × 10¹¹)</td>
<td>1.04 × 10¹² (1.2 × 10¹²)</td>
<td>1.50 × 10¹¹–4.15 × 10¹¹</td>
</tr>
<tr>
<td>60</td>
<td>2.93 × 10¹¹ (8.1 × 10¹¹)</td>
<td>2.61 × 10¹² (1.5 × 10¹²)</td>
<td>1.62 × 10¹¹–4.22 × 10¹¹</td>
</tr>
<tr>
<td>120</td>
<td>2.93 × 10¹¹ (8.1 × 10¹¹)</td>
<td>2.1 × 10¹³ (3.4 × 10¹³)</td>
<td>1.57 × 10¹¹–4.23 × 10¹¹</td>
</tr>
<tr>
<td>240</td>
<td>2.93 × 10¹¹ (8.1 × 10¹¹)</td>
<td>0</td>
<td>_b</td>
</tr>
<tr>
<td>(d) NaOCl (0.05%) treatment of E. faecalis cells in ozone demand-free water (n = 4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>7.31 × 10⁶ (1.37 × 10⁷)</td>
<td>0</td>
<td>_b</td>
</tr>
<tr>
<td>60</td>
<td>7.31 × 10⁶ (1.37 × 10⁷)</td>
<td>0</td>
<td>_b</td>
</tr>
<tr>
<td>120</td>
<td>7.31 × 10⁶ (1.37 × 10⁷)</td>
<td>0</td>
<td>_b</td>
</tr>
<tr>
<td>240</td>
<td>7.31 × 10⁶ (1.37 × 10⁷)</td>
<td>0</td>
<td>_b</td>
</tr>
</tbody>
</table>

*Significant at 5% level.

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

<table>
<thead>
<tr>
<th>Treatment time (s)</th>
<th>Stirring only (n = 4)</th>
<th>Stirring and ozone sparging (n = 4)</th>
<th>Stirring and air sparging (n = 4)</th>
<th>2.5% NaOCl stirring and ozone sparging (n = 4)</th>
<th>2.5% NaOCl stirring and air sparging (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Mean (SD) CFU/disc of bacterial cells recovered from biofilms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (Baseline count)</td>
<td>4.99 × 10⁷ (3.5 × 10⁷)</td>
<td>9.45 × 10⁷ (9.2 × 10⁷)</td>
<td>3.95 × 10⁸ (1.11 × 10⁹)</td>
<td>_b</td>
<td>_b</td>
</tr>
<tr>
<td>60</td>
<td>8.45 × 10⁹ (1.1 × 10⁹)</td>
<td>2.65 × 10⁹ (2.3 × 10⁹)</td>
<td>3.2 × 10¹⁰ (1.04 × 10¹¹)</td>
<td>5.2 × 10⁹ (5.24 × 10⁹)</td>
<td>_b</td>
</tr>
<tr>
<td>120</td>
<td>5.3 × 10⁹ (2.8 × 10⁹)</td>
<td>0</td>
<td>1.29 × 10¹⁰ (1.7 × 10¹⁰)</td>
<td>_b</td>
<td>_b</td>
</tr>
<tr>
<td>240</td>
<td>9.35 × 10⁹ (2.7 × 10⁹)</td>
<td>0</td>
<td>_b</td>
<td>_b</td>
<td>_b</td>
</tr>
</tbody>
</table>

*Significant difference between baseline count and bacteria recovered after treatment at 5% level.

Not tested.

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 (Hoigne & Bader 1976, Shin et al. 1999). As a result, hydroxyl (OH) radicals are generated, which are amongst the most...
without ozone treatment & after ozone treatment & Mean (SD) difference & 95% Confidence interval & P-value \\
2.62 × 10^{10} (2.7 × 10^{10}) & 5.75 × 10^8 (2.38 × 10^8) & 2.04 × 10^{10} (1.39 × 10^{10}) & -1.38 × 10^{-1} - 6.46 × 10^{-10} & 0.194 \\

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).
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. 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 to 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 (Hauzuka 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.

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