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ORIGINAL ARTICLE

Effect of ozone on periodontopathogenic species—an in vitro study

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Abstract The in vitro study was aimed to determine the effect of ozone on periodontopathogenic microorganisms. Ozone was generated for 6 s–2×24 s (corresponding to 0.56 mg–2×2.24 mg of ozone) against 23 mainly anaerobic periodontopathogenic species. Agar diffusion test was used as a screening method. Then, the killing activity was tested in a serum-free environment and with 25% *v/v* inactivated serum. Further, the effect of ozone on bactericidal activity of native serum was analyzed against *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, and *Aggregatibacter actinomycetemcomitans*. Agar diffusion test showed a high efficacy of ozone against microorganisms, especially against *Porphyromonas gingivalis*. This result was confirmed by the killing tests; most of the strains in a concentration of 10^5 were completely eliminated after twofold 18-s application of ozone. Only four of the six potentially "superinfecting" species (*Staphylococcus aureus*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Candida albicans*) survived in part. Addition of heat-inactivated serum reduced the killing rate of ozone by 78% after 6-s and by 47% after twofold 18-s exposures; no strain was completely eradicated after any application of ozone. The bactericidal effect of native serum was enhanced after application of ozone; no effect was visible on the included *A. actinomycetemcomitans* strain which was found to be completely resistant to the bactericidal action of serum. In conclusion, (a) ozone has a strong antibacterial activity against putative periodontopathogenic microorganisms, and (b) the bactericidal effect is reduced in the presence of serum. Ozone may have potential as an adjunctive application to mechanical treatment in periodontitis patients.

Keywords Ozone · Periodontitis · Anaerobes · Serum

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Introduction

Periodontal disease status impacts markedly on biofilm composition [1]. It is generally accepted that a small group of predominantly gram-negative anaerobic or microaerophilic bacteria is associated with initiation and progression of periodontitis. Organisms strongly implicated as etiologic agents of periodontitis include *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* [2]. Moreover, other species such as *Campylobacter rectus*, *Eubacterium nodatum*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Parvimonas micra,* and *Streptococcus constellatus* support pathogenesis of disease. *Eikenella corrodens*, enterobacteria, *Pseudomonas* spp., *Selenomonas* spp., and yeasts may play a role as superinfecting species [2, 3]. Nowadays, dental implants are widely used. Similar microbial patterns like in periodontitis are found in periimplant diseases [4, 5]. Subgingival bacterial biofilms are surrounded by gingival crevicular fluid (GCF), which is rich of serum [6]. Serum may inhibit efficacy of antimicrobials [7]. Contrary, bactericidal activity of complement being a constituent of native serum is a well-known mechanism in innate immunity.

Based on the impact of pathogens, antiinfective regimen is an important component in any treatment of periodontal and periimplant diseases. In preventing recolonization of bacteria, chlorhexidine digluconate is a widely used agent in periodontitis treatment [8, 9]. Antibiotics are recommended

for severe cases [10, 11]. As an alternative method, application of laser is discussed [12]. Another possibility might be ozone. Ozone is a naturally occurring compound consisting of three oxygen atoms. It is a powerful antimicrobial agent by destructing cell walls and cytoplasmic membranes of bacteria and fungi [13–15]. In vitro, ozone is active against salmonella contaminations on food, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Aspergillus fumigatus*, and *Candida albicans*. Ozone can enhance serum-mediated killing of bacteria [16].

As reviewed recently by Azarpazhooh and Limeback [17], application of ozone might be promising as a prophylactic antimicrobial prior to restorations and as a denture cleaner, contrary in vitro data are conflicting regarding endodontics, oral and maxillofacial surgery, oral diseases as well as implant therapy. Nearly nothing is known about application of ozone in treatment against periodontopathogens. Only one study using ozonated water showed a killing effect on *A. actinomycetemcomitans* and *Porphyromonas gingivalis* in vitro [18].

Therefore, the aims of the in vitro study were: (a) to determine the effect of ozone on microorganisms which are involved in the pathogenesis of periodontitis and periimplantitis and (b) to evaluate the possible influence of serum on the antibacterial efficacy of ozone.

Material and methods

The Prozone® device which is CE labeled (W&H, Bürmoos, Austria) was used according to the manufacturer's description. The manufacturer obligates the usage of an evacuation system with a power of 50 L/min at the application site; nevertheless, the device should be not used in patient having pacemakers and respiratory problems or in pregnant women. Times of 6, 12, 18, and 24 s were applied by using the Coro tips always in a distance of 2 mm to the surface. By using Coro tips, 140 ppm of ozone per minute is produced corresponding to 2.24 mg of ozone per 24 s. In periodontology, 18-s applications are recommended by the manufacturer. Controls were not exposed to any ozone.

Microorganisms

At a total, 23 different microbial strains were included in the study. Seventeen of these strains belong to species clearly involved in pathogenesis of periodontitis; the six others may play a role as potentially superinfecting species. The periodontopathogens were grouped into *Porphyromonas gingivalis* strains, *A. actinomycetemcomitans* strains, gram-positives as well as other gram-negatives. Three *Porphyromonas gingivalis* strains, three *A. actinomycetemcomitans* strains as well as two potentially superinfecting species originated from clinical samples obtained from patients with severe periodontitis (Table 1).

All the strains were precultivated 24–72 h prior to the experiments. Modified tryptic soy agar [19] and trypic soy agar (BD, Franklin Lakes, NJ, USA) were used as cultivation media. *A. actinomycetemcomitans* strains and the *Streptococcus constellatus* ATCC 27823 were always incubated with 5% CO_2, all potentially superinfecting species within normal atmospheric conditions and the other periodontopathogens anaerobically (80% N_2, 10%CO_2, 10% H_2), each at 37°C. The number of bacteria was adjusted to OD_{640nm}=0.1 which refers to 1.5×10^8 bacterial cells/mL. These suspensions were diluted to the necessary concentration in the experiments.

Agar diffusion test as a screening method

First, a defined amount of microbes (10^5) was spread on Wilkins-Chalgren agar plates (BD) and Mueller-Hinton agar plates (BD) respectively. For that, the suspension McFarland 0.5 was diluted 1:150, and each 100 µl is used. Prozone device using the Coro tips was placed in a distance of about 1–2 mm to the surface of the agar. Ozone was applied for 18 and 24 s. Further, in one case, the device was kept on its place and the spot of microorganisms was exposed to ozone for a second time (2×24 s). One part of the plate served as growth control. After an incubation time of 42 h (18 h aerobes), the effects including diameters of the inhibition zones were registered.

Quantitative killing activity of ozone on different microorganisms

A defined inoculum of microorganisms (10^5 and 10^3 in 10 µl NaCl 0.9% each) was given into 1.5-mL tubes. After a short centrifugation, the tubes were placed for 15 min in the laminar air flow to enable a bacterial layer containing a reduced amount of liquid. A complete dryness as recommended by the manufacturer was avoided; this would not represent an in vivo situation. Ozone was applied for 6, 12, 18, and 24 s. Two tubes were exposed to ozone for a second time (2×18 s and 2×24 s) and two served as controls (no exposure to ozone). The numbers of colony-forming units (cfu) were determined after addition of 100 µl NaCl 0.9% solution, mixing, serial dilution, and plating each 25 µl on agar plates and incubation in the appropriate atmosphere.

Influence of serum as an essential component of GCF on killing activity

Determinations of killing activity were repeated in the presence of 25% *v/v* serum (Sigma-Aldrich, Steinheim, Germany). Serum was heat-inactivated (30 min at 56°C) to

Table 1 Tested microorganisms and groupings

Species	Origin	Group
Porphyromonas gingivalis ATCC 33277	Laboratory strain	*Porphyromonas gingivalis*
P. gingivalis M5-1-2	Clinical isolate	*Porphyromonas gingivalis*
P. gingivalis MaRL	Clinical isolate	*Porphyromonas gingivalis*
P. gingivalis J430-1	Clinical isolate	*Porphyromonas gingivalis*
Aggregatibacter actinomycetemcomitans ATCC 33384	Laboratory strain	*Aggregatibacter actinomycetemcomitans*
A. actinomycetemcomitans J1	Clinical isolate	*Aggregatibacter actinomycetemcomitans*
A. actinomycetemcomitans J2	Clinical isolate	*Aggregatibacter actinomycetemcomitans*
A. actinomycetemcomitans J7	Clinical isolate	*Aggregatibacter actinomycetemcomitans*
Campylobacter rectus ATCC 33238	Laboratory strain	Other gram-negatives
Capnocytophaga gingivalis ATCC 33624	Laboratory strain	Other gram-negatives
Eikenella corrodens ATCC 23834	Laboratory strain	Other gram-negatives
Fusobacterium nucleatum ATCC 25586	Laboratory strain	Other gram-negatives
Prevotella intermedia ATCC 25611	Laboratory strain	Other gram-negatives
Tannerella forsythia ATCC 43037	Laboratory strain	Other gram-negatives
Eubacterium nodatum ATCC 33099	Laboratory strain	Gram-positives
Parvimonas micra ATCC 33270	Laboratory strain	Gram-positives
Streptococcus constellatus ATCC 27823	Laboratory strain	Gram-positives
Enterobacter cloacae JGr1	Clinical isolate	Potentially "superinfecting" strains
Klebsiella pneumoniae JGr2	Clinical isolate	Potentially "superinfecting" strains
Pseudomonas aeruginosa DSM 50071	Laboratory strain	Potentially "superinfecting" strains
Enterococcus faecalis ATCC 29212	Laboratory strain	Potentially "superinfecting" strains
Staphylococcus aureus ATCC 29213	Laboratory strain	Potentially "superinfecting" strains
Candida albicans ATCC 76615	Laboratory strain	Potentially "superinfecting" strains

exclude the bactericidal effect by complement. In these experiments, only two times (6 s, 18 s) and one repeated exposure (2×18 s) as well as one microbial concentration (10^5) were used in dependence of the results obtained before in determination killing activity of ozone without serum.

Effect of ozone on serum-mediated killing on selected bacterial species

Selected bacterial species (*Porphyromonas gingivalis* ATCC 33277, *A. actinomycetemcomitans* J7, *F. nucleatum* ATCC 25586; each 10^7/10 μl NaCl 0.9%) were exposed for 12 s to ozone before addition of 100 μl serum 25% *v*/*v* in NaCl 0.9% solution. The used higher microbial concentration was necessary to achieve about 10^5 viable cells after treatment with ozone. After an incubation of 15 min at 37°C in an anaerobic atmosphere, the numbers of cfu were determined. NaCl 0.9% solution with inactivated serum as well as samples without exposure to ozone served as controls.

All experiments were made in independent duplicates at least. Statistical analysis was made by using paired Student's *t* test and ANOVA with post-hoc Bonferroni.

Results

Agar diffusion test

In mean, the diameters of the inhibitions zones were 2.87±4.42 mm after an application of ozone for 18 s, 5.41±5.84 mm for 24 s, and 11.0±8.49 mm for the twofold 24-s application of ozone (Fig. 1). Inhibition zones with>20 mm were found against four strains by using the highest exposure of ozone. No inhibition was seen against 15 strains by using the lowest exposure, 10 strains after the moderate exposure, and three strains after the highest exposure of ozone, respectively. The highest inhibition zones were measured after exposing ozone against *Porphyromonas gingivalis* and the smallest were observed on the plates with capnophilic and anaerobic gram-positive bacterial strains (data not shown).

Killing by ozone

Ozone was efficient in killing the tested microorganisms. In mean, the killing rate was more than 90% after exposure times of 12 s for 10^3 and 18 s for 10^5 microorganisms (Fig. 2). A significant difference between the two used

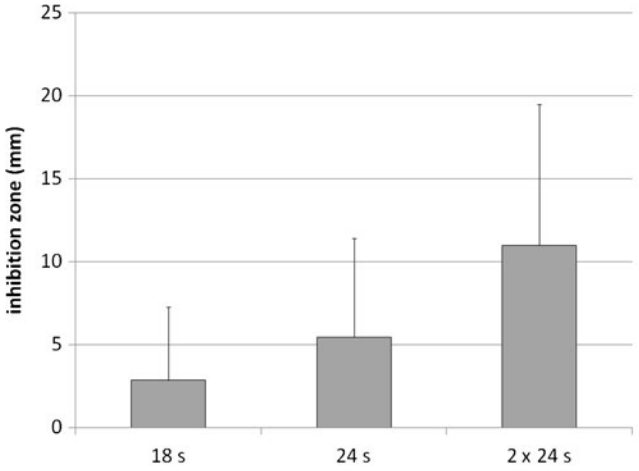

Fig. 1 Inhibition zones (mean and standard deviation) for all tested microbial strains after exposure of 16 s, 24 s as well as 2×24 s to ozone (24 s corresponds to 2.24 mg of ozone). Microbes were spread on agar plates. Prozone device was placed in a distance of about 1–2 mm to the surface. After application of ozone and an incubation time of 42 h (18 h aerobes) the effect including diameters of the inhibition zones were registered

concentrations of the microbial strains was not found at any exposure time.

Following, only results using the 10^5 microorganisms are presented.

Ozone completely eradicated most of the strains in a concentration of 10^5 after twofold 18-s application of ozone. Only four of the six potentially "superinfecting" species survived in part (Table 2).

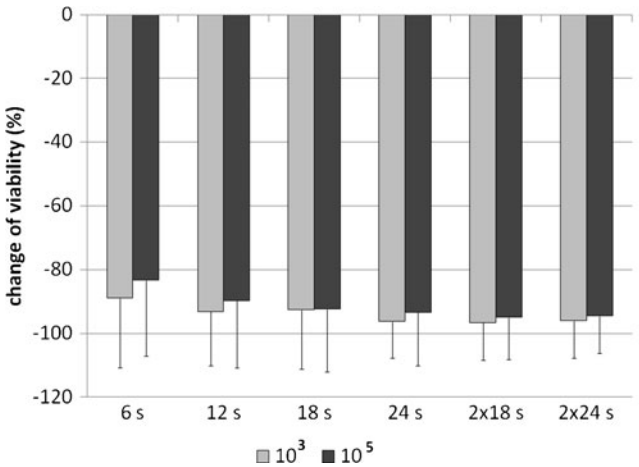

Fig. 2 Changes in viabilities of microorganisms (mean and standard deviation) by different time of exposure to ozone for all tested microorganisms. Ozone was applied to 10^3 and 10^5 microorganisms/10 µl 0.9% NaCl in tubes for 6, 12, 18, and 24 s as well as 2×18 and 24 s (24 s corresponds to 2.24 mg of ozone). The references were microorganisms without exposure to ozone (median controls: 1,220 cfu for testing 10^3 microorganisms and 79,000 cfu for testing 10^5). The numbers of cfu were determined after addition of 100 µl NaCl 0.9% solution and plating different dilutions on agar plates

Porphyromonas gingivalis was killed after any application of ozone. *A. actinomycetemcomitans* was eliminated nearly completely after 18-s exposure of ozone. The other gram-negative anaerobic and capnophilic periodontopathic strains were also highly susceptible to ozone. Only in tests with 10^5 of *F. nucleatum* ATCC 25586, the twofold 18- or 24-s application of ozone was necessary to eradicate totally that strain. The gram-positive anaerobic and capnophilic periodontopathic strains were killed to 100% by ozone in general. The potentially "superinfecting" species were less sensitive to ozone. Most resistant were the *Enterobacter cloacae* JGr1 and the gram-positive bacterial strains (killing rates of 10^5 microorganisms and twofold 18-s exposure to ozone: *Enterobacter cloacae* JGr1 51%, *Enterococcus faecalis* ATCC 29212 76%, *Staphylococcus aureus* ATCC 2921 62%). Further, *Candida albicans* ATCC 76615 survived in part after the twofold 18-s exposure to ozone (killing rate 94%). The results for 6 s, 18 s as well as 2×18 s of exposure to ozone are presented in Fig. 3; at all time-points, differences between the microbial groups were significant (6 s: $p=0.002$; 18 s: $p=0.035$; 2×18 s: $p=0.037$).

Killing in the presence of serum

Although adjusting the same concentration at the beginning of the experiments, cfu counts were higher in the control samples with serum showing a protective influence of serum on viability of bacteria. Addition of inactivated serum reduced clearly the killing rate of all selected bacterial strains by ozone. No strain was completely eradicated after any application of ozone. The killing rate was descreased by 78% after 6-s and by 47% after twofold 18-s exposures to ozone (each $p<0.001$). Twofold 18-s application enhances the killing of ozone compared to onefold usage (killing rate for all strains after twofold 18-s application, 50±25%; after onefold 18-s application, 34±26%; $p<0.001$; Fig. 4).

Also the killing rate of *Porphyromonas gingivalis* was lowered in the presence of serum. Nevertheless, 72% of the bacteria were killed after exposure of twofold 18 s of ozone. Contrary to *Porphyromonas gingivalis*, only 50% of *A. actinomycetemcomitans* and of the included other gram-negative anaerobic and capnophilic periodontopathic strains were killed after twofold 18-s exposure of ozone. Serum also reduced the efficacy of ozone against gram-positive capnophilic and anaerobic periodontopathic bacteria by about 40%. After the twofold 18-s application of ozone, only 28% of the microbial cells of the potentially superinfecting species were killed in the presence of serum (Figs. 3 and 4). Most resistant was *Candida albicans* ATTC 76615 (11%), followed by the gram-positives (*Enterococcus faecalis* ATCC 29212 (27%), *Staphylococcus aureus* ATCC 2921 (28%)) and *Klebsiella pneumoniae* JGr2 (27%); more sensitive were the *Pseudomonas aeruginosa*

Table 2 Number of strains (10^5 microorganisms) which were totally killed after different times of exposure to ozone

Group of microbes (number of strains)	Application of ozone					
	6 s	12 s	18 s	2×18 s	24 s	2×24 s
P. gingivalis (4)	4	4	4	4	4	4
A. actinomycetemcomitans (4)	0	2	3	4	3	4
Other gram-negatives (6)	4	5	5	6	5	6
gram-positives (3)	2	2	3	3	3	3
"Superinfecting" species (6)	0	1	2	2	2	2

DSM 50071 (38%) and the *Enterobacter cloacae* JGr1 (39%) strains.

Effect of ozone on bactericidal activity of serum

First, microbial concentration and exposure time of ozone had to be chosen which did not destroy all cells. In using *Porphyromonas gingivalis* ATCC 33277 and *F. nucleatum* ATCC 25886, bactericidal killing of serum was enhanced after application of ozone. No effect was visible on *A. actinomycetemcomitans* J7 which was found to be resistant to any effect of serum (Table 3).

Discussion

Ozone is used for different purposes in medicine. It may reduce acute back pain [20] and inflammation in venous ulcer [21]. Ozone is widely used for disinfection of drinking water; it can inactivate influenza viruses [22], bacilli [23], and *Escherichia coli* [24]. In dentistry, ozone was discussed in endodontic treatment since 1951 [25]; nevertheless, clinical studies are missing. Ozone was found to prevent white spot lesions in orthodontic patients [26] and to stop or reverse fissure and root caries lesions [27, 28]. Knowledge about a possible usage of ozone in periodontitis and periimplantitis is extremely limited.

Here, the effect of ozone on periodontopathogens was studied. As shown by agar diffusion test and confirmed by the killing assays, ozone was highly efficient in combating these species. Species-specific aspects were found. Ozone was more active against the major periodontopathogens, compared to the potentially super-infecting species. Especially, *Porphyromonas gingivalis* was very susceptible to the action of ozone. Although expecting a toxic effect of oxygen molecules against anaerobes or capnophilic species, data about an efficacy of ozone against anaerobes or capnophilic species are extremely rare. Nagayashi et al. [18] studied the effect of ozonated water on selected oral species; the included *Porphyromonas gingivalis* strain was extremely sensitive and even more than oral streptococci and *A. actinomycetemcomitans*. Thus, our results referring the relation *Porphyromonas*

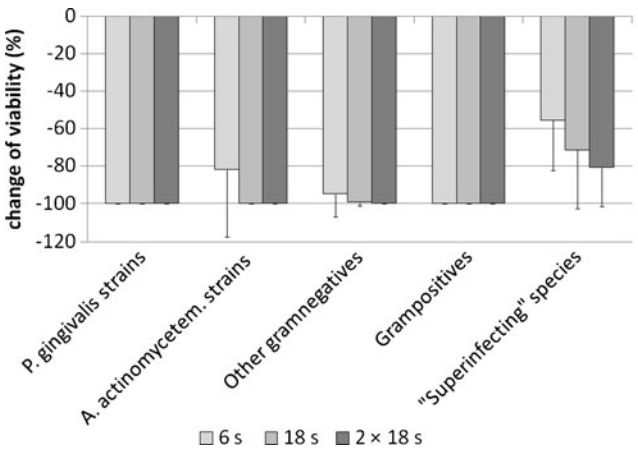

Fig. 3 Changes in viabilities (mean and standard deviation) of different groups of microorganisms in concentrations of 10^5 by 6 s, 18 s as well as 2×18 s of exposure to ozone (18 s corresponds to 1.68 mg of ozone)

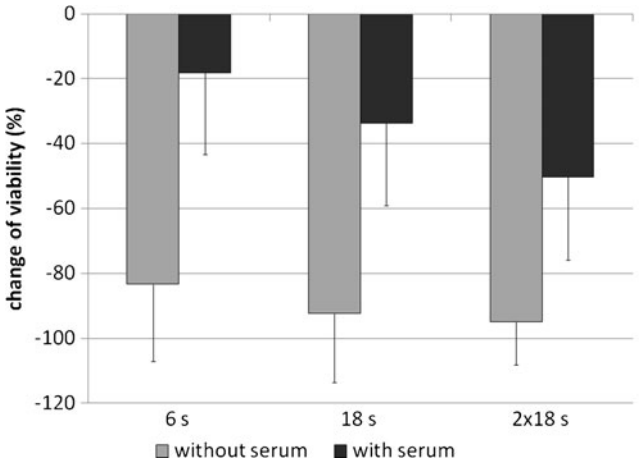

Fig. 4 Influence of 25% *v/v* serum (inactivated) on changes in viabilities (mean and standard deviation) by 10^5 by 6 s, 18 s as well as 2×18 s of exposure to ozone for all tested microorganism (18 s corresponds to 1.68 mg of ozone). Ozone was applied to 10^5 microorganisms per 10 µl NaCl 0.9% with 25% *v/v* serum. The references were microorganisms without exposure to ozone (median of 79,000 cfu without serum and 174,000 cfu with serum). The numbers of cfu were determined after addition of 100 µl NaCl 0.9% solution and plating different dilutions on agar plates. The respective results without serum are shown in Fig. 2

Table 3 Serum-mediated killing without and after 12-s application of ozone

	Bactericidal effect of serum without ozone (%)	Bactericidal effect of serum with ozone (%)
P. gingivalis ATCC 33277	21.15±4.53	61.90±13.47
A. actinomycetemcomitans J7	−12.62±17.25	−20.04±17.21
F. nucleatum ATCC 25886	22.59±28.81	86.24±3.81

gingivalis and *A. actinomycetemcomitans* and using gaseous ozone confirm this. Another study found *Parvimonas micra* as a species involved in root canal infections to be highly susceptible [29]. The destroying potential of radical ions of oxygen as another oxygen species was more pronounced on *F. nucleatum*, *Porphyromonas gingivalis*, and *Prevotella intermedia* than on *Streptococcus sobrinus* [30].

The potential superinfecting species, especially the gram-positive species *Staphylococcus aureus* and *Enterococcus faecalis* as well as *Candida albicans*, were less sensitive in general. Data about a slightly higher efficacy of ozone against *Pseudomonas aeruginosa* in comparison to *Staphylococcus aureus* and *Enterococcus faecalis* [31] were confirmed. In another study, *Enterococcus faecalis* was found to be less sensitive to ozone than to common disinfectants [32]. In endodontic infections where *Enterococcus faecalis* is a critical species [33], the effect of ozone was limited in an ex vivo model [34].

Gingival crevicular fluid contains up to 35% of the albumin found in serum [35]. Serum proteins may bind to antimicrobials and, following this, inhibit the activity of different antibiotics [36] and disinfectants [37]. Bacteria can use serum as a nutrient source [38] which may explain higher cfu counts of controls in experiments with serum. Data about the influence of serum on the activity of ozone are different. Ten percent bovine fetal serum did not interfere with the activity of ozone [31]; whereas 5–10% of human plasma reduced in a concentration-dependent manner the killing activity of ozone [39]. Our results also clearly indicate that efficacy of ozone is decreased in the presence of 25% *v/v* human serum, but still killing was found up to 90% in dependence of the strain, especially after a twofold 18-s application of ozone (Fig. 5).

Serum proteins might be essential compounds of subgingival biofilm matrix which are highly organized and adapted communities [40]. Although in this study the efficacy of ozone on biofilms was not tested, a limited effect can be assumed. It has been shown that ozone kills moderately efficiently cariogenic bacteria in saliva [41] and the effect is minimal in a cariogenic biofilm using saliva as protein source [42]. Further studies are needed to evaluate the possible effect of ozone against periodontopathogenic bacteria within biofilm. The reduced antibacterial activity of ozone in the presence of serum together with its anticipated limited effect against microorganisms within biofilm may suggest the adjunctive application of ozone to mechanical therapy in periodontitis patients.

An enhanced bactericidal activity of serum as described before [16], probably due to membrane damage by ozone leading to a more rapid penetration of the membrane attack complex of complement [43] was found for two of the three strains. Complement was found to be activated within gingival fluid [44], suggesting a possible clinical relevance of the findings. The third included strain, *A. actinomycetemcomitans* J7, was highly resistant against serum; this did not change after the application of ozone. Resistance of many periodontopathogens against killing by complement is well known [45, 46]; a complete resistance is probably not abolished by ozone.

It may be also suggested that ozone influences host response. Host response to biofilm plays a critical role in the pathogenesis of periodontitis [47]; many proinflammatory cytokines are involved in inflammation and bone loss [48]. Resolution of inflammation may be supported by ozone; ozone was found to inhibit NFκB-pathways [49] as well to increase the phagocytotic capacity of bovine PMNs in inflammation [50]. Ozone was shown to be toxic to human oral epithelial cells and gingival fibroblasts but in a lower extent than different antimicrobials [51]. The possible modulatory properties of host response by ozone might be

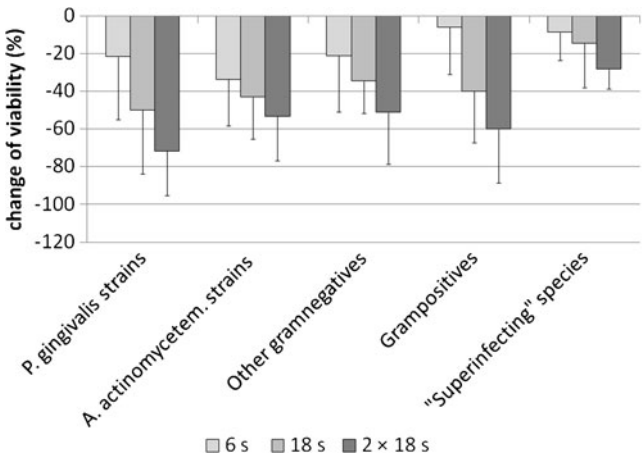

Fig. 5 Influence of 25% *v/v* serum (inactivated) on changes in viabilities of different groups of microorganisms in concentrations of 10^5 by 6 s, 18 s as well as 2×18 s of exposure to ozone (18 s corresponds to 1.68 mg of ozone)

of importance in periodontitis and should be specified in vitro and in vivo in this context.

Based on the present findings, it can be concluded that: (a) ozone has a strong antibacterial activity against anaerobic periodontal pathogenic microorganisms, (b) the bactericidal effect is reduced in the presence of serum, and, following, (c) ozone may have potential as an adjunctive application to mechanical treatment in periodontitis patients.

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Conflict of interest The authors declare that they have no conflict of interest.

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