Biotechnology & Biotechnological Equipment

ISSN: 1310-2818 (Print) 1314-3530 (Online) Journal homepage: https://www.tandfonline.com/loi/tbeq20

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To cite this article: Esra Yesiloz Gokcen, Firdevs Tulga Oz, Berrin Ozcelik, Ayse Isıl Orhan & Betul Memis Ozgul (2016) Assessment of antibacterial activity of different treatment modalities in deciduous teeth: an *in vitro* study, Biotechnology & Biotechnological Equipment, 30:6, 1192-1198, DOI: 10.1080/13102818.2016.1223556

To link to this article: https://doi.org/10.1080/13102818.2016.1223556

Published online: 26 Oct 2016.

BIOTECHNOLOGY & BIOTECHNOLOGICAL EQUIPMENT, 2016
VOL. 30, NO. 6, 1192–1198
http://dx.doi.org/10.1080/13102818.2016.1223556

Taylor & Francis
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ARTICLE; MEDICAL BIOTECHNOLOGY

OPEN ACCESS

Assessment of antibacterial activity of different treatment modalities in deciduous teeth: an *in vitro* study

Esra Yesiloz Gokcen[a], Firdevs Tulga Oz[a], Berrin Ozcelik[b], Ayse Isıl Orhan[c] and Betul Memis Ozgul[d]

[a]Department of Pedodontics, Faculty of Dentistry, Ankara University, Ankara, Turkey; [b]Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Gazi University, Ankara, Turkey; [c]Division of Pediatric Dentistry, Ministry of Health, 75th Year Ankara Oral and Dental Health Centre, Ankara, Turkey; [d]Department of Pedodontics, Faculty of Dentistry, Baskent University, Ankara, Turkey

ABSTRACT

In recent years, different biotechnological materials and modalities with antibacterial activity are being developed for oral cavity disinfection. However, the antimicrobial effects of all these materials have not been studied and understood in detail. Thus, the aim of this study was to compare the antibacterial activity of ozone therapy with dentine-bonding agents (containing antibacterial monomer 12-meth-acryloyloxydodecylpyridinium bromide (MDPB) and 10-methacryloyloxydecyl dihydrogen phosphate (MDP) and $Ca(OH)_2$ for deciduous teeth *in vitro*. The antibacterial effectiveness of the studied materials was determined by using a tooth cavity model on cylindrical cavities created in 90 deciduous second mandibular molars. *Streptococcus mutans* suspension was inoculated in the cavities. The teeth were distributed into six study groups (five different modalities and a negative control group). Dentine samples, which were collected from the cavities before and after the treatment sessions, were microbiologically evaluated and the materials' antibacterial activities were compared. There were statistically significant differences in the *S. mutans* counts before and after treatment ($P < 0.05$). In terms of antibacterial efficiency, 60-second O_3 treatment was found to be the most successful method, followed by 30-second O_3, Clearfil Protect Bond (containing MDPB), Clearfil SE Bond (containing MDP) and $Ca(OH)_2$ treatment. The results from this study suggested that longer exposure to ozone might have more beneficial effects in terms of antibacterial activity for reducing the levels of *S. mutans*.

ARTICLE HISTORY
Received 1 November 2015
Accepted 9 August 2016

KEYWORDS
Biotechnological materials; ozone therapy; deciduous teeth; disinfection; antibacterial activity; microbiology

Introduction

The removal of the soft and decayed dentine before performing any restoration of teeth is a routine protocol for caries treatment [1]. However, the condition of the remaining dentinal tissue after the cavity preparation is generally evaluated by visual traits such as the colour and hardness of the dentine tissue. Previous studies indicate that this kind of approach is quite subjective and is considered to be insufficient to reflect the bacterial status [2]. It has been suggested that decay dyes might be used for the excision of the infected tissues; however, this would not entirely eliminate the micro-organisms within the cavity and micro-organisms would still remain in 15%–40% of the teeth [3].

It has also been reported that even after the removal of the caries dentine, micro-organisms may still exist within a distance of 0.1–2.4 mm from the base of the cavity towards the dental pulp [3]. Therefore, the use of antibacterial adhesive systems, restorative materials, acids and cavity disinfectants is suggested for the prevention of the post-operative sensitivity, secondary caries and pulpal inflammation caused by micro-organisms [4]. For this purpose, calcium hydroxide ($Ca(OH)_2$) can be used especially for direct or indirect pulp-capping procedures [5–10]. Cements containing calcium hydroxide have been shown to provide antibacterial activity [2,7] due to its high pH, which has a destructive effect on bacterial cell membranes and protein structure [6].

In recent years, different biotechnological materials with antibacterial activity are being developed, such as the antimicrobial monomer MDPB (12-meth-acryloyloxydodecylpyridinium bromide) [11]. MDPB has strong bactericidal activity against oral micro-organisms [12–15] and has been reported to show antibacterial activity against *Streptococcus mutans* before and after treatment [4,15]. Moreover, self-adhesive composite cements, such as those containing MDP (10-methacryloyloxydecyl dihydrogen phosphate), are increasingly applied for cementing inlays/onlays, intraradicular posts, crowns and laminate veneers due to the simpler and faster handling procedures, similar to those of self-etching adhesives [16].

CONTACT Firdevs Tulga Oz oz@dentistry.ankara.edu.tr

Apart from biotechnological materials, the application of ozone has also been proposed for the treatment of caries due to its antibacterial activity. Previous studies have shown that ozonized water and ozone gas reduce the total cultivable microbiota significantly *in vitro* [5,17–19]. Baysan and Lynch [18] found a significant reduction in *S. mutans* and *Streptococcus sobrinus* in the ozone-treated side of the root caries lesions compared with the control side.

To our knowledge, the antimicrobial effects of all these materials have not been studied in deciduous teeth *in vitro*. Thus, the aim of this study was to compare the antibacterial activity of ozone therapy with dentine-bonding agents (containing antibacterial monomer MDPB and MDP) and $Ca(OH)_2$ *in vitro*.

Materials and methods

Experimental design

A power analysis (Power and Precision software, Biostat, Englewood, NJ, USA) was conducted in order to determine an appropriate sample size based on previous studies. It indicated that detection of differences could be obtained with at least 11 teeth at a power of 0.8 ($\alpha = 0.05$). Thus, this study was conducted using 15 teeth per group (90 teeth in total).

The study protocol was carried out according to the principles of the Helsinki Declaration, including all amendments and revisions. Collected data were only accessible to the researchers. Patients or their legal representatives gave their informed consent prior to any treatment of the teeth and the study was reviewed and approved by the institutional ethics board of the faculty (no: 118/2).

Preparation of the cavities with S. mutans

Ninety extracted mandibular second deciduous molar teeth were used in this study. The teeth were cleaned using a polishing brush and were stored in sterile physiological serum solution. The enamel layer on the occlusal part of the teeth was removed by means of a diamond bur (6879/016 Komet Medical, Brasseler, Lemgo, Germany) to the deepest point of the pit on the occlusal surface which ended up with flat dentine surfaces. As previously described, the roots of the teeth were also cut to provide a better penetration of the *S. mutans* culture suspension [19,20]. Subsequently, in each tooth, two different cavities were opened with a depth of 1.5 mm and a width of 2 mm; extensive care was taken in order not to expose the pulp cavity (Figure 1). Then, all samples were sterilized in an autoclave (10 min, 121 °C) prior to bacterial inclusion. All the cavities were dried via sterile cotton pellets.

A 10-μL culture suspension of *S. mutans* (676 RSKK; culture collection of Refik Saydam Central Hygiene Institute, Ankara, Turkey) was prepared at a density of 10^6 CFU/mL (colony-forming units). Colombia Agar Base (Merck, Darmstadt, Germany) containing 23.0 g special peptone, 1.0 g starch, 5.0 g sodium chloride, 10 g agar and 5% defibrinated sheep blood and Tryptic Soy Broth (TSB; Merck, Darmstadt, Germany) containing 17 g casein peptone, 3 g peptone from soymeal, 2.5 g glucose, 5 g sodium chloride and 2.5 g dipotassium hydrogen phosphate were used at 50 °C in order to stimulate reproduction and passage of the bacterial suspensions.

Then this suspension was placed into each cavity of all samples. The teeth were incubated under the same conditions for 3 min in order to ensure the penetration of the micro-organisms into the dentine. Each sample was then stored in a separate microplate well containing 5 mL of TSB, 50 μL of *S. mutans* suspension and 1% sucrose. These microplates were transferred for incubation at 36 °C for 48 hours in order to obtain infected cavities.

In each tooth, one of the cavities was assigned as an experimental cavity and the other one was left as a control cavity (Figure 1). In the experimental cavity, various

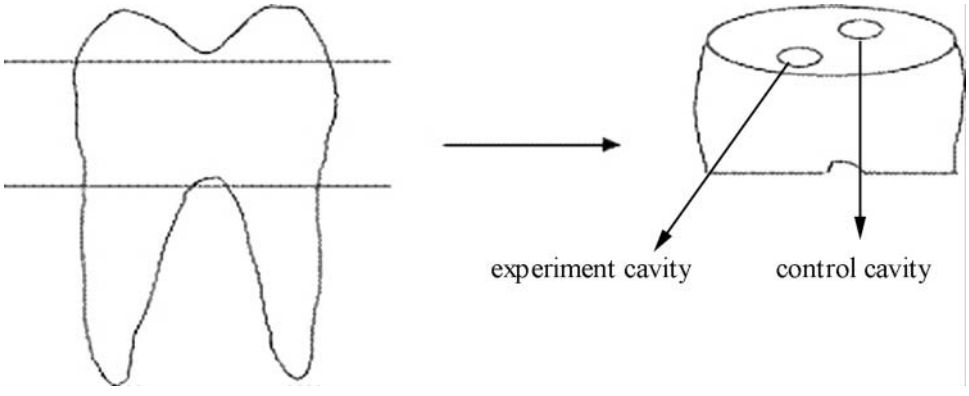

Figure 1. Preparation of the control and experimental cavities.

treatment modalities were performed in order to test the antibacterial activity, while the control cavity served as a proof of dentinal cavity *S. mutans* inclusions.

To determine the bacterial inclusions in the teeth, dentine samples were collected from the control cavities by means of sterile steel round burs (Komet no. 1.204.18, Lemgo, Germany). These samples were diluted in broth medium (TSB, 10 mL) and, subsequently, each dentine sample was homogenized in an ultrasonic bath (Ultrasonic, LC30, Singen, Germany) for 5 s under laboratory conditions and vortexed (Firlabo.S.A., 230/50, Lyon, France) for a duration of 15 s. After homogenization, 10-fold (10^{-1}–10^{-2}–10^{-3}) serial dilutions of each sample were prepared in sterile tubes and 0.1 mL of sample was taken from each group to make inoculations in five Mannitol salt agar (MSA) plates [19,20]. The microbiological evaluations of the control cavities were macroscopically done following incubation (37 °C) for two days under *microaerophilic* conditions. The colony-forming units of *S. mutans* were scored with the inoculation of each cavity and any samples with less than 10^5 *S. mutans* colonies were not included in the study [21,22].

Application of antibacterial modalities

The teeth included in this study were randomly divided into six groups, and the following modalities were applied in the experimental cavities of each group.

Group 1: $Ca(OH)_2$ (Dycal, Dentsply/Caulk, Denstsply International Inc., Milford, DE, USA) was applied to the experimental cavity by sterile ball-ended hand instrument.

Group 2: Ozone therapy was applied to the experimental cavity for 30 s, according to the manufacturer's instructions. The system used in this study was an active oxygen generator with a set of plasma probes which offers a wide array of therapeutic uses for treatment and prevention (OzonyTronX, Mymed, Münich, Germany).

Group 3: Clearfil Protect Bond (CPB; Kuraray, Europe; Düsseldorf, Germany), an antibacterial bonding agent containing MDPB and an acidic adhesion-promoting monomer MDP, was directly applied to the experimental cavities, according to the manufacturer's instructions.

Group 4: Ozone therapy was applied to the experimental cavities for 60 s with the same equipment, according to the manufacturer's instructions.

Group 5: Clearfil SE Bond (CSEB; Kuraray, Europe; Düsseldorf, Germany), an acidic adhesion-promoting monomer MDP, was directly applied to the experimental cavities, according to the manufacturer's instructions.

Group 6: No material was applied to the experimental cavities (control group).

Following the application of treatment modalities for each group (Groups 1–6), a sterile blue sponge (VDW, Munich, Germany) with no antibacterial traits was placed in the cavities. These cavities were subsequently sealed using different coloured compomers (Twinky Star, Voco, Cuxhaven, Germany) and were polymerized with a light cure (Ultralume 5; Ultradent, S. South Jordan, UT, USA) for 20 s. After these steps, the tooth samples were placed in microplates (24 well-TPP®, tissue culture test plate 92096, DNA, RNAases free, tissue culture-treated Europe, TPP Techno Plastic Products AG, Trasadingen, Switzerland) at 36 °C for 72 hours (Figure 2).

After 72 hours of incubation, the compomer fillings were removed by means of a sterile diamond bur without contacting the dentine cavity walls. Then, the dentine samples were collected for a second time by sterile steel round burs. A total CFU count was obtained through a culture (MSA plates) of dentine samples collected from each group. Counts below 20 CFU were below the limits of detection and were recorded as 0 (undetectable). All microbiological processes were carried out by a microbiologist experienced in oral microbiology (B.O.).

Figure 2. Sealing of cavities in different coloured compomer materials (a, b); tooth samples placed inside microplates for further evaluation (c).

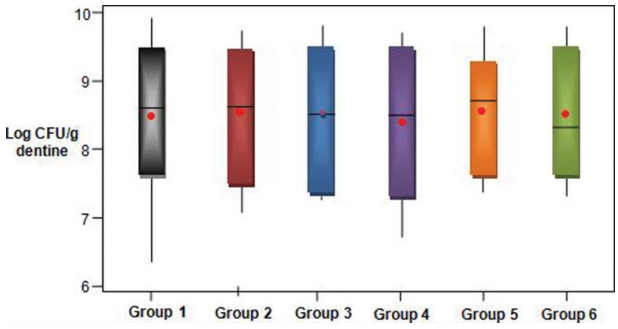

Figure 3. Microbial counts (log CFU/g dentine) in the control cavities of the six groups.

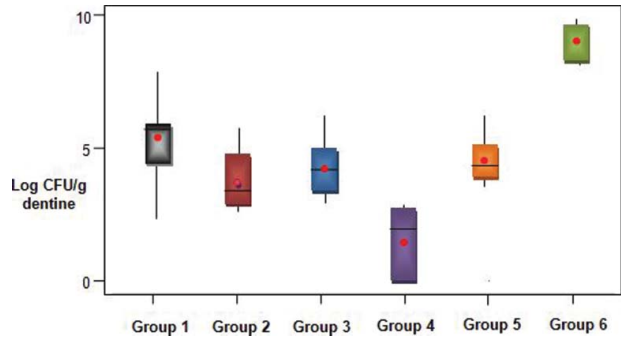

Figure 4. Microbial counts (log CFU/g dentine) in the experimental cavities.
Note: Group 1: $Ca(OH)_2$; Group 2: 30 s O_3; Group 3: CPB; Group 4: 60 s O_3; Group 5: CSEB; Group 6: control.

Statistical analysis

The results were analysed using the Statistical Package for Social Sciences (SPSS 17.0 for Windows, SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) and Kruskal–Wallis test were used for statistical analysis. *P*-values of less than 0.05 were considered to be statistically significant in all tests.

Results and discussion

The methods and materials of cavity disinfection have been discussed for several years in pediatric dentistry, where the cleaning and filling of decay cavity are particularly difficult. This *in vitro* study analysed the antibacterial effects of ozone therapy, which is recently used, antibacterial bondings (CPB, CSEB) and $Ca(OH)_2$ on deciduous teeth.

The results from the microbiological analysis of the control cavities are presented in Figure 3. According to the ANOVA and Kruskal–Wallis analysis, no statistically significant difference was found between the number of micro-organisms present in the control cavities of the six groups of teeth that were exposed to *S. mutans* culture suspensions at identical quantities under laboratory conditions ($P > 0.05$) (Table 1).

In the test cavities (Figure 4), there was an increase in the number of micro-organisms in Group 6 (control), whereas a decrease was observed in the viable microbial counts in the other groups ($Ca(OH)_2$, 30-second O_3, CPB, 60-second O_3 and CSEB). The ANOVA results indicated that the micro-organism counts in the teeth within each group were found to be comparable. ANOVA showed that the number of micro-organisms (CFU) in the collected dentine was significantly different ($P < 0.05$) between the groups. This difference was also confirmed by the Kruskal–Wallis test. It was found that the results in Group 1 ($Ca(OH)_2$), Group 2 (30-second O_3), Group 3 (CPB), Group 4 (60-second O_3) and Group 5 (CSEB) were statistically significantly different ($P < 0.05$) compared with the results in Group 6 (control). In terms of antibacterial efficiency, the 60-second O_3 treatment was observed to be the most successful method, followed by 30-second O_3, CPB, CSEB and $Ca(OH)_2$ treatment (Table 1). Our results that CPB had a higher bactericidal effect than CSEB support the findings of other studies [4,14,23] which also showed that incorporation of the antibacterial monomer MDPB has an additional bactericidal effect compared to other self-etching solutions. The antibacterial activity of MDPB has been verified before [24] and after [25] curing.

Clinical studies are considered the most appropriate method for evaluation and development of antibacterial systems. However, *in vivo* studies are costly, painstaking and time consuming [26,27]. It has been suggested that manipulation, ambient humidity and sample selection might affect the success of the therapy [23]. However, *in vitro* studies are rapid, easy and less costly tests. On the other hand, *in vitro* studies may not completely reflect the oral environment. The most trustworthy result

Table 1. Microbial counts (CFU/mL) in the control cavities of the six groups at the beginning (0 hour) and after 72 hours of incubation.

	Groups					
Time (hours)	$Ca(OH)_2$	30 s O_3	CPB	60 s O_3	CSEB	Control
0	$8.484 \pm 1.062^{a,A}$	$8.544 \pm 0.936^{a,A}$	$8.541 \pm 0.966^{a,A}$	$8.404 \pm 1.020^{a,A}$	$8.555 \pm 0.818^{a,A}$	$8.516 \pm 0.909^{a,A}$
72	$5.419 \pm 1.442^{b,A}$	$3.715 \pm 0.951^{b,B}$	$4.211 \pm 0.944^{b,B}$	$1.463 \pm 1.275^{b,B}$	$4.541 \pm 0.716^{b,B}$	$9.045 \pm 0.595^{a,C}$

Note: Different lower case letters within each column indicate values that are significantly different at $P \leq 0.05$; different upper case letters within each row indicate values that are significantly different at $P < 0.05$.

will be obtained when the two methods are jointly applied and evaluated [28].

In an *in vitro* study, an infected cavity model was used to compare the antibacterial effects of ozone therapy, CPB, CSEB and $Ca(OH)_2$ [29]. While planning our study, we conducted a pilot study in which we attempted to open four cavities in each tooth, a method used by Polydorou et al. [20]. However, in our experiments, which analysed the utilization of experimental materials in pediatric dentistry, only two cavities could be opened on the occlusal surface, since deciduous teeth were used instead of third molar teeth.

The high number of micro-organisms in the entire control cavities over a level of 10^5 CFU/mL was presumably a result of the glucose in Triptic Soy and to the immersion of each tooth in *S. mutans* culture suspension for a period of 3 min before bacterial colonization. This method provided all cavities with the bacterial colonization at desired criteria. Furthermore, the roots of the teeth were cut with the aim to ensure better bacterial colonization [20].

In both *in vivo* and *in vitro* studies, dentine samples are taken from the cavities by means of carbide round burs assembled to a slowly rotating micromotor. At this stage, carbide burs and slowly rotating micromotors, which were kept at approximately 25 °C, were used to eliminate the formation of heat [20,27]. The tissue samples are typically collected by excavators or burs in the tests conducted for the microbiological inspection of the dentine tissue. A review of the relevant studies showed that the samples are mostly collected by burs [10,30–40]. On the other hand, excavators are mostly used in studies dedicated to microbiological analysis of atraumatic restorative treatment [41–43] and the removal of decay through chemo-mechanic methods [44,45]. In our study, the dentine samples for microbiological analysis were collected through sterile burs. It was not possible to calculate the weight of the collected dentine by weighing the bur before and after taking the samples because the bur outweighs the sample of dentine. Therefore, 'samples at a quantity to fill the groves of the bur' have been accepted as standard. The repeatability of such a sampling procedure has also been shown in a number of studies [30,34,39].

Compomer, which is frequently used by pediatric dentists, was the material of choice in our study and different colours were used so as to distinguish between the groups. Finally, sterile sponge was placed on the experimental materials applied to all test cavities to prevent any mechanical damage to the base of the cavity while removing the compomer filling material during the second session and to ensure the taking of microbiological samples from the same layer [20]. At this stage, other materials could also be used, e.g. wax, gutta percha or cotton pellets [9,10].

A similar study on the antibacterial activity of different treatment modalities was conducted by Polydorou et al.,[20] using the 'tooth cavity model.' The authors compared the effects of ozone treatment (40 and 80 seconds) and antimicrobial bonding agents (CPB and CSEB) on *S. mutans*. They demonstrated that the efficacies of the two bonding systems and the 80-second ozone treatment (Heal Ozon) were significantly higher than that of the 40-second ozone treatment. Although the observation of Polydorou et al. [20] that ozone treatment has antibacterial effect was confirmed in our study under *in vitro* conditions, our results suggested that 30-second ozone therapy could exert a higher antibacterial effect than the bonding agents. This discrepancy may be attributed to differences in the concentration of ozone in the generators used in the two studies. Moreover, like several other studies that investigated the effectiveness of ozone therapy,[12,17,19] it can be suggested that this treatment modality has a strong bactericidal effect on micro-organisms within the dentinal tubules of Class I cavities. In this context, our results indicate that ozone treatment with an adequate duration could be considered to exert a satisfactory antibacterial effect in the treatment of deciduous teeth. Further research on the long-term effects of ozone on micro-organisms, and a more detailed comparison of ozone with dentine-bonding systems and $Ca(OH)_2$, is necessary.

Conclusions

The results from this study showed that longer exposure to ozone may have superior antibacterial effect in reducing the levels of *S. mutans* in deciduous teeth as compared to MDPB-and MDP-based dentine-bonding systems and $Ca(OH)_2$. Further studies are needed to throw more light on the long-term effects of ozone treatment in comparison to different dentine-bonding systems and $Ca(OH)_2$ in deciduous teeth *in vivo*.

Disclosure statement

The authors declare no conflict of interest.

Funding

This study was supported by Ankara University BAP [grant number 08B3334003].

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