SCIENTIFIC ARTICLE

Australian Dental Journal 2008; 53: 349–353

doi: 10.1111/j.1834-7819.2008.00077.x

The inability of *Streptococcus mutans* and *Lactobacillus acidophilus* to form a biofilm *in vitro* on dentine pretreated with ozone

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ABSTRACT

Background: The use of ozone therapy in the treatment of dental caries is equivocal. The aim of this study was to use an *in vitro* model to determine the effects of prior ozone application to dentine on biofilm formation and to measure any associated reduction in bacteria viability.
Methods: Twenty dentine discs were bonded to the bases of 5 mL polycarbonate screw top vials. Ten dentine discs were infused with ozone for 40 seconds, 10 samples remained untreated as a control. The vials were filled with nutrient medium, sterilized and placed into the outflow from a continuous chemostat culture of *Streptococcus mutans* and *Lactobacillus acidophilus* for four weeks. At the conclusion of the experiment bacterial growth was monitored by taking optical density readings of the growth medium in each vial and the outer surface of the dentine specimens were examined by scanning electron microscopy as shown by SEM analysis.
Results: Ozone infusion prevented biofilm formation on all the treated samples while there was substantial biofilm present on the control specimens. While the average optical density of the control specimens was almost twice that of the ozone infused dentine (0.710 for the control with a SD of 0.288 and 0.446 for the ozonated samples with a SD of 0.371), the results were not significant ($p > 0.05$).
Conclusions: This preliminary study has shown that the infusion of ozone into non-carious dentine prevented biofilm formation *in vitro* from *S. mutans* and *L. acidophilus* over a four-week period. The possibility exists that ozone treatment may alter the surface wettability of dentine through reaction with organic constituents.

Key words: Dentine, *Streptococcus mutans, Lactobacillus acidophilus,* ozone, biofilm.

(Accepted for publication 3 April 2008.)

INTRODUCTION

The formation of a biofilm overlying tooth structure is essential for the initiation and progression of caries.[1,2] *Streptococcus mutans* and *Lactobacillus acidophilus* are present in cariogenic biofilms and play a significant role in the carious process.[3] The treatment of dentine surfaces to prevent biofilm formation and reduce bacteria growth may assist in the prevention of caries initiation and progression.[4]

The use of ozone therapy in the treatment of dental caries is equivocal and literature reviews suggest there is insufficient evidence to regard ozone treatment as a viable alternative to the current management and treatment of dental caries.[5,6] Opposing this view are studies showing both *in vitro*[7] and *in vivo*[8] reduction of viable bacteria in primary root carious lesions and there is *in vitro* evidence showing ozonated water decreased levels of *S. mutans* in an experimental dental plaque.[9]

The aim of this study was to use an *in vitro* model to determine the effects of biofilm formation on dentine and any reduction in bacteria viability by the application of ozone prior to placing dentine specimens into the outflows from two chemostats inoculated respectively with *S. mutans* and *L. acidophilus*.

MATERIALS AND METHODS

Preparation of dentine slabs

The crowns of 20 recently extracted human third molar teeth had been stored in 0.5% chloramine. Horizontal mid-coronal sections were made to produce enamel and dentine sections approximately 1.5 mm thick. Only

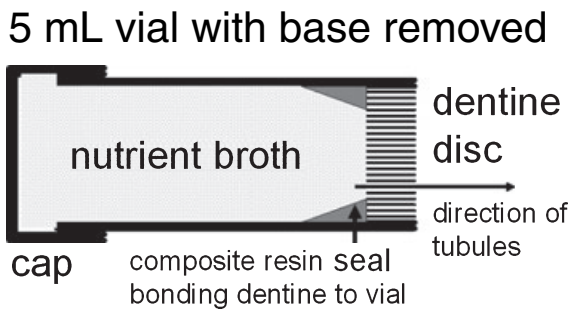

Fig 1. 5 mL vial showing the various components.

sections with flat, sound dentine on either surface were used. Teeth were collected within the guidelines set by the Committee for the Ethics of Human Experimentation, University of Adelaide.

A rim of composite resin (Glacier SDI, Melbourne, Australia) was bonded to the etched outer enamel surface after which the sections were reduced to 1 mm in thickness using a graded series of wet and dry papers to 4000 grit Silicone Carbide paper (Struers, Denmark).

Construction of the bacterial migration model apparatus

Twenty 5 mL polycarbonate vials were prepared by removing the base of each vial, leaving the lip for added retention. The cut bases were then roughened using air abrasion (Rondoflex, Kavo, Germany) (50 micron Al_2O_3 particles) around the base and over the inner lip of the container.

Specimens were attached onto the base by first etching with 37% phosphoric acid (SDI), washing and drying with oil-free air prior to applying resin bond (3M ESPE, Minnesota, USA) and then securing with composite resin (Glacier) to ensure there was no leakage through the composite seal[10] (Fig 1).

Pretreatment of dentine prior to experimentation

Samples were treated as follows: (1) 10 samples were infused with ozone on the outer surface for 40 seconds using a HealOzone 2130C device (KaVo Dental, Biberach, Germany); and (2) 10 samples had no treatment and were used as a control.

Micro-organisms

Streptococcus mutans Ingbritt was obtained from B Krasse, University of Gotëborg, Sweden. It was maintained on tryptone soy agar plates (Oxoid, Basingstoke, UK) grown at 37°C in an atmosphere of $N_2/CO_2/H_2$ (90:5:5). Starter cultures (200 ml) were grown in 3% tryptone soya broth (Oxoid, Basingstoke, UK), yeast extract 0.5% (Oxoid, Basingstoke, UK) and 20% sucrose and, after overnight incubation, was used for chemostat inoculation. Following inoculation cultures were allowed to reach mid-log phase before switching on the medium pump to allow continuous culture. The pH of the culture was not controlled.

Lactobacillus acidophilus (a human isolate) was donated by the Division of Microbiology at the Institute of Medical and Veterinary Science, Adelaide. It was maintained on rogossa agar (Oxoid, Basingstoke, UK) under the same conditions used for *S. mutans*. The starter culture was prepared using the following ingredients (in grams) added to a litre of distilled water: tryptone 10.0 (oxoid), yeast extract 5.0 (oxoid), glucose 20, sorbitan mono-oleate 1.0, potassium dihydrogen phosphate 6.0, ammonium citrate 2.0, sodium acetate (anhydrous) 17.0, magnesium sulphate 0.575, manganous sulphate 0.12 and ferrous sulphate 0.034.

One litre of solution was boiled to dissolve the ingredients and after cooling 1.32 ml glacial acetic acid was added to the solution. Chemostat inoculation followed the same protocol as used for *S. mutans*.

Preparation of the vial culture medium

A batch of nutrient solution was made up consisting of 3% tryptone soya bean broth (Oxoid, Basingstoke, UK), yeast extract 0.5% (Oxoid Basingstoke, UK) and 20% sucrose.

After dissolution in distilled water, the solution was poured into each of the 5 ml vials so as to minimize the amount of entrapped air. After the caps were screwed on and the containers dried each container was sealed around the cap with nail varnish to minimize the chance of leakage at the seal.

The containers were placed into a sealed plastic vessel and chilled in a refrigerator prior to being dispatched for gamma irradiation (Steritech Pty Ltd, Dandenong, Victoria, Australia) at a dose of 15 R to sterilize the plastic vessel and its contents.

Experimental method

Streptococcus mutans and *L. acidophilus* were grown anaerobically using two separate model C30 Bio Flo chemostats (New Brunswick Scientific Co. Inc., Edison, NJ, USA) each with a culture volume of 365 ml as described previously.[11] The sterilized vials were placed into a sterile glass container that was connected by a "T" junction to the outflow of each chemostat system. This provides a constant supply of viable *S. mutans* and *L. acidophilus*. In each chemostat growth was maintained under anaerobic conditions at an imposed dilution rate of 0.1 h –1 (td = 7 hrs) and the pH was maintained at pH 7.4 for the *S. mutans* and pH 5.0 for the *L. acidophilus* by

the automatic addition of KOH (2 N). The pH of the flask containing the vials was uncontrolled and remained at ca. pH 4.5 throughout the four-week duration of the experiment.

Optical density measurement

After four weeks, the specimens were removed from the chemostat system and the optical density of the nutrient solution in the vials was measured using a λ 5 dual beam spectrophotometer (Perkins Elmer, Uberlingen, Germany) at a wavelength of 560 nm to determine bacterial growth within each vial. There are no data available to give a correlation between the optical density readings and concentrations of *S. mutans* and *L. acidophilus* in the solution.

Preparation of sections for SEM analysis

Following optical density readings, the discs were removed from the vials, placed in a fixing solution and then dehydrated as described by Knight *et al.*[12] The surface of each specimen that was exposed to the chemostat was attached to a mounting stub and carbon coated for SEM analysis (Philips XL30 Field Emission Scanning Electron Microscope, Netherlands).

Data analysis

Calculations were performed using SAS Version 9.1 (SAS Institute Inc., Cary, NC, USA). Differences in optical density readings between the control and HealOzone groups were compared using non-parametric Wilcoxon two-sample tests. Non-parametric tests were chosen as there was some evidence of non-normality in the distribution of the outcomes.

RESULTS

Scanning electron microscopy (SEM)

Two of the 20 samples were lost during vial recovery. The SEMs showed biofilms present on all the control samples (Fig 2). Higher magnification clearly differentiated *S. mutans* and *L. acidophilus* within the biofilms (Fig 3). No biofilms were observed on any of the ozonated treated surfaces (Fig 4). At higher magnification small numbers of planktonic *S. mutans* and *L. acidophilus* were observed on the ozonated samples (Fig 5). The pH of the chemostat remained at 4.2 throughout the period of the experiment.

Optical density

The mean and standard deviation of optical density readings of the two samples were 0.710 for the control with a SD of 0.288 and 0.446 for the ozonated samples with a SD of 0.371. Although the mean value of the control specimens were almost twice that of the

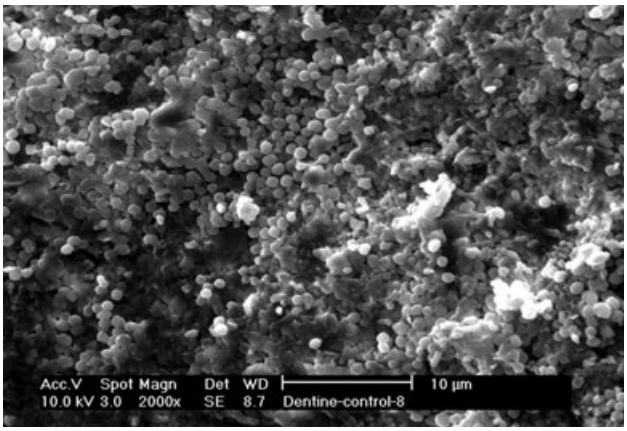

Fig 2. Typical outer surface of non-ozonated sample after four weeks exposure to *S. mutans* and *L. acidophilus*. Heavy biofilm formation was evident.

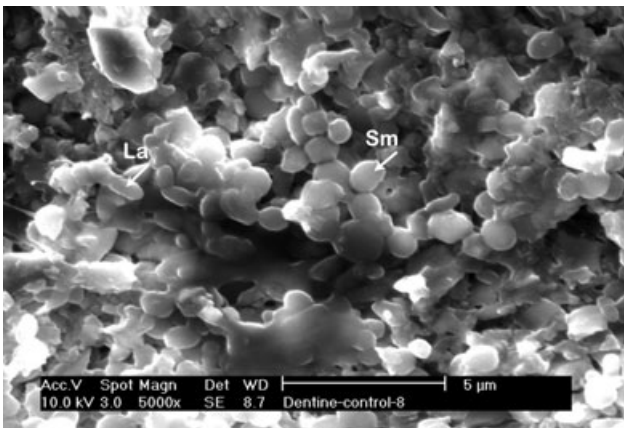

Fig 3. Higher magnification view of typical outer surface of non-ozonated sample after four weeks chemostat exposure. *S. mutans* and *L. acidophilus* were clearly identified within the biofilm.

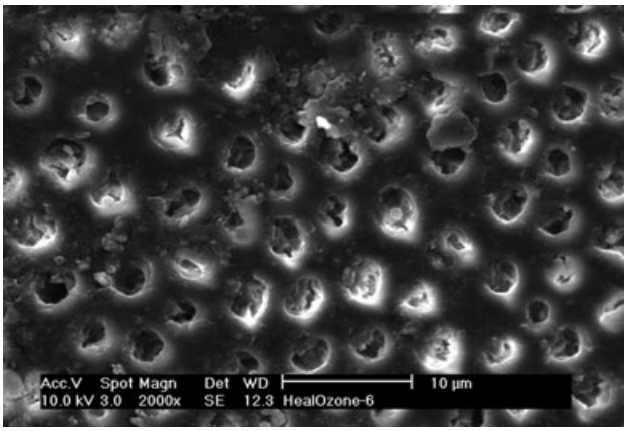

Fig 4. Typical outer surface of ozonated sample after four weeks exposure to *S. mutans* and *L. acidophilus*. A few planktonic bacteria were present but no biofilm formation was evident.

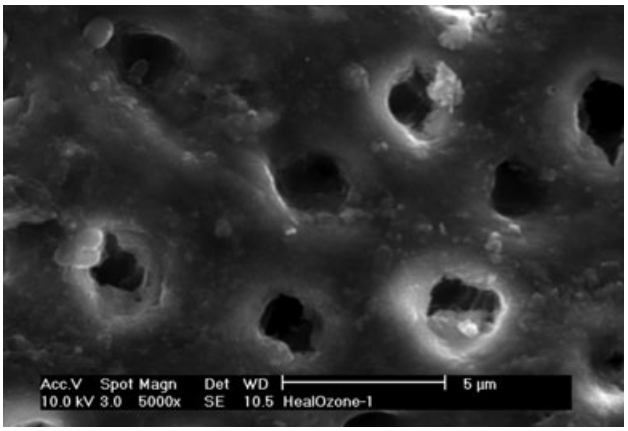

Fig 5. Higher magnification view of typical outer surface of ozonated sample after four weeks chemostat exposure. Only planktonic *S. mutans* and *L. acidophilus* were present on the dentine surface.

Table 1. Mean and standard deviation of optical density of nutritional broth of control and ozone pretreated dentine discs. The differences in the two samples were not significant (p > 0.05)

	Control		HealOzone
1	1.008	1	0.118
2	0.661	2	1
3	0.81	3	0.136
4	0.76	4	0.776
5	0.954	5	0.121
6	0.673	6	0.845
7	0.976	7	0.191
8	0.427	8	0.69
9	0.119	9	0.141
Mean	**0.710**		**0.446**
SD	**0.288**		**0.371**

ozonated specimens, the results were not significant ($p > 0.05$) The results are presented in Table 1 and presented graphically in Fig 2.

DISCUSSION

The existence of an overlying bacterial biofilm is essential for the initiation and progression of dental caries.[13] A recent paper has shown the application of ozone to an established biofilm had a minimal effect in reducing bacterial counts within the biofilm.[14]

The ozone treatment of dentine failed to have a consistent effect on the optical density readings in the chamber of the diffusion apparatus. This is not surprising given that the half-life of ozone is relatively short, especially so when dissolved in an aqueous medium. In the latter situation the half-life may be in the order of 20 minutes at 20°C.[15] Consequently, any ozone effect on bacterial viability would probably have dissipated early in the four-week exposure of the dentine discs to the test organisms.

In regard to the anti-microbial properties of ozone, Baysan *et al.*[16] found no significant differences in numbers of viable bacteria between ozonated and non-ozonated infected dentine beneath demineralized enamel.

It is noteworthy that there was no evidence of bacterial colonization on the dentine surfaces. In determining the possible mechanisms that may have operated to create this situation, the short half-life of ozone makes it highly improbable that it could have interfered with the synthesis of cellular polysaccharides produced by *S. mutans* by an inactivation of glycosyltransferase enzyme. These enzymes are responsible for the synthesis of soluble and insoluble glucans that not only contribute to the bulk of the biofilm but also play essential roles in the sucrose-dependent adhesion of the organism to tooth surfaces.[17]

A more likely mechanism may have been that the ozone treatment changed the wettability of the dentine surfaces, making it more difficult for organisms to colonize. Glantz[18] has shown that reducing the wettability of dentine surfaces can inhibit plaque formation. Ozone is a strong oxidizing agent that can react with almost every organic material. In the case of a dentine surface, the possibility exists that the treatment altered organic surface constituents, such as collagen, in some way. There is evidence for the ability of ozone to induce the release of selected low molecular mass salivary biomolecules from macromolecular binding-sites. This may have the effect of ''smoothing'' the protein molecules thus affecting the wettability of the dentine surface.[19] Additional studies are warranted to examine this aspect further.

In this study, both the smear layer and any biofilm was removed by etching the samples with 37% phosphoric acid during preparation. The ability of ozone treatment to prevent a biofilm formation by *S. mutans* and *L. acidophilus* on dentine suggests there may be therapeutic benefits of ozone application to root surfaces.

The dentine surfaces used in this study were free of a bacterial biofilm prior to exposure to the test organisms. An examination of treatment protocols for root surface caries using ozone that suggests the removal of any bacterial biofilm prior to commencing ozone therapy is necessary for a successful clinical outcome of these procedures. Holmes[20] was able to show a significant remineralization of root caries after cleaning the surfaces and applying ozone. It is suggested that as there was no biofilm forming over the surface of the lesions, the remineralization pH remained above the critical level for remineralization of the lesions to occur

Whilst the results of this preliminary investigation point to a useful role for ozone in the inhibition of bacterial biofilm formation, no attempt was made to determine the effect of varying ozone infusion times on either biofilm formation or the effects on organism

growth. Confocal laser scanning microscopy may be a useful tool to help examine some of these aspects further.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the statistical support from Mr. Thomas Sullivan (Statistician, Department of Public Health, The University of Adelaide) and Steritech Pty Ltd (Dandenong, Victoria) for gamma irradiation of samples.

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