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The Efficacy of Gaseous Ozone on Some Cariogenic Bacteria

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A B S T R A C T

The aim of this study is to analyze ozone impact on some cariogenic bacteria in ex vivo and in vitro conditions. The in vitro part of study inoculated dentine with strains of Streptococcus mutans ATCC 33402 and Lactobacillus paracasei ATCC 11974 bacteria. Samples of dentine before and after 40s ozone treatment were collected and anaerobically incubated. Samples of cariogenic dentine (N=24) were collected from permanent molars within the ex vivo segment of the study, prior and after 40s ozone treatment and a number of colonies were counted after incubation. For the in vitro part of study, results have shown a statistically significant average value of reduction of Streptococcus mutans ATCC 33402 and Lactobacillus paracasei ATCC 11974 prior and after ozone treatment ($p<0.001$). The ex vivo segment of the study has also demonstrated a statistically significant difference in the number of bacteria prior and after ozone implementation ($p<0.001$). Gaseous ozone demonstrated a strong antimicrobial effect on cariogenic bacteria in both in vitro and ex vivo conditions and it can be used as an adjuvant in caries therapy.

Key words: *cariostatic agents, colony count/microbial, dentin/drug effects, dental caries/microbiology, ozone*

Introduction

Recent molecular methods have revealed that almost all dental diseases are caused by dental biofilms that consist of a multispecies community[1–3]. Dental biofilms are characterized by surface attachment, structural heterogeneity, complex interspecies interactions, and an extracellular matrix of polymeric substance. They act as high-density micro-niches that differ dramatically from surrounding conditions[4]. Cariogenic plaques are comprised of numerous different microbial species, including *S. mutans* and other low-pH streptococci (*Streptococcus oralis, Streptococcus mitis, Streptococcus anginosus*), *Rothia, Actinomyces, Lactobacilli* and *Bifidobacterium* spp., and *Candida albicans*[5–7]. Practical use of gaseous ozone is dentistry due to its antimicrobial characteristics and effects against common oral pathogens[8–16]. Ozone, in its gaseous or aqueous form is shown to be a strong and reliable antimicrobial agent against bacteria, fungi, protozoa and viruses[17,18]. Generally, it is believed that ozone oxidation potential induces the destruction of cell membrane and cytoplasmic membranes of bacteria and fungi, leading to the damaging of glycoprotein, glycolipid and amino acids inhibiting cell enzyme systems[19]. All of this results in increased membrane permeability, leading to the additional entry of ozone molecules; resulting in cellular death[17,20]. The aim of this study is to analyze *in vitro* efficacy of elimination of *Streptococcus mutans* and *Lactobacillus paracasei* bacteria from human dentin by the use of gaseous ozone. Also, the total counts of bacteria as well as the total number of lactobacilli and Streptococcus mutans were evaluated in ex vivo part of the study.

Materials and Methods

In the *in vitro* part of the study sixty freshly extracted human non-carious third molars were used. The teeth were cleaned with a toothbrush and water for 60s each and then stored in 1% chloramine solution. Further, class 1 cavities were created on the occlusal area using fast hand piece and diamond fissure burs (2979, Komet Bras-

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seler, Lemgo, Germany) under water-cooling. The cavity base was inside dentine. Finally, the teeth were autoclaved at 121 °C. Strains of *Streptococcus mutans* ATCC 33402 and *Lactobacillus paracasei* ATCC 11974 (LGC Standards, Middlesex, UK) were used in the study. Microorganisms were grown inside the Schaedler Bouillon (Oxoid Limited, Hampshire, UK), over 24 h, in anaerobic condition at 37 °C. The previously sterilized tooth was incubated within a mixed bouillon culture for six days also in anaerobic conditions at 37 °C. After 24 h of incubation the broth medium contains 10^8 CFU/mL irrespective of the initial inoculums. After six days, the teeth were removed and the dentine preparation procedure commenced. Using a hand instrument, sterile dental excavator, a certain amount of dentine was collected, from within the mesial part of cavity, and placed in a testing tube with 2 ml of saline, representing a sample of dentine prior to ozone treatment. Finally, the tooth cavity was treated with ozone using KaVo Healozone 2130 C (KaVo, Biberach, Germany) during a period of 40 seconds. This is self-contained device that produces ozone at a fixed concentration of 2100 ppm ± 5% ozone at a flow rate of 615cc min^{-1}. The same procedure was used to collect dentine from a distal part of the cavity using a new sterile excavator, representing a sample after the ozone treatment. It was placed in a different testing tube. The above procedure was used on 60 teeth, resulting in 120 collected samples. The contents were stirred using vortex mixer (Mixomat, Boskamp, Germany), and immediately thereafter ten-fold dilutions with saline were made. A sample of 0.1 mL of each dilution (from dentine samples prior and after ozone treatment) was spread on three ROGOSA Agar plates (Difco; Becton-Dickinson and Company, Sparks, MD, USA) and three Mitis Salivarius Agar plates (Difco; Becton-Dickinson and Company, Sparks, MD, USA). The bacteria do not grow beyond 10^8 CFU/mL, so the broth always contains a predictable and identical amount of bacteria. The plates were incubated for five days in anaerobic conditions at 37 °C. Following the incubation a number of colonies on plates of specific dilutions were counted and the concentration of bacteria per mililitra of sample was calculated (Figures 1 and 2). The growth of colonies inside the specific dilution was expressed as a middle value, resulting from three spread plates, and the results were presented as $\log_{10}$(CFU +1) per mL. Lack of growth in the lowest dilution on one of the plates was calculated as 0 (e.g. 0+8+7=15:3=5 cfu/mL x dilution).

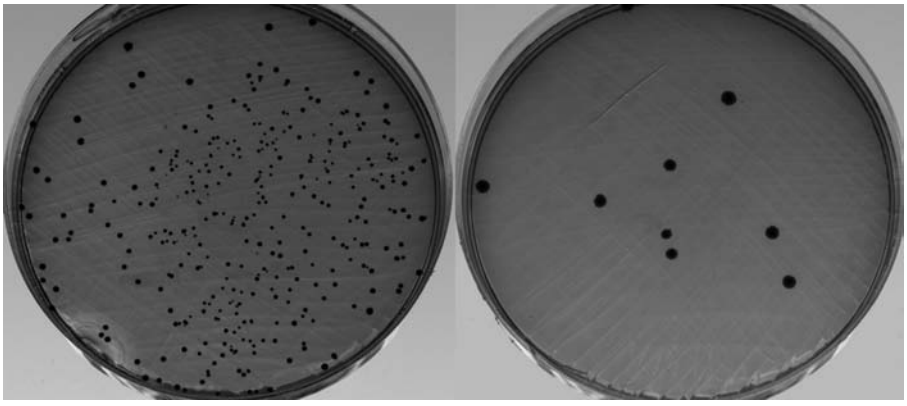

Fig. 1. Growth of Streptococcus mutans ATCC 33402 on plates before and after ozone application.

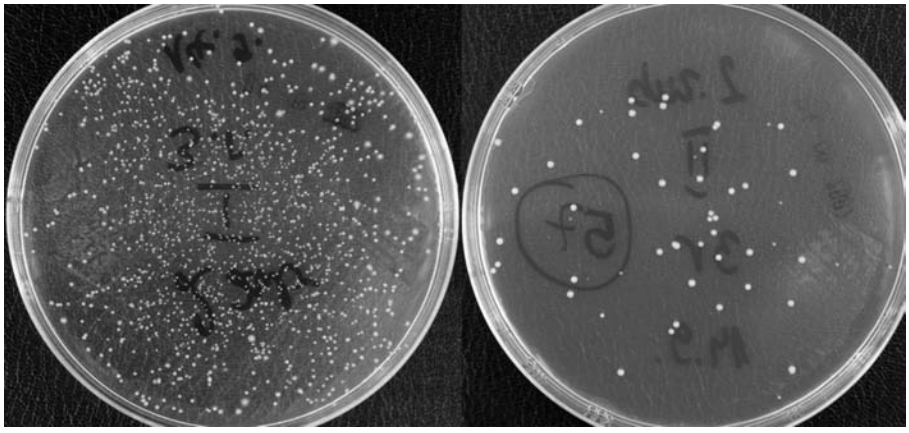

Fig. 2. Growth of Lactobacillus paracasei ATCC 11974 on plates before and after ozone application.

In the *ex vivo* part of the study the patients of the age group 7–18 years were selected at the Department of Pediatric and Preventive Dentistry, School of Dental Medicine, University of Zagreb. All of them were diagnosed with deep dentine caries lesion on vital permanent molars, which was to be treated with ozone prior to final filling. An experienced pediatric dentist conducted clinical work and all samplings. Ethical Committee of School of Dental Medicine, University of Zagreb, made approval for this clinical study and the informed written consent from participants and their parents was obtained. The overlying superficial layer and soft biological material were removed prior to therapy, using sterile steel bur (H1 021, Komet Brasseler, Lemgo, Germany) on a slow rotating hand piece without water-cooling. The cavity was rinsed with sterile distilled water and finally dried by use of an air blower over 5s. Using the modified technique for collecting dentin samples, carious dentine (on a specific spot on a tooth, different from the previous) was collected using a new sterile steel bur (H1 021, Komet Brasseler, Lemgo, Germany), on a slow rotating hand piece without water-cooling, mostly from the mesial part of the cavity and left inside a testing tube containing transport media for transportation of microbiological samples of the Stuart type (Difco; Becton--Dickinson and Company, Sparks, MD, USA)[8,21]. Finally, the same tooth was treated with ozone using Kavo Healozone device over 40s, and in the same manner using a new steel bur, a new sample of dentine was collected[22]. This time the spot was different from the first sample. Finally, a tooth was restored with a filling. Twenty-four patients were treated in this way; 48 dentine samples were collected. In all cases the samples were numerically coded in the clinic, and the microbiology laboratory did not know the treatment received by any sample until after the numbers of bacteria per sample had been determined. Within two hours of the period of time in which the sample was collected, tenfold dilutions with saline were made and each dilution was spread on three Columbia Agar Base plates with 5% sheep blood (Oxoid Limited, Hampshire, UK), three ROGOSA Agar plates (Difco; Becton-Dickinson and Company, Sparks, MD, USA) and three Mitis Salivarius Agar plates (Difco; Becton-Dickinson and Company, Sparks, MD, USA). The smeared plates were incubated for five days in anaerobic conditions at 37 °C. A number of colonies on plates of specific dilutions were counted after incubation and the concentration of bacteria per mililitra of sample was calculated. The growth of colonies inside the specific dilution was expressed as a mean value, resulting from three spread plates, and the results were presented as $\log_{10}$(CFU +1) per mL. The bacteria do not grow beyond 10^8 CFU/mL, so the broth contains a predictable and identical amount of bacteria. The amount of dentin taken was always identical – possible minor variations in both parts of the study do not influence the results significantlly as the number of bacteria is expressed logaritmically.

The results were expressed as the reduction of the total number of bacteria, and the reduction of the specific number of *Streptococcus mutans* and *Lactobacillus spp* colonies. The Kolmogorov-Smirnov test applied in the first part of the study (*in vitro*) has shown that the variables of the *log* number of bacteria before treatment, after treatment, and the reduction of bacteria (log difference before and after treatment) were distributed normally (p=0.618, p=0.899 and p=0.087), therefore, in further processing we used t-tests of mean values with significance of p=0.05. The statistical processing for the second part of the study (*ex vivo*) was conducted with the aid of the Kolmogorov-Smirnov test, which has shown that the variables of the log number of bacteria before treatment and after treatment were distributed normally (p=0.073, p=0.066), while reduction of bacteria (log difference before and after treatment) was not distributed normally (p=0.003). Therefore, in further processing, we used the t-tests of mean values, and the non-parametric tests – Kruskal-Wallis, with significance of p=0.05.

Results

The results of the first part of the study (*in vitro*), specifically impact of ozone on *Streptococcus mutans* ATCC 33402 and *Lactobacillus paracasei* ATCC 11974 are shown in Table 1. The t-test has shown that the mean values of the number of bacteria prior to ozone treatment and the number of bacteria after the treatment display significant statistical difference (p<0.001). The Pearson correlation test has also shown a correlation between the number of *Streptococcus* bacteria before and after the ozone treatment (r=0.468, p=0.009) and a strong correlation between the number of *Lactobacillus* bacteria before and after the ozone treatment (r=0.894, p<0.001). The Table 2 shows results of the second part of the study (*ex vivo*). The Kolmogorov-Smirnov test has shown that the variables of *log* number of bacteria before

TABLE 1
RESULTS FROM *IN VITRO* PART OF THE STUDY.

In vitro	Before ozone ($\log_{10}$(CFU+1))	After ozone ($\log_{10}$(CFU+1))
Streptococcus mutans ATCC 33402	5.55±0.53[a]	4.16±1.09[a]
Lactobacillus paracasei ATCC 11974	4.38±0.98[b]	3.25±1.04[b]

[*] Same letters in superscript show statistical difference

TABLE 2
RESULTS FROM *EX VIVO* PART OF THE STUDY.

Ex vivo	Before ozone ($\log_{10}$(CFU+1))	After ozone ($\log_{10}$(CFU+1))
All bacteria	7.45±1.29[a]	6.65±1.54[a]
Streptococcus mutans	6.06±1.63[b]	5.23±1.89[b]
Lactobacillus spp.	4.79±0.5[c]	3.55±1.16[c]

* Same letters in superscript show statistical difference

and after treatment was normally distributed (p=0.073, p=0.066), while reduction (*log* difference before and after treatment) was not normally distributed (p=0.003). Therefore in further processing we used t-tests of mean values, and non-parameter tests – Kruskal-Wallis, with significance of p=0.05. The t-test has shown that the mean values of the *log* number of bacteria before ozone treatment and the number of bacteria after treatment display a significant statistical difference for the total of bacteria, for *Steptococcus* and for *Lactobacillus* (p< 0.001). Kruskal-Wallis in his test failed to demonstrate that this mean value displays significant statistical difference in relation to bacteria type (χ^2=0.341, p=0.843, df=2). Pearson's correlation test has also shown a strong correlation between the total number of bacteria before and after ozone treatment (r=0.977, p<0.001) and a strong correlation between the number of Streptoccocus bacteria before and after ozone treatment (r=0.979, p< 0.001), but it has shown that the number of Lactobacillus bacteria before and after ozone treatment has no correlation (r=0.215, p=0.312).

Discussion and Conclusion

Numerous studies have shown success in the use of ozone with the aim of reduction of microorganisms[8,12,16,20,23,24]. Others studies demonstrated failure of ozone[8,15,22]. It is important to note that the majority of modern antimicrobial agents and techniques have greater or smaller eliminating effects on cariogenic bacteria in the *in vitro* conditions. One of the most notable features of dental biofilms is that oral bacteria growing in the biofilms frequently express phenotypes that are different from those of planktonic bacteria. For instance, many bacterial species in biofilms exhibit greater tolerance to antibiotics and other environmental factors, such as pH and oxygen[25,26]. Another study reported the concentration of the antibiotic for inhibiting the growth of bacterial strains within their biofilms was approximately 250 times greater than that required when the same strains were grown planktonically[25]. The results from another study have proven that there is no bacteria reduction prior and after 30s of ozone or chlorhexidine treatment, regardless of whether a biofilm was or was not removed from the cavity[22]. It is concluded that gaseous ozone treatment for more than 20 seconds effectively hinder bacterial growth on the strips and the agar plates, and ozone application of 20, 40 or 60s prevented the bacteria to grow on the different media[12]. It is possible that ozone has a different effect on different strains of cariogenic bacteria; therefore the ozone effect on most important cariogenic bacteria should be examined.

Baysan had also proven the weak effect of ozone on non-cavitated occlusal lesions[8]. During the second part of her study ozone was directly applied on cavitated teeth with carious dentine and in this case he was able to achieve a statistically significant reduction of the total number of bacteria. The author also stresses an important protective role of biofilm in terms of effects of ozone.

Müller demonstrated success in the elimination of cariogenic bacteria[15]. In his study, using ozone, he successfully eliminated *A.naeslundii, S. mutans* and *L. Casei* bacteria *in vitro* conditions. He was able to prove that salivary proteins reduce the bactericide effect of ozone, meaning that they, as is the case with biofilm, reduce the effects of ozone. It needs to be noted that contamination with salivary protein, after ozone treatment, can also lead to the recolonization of lesion with bacteria and to therapy failure.

This study has demonstrated success in elimination of *Streptococcus mutans* ATCC 33402 and *Lactobacillus paracasei* ATCC 11974 bacteria using ozone *in vitro* conditions. The t-testing of independent samples did not show that this mean value displays significant statistical difference considering the type of bacteria (p=0.178, df=41.85), hence, a conclusion cannot be made that ozone would have different impact on these types of bacteria. Elimination of *S. mutans* and *L. spp* bacteria was also successful in *ex vivo* conditions. Finally, use of ozone can be suggested as a means of reduction in the number of some cariogenic bacteria in cases of active lesions, which can result in the removal of smaller amounts of hard dental tissue during preparation, supporting the principle of minimal invasive dentistry.

The results of another study show that daily consumption of LGG yoghurt can have an inhibitory effect on oral pathogenic microflora. Results show that thirty days after yoghurt consumption percentage of the patients with high S. Mutans count dropped significantly from 80% to just 52%, and in the high caries activity group, S. Mutans count dropped from 91% to 40%, which was highly significant as well[27]. This type of diet could have some impact on carogenic flora, because analysis of dietary habits showed that jam, honey, sweets, candies and sweetened tea were consumed more often by the Croatians in contrast to the Italians[28].

The caries incidence in Croatia is high and children from urban and suburban population showed very high values of the dmf-t/DMF-T indexes (7.7/6.7), dmf-s/DMF-S (16.5/11.8), and significant index of caries (SiC=10.89)[29]. Also, another study from Zagreb showed the median DMFT and DMFS of 12-year-old children were 4 and 5, respectively. The highest median DMFT score of 7 was found among 14-year-old children[30]. Another study from neighboring country showed DMFT for capital Sarajevo of 0.57 among 6-year old children and dmft of 6.71 in the same age group[31].

Another type of study analyzed the University teachers in Croatia, and they seem to intervene operatively at a later stage of caries development and are more familiar with non-operative strategies[32]. Ozone also could be helpfull in minimally invasive dentistry, since the majority of University teachers and dentists from private practice are familiar with new materials and techniques are applied in the practice. Considering these data, ozone as a part of minimally invasive dentistry could help in the treatment of early carious lesions and preventing more severe caries lesions. More studies are necessary to support the effect of ozone on different cariogenic bacteria and its use in dentistry.

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UTJECAJ OZONA NA NEKE KARIOGENE BAKTERIJE

SAŽETAK

Svrha ovog istraživanja je analizirati utjecaj plinovitog ozona na neke vrste kariogenih bakterija u *ex vivo* i *in vitro* uvjetima. *In vitro* dio ovog istraživanja je uključivao inokulirani dentin s sojevima bakterija tipa *Streptococcus mutans* ATCC 33402 i *Lactobacillus paracasei* ATCC 11974. Uzorci dentina prije i poslije djelovanja ozona od 40s su anaerobno nasađeni. Uzorci kariogenog dentina (N=24) su prikupljeni iz trajnih molara u sklopu *ex vivo* dijela istraživanja prije i poslije djelovanja ozona od 40s, te su se kolonije bakterija brojale nakon nasađivanja. Rezultati su u sklopu *in vitro* dijela istraživanja pokazali statistički značajno smanjenje prosječnog broja *Streptococcus mutans* ATCC 33402 i *Lactobacillus paracasei* ATCC 11974 prije i poslije djelovanja ozona ($p<0,001$). Također je i *ex vivo* dio istraživanja pokazao statistički značajnu razliku u broju bakterija prije i poslije djelovanja ozona ($p<0,001$). Plinoviti ozon je pokazao snažan antimikroban učinak prema kariogenim bakterijama u *in vitro* i u *ex vivo* uvjetima te se može koristiti kao dodatak u terapiji karijesa.