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Antibacterial Effectiveness of Prophylactic Ozone Application with Full-Mouth-Tray in Orthodontic Patients with High Caries Activity

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Abstract

The objective of this study was to investigate the effectiveness of ozone and mouth rinse on *Streptococcus mutans (S.mutans)* in pediatric patients with fixed orthodontic appliances. The study was conducted on 13-18 years old patients with fulfilled the inclusion criteria of high levels of *S.mutans* in saliva. Number of *S.mutans* in plaque and saliva was determined CFU/ml terms for baseline bacteriological counts. Patients were randomly divided into three groups: Group 1: Gaseous ozone, Group 2: Mouth Rinse, Group 3: Daily oral hygiene regime. Immediately after applications, stimulated saliva samples were collected again. After 1 week, plaque and saliva samples were recorded. Mann-Whitney U, Kruskal-Wallis and Wilcoxon T tests were used for statistical analysis. The reduced levels of *S.mutans* in saliva immediately after the application in the groups of Ozone and Listerine were found to be statistically significant ($p<0,05$). The Listerine group exhibited a significantly lower *S.mutans* counts in saliva compared with baseline and other groups after one week ($p<0,05$). After one week, the reduced level of *S.mutans* in plaque as compared to baseline values in all groups were found to be statistically significant ($p<0,05$). It can be concluded that the ozone treatment may have an instantaneous lethal effect on *S.mutans*; however, a long-term preventive effect could not be observed. Listerine showed better effects on decreasing the level of *S.mutans* in saliva and plaque.

1. Introduction

Orthodontic treatment with fixed appliances may cause oral ecologic changes. Orthodontic bands and brackets play a significant role in microbial plaque accumulation because of causing retention areas for microorganisms (Dogan, Cetin, Hüssein & Adiloglu, 2009; Attin, Ilse, Werner, Wiegand & Attin, 2006). On the other hand, due to the reduced pH and increased affinity of microorganism to metallic surface, the composition of cariogenic microflora increases (Dogan, 2009; Sari & Birinci, 2007). Several studies have reported that increased level of *Streptococcus mutans (S.mutans)* species detected in the oral cavity after placement of fixed orthodontic appliances (Topaloglu-Ak, Ertugrul, Eden, Ates & Bulut, 2011; Koopman et al., 2015). The increase in the *S.mutans* level starting with the orthodontic treatment causes enamel demineralization and caries development, predominantly on sites adjacent to brackets (Derks, Frencken, Bronkhorst, Kuijpers-Jagtman & Katsaros, 2008).

A crucial step for the prevention of caries during orthodontic treatment is the reduction of cariogenic microorganism from the oral cavity. Standard caries preventive measures based on oral hygiene, established of non-cariogenic diet, and fluoride application, are insufficient to prevent the occurrence of new carious lesion in orthodontic patient with high caries activity (Dogan, Cetin, Hüssein & Adiloglu, 2009; Attin, 2006; Fard et al., 2011). Fixed orthodontic appliances such as arc wires and brackets cause difficulties in the effective control of dental plaque and satisfactory oral hygiene. Therefore, preventive efforts have focused on direct suppression of cariogenic microflora by chemotherapeutics, especially mouth rinses, for maintaining healthy teeth throughout orthodontic treatment. Although there is no specific clinical protocol, the use of mouth rinse, as a prophylactic method, has been advised for reducing the bacterial plaque accumulation and control of the level of microorganisms during the active phase of orthodontic treatment (Sari & Birinci, 2007; Fard et al., 2011; Oltramari-Navarro et al., 2009).

In this context, chlorhexidine is the potent documented antimicrobial agent against *S.mutans* (Attin, 2006). It has been shown that chlorhexidine treatment in different forms reduces the number of *S.mutans* in plaque and saliva in non-orthodontic patient, however, it has been suggested that chlorhexidine is less effective in high-risk orthodontic patient because of increased plaque retention area and a rapid recolonization of *S.mutans* (Derks, 2008; Fard et al., 2011). On the other hand, the handicaps relate to use of chlorhexidine like the need for long-term use, bacterial resistance and significant side effects have stimulated the search for new and more appropriate alternatives for use in pediatric orthodontic patients **(**Dhingra & Vandana, 2011).

Listerine (Pfizer Consumer Healthcare, Morris Plains, NJ), the essential oil-containing mouth rinse, has bactericidal activity and clinically proven effectiveness in decreasing microbial plaque. Listerine has usually been recommended as a part of home-care oral hygiene regime. Studies have shown that adding Listerine to the daily oral hygiene regime reduces plaque and gingivitis development in orthodontic patients, thus lessen the likelihood that

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white spot lesions and caries will develop (Fard et al., 2011; Tufekci, Casagrande, Lindauer, Fowler & Williams, 2008; Alves, Goursand, Zenobio & Cruz, 2010).

Ozone is the most powerful oxidizing agent for cell walls and cytoplasmic membrane of bacteria and is an alternative non-invasive treatment aiming to reduce the levels of caries-associated microorganism (Johanson, Claesson & van Dijken, 2009). The antibacterial effect of ozone on *S.mutans* has been evaluated and has been shown to kill *S.mutans* efficiently (Johanson, 2009; Kronenberg, Lussi & Ruf, 2009; Baysan & Lynch, 2004; Castillo et al., 2008; Fagrell, Dietz, Lingström, Steiniger & Noren, 2008). Azarpazhooha & Limeback (2008) reported that the application of ozone as a prophylactic antimicrobial treatment prior to the placement of restoration is useful and ozone may prove an alternative to conventional prophylactic treatments. There are very few *in-vitro* and *in-vivo* studies evaluating the effectiveness of ozone application in the field of orthodontics. The studies on this field mainly focus on the anticaries effect of ozone and shear bond strength of orthodontic brackets (Kronenberg, 2009, Al Shamsi, Cunningham, Lamey & Lynch, 2008; Cehreli, Guzey, Arhun, Cetinsahin & Unver, 2010).

The newest device on the use of ozone in dentistry is Ozonytron *(MIO International, Munich, Germany)*, which is approved by MDA and TUV. A newly designed ozone appliance has a full-mouth-tray (FMT), dual spoons of soft polymer in different size for primary and permanent teeth. FMT can be used for disinfection of all teeth, periodontal area and tongue in addition to bleaching and desensitizing the teeth. A novel application technology has a process-controlled aspiration; therefore, ozone gas cannot be inhaled (Cehreli et al., 2010; Aykut-Yetkiner, Eden, Ertuğrul, Ergin & Ateş, 2013).

To our knowledge, the antimicrobial effect of ozone in salivary and dental plaque has not been studied in children especially. The objective of this study was to evaluate the effectiveness of gaseous ozone as an antimicrobial agent in reducing bacteria in salivary and plaque of fixed orthodontic pediatric patients.

2. Material and Method

This prospective randomized controlled clinical trial was conducted at the Department of Pediatric Dentistry in collaboration with Department of Medical Microbiology. The study was approved by the Ethical Committee of the Ankara University, Faculty of Dentistry (protocol number 36290600/58).

2.1. Study sample

The volunteers and their parents were informed about the study and signed informed consent forms. Fourty eight healthy 13-18 years of aged volunteers with full fixed appliances (for at least 3 months prior to the start of the study at the Department of Orthodontics of the same university) were enrolled in the study. The inclusion criteria: 1) not take antibiotics and antibacterial mouth rinse within last month 2) nonsmoker 3) full dentition 4) non detectable frank caries or defective restorations 5) intact interproximal tooth surface in radiographs 6) high levels of *S.mutans* in saliva as demonstrated by at least a score of 2 identified with the chair-side Strip-mutans method according to Jensen and Bratthal.

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The study population finally consisted of 30 participants (16 males and 14 females) were selected who fulfilled the inclusion criteria. Participants were randomly assigned to three groups (1:1:1; two experimental and one control groups): Group 1: The application of Ozone gas *(Ozonytron OZ, MIO International, Munich, Germany)*, Group 2: The application of mouth rinse *(Listerine, Johnson & Johnson, NJ)*, Group 3: Daily oral hygiene regime (negative control).

2.2. Clinical procedures

Professional tooth cleaning with rubber cup and pumice was performed and oral hygiene instruction was given to each participant before the study. Samples were collected in the morning between 9 and 12 a.m. under standardized conditions. Before baseline examination, participants were advised to avoid eating or drinking and no tooth brushing at least 2 hours before sampling in the morning. All clinical measurements were performed by two investigators at three time points: Baseline (T0); Immediately after gaseous ozone and mouth rinse application (T1); After one week (T2). While the level of *S.mutans* were recorded both plaque and saliva at the time point T0 and T2, only the saliva *S.mutans* levels were recorded at the time point T1.

Selected teeth for plaque sampling were isolated with cotton rolls and dried. The plaque samples were carefully taken with a sterile excavator on the sites around the brackets of teeth 11,14, 22, 25, 31, 34, 42 and 45. In subjects with extracted premolars, canines were evaluated. A total of 240 sites were evaluated for 30 participants. Stimulated saliva was collected in a sterile plastic cup during 5 minutes by chewing a standardized piece of paraffin wax.

Group 1 (The application of Ozonytron, n=10): An available size of full mouth tray (FMT) was chosen for each patient and according to the manufacturer's recommendations, gaseous ozone was applied for 180 seconds by running the device on prophylaxis mode. The patients were instructed to brush their teeth at least two times per day using the Bass modified technique with a fluoride toothpaste (Colgate, 1450 ppm F, Colgate-Palmolive Company, Herstal, Belgium) and a manual toothbrush for one week.

Group 2 (The application of Listerine, n=10): For one week, in the morning and evening after brushing the teeth with toothpaste containing fluoride, participants used 15mL mouth rinse for 30 seconds, and then, not to eat or drink for 30 minutes.

Group 3 (Daily oral hygiene regime, n=10): As negative control group, basic oral hygiene regime was used. It was recommended that patients brush their teeth at least two times a day using the Bass modified technique with toothpaste containing fluoride for one week. The patients were instructed to avoid any antibacterial agent such as mouth rinse during trial procedure.

After gaseous ozone and Listerine application and only teeth brushing, stimulated saliva samples were collected (T1). After one week, plaque and stimulated saliva samples were collected as described above (T2).

2.3. Microbiological analysis

For microbiological analysis, samples collected were immediately transported within 30 minutes of collection.

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Table 1: Means and standard deviations of the level of *S.Mutans* in saliva (expressed as log_{10} CFU)

	Baseline (T0)	Immediately after applications (T1)	p-value†	Changes in Salivary *S.mutans* level	p-value	Baseline (T0)	After 1 week (T2)	p-value†	Changes in Salivary *S.mutans* level	p-value
Group 1 (Ozone)	5,68±0,27	2,88±2,08	<0,05*	2,81±2,15		5,68±0,27	5,46±0,37	>0,05	0,22±0,34	
Group 2 (Listerine)	5,81±0,47	2,29±1,54	<0,05*	3,53±1,86	<0,05	5,81±0,47	4,37±1,62[d]	<0,05*	1,45±1,81	>0,05
Group 3 (Negative Control)	5,95±0,19	5,95±0,19	-	0,00		5,95±0,19	5,69±0,40	>0,05	0,26±0,28	
p- value‡	>0,05	<0,05				>0,05	>0,05			

The plaque samples were diluted (10^{-1},10^{-2},10^{-3}) using phosphate-buffered saline solution (PBS) and homogenized by vortex mixing for 60 seconds. The saliva samples were homogenized by vortex mixing for 60 seconds, and then diluted (10^{-1},10^{-2},10^{-3}) using sterile saline. Then, 0.01 ml of each diluent was inoculated on Tryptic Soy Agar *(Merck, Germany)* plates for total bacteria counts. After 48 hours of incubation at 37^{0}C and 5% CO_2, the number of *S.mutans* colonies was counted and was reported as colony forming unit (CFU).

Statistical analysis

Statistical analysis was performed by using SPSS for Windows version 21 (SPSS Inc., Chicago, IL, United States). Data were shown as mean ± standard deviation (SD). Logarithmic transformation was used for number of microorganism in data analyses. While, the mean differences between two independent groups were compared by Mann-Whitney U test, otherwise, Kruskal-Wallis test applied for comparisons among more than two independent groups. Wilcoxon T test was used to evaluate differences within the group. For all data analysis, p< 0.05 was considered significant.

Results

The study population finally consisted of 30 participants (16 males and 14 females) were selected who fulfilled the inclusion criteria. No adverse effects were reported by the participants or their parents.

The levels of *S.mutans* in saliva samples are presented in Table 1 for all measurements (baseline (T0), immediately after applications (T1) and after one week (T2)). At baseline (T0), there were no significant differences among groups in terms of the *S.mutans* levels in saliva (p>0,05). Statistically significant reduced levels of *S.mutans* in saliva was found at T1 threshold for the groups of Ozone and Listerine (p<0,05). After one week (T2), *S.mutans*

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Table 2: Means and standard deviations of the level of *S.mutans* in plaque (expressed as log_{10} CFU)

	Baseline (T0)	After 1 week (T2)	p-value†
Group 1 (Ozone)	5,06±0,22	4,45±0,30	<0,05*
Group 2 (Listerine)	4,44±0,45	2,62±0,44	<0,05*
Group 3 (Negative Control)	4,75±0,30	4,52±0,23	<0,05*
p-value‡	>0,05	<0,05	

Table 3: Means and standard deviations of the level of *S.mutans* in selected teeth for plaque sampling (expressed as log_{10} CFU)

	Group 1 (Ozone)			**Group 2 (Listerine)**			**Group 3 (Negative Control)**		
	Baseline (T0)	**After 1 week (T2)**	**p-value**	**Baseline (T0)**	**After 1 week (T2)**	**p-value**	**Baseline (T0)**	**After 1 week (T2)**	**p-value**
14	5,22±1,01	4,23± 1,44	<0,05*	4,28±1,41	2,63±1,74	<0,05*	4,92±0,93	4,54±1,48	>0,05
11	5,07± 0,84	4,06± 1,75	>0,05	4,59±1,59	2,34±2,03	<0,05*	4,51±1,19	4,56±1,18	>0,05
22	5,30± 0,64	4,08± 1,84	>0,05	4,81±1,58	2,97±2,31	<0,05*	4,90±1,06	4,37±1,24	>0,05
25	4,76± 1,37	4,40± 1,70	>0,05	4,09±1,40	2,07±1,73	<0,05*	4,82±0,93	4,69±1,15	>0,05
34	5,29± 1,04	4,84± 1,40	>0,05	5,11±1,15	3,24±1,62	<0,05*	5,23±1,27	4,23±1,75	<0,05*
31	4,81± 1,35	4,61± 1,30	>0,05	3,79±1,83	2,16±1,96	<0,05*	4,35±1,46	4,81±0,88	>0,05
42	4,86± 1,46	4,64± 1,25	>0,05	4,11±1,92	2,46±2,03	<0,05*	4,39±1,50	4,78±0,98	>0,05
45	5,20± 1,12	4,75± 1,68	>0,05	4,76±1,50	3,10±1,59	<0,05*	4,87±1,79	4,21±1,39	>0,05

counts were reduced as compared to baseline values in Ozone group. The *S.mutans* levels increased by time and the application of Ozone did not exert any significant effect for *S.mutans* levels in saliva in this period (p>0,05). However, after 1 week (T2), Listerine group exhibited significantly lower *S.mutans* counts compared with baseline values (p<0,05). In negative control group, the value of *S.mutans* in saliva samples was reduced after the one-week period (T2), but no significant effect was observed. Statistically significant differences in the saliva *S.mutans* levels at any evaluation period (T0,

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T1, T2) in negative control group were not found (p>0,05).

In Table 2, the results of plaque samples on teeth with brackets at baseline (T0) and one week (T2) after application were demonstrated. At baseline, there were no significant differences among groups (p>0,05). After one week, *S.mutans* counts were reduced as compared to baseline values in all groups and these reductions were found to be statistically significant (p<0,05). The Listerine group exhibited significantly lower *S.mutans* counts compared with baseline values and other groups (p<0,05).

The CFU levels of *S.mutans* obtained from the plaque samples of selected teeth presented in Table 3 for all measurements (baseline and after one week). After one week (T2), *S.mutans* counts were reduced as compared to baseline values in Ozone and Listerine groups in all teeth (p<0,05). While these reductions were found to be statistically significant for the teeth in group of Listerine; significant difference was found for only one tooth in Ozone group. In negative control group, the value of *S.mutans* was reduced statistically significant for a single tooth after the one-week period (T2). On the other hand, the in plaque samples collected from some teeth it was found that levels of *S.mutans* were increased.

4. Discussion

A systematic review has demonstrated that the presence of fixed appliances influences the quantity and quality of oral microbiota and this effect depends on oral hygiene control (Freitas, Marquezan, Nojima Mda, Alviano & Maia, 2014). The presence of fixed appliances in orthodontic patients alters the structure and composition of dental plaque and increases microbial population, especially *Streptococcus*. Pathogenic bacteria colonization lead gingival inflammation, periodontal destruction and changes in enamel surfaces. In the absence of effective prevention strategies, gingival inflammation and enamel demineralization surrounding orthodontic brackets are occurred. Enamel demineralization is more prevalent in patients with fixed appliances than in those without bracket (Freitas et al., 2014; Perrini, Lombardo, Arreghini, Medori & Siciliani, 2016).

In this field, preventive strategies can be divided into two groups: The first group of methods is related to the patient and comprises strategies such as daily oral hygiene, patient motivation, mouth rinses containing essential oil or chlorhexidine and plaque staining, whereas the second group of methods is related to the appliance and includes fluoride-releasing adhesive, chlorhexidine and fluoride varnishes and ozone application (Aykut-Yetkiner, 2013; Migliorati et al., 2015).

Correctly performed daily oral hygiene is the most important preventive strategy for enamel demineralization. However, the presence of retention factors favorable to plaque accumulation aggravates home oral hygiene quality in patients with fixed orthodontic appliances. On the other hand, daily oral hygiene is not performed efficiently by the majority of the orthodontic population, mainly due to lack of motivation and manual dexterity (Haas et al., 2014). Therefore, the negative control group was composed of participants who were not using mouth rinse and continuing daily oral hygiene.

Because of difficulties to achieve ideal mechanical plaque control, other methods such as chemical

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agents have the search to control of the microbial population. Current evidence shows that when chemical agents, especially oral mouth rinses, are used as adjuvants to brushing and flossing, they can promote additional advantages (Haas et al., 2014; Pithan et al., 2016). Mouth rinses which contain active principals (such as chlorhexidine, sodium fluoride, essential oil) alter bacterial membrane permeability, contribute to its lysis; therefore, reduce its ability to adhere to the tooth surface (Pithan et al., 2015). Although mouth rinses containing chlorhexidine showed better results when they compared with others, they have adverse effects, such as tooth and soft tissue staining, which limit their long-term use. Therefore, it should be indicated only for short periods of time, especially in case of gingivitis (Haas et al., 2014).

In a systematic review of the literature regarding the use of antiseptic, among the daily-use agents, essential oils are recommended as the first option, because of no adverse effect on continuous use and significant efficacy (Haas et al., 2014). The greatest effect of daily use of Listerine in orthodontic patients was observed in two studies. Tufekci at al. (2008) evaluated the effectiveness of the adjunctive use of Listerine in reducing plaque and gingivitis. After six months, significantly less plaque and gingivitis were observed in the test group, compared with daily oral hygiene (brushing and flossing) alone. Alves et al. (2010) have demonstrated that daily use of mouth rinses containing essential oils reduce plaque and gingivitis.

The studies report inconsistent results about the effect of Listerine on reduction of cariogenic microorganisms. With regard to the effectiveness of the Listerine as antibacterial agent, Chen et al. (2013) have demonstrated that the use of the mouth rinses containing essential oil present no significant alteration in the microbiological profile of the orthodontic patient. Wiken Albertsson, Persson & Van Dijken (2013) showed that no differences were observed the reduction of *S.mutans* in saliva after rinsing with the essential oils and water. Despite that, Fine et al. (2000) reported that rinsing with an essential oil mouth rinse resulted in significant reductions *S.mutans* in plaque and saliva. Our results showed that Listerine was significantly more effective than Ozone and daily oral hygiene with the help of Listerine reduced bacterial counts in saliva and plaque samples. The mouth rinse evaluated in this study was alcohol-free because it had used by children. Marchetti et al. (2011) compared the effectiveness of essential oil mouth rinse with and without alcohol. They found that mouth rinse without alcohol seems less efficient on the plaque regrowth than conventional mouth rinse with alcohol.

The preventive methods such as mouth rinses may fail because they depend on the patient's cooperation and the need for long-term use. Therefore, new and more appropriate alternatives are needed in pediatric patients. Application of gaseous Ozone with the aid of the FMT in one session, which is applied only tooth-by-tooth can be more applicable compared to the other preventive methods (Aykut-Yetkiner et al., 2013).

Ozone therapy is an alternative and/or complementary treatment strategy in dentistry. Ozone is a highly unstable form of oxygen and has been recognized as a robust and effective antibacterial agent. The antibacterial effect of ozone

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on *S.mutans* has been evaluated in most *in-vitro* studies and has been shown to kill *S.mutans* efficiently; however, there are a few studies under *in-vivo* conditions in the presence of saliva and the dental biofilm, a barrier to be highly resistant to various types of antibacterial treatments Kronenberg et al., 2009; Baysan & Lynch, 2004; Castillo et al., 2008; Fagrell et al., 2008). Reducing the levels of caries-associated bacterial species in saliva and dental plaque is one of the most important strategies to prevent dental caries (Johanson, 2009). Therefore, in our study, the effectiveness of gaseous ozone was evaluated as an antimicrobial agent on bacteria in salivary and plaque.

Polydorou, Halili, Wittmer, Pelz & Hahn (2012) evaluated that the antimicrobial effect of ozone application on the *S.mutans* using a tooth cavity model. Ozone treatment (60 seconds) reduced significantly the amount of *S.mutans* and this antibacterial effect was able to be seen even after 4 and 8 weeks. This finding was similar to those of Kapdan, Oztaş & Sümer (2013) who found that Ozone application (80 seconds) significantly reduced the number of *S.mutans* on a tooth cavity model and there was a significant difference in terms of the amount of the microorganisms grown. Johansson et al. (2009) showed that *the gaseous ozone efficiently killed S.mutans*, but the presence of saliva hampered the bacterial killing. They evaluated that the antibacterial effect of ozone gas on cariogenic bacterial species with and without the presence of saliva. After 60 seconds ozone gas exposure, while approximately 99.9% of the *S.mutans* in salt buffer were dead, *S.mutans* less efficiently killed in saliva compared to the salt buffer. The results of our study indicate that gaseous Ozone had no effect *S.mutans* level in saliva on orthodontic patient with high caries activity.

Yetkiner A et al. (2013) investigated that the efficacy of ozone on microflora of patients with fixed orthodontic appliances. In this study, researchers used elastomeric rings to collect the biofilm and to assess microbiologically. Although the ozone treatment reduced the *S.mutans* immediately after the application, it is reported that *S.mutans* values increased after one week-period and the *S.mutans* values were significantly higher than the baseline values. It is reported that the increase in *S.mutans* might be explained by deformation of elastomeric ligature due to orthodontic forces might lead to a retention area. Unlike this study, an apparent effect of the gaseous ozone in plaque *S.mutans* level after 1 week-period was observed in our study. This difference might be explained by differences in plaque samples collection technique.

One of the limits of the carried out study was short follow-up. At the beginning of the study, long-term effects of the strategies were aimed to evaluate week by week till the saliva- plaque *S.mutans* levels become to baseline value. But, at the end of the first week the assessed CFU values were like the baseline counts, so the study was terminated at the end of the first week. Another limitation of this study, results may be affected by the participant's behavior and individual difference such as diet and salivary quality. The participants may feel motivated to perform satisfactory oral hygiene obtaining excellent results or may not use the mouth rinse properly. Another limitation is difficulties in collecting plaque samples. The plaque sampling method was not standardized concerning volume or weight. Due to

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large differences in plaque liquid contents, saliva sampling may be easier to standardize and perform.

It can be concluded that the Ozone and Listerine significantly reduced bacterial counts in plaque after the one-week period, although the Listerine mouthwash showed a further reduction in the bacterial colony count. The bacterial count in the saliva decreased after using Listerine mouth rinse. Although the gaseous ozone have an instantaneous lethal effect on *S.mutans* in saliva, it was not effective in reducing bacterial counts in saliva in long-term period. Thus, the use of Listerine mouth rinse during orthodontic treatment might be beneficial for the health status of the oral cavity.

5. Conclusion

Within the limitations of this study, ozone application with FMT significantly reduced bacterial counts in plaque samples, but in saliva samples it had only immediate lethal effect on *S.mutans*. Listerine mouth rinse caused the highest reduction in bacterial counts, followed by Ozone and negative control group. Further well-designed studies conducted within longer periods of investigation needed to confirm the influence of prophylactic ozone application.

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Conflicts of interest

The author declares no conflicts of interest related to this study.

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