

**ANTIBACTERIAL EFFECTS OF OZONE ON  
*STREPTOCOCCUS MUTANS***

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## ABSTRACT

FERNANDO ASTORGA: Antibacterial effects of ozone on  
*Streptococcus mutans*  
(Under the direction of Luiz Pimenta)

**Aims:** To investigate the antibacterial effects of ozone on *Streptococcus mutans in vitro*. **Methods:** A bacteria volume of  $10^6$  CFU/ml was inoculated on human and bovine dentin specimens and in Wilkins-Chalgren broth media (WC, Difco Laboratories. Sparks) treated with two ozone sources (Curozone X4<sup>®</sup>, Kavo; OL80A and OL80W, Yanco Industries), at various concentrations (2, 20, 200, 2000 and 2100 ppm) and exposure times (from 15 sec to 4 min). **Results:** Curozone X4 had no effect on bacteria recovery, while OL80A and OL80W showed a concentration-dependent effect on *S. mutans* recover on a Trypticase<sup>™</sup> Soy Agar with 5% Sheep Blood (Difco Laboratories. Sparks). Concentrations of 200 and 2000 ppm showed no bacteria recovery after treatment for 4 min, while 2 and 20 ppm showed significant reduction of the recovered bacteria. **Conclusions:** This study suggests that Curozone X4<sup>®</sup> generator is not capable of delivering ozone to would effectively disinfect a caries lesion.

To my mother Amarilis who taught me the pursuit of excellence in life,  
wherever you are I hope you are proud

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## LIST OF ABBREVIATIONS

*S. mutans*: *Streptococcus mutans*

*E. faecalis*: *Enterococcus faecalis*

*S. sobrinus*: *Streptococcus sobrinus*

*L. acidophilus*: *Lactobacillus acidophilus*

*L. casei*: *Lactobacillus casei*

*A. naeslundii*: *Actinomyces naeslundii*

*V. dispar*: *Veillonella dispar*

*F. nucleatum*: *Fusobacterium nucleatum*

*S. oralis*: *Streptococcus oralis*

*C. albicans*: *Candida albicans*

*S. aureus*: *Staphylococcus aureus*

*E. coli*: *Escherichia coli*

*B. atrophaeus*: *Bacillus atrophaeus*

mg/L: Milligrams per liter

mO.D.: Millioptical density

ppm: Parts per million

TSA: Trypticase soy agar

TSB: Trypticase soy broth

*V. dispar*: *Veillonella dispar*

μl: Microliter

## **1. Introduction**

Since the 1970's there has been a significant reduction in dental caries in industrialized countries for children and adolescents, due to environmental and educational factors such as the increased use of fluoride in public water supplies, dentifrices and dental products; improved oral hygiene and prophylaxis; dietary counseling; and increased access to dental care.<sup>1</sup> Nevertheless, dental caries is still a common disease, experienced by almost 80% of children by the age of 18 and by almost 90% of adults.<sup>1</sup>

Dental caries is a multifactorial disease, characterized at the tooth level by a dynamic imbalance of the demineralization/remineralization processes over time<sup>2</sup>. The mineral content of the tooth is sensitive to increases in acidity. For enamel surfaces, when the pH drops below 5.5 demineralization proceeds faster than remineralization resulting in a net loss of mineral structure. Carious lesions are caused by bacterial fermentation of dietary carbohydrates into acids (principally lactic acid) within the dental biofilm. If the acid production and accumulation exceeds the buffering capacity of the oral environment, demineralization occurs eventually leading to cavitation.<sup>2</sup>

Dental biofilm is a key requirement for carious lesions to develop. Dental biofilm results from adherence of oral microorganisms to and growth onto the tooth surface. When tooth biofilm conditions favor demineralization as a result of bacteria metabolism, as the pH

is lowered, acids diffuse rapidly into the underlying enamel or dentin.<sup>3</sup> The two most important groups of bacteria that predominantly produce acids are the mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) and the lactobacilli.<sup>3</sup> These acidogenic bacteria metabolize a variety of fermentable, dietary carbohydrates to lactic acid, the strongest naturally occurring acid produced by acidogenic bacteria during the caries process.<sup>3</sup>

Dental caries may have a significant impact on an individual's life. The most common consequences of untreated lesions are discomfort and pain.<sup>4</sup> Restorative dental treatments can now be provided pain free, apart from the pain of the local anesthetic injection. However, for some people restorative treatments are associated with fear and anxiety, which may become barriers to dental attendance as well as financial limitations.<sup>4</sup> Treatment avoidance can subsequently lead to further progression of caries lesions which, in turn, may cause more distress and long-term complications<sup>4</sup>. Gross decay may lead to disturbances in eating and sleeping patterns because of pain. Psychological distress can arise from the embarrassment and self-consciousness of having missing or decayed teeth, especially in the anterior dentition.<sup>4</sup>

According to the anatomical location of carious lesions, it is possible to differentiate between coronal lesions, which may affect the pits and fissures or the smooth surfaces of a tooth in enamel, and root lesions, which affect the exposed root surface in dentin.

The prevalence of root caries begins at about 30–40 years of age and tends to increase thereafter. Adults are keeping their teeth longer however, increased retention of teeth means that more adults are at risk for root caries. Gingival recession caused by aging puts root

surfaces especially at risk. Among dentate adults, the prevalence of root caries experience is about 8 times higher among those aged 75+ years (55.9%) compared to those aged 18 to 24 years (6.9%).<sup>5</sup>

Ozone has been proposed as a therapeutic agent for dental caries.<sup>6-18</sup> Ozone (O<sub>3</sub>) is a triatomic molecule, consisting of three oxygen atoms with a molecular weight of 47.98 g/mol. Ozone is a highly thermodynamically unstable compound that, dependent on system conditions such as temperature and pressure, decomposes to pure oxygen with a short half-life.<sup>19</sup> Ground-level ozone is an air pollutant with harmful effects on the respiratory system. Ozone in the upper atmosphere filters potentially damaging ultraviolet light from reaching the Earth's surface.<sup>19</sup> The word ozone was first introduced by Schonbein in 1840. He subjected oxygen to electrical discharges and noted "the odor of electrical matter". Schonbein concluded that odor was due to a gas which he named ozone, from the Greek Ozein, which means smell.<sup>6</sup>

Ozone, in the gaseous or aqueous phase, has been shown to be a powerful antimicrobial agent against bacteria, fungi, protozoa, and viruses.<sup>20</sup> It is generally accepted that the strong oxidizing potential of ozone initiates the destruction of cell walls and cytoplasmic membranes of bacteria and fungi.<sup>12, 21</sup> During this process, ozone reacts with glycoproteins, glycolipids, and select amino acids resulting in inhibition of the enzymatic control systems of the cell. This inhibition results in a collapse in the permeability barrier function of the membrane, permitting the ready diffusion of ozone and other toxic molecules

into the cell resulting in microbial death.<sup>21</sup> Ozone can attack many biomolecules, especially the cysteine, methionine, and histidine amino acid residues of proteins. By oxidizing critical biomolecules associated with the etiology of dental caries, ozone can have disruptive effects on the cariogenic processes of bacteria.<sup>21</sup> For example, ozone is capable of decarboxylation of pyruvic acid to the weaker acetic acid. It has been shown that remineralization of incipient carious lesions can be encouraged when the production of acetic acid, or other high *pKa* acids found in resting biofilm, buffers plaque fluid.<sup>21</sup>

The water industry has used ozone to kill microorganisms for many years.<sup>22</sup> One of the most common uses of ozone is treating drinking water for disinfection; oxidation of natural organic matter; degradation of hazardous micropollutants; algae inactivation; improvement of color, taste, and odor of water.<sup>23</sup>

Ozone has also been tested in medicine to decontaminate hospital side rooms, rooms contaminated with methicillin-resistant *S. aureus* and in auto-hemotherapy.<sup>23</sup> Ozone has been used in the medical field for over 150 years to disinfect and treat infections, wounds and multiple diseases (Table 1).<sup>24</sup> Some of the contraindications for the ozone therapy are acute alcohol intoxication, recent myocardial infarction, hemorrhage from any organ, pregnancy, hyperthyroidism, thrombocytopenia and ozone allergy.<sup>19</sup>

The first use of ozone in dentistry is attributed to Dr. E. Fisch who used ozonated water in his practice;<sup>21</sup> and Dr. Erwin Payr used it in surgery and reported his results at the 59th Congress of the German Surgical Society in Berlin (1935).<sup>21</sup> In dental surgery, ozonated

water was used to promote hemostasis, enhance local oxygen supply, and inhibit bacterial proliferation.<sup>21</sup> Theoretically, ozone in either gas or ozonated water forms, can reduce the bacterial count in biofilm as well as in active carious lesions, and therefore it may temporarily arrest the progression of caries, resulting in prevention of or delaying the need for tooth restorations.<sup>21</sup> In the dental field, ozone has been advocated for treatment in endodontics, ozonated oils as medicaments, root and cavitated caries and for its potential application in reducing bacterial counts in dental unit water delivery systems.<sup>15</sup> Cesar *et al.* (2012) tested the effectiveness of ozonated water for disinfection of the surfaces of dental diamond burs that were contaminated with *Eschericia coli*, *Staphylococcus aureus*, *Candida albicans*, or the spores of *Bacillus atrophaeus*. They found that ozonated water effectively reduced the number of CFU/mL in an exposure of 10 or 30 min at a concentration of 10 mg/L for all microorganisms.<sup>25</sup>

There is evidence that ozone can effectively kill microorganisms on surfaces<sup>26, 27</sup> but the evidence that ozone effectively kills microorganism in biofilm is contradictory. There are reports that ozone is effective in short-time exposures (10 seconds),<sup>11, 13</sup> while other studies have shown its effectiveness in short<sup>20</sup> and longer exposures (over 60 seconds).<sup>28</sup> There are also other authors who claim that ozone applied as gas has no effect on biofilm.<sup>22, 29, 30</sup> Many authors claim ozone has an anticariogenic effect not only *in vitro*<sup>11, 13, 14, 20</sup> but also *in vivo*, and clinically by reducing the number of cariogenic bacteria in the oral cavity reversing the caries process in existing lesions.<sup>7, 10-12</sup> Saini (2011) advocates the use of ozone as an

anticariogenic agent.<sup>31</sup> It has been suggested that the application of ozone, as an adjunct therapy in association with a remineralizing agent, could arrest or reverse carious lesions, providing an alternative to restorative treatment.<sup>7, 8, 10, 11</sup> Using ozone to reduce the bacterial count in active carious lesions might temporarily modify the biofilm allowing the arrestment of caries lesions<sup>11</sup>. This could prevent or delay the need for restorative procedures. The clinical protocol for application of ozone is purportedly fast and easy. It has been proposed that the application of 10 to 60 seconds of ozone could reduce the number of most of the microorganisms in leathery (active) root caries lesions.<sup>4</sup> These studies report that ozone acts rapidly and at lower concentrations than those required for chlorine, and has no side-effects such as taste and odor which are characteristic of other disinfectant agents.<sup>4</sup> Once the ecological niche of the acid-producing microorganisms has been reduced or eradicated, the area is treated with a remineralizing solution. It has been suggested that for clinical treatment the ozone exposure be repeated at 3 and 6 months after the initial application.<sup>12</sup>

Knight *et al.* in 2008 found that the infusion of ozone into non-carious dentin prevented biofilm formation *in vitro* from *S. mutans* and *L. acidophilus* over a four-week period. It has been hypothesized that ozone treatment might alter the surface wettability of dentin through reaction with organic constituents leading to a delay in bacterial colonization.<sup>14</sup>

In contrast several publications report negative results both *in vitro*<sup>22, 29, 30, 32</sup> and in clinical trials.<sup>33</sup> Two systematic<sup>4, 21</sup> and one Cochrane review<sup>34</sup> agree in “given the high risk of bias in the available studies and lack of consistency between different outcome measures,



there is no reliable evidence that application of ozone gas to the surface of carious teeth stops or reverses the caries process. There is a fundamental need for more evidence of appropriate rigor and quality before the use of ozone can be accepted into mainstream primary dental care or can be considered a viable alternative to current methods for the management and treatment of dental caries”.<sup>34</sup> The publication by Brazzelli *et al.* concluded that the current evidence base for ozone is insufficient to say that ozone is a cost-effective addition to the management and treatment of occlusal and root caries<sup>4</sup>. Before expensive and time-consuming independent randomized clinical trials are performed, well-controlled *in vitro* proof of concept study showing that ozone kills cariogenic bacteria are needed.

Hems *et al.* in 2005 showed the inability of gas ozone to effectively kill *E. faecalis* after a 240 s exposure; stating that “gaseous ozone had no significant antibacterial effect on the biofilms. This was predictable considering that the effectiveness of ozone is highest in solution and given the previous results with biofilms, gaseous ozone would not be expected to exert any great antibacterial effect”. They state that “Ozone is a selective oxidant and affects only certain compounds but when it dissolves in water, it becomes highly unstable and rapidly decomposes through a complex series of chain reactions”.<sup>22</sup> Consequently, there is a fundamental need for more evidence that ozone can be accepted into mainstream primary dental care and be considered a viable alternative as adjunctive treatment of dental caries.

## **2. Objectives**

1. To develop a mechanical model that simulates a “lesion” in dentin as a surface for reproducible recovery of inoculated bacteria to test efficacy of ozone delivery to target bacteria in a relevant environment.
2. To treat that lesion model with ozone to simulate clinical conditions.
3. To determine the conditions and circumstances where ozone can have a killing effect on *S. mutans* (concentration, exposure time, bacteria on surface or in media)
4. To investigate if ozone can be effectively delivered to a caries lesion to kill *S. mutans*.
5. To determine if *S. mutans* can be killed by ozone where the concentrations are validated.

### **3. Materials and methods**

#### **3.1 Bacterial species and culture**

The bacterial species used in this study was *Streptococcus mutans* strain ATCC 10449 (serotype c).

A stock culture of the bacteria was transferred to Wilkins-Chalgren broth media (WC, Difco Laboratories. Sparks) and then incubated at 37°C in aerobic (ambient) atmosphere. After 24 hours, an aliquot of this culture was inoculated to a fresh W-C tube, to an approximate concentration of  $10^8$  colony forming units (CFU) /ml using McFarland standards (equivalent to  $\sim 10^8$  CFU/ml).

#### **3.2 Ozone generators**

The ozone generator used for this study was the Curozone X4<sup>®</sup> (Kavo, Biberach/Riß, Germany) generator (Fig. 1). The device is designed to generate ozone either using atmospheric oxygen or with supplemental oxygen delivered with an attached oxygen tank to yield higher ozone concentration. According to the manufacturer the generated ozone concentration is 2100 ppm,  $\pm 10\%$ , delivering 65  $\mu\text{mol}$  ozone/min. The exposure time can be set from 5 to 60 sec. The ozone is delivered through a hand-piece fitted with disposable silicone tips of variable sizes as appropriate to the treatment surface, designed to seal the surface to be treated avoiding gas leakage. The silicone tip needs to completely seal the

surface, otherwise the device stops working. After the exposure time there is a two sec vacuum/ scrubbing cycle to eliminate any remaining ozone in the system thus preventing escape of ozone to the ambient atmosphere (Fig. 2).

The ozone generator OL80A (Ozone Services, Yanco Industries, Burton, BC). (Fig. 3), was used to expose the bacteria in the Mouse Exposure Chamber (ESE Design Center, Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, University of North Carolina at Chapel Hill) (Fig. 4) to atmospheres of either 2 or 20 ppm of ozone. The OL80W (Fig. 5, located at the Environmental Sciences and Engineering Laboratory located at Gillings School of Global Public Health at the University of North Carolina at Chapel Hill) was used to expose the bacteria in the Modular Incubator Chamber (Billups-Rothenberg, Inc. Del Mar, CA) (Fig. 6) to atmospheres of either 200 or 2000 ppm of ozone.

### **3.3 Effect of ozone on human dental specimens.**

Thirty-six extracted human third molars were used as dental specimens. The teeth were debrided and examined to ensure that they were free of defects (caries, fractures, cracks). The crowns were separated from the roots using an Isomet diamond saw (Buehler Ltd., Lake Bluff, IL) under running water to obtain 6 x 4 mm slabs. The specimens were ground mechanically (Ecomet 3, Buehler Ltd.) under running water using a 600-grit silicon

carbide (SiC) paper to obtain flat dentin surface (Fig. 7). Single preparations were made on the specimens with a #2 bur at high speed with water refrigeration to create a cavity of 1 mm<sup>2</sup> that would harbor bacteria; they were then sterilized with steam autoclave. The specimens were then inoculated with serial ten-fold dilutions of the bacteria culture 10<sup>8</sup> to 10<sup>6</sup> CFU/ml and incubated at 37°C for 24 hours. The specimens were exposed to ozone generated by the Curozone X4<sup>®</sup> which produces concentrations up to 2100 ppm, according to the manufacture. This was accomplished using the Curozone handpiece fitted with 3 mm silicone tips. This completely surrounded the “inoculated lesion”, creating a sealed ozone atmosphere at the treatment site. Fresh tips were used with each treatment to prevent cross-contamination. The treatment timing was begun automatically by the instrument once the desired ozone atmosphere was achieved.

The specimens were randomly divided in three groups: (n=12)

1. Low ozone group: Exposed to ozone generated using ambient air for 60 seconds.
2. High ozone group: Exposed to ozone generated using pure oxygen for 60 seconds.
3. Control group: Exposed to ambient air for 60 seconds.

After the exposure to air or ozone, dentin shavings were collected from the circumferential cavity walls to a 1.4 mm depth into 1 ml sterile bacterial transport media containing glass beads to facilitate dispersion of the bacteria clumps with vortexing, by using sterilized #4 carbide burs at low speed (NSK Ultimate 450K, Nakanishi Inc. Japan). A new sterile bur was used for each specimen to prevent over heating of dentinal walls and cross-

contamination.

Serial ten-fold dilutions of the vortexed transport media were used to determine the bacteria counts from the dentin shavings obtained. The diluted solutions were spiral plated (Spiral Systems Inc., Cincinnati, OH) on Trypticase™ Soy Agar with 5% Sheep Blood. (Becton, Dickinson and Company. Sparks, MD). After 24 hours of incubation at 37°C, the resulting colonies were counted using a ProtoCol automatic plate counter (Spiral Systems Inc., Cincinnati, OH) and Protocol software. Data were calculated as CFU/ml of the original transport medium. To normalize the CFU/ml, descriptive statistics (means and standard deviations) were performed on log transformed data.

### **3.4 Effect of ozone on bovine dental specimens**

Thirty six extracted bovine teeth were used. The teeth were debrided and examined to ensure that they were free of defects (caries, fractures, cracks). The crowns were separated from the roots using an Isomet diamond saw (Buehler Ltd., Lake Bluff, IL) under running water to obtain 10 x 6 mm slabs. The specimens were ground mechanically (Ecomet 3, Buehler Ltd.) under running water using a 600-grit SiC paper to obtain a flat dentin surface, (Fig. 8). Single preparations of 1.4 mm<sup>2</sup> were made on the specimens with a #4 bur at high speed with water refrigeration to create a cavity that would harbor bacteria. They were then sterilized with steam autoclave. The specimens were then inoculated with ten fold dilutions of the bacteria culture 10<sup>8</sup> to 10<sup>6</sup>CFU/ml and incubated at 37°C for 24 hours.

The results of Experiment 1 show that the recovered  $\log_{10}$  CFU obtained from low ozone, high ozone and control groups did not differ significantly among the three groups results. It was therefore decided to compare the results of the two test groups: high ozone and low ozone delivered using handpiece of the Curozone X4<sup>®</sup> fitted with 3 mm silicon tips as described above for the human teeth.

The specimens were randomly divided in two groups: (n=18)

1. Low ozone control group: Exposed to ozone generated using ambient air for 60 seconds.
2. High ozone: Exposed to ozone generated using pure oxygen for 60 seconds.

After the exposure to ozone generated from ambient air or using pure oxygen, dentin shavings were collected from the circumferential cavity walls of diameter to 1.8 mm depth into 1 ml sterile bacterial transport media containing glass beads to facilitate dispersion of the bacteria clumps with vortexing, sterilized #6 carbide burs at low speed. A new sterile bur was used to prevent over-heating of dentinal walls and cross-contamination.

Serial ten-fold dilutions of the vortexed transport media were used to determine the bacteria counts from the dentin shavings obtained. The solution was spiral plated on Trypticase<sup>™</sup> Soy Agar with 5% Sheep Blood (WC, Difco Laboratories. Sparks). After 24 hours of incubation at 37°C, the resulting colonies were counted using a ProtoCol automatic plate counter and Protocol software as described above.

### **3.5 Bactericidal activity as loss of recoverable CFU after exposure of *S. mutans* inoculated agar surfaces to defined atmospheres of ozone**

After showing the inability of the Curozone X4 to effectively inhibit *S. mutans* on human and bovine roots at purported levels of ozone of 2100 ppm for 1 min, it was decided to determine if the target strain of bacterium (*S. mutans* ATCC 10449) is, in fact, susceptible to ozone. To optimize delivery to the target, the bacteria were spread on a Trypticase™ Soy Agar with 5% Sheep Blood surface to allow uniform delivery of ozone in a contained atmosphere. These inoculated plate surfaces were treated by directly exposing the uncover plates to specific ozone atmospheres. Determination of the effective killing dose of ozone was accomplished by varying the exposure atmosphere to include 2, 20, 200 and 2000 ppm of ozone for 4 min under laboratory conditions using a different ozone generator. In addition, the 2 and 20 ppm groups were tested with the Petri dish covers in place to determine if the cover would be a physical barrier to ozone access to the inoculated surface.

The experiments were performed under defined conditions at the Environmental Sciences and Engineering Laboratory located in the Gillings School of Global Public Health at the University of North Carolina at Chapel Hill.

To determine the bactericidal activity of ozone, an overnight culture (early exponential) of *S. mutans* was adjusted to a suspension of  $10^6$  CFU/ml and 49.2 µl was spiral plated to replicates of seven SBA plates. Six plates were treated by varied exposures to atmospheres of ozone and one was left untreated as control. The 2000 and 200 ppm ozone atmosphere groups were exposed using the OL80A ozone generator into the Modular



Incubator Chamber. The 2 and 20 ppm ozone atmosphere groups were exposed either with or without the Petri dish cover in place using the OL80W ozone generator into the Mouse Exposure Chamber. The larger size of this chamber permitted the simultaneous treatment of multiple plates in a single plane.

The distribution of the groups was as follows:

1. Uncovered replicate plate exposed to 2000 ppm of ozone for 4 min
2. Uncovered replicate plate exposed to 200 ppm of ozone for 4 min
3. Uncovered replicate plate exposed to 20 ppm of ozone for 4 min
4. Replicate plate exposed to 20 ppm of ozone for 4 min with the Petri dish cover in place to determine if the cover would be a physical barrier to ozone
5. Uncovered replicate plate exposed to 2 ppm of ozone for 4 min
6. Replicate plate exposed to 2 ppm of ozone for 4 min with the Petri dish cover in place to determine if the cover would be a physical barrier to ozone
7. Untreated replicate plate as a control for determining recoverable CFU.

Following treatments the replicate plates were incubated in an anaerobic chamber (10% H<sub>2</sub> – 5% CO<sub>2</sub> – 85% N<sub>2</sub>) at 37°C for 18 hours to permit development of the CFU. After this period the resulting CFU were counted using a ProtoCol automatic plate counter. The log transformed data were treated as described above. The experiments exposing uncovered plates to the various atmospheres were repeated on three independent occasions. Treatment of covered plates with 2 and 20 ppm ozone atmosphere was only done once.

### **3.6 Influence of ozone exposure of *S. mutans* suspended in liquid in microtiter plate wells on bacterial viability.**

Our previous experiments with *S. mutans* in the artificial cavity preparations in either human or bovine dentin slabs determined that exposure to ozone delivered directly with the hand piece did not result in demonstrable reduction in viability of the bacteria target even at the highest concentration (2,100 ppm) for up to 60 sec. Longer treatments were technically challenging with the small dentin slabs and their relatively irregular surfaces. It was also possible that the failure to kill was due to the competitive inactivation of the ozone by the dentin. The aqueous environment of the suspended bacteria in the “cavity” could also have limited accessibility of the ozone to the target bacteria. In the next set of experiments, a more uniform approach for testing the effects of ozone atmospheres on the viability of *S. mutans* in an aqueous environment was sought. It was determined that the 3 mm silicone tip fit formed a tight seal with the well of a 96-well microtiter plate. This permitted the creation of individual ozone atmospheres in the micro-format of each well. A liquid volume containing the target bacteria could be uniformly delivered to the bottom of each well and exposed to a defined ozone atmosphere. After exposure the treated bacteria could be resuspended in culture medium and incubated to permit growth. Total killing of the bacteria would result in a failure to grow (no change in optical density). Logarithmic killing of the bacteria would result in delays in attaining discernible growth and consequent reductions in attained optical densities when compared to untreated controls. The effectiveness of Curozone X4® on bacteria

suspended in liquid was therefore tested by comparing the post-treatment growth as optical density (mO.D.at  $\lambda$ 610 nm) 18 to 24h after exposure to 15, 30, 45 and 60 seconds of ozone with that of untreated controls.

A 96-well microtiter plate (Fig.9) was used to receive ten microliter ( $\mu$ l) of  $10^6$  CFU/ml of bacteria to be treated with ozone through the Curozone X4<sup>®</sup> unit using a 3 mm silicone tip in high and low ozone modes. The controls consisted of two groups of 200 $\mu$ l of media without bacteria, one group was treated for 1 min and the other was left untreated. The positive control was 10 $\mu$ l of the bacteria culture that was not treated.

Each bacteria and control group, with the exception of the untreated media group, was divided in 2 groups of 4 wells each. One received the low ozone treatment and the other high ozone treatment.

The group distribution was as follows:

1. Media untreated. 200 $\mu$ l.
2. Media treated for 1 min. 200 $\mu$ l.
3. Bacteria untreated. 10 $\mu$ l of  $10^6$  CFU/ml.
4. Bacteria treated for 15 sec. 10 $\mu$ l of  $10^6$  CFU/ml..
5. Bacteria treated for 30 sec. 10 $\mu$ l of  $10^6$  CFU/ml.
6. Bacteria treated for 45 sec. 10 $\mu$ l of  $10^6$  CFU/ml.
7. Bacteria treated for 60 sec. 10 $\mu$ l of  $10^6$  CFU/ml.

After treatment, the 10 $\mu$ l aliquots of bacteria were resuspended in 200 $\mu$ l of fresh W-C

medium broth and incubated in an anaerobic chamber at 37°C. The optical densities ( $\lambda 610$  nm) of the quadruplicate samples were determined after 18 hours by using a Vmax kinetic microplate reader and companion SoftMax Pro 3.1 software (Molecular Devices, Sunnyvale CA). Data were expressed as mOD and means and standard deviations determined on replicate wells. Each experiment was independently repeated at least three times.

### **3.7 Effect of ozone in *S. mutans* suspended in media in exposures times of 1, 2, 3 and 4 min**

This experiment was designed to determine ozone effectiveness on bacteria in media after different exposures times ranging from 1 to 4 min.

A 96 well microtiter plate was used to receive ten microliter ( $\mu$ l) of  $10^6$  CFU/ml of bacteria to be treated with ozone through the Curozone X4<sup>®</sup> unit using a 3 mm silicone tip in high and low ozone modes in exposures time of 1, 2, 3 and 4 min. The controls consisted of two groups of 200  $\mu$ l of medium without bacteria, one group was treated for 1 min and the other was left untreated. The positive control was 10 $\mu$ l of the bacteria culture that was not treated.

Each bacteria and control group, with the exception of the untreated media group, was divided in 2 groups of 4 wells. One received the low ozone treatment and the other high ozone treatment.

The group distribution was as follows:

1. Media untreated. 200µl
2. Media treated for 1 min. 200µl
3. Bacteria untreated 10µl of  $10^6$  CFU/ml.
4. Bacteria treated for 1 min. 10µl of  $10^6$  CFU/ml.
5. Bacteria treated for 2 min. 10µl of  $10^6$  CFU/ml.
6. Bacteria treated for 3 min. 10µl of  $10^6$  CFU/ml.
7. Bacteria treated for 4 min. 10µl of  $10^6$  CFU/ml.

After treatment, the 10µl aliquots of bacteria were resuspended in 200µl of fresh W-C medium broth and incubated in an anaerobic chamber at 37°C. The optical densities ( $\lambda 610$  nm) of the quadruplicate samples were determined after 18 hours by using a Vmax kinetic microplate reader and companion SoftMax Pro 3.1 software (Molecular Devices, Sunnyvale CA).

### **3.8 Effect of ozone in *S. mutans* suspended in media in concentrations of $10^6$ , $10^5$ , $10^4$ and $10^3$ for 1 min**

This experiment was designed to determine ozone effectiveness on titrated bacteria in concentration of  $10^6$ ,  $10^5$ ,  $10^4$  and  $10^3$  CFU/ml in media for 1 min.

This experiment was designed to determine ozone effectiveness on titrated bacteria in concentration of  $10^6$ ,  $10^5$ ,  $10^4$  and  $10^3$  CFU/ml in media for 1 min. A 96 well microtiter plate

was used to receive ten microliter ( $\mu\text{l}$ ) of  $10^6$  CFU/ml of bacteria to be treated with ozone through the Curozone X4<sup>®</sup> unit using a 3 mm silicone tip in high and low ozone modes. The controls consisted in two groups of 200  $\mu\text{l}$  of media without bacteria, one group was treated for 1 min and the other was left untreated. The positive control was 10 $\mu\text{l}$  of the bacteria culture that was not treated.

Each bacteria and control group, with the exception of the untreated media group, was divided in two groups of four wells. One received the low ozone treatment and the other high ozone treatment.

The group distribution was as follows:

1. Media untreated. 200 $\mu\text{l}$
2. Media treated. 200 $\mu\text{l}$
3. Bacteria untreated 10 $\mu\text{l}$  of  $10^6$  CFU/ml.
4. Bacteria treated for 1 min. 10 $\mu\text{l}$  of  $10^6$  CFU/ml.
5. Bacteria treated for 1 min. 10 $\mu\text{l}$  of  $10^5$  CFU/ml.
6. Bacteria treated for 1 min. 10 $\mu\text{l}$  of  $10^4$  CFU/ml.
7. Bacteria treated for 1 min. 10 $\mu\text{l}$  of  $10^3$  CFU/ml.

After treatment, the 10 $\mu\text{l}$  aliquots of bacteria were resuspended in 200 $\mu\text{l}$  of fresh W-C medium and incubated in an anaerobic chamber at 37°C. The optical densities ( $\lambda 610$  nm) of the quadruplicate samples were determined after 18 hours by using a Vmax kinetic microplate reader and companion SoftMax Pro 3.1 software (Molecular Devices, Sunnyvale CA).

### **3.9 Effect of ozone in *S. mutans* suspended in media and on agar under controlled conditions**

The ozone generator OL80W was used to expose the bacteria in the Modular Incubator Chamber at Environmental Sciences and Engineering Laboratory located at Gillings School of Global Public Health at the University of North Carolina at Chapel Hill.

This experiment was designed to test the effectiveness of exposing *S. mutans* in media and on a surface to ozone gas, at the same time for four min in an atmosphere of 2000 ppm ozone. To accomplish this objective two groups were designed:

**Media Group:** A total of 15 wells of a 96 well microtiter plate were inoculated with 10 $\mu$ l of 10<sup>3</sup> CFU/ml. Five wells were inoculated with 200 $\mu$ l of media serving as controls. (Fig.10). Two sets were prepared one was treated and the other left untreated as control.

**Agar Group:** For this group two agar plates with 49.2 $\mu$ l from a culture 10<sup>6</sup> CFU were spiral plated on Trypticase™ Soy Agar with 5% Sheep Blood. One was treated with ozone and the other left untreated as control.

After being exposed to ozone at 2000 ppm for four min, the 10 $\mu$ l suspension of the treated bacteria in the media group was brought to a volume of 210 $\mu$ l with fresh W-C medium and incubated in an anaerobic chamber at 37°C. The optical densities ( $\lambda$ 610 nm) of the quadruplicate samples were determined after 18 hours by using a Vmax kinetic microplate reader and companion SoftMax Pro 3.1 software (Molecular Devices, Sunnyvale CA).

## **4. Results**

### **4.1 Effect ozone in *S. mutans* on human dental specimens**

Results are presented in Table 2. The mean recoverable CFU/ml obtained from low ozone ( $5.25 \pm 0.87$ ), high ozone ( $5.47 \pm 0.92$ ) and control ( $5.58 \pm 0.67$ ) groups did not differ significantly among the three groups. (Kruskal-Wallis test,  $p=0.58$ ).

During the experiment it was noticed the handling difficulties of the Curozone X4<sup>®</sup>, keeping the seal of the silicone tip turn to be problematic, changings in pressure and holding angle of the hand-piece lead to seal loss, also the irregularities of the tooth surface made the dentin shaving proccess challenging. Due to this inconvenient it was decided to utilize bovine teeth to minimize the mechanical problems found with human teeth.

### **4.2 Effect ozone in *S. mutans* on bovine dental specimens**

Results are reported in Table 3. There was no statistically significant difference between the mean of recovered  $\log_{10}$  CFU obtained from low ozone control ( $5.37 \pm 0.71$ ) and high ozone ( $5.32 \pm 0.82$ ) (Wilcoxon test,  $p=0.53$ ).

Having a larger tooth surface did not prevent the mechanical problems explained in 4.2, even though the surface was grinded to a flat shape. These findings suggest that the



Curozone X4<sup>®</sup> is not delivering ozone to the lesion site in a concentration that is sufficient to kill or that the test bacterial strain is inherently resistant to ozone. A different approach is proposed, to test the antibacterial effect of ozone under control conditions at the Environmental Sciences and Engineering Laboratory located at Gillings School of Global Public Health at the University of North Carolina at Chapel Hill.

#### **4.3 Effect ozone in *S. mutans* suspended in media under controlled conditions**

Results are reported in Table 4. The plates exposed to 2000 and 200 ppm had no recoverable colony forming units (total inhibition). The 20 ppm group that was left uncovered showed 99.99% bacteria inhibition (1.59 log<sub>10</sub> CFU/ml recovered), while the covered group showed 99% bacteria inhibition (3.55log<sub>10</sub> CFU/ml recovered). The 2 ppm group that was left uncovered showed 99.99% bacteria inhibition (2.81 log<sub>10</sub> CFU/ml recovered), while the covered groups showed 91.41% bacteria inhibition (4.58 log<sub>10</sub> CFU/ml recovered). The untreated control group showed a bacteria value of 5.64 log<sub>10</sub> CFU/ml. Logarithmic reduction in recoverable CFU greater than 1 compared to control was considered significant.

These results show the antibacterial effect of ozone on *S. mutans* while spread on a surface, during the test another agar plate was spot assay with 10µl of a titration of *S. mutans*, *L. acidophilus* and *E. faecalis* from 10<sup>9</sup> to 10<sup>3</sup> and exposed to ozone at 2000 ppm for 4 min, resulting in complete killing of the bacteria. This experiment was made in collaboration with Dr. Marcos Ximenes.

A new experiment for the Curozone X4<sup>®</sup> is designed. The tooth model could bring limitations in the recovery of the treated bacteria which can diffuse through the dentin tubules and become unreachable to ozone; in this approach a 96 microtiter well was used to avoid the bacteria recover process with the tooth model.

#### **4.4 Effect ozone in *S. mutans* suspended in media for 15, 30, 45 and 60 s**

Results are reported on Table 5. No killing was observed. Bacteria counts are not different among the high ozone and low ozone groups (Wilcoxon rank sum test,  $p=0.42$ ).

Being unable to reduce the bacteria recover in different exposure times a new experiment was run increasing the time for 1, 2, 3 and 4 min.

#### **4.5 Effect ozone in *S. mutans* suspended in media for 1, 2, 3 and 4 min**

Results are reported on Table 6. No killing was observed. Bacteria counts are not different among the high ozone and low ozone groups but they are significantly different compared to media control groups (Wilcoxon rank sum test,  $p=0.53$ ).

Being unable to reduce the bacteria recover in exposures up to 4 min, a new experiment was designed titrating the bacteria concentration one log from  $10^6$  to  $10^3$ .

#### **4.6 Effect ozone in *S. mutans* suspended in media in concentrations of $10^6$ , $10^5$ , $10^4$ and $10^3$**

Results are reported on Table 7. No killing was observed. Bacteria counts are not

different among the high ozone and low ozone groups but they are significantly different compared to media control groups (Wilcoxon rank sum test,  $p=0.56$ ).

These results demonstrate the inability of the Curozone X4<sup>®</sup> to effectively reduce the CFU/ml of bacteria on suspension. A new experiment testing the ozone effect under control conditions was run exposing bacteria in a microtiter plate and a spiral plate and the same time to 2000 ppm for 4 min.

#### **4.7 Effect ozone in *S. mutans* in media and on agar**

Results are shown on Table 8. The bacteria density of the treated (424 mO.D.  $\lambda 610$  nm) and untreated group (432 mO.D.  $\lambda 610$  nm) showed no bacteria killing.

For the agar group, the plate that was treated with ozone gas showed 100% bacteria inhibition, the untreated control group showed 5.63 log<sub>10</sub> CFU/ml. Bacteria counts are different among the 3 mutually exclusive groups of non-treated, ozone treated, treated media. (Wilcoxon.  $p<.0001$ ).

## 5. Discussion

The association between plaque levels of *S. mutans* and caries is well described<sup>35-38</sup>. Bowden et al (1990) found the strongest association when plaque was removed from single occlusal fissures. Seventy-one percent of the carious fissures had *S. mutans* accounting for more than 10% of the viable flora, whereas 70% of the fissures that were caries free had no detectable *S. mutans*. Sixty-five percent of the pooled plaque samples from the children with rampant caries had *S. mutans* accounting for more than 10% of the viable flora, whereas 40% of the pooled samples from children that were caries free had no detectable *S. mutans*<sup>35</sup>. Therefore methods to eliminate or inhibit *S. mutans* in the oral cavity must be the main objective in caries prevention. Ozone is considered a strong oxidizer of the cell walls and cytoplasmic membranes of bacteria and is considered a potent bactericidal, antiviral, and antifungal agent<sup>39</sup>. It is important to point out that this statement is based on the use of ozone in blood not gassing ozone to a microfilm.<sup>39, 40</sup> This is a common finding in the literature, extrapolating the use and effect of systemic ozone dissolving it in the bloodstream, to the dental field.<sup>18, 39</sup> These articles explain the indications and mechanism of action of ozone dissolved in blood but there is no reference to the effect in the oral cavity. Therefore the real ability of ozone to kill cariogenic bacteria is yet to be determined.

The majority of microorganisms in nature are found attached to surfaces, where they

grow to form biofilms. Biofilms have been defined as matrix embedded microbial populations, adherent to each other and/or to surfaces or interfaces.<sup>2, 41, 42</sup> This is especially true in the oral cavity which is permanently in wet conditions. One property of biofilms is the protection from host defenses, desiccation etc. by production of extracellular polymers to form a functional matrix<sup>2</sup> this is a key factor understanding the effect of antimicrobial agents, they have to be able to reach the bacteria through the biofilm. There is evidence that bacteria living in biofilms as dental plaque on tooth surfaces are generally more resistant to antimicrobial agents than bacteria in batch culture normally used for “in vitro” susceptibility testing<sup>43</sup>. The bacteria culture used in this study was grown in a sucrose free media; therefore the bacteria could not secrete a glucan layer that would allow creating a protective biosphere.

The antibacterial effect of ozonated water and ozone bubbled in liquid has been extensively published.<sup>20, 23, 39, 40, 44</sup> There are numerous publications that claim an antibacterial effect ozone *in vivo*<sup>7, 12</sup> and *in vitro*<sup>8, 9, 11, 13, 14, 16, 17, 20</sup> but several authors have reported the inability of gas ozone to reduce the number of bacteria as a result of treatment<sup>4, 22, 29, 30, 32, 33</sup> which are in accordance with the results obtained in this investigation when using the Curozone X4<sup>®</sup> on both root surfaces and microtiter plates and the ozone generator OL80A on the microtiter plate. Reviewing the literature that supports the use of ozone in dentistry it was found that not all the cited “*in vitro*” studies tested ozone in the same conditions.<sup>11, 13, 14, 20</sup> Baysan et al. (2000) tested the effect of ozone gas and ozonated water on *S. mutans* and *S. sobrinus*, the bacteria was collected from primary carious lesions on freshly extracted teeth;

they concluded that ozone gas application for a period of 10 s was capable of reducing the numbers of *S. mutans* and *S. sobrinus* on saliva-coated glass beads “in vitro”. The article by Nagayoshi *et al.* (2005) on *S. mutans* tested the effect of ozone on dental plaque samples from human subjects showing that *S. mutans* was killed immediately when exposed to ozone water; however there was no exposure to ozone gas in this study. Johansson *et al.* (2009) showed 99.9% killing of *S. mutans* in saliva after being exposed to ozone gas for 60 s. It is important to point out that the ozone generator used in this investigation KaVo Healozone™ 2130C (Curozone; KaVo Biberach/Riss, Germany) was modified to bubble the bacteria culture. The device was not used as proposed by the clinical protocol in the oral cavity.

In contrast, there are other “in vitro” studies showing the inability of ozone gas to effectively reduce the bacteria number<sup>22, 29, 30, 32, 45</sup> Hems *et al.* (2005) compared the effect of sodium hypochlorite (NaOCl ) and ozone gas generated by Purezone (Ipswich, UK). They concluded that “Ozone had an antibacterial effect on planktonic *E. faecalis* cells and those suspended in fluid, but little effect when embedded in biofilms. The antibacterial efficacy of ozone was not comparable with that of NaOCl under the test conditions used”. In 2007 Muller *et al.* published an article where ozone gas, photodynamic therapy, chlorhexidine in 0.2 and 2% and NaOCl in 0.5 and 5% were applied to *A. naeslundii*, *V. dispar*, *F. nucleatum*, *S. sobrinus*, *S. oralis* and *C. albicans* biofilms on bovine teeth. The HealOzone 2130C device generator was used. They found that “only the 5% sodium hypochlorite solution was able to totally eliminate the microorganisms in the biofilm. The observed reduction of viable counts

by vacuum-ozone application and PDT was less than one  $\log_{10}$  step. Under the conditions of the study, gasiform ozone and PDT had a minimal effect on the viability of microorganisms organized in a cariogenic biofilm". Polydorou et al. (2012) tested the HealOzone 2130C device by inoculating *S. mutans* and *L. cassei* in artificially made cavities in caries-free third molars and then treat them for 60 s with ozone. The cavities were filled with composite. After 4 and 8 weeks, the bacteria number was determined. The conclusion brought is the exposure of ozone through the "HealOzone 2130C device for 60 s can provide some antibacterial treatment against *S. mutans* even after 8 weeks. However, an elimination of the microorganisms seems not to be possible. *L. cassei* was more resistant to ozone. Although ozone exerts a significant antibacterial effect against *S. mutans*, it is probably not enough as the only antibacterial method, during the fillings therapy".<sup>30</sup> The article published by Estrela et al (2007) tested the effect of ozonated water, gaseous ozone (PXZ3507; Eaglesat Tecnologia em Sistemas Ltda., Sao Jose dos Campos, SP, Brazil), sodium hypochlorite (2.5%) and chlorhexidine (2%) on *E. faecalis* inoculated in human roots. They concluded that "The irrigation of infected human root canals with ozonated water, NaOCl, chlorhexidine and the application of gaseous ozone for 20 min was not sufficient to inactivate *E. faecalis*". Iman et al. (2008) tested the effect of 5.25% sodium hypochlorite (NaOCl), 2% chlorhexidine (CHX) and ozonated water on *E. faecalis* and *C. albicans* biofilms. They found that NaOCl succeeded in complete degradation of the biofilm. This effect was less with CHX and even less with ozonated water. Their conclusion is that the antimicrobial effectiveness of 5.25%

NaOCl was reinforced, whereas that of ozonated water was questionable.

Previous studies with positive results have been presented in Europe but there are several studies that question the effectiveness of ozone as an anticariogenic agent. A Cochrane Database of Systematic Reviews<sup>34</sup> on three clinical studies reported that the analyses of all the studies were conducted at the level of the lesion, which is not independent of the person, for this reason pooling of data was not appropriate or attempted. Few secondary outcomes were reported, but one trial reported an absence of adverse events. Also, masking of participants was absent (as no placebo was provided), and there are doubts about the randomization. Therefore, the Cochrane study concluded that given the high risk of bias in the available studies and lack of consistency between different outcome measures, there is no reliable evidence that application of ozone gas to the carious lesion stops or reverses the process<sup>30,46</sup>.

Several investigations show that different killing methods have demonstrated dose effectiveness.<sup>29, 47</sup> Gomes *et al.* (2001) compared sodium hypochlorite (NaOCl) (0.5%, 1%, 2.5%, 4% and 5.25%) and chlorhexidine (CHX) liquid and gel at (0.2%, 1% and 2%). it was concluded that all irrigants were effective in killing *E. faecalis*, but at different times. CHX in the liquid form at all concentrations tested (0.2%, 1% and 2%) and NaOCl (5.25%) were the most effective irrigants. However, the time required by 0.2% CHX liquid and 2% CHX gel to promote negative cultures was only 30 s and 1 min, respectively in contrast NaOCL 5.25% required less than 30 s to produce 100% inhibition growth.<sup>47</sup> Their conclusion was that all irrigating solutions had antibacterial activity but its effectiveness was depended on its



concentration. The results in the present study show that ozone is not dose dependent, there was no effective bacteria killing either after an exposure of 15 seconds or 4 minutes in the two different experimental designs. In the root experiments, the results were similar among the low and high ozone modes compared to the control while exposing 7µl of 10<sup>6</sup> CFU/ml for up to 60 sec; the microtiter experiment show similar result where effective killing could not be achieved in exposition that were form 15 to 4 min and the results were not different from the control.

The present *in vitro* study could not demonstrate any reduction in the number of *S. mutans* after being treated with gasiforme ozone using the Kavo Curozone X4<sup>®</sup> generator in exposures that went from 15 sec to 4 min; the results were similar on roots as well as in microtiter plates. It is interesting to mention that while using the Curozone X4<sup>®</sup> it was found that the pressure required to keep the seal of the silicone tip to the surface being treated was minimal, when increasing the pressure, the device stopped working making this procedure very sensitive and not easy to perform even on the bench in the laboratory, so we presume that clinically would be even more difficult to obtain good sealing and consequently easy application. Even though some publications suggest that “It is recommended to deliver ozone be under pressure into a lesion by pressing the delivery tube onto the carious surface so that it can penetrate the lesion”.<sup>15, 17</sup> It was also noticed that after the ozone exposures some biofilms were desiccated and some other were wet; but the bacteria recovery did not show any difference compared to the control, this finding is in accordance with the study published by

Hems *et al.* (2005). It has been published that “ozone will react immediately with the reductants in culture media, and the authors did not bubble the ozone into the biofilm”<sup>15, 16</sup> During the present investigation the bubbling effect was not seen when the ozone was applied to bacteria in media. Actually while using the Curozone X4<sup>®</sup> when pressure was applied the device immediately stopped the ozone delivery.

The ozone concentrations tested were 2 and 20 using the ozone generator OL80A in the Mouse Exposure Chamber; for the 200 and 2000 ppm the ozone generator OL80W in the Modular Incubator Chamber for exposure time of 4 min. It is important to mention that the samples were exposed longer than the mentioned times, the chamber took around 20 min to reach the concentration levels and then the 4 min were counted, therefore the exposure time to ozone was longer but it is important to emphasize that the effect on bacteria in the microtiter plate was still negative. The inability of ozone gas to diffuse in liquid has been proposed by different authors.<sup>22, 48</sup> In 1976 Hoigné and Bader stated that “Ozone is a selective oxidant and affects only certain compounds but when it dissolves in water, it becomes highly unstable and rapidly decomposes through a complex series of chain reactions”. It has also been stated that the stability of ozone in water is low and the ozone dissipates very quickly at room temperature.<sup>49</sup>

Separate experiments in collaboration with Dr. Marcos Ximenes were conducted at the same time as the spiral plated assays described above. Instead of spiral plating 49.2ml volumes, 10µl volumes of *S. mutans*, *Lactobacillus acidophilus* and *Enterococcus faecalis* in concentration ranging from 10<sup>8</sup> to 10<sup>3</sup> CFU/ml were delivered as drops on the agar surface

prior to exposure to atmospheres of 2, 20, 200 and 2000 ppm of ozone for 4 min. This simulated the volumes in the microtiter plate wells. As with the spiral plate studies with *S. mutans*, the results obtained showed 100% killing at 2000 and 200 ppm and logarithmic reductions in 20 and 2 ppm atmospheres with all three test species. In addition an experiment exposing 10µl of 10<sup>6</sup> CFU/ml of *S. mutans* on the bottom of an agar free Petri dish to 2000 ppm of ozone for 4 min, showed 100% killing (Unpublished data). This may suggest that ozone gas does not equilibrate with the atmospheric air into a small space such as a well in a microtiter plate as a result it is not capable to have an antimicrobial effect. The results obtained in the experiment conducted by Dr. Marcos Ximenes show that ozone can effectively kill bacteria when it has access to the microorganism and while being on a surface in low volume of liquid (unpublished data) and probably ozone is ineffective in larger liquid amounts.<sup>22, 48</sup> These results may explain why the Curozone X4 could not kill bacteria, the ozone generated by the instrument was not able to adequately displace the air either in the artificial lesion preparation in the dentin slab or in the microtiter plate to achieve bactericidal concentrations of ozone in the target bacterial suspension.

The negative results of this and other *in vitro* investigations along with the fact that bacteria living in biofilms as dental plaque on tooth surfaces are generally more resistant to antimicrobial agents than bacteria in batch culture normally used for *in vitro* susceptibility testing<sup>43</sup>; raise questions about the antimicrobial capacity of the Curozone X4 for clinical use. In a communication received by the manufacture it was assured that the device used for this

investigation had been tested by the quality control department of the company and was properly working before being sent to us. (See appendix A).

The results of the present study suggest that ozone in controlled conditions is not capable to effectively reduce the bacteria number inoculated in media in long exposures when placed in a microtiter plate. In contrast ozone was able to reduce the bacteria number when it had adequate access to the bacteria (Petri dish (100 mm diameter) *vs.* microtiter well (6 mm diameter) *vs.* the ~ 1 mm diameter simulated lesion in the dentin slab. The latter result confirms the finding of previous studies that ozone gas is effective in killing *S. mutans*.<sup>25, 27</sup>

## **6. Conclusions**

Without the obvious limitations that exist in a clinical environment, it can be concluded that the Curozone X4<sup>®</sup> generator is not capable to effectively reduce the number of *S. mutans* in exposure times up to 4 min.

When the bacteria exposure is in an open surface, ozone at 2000 ppm for 4 min can completely kill *S. mutans*. Concentrations of ozone as low as 2 ppm result in biologically significant (exponential) killing of *S. mutans*.

This study suggests that Curozone X4<sup>®</sup> generator is not capable of delivering ozone in a way that would effectively disinfect a caries lesion.

## 7. Tables

**Table 1. Indication of the ozone therapy in medicine.**<sup>19</sup>

Indications	Mechanisms
External ulcers and skin lesions	Disinfection, wound cleansing and improved wound healing
Arterial circulatory disorders	Activation of red blood cell metabolism with an improvement of oxygen release Activation of reactive oxygen species and radical scavengers
Immunodeficiency and immunodysbalance: Chronic forms of hepatitis B and C Supportive therapy in cancer patients	Activation of immunocompetent cells with release of cytokins such as interferons and interleukins.
Supportive therapy in rheumatoid arthritis	Modulation of the immune system Increase of antioxydative capacity by activation of biological antioxidants
Inflammatory condition such as: Knee arthrosis Gonarthrosis Traumatic knee disorders	Antiinflammatory effect Activation of antioxidative enzymes as radical scavengers Activation of immunocompetent and cartilage cells with release of TGF- $\beta$

**Table 2. Number of bacteria recovered after the different treatment protocols applied with the Kavo Curozone X4<sup>®</sup> ozone generator to human dentin specimens for 60 s (log<sub>10</sub> CFU)**

<b>Treatment protocol (n=36)</b>	<b>log<sub>10</sub> CFU (Mean ± SD)</b>
<b>Low ozone<sup>1</sup></b>	5.25 ± 0.87
<b>High ozone<sup>2</sup></b>	5.47 ± 0.92
<b>Control<sup>3</sup></b>	5.58 ± 0.67

(Kruskal-Wallis test, p=0.58)

1. Exposed to ozone in ambient air for 60 seconds. n=12
2. Exposed to ozone in oxygen using the attached tank for 60 seconds. n=12
3. Exposed to ambient air for 60 seconds. n=12

**Table 3. Number of bacteria recovered after the different treatment protocols applied with the Kavo Curozone X4<sup>®</sup> ozone generator to bovine dentin specimens for 60 s (log<sub>10</sub> CFU)**

<b>Treatment protocol (n=36)</b>	<b>log<sub>10</sub> CFU</b>
<b>Low ozone control<sup>1</sup></b>	5.37 ± 0.71
<b>High ozone<sup>2</sup></b>	5.32 ± 0.82

(Wilcoxon rank sum test, p=0.53)

1. Exposed to ozone in ambient air for 60 seconds. n=18
2. Exposed to ozone in oxygen using the attached tank for 60 seconds. n=18



**Table 4. Number of bacteria recovered and killing rates after exposing *S. mutans* on agar ( $\log_{10}$  CFU/ml) to ozone in the Modular Incubator Chamber at different concentrations for 4 min in covered and uncovered Petri dishes.**

After 24 hours	Control	2 ppm	2 ppm cover on	20 ppm	20 ppm cover on	200 ppm	2000 ppm
mean	5.64	2.81	4.58	1.59	3.55	0	0
$\log_{10}$ reduction		2.83	1.06	4.05	2.09	5.64	5.64

logarithmic reduction in recoverable CFU greater than 1 compared to control was considered significant.

**Table 5. Bacterial growth as optical density (mO.D. at  $\lambda$ 610 nm) attained following incubation at 37°C for 18 hr after the different treatment protocols applied with the Kavo Curozone X4<sup>®</sup> ozone generator on 10 $\mu$ l of 10<sup>6</sup> CFU/ml of *S. mutans***

		10 <sup>6</sup> CFU untreated	10 <sup>6</sup> CFU 15 sec	10 <sup>6</sup> CFU 30 sec	10 <sup>6</sup> CFU 45 sec	10 <sup>6</sup> CFU 1 min
<b>Low Ozone</b>	<b>mean sd</b>	281 38	278 45	289 9	282 7	299 13
<b>High Ozone</b>	<b>mean sd</b>	312 14	308 2	297 7	302 15	306 4

(Wilcoxon rank sum test, p=0.42)

**Table 6. Bacterial growth as optical density (mO.D.  $\lambda$ 610 nm) attained following incubation at 37°C for 18 hr after the different treatment protocols applied with the Kavo Curozone X4<sup>®</sup> ozone generator on 10 $\mu$ l of 10<sup>6</sup> CFU/ml of *S. mutans***

		10 <sup>6</sup> CFU untreated	10 <sup>6</sup> CFU 1 min	10 <sup>6</sup> CFU 2 min	10 <sup>6</sup> CF 3 min	10 <sup>6</sup> CFU 4 min
<b>Low Ozone</b>	<b>mean</b>	229	257	251	244	245
	<b>sd</b>	13	8	15	18	20
<b>High Ozone</b>	<b>mean</b>	268	256	233	257	236
	<b>sd</b>	14	2	7	15	4

(Wilcoxon rank sum test, p=0.53)

**Table 7. Bacterial growth as optical density (mO.D.  $\lambda$ 610 nm) attained following incubation at 37°C for 18 hr after the different treatment protocols applied with the Kavo Curozone X4<sup>®</sup> ozone generator on 10 $\mu$ l of a bacteria titration from 10<sup>6</sup> to 10<sup>3</sup> CFU/ml of *S. mutans***

		10 <sup>6</sup> CFU untreated	10 <sup>6</sup> CFU 1 min	10 <sup>6</sup> CFU 2 min	10 <sup>6</sup> CFU 3 min	10 <sup>6</sup> CFU 4 min
<b>Low Ozone</b>	<b>mean</b>	267	263	251	244	200
	<b>sd</b>	10	9	15	18	19
<b>High Ozone</b>	<b>mean</b>	265	266	233	257	168
	<b>sd</b>	6	14	20	6	9

(Wilcoxon rank sum test, p=0.56)

**Table 8. Bacterial growth as optical density (mO.D.  $\lambda$ 610 nm) recovery in media and recovery ( $\log_{10}$  CFU/ml) on agar attained following incubation at 37°C for 18 hr after the different treatment protocols applied with the ozone generator OL80W in the Modular Incubator Chamber at 2000 ppm for 4 min**

At 24 hours	Non treated bacteria in media <sup>1</sup>	Ozone treated bacteria in media <sup>1</sup>	Non treated bacteria in agar <sup>2</sup>	Ozone treated bacteria in agar <sup>2</sup>
<b>mean</b>	432	424	5.63	0
<b>SD</b>	108	158		

(Wilcoxon.  $p < .0001$ ).

1. mO.D. at  $\lambda$ 610 nm

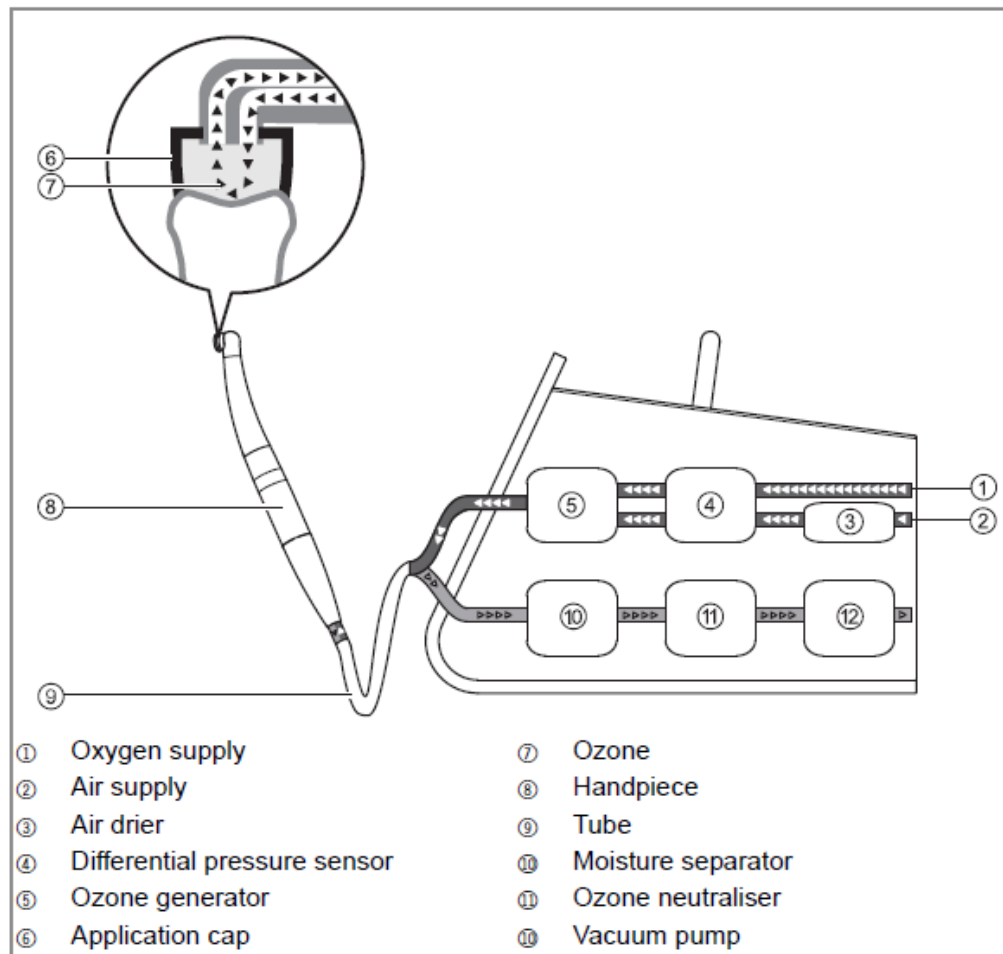
2.  $\log_{10}$  CFU/ml

**8. List of Figures.**

**Figure 1. Kavo Curozone X4<sup>®</sup>.**



**Figure 2. Curozone X4<sup>®</sup> schematic functioning.**



**Figure 3. OL80A Ozone generator.**





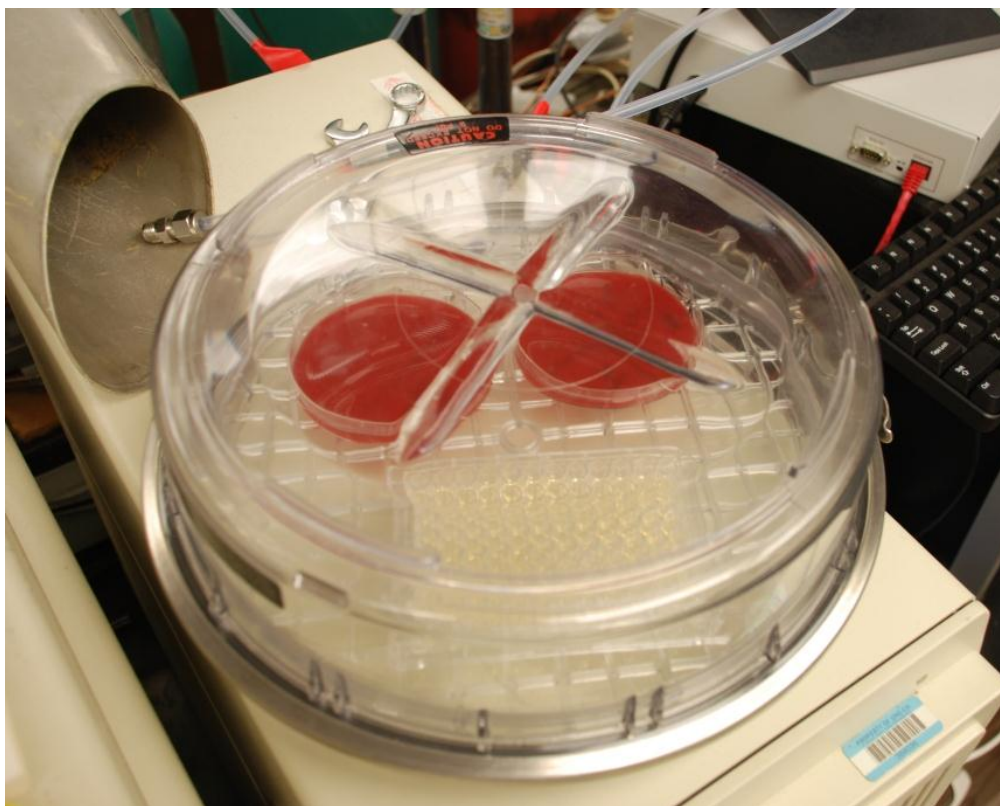
**Figure 4. Mouse Exposure Chamber.**



**Figure 5. OL80W Ozone generator.**



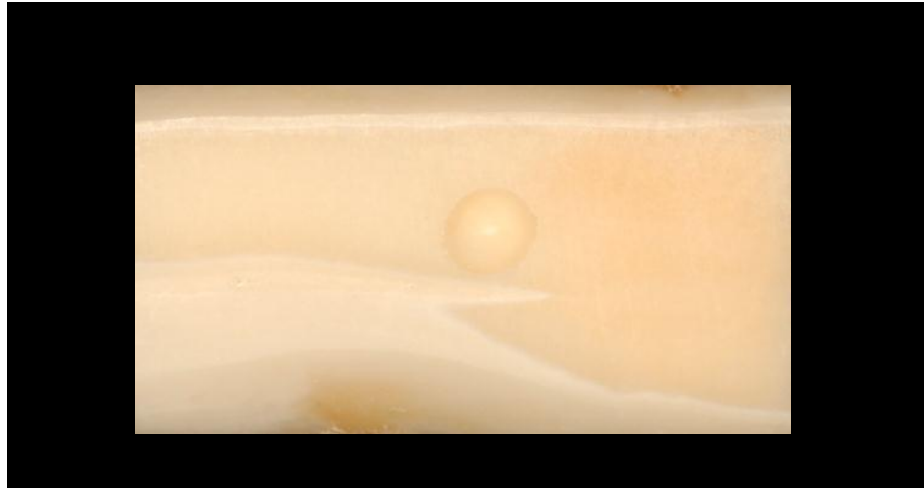
**Figure 6. Modular Incubator Chamber.**



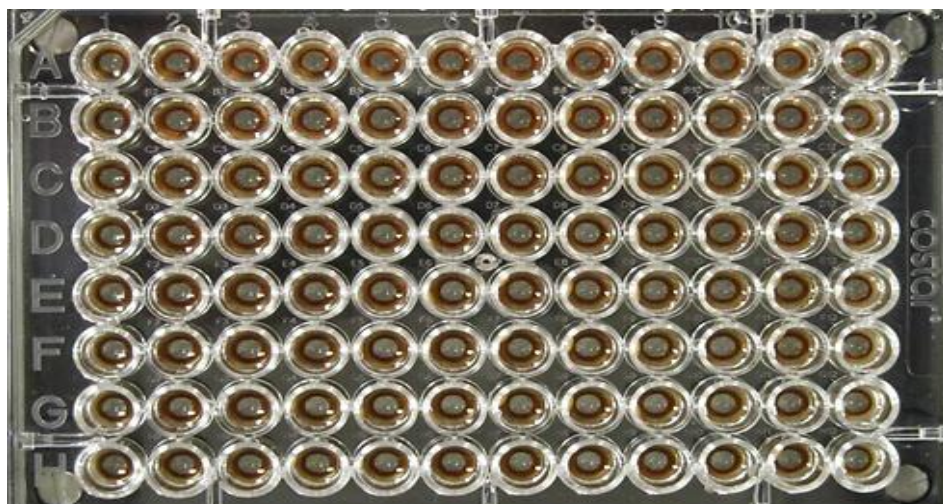
**Figure 7. Human root specimen.**



**Figure 8. Bovine root specimen.**

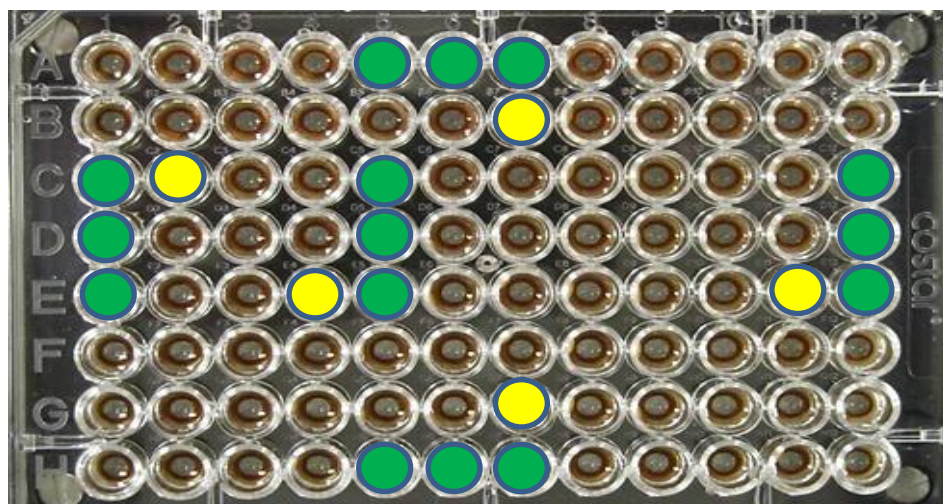


**Figure 9. 96 well microtiter plate.**





**Figure 10. Experiment 7 sample distribution.**



● 10µl of  $10^3$  CFU/ml

● 200µl of media

## Appendix A.

Snapshot of the report of the Curozone X4® (Kavo, Biberach/Riß, Germany) tested in this investigation, showing the ozone values for low and high mode.

Testschritt	Antwort	erwartet	Min	Max	Ergebnis
Prüfer	stuwe	-	-	-	-
Datum	14.06.2011 17:43:47	-	-	-	-
Programmversion	1.0.6.0	-	-	-	-
Prüfdatei	Healozone X4_Pruefung_Rev1.skr	-	-	-	-
Seriennummer	4102497	-	-	-	-
Version auslesen	4.03 2011-06-10b	4.03 2011-06-10b	-	-	OK
Healozone in Testmodus	TESTO2>@#echo off	-	-	-	-
Prüfdatum setzen	10.06.2011	10.06.2011	-	-	OK
O2 Version auslesen	O2>V20110315.1413	O2>V20110315.1413	-	-	OK
Sollwert setzen 410	O2>ACK	O2>ACK	-	-	OK
Reglerparameter Kp 0,05	O2>ACK	O2>ACK	-	-	OK
Reglerparameter Kd 0,02	O2>ACK	O2>ACK	-	-	OK
Healozone Reset	-	-	-	-	-
Fluss Low	33,0	35	31,5	38,5	OK
Ozon Low	5,4	5,5	5,25	5,75	OK
Fluss High	14,8	15	14,30	15,7	OK
Ozon High	40,2	43	38,7	47,3	OK
Feuchte auslesen	0; 1	-	-	-	-
Fluss Low Handstück	32,2	35	31,5	38,5	OK
Ozon Low Handstück	5,4	5,5	5,25	5,75	OK
Fluss High Handstück	14,8	15	14,2	15,7	OK
Ozon High Handstück	40,5	43	38,7	47,3	OK
Feuchte auslesen	0; 1	-	-	-	-
Fluss Low Endo	11,8	11	9,50	12,50	OK
Ozon Low Endo	5,5	5,5	4,4	6,60	OK
Fluss High Endo	10,4	9	7,5	10,5	OK
Ozon High Endo	38,6	43	34,4	48,00	OK
Feuchte auslesen	0; 0	-	-	-	-
Reset Behandlungszähler Service	P	P	-	-	OK
Reset Behandlungszähler Total	P	P	-	-	OK
Reset Betriebszeit	P	P	-	-	OK
Reset Behandlungszeit	P	P	-	-	OK
Flusswerte auslesen	494; 0; 189; 53	-	-	-	-
PWM auslesen	250;7;140;113;3;250;3	-	-	-	-
Temperatur auslesen	23; 23	-	-	-	-



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