

Effectiveness of ozone against endodontopathogenic microorganisms in a root canal biofilm model

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Abstract

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Aim To assess the antimicrobial efficacy of aqueous (1.25–20 µg mL⁻¹) and gaseous ozone (1–53 g m⁻³) as an alternative antiseptic against endodontic pathogens in suspension and a biofilm model.

Methodology *Enterococcus faecalis*, *Candida albicans*, *Peptostreptococcus micros* and *Pseudomonas aeruginosa* were grown in planctonic culture or in mono-species biofilms in root canals for 3 weeks. Cultures were exposed to ozone, sodium hypochlorite (NaOCl; 5.25%, 2.25%), chlorhexidine digluconate (CHX; 2%), hydrogen peroxide (H₂O₂; 3%) and phosphate buffered saline (control) for 1 min and the remaining colony forming units counted. Ozone gas was applied to the biofilms in two experimental settings, resembling canal areas either difficult (setting 1) or easy (setting 2) to reach. Time-course experiments up to 10 min were included. To compare the tested samples, data were analysed by one-way ANOVA.

Results Concentrations of gaseous ozone down to 1 g m⁻³ almost and aqueous ozone down to 5 µg mL⁻¹ completely eliminated the suspended microorganisms as did NaOCl and CHX. Hydrogen peroxide and lower aqueous ozone concentrations were less effective. Aqueous and gaseous ozone were dose- and strain-dependently effective against the biofilm microorganisms. Total elimination was achieved by high-concentrated ozone gas (setting 2) and by NaOCl after 1 min or a lower gas concentration (4 g m⁻³) after at least 2.5 min. High-concentrated aqueous ozone (20 µg mL⁻¹) and CHX almost completely eliminated the biofilm cells, whilst H₂O₂ was less effective.

Conclusion High-concentrated gaseous and aqueous ozone was dose-, strain- and time-dependently effective against the tested microorganisms in suspension and the biofilm test model.

Keywords: antimicrobials, biofilm, endodontics, microbiology, ozone, root canal.

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Introduction

The successful treatment of an infected root canal, especially those with persistent apical periodontitis remains a clinical challenge (Nair 2006). The main aim of endodontic treatment is to eradicate or substantially reduce the microbial load in the root canal

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system, which is conventionally achieved by chemo-mechanical instrumentation followed by canal filling to prevent recolonization (Nair 2006). Endodontic irrigants must have effective antimicrobial activity but also exhibit relatively no cytotoxicity toward periapical and oral mucosal tissue. An anti-inflammatory action especially in cases of persistent apical periodontitis might also be advantageous.

Enterococcus faecalis and *Candida albicans* have been reported to be of particular interest in cases of persistent periodontitis (Molander *et al.* 1998, Sundqvist *et al.* 1998, Siqueira & Rôças 2004, Fouad *et al.* 2005). Anaerobic bacteria, such as *Peptostreptococcus micros* or Gram-negative bacteria including *P. species* have also been associated with persistent infections (Siqueira 2002). These microorganisms grow in highly resistant biofilms (Pinheiro *et al.* 2003), but also as planctonic cells suspended in the fluid phase of the root canal or as remnants after mechanical canal preparation (Distel *et al.* 2002, Nair 2006).

Sodium hypochlorite (up to 5.25%) is the most commonly used root canal irrigant and has been used alternately with H₂O₂ (3%) (Takeda *et al.* 1999). Chlorhexidine digluconate (2%) has also been recommended for root canal irrigation in combination with mechanical debridement (Siqueira *et al.* 1998, Gomes *et al.* 2001, Basrani & Lemonie 2005). However, the success rate for conventional treatment of persistent and refractory apical periodontitis is only in the order of between 50% and 70% (Weiger *et al.* 2001) and consequently NaOCl up to 3% has been reported to have limited efficacy against high-pathogenic endodontic microorganisms and CHX 2% has demonstrated inconsistent results (Siqueira *et al.* 1998, Gomes *et al.* 2001). In addition, side effects such as haemorrhage, oedema and skin ulceration have been reported when high concentrations of NaOCl and H₂O₂ come into contact with oral tissues (Pashley *et al.* 1985, Önçağ *et al.* 2003, Gernhardt *et al.* 2004). A significant degree of cytotoxicity towards oral cells has been found *in vitro* as well (Hyslop *et al.* 1988, Nagayoshi *et al.* 2004, Huth *et al.* 2006). Chlorhexidine (2%) may cause mucosal desquamation, impaired wound healing and tooth staining (Bassetti & Kallenberger 1980, Cline & Layman 1992) and a high cytotoxic potential has been demonstrated on epithelial cells (Huth *et al.* 2006). Therefore, an alternative endodontic antiseptic with high antimicrobial potential and fewer side effects would be valuable.

Ozone is currently being discussed as a possible alternative antiseptic agent in dentistry because of its

reported high antimicrobial power without the development of drug resistance (Restaino *et al.* 1995, Paraskeva & Graham 2002). Ozone gas in a concentration of $\sim 4 \text{ g m}^{-3}$ (HealOzone; KaVo, Biberach, Germany) is already being used clinically for endodontic treatment. However, results of studies into its efficacy against endodontic pathogens has been inconsistent, and there is little information regarding the most appropriate application time and concentration to use (Nagayoshi *et al.* 2004, Arita *et al.* 2005, Bezrukova *et al.* 2005, Hems *et al.* 2005). Regarding the demand on relative non-toxicity toward periapical and oral mucosal tissue for the endodontic irrigants (Nair 2006), the ozone gas concentration currently used in endodontics (4 g m^{-3}) has been shown to be slightly less cytotoxic than NaOCl (2.5%) and aqueous ozone (up to $20 \mu\text{g mL}^{-1}$) showed essentially no toxicity to oral cells *in vitro* (Filippi 2001, Ebersberger *et al.* 2002, Nagayoshi *et al.* 2004, Huth *et al.* 2006). The aim of this study was to investigate the antimicrobial efficacy of gaseous and aqueous ozone against specific endodontic pathogens in suspension and in biofilms grown in human root canals.

Materials and methods

Microorganisms

Freeze-dried microorganisms: *E. faecalis* (ATCC 14506; LGC Promochem, Wesel, Germany), *C. albicans* (ATCC MYA-273), *P. micros* (ATCC 33270) and *P. aeruginosa* (ATCC 15442) were suspended in brain heart infusion medium (BHI) and recultivated on Schaedler agar plates (vitamin K₁ and 5% sheep blood; BD Diagnostic Systems, Heidelberg, Germany).

Test agents

Dose–response experiments were performed for gaseous and aqueous ozone covering a concentration range as wide as possible to evaluate if there was a concentration that could possibly compete with the established endodontic irrigants in antimicrobial effectiveness. Basically following a log₂ scale, the concentration ranges were limited because of the experimental setting and equipment. Ozone gas (Ozonosan photonic, Dr Hänsler, Iffezheim, Germany) in concentrations between 1 g m^{-3} (the minimum concentration to measure by the available ozone gas measuring device) and 53 g m^{-3} (the highest achievable concentration because of the experimental set-up and the limitation

of the ozone generator) was applied to the test microorganisms in a self-constructed glass chamber with simultaneous concentration measurement (GM-6000-NZL; Anseros, Tübingen, Germany). The analytical method of the concentration measuring device is based on UV light absorption at a wavelength of 253.7 nm where gaseous ozone has its maximum absorbance (Bocci 2002). For aqueous ozone, bi-distilled water was treated with ozone gas ($75 \mu\text{g mL}^{-1}$, 15 min) using the ozone generator, which resulted in a final photometrically confirmed (Palintest 1000 Ozone Meter, Palintest Ltd, Gateshead, UK) ozone concentration in water of $20 \mu\text{g mL}^{-1}$ (saturation point), which was diluted to $1.25 \mu\text{g mL}^{-1}$. The ozone concentration measurement in water involves the oxidation of a colourless indicator (diethyl-*p*-phenylene diamine) to a pink compound by ozone in comparison with a reference sample without ozone (manufacturer's information, operating wavelength of the photometer is 505 nm). Ozone was compared with freshly prepared solutions of NaOCl (5.25%, 2.25%), CHX (2%), H_2O_2 (3%) and phosphate buffered saline (PBS) as a control. As ozone is an endothermic, highly instable oxygen compound (Sehested *et al.* 1991, Hoigne 1998, Stübinger *et al.* 2006), both the gas and the ozonated bi-distilled water were freshly prepared before each experiment. During production and processing of the ozone experiments only ozone-resistant materials were used (e.g. ozone demand-free glass, ozone-resistant piping material).

Testing ozone against microorganisms in suspension

Microorganisms were grown overnight (37°C , 10 mL of BHI), centrifuged, resuspended in PBS to a turbidity of McFarland 1 [3×10^8 colony forming units (CFU) mL^{-1}] and diluted 1 : 3. Ten microlitres were suspended in 1 mL of agent for 1 min followed by immediate, appropriate dilution with PBS as evaluated by preceding experiments. Thereof, 10 μL were plated out on agar plates and incubated aerobically (48 h, 37°C). For the obligate anaerobic *P. micros*, all experimental steps were completed in an anaerobic work bench (Bactron, Sheldon Manufacturing Inc., Cornelius, OR, USA; 85% N_2 , 5% H_2 , 10% CO_2 ; 37°C). Again, 10 μL of an equal dilution of the specific microorganism suspension were plated out on agar plates and exposed to ozone gas whilst the control plates were exposed to ambient air (1 min). After incubation of the agar plates (48 h, 37°C), the number of CFU mL^{-1} was determined.

Testing ozone against microorganisms in biofilms grown in human root canals

Crowns of freshly extracted single rooted permanent teeth (root length 18–19 mm), were removed at the level of the cemento-enamel junction. The use of the teeth for these experiments had been agreed upon by informed consent of the patients. The root canals were instrumented to the size ISO 40 (K-files; Dentsply Maillefer, Ballaigues, Switzerland), the apical regions to size 30 (ProFile[®].04; Dentsply Maillefer) with intermittent canal irrigation following each file size (3 mL of NaOCl 5.25%) (Takeda *et al.* 1999, Zehnder *et al.* 2003). Finally, the canals were irrigated with EDTA 10% (5 min, 10–30 mL) followed by normal saline (Zehnder *et al.* 2003), dried with paper points and the roots sterilized (121°C , 2 bars, 5 min).

The biofilm growth assembly (Fig. 1) contained a programmable peristaltic pump (IPC-8; Ismatec, Wertheim-Mondfeld, Germany), freshly prepared autoclaved artificial complete saliva (Pratten *et al.* 1998), 10% aqueous sucrose solution (Sigma-Aldrich, Schnellendorf, Germany), flexible silicone tubes (diameter 1 or 2.06 mm; Hartlmaier, Munich, Germany), several flasks and the prepared dental roots. The ingredients for the saliva were from Oxoid (Wesel, Germany), Sigma-Aldrich and BD Diagnostic Systems. All the equipment was sterilized before use. Overnight cultures of *E. faecalis*, *C. albicans* or *P. aeruginosa* were used. The latter species substituted the anaerobic *P. micros* which could not be evaluated since the growth assembly was too large to be incorporated into the anaerobic work bench. The experiments with *P. aeruginosa* were confined to the biofilm trials because of the greater relevance than the suspension experiments already undertaken for the other three strains.

The artificial saliva was constantly pumped through a flexible tube into a 50-mL reservoir, supplemented with the sucrose solution three times a day (30 min, $3 \times 33 \text{ mL}$) (Wilson *et al.* 1998). For the first week, an overnight culture (37°C in 10 mL of BHI) of the respective strain was added daily to the saliva. The nutrient broth from the reservoir was pumped (720 mL day^{-1}) (Wilson *et al.* 1998) through the canals of four parallel-mounted dental roots each hanging in a flask, the coronal canal orifice connected to the flexible tube by a 10- μL micropipette tip (Eppendorf, Hamburg, Germany). To avoid a contamination of the root surface, the used saliva, which dropped from the roots' apical region to the bottom of the flask, was constantly pumped off into a waste flask

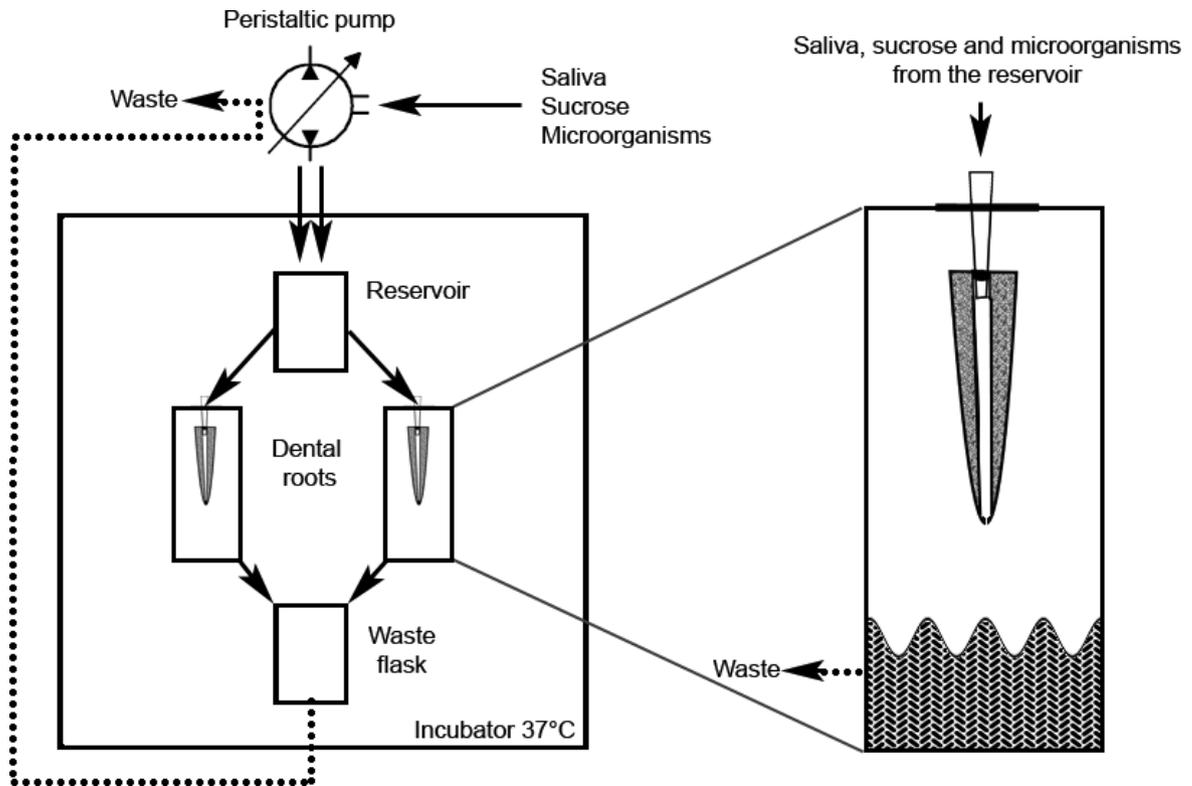


Figure 1 Growth assembly for the mono-species biofilms in root canals. Mono-species biofilms of endodontic pathogens were grown for 3 weeks in prepared dental root canals of extracted single-rooted permanent teeth. For this purpose, a peristaltic pump carried pre-warmed artificial saliva supplemented with sucrose and microorganism broth from a reservoir through the roots to a waste flask at a rate of 720 mL day^{-1} under aerobic conditions at 37°C . The right part of the drawing is a magnified view of an exemplary root hanging in a flask.

via a wider flexible tube (diameter 2.06 mm). After 3 weeks, the roots were removed and cut into 5-mm-thick horizontal slices, and the apical root portions were disposed of.

For each test condition, one slice was carefully transferred to a flask and 1 mL of the test agent added (four independent trials). For the ozone gas exposure, two experimental settings were used: setting 1, the slices were laid flat on glass beads into the gas box, that the gas streamed over the canal space (resembling canal areas that are difficult to reach); setting 2, the slices were positioned upright so as to allow the gas to stream through the root canal (resembling canal parts that are easy to reach). After 1 min, the agent was removed or the slice was removed from the gas box, 1 mL of PBS was immediately added and the slice vortexed for 1 min (Wilson *et al.* 1998). Restrained reactions beyond the 1 min contact time could have occurred as no chemicals were used to stop the action. Rather, the vast majority of the test agents were

removed immediately after 1 min and PBS added for appropriate dilution. Thereafter, $100 \mu\text{L}$ were plated out on agar plates and incubated (48 h, 37°C), and the CFU per plate were counted. Additionally within setting 2, ozone gas (4 g m^{-3}) was applied for longer time intervals, i.e. 2.5, 5 and 10 min. The counted number of CFU were calculated as a percentage of the respective control (mean \pm SD; $n = 3\text{--}4$).

For each of the independent trials, one slice was checked for the presence of a biofilm inside the root canal and for possible microbial contamination of the outer root surface by scanning electron microscopy (JSM-35 CF; Jeol, Echting, Germany and SmartSEM; Zeiss, Oberkochen, Germany).

Statistical methods

As a result of the large number of test agents, the experiments were conducted in several stages each with its own control. To compare the antimicrobial

activity of the agents, the counted CFU were calculated in percentage of the respective controls (mean \pm SD; $n = 3-4$). For all experiments, the absolute numbers of CFU, the percentage values and the means with standard deviation of the independent trials are given in the accompanying Supporting Information. Data were analysed by one-way ANOVA with Tamhane *post hoc* tests to compare independent samples (two-tailed tests, α -level 0.05) (SPSS software 12; SPSS Inc., Chicago, IL, USA).

Results

Effect of ozone on microorganisms in suspension

Firstly, the effect of aqueous and gaseous ozone on the specific endodontic pathogens in planctonic culture was evaluated (see Supporting Information, Tables A–C). Aqueous ozone completely eliminated *E. faecalis* and *C. albicans* when used in concentrations down to $5 \mu\text{g mL}^{-1}$, whereas lower concentrations (2.5 and $1.25 \mu\text{g mL}^{-1}$) reduced substantially but did not eliminate them totally (Fig. 2a,b). In the case of *P. micros*, aqueous ozone down to $2.5 \mu\text{g mL}^{-1}$ led to complete eradication whilst $1.25 \mu\text{g mL}^{-1}$ was less effective (Fig. 2c). In comparison, NaOCl and CHX led to a total elimination of the tested microorganisms, whereas H_2O_2 reduced but did not eliminate them. Ozone gas in concentrations down to the tested minimum of 1 g m^{-3} for 1 min almost completely eliminated the tested strains with a mean reduction of more than 99% (Fig. 2a–c, Supporting Information, Tables A–C). Statistically, no differences in effectiveness of the different agents were seen for *E. faecalis* (ANOVA, $P > 0.05$). Regarding *C. albicans*, H_2O_2 and low concentrations of ozonated water (2.5 and $1.25 \mu\text{g mL}^{-1}$) were significantly less effective than all other agents ($P < 0.05$). Against *P. micros*, low dose ozonated water ($1.25 \mu\text{g mL}^{-1}$) was less effective than the other antiseptics ($P < 0.05$).

Establishment of the anatomical biofilm model

The experimental set-up (Fig. 1) allowed the growth of mono-species biofilms of *E. faecalis*, *C. albicans* and *P. aeruginosa* over 3 weeks in an anatomically correct form inside the canal of tooth roots. The roots were sectioned into horizontal slices before exposure to the gas/agents. The formation of biofilms was checked for the different species by SEM of one slice for each independent trial as well as the outer root surfaces, which showed no

bacterial contamination or biofilm formation (pictures not shown). *P. aeruginosa* was substituted for the anaerobic *P. micros* because the growth assembly was too large for the anaerobic chamber.

Effect of ozone on microorganisms in biofilms

The antimicrobial action of ozone against *E. faecalis*, *C. albicans* and *P. aeruginosa* mono-species biofilms was tested (see Supporting Information, Tables D–F). Application of aqueous ozone for 1 min was dose-dependently effective against the microorganisms, its highest concentration of $20 \mu\text{g mL}^{-1}$ revealing mean CFU reductions of over 96%, similar to CHX 2% (Fig. 3a–c, Supporting Information, Tables D–F). Sodium hypochlorite (5.25%) completely eliminated the microorganisms, whilst H_2O_2 was less effective. In this series of experiments, ozone gas was applied to the root slices laying flat in the gas box (setting 1), which revealed a dose-dependent effectiveness of ozone gas against the different species (Fig. 3a–c). *E. faecalis* and *C. albicans* was almost eliminated by the highest gas concentration achievable within the experimental setting (53 g m^{-3}) (Fig. 3a,b) and *P. aeruginosa* by the highest and the second highest concentration (Fig. 3c). Statistically, no significant differences in effectiveness could be found between the antiseptics for *E. faecalis* and *C. albicans* (ANOVA, $P > 0.05$). Against *P. aeruginosa*, ozone gas 4 g m^{-3} was significantly less effective than NaOCl, CHX and ozonated water down to $10 \mu\text{g mL}^{-1}$ and ozonated water $10 \mu\text{g mL}^{-1}$ less effective than CHX 2% (ANOVA, $P < 0.05$). This was mainly because of a very small standard deviation in comparison with lower gas and ozone water concentrations, which showed no significant differences.

Exposure of the biofilm to ozone gas in a different setting and with longer contact times

In the following, the experimental conditions were changed by positioning the slices with *E. faecalis* biofilms upright with their cut surfaces in front of the inlet of the gas box as to allow the gas to stream through the root canal (setting 2) rather than over the canal space as in the setting before. Two concentrations were selected, i.e. one high gas concentration (32 g m^{-3}) as well as a lower concentration, which is currently used in dentistry (4 g m^{-3} ; HealOzone). Comparing the outcome of the two settings, the high gas concentration led to complete eradication of viable cells after 1 min in the new setting whilst in the old

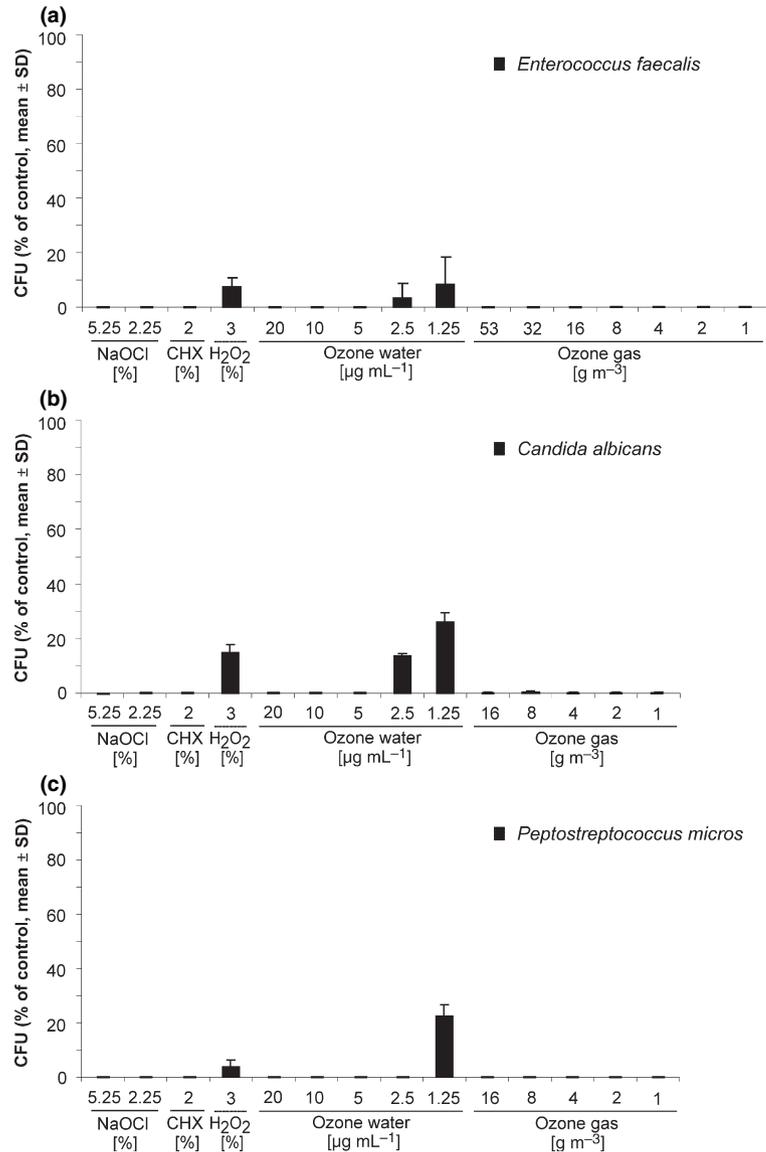


Figure 2 Antimicrobial efficacy of ozone against endodontic pathogens in suspension. The suspended microorganisms were exposed to aqueous or gaseous ozone in different concentrations or established endodontic irrigants (NaOCl, CHX and H₂O₂) for 1 min. The numbers of CFU after contact with PBS for 1 min were defined as 100% control (dotted line). The remaining CFU after agent/gas exposure were counted and calculated as a percentage of the control ($n = 3-4$, mean \pm SD) (see Supporting Information, Tables 1-3). (a) shows the antimicrobial activity against *E. faecalis*. (b) shows the antimicrobial activity against *C. albicans*. (c) Shows the antimicrobial activity against *P. micros*.

setting only a reduction was observed (Fig. 4a). Ozone gas in the lower concentration (1 min) reduced the cell count more than before, but not to zero. Therefore, as a last step, the effect of longer exposure times (2.5, 5 and 10 min) of this concentration was tested on the bacterial biofilms. Contact times of 2.5 min and more with 4 g m^{-3} ozone gas led to complete elimination of the microorganisms (Fig. 4b), but without being sig-

nificantly different to the cell count after 1 min ($P > 0.05$) (see Supporting Information, Table G).

Discussion

In this study, gaseous ozone in concentrations down to 1 g m^{-3} substantially and aqueous ozone down to 5 $\mu\text{g mL}^{-1}$ completely eliminated the tested planctonic

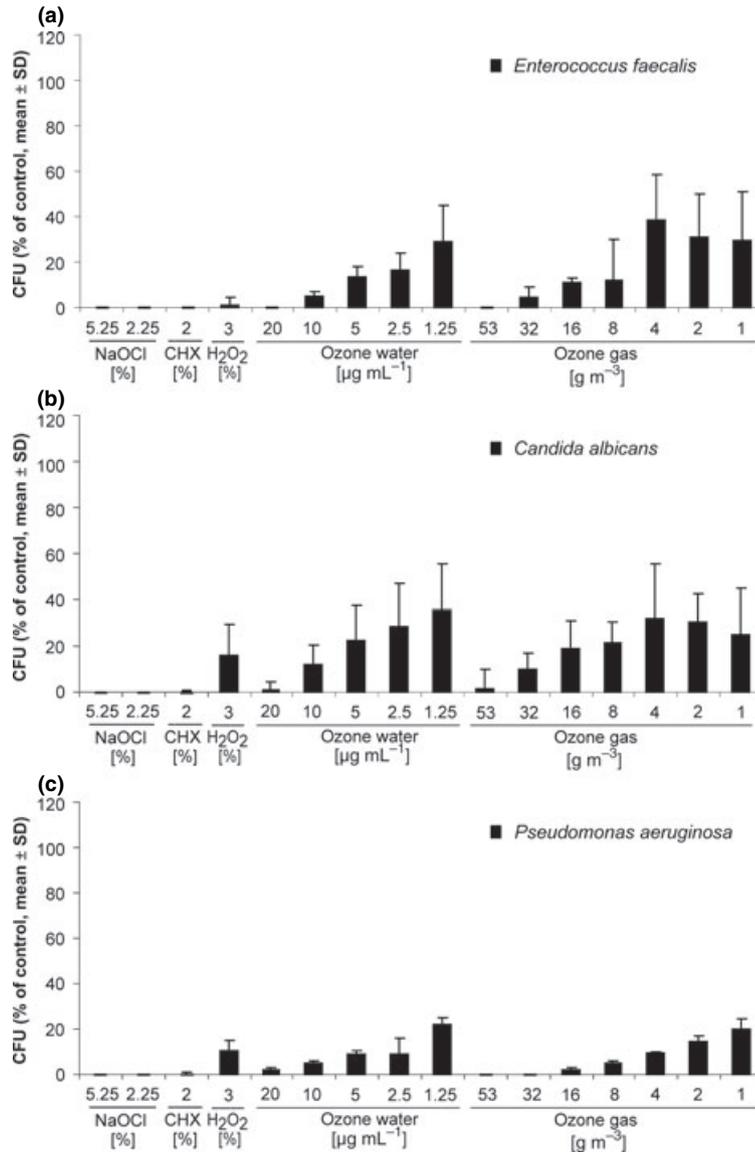


Figure 3 Antimicrobial efficacy of ozone against endodontic pathogens associated in mono-species biofilms in a root canal model. Mono-species biofilms were grown inside of dental root canals of extracted single-rooted teeth for 3 weeks. In the following, the roots were cut into horizontal slices that were exposed to aqueous or gaseous ozone in different concentrations or established endodontic irrigants (NaOCl, CHX, H₂O₂) or PBS as a control for 1 min. After removal and suspension of the biofilms, the remaining number of CFU were counted and calculated in % of the CFU counts after contact with the PBS control (100%, dotted line) ($n = 4$, mean \pm SD) (see Supporting Information, Tables 4–6). (a) Antimicrobial efficacy against *E. faecalis* biofilm. (b) Antimicrobial efficacy against *C. albicans* biofilm. (c) Antimicrobial efficacy against *P. aeruginosa* biofilm.

pathogens. Gaseous and aqueous ozone were dose- and strain-dependently effective against the microorganisms in biofilms. Total elimination of the microorganisms in terms of the methods used here could be achieved by ozone gas at 32 g m⁻³ for 1 min or a lower concentration (4 g m⁻³) for longer contact

times (≥ 2.5 min) in case of *E. faecalis* (setting 2). Aqueous ozone in the highest concentration (20 $\mu\text{g mL}^{-1}$, 1 min) nearly eliminated *E. faecalis*, *C. albicans* and *P. aeruginosa* biofilms.

The root canal model used in these experiments allowed for the growth of biofilms inside the canal. To

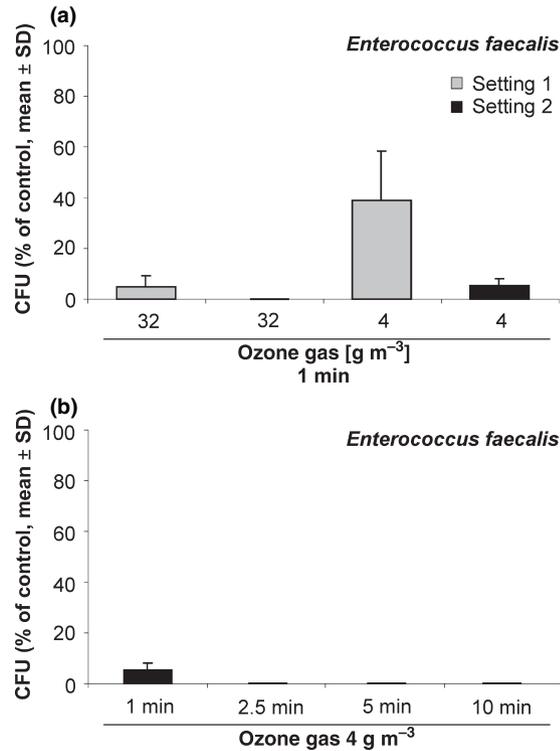


Figure 4 Antimicrobial efficacy of ozone gas applied in two experimental settings to *E. faecalis* biofilms and the effect of prolonged exposure times. The biofilms were grown as described in Fig. 3. In setting 1, the horizontal root slices were laid flat on glass beads in the gas box (see experiments in Fig. 3). In setting 2, the horizontal root slices were positioned upright with their cut surfaces in front of the gas inlet as to allow the gas streaming through the canals. (a) The antimicrobial effect of gaseous ozone in concentrations of 32 g m^{-3} and 4 g m^{-3} for 1 min on the *E. faecalis* biofilms according to setting 1 (grey bars) and 2 (black bars) is shown in comparison. PBS served as control. The remaining CFU were counted and calculated in % of the PBS control which was defined as 100% (dotted line) ($n = 3$, mean \pm SD). (b) The antimicrobial effect of ozone gas (4 g m^{-3}) according to setting 2 for 1 min and prolonged contact times (2.5 min, 5 min and 10 min) is depicted (see Supporting Information, Table 7).

determine the efficacy of ozone as alternative antiseptic, it was compared with traditional endodontic irrigants (NaOCl, CHX and H_2O_2) by adding the agents for 1 min. The dose–response experiments for ozone and additionally the time-course experiments for the ozone gas concentration currently used in dentistry (4 g m^{-3} , HealOzone) were aimed at finding a dose-time-concentration that could completely eliminate the microorganisms in the test model as a basis for clinical study designs in the future. As a source of impreciseness in the present study, no chemicals were used to arrest the action of the agents. Therefore, the contact times, e.g. for CHX, which is known for its substantivity (Khademi et al. 2006), might be prolonged similar as in the clinical situation.

Earlier studies reported in part contradictory results regarding the efficacy of ozone against endodontic pathogens: one group tested ozonated water

($4 \mu\text{g mL}^{-1}$, 10 min) against *E. faecalis* incubated on dentine blocks for 6 days (Nagayoshi et al. 2004). A significant reduction was found but complete elimination was not observed as was the case with NaOCl 2.5%, which is consistent with the present results. Additionally, the trials reported here revealed that the highest concentration of ozonated water ($20 \mu\text{g mL}^{-1}$) led to a near eradication of the microorganisms in the 3-week-old biofilm and a complete elimination by gaseous ozone at a concentration of 32 g m^{-3} for 1 min or a lower concentration (4 g m^{-3}) for contact times of at least 2.5 min (setting 2). Further, the biofilm experiments revealed a near eradication of *E. faecalis* by CHX 2% whereas H_2O_2 was less efficient throughout. Another study found no significant reduction of *E. faecalis* biofilms (grown on membranes for 48 h) using ozonated water, but did so against planctonic bacteria (Hems et al. 2005). A reason for these differing

results compared with the study mentioned above (Nagayoshi *et al.* 2004) and the present experiments revealing a CFU reduction when exposed to high concentrated ozonated water might be that a rather low ozone concentration was used in the other study (Hems *et al.* 2005). That is, ozone gas was bubbled through the water containing the biofilm for only 4 min. The maximum concentration of ozonated water ($20 \mu\text{g mL}^{-1}$) was achieved in the present study only after 15 min of ozonation (data not shown). Another recent study grew *E. faecalis* biofilms over 60 days in root canals and applied ozonated water, ozone gas, NaOCl 2.5% or CHX 2% for 20 min (Estrela *et al.* 2007). Contrasting to the present results, none of the irrigants were found to have an antimicrobial effect.

The effect of ozone against *C. albicans* has been reported primarily for denture cleaning (Murakami *et al.* 1996, Oizumi *et al.* 1998). More recently, *C. albicans* incubated on resin plates for 120 min was almost eliminated by use of ozonated water (2 and $4 \mu\text{g mL}^{-1}$, 1 min) with or without ultrasonication (Arita *et al.* 2005). As 120 min represents a short time interval for biofilm formation, that study might be better compared with the present suspension experiments, in which a mean reduction of about 86% of *C. albicans* by $2.5 \mu\text{g mL}^{-1}$ ozonated water and a total elimination by $5 \mu\text{g mL}^{-1}$ ozonated water and a reduction of over 99% by ozone gas down to 1g m^{-3} was achieved. In the present biofilm experiments, *C. albicans* was found to be completely eliminated only by NaOCl (5.25%) and to over 96% by 53g m^{-3} gaseous ozone (setting 1), $20 \mu\text{g mL}^{-1}$ ozonated water and CHX 2%.

The effect of ozone against the anaerobe *P. micros* has not been evaluated before. Ozone gas in the tested minimum concentration (1g m^{-3} , setting 1) and aqueous ozone ($\geq 2.5 \mu\text{g mL}^{-1}$) completely eliminated the suspended microorganisms. Biofilm experiments were not performed with *P. micros* as the growth assembly could not be maintained in anaerobic conditions.

The use of ozone as a disinfectant against *P. aeruginosa* in dental unit water lines has been reported, but there is no information about the required time and concentration for total elimination (Filippi 1995, Al Shorman *et al.* 2003). In present biofilm experiments, total eradication was achieved by ozone gas concentrations of 32g m^{-3} (setting 1) and NaOCl (2.25%, 1 min). High-concentrated aqueous ozone ($20 \mu\text{g mL}^{-1}$, 1 min) and CHX 2% almost eliminated the viable microorganisms.

Conclusions

High-concentrated gaseous and aqueous ozone was dose-, strain- and time-dependently effective against the tested microorganisms in suspension and the biofilm test model. However, NaOCl was the only method that completely eliminated all types of microorganisms.

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Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 Antimicrobial efficacy of ozone and established endodontic irrigants (1 min) against the tested microorganisms in suspension or associated in biofilms. The absolute number of remaining colony forming units (CFU abs) of 3 to 4 independent trials ($n = 3-4$) are given. The CFU are also given in % of the respective controls in parentheses and their means with the standard deviations of the independent trials (% control, mean \pm SD) which correspond to Fig. 2, 3 and 4. NaOCl, sodium hypochlorite; CHX, chlorhexidine digluconate; H_2O_2 , hydrogen peroxide; O_3 , ozone. The antimicrobial effects against *E. faecalis* (**A**), *C. albicans* (**B**), and *P. micros* (**C**) in suspension are shown as well as against *E. faecalis* (**D**), *C. albicans* (**E**) and *P. aeruginosa* (**F**) associated in biofilms. Table **G** shows the antimicrobial efficacy of ozone gas in concentrations of 32g m^{-3} and 4g m^{-3} applied in two experimental settings to *E. faecalis* biofilms and the effect of prolonged exposure times in setting 2 (1 min, 2.5 min, 5 min, 10 min).

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