

## The antimicrobial effect of 0.1 ppm ozonated water on 24-hour plaque microorganisms *in situ*

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**Abstract:** Ozone is a known oxidant present in the atmosphere and is commercially produced by simple ozonizer machines. It is a powerful antimicrobial agent in its gaseous and aqueous forms. Ozone readily dissolves in water and retains its antimicrobial property even in the dissolved state. In this study, the effect of 0.1 ppm ozonated water was analyzed on 24-hour supragingival plaque (SP) samples *in situ*. SP was collected from the two most posterior teeth in the contra-lateral quadrants before and after a 30-second rinse with either distilled water (control group) or 0.1 ppm ozonated water (test group). The plaque was used to count the number of total bacteria, total anaerobic bacteria, *Streptococcus mutans*, and *Candida albicans* on selective agar media. The statistical analysis of the number of colony forming units (CFUs) obtained demonstrated a significant antimicrobial effect of ozonated water on the total bacteria ( $p = 0.01$ ) and anaerobes ( $p = 0.02$ ). A reduction in the post-rinse CFU count for *Streptococcus mutans* was also observed, but the effect was not statistically significant ( $p = 0.07$ ). The *Candida* species was only grown from one sample. Ozonated water at the 0.1 ppm concentration was effective in reducing the load of 24-hour plaque bacteria, but it did not eliminate them completely.

**Descriptors:** Ozone; Dental Plaque; Bacteria.

### Introduction

Bacteria in a biofilm are more resistant to antimicrobial agents<sup>1</sup> because their organized nature enables them to behave as a quorum. Within its layers, a biofilm has dynamic interactions between its biotic and abiotic components.<sup>2</sup> Dental plaque biofilm is a known etiological factor that causes oral diseases such as dental caries,<sup>3</sup> gingivitis,<sup>4</sup> and periodontitis.<sup>5</sup> Oral microorganisms have also been associated with systemic problems such as pneumonia<sup>6</sup> and cardiovascular diseases.<sup>7</sup> To control the accumulation of dental plaque, antiseptics, antibiotics, oxidizing agents, herbal extracts and enzymes are used as antiplaque agents.<sup>8</sup>

Ozone (O<sub>3</sub>) in a gaseous or aqueous phase has been shown to be a powerful and reliable antimicrobial agent against bacteria, fungi, protozoa, and viruses.<sup>9</sup> It is an unstable gas capable of oxidizing any biological entity.<sup>10</sup> Its oxidative capacity at 100 ppm, 200 ppm and 400 ppm can also induce serious toxicity due to lipid peroxidation and ultimately cause DNA damage.<sup>11</sup> A low concentration of ozonated water is sufficient

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to inactivate bacterial cells (0.12–0.19 mg/l) and their spores (2.29 mg/l).<sup>12</sup> It has been shown that *Streptococcus mutans*, *Lactobacilli casei* and *Actinomyces naeslundii* suspended in a salt buffer can be completely killed within 60 seconds<sup>13</sup> following exposure to ozone gas. Ozone readily dissolves and forms ozonated water when introduced into water. Ozonated water is also a powerful oxidizing and antimicrobial agent.<sup>14</sup> In both gaseous and aqueous forms, ozone is potentially effective as a disinfecting agent for the removal of biofilms and their related microorganisms.<sup>15</sup> The powerful disinfecting property of gaseous ozone has been utilized in dentistry to treat primary root caries,<sup>16</sup> occlusal caries,<sup>17</sup> dentine hypersensitivity<sup>18</sup> and cervical sensitivity.<sup>19</sup> It is accepted that its application at doses between 90 µg and 120 µg does not affect the physical properties of enamel.<sup>20</sup> Ozonated water has been used in the sterilization of dentures (10 ppm)<sup>21</sup> and dental unit water-line systems.<sup>22</sup> Plaque microorganisms have shown vulnerability to ozonated water under *in vitro* conditions<sup>23</sup> (4 mg/l for 10 seconds). The main objective of this study was to determine the *in situ* antimicrobial effect of 0.1 ppm ozonated water on 24-hour plaque microorganisms following a 30-second rinse.

## Methodology

The study involved 40 healthy volunteers between the ages of 18 and 40, who had at least 20 permanent teeth that were periodontally healthy. Volunteers who had taken antibiotics or other antibacterial agents less than one month prior to the study were not included. Ethical approval was obtained from the Dental School Ethics Committee, School of Dentistry, University of Malaya.

### Sample collection

The study was designed to utilize convenient sampling. The volunteers were randomly distributed and grouped as either Group 1 (20 volunteers) or Group 2 (20 volunteers). The volunteers were asked to not brush their teeth or use any form of oral hygiene for 24 hours before sample collection. Pre- and post-rinse 24-hour supragingival plaque (SP) samples from Group 1 and Group 2 were collected.

The pre-rinse samples provided the baseline data for this study. The post-rinse SP samples were collected 20 minutes after a 30-second rinse with either distilled water (Group 1) or 0.1 ppm ozonated water (Group 2). Thus, Group 1 was the control group, and Group 2 was the test group. The pre- and post-rinse SP samples were collected from the buccal and lingual surfaces of the teeth using a sterile stainless steel excavator. To standardize the amount of SP collected, the excavator was used in several gentle scooping motions to avoid contact with the marginal gingiva. Each time a scoop was made, the SP was transferred into a microfuge vial containing sterile reduced transport fluid (RTF). The oxygen content of the RTF was reduced with the addition of trichloroacetic acid (TCA). The pre-rinse SP samples were collected from the three most posterior teeth on the upper right and lower left quadrants, while the post-rinse SP samples were collected from the three most posterior teeth on the upper left and lower right quadrants (contralateral teeth).

### Preparation of ozonated water

The ozonated water was freshly prepared using the ORM AW600 ozone gas generator machine (ORM Beauty and Health care Sdn Bhd, Petaling Jaya, Malaysia). Ozone gas produced from this ozonizer was introduced into 1 liter of sterile distilled water for 20 minutes. The concentration of dissolved ozone in the water was measured using an EcoZone™ EZ10W portable dissolved ozone meter (Ecosensors Inc., Newark, USA). The concentration of ozonated water used for this study was between 0.08 ppm and 0.1 ppm. The ozonated water was used within 20 minutes after its preparation.

### Determination of microbial population

The wet weight of the plaque was calculated by subtracting the weight of the microfuge vial containing the RTF from the weight of the same vial after the addition of the plaque sample. The population of plaque bacteria was expressed as colony forming units per unit of plaque (CFU/mg plaque). Selective agar media was used to culture, isolate and enumerate specific plaque bacteria. The media used were:

- brain heart infusion broth (BHI, Oxoid Limited,

- Hampshire, United Kingdom),
- BHI agar (Fluka/Sigma-Aldrich Corporation, Bangalore, India),
  - Schaedlers agar (SA, Oxoid Limited, Hampshire, United Kingdom),
  - Columbia nutrient agar (CNA, Difco/Voigt Global Distribution Inc., Kansas, USA) and
  - Sabourauds dextrose agar (SDA, Oxoid Limited, Hampshire, United Kingdom).

BHI broth was used in the preparation of the plaque bacterial suspension.

The bacterial plaque suspension was serially diluted five-fold in RTF before inoculation onto the selective agar plates to reduce the bacterial population to a level where the growth could be detected easily. The inoculation of each sample was performed in triplicate in a laminar flow cabinet. The BHI agar was used to culture fastidious Gram positive and Gram negative plaque bacteria aerobically. The SA was used to culture and isolate the anaerobic plaque bacteria. The CNA was mixed with sterile human blood and potassium tellurite to make the media selective for *Streptococcus mutans*. The SDA was used to selectively grow the *Candida* species.

Following inoculation, all of the agar plates were incubated for 48 hours at 37 °C aerobically, with the exception of the SA plates, which were incubated in an anaerobic jar. The CFU of the pre-rinse and post-rinse SP samples of Group 1 and Group 2 were compared to evaluate the effect of rinsing with ozonated water. The results were statistically analyzed by a one-way analysis of variance (ANOVA) using the Minitab14 software (Minitab, State College, USA). A p value < 0.05 was considered significant.

## Results

None of the volunteers expressed discomfort or any kind of adverse reaction to the ozonated water rinse. Three samples were discarded because the media plates were feared to be contaminated.

### Effect of rinsing with distilled water on microbial components (Group1)

The post-rinse CFU counts for total bacteria, anaerobes and the *Streptococcus* species showed a dif-

**Table 1** - Tabulation of the percentage of samples that displayed a reduction in the CFU counts following rinsing.

	BHI agar (total bacteria)	SA (anaerobes)	CNA ( <i>Streptococcus</i> )
Group 1 - rinsing with distilled water	47% (8 out of 17 samples)	52% (9 out of 17 samples)	58% (10 out of 17 samples)
Group 2 - rinsing with ozonated water	100%	100%	100%

**Table 2** - Tabulation of the results of Group 2 following the ozonated water rinse.

	Average reduction of CFU	Significance (ANOVA)
Total bacteria	45.3%	0.01*
Anaerobes	51.7%	0.02*
<i>Streptococcus</i> sp.	56.4%	0.07
<i>Candida</i> sp.	-	-

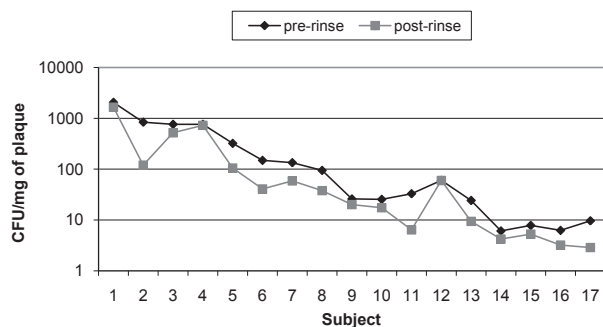
\* Significant at p < 0.05.

ference in a few samples, but many samples were not affected by the distilled water rinse. Only 47% of the BHI agar, 52% of the SA and 58% of the CNA media plates inoculated with post-rinse SP had fewer CFUs than the pre-rinse SP media plates (Table 1). For the few samples that displayed a difference between pre- and post-rinsing, the effect was determined by the ANOVA test to not be statistically significant. Only one of seventeen Group 1 volunteers showed positive *Candida* growth.

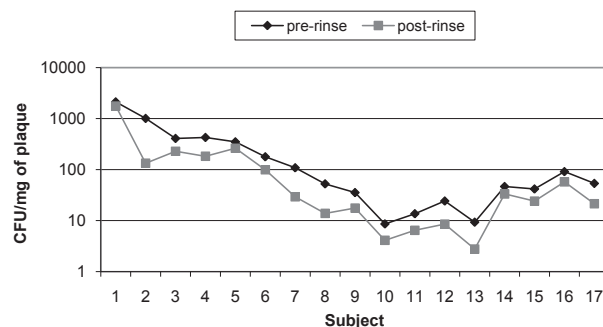
### Effect of rinsing with ozonated water on microbial components (Group 2)

Unlike Group 1, a reduction in the CFU count of SP was observed in all of the post-rinse samples of Group 2 following the ozonated water rinse (Table 1). The pre-rinse and post-rinse CFU values of the total microbes for Group 2 are plotted in Figure 1. The average reduction of the total microbial count observed was 45.3%. This difference was statistically significant (Table 2).

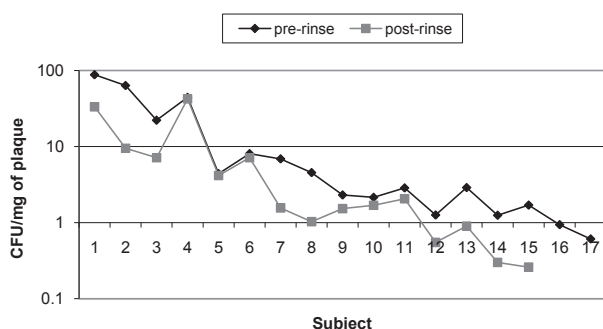
For the anaerobes, the reduction was 51.7% and was shown to be statistically significant (p = 0.02,



**Figure 1** - Pre-rinse and post-rinse CFU counts of the total microbes of Group 2 following the ozonated water rinse.



**Figure 2** - Pre-rinse and post-rinse CFU counts of the anaerobes of Group 2 following the ozonated water rinse.



**Figure 3** - Pre-rinse and post-rinse CFU counts of the streptococci of Group 2 following the ozonated water rinse.

Table 2). The pre-rinse and post-rinse CFU values of the anaerobes for Group 2 are shown in Figure 2.

The streptococci count was reduced by 56.4% for Group 2. However, ozonated water at 0.1 ppm did not show any significant effect ( $p = 0.07$ ) on the CFU counts (Table 2). The pre-rinse and post-rinse CFU values of the streptococci for Group 2 are shown in Figure 3.

The *Candida* species was not isolated from any of the Group 2 samples. Therefore, the effect of ozonated water (0.1 ppm) on the *Candida* species could not be analyzed.

## Discussion

The methodology of this study was generally based on that performed by Pan *et al.*,<sup>24</sup> with some modifications. This pilot study was performed to determine the effect of 0.1 ppm ozonated water on *in situ* plaque formation and to identify any immediate discomfort or adverse reaction an individual may have to the gas. The commonly employed method of evaluating the effectiveness of oral antiseptics

by cleaning the teeth, asking the volunteers to follow a prescribed regimen of mouth rinsing, and then testing the effectiveness after a period of time was not used in this study because ozone machines could not be given to all of the volunteers to prepare fresh ozonated water for rinsing. Taking into consideration the safety of the subjects, this study did not include concentrations of ozonated water higher than 0.1 ppm.

The bacteria grown on selective media from all of the plaque samples were fastidious and required a 24- to 48-hour incubation period. The microbiological tests performed in this study suggested that the ozonated water exhibited some antimicrobial activity on the bacterial population of the 24-hour plaque. Exposure to the ozonated water for 30 seconds reduced the total bacteria population of the 24-hour plaque by 45.3% (Table 2). This reduction may have been due to the activity of the ozonated water, which would have affected the viability of these microorganisms in two ways:

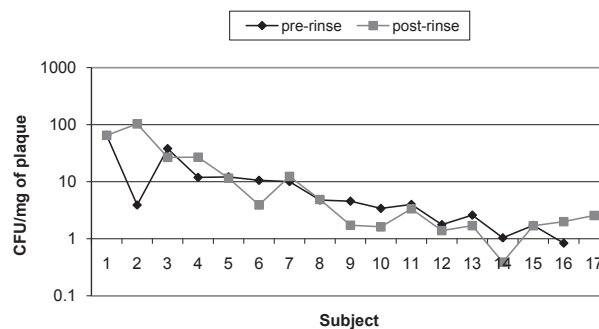
- by directly inactivating the bacterial cells by oxidation and
- by disturbing the normal ecosystem of the plaque by creating an oxygen-rich environment after the dissociation of ozone into oxygen.

A similar observation<sup>25</sup> has been reported for peroxycarbonate, which has an active post-rinse oxygen concentration of 11.4% and was shown to reduce the bacterial count in plaque. In a soft-textured 24-hour plaque, an additional mechanism that can result in the reduction of its bacterial population may be the dislodging effect caused by rinsing.

A few post-rinse samples showed a reduction in the CFU count following rinsing with distilled water (Table 1). However, this observation was erratic, with a few samples showing a reduction in the CFU counts and the remainder of the samples showing either an increase or no difference at all. The reduction in the CFU counts in this group may be attributed to the mechanical dislodging or “washing away” of loose supragingival plaque that formed within the 24-hour period or to the inactivation of viable bacteria by the distilled water. A decrease in the cell viability of bacteria has been reported<sup>23</sup> for bacteria grown on agar plates and treated with distilled water. The finding that post-rinse samples isolated from the group treated with distilled water exhibited more CFUs compared to the pre-rinse samples could be due to differences in the bacterial concentrations in the plaque samples collected from the different quadrants of the upper and lower teeth. However, any major discrepancy related to this difference in bacterial concentrations would have been minimized by collecting pre-rinse and post-rinse plaque from the contralateral quadrants.

In contrast to the erratic results obtained by the distilled water-rinsed SP samples, a clear reduction in the CFU counts was observed in all of the ozonated water-rinsed SP samples (Table 2). The average reduction of the anaerobes (51.7%) in the subjects treated with ozonated water was greater than the reduction of the total bacterial load (45.3%). The enhanced effect of ozonated water on the anaerobes could be because the obligate anaerobes are sensitive to and become inactive in oxygen-rich conditions.<sup>26</sup>

The average reduction observed for the streptococci load (56.4%) was more than that observed for the total bacteria (45.3%) and the anaerobes (51.7%). However, it is interesting to note that this difference was not statistically significant when compared with the CFU count of streptococci following the distilled water rinse. The most plausible reason for this is that *Streptococcus* showed the least resistance to the distilled water rinse. 10 of 17 streptococci samples treated with distilled water showed a reduction in CFUs, whereas total bacteria (8 of 17 samples) and anaerobes (9 of 17 samples) showed greater resistance to the distilled water rinse. This



**Figure 4** - Pre-rinse and post-rinse CFU counts of the streptococci of Group 1 following the distilled water rinse.

suggests that streptococci are somewhat sensitive to distilled water and much more sensitive to ozonated water rinsing than other microbes, and therefore, a statistically insignificant result was obtained for the streptococci for the comparison between the distilled and ozonated water rinses. The pre-rinse and post-rinse CFU values of the streptococci for Group 1 are shown in Figure 4.

The results of this study are comparable to those of previous studies on oxidizing agents and antiseptics. Moran *et al.*,<sup>25</sup> studied the effect of a single dose of two mouth rinses containing either peroxyborate or peroxyborate oxidizing agents on salivary bacteria. The methodology of the present study was similar to that of their study except that salivary bacterial counts instead of plaque bacterial counts were assessed in their study after the single rinse. Both peroxyborate and peroxyborate reduced the bacterial count, but the results were not significant when compared to those of a negative control saline rinse. Additionally, both rinses showed an inhibition of plaque accumulation over time.

## Conclusion

This study suggests that ozone at a concentration of 0.1 ppm is effective in reducing the plaque microbial load but does not eliminate the entire plaque microbial population. The prevention of plaque accumulation is more desirable than plaque elimination. Therefore, ozonated water rinsing may be an extremely useful addition to tooth brushing and flossing because it is bactericidal, easy to prepare and cost effective. There is also convincing evidence demonstrating its bio-compatibility (at 1.25–20 µg/



ml) with human oral epithelial, gingival and periodontal cells.<sup>27</sup> The magnitude of bacterial inactivation that it produces, however, needs further investigation. The need for an ideal tool to maintain oral

health supports the rationale for further study of its benefits in inhibiting the accumulation and growth of plaque microorganisms.

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