

## ANTIMICROBIAL ACTIVITY OF OZONE GAS AND COLLOIDAL SILVER AGAINST ORAL MICROORGANISMS

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The aim of this study was to evaluate antimicrobial activity of ozone gas and colloidal silver suspension against selected oral microorganisms. Gaseous ozone was used in the ozone study. Suspension of reference and clinically isolated strains of oral microorganisms were exposed to ozone gas for 30, 60 and 120 s. The number of colonies was counted and the killing rate for each microorganism was calculated. In order to determine minimum inhibitory (MICs) and minimum bactericidal/fungicidal concentrations (MBCs/MFCs) of colloidal silver microdilution method was used. Results showed that after 120 s exposition to gaseous ozone approximately 82.68-99.9% of all strains were eliminated. The colloidal silver exhibited strong antimicrobial activity with MICs and MBCs/MFCs values range from 2.0 to 4.0 µg/ml. The present study confirmed the efficacy of ozone gas and colloidal silver against selected and isolated oral microorganisms. The results obtained in this study should provide additional evidence for their potential application in reducing the infection caused by microorganisms in the oral cavity.

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### 1. Introduction

Dental caries and periodontitis are among the most prevalent oral diseases in the world [1]. Among other *Streptococcus* species, especially *S. mutans* are identified as major causative agent [2]. *Streptococcus* species are known for their capacity to take the opportunity of shifted conditions in oral cavity in their favour, rapidly metabolising fermented carbohydrates, at low pH and acid conditions. Several antibacterial agents are used in control of dental plaque, with chlorhexidine being the most effective [3]. However, extrinsic tooth staining and perturbation of the taste limit its long term use. In addition, chlorhexidine effectively reduces the number of *S. mutans* and controls gingivitis, but there is limited evidence for the effectiveness of chlorhexidine in preventing caries [4]. Certain species of fungi also pose a serious threat to oral health. Yeasts from the genera *Candida* are the main cause of invasive fungal infections in hospitals [5]. Oral candidiasis is presented as infection of mucose membrane of the oral cavity. Oral candidiasis may easily progress to oesophageal candidiasis and even more harmful complications [6]. *C. albicans* is known as the most virulent species and predominantly isolated species from oral infections [7]. Once again, excessive use of antimycotics, particularly azoles, significantly increases azole resistance strains [8], such as *C. krusei*, which is totally resistant to azoles therapy.

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These problems are the reasons for further research and effective antimicrobial agents that are safe for humans and specific for oral pathogens.

Several investigations have shown that ozone gas exhibits strong antimicrobial activity against oral bacteria and fungi, even in resistance strains [9]. Ozone gas has strong oxidation capacity and it is used for variety of applications. There are many advantages of ozone application in food industry, water purification, medicine and cosmetic treatments [10].

Antimicrobial activity of silver was recognized in 19<sup>th</sup> century, and colloidal silver was approved by FDA for wound management in 1920s [11]. Mechanism of antimicrobial activity of colloidal silver is unclear. It is speculated that silver works as catalyst in disabling enzyme activity, or react with proteins resulting in forming thiol group -SH which inactivates them. Silver's strong antimicrobial activity was confirmed by number of studies in which more than 650 microorganisms were found to be susceptible to silver or silver ions [12].

Aim of this study was to evaluate ozone gas therapy and colloidal silver water against selected oral microorganisms as safe alternatives and non-invasive treatment in oral hygiene.

## **2. Experimental**

### **2.1 Microorganisms**

Representative microorganisms of oral microbiota were chosen for this investigation. The reference sample of commonly tested oral microorganism was *Staphylococcus aureus* (ATCC 25923) and *Candida albicans* (ATCC 10231). The other three isolates (*Streptococcus mutans*, *Streptococcus salivarius* and *Candida krusei*) were human isolates obtained by rubbing a sterile cotton swab over oral mucosa from patients at the Department of Pediatric and Preventive Dentistry, Faculty of Dentistry, University of Belgrade, Serbia. The swabs were transferred to Triptone Soya Broth (Merck, Germany) medium and thoroughly mixed using vortex mixer and 50 µl of suspension inoculated on various selective and non-selective medium and incubated microaerobically for 48 h at 37<sup>o</sup> C. Isolates were identified using biochemical profiles (API 20 Strep kit for *Streptococcus spp.* identification, API 20 C and Chrom-agar for *Candida spp.* identification) and other standard microbiological methods.

### **2.2 Cultivation media**

*S. mutans* and *S. salivarius* were cultured on Mitis Salivarius Agar (MSA, Difco, USA), *S. aureus* was cultured on Mueller Hinton Agar (MHA, Merck, Germany) and *C. albicans* and *C. krusei* on Sabourand Dextrose Agar (SDA, Merck, Germany), all at 37<sup>o</sup> C for 24 h. Fresh 24 h cultures were prepared for each experiment.

### **2.3 Antimicrobial activity of ozone gas**

The ozone generator O<sub>3</sub> Intensive (LAH d.o.o., Velika Gorica, Croatia) was used in this study. Suspension of each isolates (100 µl) was added in 900 µl PBS in experimental tubes, for final concentration of 1 x 10<sup>4</sup> CFU/ml. The inoculums were prepared prior to experiment and stored at +4<sup>o</sup> C until use. Dilutions of the inoculums were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculums. Each microorganism was exposed to four concentration of ozone gas for 30, 60 and 120 s which correspond to concentration of ozone gas of 6, 11 and 26 µmol of O<sub>3</sub> (0.50, 1.05, 1.24 mg O<sub>3</sub>). The ozone exposure of microorganisms suspension were monitored on the ozone generator screen. Control tubes were not exposure to ozone gas. After the treatment, serial dilution of 100 µl of content was immediately processed by spreading on MSA, SDA and MHA plates which were incubated for 24 h at 37<sup>o</sup> C. The number of colonies was counted and the killing rate for each microorganism was calculated. Experiments were done in triplicate and final results were presented in percentage.

### 2.4 Antimicrobial activity of colloidal silver

Colloidal silver with concentration of 5 mg/l (A) was purchased from “Eko solar” Company, Serbia and used in this study. Antimicrobial activity of colloidal silver water was carried out by microdilution method [13]. Sterile 96-well microplates were used. Each well contained  $1.0 \times 10^5$  CFU/ml of microorganism, serially diluted colloidal water and the respective growth medium. Trypticase Soy broth (TSB, Merck) for bacteria species and Sabourand Dextrose broth (SDB, Merck) for *Candida* species were used. The microplates were incubated for 24 h at 37° C. The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations which completely inhibited microorganisms growth (MICs). The minimum bactericidal/fungicidal concentrations (MBC/MFC) were determined by serial subcultivation of a 2 µl into microtitre plates containing 100 µl of broth per well and further incubation for 24 h at 37° C. The lowest concentration with no visible growth was defined as the MBC/MFC, indicating 99.5% killing of the original inoculum. Ampicillin and Nystatin (Sigma-Aldrich, USA) were used as control. All experiments were done in triplicate.

### 2.5 Statistical analysis

For each treatment, data from independent replicate trials were pooled and the mean value and standard deviation determined.

## 3. Results

The antimicrobial activity of the ozone gas was initially evaluated using two strains of *Streptococcus* species (*S. mutans* and *S. salivarius*), common oral bacteria *S. aureus*, and two oral fungi specimens (*C. albicans* and *C. krusei*). The results obtained in this assay are shown in Fig. 1.

Initial number of starting inoculum (79.05, 84.99, 91.14, 51.00 and 60.83%) was eliminated after 30 s of ozone treatment. After 120 s approximately 82.68-99.90% of all strains were dead. Among bacteria, *S. mutans* was the most susceptible to ozone gas treatment while *S. aureus* was the most resistant one. Yeasts proved to be less sensitive to ozone gas than bacteria. Ozone gas was more efficient in killing *C. krusei* than *C. albicans* (ATCC 10231).

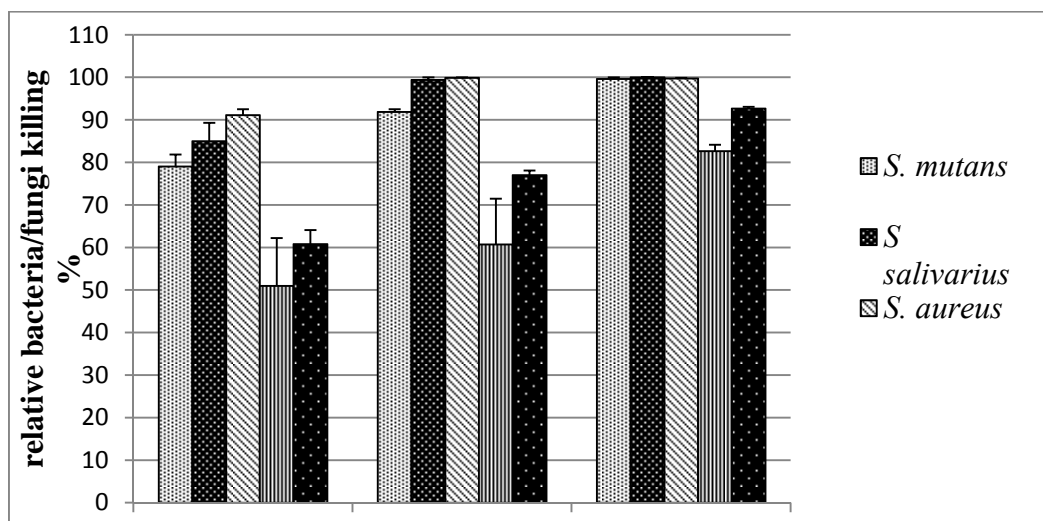


Fig. 1. Relative numbers of killed oral bacteria and yeasts in PBS, after exposure to ozone gas for 30, 60 or 120 s equivalent to mg O<sub>3</sub>.

Next we tested colloidal silver as antimicrobial agent against target oral microorganisms. MICs and MFCs were determined using microdilution methods and all isolates were susceptible to colloidal silver in concentration dependent manner (Fig. 2 and Fig. 3).

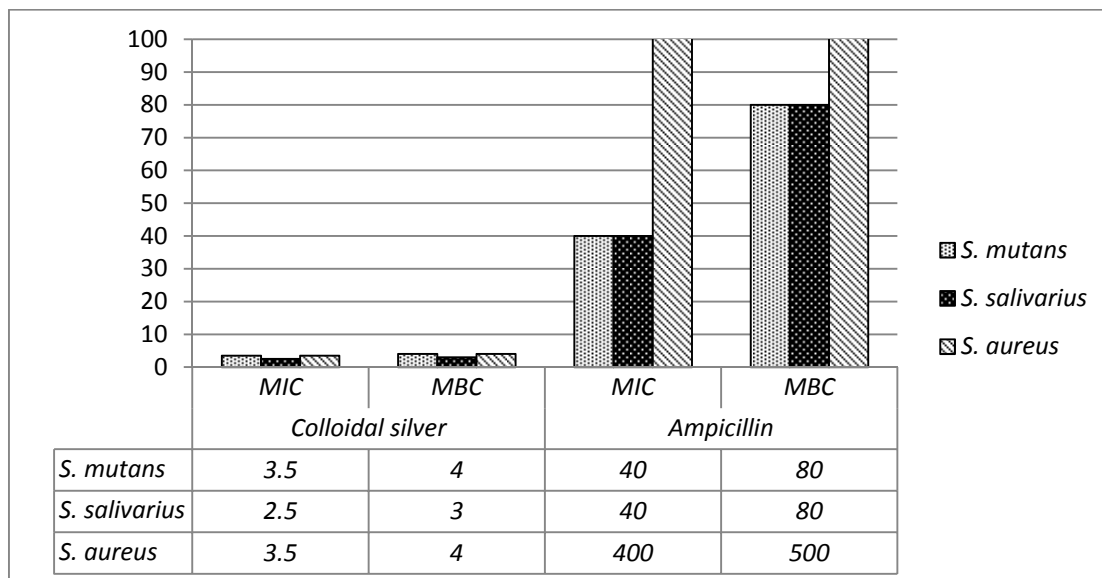


Fig. 2. Antibacterial activity of colloidal silver.

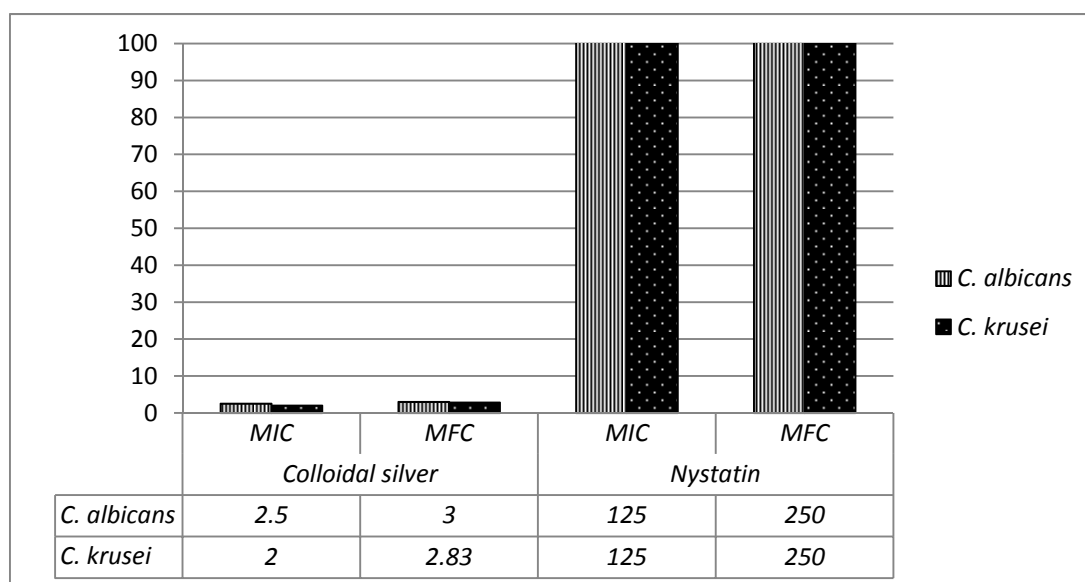


Fig. 3. Antifungal activity of colloidal silver.

Colloidal silver (A) showed bactericidal/fungicidal activity with MICs and MBCs/MFCs values range from 2.0 to 4.0  $\mu\text{g/ml}$ . Fungi were slightly more sensitive to colloidal silver solution. MICs and MFCs for fungi were 2.0-3.0  $\mu\text{g/ml}$ , while MICs and MBCs for bacteria were 2.5-4.0  $\mu\text{g/ml}$ . The most susceptible species among selected oral microorganisms was *C. krusei*, while the most resistant were bacteria *S. aureus* and *S. mutans*. Ampicillin showed better results against *S. mutans* and *S. salivarius* clinical isolates (MIC 40  $\mu\text{g/ml}$ , MFC 80  $\mu\text{g/ml}$ ) but failed to inhibit the growth of *S. aureus* in lower concentrations (MIC 400  $\mu\text{g/ml}$  MBC 500  $\mu\text{g/ml}$ ). Colloidal silver exhibited better activity against *Candida* compared to nystatin MIC 125  $\mu\text{g/ml}$  MFC 250  $\mu\text{g/ml}$ .

#### 4. Discussion

During the last years, there is a growing interest in alternatives to synthetic drugs and their application in medicine and pharmacy. The potential of the ozone gas and colloidal silver could be presented as a new strategy because of their high efficiency, low cost and rare side effects [14, 15].

The data from the ozone experiment suggest that ozone gas could be useful for killing oral infectious microorganisms. According to our results, the effect of ozone significantly reduce the number of viable *S. mutans* cells after only 30s of treatment. Previous reports [16, 17, 18, 19, 20, 21] also proves that ozone have a strong effect on *S. mutans*. The study of Polydorou et al., 2012 [21] proves prolonged effect of ozone treatment up to 8 weeks after treatment, but this results should be accept with caution. They showed lower number of *S. mutans* compared to control group but failed in total elimination of *S. mutans*. Different dosage and application times are used by different research groups in order to eliminate target microorganisms. *S. aureus* and *S. salivarius* need slightly higher dosage of ozone for elimination in our experiment. Previous report of Lescano et al., 1999 [22] showed that time required for total inactivation of *S. aureus* is 10 min. As different methodology is used in these two studies the results aren't comparable. It is evident that higher dosage or longer exposure to ozone gas should lead to efficient elimination of target organisms. Bezirtzoglou et al., 2008 [23] showed that low dosage ozone treatment need 30 min for complete decontamination while other studies showed that high dose of ozone need less time to efficiently kill the bacteria or fungi. There are no studies so far conducted to our knowledge proving which tactic is not only more efficient but also less toxic and safer. Experimental design for our experiment may influence the results. In our study we did not use the positive control, which can be interpreting as a drawback for the study. However, direct comparison of ozone treated and non treated group should provide enough information of ozone treatment effect against oral microorganisms. Both *Candida* species proved less sensitive to ozone. There are reports of *C. albicans* susceptibility to ozone, but different methods were used so the results cannot be directly compared [24]. Azole-resistant strain such as *C. krusei* is also included in this study for the first time. There was no significant difference between susceptibility of both *Candida* species, but ozone treatment against *C. albicans* proves to be more efficient. The difference between bacteria and yeast susceptibility to ozone can be explained by different cell organisation. Oxidation power of ozone is responsible for the destruction of cell walls and cytoplasmic membranes, changing the permeability of membrane and thus clears the path for ozone molecules easily finding their targets.

Ozone proves very efficient against not only individual species, but also against oral biofilm [25]. Structures such as biofilm and saliva are known to limit the efficiency of ozone treatment [16]. Biofilm structure is characterized by increased resistance to detergents and antibiotics, as the dense extracellular matrix and the outer layer of cells protect the interior of the community. There are few papers explaining good activity of ozone treatment of biofilm formations, but Mueller et al., 2007 [25] suggest that we should be careful with such interpretation. Application of gaseous ozone has been documented so far by few authors for its *in vivo* antimicrobial activity [26, 27]. Ozone has a number of the advantages such as: microbicidal effect, potency, ease of handling and lack of mutagenicity [28]. Much is speculated of safe application of ozone. Ozone gas was found to have toxic effects on some cell types, while no cytotoxic signs were observed for aqueous ozone [29]. Further studies are needed to reveal some more facts on the toxicity of ozone application.

Nevertheless, the results obtained in this study showed that ozone gas was effective against tested oral microorganisms. Taking all in consideration, ozone treatment should not be used alone as antimicrobial method, but should be used along with other antimicrobial agents for effective elimination of infectious microorganisms from oral cavity.

Antimicrobial activity of silver has been known for more than 100 years [30]. The results from both colloidal silver solutions from our test showed strong antimicrobial activity against tested microorganisms.

In our study *S. mutans* along with *S. aureus* was slightly less sensitive to colloidal silver solution compared to *S. salivarius*. In previous reports [32] the antimicrobial sensitivity of *S. mutans* to nanoparticles of silver, zinc oxide, and gold showed an average MIC of  $4.86 \pm 2.71$   $\mu\text{g/ml}$  and MBC of  $6.25$   $\mu\text{g/ml}$ . Colloidal silver activity against cariogenic *Streptococcus* species, fungi and wide range of gram-positive and gram-negative bacteria is reported by different groups [33, 34, 35]. Various modified techniques were used for determining antimicrobial activity of silver and range of different results are presented by some authors, for example, MIC values for *S. aureus* vary in some studies from 8 to 80  $\mu\text{g/ml}$  [36, 37] compared to our study (3.5  $\mu\text{g/ml}$ ). Kim et al., 2008 [38] also report the very strong antifungal activity of nano  $\text{Ag}^+$  particles of several

*Candida spp.* including *C. albicans* and *C. krusei*. In contrast to their studies our clinical isolate of *C. krusei* was slightly susceptible than *C. albicans* (ATCC 10231) strain. This could be explained in using other strains and different experimental design. The same study showed inhibition effect of Ag<sup>+</sup> nano-particles on dimorphism transition of *C. albicans* also.

Silver also showed very good biological activity against some viruses also [39]. Strong antimicrobial activity below 100 µg/ml and low toxicity reported especially to mammalian cells along with very few cases of resistance to silver [40] makes silver very useful agent for application in various products for treatment of infection and diseases.

Number of conducted studies demonstrated the possible mechanism of silver antimicrobial activity. There are reports of possibility of accumulation in the microorganisms membrane, thus increasing the permeability resulting in destruction and structural changes of membrane. Other theories stand for that silver ions interact with sulfhydryl –SH groups of proteins as the bases of DNA leading either to the inhibition of respiratory process [41] or DNA unwinding [42]. Disabling enzyme reaction and inhibition of cell division are also recorded and interaction with hydrogen bonding process also. It was reported that exposure time, temperature and pH also impact on the rate and extent of antimicrobial activity [43].

The development of resistance to synthetic drugs poses a serious long-term trait to public health. The present study confirmed the antimicrobial activity in *in vitro* conditions of two potentially alternative strategies, ozone and especially colloidal silver, against oral microorganisms associated with most common oral diseases. Therefore, its' use could be recommended for the prevention and early treatment of caries, periodontitis and mucositis, but further and more detailed studies are needed before their routine application in clinical dental practice.

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