Original Article / Özgün Araştırma

Antimicrobial Efficacy of Ozone Therapy on Cariogenic Bacteria

Ozon Tedavisinin Karyojenik Bakteriler Üzerindeki Antimikrobiyal Etkinliği

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Abstract

Objective: This study aimed to evaluate the antimicrobial effectiveness of ozone therapy on cariogenic bacteria.

Materials and Methods: We evaluated 40 children with deep caries in the permanent first molar. Patients were divided into ozone and chlorhexidine digluconate (CHX) treatment groups. Cariogenic dentin samples were collected from permanent molars before and after treatment: 120 seconds with ozone and 60 s with 2% CHX solution. *Streptococcus* mutans (S. mutans) and *Lactobacillus sp.* colonies were counted after 48 hours of incubation in phosphate buffer. Obtained data were statistically analysed.

Results: Significant differences between antimicrobial activities of ozone and CHX against *S. mutans* and *Lactobacillus sp.* were observed (p<0.05). In the *Lactobacillus sp.* group, the amount of growth in the CHX group was significantly lesser than that in the ozone group (p<0.05).

Conclusion: When applied appropriately and cautiously, ozone treatment can be used for disinfection against cariogenic bacteria.

Keywords
Ozone, chlorhexidine, gluconate, cavity disinfection, *Streptococcus mutans*, *Lactobacillus*

Öz

Amaç: Ozon tedavisinin karyojenik bakteriler üzerindeki antimikrobiyal etkinliğinin değerlendirilmesi amaçlanmıştır.

Gereç ve Yöntemler: Çalışma derin dentin çürüklü daimi birinci azı dişe sahip 40 çocuk üzerinde gerçekleştirilmiştir. Hastalar klorheksidin diglukonat glukonat (CHX) grubu ve ozon grubu olarak iki gruba ayrılmıştır. Daimi büyük azı dişlerden uygulama öncesi ve sonrası dentin çürüğü örnekleri toplanmıştır (120 sn ozon ile tedavi ve 60 sn %2 CHX çözeltisi uygulanmıştır). Kırk sekiz saat anaerob şartlarda etüvde bekletilen besiyerlerinde *Streptococcus mutans (S. mutans)* ve *Lactobacilus* kolonileri sayılmış ve değerler kaydedilmiştir. Elde edilen veriler istatistiksel olarak değerlendirilmiştir.

Bulgular: Çalışmamızda elde edilen verilere göre, ozon ve CHX uygulamasının antimikrobiyal etkinliği *S. mutans* ve *Lactobacilus*'lar üzerinde istatistiksel açıdan anlamlı bulunmuştur (p<0,05). *Lactobacillus sp.* grubunda, CHX grubundaki koloni büyüme miktarı ozon grubundan önemli ölçüde daha azdı.

Sonuç: Ozon tedavisi, uygun ve dikkatli bir şekilde kullanıldığında dezenfektan bir seçenek olabilir.

Anahtar Kelimeler
Ozon, klorheksidin glukonat, kavite dezenfeksiyonu, *Streptococcus mutans*, *Lactobacillus sp.*

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Introduction

Dental caries is a biofilm-supported, diet-regulated, multifactorial, non-infectious, and dynamic disease that causes mineral loss in dental hard tissues (1). Dental pellicle is an accumulation of organic matter on the tooth surface as a result of the absorption of proteins found in the saliva. A pellicle is formed when the surface of cleaned teeth comes in contact with saliva. Dental pellicle allows microorganisms inside the mouth to adhere to the surface of the teeth (2,3). Dietary carbohydrates are used by microorganisms inside the biofilm present on the tooth surface, and these microorganisms produce acid. As the neutral pH of the biofilm on the enamel surface (7.0) rapidly decreases to below a critical pH (5.5), the enamel surface begins to break down (4-6). Pellicle formation is followed by the adherence of gram-positive [Gr(+)] cocci on the surface of teeth, followed by formation of Gr(+) bacillus, gram-negative anaerobic cocci, and fusiform bacteria (7-10).

Streptococcus mutans (*S. mutans*) accounts for most of the flora present immediately before tooth caries occurrence and is abundant in caries lesions with cavitation. Thus, *S. mutans* plays an important role in the initiation and progression of caries lesions (11). The ability of *S. mutans* to adhere to tooth enamel and plaques and its ability to produce acid are among the factors responsible for its virulence. *S. mutans* ferments the sucrose in food into lactic acid, causing dissolution of the enamel matrix. This allows the bacteria to adhere to the surface of the teeth via the extracellular dextrans (12).

Lactobacilli are Gr(+) bacteria present in the normal oral flora. As *Lactobacilli* are acidogenic and aciduric, they grow in environments with a low plaque pH and on active caries lesions. Studies have demonstrated reduced levels of *Lactobacilli* within the saliva after teeth are restored (13). *Lactobacilli* are responsible for destroying the dentin texture rather than initiating caries, and are more active in the later stages of caries (14). *S. mutans* comprises most of the flora immediately before cavity formation and is present in high abundance in lesions with cavitation. *Lactobacilli* are predominantly present in deeper cavities (11).

As a result of ultraconservative dentistry procedures, ongoing efforts seek to eliminate or reduce the remaining bacteria in the affected dentine. These procedures aim to reduce the risk of secondary caries formation, and include using antimicrobial solutions to clean the cavity before restoration (15). Among currently available antimicrobials, chlorhexidine (CHX) remains the most commonly used agent to reduce plaques and control caries (16). CHX is widely used as an antimicrobial agent for disinfection before restoration (15). Owing to its broad antimicrobial spectrum, CHX has been used to treat endodontic or periodontal diseases and to stop or prevent caries progression. The biocompatibility of CHX with low toxicity levels is also acceptable at clinical concentrations (16).

Liquid or gas ozone has strong antimicrobial effects against bacteria, fungi, protozoa, and viruses, and disrupts the cellular membranes and walls of bacteria and fungi. Ozone inhibits the functions of glycolipids, glycoproteins, and amino acids in cells. This increases membrane permeability, enabling ozone molecules to enter cells and kill microorganisms (17,18). Ozone is particularly effective against antibiotic-resistant bacteria. Ozone activity inhibits viral infections via inhibiting peroxide and viral protein synthesis in infected cells by altering reverse transcriptase enzyme activity (18). As a result of recent studies on new methods for caries treatment, ozone application has begun to be used. Painless ozone application to eliminate bacteria in carious lesions may provide an alternative to traditional treatment methods. Since ozone therapy is a non-invasive method of disinfection, it has the potential to be widely applicable in the field of pediatric dentistry. This study was conducted to evaluate the antimicrobial effectiveness of ozone application, together with a cavity disinfectant containing CHX gluconate, on *S. mutans* and *Lactobacillus*.

Materials and Methods

This study was compared the antimicrobial effectiveness of an agent containing CHX, which is used for cavity disinfection (Cavity Cleanser, Bisco, Schaumburg, IL, USA), and ozone treatment (OzonyTronXP, MIO International OzonyTron, Rosenheim, Germany) against *S. mutans* and lactobacilli. This was an *ex vivo* study.

Study Design

The study protocol was approved by the Ethics Committee of the Atatürk University Faculty of

Dentistry (decision no: 145, date: 04.03.2015). The parents of the children who participated in the research were informed about the study. The necessary permissions and informed consent were obtained before beginning the experiments.

We examined the antibacterial effectiveness of ozone application against *S. mutans* and *Lactobacillus sp.,* which are known to form caries. This was conducted under *ex vivo* conditions for 120 s with an OzonyTronXP device. A cavity disinfectant (Cavity Cleanser) containing 2% CHX, which has been shown to have antimicrobial effects for a period of 60 seconds (s) (19), was administered to a second group of patients.

The study included 40 healthy (ASA 1) children aged 8-15 years who visited the clinic at the Atatürk University Faculty of Dentistry Department of Pediatric Dentistry. All participants had deep dentin caries on their permanent first molars. Procedures were carried out on one tooth of each patient. A total of 40 active caries on the permanent first molars were evaluated. The patients were randomized into two groups of 20 subjects each. CHX was administered to the first group for 60 s, whereas ozone treatment was administered to the second group for 120 s. Local anesthesia was administered to the teeth prior to the disinfection procedures, and a rubber dam was applied to isolate the teeth from the oral environment.

Collection and Preparation of Samples

The overlying superficial layer and soft biological material were removed prior to therapy using a sterile steel bur (Steel bur No.14, Thomas Dental Products, Bourges Cedex, France) on a slow rotating handpiece without water cooling. After the cavity was washed with sterile distilled water, it was air-dried for 5 s. To ensure standardization, soft dentin tissue was removed with a new sterile steel round bur at low-speed from the mesial section of the cavity to fill the grooves of the bur. The tissue was placed in tubes containing phosphate-buffered saline (PBS, Sigma, St. Louis, MO, USA). An OzonytronXP device was used to apply ozone to the same cavity for 120 s according to the manufacturer's instructions. The device delivered gas at a flow rate of 1 L/min. According to the manufacturer, the ozone concentration of the gas was 2100 ppm±10%. Using a new steel round bur, a soft dentin specimen was collected from the mesial section of the cavity adjacent to the site from which the first specimen was collected. Finally, the bur was placed in a separate tube containing PBS. After numbering, the tubes were sent to the laboratory on the same day and plated within 2 h. The same procedures were carried out for subjects treated with the cavity disinfectant containing CHX. Dentin specimens were collected with a sterile steel round bur from the mesial section before the procedure and placed inside a tube containing PBS. After Cavity Cleanser was applied using sterile cotton pellets, excess solution was absorbed with the sterile cotton pellet. The pellet was kept in place for 60 s. A dentin specimen that was large enough to fill the grooves of the drill was collected with a sterile steel round bur. The specimen was collected from the site adjacent to where the first specimen was collected and then placed in tubes containing PBS. The tubes were numbered and sent to the laboratory. Routine treatment procedures were applied to the teeth after collecting the dentin specimens.

Microbiological Method

On the day of specimen collection from the cavities, fresh Mitis Salivarius Agar (Fluka-01337/Sigma-Aldrich) and Rogosa SL Agar (Fluka-R1148/Sigma-Aldrich) media were prepared for each specimen.

After the dentin specimens were mixed with a test tube rotator (GFL SH 3025, GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany) for 60 min, different dilutions (10^{-2}, 10^{-3}, and 10^{-4}) of each specimen were prepared in PBS in sterile tubes. The samples (0.1 mL) from each group were plated onto the media. One plate was left empty as a control. The media were incubated in an anaerobic environment (inside a jar) for 48 h at 37 °C.

The colonies that formed on the media after 48 h were examined macroscopically under a light and counted. As 0.1-mL samples were plated from the specimens prepared at a 10^{-4} dilution, the number of colonies was multiplied by 10^5. Colony numbers were expressed in units of colony-forming units (CFU)/mL.

The dentin specimens collected from the caries cavity before and after ozone and CHX were placed in sterile tubes and examined with a scanning electron microscope (SEM) (QUANTA FEG 250, FEI, Hillsboro, OR, USA). Images were collected at 30,000x magnification level.

Statistical Analysis

SPSS version 20.0 software for Windows (SPSS, Inc., Chicago, IL, USA) was used for data analysis. For statistical analysis, colony numbers of *S. mutans* and *Lactobacilli* were calculated as log CFU/mL to obtain a normal distribution.

The difference between *S. mutans* and *Lactobacilli* colony numbers before and after treatment was compared via a paired sample t-test.

To determine whether there was a significant difference between the antimicrobial effectiveness of ozone and CHX administration on *S. mutans* and lactobacilli, analysis of variance was performed using a 2×2 factorial design. $P<0.05$ was considered statistically significant for all analyses.

Results

Colonies obtained from dentin samples of carious cavities and reproduced in medium were determined by the number of *S. mutans* and lactobacilli. The means, standard deviations, and statistical differences (log CFU/mL) of the microbiological results obtained in the study are presented in Table 1. There was a significant difference in the growth of microorganisms in all groups ($p<0.05$). The observed microorganisms were significantly reduced ($p<0.05$) in the ozone and CHX groups compared to in the control group. Although CHX appeared to be numerically more effective than ozone on *S. mutans*, this difference was not statistically significant. Lactobacillus growth was significantly lower in the CHX group than in the ozone group ($p<0.05$).

SEM images show the colonies before and after antimicrobial application (Figure 1). Dentin tubules became more obvious after CHX application, which may be due to the chelation properties of CHX. Only the bacterial components disappeared upon ozone application, and a chelation effect was not observed.

Discussion

Despite advances in protective treatments, fluorine usage and an emphasis on dental health education have guided the practices of pediatric dentistry. Furthermore, the treatment of caries lesions in both primary and permanent teeth is the most common procedure performed on children (20).

Infected dentin tissue must be completely removed before a tooth with caries can be restored. This procedure is extremely important for preventing secondary caries formation (21). However, the inability to clinically distinguish between infected dentin and affected dentin tissue prevents precise determination of the amount of dentin caries. Using a mirror and a probe, it can be partially determined whether the dentin tissue remaining after cavity preparation is healthy. As this method requires the dentist to determine the hardness and color of the dentin tissue, this method is not objective and is insufficient for determining bacterial status (22). Previous studies

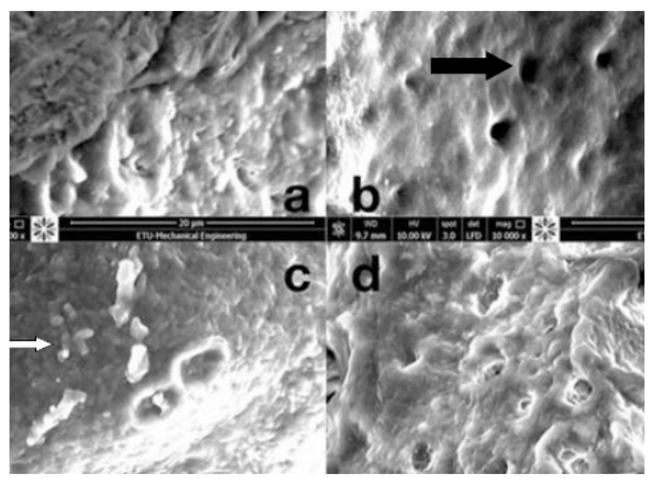

Figure 1. Scanning electron microscopy images before and after antimicrobial application (30,000x magnification)
a. Before chlorhexidine digluconate (CHX) treatment, b. After CHX treatment, c. Before ozone treatment, d. After ozone treatment. White arrow: Bacterial colonies. Black arrow: Dentin tubules after antimicrobial agent application.
CHX: Chlorhexidine digluconate

Table 1. The mean logarithmic values of the number of microorganisms (log colony forming units/mL)

	Before ozone [$\log_{10}$ (CFU/mL)]	After ozone [$\log_{10}$ (CFU/mL)]	Before CHX [$\log_{10}$ (CFU/mL)]	After CHX [$\log_{10}$ (CFU/mL)]
Streptococcus mutans	6.11 ± 0.15[a]	5.37 ± 0.29[a]	6.22 ± 0.25[b]	5.46 ± 0.25[b]
Lactobacillus sp.	6.17 ± 0.16[c]	5.57 ± 0.25[c]	6.36 ± 0.20[d]	5.46 ± 0.26[d]

*Same letters in superscript indicate a significant difference. CHX: Chlorhexidine digluconate, CFU: Colony-forming units indicate that even if the infected dentin tissue of the affected teeth is completely removed, the dentin specimen collected from the cavity base still contains bacteria (23,24).

Several materials, such as CHX, are used as cavity disinfectants. However, administration of cavity disinfectants or adhesive systems can negatively impact the ability of hydrophilic resin to bind to dentin, thus altering its permeability (25,26). Ozone gas is considered an alternative non-invasive antibacterial, antiviral, and antifungal agent and can reduce the number of bacteria in caries. Ozone is effective at lower concentrations and acts more rapidly and without side effects such as taste or odor, which are typical of other disinfectant agents such as chlorine and ammonium compounds. Moreover, ozone treatment is less costly, easier, and faster, and can be applied as a substitute for traditional caries cleaning methods and antimicrobial agents (27).

Ozone treatment is considered appropriate for use in pediatric dentistry owing to its painlessness, and is thought to have antimicrobial features. It is therefore used as an alternative to existing methods for cavity disinfection and their negative effects on the bonding force of resins (27).

S. mutans is the primary bacterium responsible for human tooth caries. The antimicrobial effectiveness of different treatments against *S. mutans* and *Lactobacilli* was therefore evaluated in deep dentin cavities (28). *Lactobacillus acidophilus* and *Lactobacillus casei* are isolated from the saliva, vestibular mucosa, back of the tongue, and hard palate in the oral cavity of humans. All types of *Lactobacilli* catalyze mixed acid fermentation reactions, in which carbohydrates, particularly lactic acid, are transformed into caries-causing acids (29). The cavity disinfectant with CHX and ozone used in this study showed significant antimicrobial effectiveness against *S. mutans* and *Lactobacilli*.

The antimicrobial efficacy of Cavity Cleanser was statistically significant for both types of microorganisms. By comparing antimicrobial activity of Cavity Cleanser on these microorganisms, it was observed that this was more effective against *Lactobacilli* than against *S. mutans*, with a larger number of bacteria being eliminated in the *Lactobacilli* group. SEM showed that microorganisms and the exposed dentin tubules. In contrast, another study used the dental cavity method to evaluate the antibacterial effectiveness of CHX, and found that it was more effective against *S. mutans* than *Lactobacilli* (17).

Ozone treatment has antibacterial effects and has become widely used in the dentistry field in recent years. Ozone treatment has easier and faster application compared to other traditional caries cleaning methods and antimicrobial agents. Caries lesions have been suggested to be reversible, although it is difficult to estimate the extent to which this is possible (30). Polydorou et al. (31) applied ozone on the dentin surface for 80 s under *in vitro* conditions and observed reduced numbers of microorganisms. The biomolecular structure of the caries lesion was examined before and after ozone application by Holmes (30). Holmes (30) reported that when oxidized, the acids formed by the bacteria become more alkaline, which is not a suitable environment for bacterial growth. It was reported that ozone transforms the microbial flora, consisting of acidogenic and aciduric microorganisms, into normal oral flora. Moreover, it ensures remineralization by removing the proteins in the caries lesion, and causes calcium, phosphate, and fluorine ion diffusion in the caries lesion. The use of remineralizing solutions following ozone administration after active caries is stopped, along with good oral hygiene and reduced sugar intake, can make remineralized tissue more resistant to caries attack (30). Other studies reported that the antimicrobial effectiveness of ozone may be affected by various factors such as the administration period, concentration, medium temperature, bacteria types, and colonization. Thus, further *in vivo* and *in vitro* studies of the effects of different treatment parameters on ozone treatment are needed (32,33).

Ozone treatment is pain-free and shows antibacterial properties, making it suitable for use in pediatric dentistry (34-36). We used an *ex vivo* technique to investigate the efficacy of ozone for cavity disinfection. Ozone administration was performed using an OzonyTronXP, which is the latest-generation ozone device. The OzonyTronXP device does not produce ozone gas. The probe of the device degrades oxygen molecules (O_2) present in the environment once the probe is in contact with the patient. Atomic oxygen (O^-) kills bacteria and combines with oxygen in the environment to temporarily create ozone

(O_3) gas, which has strong disinfectant effects. The manufacturer recommends a disinfection period of 120 s for deep dentin cavities.

Baysan and Lynch (37) showed that in primary root surface caries, *in vitro* ozone administration for 10 or 20 s significantly reduced the number of microorganisms. They reported that *in vitro* ozone administration on the surface of caries dentin for 10 s reduced the number of all bacteria. along with *S. mutans* and *S. sobrinus*, to less than 1%. Furthermore, 20 s of administration eliminated 99.9% of microorganisms (37).

We utilized SEM to evaluate changes in dentin tissue, allowing the detection of microorganisms and their accessories following ozone treatment and CHX administration. The SEM images showed that microorganisms and their accessories were removed from the ozone-treated dentin specimens compared to before administration, and that the dentin tubules were exposed.

Conversely, in a study measuring the efficacy of ozone treatment on infected dentin underneath the lesions without cavitation, Baysan and Beighton (38) found that ozone treatment had weak antibacterial effects. The researchers carried out ozone treatment for 40 s (HealOzone). They reported that these results may have been obtained because the ozone did not directly contact the dentin. Additional microbiological evaluation, in which ozone was applied to infected dentin for 40 s after removing the enamel tissue, showed that the microbial counts were significantly reduced. In an *in vitro* study conducted using the "Tooth Cavity Model" technique on permanent teeth, Polydorou et al. (31) compared the effects of ozone treatment (HealOzone 40 s, 80 s) and antibacterial bonding agents (Clearfill Protect Bond, Clearfill SE Bond) on *S. mutans*. They reported that both bonding systems and 80-s ozone treatment were significantly more effective than 40-s ozone treatment. In this study, 120 s of ozone treatment with the OzonytronXP device was effective for reducing bacterial counts.

Conclusion

In clinical practice, applying antimicrobial agents before restoring cavities may reduce the risk of secondary caries. This study showed that the chemical cavity disinfectant (Cavity Cleanser) containing CHX is the most effective antibacterial treatment against *S. mutans* and *Lactobacillus*. However, ozone therapy can also be an effective disinfectant when used carefully and for an appropriate period of time. More extensive clinical studies are needed to determine the effects of ozone treatment on infected dentine tissues, either alone or in combination with other cavity disinfectants. The data obtained in our study show that ozone treatment has antimicrobial efficacy. However, the effect of antimicrobial agents on the restoration of dental joints remains to be investigated.

Ethics

Ethics Committee Approval: The study protocol was approved by the Ethics Committee of the Atatürk University Faculty of Dentistry (decision no: 145, date: 04.03.2015).

Informed Consent: The necessary permissions and informed consent were obtained before beginning the experiments.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: T.G., Design: E.D., Supervision: Ö.B., Fundings: E.D., T.G., Ö.B., Data Collection or Processing: E.D., Ö.B., Analysis or Interpretation: T.G., Literature Search: E.D., Critical Review: E.D., Writing: E.D.

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