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Antimicrobial Effect of a Novel Ozone-Generating Device on Micro-Organisms Associated with Primary Root Carious Lesions in vitro

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Key Words
Micro-organisms · Ozone · Root caries

Abstract
The aims of this present study were (1) to assess the antimicrobial effect of ozone from a novel ozone-generating device (Heolozone, USA) [0.052% (v/v) in air delivered at a rate of 13.33 ml·s^{-1}] on primary root carious lesions (PRCLs) and (2) to evaluate the efficacy of ozone specifically on *Streptococcus mutans* and *Streptococcus sobrinus*. In study 1, 40 soft PRCLs from freshly extracted teeth were randomly divided into two groups to test the antimicrobial effect on PRCLs from exposure to ozonated water for either 10 or 20 s. Half of a lesion was removed using a sterile excavator. Subsequently, the remaining lesion was exposed to the ozonised water for a period of either 10 or 20 s (corresponding to 0.069 or 0.138 ml of ozone, respectively). Using paired Student t tests, a significant ($p<0.001$) reduction (mean ± SE) was observed in the ozone-treated groups with either a 10-second ($\log_{10}$ 3.57±0.37) or 20-second ($\log_{10}$ 3.77±0.42) ozone application compared with the control groups ($\log_{10}$ 5.91±0.15 and $\log_{10}$ 6.18±0.21, respectively). In study 2, 40 sterile saliva-coated glass beads were randomly divided into two groups for each micro-organism. One glass bead was put into each bijou bottle with 3 ml of Todd-Hewitt broth. *S. mutans* and *S. sobrinus* were inoculated anaerobically overnight. Each glass bead was then washed with 2 ml of phosphate-buffered saline. Immediately, 10 s of ozone gas was applied to each glass bead in the test groups. There was a significant ($p<0.0001$) reduction (mean ± SE) in ozone-treated samples for *S. mutans* ($\log_{10}$ 1.01±0.27) and *S. sobrinus* ($\log_{10}$ 1.09±0.36) compared with the control samples ($\log_{10}$ 3.93±0.07 and $\log_{10}$ 4.61±0.13, respectively). This treatment regime is an effective, quick, conservative and simple method to kill micro-organisms in PRCLs. Ozone gas application for a period of 10 s was also capable of reducing the numbers of *S. mutans* and *S. sobrinus* on saliva-coated glass beads in vitro.

Root caries is an emerging challenge to the dental profession as the risk factors for developing root caries point to both intra-oral and environmental factors, making management complex and multidisciplinary [Shay, 1997]. The microbiology of primary root carious lesions (PRCLs) is well established [Beighton et al., 1993; Lynch and Beighton, 1994]. The primary initiating agent of root caries is generally accepted to be *Streptococcus mutans* [Sumney et al., 1973; Billings et al., 1985; Brown et al., 1986; van Houte et al., 1990], although animal models and clinical data suggest

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strongly adjunctive roles for *Lactobacillus* and *Actinomyces* species [Bowden, 1990; Beighton et al., 1993; Ravald, 1994; Schüpbach et al., 1995]. Based on numerous microbiological studies performed over the past three decades, it is clear that Mutans streptococci and lactobacilli are high-risk factors. Soft lesions are the most severe type of PRCLs according to the clinical severity index and contain a great number of micro-organisms. Observations suggest that soft lesions can undergo remineralisation and may become hard under favourable conditions [Hellyer and Lynch, 1991].

As an alternative management strategy, ozone may be considered. The powerful ability of ozone to inactivate micro-organisms has led to its use in water purification [Pope et al., 1984] and there is growing evidence that it can be employed as a useful therapeutic agent in both dentistry and medicine.

S. mutans and *Streptococcus sobrinus* were chosen as test micro-organisms. These are the most common species of Mutans streptococci in humans. *S. sobrinus* seems to be more acidogenic than the other species of mutans streptococci [de Soet et al., 1989; Köhler et al., 1995]. Lundgren et al. [1998] showed that the prevalence of root caries was significantly higher in *S. sobrinus* carriers than in non-carriers. Moreover, numbers of root carious lesions were higher in patients who harboured both *S. mutans* and *S. sobrinus* than those who harboured only *S. mutans*. Therefore, application of ozone was evaluated specifically on these two micro-organisms in vitro and further in vitro studies will test the susceptibility of ozone for all different microbial species from PRCLs.

The aims of this present study was (1) to assess the antimicrobial effect of ozone from a novel ozone-generating device on PRCLs and (2) to evaluate the efficacy of ozone specifically on *S. mutans* and *S. sobrinus* in vitro.

Materials and Methods

The Ozone Delivery System

In this study, a novel ozone-generating device (Heolozone, Tonawanda, NY, USA) [0.052% (v/v) in air delivered at a rate of 13.33 ml. s^{-1}] was employed. This portable ozone generator produced ozonated water/ozone gas through a nozzle, which was connected to the device. Ozonated water/ozone gas was passed through a suction system using a granular activated carbon filter to remove all traces of ozone after contact with the carious lesions. Ozone gas was delivered into the middle of the lesion with a shroud of water surrounding it. The ozone generator conforms to all European Union legislation covering medical devices (93-42-EEC), as no ozone is detectable in the surrounding air during use, and also with the more stringent regulations applicable to equipment in the office environment (COSHH EH40-96).

Study Design

Study 1

40 freshly extracted teeth with soft PRCLs requiring restorations were collected from the Department of Oral and Maxillofacial Surgery at St. Bartholomew's and the Royal London School of Medicine and Dentistry. Immediately after extraction, these teeth were randomly divided into two groups to test the antimicrobial effect on PRCLs from exposure to ozonated water for either 10 or 20 s (corresponding to 0.069 or 0.138 ml of ozone, respectively). Plaque was removed using a hand-held standard fine nylon fibre sterile toothbrush with water as a lubricant. Each tooth was dried using dry sterile cotton wool rolls and a dental 3 in 1 air syringe. Half of each lesion was removed using a sterile excavator. The excavator blade was used to traverse the lesion in line with the long axis of the tooth across the maximum gingival/occlusal dimension. Following sampling, water without ozone from the ozone generator was applied to the samples in the control group. Subsequently, the remaining lesion was exposed to the ozonised water for a period of either 10 or 20 s at room temperature (23 °C) and a further sample was taken. Each sample was immediately put into a preweighed sterile vial and weighed. 1 ml of fastidious anaerobe broth (FAB, Lab M Ltd., FAB Bury, Lancs, UK) with sterile glass beads (3.5-4.5 mm in diameter, BDH, Poole, Dorset, UK) was added to these vials and vortexed for 30 s to facilitate the extraction of any micro-organisms from carious dentine and disperse any aggregates. Dilutions were performed by transferring 1 ml of the resulting suspensions into 9 ml of FAB and this process was repeated in 10-fold dilutions to 10^4. After decimal dilution with FAB, 100-µl aliquots (for both test and control groups) were spread on fastidious anaerobe agar (Lab M, Bury, Lancs, UK) supplemented with 5% (v/v) horse blood and placed in an anaerobic chamber at 37 °C for 4 days. The total number of colony-forming units (cfu) was calculated.

Study 2

Test Micro-Organisms. *S. mutans* (NCTC 10449) and *S. sobrinus* (TH 21) were maintained by subculturing on 5% blood agar (Oxoid, Basingstoke, UK) every 7 days. Cultures were grown anaerobically for 16 h at 37 °C in 5 ml Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, Md., USA).

Preparation of Saliva-Coated Glass Beads. 5 ml of unstimulated human saliva from 1 donor was collected into a sterile container for each experiment. 1 ml of saliva was clarified by centrifugation for 2 min. The saliva supernatant was pipetted into a sterile universal bottle and filtered using 0.45- and 0.2-µm filters. Three glass beads (3.5-4.5 mm in diameter, BDH, Poole, Dorset, UK) were put into a sterile bijou bottle. Immediately, 0.5 ml filtered saliva was added and left for 5 min.

Test procedure. 40 sterile saliva-coated glass beads were randomly divided into two groups (test and control) for *S. mutans* and *S. sobrinus*. Each glass bead was put into a sterile bijou bottle with 3 ml of Todd-Hewitt broth for control and test groups and agitated for 2 s. Each bijou was inoculated with either *S. mutans* or *S. sobrinus* and inoculated anaerobically overnight. The glass beads were then washed with 2 ml of PBS and transferred to sterile bijoux. Immediately, ozone gas was applied for 10 s to each glass bead in the test groups for either *S. mutans* or *S. sobrinus* at room temperature (23 °C) while shaking the bijoux to deliver the ozone gas to all surfaces of the test glass beads, whilst control glass beads for each micro-organism were left in the sterile bijoux and shaken for 10 s. Subsequently, these glass beads (control and test groups) were placed in 3 ml of Todd-Hewitt broth

with six more sterile glass beads and vortexed for 30 s. Samples were serially diluted with FAB up to 10^8, and 100-µl aliquots were spread on blood agar plates supplemented with 5% (v/v) horse blood and placed in an anaerobic chamber at 37 °C for 2 days. The number of each colony type was counted and calculated.

Statistical Analyses

Microbiological counts from test and control groups for each study were transformed as $\log_{10}$ (cfu + 1) prior to statistical analyses in order to normalise their distributions. Statistical analyses of the data were obtained by paired Student t tests to determine differences between test and control groups, with the threshold of significance chosen at 0.05 for each study. Means and standard errors were also recorded for each experimental condition. All analyses were performed using the SPSS statistical package for MS Windows 6.1.

Results

Study 1

The total cultivable microflora was assessed by counts of the colony-forming units recovered. The colony-forming units were divided by the samples' weights. There was a significant ($p<0.001$) difference between the control and test samples for either 10 or 20 s in $\log_{10}$ (cfu + 1)/mg (table 1).

Study 2

The means ± SE of test and control groups for both micro-organisms are shown in table 2. There was a significant ($p<0.0001$) reduction in ozone-treated samples compared with the control samples for both micro-organisms.

Discussion

It was demonstrated that exposure of carious dentine to ozone produced by a novel ozone-generating device for periods of either 10 or 20 s substantially reduced the levels of total micro-organisms to <1% of the control values.

Ozone exposure of PRCLs consistently achieved microbial reduction in the test groups regardless of the application time. The rapid inactivation of micro-organisms is one of ozone's outstanding characteristics. Presumably, ozone dissipates quickly in water [Bocci et al., 1993] and kills micro-organisms via a mechanism involving the rupture of their membranes in the lesions. In this study, the mechanism of killing of ozone on PRCLs was not investigated. However, Yamayoshi and Tatsumi [1993] demonstrated that ozone was a strong oxidiser to cell walls and cytoplasmic membranes of bacteria. Such an ozone solution could be used to disinfect medical instruments and similar equipment.

Table 1. Mean ± SE $\log_{10}$ (cfu + 1)/mg in PRCLs before and after ozone application for either 10 or 20 s

Groups	10 s $\log_{10}$ (cfu + 1)/mg	20 s $\log_{10}$ (cfu + 1)/mg
Control samples	8.99 ± 0.39	9.19 ± 0.36
Test samples	6.79 ± 0.39	6.31 ± 0.12

Table 2. Mean ± SE $\log_{10}$ cfu before and after ozone application for *S. mutans* and *S. sobrinus*

Groups	*S. mutans*	*S. sobrinus*
Control	3.92 ± 0.07	4.61 ± 0.13
Test	1.01 ± 0.27	1.09 ± 0.36

Saliva-coated glass beads were used to demonstrate the efficacy of ozone specifically on *S. mutans* and *S. sobrinus* with the bacteria adherent to a solid surface as in the oral environment. The use of PBS eliminated the effect of reductants present in culture media.

Results in test groups obtained from saliva-coated glass beads showed a greater reduction compared with the carious dentine samples following ozone application. It can be speculated that in PRCLs, ozone clearly interacted with the abundant organic material in the lesions, which may reduce the antimicrobial effect of ozone. However, there were less organic materials from some salivary proteins on the glass beads than in PRCLs.

Bocci [1992] suggested that treating human blood with low ozone concentrations for the management of vascular disorders, chronic viral and autoimmune diseases can actually activate cells of the immune system and this treatment regime would be beneficial. However, it should be noted that ozone concentrations and time of exposure should be considered. In principle, the potential toxicity of ozone should not prevent its use as a therapeutic agent. At the correct dose, ozone can be useful as a therapeutic agent. Furthermore, Bocci [1994] reported that human blood treated with the correct dose of ozone can minimise the formation of free radicals and convert oxidants to less toxic species.

Ozone is approximately 10 times more soluble in water than oxygen. Mixed into pyrogen-free water, the half-life of ozone is 9–10 h (at pH 7 and 20 °C), and at 0 °C, this value is doubled. Ozonated water was effective in dental surgery where it was reported to promote haemostasis, enhance local oxygen supply and inhibit bacterial proliferation. There-

fore, ozonated water was employed as a mouthrinse during dental surgery, or following tooth extraction procedure [Türk, 1985; Filippi, 1997].

This proposed treatment regime by the employment of an ozone generator to kill the micro-organisms associated with PRCLs may therefore be considered to be potentially an effective alternative to conventional 'drilling and filling' for the management of primary root caries. This ozone generator system derived for in vivo uses a shroud of water, which immediately dissipates the ozone and does not permit any ozone to be detected in the oral cavity or surrounding area.

In conclusion, ozone exposure for either 10 or 20 s under the experimental conditions reduced micro-organisms in the PRCLs. Ozone application for a period of 10 s was also capable of reducing the numbers of *S. mutans* and *S. sobrinus* on saliva-coated glass beads in vitro. Further in vitro studies with different species and clinical trials designed to investigate the effects of ozonised water compared to conventional 'drilling and filling' are now in progress.

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