



Antibacterial Effect of Ozonated Water on Adherent Mutans Streptococci (In Vitro)

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Abstract

To evaluate the antibacterial efficiency of ozonated water against adherent bacteria (in vitro). Ten dentin samples per group were inoculated with bacterial suspension and treated as follows: (I) untreated served as -ve control, (II) sterile distilled water for 10 seconds served as +ve control, (III) 5.25% sodium hypochlorite (Sultan-USA) for 10 seconds, (IV) 0.2% chlorhexidine (Corsodyle[®], England) for 10 seconds, and (V) 4mg/L ozonated water (Ozonesolution-Enaly, USA) for 10 seconds. Swab taken from all samples and an inoculum was spread on the selective medium MSB (HiMedia, India). Count of bacteria was recorded expressed in colony forming unit (CFU) taking in consideration the dilution factor.

There was high significant reduction in viable count of adherent M.S treated with ozonated water 4mg/L compared with the other groups at $P < 0.01$ level.

According to the circumstances of this study, ozonated water was very potent antibacterial agent against mutans streptococci.

Introduction

During cavity preparation, complete caries excavation based on clinical judgment (color and texture of dentin) does not provide certainty as to whether bacteria remain, i.e., the therapeutic procedures used in the treatment of caries do not always eliminate all microorganisms in the residual tissues ⁽¹⁾. Studies have examined penetration of coronal or root dentin by a limited number of oral bacteria that are associated with carious or non-carious dentin. Cells of *Streptococcus mutans*, *Streptococcus sanguis*, and *Actinomyces naeslundii* have all been shown to penetrate dentin

discs in vitro ⁽²⁾. Today, mutans streptococci are considered to be the main etiological microorganisms in caries disease, with lactobacilli and other microorganisms participating in the disease progression. Occasionally, some other microorganisms have been traced as initiator microorganisms ⁽³⁾. The Mutans Streptococci (MS) comprise a group of seven species, of which *streptococcus mutans* and *streptococcus sobrinus* are the predominant species isolated from human saliva and dental plaque ⁽⁴⁾. The mutans group includes. *S. mutans*, *S. sobrinus*, *S. cricetus*, *S. rattus*, *S.*

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downeii and *S. macacae*. The MS represent eight serotypes ^(5,3). It is therefore evident that other means additional to physical removal of caries dentin would be advantageous before placing a restoration (the sterilization of a cavity preparation before inserting a final restoration seems to be a logical procedure ⁽⁶⁾. It has been suggested that chlorhexidine or sodium hypochlorite be applied to prepared dentin surfaces for this purpose ⁽⁷⁾. As an alternative approach, ozonated water may be used as an antimicrobial agent ⁽⁸⁾.

Ozone is a powerful oxidant among chemicals typically used in water treatment. Therefore, it is capable of oxidizing many organic and inorganic compounds in water.

These reactions with organic and inorganic compounds cause an ozone demand in the water treated, which should be satisfied during water ozonation prior to developing a measurable residual ⁽⁹⁾.

The generation of ozone involves the intermediate formation of atomic oxygen radicals which can react with molecular oxygen ⁽¹⁰⁾. In the present study, we examined the effect of ozonated water on the viability of adherent mutans streptococci on human dentin.

Materials and Methods

Fifty freshly extracted intact human maxillary premolar teeth were used in this study. All teeth were then scaled with scalpel and polished with pumice using rubber cap at low speed and rinsed under tap water for 15 minutes, then immediately placed in distilled water at room temperature throughout the experiment ⁽¹¹⁾.

All teeth were embedded in a mold of 2×2×3cm dimensions filled with auto-polymerizing acrylic resin in such a way that the teeth were parallel to the long axis of the mold leaving 2 mm of

the crown uncovered, then the blocks were left to polymerize under 6 bar pressure and 50°C for 10 minutes in the Ivomet (Japan) ⁽⁷⁾, then a loop of acrylic was added at center base of the blocks, this will aid as an attachment mean for the hangers.

The teeth samples were then sectioned horizontally using diamond cutting disc and low speed hand piece (MF-Tectorque, W&H, Austeria) to expose dentin surface. Each disc was used for 10 specimens, then the dentin surfaces were grounded using fine stone bur under running water to gain flat surface ⁽¹²⁾.

All teeth samples were then abraded using Rotofix machine with silicon carbide abrasive paper of a grit size of P600A under water irrigation, to create a standard surface i.e. a uniform smear layer ⁽¹²⁾, the teeth were then inspected under dissecting microscope at 2X magnification to exclude any tooth containing perforations into the pulp (Fig.1). All abraded teeth were then placed in distilled water at 37°C in the incubator ⁽¹¹⁾.

Ozonated water was prepared using ozone generator based on corona discharge which installed according to the manufacturer instruction and the bubbling stone of the device was immersed into the bottom of 500 ml measuring cylinder filled with cooled distilled water. In this current study we used 4mg/L.

Since the concentration of ozonated water decreases with time ^(13,8) and to insure it is correct, and keep it constant during the work, a chemical diagnostic test by specialized kit (CHEMets® Kit) was used (Fig. 2).

Saliva samples were collected from young-age volunteer and dispersed with vortex for 2 minutes. Ten folds dilutions were prepared (10⁻¹-10⁻⁴) using sterile normal saline solution then 0.1 ml from 10⁻¹-10⁻⁴ was taken

and spread in duplicate on MSB. Plates were then incubated anaerobically using a gas pack (bio Mérieux, France) for 48 hours at 37 °C then aerobically for 24 hours at room temperature ^(14,15).

The colonies of Mutans Streptococci were then identified first by colony morphology under dissecting microscope 20×magnification (Hamilton, ALTAY), second by gram's stain and investigated under light microscope (Olympus-XSZ-N107, Japan) with 1000×magnification ⁽¹⁶⁾ and last by biochemical test using Cystine Trypticase agar media (CTA) ⁽¹⁷⁾.

A glass trough of 4.5×7.5×5.5 cm dimensions was modified to be used as adhesion model for samples (Fig. 3) filled with 50 ml Sucrose-Brain Heart Infusion Broth (SBHI-B) and inoculated with 1 ml activated bacterial inoculum then sterilized together at 15 lbs pressure and 121°C for 15 minutes.

All dentin surfaces were rinsed by immersion the samples in sterile distilled water (25ml) for 10 seconds to get rid of bacterial by-products, followed by gentle drying with filter paper. An adhesive tape with 4 mm hole was affixed on dentin surface to achieve swab area standardization, 10 samples for each group were randomly selected (n=10) and treated as follows: (I) untreated (negative control), (II) sterile distilled water for 10 seconds served as positive control, (III) 5.25% sodium hypochlorite (Sultan-USA) for 10 seconds, (IV) 0.2 % chlorhexidine (Corsodyle, England) for 10 seconds, and (V) 4 gm/L ozonated water for 10 seconds. Immediately swab by scraping dentin surfaces using absorbent paper point #140 (Roeko-Geramny) and transferred to 10 ml sterile normal saline (Fresenius Kabi, Germany). Serial dilutions in saline were performed (10^{-1} - 10^{-4}). 0.1 ml inoculum from each dilution was cultured onto MSB agar plates using a

sterile microbiological spreader and incubated at 37°C for 48 hours anaerobically followed by 24 hours aerobically. The colony-forming units grown were then counted in each plate and then recorded taking in consideration the dilutions factor (no. of colonies × reverse the dilution) and expressed as colony forming unit (CFU/sample) of M.S. The final result for the mean number of M.S binding to the dentin was obtained by calculating the mean scores of cultures from each dilution.

Results

Two forms of colonies were revealed, spherical or ovoid in shape with raised or convex surface, light blue in color about 1-2 mm in diameter (Smooth type), the other form of colonies appeared as irregular with rough or frosted glass surface (Rough type). Most of M.S. colonies had a depression at the middle with a drop of polysaccharide in it, or sometimes the whole colony submerged in a pool of polysaccharide. Both types of M.S. colonies were adhered well to the agar surface (Fig.4, Fig.5).

The count of Mutans streptococci bacteria adhered on the dentin surface was expressed as colony forming unit (CFU $\times 10^4$). The number of colonies recorded multiplied by reverse of the dilution factor. The differences in M.S growth on MSB agar that were cultured from dentin surfaces of all groups are shown in figure (6).

Dentin surfaces treated with 4mg/L ozonated water exhibited the least viable count of adherent M.S, followed by the dentin surfaces rinsed with 0.2% CHX, then the dentin surfaces rinsed with 5.25% NaOCl, next the dentin surfaces treated with SDW (Table 1). This is clearly shown in figure (7).

Ozonated water exhibited high significant correlation at $P < 0.001$ level

among groups (t: 15.35, 24.66, 18.07 and 14.06) when compared with -ve control (GI), +ve control (GII), sodium hypochlorite (GIII) and chlorhexidine (GIV) respectively.

Discussion

Members of the mutans group streptococci, in particular *S. mutans* and *S. sobrinus*, are considered to be the primary etiological agents in the induction of coronal and of root caries⁽¹⁸⁾.

It is found that mutans group streptococci were the predominant bacteria within dentin from fissure and smooth-surface coronal caries, with higher numbers in the shallow and middle layers of dentin compared with deep dentin⁽¹⁹⁾.

Thus, the bacteria used in the study are a good representative of the microbiology of mouth.

Since an important cariogenic determinant of Mutans Streptococci is its ability of adherence on tooth surface^(20,21), our study was based on adherent M.S on dentin surface.

Adhesion usually takes place through bacterial surface located adhesins, or fimbriae (proteinaceous adhesive filaments, protruding from the bacterial surface), which interact with specific target molecules⁽²²⁾.

There are important factors that influence in vitro early bacterial adhesion to hard surfaces including: the type of culture medium, culture conditions, incubation time, and the growth phase of the bacteria⁽²³⁾. In the present study, all bacterial colonies were identically prepared in order to eliminate the effect of different growth conditions.

The most frequently used medium for isolation of M.S is mitis salivarius bacitracin (MSB) agar, which is composed of mitis salivarius agar (MSA) with sucrose and bacitracin⁽²⁴⁾.

Success in operative dentistry depends on the total removal of the infected structures and on the achievement of a good sealing against microleakage to avoid bacterial contamination, which could cause secondary caries and pulp alterations⁽²⁵⁾.

Secondary caries could also result from inadequate removal of bacteria after the initial preparation. Brannstrom (1986) indicated that bacteria can multiply from within the smear layer even with a good seal from the oral cavity, and this can be a source of bacterial toxins, which can diffuse through the dentin causing irritation of the dental pulp⁽²⁶⁾.

An alternative approach to eliminate residual bacteria left in a cavity preparation would be the treatment with a disinfectant wash⁽²⁵⁾.

The newly designed tooth adhesion model was used to test the antibacterial effect of ozonated water 4mg/L on dentin adherent Mutans Streptococci.

Various methodologies can be used to assess the antimicrobial activity of cavity cleansers. Some methodologies allow direct contact of the substances with the microorganisms (as in agar diffusion test). In present study, microorganisms (M.S) adhered on dentin surfaces of the teeth using a modified teeth adhesion design for M.S adhesion, the assembly was examined and developed to be logic as possible.

We select most popular disinfectant agents to represent the experimental agents in this study. Sodium hypochlorite was one of the materials used as a disinfectant solution before the acid etching which shows an efficient antibacterial effect (broad spectrum in the aqueous solutions of 4 to 6% caused by its high pH and by the release of oxygen and chlorine when contacting organic substrate^(27,28), it presents antimicrobial activity with action on bacterial essential enzymatic

sites promoting irreversible inactivation originated by hydroxyl ions and chloramination action ⁽²⁹⁾.

Chlorhexidine is a cationic agent which exhibits antibacterial activity. The cationic nature of the compound promotes connection with anionic compound at the bacterial surface (phosphate groups from teichoic acid at gram-positive) capable of altering its integrity. The potassium ion, being a small entity, is the first substance to appear when the cytoplasmic membrane is damaged. The alteration of the cytoplasmic membrane permeability promotes precipitation of cytoplasmic proteins, alters cellular osmotic balance, interferes with metabolism, growth, cell division, inhibits the membrane ATPase and inhibits the anaerobic ⁽²⁹⁾.

To reach the maximum effect of the agents mentioned before, one minute and up exposure time needed based on several studies ^(27,30,31). The exposure time set in this study was 10 seconds, to find if these agents able to eliminate adherent M.S properly.

The results of study showed that the sensitivity of M.S to ozonated water 4mg/L was the highest among the other groups, chlorhexidine and sodium hypochlorite in other hand exhibit a sensitivity toward M.S too (4.25 ± 0.48 , 3.02 ± 0.49) respectively when compared with -ve control and +ve control (11.91 ± 2.27 , 6.470 ± 0.68) respectively as seen in figure (7), but when compared with ozonated water using ANOVA, there was a high significance correlation ($P < 0.01$) between this group and the others, also ozonated water was compared with all groups separately using Student t-test and again this group exhibited a high significance difference ($P < 0.001$).

From present results, it is quite obvious that ozonated water (4 mg/L) was highly effective in killing adherent M.S in 10 seconds. This result is in

agreement with that of Nagayoshi et al. (2004a), who test ozonated water in different concentrations and different time on various species of oral microorganisms and found 4mg/L concentration for 10 seconds is enough to kill 99.9% of bacteria ⁽⁸⁾, also the results of current study coincides with that of Nagayoshi et al. (2004b), who found the remarkable killing ability of ozonated water against bacteria invading dentinal tubules ⁽³²⁾.

Ozone is a powerful oxidant able to achieve disinfection with less contact time and concentration than all weaker disinfectants, such as chlorine, chlorine dioxide, and monochloramine ⁽³²⁾.

The advantages of ozone in the aqueous phase are its potency, ease of handling, lack of mutagenicity, rapid microbicidal effects, and suitability for use as a soaking solution for medical and dental instruments ⁽³³⁾. In the present study, we found that ozonated water had a rapid antimicrobial effect on adherent M.S, and that an ozone concentration of 4 mg/L was needed to kill the cells.

Membrane permeability is a key element to cell viability, and the changes in permeability involve the loss of several vital processes linked to the cytoplasmic membrane. It is generally accepted that oxidation due to ozone induces the destruction of cell walls and cytoplasmic membranes of microorganisms, and that differences in the sensitivity to ozonated water are probably due to differences in the structure of the cell walls of microorganisms. After the membrane is damaged by oxidation, the permeability of the membrane increases, and ozone molecules can readily enter the cells ^(8,32).

In addition, ozone disrupts enzymatic activity of bacteria by acting on the sulfhydryl groups of certain enzymes. Beyond the cell membrane

and cell wall, ozone may act on the nuclear material within the cell ⁽³⁴⁾.

These findings suggest that the bactericidal activity of ozonated water is through functional and structural disorder in the cytoplasmic membrane. Ozone increases the metabolism without the expenditure of vital energy and special stress should be laid on the fact that ozone is a natural remedy.

Conclusions

Under the conditions of this in vitro study, it is revealed that ten seconds exposure to ozonated water 4mg/L had strong bactericidal activity against adherent Mutans Streptococci on dentin surfaces of the teeth that made it the best antimicrobial agent among the other tested antimicrobial agents (Sodium hypochlorite 5.25% and chlorhexidine 0.2%).

The efficacy of ozonated water as cavity cleanser against different species of oral microorganisms in vivo, as endodontic irrigant alone and/or with sodium hypochlorite or with chlorhexidine must be further investigated.

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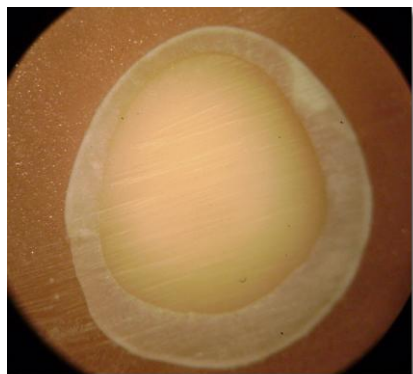


Figure 1: Complete tooth preparation (2X under dissecting microscope).



Figure 2: Ozone generating device with tubing and stone and the ozone CHEMets®Kit.



Figure 3: Glass trough and teeth attached to the suspenders (20 suspenders receiving 20 sample).

Group	N	Viable adherent MS Mean \pm SD*
I	10	11.91 \pm 2.2
II	10	6.47 \pm 0.68
III	10	4.25 \pm 0.48
IV	10	3.02 \pm 0.49
V	10	0.65 \pm 0.19
* Highly significant difference at level P<0.001		

Table 1: Viable count of adherent M.S (values expressed in CFU $\times 10^4$).

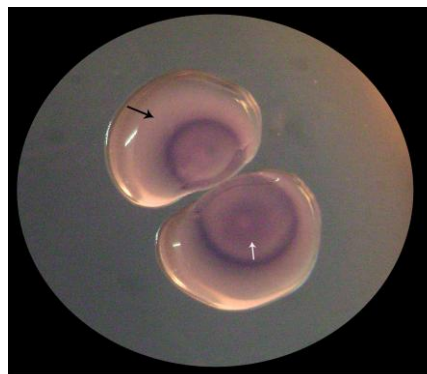


Figure 4: Smooth form of M.S. colonies (white arrow) submerged in polysaccharide (black arrow) (20 \times).

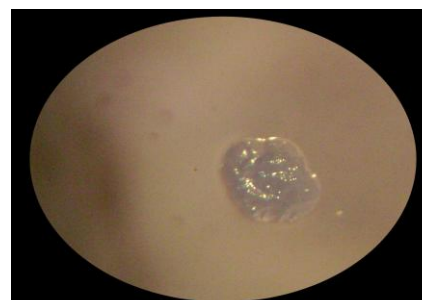


Figure 5: Rough form of M.S. (20 \times).

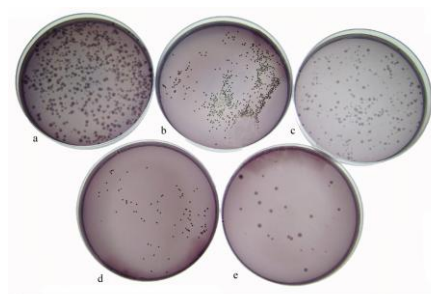


Figure 6: M.S. grown on MSB from dentin Surfaces of each group. a) untreated, b) distilled water, c) Naocl, d) CHX, e) ozonated water.



Figure 7: Mean of viable count of adherent M.S (CFU $\times 10^4$)