Original Article

Efficacy of an experimental gaseous ozone-based sterilization method for clear aligners

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ABSTRACT

Objectives: To assess effectiveness of an experimental sterilization method based on the exposure of an O_3/O_2 gas mixture directly inside the packaging for clear aligners.

Materials and Methods: Fifty samples consisting of pieces of polyethylene terephthalate glycol (PET-G) aligners were contaminated by manual handling and subsequently divided into different groups (n = 30 for exposure to O_3/O_2 gas at different times, n = 10 for positive control with 2% chlorhexidine digluconate, n = 10 for negative control). The measurement of optical densities (OD) of the initial and final microbial cultures was recorded for all groups. Kruskal-Wallis test was used for differences between groups while Wilcoxon test was used to compare initial and final OD values within groups. Statistical significance was set at $P < .05$.

Results: Comparison within the groups showed statistically significant differences for exposure to the gaseous mixture (72 hours), for positive and negative controls. Other significant differences were found in the multiple comparisons between the application of gaseous ozone (48 hours and 72 hours) and the negative control.

Conclusions: The direct exposure of gaseous ozone on the aligners inside their packaging showed microbicidal capacity at 72 hours, which was equivalent to the positive control with immersion in chlorhexidine digluconate. This innovative sterilization procedure could be considered in the final manufacturing processes of clear aligners to eliminate the potentially pathogenic microorganisms that are deposited on surfaces of these orthodontic devices. (*Angle Orthod*. 2024;94:194–199.)

KEY WORDS: Clear aligners; Microbicidal capacity; Sterilization; O_3/O_2 gas mixture; Gaseous ozone

INTRODUCTION

Environmental contamination is caused by the proliferation of pathogenic microorganisms, particularly in healthcare and professional settings, due to lapses in hygiene and sanitation standards.[1] Pathogens can spread through bioaerosols and dust particles with consequent risk of exposure by inhalation or by contact with contaminated surfaces.[2,3] In recent years, the healthcare system has been exploring new disinfection and sanitization procedures to enhance safety and minimize pathogen transmission.[4–6]

Various sterilization methods are already known and may be used (dry heat, steam, hydrogen peroxide, ethylene oxide, and irradiation) to attain germ-free environments and devices to preserve human health.[7] However, some of these processes can have undesirable effects on certain materials, such as melting and oxidation of some metals or the degradation of polymers.[7] Recently, the use of ozone (O_3) has been proposed as a sterilizing agent[8,9] and for the decontamination of various environments.[10–12] The oxygen atom (O), which derives from the degradation of ozone, acts on microorganisms by direct and indirect oxidation and peroxidation of substrates, causing alteration of the structure and functionality of biomolecules.[9,13] Sterilization by ozone can be accomplished by a mixture of O_3/O_2 gas, ozonized

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water, ozonized saline solution, or ozone associated with other substances,[14–17] and is particularly effective in eliminating bacteria, viruses and fungi.[18,19] Several factors can influence the bactericidal and virucidal action of ozone, such as concentration and exposure time, temperature and humidity of the environment, material being sterilized, and presence of biofilm.[20]

In orthodontics, the demand for esthetic appliances, such as clear aligners, has increased significantly[21] and sterility of these devices is important. After virtual treatment planning and manufacturing with stereolithography technology,[22] the aligner production process involves thermoforming of commercial polymer discs on dental models.[23–25] However, microbial contamination may occur during the final phase of manual finishing and polishing the contours of the aligners. Decontamination procedures should be used in production laboratories of orthodontic aligners to eliminate the microbial load that can be found in the working environment. Therefore, the purpose of this study was to evaluate the effectiveness of an innovative sterilization procedure based on an O_3/O_2 gaseous mixture that would eliminate microorganisms deposited on the surface of the aligners directly within their packaging.

MATERIALS AND METHODS

Subdivision and Contamination of Aligner Samples

Fifty pieces of thermoplastic material of polyethylene terephthalate glycol (PET-G), corresponding to the shape of molars and premolars, were obtained from 10 clear aligners (Lineo, Micerium Lab, Avegno, Italy). All samples were randomly divided into the following groups: gaseous ozone treatment (n = 30), positive control with 2% chlorhexidine digluconate (n = 10), and negative control without treatment (n = 10). In the gaseous ozone treated group, exposure times were 24 hours (n = 10), 48 hours (n = 10), and 72 hours (n = 10).

To achieve the level of microbial contamination that can occur during the manufacturing processes of the aligners in laboratories, all samples were contaminated with a one-time handling by an operator for a short time up to 10 minutes. Handling was performed simultaneously for all specimens to reduce the risk of error in subsequent results. Subsequently, each piece was incubated in 4 mL of sterile Lysogen nutrient broth (LB) at 37°C under vibration for 20 hours and, finally, the optical density (OD) of the initial microbial culture in all groups was recorded.

Ozone Generator

The O_3/O_2 gas mixture was produced by a commercial ozone generator (CUBO, Terminter Company, Messina, Italy) using ambient air as a source of oxygen. This equipment has different functions as a sanitized air generator with high oxidizing power, high capacity to degrade complex nonbiodegradable organic compounds, and disinfection of environments with reduction of bacterial load. Ozonated air produced at a constant flow rate (10gr/h) by the appliance was passed from a silicone tube to a diffuser. Cyclic timing of ozonation was six cycles of 45' with 15' standby. Treated samples were exposed to the gas mixture at room temperature (28°C).

Sterilization Procedure With Gaseous Ozone

The sterilization method with the ozone generator allowed for exposure of the O_3/O_2 gaseous mixture directly inside the packaging prepared for a single aligner to be sent to a clinician. For this, a silicone diffusion tube was used that was connected on one side to the ozone generator and, on the other, to a small tip to facilitate entry of the gas inside the packaging. The protocol designed for this experimental system included the following steps:

1. First, a hole of the same size as the tip was made on one of the two flat surfaces of the packaging for the aligners;
2. Each contaminated aligner piece was inserted inside the package, which was carefully sealed using a portable heat sealer;
3. All packages containing the samples were filled with gaseous ozone through the tip correctly inserted into the hole provided;
4. After filling all the packages completely, the holes were immediately sealed.

Once the exposure times established for each group were reached (24 hours, 48 hours, and 72 hours), each sample was taken from its packaging and incubated in 4 mL of sterile LB at 37°C under vibration for 20 hours.

For the positive control, specimens were immersed in 4 mL of 2% chlorhexidine digluconate for 2 hours[26] and then incubated at 37°C under vibration for 20 hours. For the negative control, untreated samples remained at room temperature for 2 hours and subsequently incubated at 37°C under vibration for 20 hours. Finally, the OD of the final microbial culture in all groups was recorded.

Optical Density Measurement

A Genesys 10S UV-vis spectrophotometer (Thermo Scientific, Waltham, MA, USA) and semimicro cuvettes (PS-UV, 1.5 mL, Brand, Biogenerica SRL, Catania, Italy) were used for all optical density measurements of microbial cultures at a wavelength of 600 nm (OD_{600}).[27]

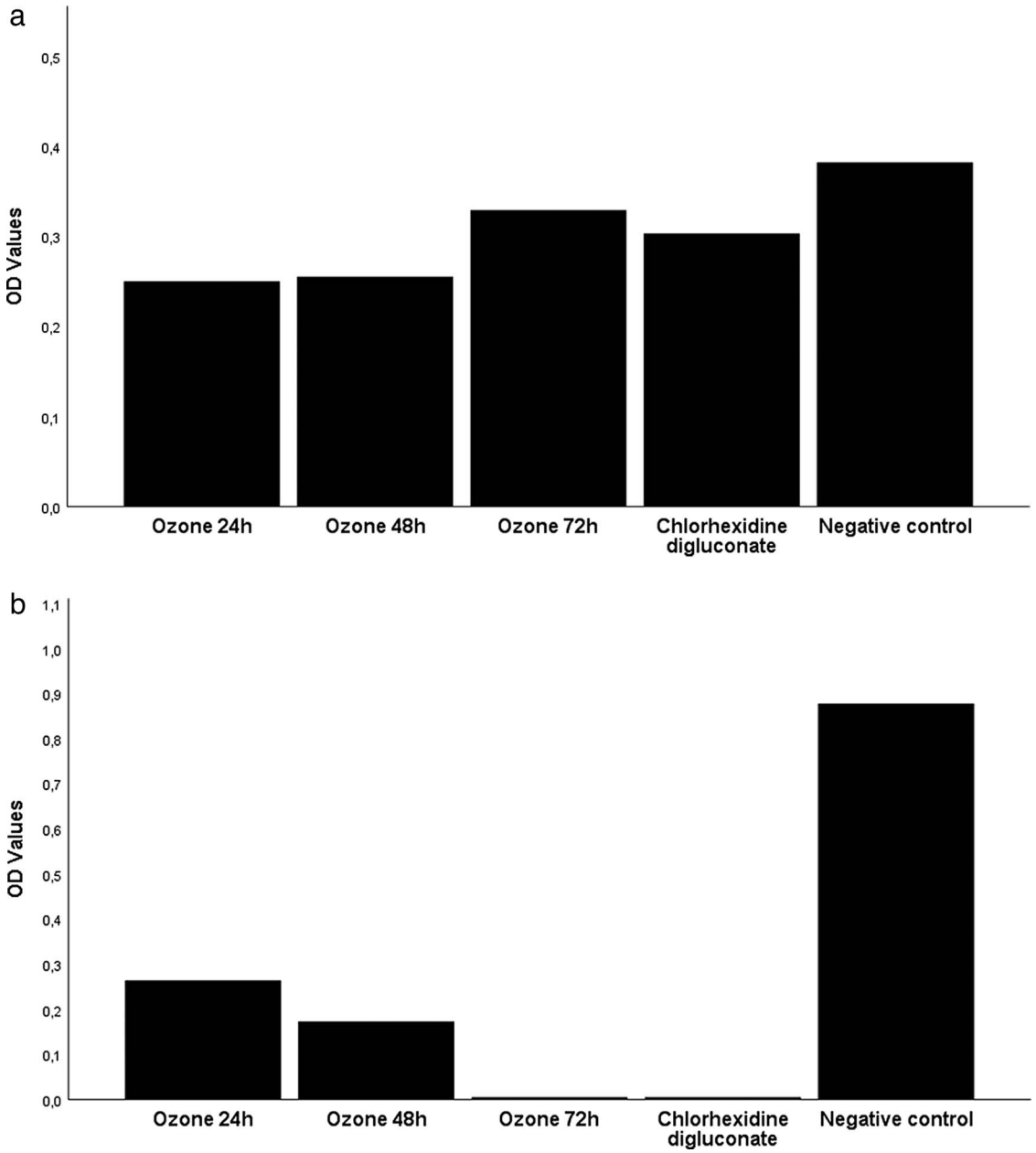

Figure 1. Descriptive statistics for the groups treated with O_3/O_2 gas mixture for different amounts of time (24 hours, 48 hours, and 72 hours) and for the positive (2% chlorhexidine digluconate) and negative controls: (a) Initial mean OD values of microbial cultures; (b) Final mean OD values of microbial cultures.

Statistical Analysis

All data are presented as mean ± standard deviation (SD). Numeric variables did not have a normal distribution from the Kolmogorov-Smirnov test, so the nonparametric method was used. The Kruskal-Wallis test was used to compare OD measurements between groups treated with gaseous ozone and those of positive and negative controls. Significance values were adapted based on the Bonferroni correction for multiple tests. In addition, the Wilcoxon signed-rank test was used to compare OD values before and after ozone treatment at different times and in control groups. A $P < .05$ was considered statistically significant. All statistical analyses were performed using SPSS 25.0 software (IBM SPSS Statistics, New York, USA) for Windows.

RESULTS

Descriptive statistics based on the mean OD values within each group are shown in Figure 1.

The mean OD values and standard deviations (SD) of microbial cultures in all groups are described in Table 1.

Table 1. OD Measurements of Microbial Cultures

Groups	N°	OD Initial Values (Mean ± SD)	OD Final Values (Mean ± SD)	*P* Value (Wilcoxon Signed-Rank Test)
Gaseous ozone exposure (24 h)	10	0.25 ± 0.82	0.26 ± 0.25	.959
Gaseous ozone exposure (48 h)	10	0.25 ± 0.07	0.17 ± 0.18	.262
Gaseous ozone exposure (72 h)	10	0.33 ± 0.09	0	.005*
Positive control	10	0.30 ± 0.05	0	.005*
Negative control	10	0.38 ± 0.09	0.88 ± 0.07	.005*

* Statistical significance.

The Kruskal-Wallis test showed that the distribution of OD was significantly different between groups. In pairwise comparisons, statistically significant differences were found between initial OD measurements of the groups exposed to the O_3/O_2 gaseous mixture for 24 hours and 48 hours compared to the negative control ($P = .004$ and $P = .032$, respectively) (Table 2).

When comparing the initial and final mean OD values within the groups, the Wilcoxon test showed significant differences in the treatment with gas mixture for 72 hours ($P = .005$) and in both control groups ($P = .005$) (Table 1).

Pairwise comparison between the final OD measurements in the different groups revealed highly significant differences between the group exposed to the gaseous mixture for 72 hours and the negative control and between the positive control treated with chlorhexidine digluconate and the negative control ($P < .001$). Statistically significant differences were also found between the final mean OD values of the groups exposed to the O_3/O_2 gas for 24 hours and 72 hours ($P = .035$), between the group exposed for 24 hours and the positive control ($P = .043$), and between the negative control and the group exposed to the gaseous mixture for 48h ($P = .048$) (Table 2).

DISCUSSION

It is important to consider the effects of various sterilization processes on the materials with which medical devices are made. Traditional heat-based sterilization can lead to thermal degradation and hydrolysis, resulting in a structural loss in thermoplastic polymers, making them unsuitable for such processes.[28] Ethylene oxide is capable of sterilizing most polymers used for medical devices, but its use is limited due to its carcinogenic potential for the release of toxic residue and byproducts.[7] Radiation sterilization could be a viable alternative for polymers that do not tolerate heat-based procedures.[29] In recent years, new sterilization methods have been developed such as hydrogen peroxide (H_2O_2) and O_3. H_2O_2 does not release residue or carcinogens, but has limitations in medical device manufacturing industry as it cannot be used for polymers with absorbent capacity.[7] Ozone-based sterilization utilizing an O_3/O_2 gas mixture has gotten attention for its surface oxidation properties and compatibility with various resistant polymers, especially in high humidity environments (>80%).

Previous literature ascertained the biocidal efficacy of O_3. Thanomsub et al.[30] found significant ultrastructural changes of certain bacterial cultures at 60 minutes of O_3 exposure and inactivation with cell membrane disruption and lysis after 90 minutes. However, O_3 did not show bactericidal efficacy against all cells after 150 minutes. Sharma et al.[10] tested O_3 efficacy at 25 ppm on plastic surfaces for bacterial strains responsible for nosocomial infections over a short period of exposure. Similarly, a pilot study by Fontes et al.[31] showed how a single exposure application of 20 μg of O_3/mL in O_3/O_2 gas mixture

Table 2. Pairwise Comparison of the Initial and Final Mean OD Values Between Groups Based on Kruskal-Wallis Test With Bonferroni Correction

Initial OD Measurements		Final OD Measurements	
Group 1-Group 2	*P*-Value	Group 1-Group 2	*P*-Value
Ozone 24 h – Ozone 48 h	1.000	Ozone 24 h – Ozone 48 h	1.000
Ozone 24 h – Ozone 72 h	.234	Ozone 24 h – Ozone 72 h	.035*
Ozone 24 h – Positive control	.432	Ozone 24 h – Positive control	.043*
Ozone 24 h – Negative control	.004*	Ozone 24 h – Negative control	.197
Ozone 48 h – Ozone 72 h	.968	Ozone 48 h – Ozone 72 h	.150
Ozone 48 h – Positive control	1.000	Ozone 48 h – Positive control	.181
Ozone 48 h – Negative control	.032*	Ozone 48 h – Negative control	.048*
Ozone 72 h – Positive control	1.000	Ozone 72 h – Positive control	1.000
Ozone 72 h – Negative control	1.000	Ozone 72 h – Negative control	<.0001*
Positive control – Negative control	1.000	Positive control – Negative control	<.0001*

* Statistical significance.

for 5 minutes eliminated mainly bacterial strains with a known resistance to antibiotics in nosocomial infections. Finally, a recent study by Rangel et al.[32] found that exposure of a low concentration of O_3 gas at 10 hours and 12 hours did not inhibit growth of selected gram-positive and gram-negative bacteria but interfered with their cell viability, resulting in an increase of reactive oxygen species (ROS) and significant structural changes.

The current study focused on the efficacy of an experimental method based on O_3/O_2 gas mixture produced by a generator at low concentrations. The gas produced was transferred directly into the aligner packaging through a silicone tube with a tip to prevent environmental leakage. The results obtained from the pairwise comparison between the initial OD measurements (Table 2) indicated that microbial contamination due to handling did not significantly differ between groups, except for the negative control. However, this difference did not affect the study's objectives but highlighted the presence of microbial growth on sample surfaces. Comparison between the initial and final OD values within all the groups showed how exposure times affected the antimicrobial capacity of the O_3/O_2 gaseous mixture (Table 1). This experimental procedure exhibited microbicidal properties at 72 hours, a result similar to the positive control group. At 48 hours of exposure, the gas mixture caused a reduction of the final OD values, suggesting a microbiostatic effect and, at 24 hours, no significant change was observed. The negative control demonstrated exponential microbial growth (Table 1). Comparison of the final mean OD values between the different groups (Table 2) further highlighted how exposure for 24 hours did not inhibit microbial growth compared to 72 hours and to the positive control. In fact, there was no difference between 24 hours and the negative control. The result obtained from the 48-hour exposure also did not differ from the 24 hours, but showed antimicrobial effects compared to the negative control. Finally, the microbicidal capacity of 72-hour exposure was again detected by comparison with the final OD value of the negative control and was equivalent to that of the comparison between the control groups.

The gas ozone sterilization method has several clinical applications. First, it demonstrates significant potential for improving infection control and patient safety, reducing the risk of oral infections that could potentially interfere with advances in orthodontic treatment. Patients are likely to appreciate the extra level of care and safety provided using sterilized aligners, improving satisfaction and compliance. Additionally, if this sterilization method proves to be consistently effective and safe over time, it could become an industry standard for clear aligner manufacturing. Standardization would ensure uniform quality and safety across various clear aligner brands. Extension of this procedure to other devices is also possible, improving infection control practices in various areas of dentistry.

This innovative system offers several advantages compared to other existing procedures. In addition to its efficacy against potentially pathogenic microorganisms, direct application of gaseous ozone inside the package is a simple procedure for manufacturers as it does not require extensive equipment or additional processing steps. In fact, the risk of recontamination is reduced since the aligners remain sterile until the packaging is opened by the clinician or the patient. Unlike other chemical-based sterilization methods, gaseous ozone sterilization does not leave chemical residue on the aligners, which is beneficial for patient safety and comfort. Finally, ozone is a sterilization agent that decomposes naturally into oxygen, leaving no harmful byproducts or chemical waste, aligning with the increasing focus on environmentally sustainable practices in healthcare. Further research studies are warranted to validate its long-term efficacy and safety. In addition, given the well-known influence of the type of microbes on the efficacy of O_3,[33,34] other investigations should be undertaken to detect any cellular changes. Cell viability, ROS levels, ultrastructural changes, or membrane permeability should be investigated, especially where O_3 exposure has not inhibited microbial growth.

CONCLUSIONS

- This preliminary study introduces new research objectives to find the most suitable method to sterilize clear aligners in orthodontic laboratories, preventing cross-infection.
- The direct application of O_3/O_2 gaseous mixture to the clear aligners has a microbicidal effect at the longest exposure time tested. A possible microbiostatic capacity for a shorter exposure time (48 hours) was also detected.
- The sterilization procedure using an O_3 generator with a customized connection system to facilitate the direct entry of the gas mixture inside the packages is possible as a final step in the aligner production processes. It is a safe method because it is a closed system with no danger of toxicity and any inhalation is insignificant.

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