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Effect of ozone on oral cells compared with established antimicrobials

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Ozone has been proposed as an alternative antiseptic agent in dentistry based on reports of its antimicrobial effects in both gaseous and aqueous forms. This study investigated whether gaseous ozone (4×10^6 $\mu g\ m^{-3}$) and aqueous ozone (1.25–20 $\mu g\ ml^{-1}$) exert any cytotoxic effects on human oral epithelial (BHY) cells and gingival fibroblast (HGF-1) cells compared with established antiseptics [chlorhexidine digluconate (CHX) 2%, 0.2%; sodium hypochlorite (NaOCl) 5.25%, 2.25%; hydrogen peroxide (H_2O_2) 3%], over a time of 1 min, and compared with the antibiotic, metronidazole, over 24 h. Cell counts, metabolic activity, Sp-1 binding, actin levels, and apoptosis were evaluated. Ozone gas was found to have toxic effects on both cell types. Essentially no cytotoxic signs were observed for aqueous ozone. CHX (2%, 0.2%) was highly toxic to BHY cells, and slightly (2%) and non-toxic (0.2%) to HGF-1 cells. NaOCl and H_2O_2 resulted in markedly reduced cell viability (BHY, HGF-1), whereas metronidazole displayed mild toxicity only to BHY cells. Taken together, aqueous ozone revealed the highest level of biocompatibility of the tested antiseptics.

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Ozone is currently being discussed in dentistry as a possible alternative antiseptic agent. Its high antimicrobial power without the development of drug resistance has been noted in water purification and food preservation (1–3). Recent investigations have reported antimicrobial effects on oral pathogens of both gaseous and aqueous forms of ozone (4–11), and the effectiveness of ozone in the treatment of oral diseases is currently a subject of intensive research (12–15).

In dentistry, ozone has been used either in gaseous (4.2×10^6 $\mu g\ m^{-3}$; HealOzone, KaVo, Biberach, Germany) or in aqueous form for the elimination of caries pathogens, in the disinfection of root canals, as a rinse for avulsed teeth, and for enhancing epithelial wound healing (6,8,16–18). Currently, the established oral antiseptics for caries prevention, endodontic irrigation or adjunctive periodontal treatment include chlorhexidine digluconate (CHX) (0.2–2%), sodium hypochlorite (NaOCl) (2.25–5.25%), and hydrogen peroxide (H_2O_2) (3%) (19–21). Regarding side-effects, it is known that CHX may cause mucosal desquamation, impaired wound healing and fibroblast attachment to root surfaces, tooth staining, and altered taste sensation (22, 23). NaOCl or H_2O_2 may result in hemorrhage, edema, and skin ulceration in oral tissues (24–26). In proposing ozone as another potential antimicrobial for use in the oral cavity, it is important to compare possible toxic effects of ozone on resident oral cells with those of established agents.

Therefore, the aim of this *in vitro* study was to investigate whether gaseous or aqueous ozone exert any toxic effects on human oral epithelial and gingival fibroblast cells compared with established antiseptics and one clinically topical applied antibiotic (metronidazole), by using several independent biochemical techniques.

Material and methods

Cell culture

Human oral epithelial cells (BHY; DSMZ, Braunschweig, Germany) and gingival fibroblasts (HGF-1; LGC Promochem, Teddington, UK) were cultured under standard conditions in Dulbecco's modified Eagle's minimal essential medium (DMEM) (PromoCell, Heidelberg, Germany) containing 7% fetal calf serum, 100 U ml^{-1} penicillin, and 100 $\mu g\ ml^{-1}$ streptomycin (Biochrom, Berlin, Germany).

Agents and mode of cell exposure

Ozone gas (in medical oxygen, Ozonosan photonic, Dr. Hänsler, Iffezheim, Germany) was used at concentrations of $0.2–53 \times 10^6$ $\mu g\ m^{-3}$ with pure oxygen as the control (27). After incubation of the cells in 96-well plates, medium was removed (28) and the moist cell layer exposed to the gas for 1 min within a self-constructed glass chamber under simultaneous concentration measurement (GM-6000-NZL; Anseros, Tübingen, Germany).

Separately, aqueous ozone was applied to the cells (1 min) in the form of ozonated phosphate-buffered saline (ozone PBS) in photometrically confirmed concentrations of 1.25–20 $\mu g\ ml^{-1}$ (Palintest, Gateshead, UK) with pure PBS as the control. In comparison, the following established antiseptics dissolved in PBS were used: CHX (2%, 0.2%),

NaOCl (5.25%, 2.25%), and H_2O_2 (3%). Additionally, the effect of the antibiotic, metronidazole, over 24 h was evaluated at concentrations found in gingival crevicular fluid after insertion of Elyzol Gel: 30 μg ml^{-1} for 1.5 h followed by 250 μg ml^{-1} (1.5 h), 450 μg ml^{-1} (2 h), 250 μg ml^{-1} (3 h), and 30 μg ml^{-1} (16 h) (29).

Cell count and metabolic activity

Besides the total cell count and the number of dead cells (determined using the Trypan blue exclusion test), cell viability was monitored by assessing their metabolic activity after agent exposure. First, using the colorimetric WST-1 assay (Roche, Penzberg, Germany), cells were cultured in 96-well plates and the gas/agent applied for the specified time. After removal of the agent, 10 μl of WST-1 Reagent in 100 μl of medium was added for 1 h and the absorbance was measured in an enzyme-linked immunosorbent assay (ELISA) reader.

Second, the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) was used to quantify ATP, which directly correlates with the amount of metabolically active cells. After exposure of the cells to gas/agent, 100 μl of CellTiter Glo Reagent in 100 μl of medium was added. The cells were lysed and incubated (10 min) before luminescence signals were recorded (System Luminometer 160; Nichols Institute Diagnostics, Teterboro, NY, USA).

Electrophoretic mobility shift assay (EMSA)

DNA-binding activity of the transcription factor, Sp-1, was determined by EMSA using a consensus oligonucleotide (Promega) labeled with [γ-^{32}P]ATP (PerkinElmer, Brussels, Belgium) by T4 polynucleotide kinase (Roche) (30). Nuclear extracts were incubated with radiolabeled DNA probes in 20 μl of binding buffer for 30 min. Samples were run on non-denaturating 4% polyacrylamide gels, followed by audioradiography.

Western blot analysis

For measuring the actin levels, cytosolic extracts were isolated and electrophoresis was performed on 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) (30). Proteins were transferred to a nitrocellulose membrane, which was incubated with anti-actin (Sigma, Deisenhofen, Germany) and a horseradish-peroxidase-conjugated secondary antibody (Dianova, Hamburg, Germany). Antibody binding was visualized on X-ray film using the Chemiluminescent Reagent Plus (PerkinElmer).

Apoptosis

The level of apoptosis was monitored by the Caspase-Glo 3/7 Assay (Promega). After treatment of the cells, 100 μl of Caspase-Glo 3/7 Reagent in 100 μl of medium was added (1 h) and the luminescence measured to determine caspase activity. Apoptosis was determined by monitoring DNA fragmentation using the Apoptotic DNA Ladder Kit (Roche).

Statistical methods

Data were analyzed by one-sample *t*-tests to compare the test gas/agents with the control [*P*-value, 95% confidence interval (CI)] and by one-way analysis of variance (ANOVA) with Bonferroni post hoc tests to compare independent samples (two-tailed tests, α-level 0.05) (SPSS software 12; SPSS, Chicago, IL, USA). Assumptions of parametric tests were checked descriptively (QQ-plots, histograms).

Results

Effect of ozone gas

First, the effect of ozone gas on oral cells was characterized, focusing on the currently used concentration of 4×10^6 μg m^{-3}, and on higher (6×10^6 μg m^{-3}) and lower (2×10^6 μg m^{-3}) concentrations. Clinically relevant, the application time for both gaseous and aqueous ozone (see below) was 1 min (31). There was a statistically significant decrease in total cell counts after exposure to 4×10^6 μg m^{-3} ozone compared with the control [BHY: $P = 0.002$, CI (%): −77 - (−55); HGF-1: $P = 0.031$, CI: − 96- (−12)] and a clear increase in the number of dead cells (Fig. 1A). Essentially the same data were obtained using higher (6×10^6 μg m^{-3}) and lower (2×10^6 μg m^{-3}) concentrations of ozone. To assess cell viability further, metabolic activity was monitored (by using the WST-1 assay) for the whole concentration range achievable within our experimental setting (0.2–53 $\times 10^6$ μg m^{-3}). A decrease in enzyme activity to $\approx 50\%$ of control values for both cell lines was observed, even at the lowest concentration, followed by a dose-dependent decrease, down to almost 0%, at the highest concentration (Fig. 1B). The outcome was confirmed by the cell viability assay, showing the ATP levels in both cell lines to be significantly decreased after contact with 4×10^6 μg m^{-3} ozone gas [BHY: $P = 0.008$, CI (%): − 84- (− 37); HGF-1: $P = 0.001$, CI: − 66- (− 50)] (Fig. 1C).

Effect of ozone PBS compared with established antimicrobials

The effect of aqueous ozone on cells was investigated. We focused on 10 μg ml^{-1} as a high-dose representative for the concentrations used, to date, in dentistry (8,9,17,18), but also considered lower and higher concentrations. Following exposure to aqueous ozone (1.25–20 μg ml^{-1}), BHY and HGF-1 cell counts showed no change compared with PBS controls (Fig. 2). Contact with CHX (2%, 0.2%) resulted in markedly reduced BHY, but only slightly reduced HGF-1, cell counts. In contrast, exposure to NaOCl (5.25%, 2.25%) or to H_2O_2 (3%) led to a strong decrease in cell counts (BHY, HGF-1) and a marked increase in dead cells (data not shown).

Using the WST-1 assay, cells showed no change in metabolic activity after contact with ozone PBS up to 10 μg ml^{-1} and a minimal decrease at 20 μg ml^{-1} in comparison with PBS controls (Fig. 3A). Similar results were obtained after an exposure of 1 h (data not shown). After contact with CHX, the metabolic activity in BHY cells was significantly decreased [CHX 2%: $P = 0.001$, CI (%): −107- (−85); CHX 0.2%: $P < 0.0001$, CI: −94-

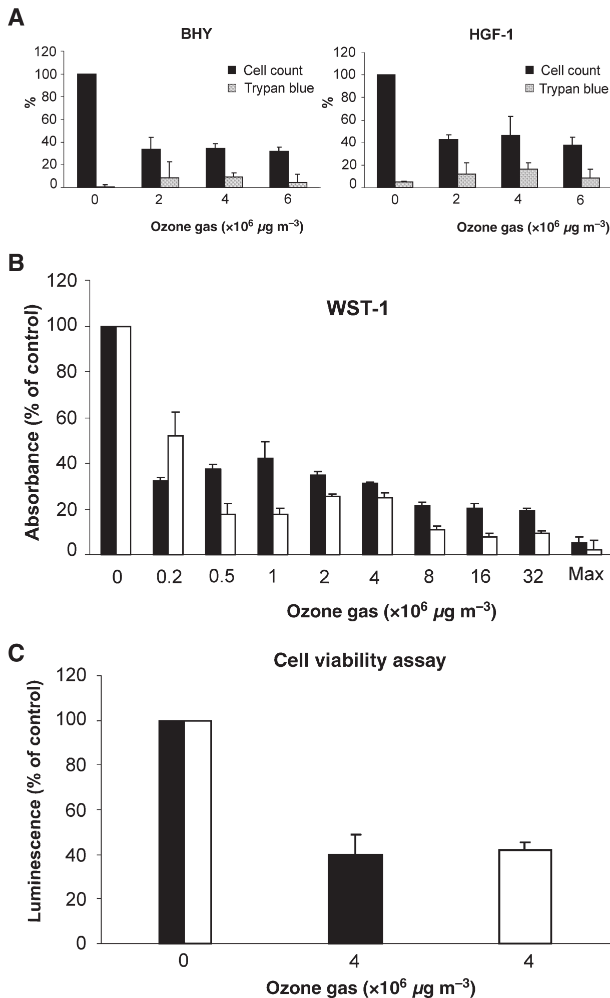

Fig. 1. Effect of ozone gas on oral cells. (A) Total cell count (BHY, HGF-1) and number of dead cells (Trypan blue exclusion test) following exposure to ozone/oxygen gas for 1 min. Pure oxygen served as the 100% control. The number of dead cells is given as a percentage of the total cell count [$n = 3$, mean ± standard deviation (SD)]. A detailed statistical analysis is given in the Results section. (B) Metabolic activity of cells (WST-1 assay) exposed to increasing concentrations of ozone gas for 1 min. Data are given as the percentage absorbance of the control wells ($n = 5$, mean ± SD). Black bars = BHY; white bars = HGF-1. (C) ATP level of cells (cell viability assay) after exposure to ozone gas for 1 min. Data are given as percentage luminescence of the control wells ($n = 3$, mean ± SD) Black bars = BHY; white bars = HGF-1.

(−78)], whereas the HGF-1 cells were only affected by CHX 2% [$P = 0.020$, CI: −40- (−9)]. The metabolic activity (BHY, HGF-1) was significantly inhibited after contact with NaOCl or H_2O_2 (BHY: $P < 0.0001$; HGF-1: $P = 0.006$, CI not shown). The 24 h exposure to metronidazole resulted in decreased metabolic activity in BHY cells [$P = 0.002$, CI: − 18- (− 7)] and no change in HGF-1 cells. Following exposure to NaOCl, H_2O_2 (BHY, HGF-1) or CHX (only BHY), a significantly lower enzyme activity was found than after exposure to 10 $\mu g\ ml^{-1}$ ozone (ANOVA post hoc tests, $P < 0.0001$).

Similar results were obtained when analysing the ATP levels. There was no change after contact with ozone PBS

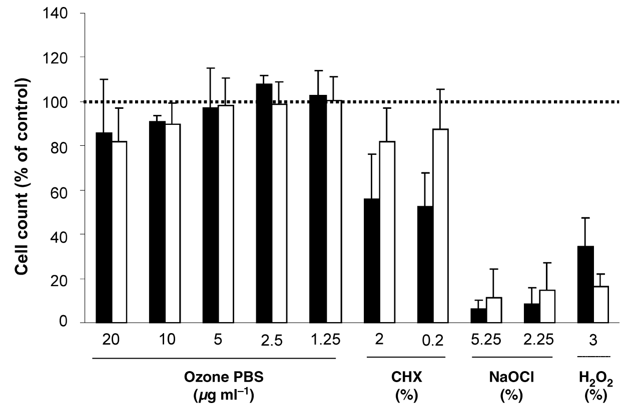

Fig. 2. Effect of aqueous ozone on cell counts in comparison to established antiseptics. Total cell counts are shown of BHY and HGF-1 cells after 1 min of exposure to ozonated phosphate-buffered saline (ozone PBS), chlorhexidine digluconate (CHX), sodium hypochlorite (NaOCl) or hydrogen peroxide (H_2O_2). Cell counts of the PBS control were defined as 100% (dotted line) [$n = 3$, mean ± standard deviation (SD)]. Black bars = BHY; white bars = HGF-1.

up to 10 $\mu g\ ml^{-1}$, and only a slight decrease at 20 $\mu g\ ml^{-1}$, compared with PBS controls (Fig. 3B). CHX showed a significant ATP depletion only in BHY cells [CHX 2%: $P = 0.014$, CI (%): −112- (−35); CHX 0.2%: $P = 0.001$, CI: −92- (−73)]. The ATP levels (BHY, HGF-1), after contact with NaOCl or H_2O_2, dropped to almost zero ($P \leq 0.004$, CI not shown). Cells exposed to NaOCl, H_2O_2 (BHY, HGF-1) or CHX (only BHY) showed significantly lower ATP levels than cells after contact with 10 $\mu g\ ml^{-1}$ ozone (ANOVA post hoc tests, $P < 0.0001$).

To exclude more subtle effects of ozone PBS on cells, the DNA-binding activity of the constitutive transcription factor, Sp-1, the level of constitutively expressed actin, as well as the potential apoptotic effects, were evaluated. EMSA revealed basically stable Sp-1 signals in BHY and HGF-1 cells exposed to ozone PBS (Fig. 4A). Sp-1 binding was clearly reduced after contact with CHX 2% (BHY) and completely abolished when NaOCl or H_2O_2 was added. The actin levels in BHY and HGF-1 cells did not change after contact with ozone PBS up to 20 $\mu g\ ml^{-1}$, decreased moderately following CHX or H_2O_2 exposure, and could not be detected after NaOCl treatment (Fig. 4B, data not shown).

No differences in caspase-3/7 activity, measured to monitor apoptosis (32), were observed (BHY, HGF-1) after contact with ozone PBS (1.25–20 $\mu g\ ml^{-1}$) compared with the control (Fig. 4C, data not shown). Incubation with tumor necrosis factor (TNF)/cyclohexamide as a positive control, however, demonstrated a marked increase in caspase activity. These data were confirmed by a second apoptosis test, revealing no DNA fragmentation in ozone-treated cells (data not shown).

Discussion

The experiments gave a consistent picture of reduced viability in BHY and HGF-1 cells following a 1-min

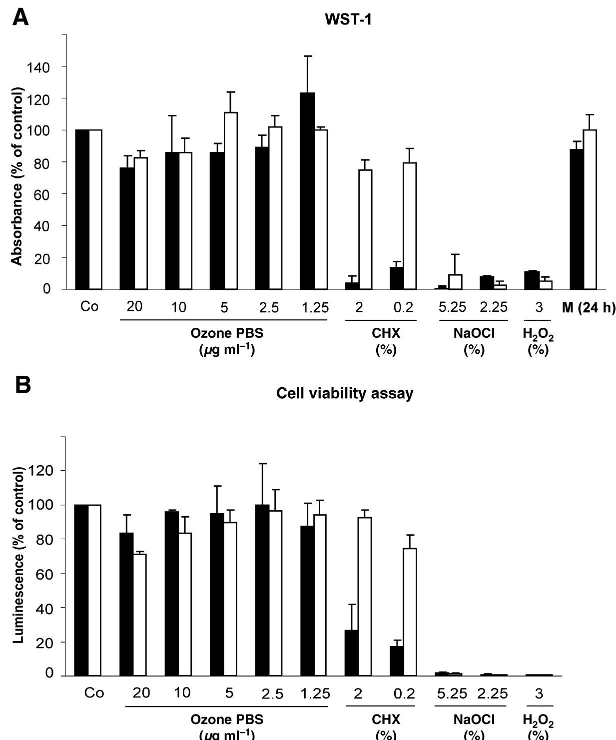

Fig. 3. Metabolic activity of cells following exposure to aqueous ozone or established antimicrobials. (A) Metabolic activity (WST-1 assay) of the BHY and HGF-1 cells after 1 min of exposure to phosphate-buffered saline (PBS) (100% control; Co), ozonated PBS (ozone PBS), chlorhexidine digluconate (CHX), sodium hypochlorite (NaOCl) or hydrogen peroxide (H_2O_2). Data are given as percentage absorbance of the control [n = 3, mean ± standard deviation (SD)]. As a control for the metronidazole-treated sample (M, exposure time 24 h) cells treated with medium change without drug over 24 h were used (n = 6, mean ± SD). A detailed statistical analysis is given in the Results section. Black bars = BHY; white bars = HGF-1. (B) ATP levels (cell viability assay) in cells exposed to ozone PBS chlorhexidine digluconate (CHX), sodium hypochlorite (NaOCl) or hydrogen peroxide (H_2O_2) for 1 min. Data are given as percentage luminescence of the PBS control (n = 3, mean ± SD). A detailed statistical analysis is given in the Results section. Black bars = BHY; white bars = HGF-1.

exposure to ozone gas at the concentration currently used in dentistry. Earlier experiments of the effect of ozone gas, investigated long-lasting contact (hours or days) with ambient ozone levels lower than the minimal concentration used in the present study. These lower concentrations are relevant for investigating the effect of ozone on the respiratory system and have been reported to cause dose-dependent impaired lung function and pathological changes in the lower airways. The cellular damage by ozone gas has been attributed to simultaneous processes such as inhibition of intracellular enzymes, glutathione depletion, and membrane damage occurring either by direct reaction between target molecules and ozone or via oxidizing intermediates (27, 33–35).

A 1-min contact with a large concentration range of aqueous ozone (1.25–20 μg ml^{-1}) revealed essentially no toxic effects to BHY and HGF-1 cells, as assessed by cell count, metabolic activity (enzyme activity, ATP level),

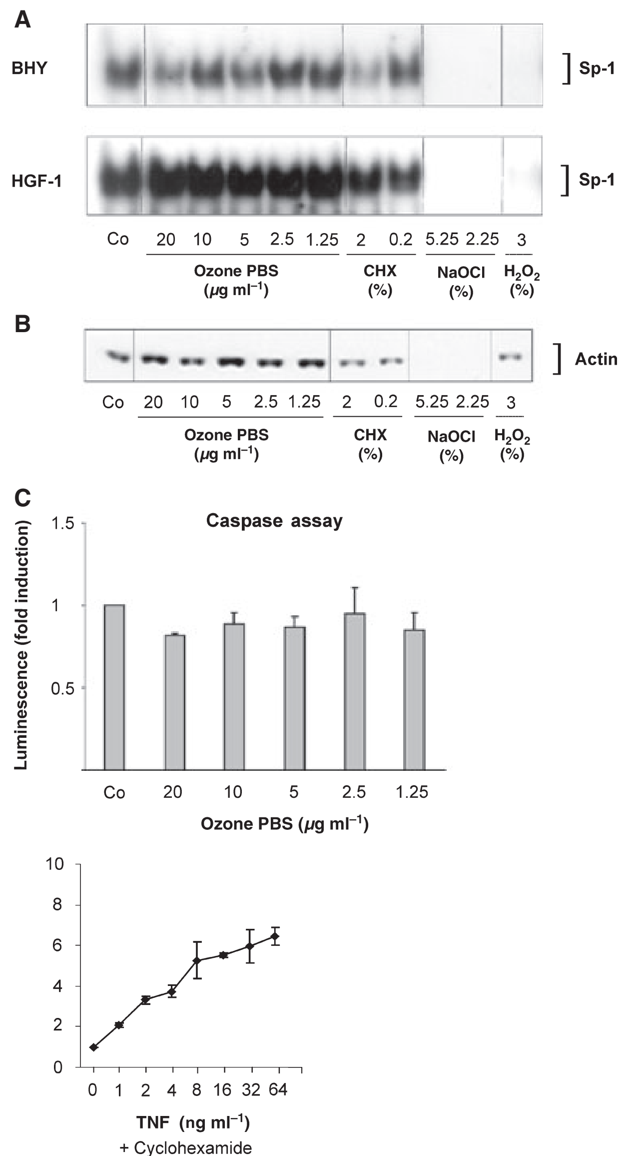

Fig. 4. Effects of ozone and other antimicrobial agents on Sp-1 binding, actin expression and caspase activity. (A) The binding activity of Sp-1 in BHY and HGF-1 cells was determined by electrophoretic mobility shift assay (EMSA) after a 1-min contact with phosphate-buffered saline (PBS) (control; Co), ozonated PBS (ozone PBS), chlorhexidine digluconate (CHX), sodium hypochlorite (NaOCl) or hydrogen peroxide (H_2O_2). Representative data of three independent experiments for each cell line are shown. (B) The levels of actin in BHY cells after a 1-min exposure to PBS (Co), ozone PBS, CHX, NaOCl or H_2O_2 were examined by western blot analysis. Representative data from three independent experiments are shown. (C) The activity of caspase-3/7 after a 1-min exposure of BHY cells to ozone PBS followed by a 6-h incubation period (caspase assay). Data are given as fold induction above the luminescence signal deriving from the PBS control [n = 3, mean ± standard deviation (SD)] (left panel). As a positive control, a dose-dependent induction of apoptosis by tumor necrosis factor (TNF)/cyclohexamide (40 μM) is depicted (right panel).

Sp-1-binding activity, actin level, and apoptosis assays. An earlier study reported unchanged metabolic activity in mouse fibroblasts after contact with 4 μg ml^{-1} aque-

ous ozone (8). Two other reports also suggest a high biocompatibility of aqueous ozone: again, when applying rather low concentrations of aqueous ozone (2.5–3.5 μg ml^{-1}), irrigation of the root surface of avulsed teeth did not reveal a negative effect on periodontal ligament cell proliferation (17). A clinical report regarding the healing-accelerating effect of ozonated water (11–12 μg ml^{-1}) did not indicate detrimental effects on cells (18).

To rank ozone among the established antiseptics regarding cytotoxicity, a direct comparison between these substances was necessary. CHX induced clear toxic effects on BHY cells, and slight (2%) or no (0.2%) toxicity on HGF-1 cells. Stronger toxic effects of CHX on fibroblasts have been reported after a short exposure time using other viability assays, for example, amino acid incorporation (0.12% or 0.2% CHX for 1 or 30 min) (36). The mode of cytotoxicity of CHX has been explained by disruption of the cell membrane following non-specific electrostatic binding to the negative moieties of proteins and phospholipids (37). Exposure to NaOCl and to H_2O_2 resulted in marked cytotoxic effects, consistent with the literature linking the cytotoxicity with ATP depletion (also found here), interference with glycolytic and mitochondrial enzymes, and inhibitory effects on DNA synthesis (25, 38). The 24-h exposure to metronidazole revealed either only slight (BHY) or no (HGF-1) toxicity. This finding might fit with the results of an animal study that reported suppression of healing by topically administered metronidazole on burned skin and cytotoxic effects in mammalian cell culture (39).

In dentistry, the cytotoxicity of antimicrobials is clinically relevant only if contact with resident oral cells takes place. Cytotoxicity is not relevant when applying ozone gas onto carious tooth hard substance via a sealing suction system as a prerequisite to avoid inhalation. For root canal disinfection, cytotoxicity is relevant owing to probable contact with cells in the apical region. Nonetheless, ozone gas has performed well compared with the established endodontic irrigants (CHX 2%; NaOCl 5.25%, 2.25%; H_2O_2 3%), which show equal or even higher cytotoxic potentials than ozone. In addition, it is also possible that the ozone gas applied into the moist root canal, as currently performed with the HealOzone device (KaVo), dissolves in canal fluids, thereby resulting in aqueous ozone which then comes into contact with tissues. In contrast to ozone gas, aqueous ozone revealed essentially no toxic effects, demonstrating a higher biocompatibility than even CHX 0.2% (BHY), not to mention the high cytotoxicity of NaOCl and H_2O_2. The latter findings may be relevant in assessing the usefulness of ozone for endodontic disinfection, adjunctive periodontal treatment or caries-preventive mouth rinse.

The relevance of ozone for the treatment of oral diseases is currently the topic of intensive investigation requiring further clinical evidence (12–15). The present research efforts can be differentiated into clinical trials, so far mainly covering fissure and root caries treatment (6, 12, 16), and *in vitro* studies dealing with caries, and endodontic or periodontal pathogens, either in planctonic culture or associated in biofilms (4, 5, 7–11). The potential influence of ozone on the physical properties of tooth hard substances, such as bond strength or sealing ability, is also under investigation (40, 41).

Taken together, both gaseous and aqueous ozone have been reported to exert antimicrobial effects (1–11). In the present study, the aqueous form of ozone, as a potential antiseptic agent, showed less cytotoxicity than gaseous ozone or established antimicrobials under most conditions. Therefore, aqueous ozone fulfils optimal cell biological characteristics in terms of biocompatibility for oral application.

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