

Ozone Gas Effect on Mineral Content of Dentin exposed to *Streptococcus mutans* Biofilm: An Energy-dispersive X-ray Evaluation

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ABSTRACT

Aim: This study aims to assess the effect of ozone gas on dentin exposed to *Streptococcus mutans* biofilm by evaluation of mineral content [log calcium-to-phosphorus (Ca/P)] using energy-dispersive X-ray (EDX) spectroscopy.

Materials and methods: Five human third molars were sectioned into four slices of dentin and distributed in four groups: I – control (no treatment); II – ozone therapy; III – biofilm development; IV – ozone therapy followed by biofilm development. Mineral content (log Ca/P) was evaluated by EDX. Data were analyzed by analysis of variance and Tukey's test ($p < 0.05$).

Results: Results showed that the mineral content of control group (I) was similar to ozone group (II), and was statistically higher than biofilm (III) and ozone + biofilm (IV). The lowest log Ca/P was determined in biofilm group (III).

Conclusion: It can be concluded that ozone gas did not grant preventive effects of demineralization by *S. mutans* biofilm on dentin surface.

Clinical significance: Ozone gas therapy may be an alternative noninvasive treatment aiming to reduce the levels of caries-associated microorganisms. This therapy may, thereby, be an alternative and/or complementary treatment strategy in preventive dentistry.

Keywords: Biofilm, Dental caries, Dentin, Laboratory research, Ozone.

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INTRODUCTION

Dental caries is a multifactorial disease, which is characterized by a local destruction of the tooth.¹ Cavities arise as a result of complex biological processes and are an interplay of four principal factors: Tooth, saliva, bacterial microbiota, and diet.² Initiation and progression of caries depend on biofilm formation over the tooth structure.³ *Streptococcus mutans* form cariogenic biofilms and play a significant role in development of the carious process.^{1,4,5} Dentin surfaces treatment to prevent biofilm formation and reduce the levels of caries-associated bacterial species in dental biofilm may assist in prevention of caries initiation and progression.⁵

During the last years, ozone gas therapy has been suggested as an alternative noninvasive treatment aiming to reduce the levels of caries-associated microorganisms.^{1,6,7} This therapy may, thereby, be an alternative and/or complementary treatment strategy in preventive dentistry.⁸⁻¹³

Ozone therapy has shown favorable results for conservative treatment of tooth decay due to the antimicrobial property of this agent in inhibition and/or destruction of many oral bacteria, and the ability to oxidize the organic material in infected and affected dentin.^{6-10,14}

The oxidant potential of ozone induces destruction of cell walls and cytoplasmic membranes of bacteria.^{8,11} During this process, ozone attacks glycoproteins,

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glycolipids, and other amino acids, inhibiting and blocking the enzymatic cell's control system, resulting in increased membrane permeability, the key element of cell viability, leading to immediate functional cessation.^{8,11,15} Then, ozone molecules can readily enter the cell and cause bacteria's death.^{8,5} Ozone can attack many biomolecules, such as cysteine, methionine, and histidine residues of proteins. Ozone has a severe disruptive effect on cariogenic bacteria by oxidization of biomolecules featured in dental diseases, resulting in elimination of acidogenic bacteria, which allows buffer plaque fluid and penetration of calcium and phosphate ions for tissue remineralization.^{1,8-10,13,16-18}

Various substances, including ozone,¹⁹⁻²² may influence the adhesive materials that restore lost parts of teeth. Literature lacks studies evaluating the effect that this oxidizing agent can induce on dental substrate. The aim of this study was to assess the mineral content of dentin exposed to bacterial biofilm model after using ozone therapy. The first null hypothesis was that ozone gas has no effect on the mineral content of dentin surface. The second null hypothesis was that ozone did not grant preventive effect on demineralization of dentin surface mineral content under bacterial biofilm challenge.

MATERIALS AND METHODS

Teeth Preparation

Five extracted, human caries-free third molars were used. Teeth were collected after obtaining patients' informed consent approval issued by the review board of Federal University of Goiás, Brazil (Protocol number 311/10).

Residual soft tissue was removed from tooth surface with a hand scaler (13/14 curette). Teeth were stored in a 0.2% aqueous thymol solution for no longer than 3 months. They were cut perpendicular to tooth's long axis to obtain slices of dentin approximately 1 mm thick. The first cut was obtained by transversally cutting the specimens with a diamond saw at about 2.5 mm above cement-enamel junction, and the second cut was done 1 mm below the first one. For this procedure, a water-cooled diamond blade in Isomet 1000 unit (Buehler Ltd, Lake Bluff, Illinois, USA) was used at low speed (250 rpm). Then, each dentin disk was sectioned into four parts to produce four samples per tooth (Fig. 1). Slices obtained were further polished using wet 600-, 1000-, and 1200-grit silicon carbide paper. They were stored in deionized water and then divided into four groups (one control and three experimental groups), so each group received a fragment (slice) of each dentin disk to ensure uniformity of mineral content in the groups. Groups were distributed as shown in Figure 1: I – control (C) (without treatment); II – ozone (O) (ozone therapy); III – biofilm

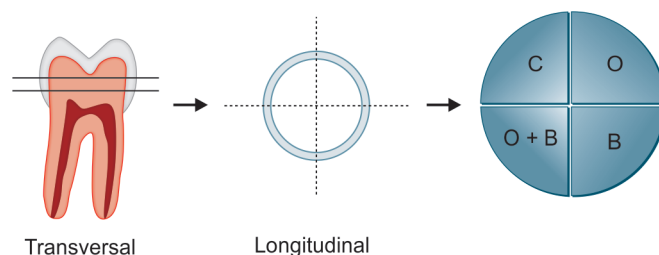


Fig. 1: Presentation of experimental groups and procedures in their respective groups: Group I: Control (C) – without treatment; group II: Ozone (O) – ozone therapy; group III: Biofilm (B) – biofilm development; group IV: Ozone + biofilm (O + B) – ozone therapy and biofilm development

(B) (biofilm development); IV – ozone + biofilm (O + B) (ozone therapy and biofilm development).

Ozone Therapy

In groups II and IV, ozone gas was applied to the dentin slice surface at a concentration of 7.0 g L^{-1} for 40 s at 5 g h^{-1} of ozone flow rate.²⁰ It was produced by electric discharge through oxygen current (Generator PXZ3507; Eaglesat Tecnologia em Sistemas Ltda., São José dos Campos, São Paulo, Brazil). This part of the study was conducted in a dry environment (inside a modified autoclave to receive ozone only). Specimens were then stored in glass tubes containing sterile deionized water and kept in a bacteriological incubator at 37°C for 24 hours until preparation of specimens for evaluation with energy dispersive X-ray (EDX) spectroscopy.

Biofilm Development

In groups III and IV, a reference strain of Gram-positive facultative coccus (*S. mutans*; ATCC 25175) obtained from the American type culture collection was used for biofilm formation. Bacterial strain was inoculated in 5 mL of brain heart infusion broth (BHI; Difco Laboratories, Detroit, MI, USA) and incubated at 37°C for 24 hours. Experimental suspensions were prepared by cultivating the biological marker on the surface of BHI agar (Difco Laboratories), following the same incubation conditions. Bacterial cells were suspended again in saline to reach a final concentration of about 3×10^8 cells/mL, adjusted to No. 1 McFarland turbidity standard. In the experimental model, each dentin slice was introduced into a glass tube containing 5 mL of sterile BHI. Five milliliters of sterile BHI broth was mixed with 5 mL of bacterial inoculum containing *S. mutans* and were inoculated using sterilized syringes of sufficient volume to cover the dentin slice during a 60-day period. This procedure was repeated every 72 hours, always using 24 hour prepared pure culture and adjusted to No. 1 McFarland turbidity standard. Teeth were maintained in a humid environment at 37°C .

At 60 days, each slice was removed from its tube under aseptic conditions and irrigation was done with 5 mL of sterile deionized water with a sterile syringe. Slices were dried and refilled with sterile deionized water.

Preparation for EDX Analysis

The objective of scanning electron microscope (SEM)-EDX was to study the changes in mineral content (in terms of log calcium-to-phosphorus [Ca/P] ratio) of calcified tooth tissue.⁷ Specimens of all groups were first prepared and examined under a SEM (Gemini, Leo 1530, Germany) set at 10 kV. An assessment of the log Ca/P of demineralized and sound areas adjacent to demineralized areas was made by EDX spectroscopy (model 7426; Oxford Instruments, Oxford, UK). Elemental analysis was carried out across the flat dentin slice in areas of 100 mm in the center of the specimen.

Statistical Analysis

Normality of data distribution and the homogeneity of group variances were verified for investigated mineral content (log Ca/P) with Shapiro–Wilk test and Levene test respectively. Differences in mineral densities were assessed by repeated measures analysis of variance (ANOVA). Tukey–Kramer multiple comparisons test was used to identify values that differed significantly from each other. Analyses were performed with GraphPad InStat 3 software. The significance level was set at 5% for all analysis.

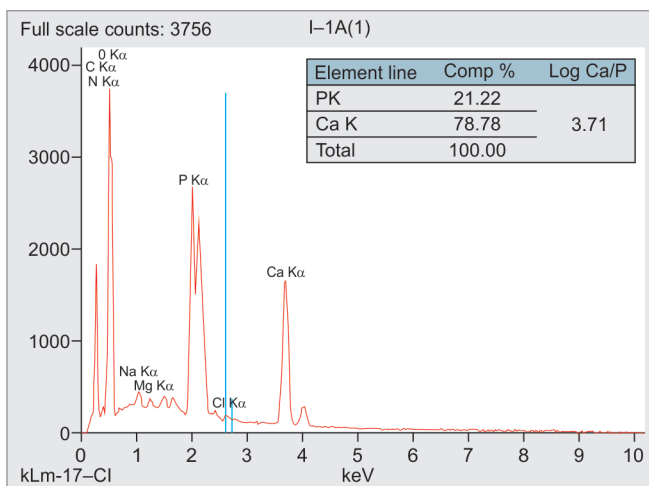
RESULTS

Means and standard deviations of each group (log Ca/P) are shown in Table 1. The lowest log Ca/P was determined in biofilm group. Repeated measures ANOVA showed significant differences between groups

Table 1: Means and standard deviation (±SD) values (log Ca/P) for each group

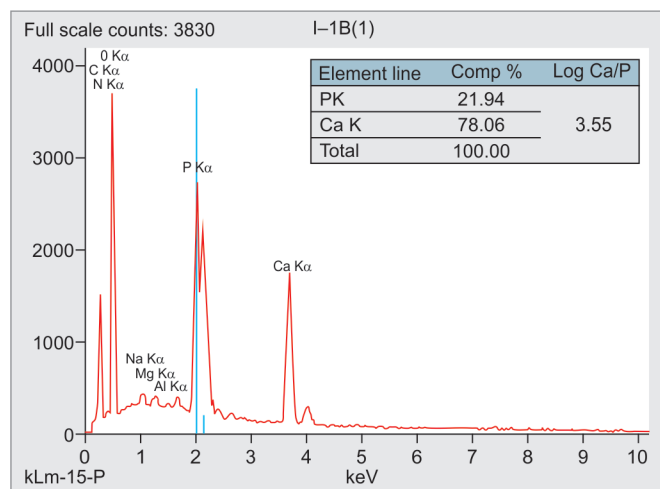
Group	n	Log Ca/P*
Control	5	3.992 ± 0.4888
Ozone	5	3.646 ± 0.5795
Biofilm	5	2.094 ± 0.1764
Ozone + Biofilm	5	2.580 ± 0.5773

*Same letters are not statistically different (p>0.05).

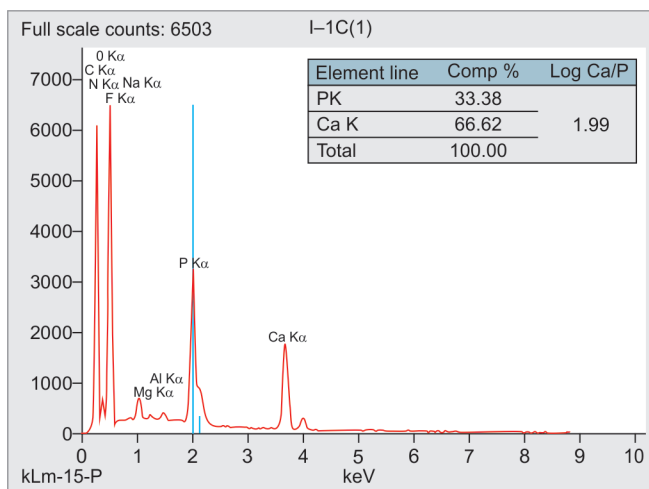


Graph 1: Distribution of Ca and P of one specimen of each group I – control without treatment (data provided by EDX)

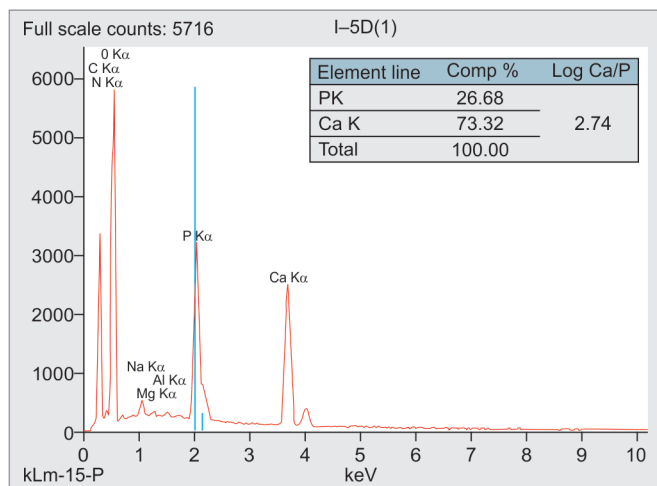
(p < 0.0001). Graphs 1 to 4, show distribution of Ca and P for all groups in the samples obtained of a same dentin disk submitted to EDX analysis. There were significant differences among control group (I) and biofilm group (III) (p < 0.001) and ozone + biofilm group (IV) (p < 0.01). There was no significant difference between control group (I) and ozone group (II) (p > 0.05). Ozone group (II) showed values statistically higher than those of biofilm group (III) (p < 0.001) and ozone + biofilm group (IV) (p < 0.05).



Graph 2: Distribution of Ca and P of one specimen of each group II – ozone therapy (data provided by EDX)



Graph 3: Distribution of Ca and P of one specimen of each group III – biofilm development (data provided by EDX)



Graph 4: Distribution of Ca and P of one specimen of each group IV – ozone therapy and biofilm development (data provided by EDX)

DISCUSSION

Findings showed ozone did not have an effect on dentin surface, and it did not grant a preventive effect on demineralization of mineral content of dentin surface under *S. mutans* biofilm, accepting both null hypotheses. When only ozone therapy was applied, there was no statistically significant difference from ozone and control groups in mineral content. In contrast with this finding, Celiberti et al⁶ reported that ozone enhanced enamel microhardness as a consequence of its dehydration. While in this study, a decrease of Ca ion was observed in the dentin surface in ozone group (II) but it was without statistical differences.

This study simulated a situation in which ozone gas was applied on dentin surface before exposition to *S. mutans* biofilm to evaluate the role of this gas with oxidizing potential against the demineralizing action of biofilm. A biofilm challenge was simulated, where bacterial suspension did not receive any intervention from oral therapeutic agents in the experimental period. The SEM was used to assess the mineral content. This tool allows for a qualitative and quantitative analyses. Yip et al⁷ used the EDX tool to study the effects of different restorative materials on root surfaces incubated in an oral biofilm generated in an artificial mouth. Only glass-ionomer restorations presented a significant increase in log Ca/P ratio on root surface after incubation in the artificial mouth.

There is controversy about the antibacterial nature of ozone tested on bacteria in planktonic cultures and organized as a biofilm. Nagayoshi et al²³ found that ozonated water was effective against Gram-positive and Gram-negative oral microorganisms and oral *Candida albicans* in pure culture. Ozonated water also had strong bactericidal activity against the bacteria in biofilm. However, Müller et al² observed that gasiform ozone had a minimal effect

on the viability of microorganisms organized in a cariogenic biofilm. This study developed a mature biofilm model during 60 days. Results found no significant difference in mineral content (log Ca/P) between biofilm group (III) and when ozone therapy (IV) was applied before demineralizing action of *S. mutans* biofilm. However, isolated values of Ca ions of ozone therapy group (IV) were higher than observed in biofilm group (III), but lower than in control group. In addition, isolated values of P ions in ozone group (II) and ozone + biofilm group (IV) were higher than in the control group, which is inversely proportional to the final log value. This finding is explained because ozone can activate Krebs cycle by increasing decarboxylation of pyruvic acid leading to formation of adenosine triphosphate.¹⁵ Pyruvic acid is the strongest acid produced naturally by cariogenic bacteria. Decarboxylation of this acid by ozone produces the high pKa acetic acid that can induce buffer effect in biofilm fluid, leading to remineralization of incipient caries lesions.^{8,12} In addition, ozone reacts with unsaturated fatty acids in cell membranes to produce secondary reactive species, such as aldehyde derivatives and lipid peroxides, which may reach the nuclei of intact cells and interact with deoxyribonucleic acid (DNA), leading to breakdown of nucleic acid and release of P ions.²⁴ This mechanism of DNA damage induced by ozone and/or its secondary reactive species remains mostly unknown, but this could explain the increase of P ions that was observed in group IV where ozone therapy was applied after biofilm.

Antimicrobial effect of ozone on oral microorganisms has been investigated in both *in vitro* and *in vivo* studies.^{9,14} However, there is limited information about the treatment time needed to inhibit bacterial growth. According to Polydorou et al,¹⁸ application of ozone for 80 seconds on an *in vitro* infected dentinal cavity model has been reported to be successful in reducing the number of microorganisms, thus confirming the potential of this treatment to disinfect carious cavities. Johansson et al¹ evaluated the antibacterial effect of ozone on cariogenic bacterial species and observed that the cariogenic species *S. mutans*, *Lactobacillus casei*, and *Actinomyces naeslundii* were almost eliminated following 60 seconds of ozone treatment. In this study, ozone was applied for 40 seconds, and it did not grant a protective effect to dentin, leading to suppose that this oxidant agent was not able to inactivate *S. mutans* biofilm action.

Therapy proposed in this study led to an increase in log Ca/P when applied before exposure to demineralizing action of *S. mutans* biofilm, though this difference was not statistically significant. Both null hypotheses were accepted. However, more studies are necessary to allow inference of these results for clinical practice since

ozone therapy may be, therefore, an alternative and/or complementary strategy for treatment of dental caries.

CONCLUSION

Based on the methodology used, it can be concluded that ozone gas did not grant preventive effect of demineralization by *S. mutans* biofilm on dentin surface.

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