Microbiological Evaluation of Ozone on Dentinal Lesions in Young Permanent Molars using the Stepwise Excavation

Osama Safwat*/ Mona Elkateb **/ Karin Dowidar ***/ Hala Abdel Salam****/Omar El Meligy *****

Aim: *To assess the microbial effect of ozone gas on dentinal lesions in young permanent molars using the stepwise excavation.* ***Study design:*** *An experimental, controlled clinical trial was performed. The sample included 80 immature first permanent molars, showing deep occlusal carious cavities that were indicated for stepwise excavation. Following first step of dentin excavation, the sample was divided into test (ozone gas) and control (calcium hydroxide ($Ca(OH)_2$) base material) groups. One half of the cases in each group were evaluated for microbiological changes after 6 months, and the other half after 12 months.* ***Results:*** *Mutans streptococci (MS), Lactobacilli, and Candida counts were significantly reduced immediately after ozone application in the test group ($P \leq 0.05$). At the final assessment period, MS and Lactobacilli were significantly reduced in the test group ($P \leq 0.05$). Meanwhile, the Candida counts were significantly reduced only in the test group of the 6 and 12 month-cases ($P \leq 0.05$). Regarding the control group, the significant reduction in microbial count was observed with MS after 6 and 12 months ($P \leq 0.05$). No significant differences were observed between test and control groups at different evaluation periods ($P > 0.05$).* ***Conclusions:*** *Ozone gas had a significant antimicrobial effect in deep class I carious lesions.*

Keywords: *Ozone, Stepwise excavation, Young permanent molars.*

INTRODUCTION

Dental caries is an infectious bacterial disease characterized by a drop of the pH, demineralization of tooth structure with loss of minerals which diffuse out of the tooth. The result is cavitation, discomfort, pain which may lead to tooth loss [1]. These processes are induced by organic acids such as lactic and pyruvic acids generated by bacteria, predominantly MS [2]. For identification of persons at high risk for caries, MS and Lactobacilli have been used. Different methods and trials were directed against these bacteria, to reduce their number and, subsequently the severity of dental caries [3].

The maintenance of integrity and health of an immature permanent tooth and its supporting tissue in case of deep carious lesion is based on the careful diagnosis and the appropriate treatment procedure which varies from indirect pulp treatment, direct pulp capping, and pulpotomy for both carious and traumatic exposures, to allow the continued physiologic development and formation of the root apex [4].

Ozone has been introduced to medicine since 1885 [5]. As regard the biological effect of ozone, this gas is considered a very effective disinfecting agent [6]. Technical advances have made it possible for ozone to be applied to small areas of dental hard tissues using a device known as the HealOzone and considered as a pharmaceutical approach for treatment of caries [7], and tooth sensitivity [8].

The classical method of treatment of dental caries is the excavation of carious lesions to remove soft, discolored and infected dentin to approach a hard cavity bottom in the translucent or hypermineralized dentin [9]. In the absence of any signs and symptoms of

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irreversible pulpitis, in case of deep carious lesions, and in attempt to avoid pulp exposure, two different techniques had been advocated. The first is indirect pulp capping [10] and the second procedure known as stepwise excavation [11].

Based on the reported antibacterial effect and dentinal sealing ability of ozone, the use of ozone gas in treatment of deep carious lesions seems to be promising. The null hypothesis tested was that there would be no changes in cultivable microflora before and after using ozone gas on dentinal lesions in young permanent molars using the stepwise excavation.

The aim of this study was to assess the microbial effect of ozone gas on dentinal lesions in young permanent molars using the stepwise excavation.

MATERIALS AND METHOD

The Ethical Committee at the Faculty of Dentistry, Alexandria University approved the research protocol.

Study Design

An experimental, controlled clinical trial was performed.

Sample Size Calculation

Sample size was estimated using the following assumptions: alpha error = 5%, beta error = 20%, DIAGNOdent reading in control group = 30, DIAGNOdent reading in test group = 20, common standard deviation = 10[12]. The minimum required sample size was calculated (http://powerandsamplesize.com/Calculators/Compare-2-Means/2-Sample-Equality) to be 16 which was increased to 20 to make up for cases lost to follow up. The minimum required sample size per group was thus set at 20.

Study Sample

A sample of 80 teeth in 40 healthy children (23 males and 17 females) aged 7-9 years with a mean age of 8.25 ± 0.69 years were selected for the present study. Each child had contralateral immature first permanent molars, with deep class I carious lesions. Fifty-four mandibular first permanent molars and 26 maxillary first permanent molars were included in the study. Children were recruited from either those attending the clinic of the Pediatric Dentistry and Dental Public Health Department, Faculty of Dentistry, Alexandria University, or children from different junior schools in Alexandria.

Inclusion Criteria

1. Bilateral vital asymptomatic carious first permanent molars with no pulp involvement, as evident by clinical examination (no spontaneous throbbing pain, no pain on percussion, no gingival redness or swelling, and no tooth mobility).
2. Cavitated class I carious lesions with opaque or discolored enamel exposing the dentin beneath according to the clinical severity index (CSI) score 4 of Ekstrand *et al* [13].
3. DIAGNOdent reading equal or more than 31 (score 4) indicating deep dentinal caries [14].
4. Immature teeth with open apex, having no periapical pathosis as evident by periapical radiographs.
5. No proximal caries as evident by bitewing radiographs.
6. Patients were free from any systemic diseases that might affect the treatment prognosis.

Table 1 shows the used equipments, materials and their manufacturers.

Table 1. Equipments, materials and their manufacturers.

Equipments and materials	Manufacturers
HealOzone device	HealOzone KaVo Co., GmbH, D-88400 biberach/ Riss-Germany
DIAGNOdent	DIAGNOdent KaVo Co, Biberach/Riss, GmbH, D-88400 Germany
Calcium hydroxide paste -Dycal	Dycal: Dentsply Co. Rua Alice Herve, 86-25665-010-Petropolis-RJ.Brasile
OpalDam	OpalDam: Ultradent products USA.505 West 10200 South South Jordan, UT 84095 1.888.230.1420
One-step self-etching adhesive	Xeno III:Dentsply Co. De-Trey-Str.1 78467 Konstanz, Germany
Light activated composite	TPH Spectrum: Dentsply Co. Rua Alice Herve,86-25665-010-Petropolis-RJ.Brasile
Light activated glass ionomer cement lining	Vivaglass Liner. Ivoclar Vivadent AG FL-9494 Schaan/ Liechtenstein. Germany
Mitis Salivarius Agar, Rogosa Agar, Sabouraud 2% Dextrose Agar	Oxiod, Basingstroke, hants, UK
Sterile no. 3 round carbide burs	SS White. USA
A 2ml sterile cryotube	2ml cryotube, Nunc, Renfrewshire, Scotland
Auto-vortex vibrator	Autovortex Mixer SA2, Stuart Scientific, UK
Agar plates	Hi-Tec Industrial Estate, Tambol Baanpo, Amphur Bangpa-in, Ayutthaya Province 13160 Thiland
Anaerobic jar	Mart Microbiology B.V. Kelvinlaan 3, 9207 JB Drachten the Netherlands
Gas pack	Oxiod, Basingstroke, hants, UK
Transport media: Wilkins-Chalgren Anaerobic Broth	BDH, Roa Poole, Dorset, UK

For each patient, one tooth was treated with ozone gas and the contralateral tooth was treated using $Ca(OH)_2$ base material and served as control.

Prior to treatment, a written informed consent was obtained from the parents.

An operator performed the clinical procedures and a microbiologist who was blinded to the method of treatment performed the microbiological assessment of dentin samples. The microbiologist was a faculty staff member from the Microbiology Department, Faculty of Medicine, Alexandria University. Regarding caries diagnosis and microbiological assessment of dentin samples, intra-examiner agreement was determined using the Kappa statistic and was considered excellent (K = 0.92).

Clinical Procedure

After application of topical anesthesia, local anesthesia was administrated; infiltration for the upper molars and nerve block for the lower molars. Complete isolation was performed using rubber dam and saliva ejector. All selected teeth were cleaned using a rotating bristle brush with copious of water. Stepwise excavation was performed according to Bjorndal *et al* [11]: Caries from the enamel walls and dentino-enamel junction (DEJ) was removed using a sterile diamond fissure bur no. 3 in a high-speed handpiece. The central cariogenic biomass and only the superficial layer of the necrotic and infected dentin were removed, using a sharp excavator.

According to the method of treatment used, the selected teeth were randomly divided into 2 groups.

Test group: Forty carious lesions were exposed to ozone gas generated for 40 seconds. Control group: Forty carious lesions on the contralateral side were treated using $Ca(OH)_2$ base material.

One half of the cases in each group were evaluated for microbiological changes after 6 months, and the other half after 12 months.

Base line sample collection for microbiological assessment

The first dentin sample was collected from the inner most layer of dentin left after the first step of caries excavation, of both the test and control subgroups, using a sterile no. 3 slow rotating round carbide bur. A sterile bur was used for each tooth till the bur blades were filled with dentin particles (bur full) [15]. When the dentin particles didn't adhere properly to the blades of the bur, few drops of sterile saline were put onto the dentin to ensure the adherence of the dentin particles onto the blades of the bur [16]. The bur was removed from the handpiece by sterile tweezers, and placed in a sterile vial containing one ml of a reduced transport fluid. The vial was shaken to dislodge the adherent dentin, then the bur was removed with sterile tweezers [15]. Photographs were taken for the sampling area in order to identify the same area in the next samples [11]. Each photograph was kept in the patient record. The dentin samples were immediately transported for microbiological study.

For the test group, ozone gas was applied for 40 seconds according to the manufacturer's instructions [17].

Second dentin sampling for microbiological assessment in the test group

The second dentin sample was collected from the same place of the first samples immediately after ozone application.

In the test group, a temporary seal of a thin layer of OpalDam, light activated resin material, was applied to isolate the dentin surface for further examinations. It was cured for 20 seconds using a high intensity visible light source device. In the control group, cavities were lined with $Ca(OH)_2$ base materials. The teeth were sealed temporarily using one-step self-etching adhesive and light activated composite in all subgroups according to the manufacturer's instructions.

The second session at 6 and 12 months

For both test and control groups, following local anesthesia administration, complete isolation and tooth polishing were performed. Composite restoration was completely removed and the cavity was re-opened using no. 3 sterile diamond fissure bur and sharp excavators. In the test group, the OpalDam was removed to expose the carious dentin, while in the control group, the $Ca(OH)_2$ was removed.

Last dentin sampling for microbiological assessment

Dentin samples were collected following the same previous procedures; third sample for the test group, and the second one for the control group.

Before permanent restoration, final excavation was performed using sharp excavators or low speed rotating round carbide burs. The ozone gas was reapplied for 40 seconds and the cavity was sealed with light activated glass ionomer lining cement. Then, the cavity was permanently restored with one-step self-etching adhesive and the light activated composite.

Microbiological Study

Sample Processing [18]

a) Sample dilution: Dentin samples were vortexed for 15 seconds with sterile glass beads. The samples were then serially diluted in a sterile saline to obtain 1/10 dilution (10^{-1}). This procedure was repeated until 10^{-4} dilution was obtained.

b) Culture preparation and incubation: A 100µl, of each dilution, was plated into the freshly prepared following media: (1) Mitis Salivarius agar supplemented by sucrose, bacitracin and potassium tellurite at a final concentration of 20% (W/V), 0.2 units/ml and 0.1% respectively, (2) Rogosa agar and (3) Sabouraud Dextrose agar.

Each agar plate was divided into 4 sectors, and 100µl volumes of appropriate dilutions were spread on each sector using an automatic pipette. Inoculated Mitis Salivarius, Rogosa agar and Wilkins-Chalgren agar plates were incubated at 37˚C for 72 hours in an anaerobic jar. The anaerobic state was generated through the hydration of a single use anaerobic gas pack in the presence of activated self-catalyst. Blood agar plates, showing no growth, were further reincubated for up to 7 days. On the other hand, Sabouraud Dextrose agar plates were incubated aerobically at 37˚C and examined after 48 hours.

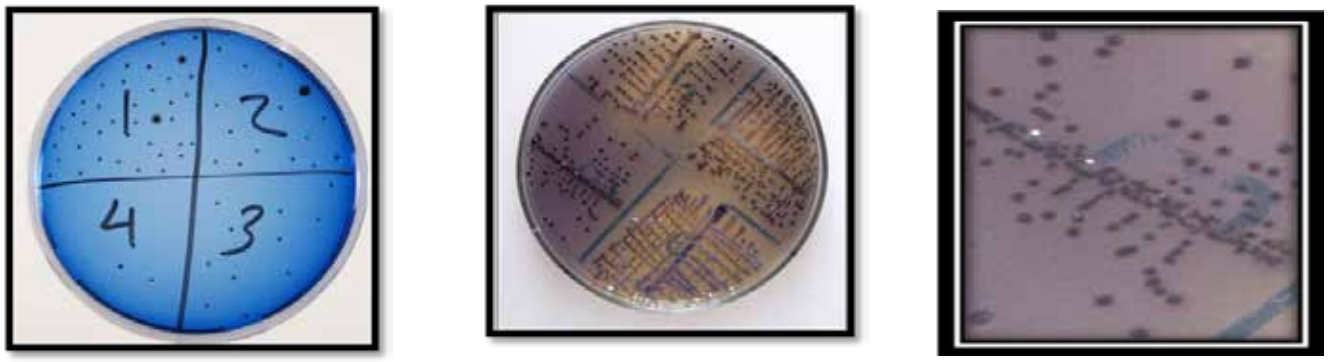
c) Isolation and enumerations: Following the predetermined incubation period, the colony forming units (CFU) were calculated on each type of media utilized. Colonies of different microorganisms were identified by their characteristic colonial and microscopic morphologies in light microscopy (power of the lens x 40). MS were identified on Mitis Salivarius agar plate (Figure 1). Opaque colonies on Rogosa agar, that appeared as Gram positive rod shaped organisms were considered to be Lactobacilli (Figure 2). Candida appeared as opaque dome shaped colonies on Sabouraud Dextrose agar, and microscopically as Gram-positive large ovoid cells (Figure 3).

d) Colony count: The number of the colonies was determined, and expressed as CFU. The CFU were estimated by multiplying the number of colonies by the reciprocal of dilution at which the colony count was estimated, and the reciprocal of the volume utilized for inoculation, expressed as a fraction of one ml:

$$\text{Count} = \text{No. of colonies x reciprocal of dilution } \frac{1\text{ml}}{.01\text{ml}} \text{ x}$$

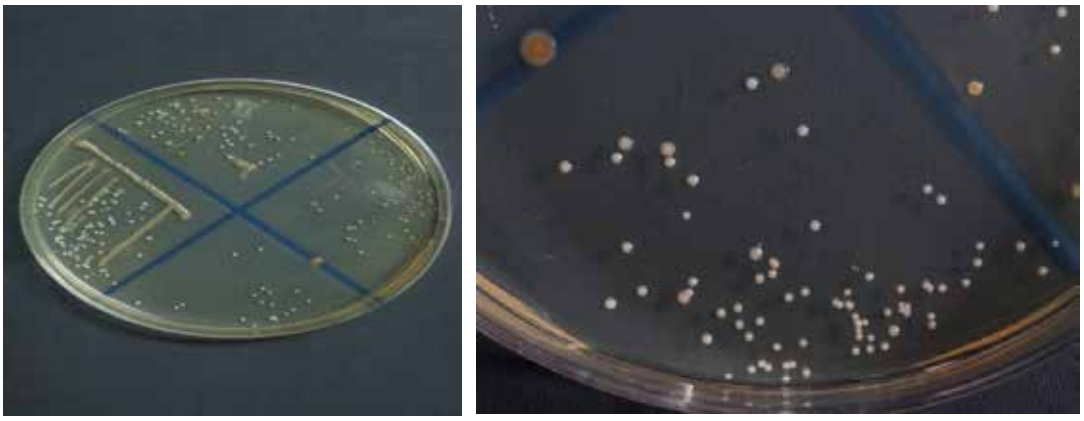
In case of no bacterial growth on any media, the value was used in subsequent analysis as zero. All bacterial counts were recorded in the patient's charts for statistical analysis.

Figure 1. A- Mitis Salivarius plate showing serial decimal dilutions of a sample positive for MS. B- Mitis Salivarius agar plate showing high colony count. C- MS colonies on Mitis Salivarius agar at a high magnification (x 400).



A B C

Figure 2. A- Rogosa agar plate inoculated with different dilutions of sample positive for Lactobacilli. B- Colonies of Lactobacilli at high magnification (x 400).



A B

Figure 3. A- Sabouraud Dextrose agar plate showing Candidal growth. B- Candida at a high magnification (x 400).

A B

Statistical Analysis

Comparison of nominal and ordinal variables between the two groups was done using chi square, and Mann Whitney U test respectively, while comparison of the quantitative variables was done using t test, or Mann Whitney (after Kolmogrov Smirnov test proved they were not normally distributed). Comparisons of subgroups and changes across time in the same subgroup was done using Wilcoxon signed ranks test (for ordinal variables or non-normally distributed quantitative variables), or paired t test (for normally distributed quantitative variables) [19].

RESULTS

All 80 (100%) teeth were available for the 6- and 12-month evaluations.

Table 2 shows comparison of mean absolute MS count and log values between dentin samples after first excavation with those of the second dentin samples, immediately after ozone application and at the final assessment after 6 and 12 months in the test group. The mean absolute MS count and log values decreased directly after ozone application and after 6 months. Statistically significant differences were found between the three dentin samples where P <0.001, <0.0001 and 0.002 respectively. In addition, the mean absolute MS count decreased directly after ozone application and after 12 months. There were statistically significant differences between the three dentin samples, where P <0.01, <0.001 and 0.031 respectively.

Table 3 shows comparison of mean absolute Lactobacilli count and log values between base line dentin samples, second ones immediately after ozone application and at the final assessment after 6 and 12 months in the test group. The mean absolute Lactobacilli count and log values significantly decreased directly after ozone application and in the final assessment after 6 months. The P values between the three dentin samples were 0.01, 0.005 and 0.03 respectively. The values also decreased in the different evaluation periods for 12 month-test cases. There were statistically significant differences between the base line and second dentin samples (P = 0.02) and the base line and final ones (P = 0.03), while there was no significant difference between the second and third ones after 12 months (P = 0.14).

Table 4 shows comparison of mean absolute Candida count and log values between base line dentin samples, second ones directly after ozone application and at the final assessment after 6 and 12 months in the test group. For 6 month-cases, the mean absolute

Table 2. Comparison between mean MS values at different evaluation periods in the test group.

Test group		First dentin sample (base line) Mean ± SD	Second dentin sample (after ozone) Mean ± SD	Third dentin sample (final sample) Mean ± SD	WSR test$_1$ P value	WSR test$_2$ P value	WSR test$_3$ P value
6m-cases	**Absolute MS count**	3.03 ± 6.90 x 10^4	4.68 ± 11.9 x 10^2	3.00 ± 13.42	3.11	3.92	3.07
	Log	3.58 ± 1.06	1.35 ± 1.27	0.09 ± 0.40	<0.001*	<0.0001*	0.002*
12m-cases	**Absolute MS count**	8.88 ± 14.8 x 10^4	3.11 ± 7.04 x 10^3	2.25 ± 7.34 x 10^2	2.65	3.11	2.91
	Log	3.67 ± 1.63	1.80 ± 1.67	0.33 ± 1.03	0.01*	<0.001*	0.031*

WSR test$_1$: between base line (after first step of caries excavation) and immediately after ozone application.

WSR test$_2$: between base line and the final samples.

WSR test$_3$: between immediately after ozone and the final samples.

***** Statistically significant at P ≤0.05.

WSR test: Wilcoxon signed ranks test.

Table 3. Comparison between mean Lactobacilli values at different evaluation periods in the test group.

Test group		First dentin sample (base line) Mean ± SD	Second dentin sample (after ozone) Mean ± SD	Third dentin sample (final sample) Mean ± SD	WSR test$_1$ P value	WSR test$_2$ P value	WSR test$_3$ P value
6m-cases	Absolute Lactobacilli **count**	134.50 ± 342.67	37.00 ± 111.93	1.00 ± 4.47	2.52	2.81	2.12
	Log	1.02 ± 1.12	0.54 ± 0.89	0.07 ± 0.30	0.01*	0.005*	0.03*
12m-cases	**Absolute Lactobacilli** count	344.00 ± 1145.23	12.50 ± 38.78	9.50 ± 29.64	2.37	2.20	1.49
	Log	0.80 ± 1.21	0.35 ± 0.68	0.20 ± 0.61	0.02*	0.03*	0.14 NS

WSR test$_1$: between base line (after first step of caries excavation) and immediately after ozone application.

WSR test$_2$: between base line and the final samples.

WSR test$_3$: between immediately after ozone and the final samples.

NS: Not statistically significant.

***** Statistically significant at P ≤0.05.

Candida count and log values decreased immediately after ozone application, while the values dropped to zero in the final assessment after 6 months. Statistically significant differences were found between the base line and second dentin samples (P = 0.030) and between the base line and final dentin samples (P = 0.036), whereas there was no significant difference between the second and final ones (P = 0.18). The values decreased immediately after ozone application and after 12 months. Statistically significant differences were found between the base line and second dentin samples (P = 0.02), and between the base line and final dentin samples (P = 0.013), while there was no significant difference between the second dentin samples and final ones after 12 months (P = 0.31).

Table 5 shows comparison between the percent reduction of the mean microorganism values after direct ozone application and at the different evaluation periods among the test group. For 6 months-cases, the mean percent reduction in the mean MS values between the base line and directly after ozone application (percent change$_1$) was 65.50 ± 30.91% for the test group. After 6 months, the percent reduction (percent change$_2$) was 97.16 ± 12.69% for the test group. The differences between the percent changes 1 and 2 in mean MS values were statistically significant (P = 0.002). Regarding Lactobacilli, the percent change$_1$ was 51.61 ± 43.41% for test group. After 6 months, the percent reduction was 91.13 ± 28.04% for the test group. The differences between the percent changes 1 and 2 in

Table 4. Comparison between mean Candida values at different evaluation periods in the test group.

Test group		First dentin sample (base line) Mean ± SD	Second dentin sample (after ozone) Mean ± SD	Third dentin sample (final sample) Mean ± SD	WSR test$_1$ P value	WSR test$_2$ P value	WSR test$_3$ P value
6m-cases	Absolute Candida count	38.00 ± 74.81	16.50 ± 67.06	0 ± 0	2.47 0.030*	2.25 0.036*	1.34 0.18 NS
	Log	0.67 ± 0.96	0.20 ± 0.63	0 ± 0			
12m-cases	Absolute Candida count	66.90 ± 222.76	10.65 ± 34.06	7.50 ± 33.54	2.37 0.02*	2.58 0.013*	0.89 0.31 NS
	Log	0.64 ± 0.94	0.29 ± 0.64	0.11 ± 0.49			

WSR test$_1$: between base line (after first step of caries excavation) and immediately after ozone application.

WSR test$_2$: between base line and the final samples.

WSR test$_3$: between immediately after ozone and the final samples.

NS: Not statistically significant.

* Statistically significant at P ≤0.05.

Table 5. Comparison between the percent reduction of the mean microorganism values after direct ozone application and at the different evaluation periods in the test group.

	MS	Lactobacilli	Candida
% change	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
		6m-cases	
% change$_1$	65.50 ± 30.91	51.61 ± 43.41	74.21 ± 44.41
% change$_2$	97.16 ± 12.69	91.13 ± 28.04	100.00 ± 0
WSR test P value	3.06 0.002*	3.42 0.001*	2.60 0.031*
12m-cases			
% change$_1$	56.52 ± 36.70	60.31 ± 28.31	61.95 ± 42.16
% change$_2$	91.73 ± 24.76	74.31 ± 47.14	100.00 ± 0
WSR test **P value**	3.06 0.002*	1.07 0.36 NS	4.85 0.016*

Percent change$_1$: between values before and immediately after ozone application.

Percent change$_2$: between values immediately following ozone application and third dentin sample (final assessment).

NS: Not statistically significant.

* Statistically significant at P ≤0.05.

WSR test: Wilcoxon signed ranks test.

mean Lactobacilli values were statistically significant (P = 0.001). Concerning Candida, the percent change was 74.21 ± 44.41% for the test group. After 6 months the percent reduction was 100. The differences between the percent changes 1 and 2 in mean Candida values were statistically significant (P = 0.031). For 12 month-cases, the mean percent reduction in the mean MS values between the base line and directly after ozone application (percent change$_1$) was 56.52 ± 36.70% for the test group. After 12 months, the percent reduction (percent change$_2$) was 91.73 ± 24.76% for the test group. The differences between the percent change 1 and 2 in mean MS values were statistically significant (P = 0.002). As regard the mean Lactobacilli values, the percent change$_1$ was 60.31 ± 28.31% for the test group. After 12 months, the percent reduction was 74.31 ± 47.14% for the test group. The differences between the percent changes 1 and 2 in mean Lactobacilli values were not statistically significant for the test group (P = 0.36). Regarding the mean Candida values, the percent changes$_1$ was 61.95 ± 42.16 % for the test group. The mean percent change was zero. After 12 months, the percent reduction was 100 for the test group. The difference between the percent changes 1 and 2 in mean Candida values was statistically significant in the test group (P = 0.016).

Comparison between base line and final dentin samples in the control group

Table 6 shows comparison of mean values of different microorganisms between base line and final dentin samples in the control group for the 6 and 12 month-cases. After 6 months, the mean absolute MS count decreased. A statistically significant difference was found between the base line and final dentin samples (P = 0.001) and the percent reduction was 96.40 ± 15.70%. In the final dentin samples, the mean absolute Lactobacilli count and log values dropped to zero after 6 months. Statistically significant difference was found between the base line and final dentin samples (P = 0.046), with a percent reduction of 100. The mean absolute Candida count and log values were zero at base line and after 6 months with no significant difference between the samples (P = 1.00) and no percent change can be computed. Regarding the control group 12 month-cases, the mean absolute MS count decreased with statistically significant difference between the base line and final dentin samples (P = 0.0001), and the percent reduction of 95.25 ± 13.88%. After 12 months, the mean absolute Lactobacilli and Candida counts decreased. There were no statistically significant differences between the base line and final dentin samples (P = 0.41 and 0.58 respectively) with 100% reduction for both.

Comparison between test and control groups regarding different types of microorganisms

Table 7 shows comparison of mean values of different microorganisms between test and control groups at the final dentin assessment. For 6 month-cases, when comparing between test and control groups at the final dentin assessment, no significant differences were found regarding the MS, Lactobacilli and Candida (P = 0.68, 0.32 and 1.00 respectively). Also, no significant differences were found between test and control groups after 12 months in the MS, Lactobacilli and Candida (P = 0.34, 0.18 and 0.32 respectively).

DISCUSSION

The operative tradition or the classical caries excavation is to remove the softened dentin in order to eliminate infected tissue. This approach assumes that both the biofilms and the microorganisms within the carious dentin drive the caries process. In fact, it is not possible to eliminate all the microorganisms because a few will remain, even if all soft dentin is removed [20]. Over the pulpal surface, contemporary trends recommended that carious dentin that is 'firm and leathery' should be left, where its removal might expose the pulp [21]. For this reason, other new techniques such as the use of ozone may help in elimination of infection in this firm dentin layer; prevent pulp exposure and simplify the treatment plan.

Table 6. Comparison of mean values of different microorganisms between base line and final dentin samples in the control group.

	MS		Lactobacilli		Candida	
Count of micro organisms	**First dentin sample**	**Final dentin sample**	**First dentin sample**	**Final dentin sample**	**First dentin sample**	**Final dentin sample**
6m-cases						
Absolute count	7.59 ± 19.9 x 10^2	1.50 ± 6.71	6.00 ± 16.03	0 ± 0	0 ± 0	0 ± 0
Log	2.14 ± 0.84	0.07 ± 0.33	0.24 ± 0.58	0 ± 0	0 ± 0	0 ± 0
WSR test	3.83		2.03		0	
P value	0.001*		0.046*		1.00 NS	
% change	-96.40 ± 15.70		-100.00 ± 0		-	
12m-cases						
Absolute count	6.86 ± 18.2 x 10^2	11.50 ± 41.20	13.00 ± 43.30	4.50 ± 16.05	8.00 ± 23.75	5.00 ± 22.36
Log	2.45 ± 1.34	0.20 ± 0.62	0.27 ± 0.67	0.16 ± 0.50	0.25 ± 0.61	0.10 ± 0.45
WSR test	3.73		0.84		0.55	
P value	0.0001*		0.41 NS		0.58 NS	
% change	-95.25 ± 13.88		-100.00 ± 0		-100.00 ± 0	

NS: Not statistically significant.

* Statistically significant at P ≤0.05.

WSR test: Wilcoxon signed ranks test.

Table 7. Comparison of mean values of different microorganisms between test and control groups at the final dentin assessment.

	MS		Lactobacilli		Candida	
Count of microorganisms	**Test**	**Control**	**Test**	**Control**	**Test**	**Control**
6m-cases						
Absolute count	3.00 ± 13.42	1.50 ± 6.71	1.00 ± 4.47	0 ± 0	0 ± 0	0 ± 0
Log	0.09 ± 0.40	0.07 ± 0.33	0.07 ± 0.30	0 ± 0	0 ± 0	0 ± 0
WSR test	0.17		1.00		0	
P value	0.68 NS		0.32 NS		1.00 NS	
12m-cases						
Absolute count	2.25 ± 7.34 x 10^2	11.50 ± 41.20	9.50 ± 29.64	4.50 ± 16.05	7.50 ± 33.54	5.00 ± 22.36
Log	0.33 ± 1.03	0.20 ± 0.62	0.20 ± 0.61	0.16 ± 0.50	11 ± 0.49	0.10 ± 0.45
WSR test	0.84		1.34		1.00	
P value	0.34 NS		0.18 NS		0.32 NS	

NS: Not statistically significant.

WSR test: Wilcoxon signed ranks test.

For all patients in this study, deep dentinal lesions were diagnosed initially by visual examination using score 4 CSI according to Ekstrand *et al* [13]. They found that, when the carious lesion appeared clinically as cavitations in opaque or discolored enamel with exposing dentin beneath, the histological picture was demineralization and heavily infected dentin, up to its inner third layer. Visual examination was used as it is non-invasive and clinically acceptable. It also preserves the surface structure of the tooth comparing to tactile examination, which may transfer microorganisms from one site to another, with possibility of further spread of the disease [22]. However, a visual diagnosis alone has low sensitivity (ability to correctly identify decayed surfaces) for detection of carious lesions, even though the specificity (ability to correctly identify sound surfaces) is high [23]. For this reason, the DIAGNOdent was selected as an adjunctive diagnostic method to the visual examination, to combine the high specificity of visual examination with the high sensitivity of DIAGNOdent [24].

The concept of this study in using the stepwise excavation in young first permanent molars was based on the results of several previous studies [11,25]. They found that, stepwise technique reduces the risk of pulp exposure, controls caries progression and promotes dentin-pulp complex reaction. According to Bjorndal *et al* [11], the first important step in declaration of lesion progression was the initial removal of the cariogenic microbial biomasses in the cavity, together with infected superficial necrotic dentin.

The ability to treat a carious lesion without the need of amputation of the diseased tissue would be one of the greatest achievements in the history of dentistry. Ozone therapy has the potential to move toward this goal. The ozone's oxidative reaction can destroy bacteria by oxidizing bacterial cell walls and membranes. This has a disruptive effect on the bacterial population in the carious lesion, which may result in swinging the equilibrium in favor of remineralization [26,27].

Split-mouth design was used, so that patient served as their own control. In the control group, $Ca(OH)_2$ was used as a gold standard capping material of indirect pulp treatment [28]. Stepwise excavation technique using $Ca(OH)_2$ has proved to be a suitable and safe method in management of deep dentinal lesions without pulpal symptoms [11].

Correlation between ozone and dentin microbiological count

Immediate effect of ozone on dentin microflora counts

In the present study, the direct effect of ozone was evaluated after initial removal of the cariogenic biomass, together with the superficial layer of infected carious dentin. For the test group, the absolute and log values of MS, Lactobacilli and Candida reduced significantly after the direct application of ozone for 40 seconds. This significant reduction in bacterial count could have resulted from cellular damage by ozone gas. The effect of ozone could be attributed to simultaneous processes such as inhibition of intracellular enzymes, glutathione depletion, and membrane damage occurring either by direct reaction between target molecules and ozone, or via oxidizing intermediates [29].

On the other hand, no significant differences were observed in the mean log value of Lactobacilli between base line and directly after ozone application of the 12-month cases. The results might be attributed to the low level of microorganisms initially reported before ozone application.

The results of the present study are in agreement with that of Polydorou *et al* [30], who showed a significant reduction in the dentinal count of MS and viability of bacteria, following 40 seconds ozone gas application in-vitro with a HealOzone device.

The percent reduction in microorganisms, in the present study, differed from one type to another. The reduction of MS, Lactobacilli and Candida were 65.50 ± 30.91%, 51 ± 43.41% and 74.21 ± 44.41% respectively. These approach the reduction reported by Dukic and Juric [31] who found 71.5% reduction of MS count and 61.4% in Lactobacilli, in samples of deep carious dentin after ozone treatment ex-vivo.

The present study is in agreement with Baysan and Beighton [32]. The results of their study showed significant reduction in the total bacterial counts, after exposure to 40 seconds ozone.

However, the results of the present study were at variance with the study done in primary molars by Hauser-Gerspach *et al* [33]. They found that 30 seconds gaseous ozone application to cavitated occlusal lesions failed to significantly reduce the number of bacteria in-vivo. In Hauser-Gerspach et al study [33], the base line samples

were taken from the mesial part of the lesions using a new sterile dental excavator, while the second samples were taken from the distal part of the lesion. They contributed the results to failure of ozone gas to access the bacteria in the tissue. Ozone diffusion within the tissue might be hampered because of strong interactions of the gas, with organic materials in the superficial layers. The possible reasons for the discrepancies between this study and the present one could be related to the different sampling techniques, sizes of the lesions, type of treated teeth, and the time of ozone application on the carious lesion.

Effect of ozone on microorganisms after 6 months

Tooth was reentered, and another dentin sample was taken for microbial evaluation after 6 months, followed by removal of residual carious dentin. Some types of microorganisms yielded low microbial count, and other became totally sterile.

For the test group, the absolute and log values of MS, Lactobacilli and Candida reduced significantly after 6 months. Moreover, the culture of Candida became sterile with complete reduction.

The high percent reduction in microorganisms in the ozone group after 6 months could be attributed to the oxidative effect of ozone. It has been suggested that delivering ozone into a carious lesion might reduce the number of cariogenic bacteria [34]. In addition, the newly erupted teeth are more permeable and less mineralized [35] allowing rapid diffusion of ozone into the deep layer of carious dentin.

In the present study, the significant reduction in the different microorganisms mean values after 6 months might also be contributed to different factors such as the stepwise excavation technique which promoted the reduction in microbial count [36], besides the possible antibacterial effect of the bonding agent [37] and the cavity seal provided by the adhesive restoration [38].

Recently, Polydorou *et al* [39] showed that HealOzone was more effective against MS than Lactobacillus cases, 4 to 8 weeks after treatment, using a tooth cavity model. However, the authors suggested that the addition of other antibacterial methods to ozone, after caries removal, might be more efficient to eliminate the remaining bacteria under a restoration.

Effect of ozone on microorganisms after 12 months

When the test group of the 12 month-cases were reentered, the absolute and log values of MS reduced significantly. Also, the mean Lactobacilli and Candida values showed significant reduction in the test group. The reduction in microorganisms counts at the final assessment period after 12 months would be an indicator to success of the clinical procedure, and the antibacterial effect of ozone. It could be also related to the stepwise technique applied in this study. The sealing ability of adhesive restoration [31,40] could also be responsible in the deceleration of the lesion progression reported in this study.

Regarding the long-term effect of ozone on different microorganisms, in comparison with other studies was not possible, because the data was not available in the literature. Most of the published articles have been related to the direct antimicrobial effect of ozone. The only available study was that conducted by Polydorou *et al* [39], who evaluated the effect of ozone on the bacterial count for a period of 8 weeks.

Effect of $Ca(OH)_2$ on microorganisms

The control cases were re-opened after 6 and 12 months. The absolute and log values of MS reduced significantly, with high percent reduction. Whereas with Lactobacilli, the significant reduction was only observed after 6 months, with 100% reduction in the mean Lactobacilli value. The results of the present study are in line with other studies which showed that $Ca(OH)_2$ were effective in promoting reparative dentin formation, as well as having the ability to sterilize the remaining carious lesion [41,42]. It is noted that bacteria like streptococci can grow only in a pH between 3 and 8, whereas, the pH under a $Ca(OH)_2$ liner is above 10. This high pH value of $Ca(OH)_2$ has a bactericidal effect on remaining microorganisms [43]. This assumption was also supported by Bjorndal *et al* [11] when they examined the effect of $Ca(OH)_2$ on the residual dentin, after an interval of 6 to 12 months. They evaluated the microbiological status of the residual carious dentin, and found a decrease in the total microorganisms count, in spite of its a high solubility, and poor seal.

In the present study, the significant reduction of the MS could also be due to the added effect of the sealing ability of the bonding agent that was applied over the $Ca(OH)_2$ base. The self-etching adhesive interacted with dentin substrate in the wall, and floor of the cavity uncovered with $Ca(OH)_2$. This interaction might have produced crystal plugs in the dentinal tubules, which in turn might reduce dentin permeability, and decrease the bacterial count [44,45].

When comparing the mean microorganisms count between test and control groups at different evaluation periods, no significant differences in the absolute and log values were observed. The results in the present study pointed that the application of ozone, or $Ca(OH)_2$ have comparable effect on affected dentin in deep cavities.

The null hypothesis was rejected, since there were significant changes in cultivable microflora before and after using ozone gas on dentinal lesions in young permanent molars using the stepwise excavation.

This study has some limitations that should be considered. First, patient compliance was difficult to achieve. That's why children were recruited from different junior schools in Alexandria in addition to those attending the clinic of the Pediatric Dentistry and Dental Public Health Department, Faculty of Dentistry, Alexandria University. Another limitation of this study was the lack of funding of the research project.

CONCLUSIONS

Ozone gas application for 40 seconds via the HealOzone device had a significant antimicrobial effect especially against MS, in deep class I carious lesions.

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