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Dentistry

EVALUATION OF THE ANTIMICROBIAL EFFICACY OF HERBAL ALTERNATIVES AND OZONE IN ROOT CANAL DISINFECTION

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ABSTRACT The aim of this study was to evaluation of antimicrobial efficacy of gaseous ozone, herbal alternatives and ozone against E.faecalis biofilm in root canal disinfection. Extracted mandibular premolar teeth (n=119) were infected with E.faecalis to form a biofilm. At the end of the 3rd and 6th weeks, all groups were treated with the disinfecting agents (morinda citrifolia, triphala, gaseous ozone, morinda citrifolia + gaseous ozone, triphala + gaseous ozone, sodium hypochlorite and saline) and were analyzed using colony forming unit (CFU) counting and confocal laser scanning microscopy (CLSM) analysis, Data was analyzed Student's t test, One-Way ANOVA and post hoc Tukey HSD test. All the disinfecting agents killed significantly more bacteria than the saline. Sodium hypochlorite showed maximum antibacterial activity against both the 3-week-old and 6-week-old biofilm. The use of gaseous ozone alone and combined with herbal agents be alternative as a root canal irrigant.

KEYWORDS : Confocal laser scanning microscopy; Morinda citrifolia; Ozone; Triphala

INTRODUCTION

Irrigation is an essential part of a successful root canal treatment; because it is the only way to reach untouched areas of the root canal system and dentinal tubules by mechanical instruments 1-3. Especially root canal irrigation in regenerative endodontics is considered a critical step because of the contraindication of mechanical instrumentation 4. Although the main root canal microorganisms should be eliminated, several can survive as biofilm deeper in the dentinal tubules 5. Enterococcus faecalis (E.faecalis) has frequently been isolated from persistent infections and treatment failure in root canals; because it can adhere to dentin, invade dentinal tubules, form a biofilm, and in this way, becomes more resistant to irrigation agents 6.

Sodium hypochlorite (NaOCl) is the most widely used irrigation solution due to its broad antimicrobial effect and tissue-dissolving effect. Besides its well-known advantages, the major disadvantages are damaging effects on dentin elasticity, its cytotoxicity, its negative impact on stem cells, and its high surface tension, which affects tubular penetration 7-9.

Because to overcome undesirable properties of chemical irrigation agents and to minimize the adverse impact on biological tissue, the importance given to herbal alternatives has increased in endodontic treatment. The rise in the use of herbs is due to their many advantages, such as low toxicity, potent antimicrobial, anti-inflammatory, and antioxidant properties, readily available, and cost-effectiveness 10-12. Morinda citrifolia and Triphala have been previously investigated as potential endodontic irrigants, and the studies have reported that the use of these herbal alternatives as irrigation agents has promising results against E.faecalis 10,13,14-16.

In recent years, research on root canal irrigation has focused on the synergism between antimicrobial agents for maximum efficiency in root canal treatment 17-19. For this reason, the combined use of irrigation agents with adjunctive disinfection methods, such as gaseous ozone, which can offer high penetration ability to dentinal tubules and is low cytotoxicity, has come to the fore 20. In a study evaluating the cytotoxicity of ozone, it was reported that the dental pulp cells showed a high proliferation rate due to the biocompatibility of ozone; therefore, it could be a possible adjuvant as an irrigation agent in regenerative endodontic procedures 21. However, in the literature review, it is seen that there are very few studies on this subject 22,23.

This study aims at a new approach for root canal infections by taking advantage of the synergy between herbal alternatives and gaseous ozone against E.faecalis biofilm. To date, any research has compared the antimicrobial efficacy of the herbal alternatives (Morinda Citrifolia and Triphala) and Gaseous Ozone on infected root canal and dentinal tubules using the cultural method and Confocal Laser Scanning Microscope (CLSM) analysis. The null hypothesis tested was that there are no significant differences among the antimicrobial effectiveness of irrigation agents, both alone and in combination.

MATERIAL AND METHOD

Part 1: Culture Method Analysis

Preparation of the Samples

This study was approved by Institutional Review Board at the Gazi University (#36290600/42). 119 extracted human single-rooted teeth with no previous endodontic treatment, internal resorption, or calcifications were selected. The coronal part was removed with diamond discs with water cooling to standardize the root length to 8 mm. The root canals were enlarged to apical size F3 (ProTaper; Dentsply Sirona Endodontics, Tulsa, OK). 3% NaOCl was used as an irrigation solution during the preparation. The cylindrical root specimens vertically fractured the midsagittal plane into two semi-cylindrical halves. The outer surfaces of 238 halves were covered with two layers of nail varnish. The samples were rinsed with 17%EDTA (DiaPrepTM Plus, DiaDent, Korea) for 2 minutes to remove the smear layer. All samples were the canal side up placed in tissue culture plates (TPP® tissue culture plate, Sigma) and were sterilized in an autoclave (Nüve, OT 4060; ISO 9001) for 15 minutes at 1210C.

Biofilm Formation and Application of Irrigation Agents

E.faecalis (ATCC 29212; American Type Culture Collection, Rockville, MD) was grown on Muller-Hinton Agar (Merck, Germany) plates at 370C in the air overnight. The bacteria were suspended in 5 ml Tryptone Soya Broth (Lab M, UK). The cell density was adjusted to 1.5 x108 bacterial cells/mL. The root samples were infected using 10 L of the bacterial solution. The samples were inoculation and incubated using 50 L Muller-Hinton medium. The inoculum was refreshed in the medium every two days, and the samples were incubated in an incubator at 370C for 3 and 6 weeks. After 3 and 6 weeks of biofilm growth, the samples were replaced with 3 mL of sterile saline to remove the medium. The samples were divided into seven experimental groups (n=17) for each biofilm formation:

Group 1: 6% Morinda citrifolia (Tahitian Noni International Inc, American Fork, UT)

Group 2: Triphala (60 mg/ml) (IMPCOPS Ltd, Chennai, India)

Group 3: Gaseous Ozone (OzonyTronX, Mymed, Germany)
Group 4: 6% Morinda citrifolia + Gaseous Ozone
Group 5: Triphala (60 mg/ml) + Gaseous Ozone
Group 6: 5.25% NaOCl (positive control) (Endosolve-Hp, IMICRYL, Turkey)
Group 7: Saline (negative control) (Osel, Turkey)

30 L of the irrigation solutions were placed on the root canal lumen for 10 minutes. In groups 3, 4, and 5, gaseous ozone was applied for 120 seconds in the endodontic program of the device. After using irrigation agents, the samples were placed in 30 L of the neutralizing broth (D/E Neutralizing Broth, Difco™, France) for 5 minutes. Then the samples were washed with 30 L of sterile saline to remove the neutralization solution.

Examination with Culture Method

The biofilm on the root canal wall surface was sampled with a sterile excavator and inoculated on Müller-Hinton agar plates. After 24 hours of incubation at 370C, the bacterial count was determined by calculating colony-forming units (CFUs)/mL for each sample, and the results were transformed into log10 CFU values.

Part 2: Confocal Laser Scanning Microscopic Analysis

Preparation of the samples

Extracted human mandibular premolars for orthodontic reasons with single canals were used for this part (n=14). The coronal part was removed at 1 mm below the cementoenamel junction with diamond discs, and the root canals were enlarged to apical size F3. During the preparation, 3% NaOCl was used as an irrigation solution. The apical part was removed with discs to standardize the sample length to 4 mm, and the cylindrical specimens were fractured into two semi-cylindrical halves. The outer surfaces of 28 halves were covered with two layers of nail varnish. 17% EDTA was applied for 2 minutes to remove the smear layer. Finally, all samples with the canal side up were placed in tissue culture plates and were sterilized in an autoclave for 15 minutes at 121^{0}C.

Biofilm formation and Application of Irrigation Agents

The samples were infected with E. faecalis, like the methodology used in the culture method. Unlike this method, the microplates were centrifuged at 5000 rotations per minute (rpm) for 5 minutes to facilitate the invasion of microorganisms into the dentin tubules. The samples were incubated at 37°C for 3 and 6 weeks to allow biofilm growth in the dentinal tubules. The medium was changed to a fresh medium every two days. After incubation for 3 and 6 weeks, the infected samples were washed with 3 ml of sterile saline. Irrigation agents were applied as described above. The samples were washed with sterile water for 1 minute and fractured vertically through the root canal into two halves to expose a fresh surface of dentinal tubules.

Examination with CLSM

As previously described, the fractured dentin surfaces were examined with CLSM and viability staining 30. According to the manufacturer's instructions, 30 L of the LIVE/DEAD BacLight Bacterial Viability stain (Molecular Probes, Eugene, OR) was added. The samples were incubated at room temperature for 10 minutes. The stained cells' fluorescence was viewed using confocal laser scanning microscopy (Zeiss Lsm 510 Meta, Zeiss GmbH, Jena, Germany). The mounted specimens were observed by using a 10X and 40X lens. Eight different images were obtained from each group by taking two images from different areas randomly selected from 4 samples in each group. The volume ratio of red fluorescence to green-and-red fluorescence in the 3-dimensional reconstructions indicated the dead portion for each disinfecting agent using the Imaris 6.4 (Bitplane Inc, St Paul, MN).

Statistical analysis

Data analysis was performed using SPSS for Windows, version 11.5 (SPSS Inc., Chicago, IL, United States). Data were shown as mean ± standard deviation (SD). While Student's t-test compared the mean differences between two independent groups, otherwise One-Way ANOVA was applied for comparisons among more than two independent groups. When the p-value from One-Way ANOVA is statistically significant, the post hoc Tukey HSD test was used to determine which group differs from others. Logarithmic transformation was used for the number of microorganisms in data analyses. A p-value less than 0.05 was considered statistically significant. However, the Bonferroni Correction was applied for control Type I error for all possible multiple comparisons.

Groups	3-week	6-week
Group 1 (Morinda citrifolia)	$0,33\pm0,059^{A,a}$	$0,30\pm0,030^{A,C,a}$
Group 2 (Triphala)	$0,47\pm0,203^{A,B,C,a}$	$0,34\pm0,089^{A,C,a}$
Group 3 (Gaseous Ozon)	$0,80\pm0,271^{B,C,a}$	$0,50\pm0,195^{A,a}$
Group 4 (M.citrifolia + Gaseous Ozon)	$0,49\pm0,071^{A,B,C,a}$	$0,16\pm0,063^{C,b}$
Group 5 (Triphala + Gaseous Ozon)	$0,53\pm0,241^{A,B,C,a}$	$0,24\pm0,096^{A,C,a}$
Group 6 (NaOCl)	$0,88\pm0,250^{C,a}$	$0,83\pm0,214^{B,a}$
Group 7 (Saline)	$0,25\pm0,031^{A,a}$	$0,07\pm0,021^{C,b}$

RESULTS

Culture Method

The number of microorganisms in the root canal was determined by counting the CFUs. The results were log10 transformed (Log CFU), and the means standard deviations were summarized (Tables 1). The whole group has significantly higher CFU in the 6-week week biofilm compared to the 3-week ($p<0.001$). All the groups showed a statistically significant reduction in both 3-week and 6-week E.faecalis biofilm compared to the negative control group, saline ($p<0.001$). However, between the groups in the 3-week-old biofilm, the maximum reduction was seen with 5.25% NaOCl, followed by Gaseous ozone, Morinda citrifolia + Gaseous ozone, Triphala + Gaseous Ozone, Morinda citrifolia, Triphala, and least by Saline. In the 6-week biofilm, the maximum reduction between the groups was seen with 5.25% NaOCl, followed by Morinda citrifolia + Gaseous ozone, Triphala + Gaseous Ozone, Gaseous Ozone, Morinda citrifolia, Triphala and least Salin ($p<0.001$).

Table 1. The number of microorganisms grown according to groups and follow-up times after logarithmic transformation (Mean ± Standard deviation)

When the follow-up times were kept constant, the difference between the groups shown with different capital letters in the same column was statistically significant ($p<0.001$), When the groups were kept constant, the difference between the follow-up times shown with different lowercase letters in the same line is statistically significant ($p<0.001$).

CLSM Examination

The killing ratio in 6-week-old biofilms was lower than in 3-week-old biofilms in each group; however, this difference was statistically significant only in the Morinda citrifolia and saline groups (Table 2). The proportion of dead E.faecalis cell volume in the infected dentin tubules to total cell volume in the 3-week biofilm was significantly higher in NaOCl and Gaseous Ozone than in the Morinda citrifolia and negative control group ($p<0.001$). Although there was no statistically significant difference between the other groups, their effectiveness can be listed as follows: 5.25% NaOCl, Gaseous ozone, Triphala + Gaseous Ozone, Morinda citrifolia + Gaseous ozone, Triphala, Morinda citrifolia and Saline. Between the groups in 6-week-old biofilm, the ratio of dead cell volume to total cell volume of the 5.25% NaOCl group was similar to the ozone gas group but statistically significantly higher than the other groups. In the 6-week biofilm, the maximum killing ratio between the groups was seen with 5.25% NaOCl, followed by Gaseous Ozone, Triphala, Morinda citrifolia, Triphala + Gaseous Ozone, Morinda citrifolia + Gaseous Ozone, and least Salin.

Table 2. Ratio of dead cell volume to total cell volume accordingto groups and follow-up times (Mean ± Standard deviation)

When the follow-up times were kept constant, the difference between the groups shown with different capital letters in the same column was statistically significant ($p<0.001$), When the groups were kept constant, the difference between the follow-up times shown with different lowercase letters in the same line is statistically significant ($p<0.001$).

DISCUSSION

Recently, there has been an increasing interest in using herbal alternatives in dental treatments. Due to their safety profile, herbal alternatives in endodontics can replace chemical irrigants in pediatric patients where open apexes limit the use of chemical irrigants. Many in vitro studies have concluded that Triphala and Morinda Citrifolia produce promising results when used as endodontic irrigation agents 24-26.

In the literature, it is seen that most of these studies were carried out on planktonic microorganisms. However, the antibacterial activity of herbal irrigation agents in these studies does not reflect the agents' effect on microorganisms in vivo biofilms. Because root canal biofilms are usually likely to be several weeks or months old when treatment is started 27,28. Therefore, the effect of biofilm age on the effectiveness of antibacterial agents is essential. Biofilms up to 3 weeks old are defined as young biofilms, 6 weeks old as mature biofilms, and ten weeks and over as aged biofilms 29.

In the first part of this study, we evaluate the antibacterial properties of irrigation agents on 3 and 6-week-old E.faecalis biofilm using the culture method. The most potent antibacterial effect was observed in the 5.25% NaOCl group on 3 and 6-week-old biofilm. All tested irrigation agents resulted in a statistically significant bacterial reduction in the main canal compared to the negative control group. While Morinda citrifolia and Triphala showed minimal antibacterial efficacy, but these herbal alternatives combined with gaseous ozone show significantly higher antimicrobial efficacy than isolated treatments. The null hypothesis of the present study was rejected because a significant difference was found between the isolated and combined use of the tested irrigation agents.

In the 6-week biofilm, the Morinda citrifolia and gaseous ozone group showed the highest antibacterial activity, followed by the %5.25 NaOCl. After applying irrigation agents, it was observed that there were statistically significantly more microorganisms in the 6-week-old biofilm than in the 3-week-old biofilm in all the groups. This is related to the biofilm's degree of maturation; as the biofilm's maturation increases, the effectiveness of irrigation agents decreases 30.

Groups	3-week	6-week
Group 1 (Morinda citrifolia)	$6,92\pm0,022^{A,a}$	$6,96\pm0,007^{A,b}$
Group 2 (Triphala)	$6,98\pm0,006^{A,a}$	$7,86\pm0,005^{B,b}$
Group 3 (Gaseous Ozon)	$0,26\pm0,125^{B,a}$	$0,98\pm0,006^{C,b}$
Group 4 (M.citrifolia + Gaseous Ozon)	$0,56\pm0,125^{C,a}$	$0,67\pm0,009^{D,b}$
Group 5 (Triphala + Gaseous Ozon)	$0,75\pm0,112^{D,a}$	$0,97\pm0,008^{E,b}$
Group 6 (NaOCl)	$0,10\pm0,000^{E,a}$	$0,37\pm0,009^{F,b}$
Group 7 (Saline)	$8,08\pm0,052^{F,a}$	$8,96\pm0,006^{G,b}$

It has been reported that using ozone alone is not an alternative to other irrigation agents, and its use will be more beneficial after applying other irrigation agents 22,23. The antibacterial effect of ozone gas alone on the 6-week biofilm was significantly lower than in the groups in which ozone was used in combination with Triphala and Morinda citrifolia. Additionally, in this study, it was found that gaseous ozone showed significantly higher antibacterial activity in both biofilms than in the negative control group. The antibacterial activity of gaseous ozone is dose and time-dependent, and therefore, it is thought that there are differences between studies.

Evaluation of the antimicrobial activity of irrigation agents by culture method is the gold standard. However, this method can only be used to detect microorganisms that can initiate cell division at a rate fast enough to form colonies in a culture medium 31,32. Confocal laser scanning microscopy (CLSM) allows the determination and quantification of the viability of microorganisms inside the dentinal tubules. The viable-but-non-culturable state of old biofilm bacteria contributes to the higher resistance of bacteria in mature biofilm. Confocal microscope examination of the biofilm could provide better insight into the irrigants 27,31. Therefore, the antibacterial efficacy of irrigation agents in this study was evaluated using two different quantification methods to assess better the bacterial reduction in the dentinal tubules and the main canal space.

In the second part, we evaluated the antibacterial properties of irrigation agents on 3 and 6-weeks-old E.faecalis biofilm using CLSM analysis. The noninvasive dentine model technique was used in this study for the dense and deep presence of bacteria in dentin tubules for CLSM scanning 30 . The significant bacterial reduction achieved by NaOCl in the main canal space was also complemented with better tubular disinfection, as presented in the CLSM analysis. NaOCl is the most effective agent, consistent with previous studies results 1,28,29,33. The antibacterial effect of gaseous ozone on biofilm at 3 and 6-week biofilms showed the highest antibacterial activity, followed by the 5.25% NaOCl. The effectiveness of gaseous ozone appears to be lower than that of NaOCl, but this difference was not statistically significant. In the literature review, few studies evaluate gaseous ozone's antibacterial activity against the dentinal tubules' biofilm 34,35. The ozone device used, ozone type and concentration, incubation time, and sampling method are different from our study, which does not make it possible to compare these studies.

In the present study, infected samples were embedded in irrigation agents, and static irrigation was applied. Fresh irrigation solution was not added. The reduction in antibacterial activity can be explained by the weakening of the effect of the irrigation agents during the 10 minutes. (Limitation) Therefore, it is predicted that the efficacy of the agents will be higher with the application of continuous irrigation in clinical practice.

Biofilm formation was not confirmed by culture method and CLSM, which can be considered a limitation of this study. However, physiological saline does not have any antibacterial and tissue-dissolving properties; therefore, the contamination of dentin was confirmed with samples taken from this group as in the study by Bhardwaj et al. 25.

It was determined that when herbal alternatives were used with ozone gas, they showed significant antibacterial activity compared to the negative control group. The resulting synergistic effect may be an excellent alternative to sodium hypochlorite. Further studies will provide more information on the activity of the combination of irrigation agents in different canal anatomies and on the complex biofilm models until a better performance is achieved, making its application to be recommended in clinical practice.

CONCLUSIONS

The main findings of the present study indicate that this protocol involving the synergism between gaseous ozone and herbal alternatives that do not have a cytotoxic effect on stem cells may have the potential to be used in clinical practice, particularly for regenerative endodontics.

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