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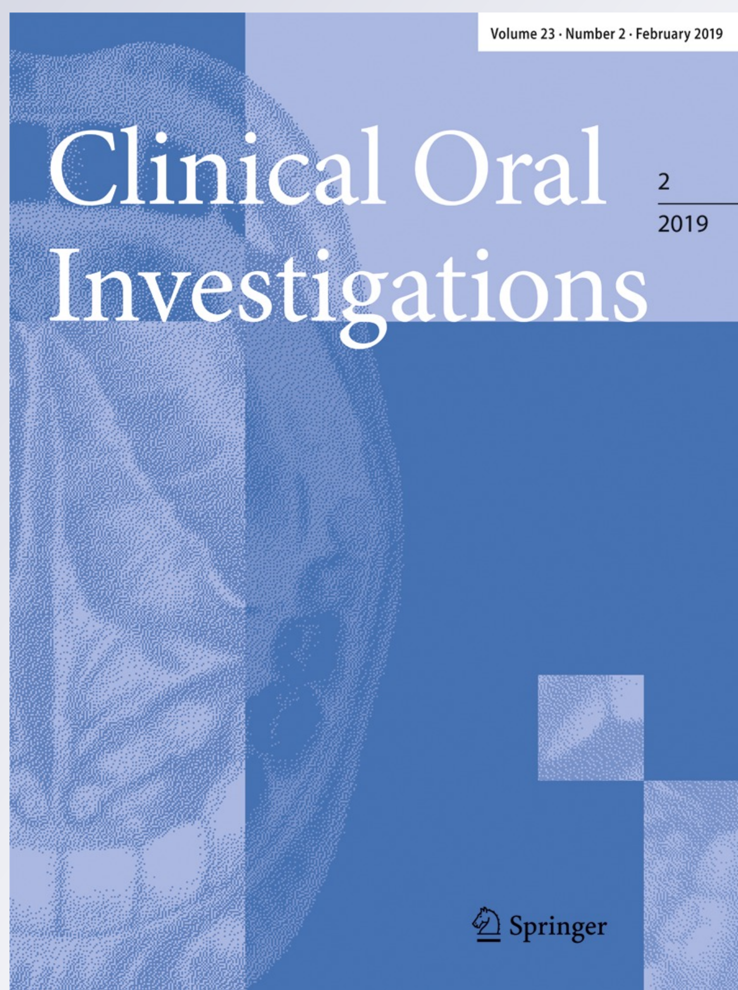

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ORIGINAL ARTICLE

Clinical antibacterial effectiveness and biocompatibility of gaseous ozone after incomplete caries removal

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Abstract

Objectives To evaluate local effect of gaseous ozone on bacteria in deep carious lesions after incomplete caries removal, using chlorhexidine as control, and to investigate its effect on pulp vascular endothelial growth factor (VEGF), neuronal nitric oxide synthase (nNOS), and superoxide dismutase (SOD).

Materials and methods Antibacterial effect was evaluated in 48 teeth with diagnosed deep carious lesion. After incomplete caries removal, teeth were randomly allocated into two groups regarding the cavity disinfectant used: ozone (open system) or 2% chlorhexidine. Dentin samples were analyzed for the presence of total bacteria and *Lactobacillus* spp. by real-time quantitative polymerase chain reaction. For evaluation of ozone effect on dental pulp, 38 intact permanent teeth indicated for pulp removal/tooth extraction were included. After cavity preparation, teeth were randomly allocated into two groups: ozone group and control group. VEGF/nNOS level and SOD activity in dental pulp were determined by enzyme-linked immunosorbent assay and spectrophotometric method, respectively.

Results Ozone application decreased number of total bacteria ($p = 0.001$) and *Lactobacillus* spp. ($p < 0.001$), similarly to chlorhexidine. The VEGF ($p < 0.001$) and nNOS ($p = 0.012$) levels in dental pulp after ozone application were higher, while SOD activity was lower ($p = 0.001$) comparing to those in control pulp.

Conclusions Antibacterial effect of ozone on residual bacteria after incomplete caries removal was similar to that of 2% chlorhexidine. Effect of ozone on pulp VEGF, nNOS, and SOD indicated its biocompatibility.

Clinical relevance Ozone appears as effective and biocompatible cavity disinfectant in treatment of deep carious lesions by incomplete caries removal technique.

Keywords Ozone · Incomplete caries removal · Antibacterial effect · Residual caries · Biocompatibility

Introduction

In contrast to traditional treatment of dentinal carious lesions, which include complete removal of carious tissue, there is an increasing interest into less invasive techniques with incomplete caries removal. The biological rationale for this preservative approaches is that cariogenic bacteria die or remain dormant when isolated from nutrition by sufficient seal, and therefore, caries arrest and carious dentin remineralizes. Incomplete caries removal, performed in one or two steps, seems advantageous for the treatment of deep carious lesions. Namely, growing number of clinical studies showed that incomplete excavation reduces the risk of pulpal exposure compared with complete caries removal [1, 2]. However, most of currently available direct restorative materials cannot provide adequate seal and prevent leakage for a sufficient long period of time. Therefore, residual bacteria may proliferate in the smear layer and dentinal tubules of the cavity causing pulp irritation, secondary caries, and restoration failure over time [3, 4]. For solving this issue, there are recommendations to use

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cavity disinfectants such as chlorhexidine and sodium hypochlorite solutions, dental bonding agents with antimicrobial activity, antibiotics, laser, and ozone [5, 6].

The use of ozone as an antibacterial agent in residual caries is justified by the following findings from in vitro and clinical studies. The clinical antibacterial effectiveness of ozone after incomplete caries removal has been evaluated in limited number of studies. Dukić et al. [7] demonstrated a significant reduction in the number of bacteria in caries cavities after ozone application in permanent teeth while Hauser-Gerspach et al. [5] showed that ozone application as well as chlorhexidine had no significant immediate antibacterial effect in carious lesion of primary teeth. In vitro studies found that gaseous ozone is effective against cariogenic bacteria *Streptococcus mutans*, *Lactobacillus casei*, *Lactobacillus paracasei*, and *Actinomyces naeslundii* [7–11]. In addition, there are in vitro data showing that ozone therapy does not adversely affect the enamel and dentin resin-bond strength [12] and show biocompatibility [13].

There are evidences that, besides antimicrobial action, ozone stimulates tissue regeneration in wound healing process [14, 15]. Among the mechanisms involved in this process are increased expression of vascular endothelial growth factor (VEGF), connected with greater wound reduction in experimental or clinical conditions [16, 17], increased level of nitric oxide (NO) [18], and decreased oxidative stress due to increased action of antioxidative enzymes [19].

Among various cytokines and growth factors regulating angiogenesis, the most potent is VEGF, inducing mitogenic activity, endothelial cell proliferation, increase in microvascular permeability, and expression of antiapoptotic proteins in endothelial cells [20]. NO, intracellular messenger implicated in a wide variety of physiological and pathophysiological processes, is produced by three isoenzymes termed nitric oxide synthases (NOS): constitutive endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) [21]. Reactive oxygen species (ROS) (superoxide anion, hydrogen peroxide, hydroxyl radical) are a group of oxidative molecules released by host cells during normal or pathological oxidative metabolism. To avoid excessive oxidative tissue damage caused by elevated concentrations of ROS, antioxidative defense mechanism, consisting of several antioxidative enzymes (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px)), is activated.

Different ozone producing devices were utilized for topical administration of gaseous ozone in dentistry. HealOzone device (KaVo, Biberach, Germany) produces ozone gas at higher concentration (2100 ± 200 ppm) in closed system. The device has a built-up suction scavenging system designed to create the tight seal around the delivery cup. Therefore, ozone application is only possible on surfaces where such air tightness can be achieved, avoiding the ozone leakage. The other types of ozone devices are delivery ozone by open system such as Ozonytron (Bioozonix, Munich, Germany). This device allows application of ozone at a lower concentration (~ 525 ppm) with no apparent need for scavenging. Although there is leakage of ozone on site of application, this device was shown to be safe to use. Also, absence of built-up evacuation system allows its application in sites that are difficult to reach (e.g., interproximal caries lesion).

This clinical study was aimed firstly to evaluate local effect of gaseous ozone on bacteria in deep carious lesions after incomplete caries removal, using chlorhexidine as control, and secondly to investigate its effect on pulp VEGF, nNOS, and SOD. The hypothesis was that immediate effect of ozone application is reduction of bacteria in deep carious lesions and modulation of VEGF, nNOS level, and SOD activity in human pulp tissue.

Material and methods

The study protocol was approved by the Ethic Committee of the Faculty of Medicine and conducted in accordance with accepted ethical standards for research practice (guidelines of the Helsinki Declaration). All enrolled patients provided written informed consent before inclusion in research.

Patients—study I

Sample was selected among patients appearing for dental treatment at the Department of Oral Pathology, Faculty of Medicine. Inclusion criteria were as follows: age > 18 years, vital posterior tooth (positive response to cold (Endo-Frost-50C; Coltene/Whaledent, Altstatten, Switzerland), and electric stimuli (Analytical Technologies, Redmond, WA, USA) with primary carious lesion on occlusal or proximal surface radiographically involving > 2/3 dentin thickness, without spontaneous pain, swelling, fistula, and radiographic signs of pulp and periapical abnormalities, and with periodontal probing depth < 4 mm. Exclusion criteria included contraindications to ozone therapy (pregnancy, favism, hyperthyroidism, anemia, myasthenia, hemorrhage, alcohol intoxication, myocardial infraction, pacemaker, ozone allergy), systemic disease, and medication intake. A total of 48 patients were included in the study, 22 females and 26 males aged 20–48 years.

Patients—study II

Consecutive patients with intact molar teeth indicated for pulp removal due to prosthetic rehabilitation or extraction due to orthodontic reason were recruited at the Department of Oral Rehabilitation, Faculty of Medicine. Inclusion criteria were as follows: age > 18 years, intact and vital posterior tooth (positive response to cold (Endo-Frost-50C; Coltene/Whaledent, Altstatten, Switzerland), and electric stimuli (Analytical

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Technologies, Redmond, WA, USA) without clinical and radiographic signs of pulp and periapical abnormalities, without history of dental trauma, and with periodontal probing depth < 4 mm. Exclusion criteria were the same as for study I. Final sample consisted of 38 patients, 30 females and 8 males, aged 18–40 years.

Each patient in both studies contributed with only one tooth.

Clinical procedures and sampling—study I

Teeth were anesthetized and isolated with a rubber dam. Carious lesions were assessed by high-speed sterile diamante bur. Carious dentin was removed from enamel-dentine junction and lateral walls with sterile round carbide bur, leaving a layer of soft dentin on the pulpal wall to prevent pulp exposure. In order to obtain that the microbiological samples were taken at the same depth, the lesions were divided into two halves: buccal/mesial and oral/distal parts. First dentin sample (S1) was taken from buccal/mesial part of lesion by using sterile round carbide bur dampened with sterile saline. The bur was placed into pre-weighted Eppendorf tube (Adventure™ OHAUS, Corp., Pine Brook, NY, USA) with 1 ml of thioglycollate. Tube was shaken to dislodge the adherent dentin sample and reweighted. Then, the bur was removed with sterile tweezers and the tube was frozen (− 80 °C) until further analysis. Bur weight was measured before sterilization. Dentin weight was calculated by the difference between the weight of the set (Eppendorf tube with thioglycollate and bur with sample) and the previously determined weight of the set without dentin.

After obtaining the first samples, cavities were randomly divided into two groups by selection of sealed envelopes: ozone and chlorhexidine groups. Operator blinding could not be obtained after this step due to difference in disinfectant appearance. The ozone disinfection was performed using an ozone generator (Ozonytron X-Bioozonix, Munich, Germany). The ozone was applied to the cavity for 40 s by the special disposable silicone cup provided by the manufacturer. This cup permitted a completely seal and prevents escape of ozone gas. In chlorhexidine group, 2% solution (Consepsis, Ultradent, South Jordan, UT, USA) was applied in the cavity for 60 s with brush tip (Black Mini® Brush, Ultradent, South Jordan, UT, USA) and gently air-dried. Subsequently, second microbiological sample (S2) was taken from oral/distal part in the same way as first sample. In both groups, pulpal wall was lined with calcium-hydroxide base material and cavity was sealed with temporary filling.

Clinical procedures and sampling—study II

Patients were anesthetized using 1.8 ml 3% mepivacaine (Scandonest 3% Plain; Septodont, Paris, France) in order to avoid vasoconstriction and consequent hypoxia of pulp tissue. Teeth were disinfected with 70% ethanol and 0.2% chlorhexidine and placed under rubber dam. Class I cavities were prepared using high-speed handpiece and slightly tapered diamond bur, changed after every forth preparation to prevent overheating. The depth of cavity preparation was standardized to 2/3 of dentin thickness, based on radiographic images. Cavities were than randomly assigned to either ozone or control group by selection of sealed envelopes. The ozone gas was applied in the prepared cavity for 40 s as mentioned before. In the control group, sterile cotton pellet was placed in the cavity for the same time. After that, pulp chamber was unroofed and rinsed with sterile saline to remove dentin debris. Pulp tissue was removed by barbed broach and transferred to previously weighed (Adventure™ OHAUS, Corp., Pine Brook, NY, USA) Eppendorf tube. Eppendorf tube with pulp tissue was reweighed and stored at − 80 °C until further analysis. The weight of pulp was calculated by subtracting the weight of the tube containing tissue from that of the empty tube. Following pulp removal, endodontic therapy or tooth extraction was performed.

After thawing, the pulp tissue in the Eppendorf tube was homogenized mechanically in lysis buffer (20 ml/1 g of tissue; CellLytic AT, Sigma Aldrich, Buchs, Switzerland) at the temperature of 4 °C. The samples were centrifuged at 12000–20000×*g* for 10 min in a microcentrifuge (Heraeus Primo R* Biofuge, Thermo Fisher Scientific, Waltham, MA, USA), and supernatant of each sample was then divided into three aliquots and stored at − 80 °C until further analysis.

Real-time quantitative polymerase chain reaction and quantification of bacteria

Quantitative polymerase chain reaction (qPCR) was performed to quantify the total bacterial load and level of *Lactobacillus* spp. in caries samples. Frozen samples were thawed and vortexed for 15 s. The DNA was isolated by with "QIAamp DNA Mini Kit" (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration and purity were evaluated by spectrophotometer. Primers for total number of bacteria and *Lactobacillus* spp. previously described and validated by Nadkarni et al. [22] and Byun et al. [23], respectively, were used for 16S rDNA (Table 1). For each qPCR carried out in duplicate, 20 μl of reaction mixture containing SYBR Green Mastermix, 0.2 μM of each primer, and 2 μl of DNA sample was placed in each well. Amplifications of qPCR were performed using Line-Gene PCR (Hangzhou Bioer Technology Co., Ltd., Shanghai, China). The reaction condition for total bacteria quantification was 94 °C for 10 min, followed by 40 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min and 30 s, and 72 °C for 5 min. The reaction condition for *Lactobacillus* spp. quantification was 95 °C for 10 min followed by 40 cycles

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Table 1 Primers used for bacterial quantification in carious samples by real-time PCR assays

Taxa	Primer sequence	Annealing temperature (°C)	Amplicon length (bp)	Reference
Bacteria 16s rDNA	5′-TCCTACGGGAGGCAGCAGT-3′ 5′GGACTACCAGGGTATCTAATCCTGTT -3′	57	466	[22]
Lactobacillus spp.	5′-TGGAAACAGRTGCTAATACCG-3′ 5′-GTCCATTGTGGAAGATTCCC-3′	62	223	[23]

of 95 °C for 15 s, 62 °C for 1 min, and 72 °C for 5 min. The same procedures were performed for negative controls containing no template DNA. Tenfold dilutions of DNA extracted from *Prevotella melaninogenica* (ATCC 25845) and *Lactobacillus casei* (ATCC 393) were used for making standard curves for quantification of total bacteria and total number of *Lactobacillus* spp., respectively. Data analysis was done using Line-Gene K software (Hangzhou Bioer Technology Co., Ltd., Shanghai, China). The amount of DNA was calculated by comparing the threshold cycle (Ct) values of standard curve. The total bacteria and *Lactobacillus* spp. were expressed as number of gene copy and number of cells per milligram (wet weight) of dentin, respectively.

VEGF, nNOS, and SOD determination

The concentrations of VEGF (pg/ml) and nNOS (ng/ml) in pulp tissue were measured by commercially available enzyme-linked immunosorbent assay (ELISA) Human VEGF ELISA Kit (Sigma Aldrich, Buchs, Switzerland) and Human ELISA Kit for Nitric Oxide Synthase 1 (Cloud-clone Corp., Houston, TX, USA), respectively, according to the manufacturer's protocols. Optical densities were measured at 450 nm with a microplate reader (Multiskan EX, Thermo Fisher Scientific, Waltham, MA, USA).

SOD activity was determined using SOD Assay Kit-WST (Sigma Aldrich, Buchs, Switzerland) (an indirect assay method based on xanthine oxidase activity) following the manufacturer's instructions. An inhibition activity of SOD was quantified by measuring the decrease in the color development at 440 nm. Results were expressed as inhibition rate percentage (SOD activity).

Statistical analysis

Statistical analyses were performed using IBM SPSS 20 software (IBM SPSS Inc., Chicago, IL, USA). The distribution of the variables: gender, cavity, dental arch involved, and tooth type was evaluated using chi-square test, while age was analyzed using Student t test. Data related to total bacteria, *Lactobacillus* spp., VEGF, nNOS, and SOD were analyzed using Mann-Whitney U and Wilcoxon signed-rank tests, for intergroup and intragroup comparison, respectively (Kolmogorov-Smirnov tests $p < 0.05$). A significance level was preset at $p < 0.05$ for all statistical tests.

Results

Tables 2 and 3 present the demographics of the study population and clinical characteristics of the samples in study I and study II, respectively. No significant difference in distribution of age, gender, tooth type (only study I), and caries localization (only study I) was found between groups. All samples were included in the final analysis. There was no adverse effect reported during the investigation.

Bacteria were found in all cavities before and after application of either ozone or chlorhexidine. Before disinfectant application, no significant difference in total microbial load was observed ($p = 0.695$). Ozone and chlorhexidine application significantly decreased the number of total bacteria for 68 and 34.5%, respectively (for both $p = 0.001$), with no difference between the antiseptics ($p = 0.805$) (Table 4).

Lactobacillus spp. were isolated from all cavities before and after application of disinfectants. There was no significant difference in the number of *Lactobacillus* spp. before the treatment ($p = 0.869$). Ozone and chlorhexidine application significantly decreased counts of *Lactobacillus* spp. for 30 and 66%, respectively (for both $p < 0.001$), with no difference between the antiseptics ($p = 0.837$) (Table 4).

Results concerning the effect of ozone on VEGF, nNOS, and SOD in healthy pulp are presented in Table 5. The VEGF and nNOS concentrations in pulp after ozone application were 24% ($p < 0.001$) and 57% ($p = 0.012$) higher, respectively, while SOD activity was 6% lower ($p = 0.001$) in comparison to those in the control pulp.

Discussion

The present study demonstrated that in clinical conditions, application of ozone in deep carious lesions after incomplete dentin caries removal caused significant antibacterial effect (study I) and, for the first time, that its application in deep cavities of healthy teeth increased VEGF and nNOS levels and decreased SOD activity in dental pulp tissue (study II),

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Table 2 Demographic characteristics of study population and clinical characteristics of sample by group—study I

Characteristics	Total ($n = 48$)		Ozone ($n = 24$)		Chlorhexidine ($n = 24$)		
	n	%	*n*	%	*n*	%	*p*
Demographic							
Age (mean ± SD)	28.40 ± 7.24		29.75 ± 8.07		27.04 ± 6.18		0.198
Sex							
Women	22	45.8	12	50.0	10	41.7	0.772
Men	26	54.2	12	50.0	14	58.3	
Clinical							
Tooth type							
Maxillary premolars	22	45.8	11	45.8	11	45.8	0.829
Maxillary molars	13	27.1	6	25.0	7	29.2	
Mandibular premolars	7	14.6	3	12.5	4	16.7	
Mandibular molars	6	12.5	4	16.7	2	8.3	
Caries localization							
Occlusal	26	54.2	10	41.7	16	66.7	0.147
Proximal	22	45.8	14	58.3	8	33.3	

showing its potential as biocompatible antiseptic to pulp tissue.

In study I, the antiseptic effect of ozone is compared to chlorhexidine, as one of the most widely used antiseptics for cavity disinfection [24]. Total bacteria and *Lactobacillus* spp. (bacteria found to be abundant in deep carious lesion [23]) were determined in a homogeneous group of patients according to demographic (age, gender), clinical (tooth type, caries localization), and microbiological variables. Single ozone application by open system device in concentration of ~ 525 ppm for a period of 40 s significantly reduced the number of total bacteria and number of *Lactobacillus* spp. Antibacterial effects of 2% chlorhexidine application during 60 s on total bacteria and *Lactobacillus* spp. were similar to those of ozone. The application time of used antiseptics was recommended by manufacturers. Clinical study of Dukić et al. [7] in the same condition, after incomplete caries removal, showed that application of ozone by a closed system device in dose of 2100 ppm for 40 s reduced the total number of bacteria, *Streptococcus mutans*, and *Lactobacillus* spp. measured by cultivation method. Altogether, these results suggest that ozone in dose range from 525 to 2100 ppm is clinically effective against bacteria and *Lactobacillus* spp. from deep carious lesions.

In study II, we investigated immediate biological response of healthy dental pulp to ozone application measuring level of VEGF and nNOS and SOD activity. Since there is evidence that age influences expression of VEGF [25], SOD activity [26], and dental pulp functions and responses to injury [27], we included only young subjects with narrower age range. Because heat produced during cavity preparation may cause pulp damage [27], cavities were performed in both groups under water spray coolant with bur, replaced every four preparations, using the least pressure; thus, all changes in dental pulp tissue can be considered to be primarily due to ozone application.

VEGF is present in healthy human dental pulp [28, 29] and is modulated by mechanical injury, pulp capping, irreversible inflammation, and diabetes mellitus [28, 30, 31]. In the present study, the concentration of VEGF in healthy human pulp tissue was 776.2 ± 207.13 pg/ml. This level corresponds to those obtained in vitro condition 770.0 ± 20.9 pg/ml in supernates of cultured human intact pulp cells [31]. For teeth exposed to ozone gas, pulp VEGF level was significantly

Table 3 Demographic characteristics of study population by group—study II

Characteristics	Total ($n = 38$)		Ozone ($n = 19$)		Control group ($n = 19$)		
	n	%	*n*	%	*n*	%	*p*
Demographic							
Age (mean ± SD)	25.97 ± 6.07		26.95 ± 6.53		25.00 ± 5.59		0.330
Gender							
Women	30	78.9	14	73.7	16	84.2	0.693
Men	8	21.1	5	26.3	3	15.8	

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Table 4 Total bacteria and *Lactobacillus* spp. in deep carious lesions before (S1) and after (S2) cavity disinfection with ozone or chlorhexidine—study I

Treatment		Total bacteria (gene copy/mg dentin) (mean ± SD)		*Lactobacillus* spp. (cells/mg dentin) (mean ± SD)	
	n	S1	S2	S1	S2
Ozone	24	$7.5 \times 10^5 \pm 1.2 \times 10^{6A,a}$	$2.4 \times 10^5 \pm 2.1 \times 10^{5B,a}$	$5.0 \times 10^4 \pm 1.4 \times 10^{5A,a}$	$3.5 \times 10^4 \pm 1.0 \times 10^{5B,a}$
Chlorhexidine	24	$8.7 \times 10^5 \pm 1.2 \times 10^{6A,a}$	$5.7 \times 10^5 \pm 1.6 \times 10^{6B,a}$	$5.0 \times 10^4 \pm 1.5 \times 10^{5A,a}$	$1.7 \times 10^4 \pm 5.1 \times 10^{4B,a}$

Mean values represented with the same superscript uppercase letters (row) or lowercase letters (column) are not significantly different ($p > 0.05$)

higher than that in control pulp. Having in mind that dental pulp upregulation of VEGF is responsible for angiogenesis, activity, and differentiation of odontoblasts [32], we postulated that increase level of pulp tissue VEGF in our study is the result of increase of pulp vascularization induced by single application of ozone. There is evidence that increase of VEGF level in tissue can signify beneficial or pathological response to tissue [20, 33]. It has been proposed that rapid increase in VEGF expression may cause pulp inflammation and necrosis due to increase of intrapulpal pressure and edema, caused by VEGF-induced vascular permeability and vascularization [34, 35]. Quantifying the release of angiogenic growth factors from cultured human dental pulp cells, Tran-Hung et al. [31] showed that concentration of VEGF increased (21%) shortly after mechanical injury of cells and returned to initial values after 1 day. On the other hand, during diabetes mellitus type 2, pathological increase of VEGF in dental pulp tissue is 33% higher than values found in health pulp tissue [28]. Considering the amount of VEGF increase in the present study (24%) and previously demonstrated positive effect of ozone on wound healing obtained in vitro and in vivo studies [16, 17], attributed to increase of VEGF, it can be assumed that observed VEGF level in pulp tissue is without pathological consequences on pulp tissue.

There are data indicating that the NO is involved in dental pulp healing as regulator of vascular homeostasis, indicator of cell differentiation, and mediator of pro-inflammatory activity and dental pulp afferent sensitivity [36, 37]. In dental pulp, nNOS is mainly localized on nerve fibers [38], odontoblasts, and vessel walls and in pulp cells [36, 39], and eNOS on endothelial cells and odontoblasts [36, 40], while iNOS is expressed during pathological processes [40]. The presence of nNOS in healthy human pulp tissue, shown for the first time in the present study, confirms its participation in pulp homeostasis. Clinical application of ozone into deep cavities of healthy teeth enhances about twofold the level of nNOS. The fact that NO could be part of the first line of dentin defense against cariogenic bacteria [41, 42], it seems likely that NO deriving from ozone-induced increase of nNOS could be involved in observed antibacterial effect of ozone. Recent studies found that ozone has analgesic effect and effectively relieves postoperative pain in dental patients [14, 43, 44]. Concerning the fact that ozone modulates nociception—low levels of peripherally generated NO being algesic while high levels analgesic [45], increased level of nNOS in pulp tissue after ozone application in our study could indicate that therapeutic use of ozone as antiseptic could be followed by analgesic effect due to NO synthetized by nNOS.

It has been shown that immediately after application ozone decomposes into hydrogen peroxide and other ROS such as hydroperoxy, hydroxyl, and superoxide radicals, producing oxidative stress [46, 47]. SOD, metallo-enzyme catalyst of superoxide radical dismutation, is one of the most common defenses against ROS. SOD is present in normal pulp tissue and inflammation may modulate its activity [26, 48, 49].The present results showed that pulp tissue SOD activity is lower after ozone application compared to untreated control pulp tissue. Most probably, the decrease of SOD activity in pulp is a result of its depletion in response to oxidative stress induced by ozone application.

It is noteworthy to mention that results concerning the effect of ozone on VEGF and nNOS levels and SOD activity in dental pulp tissue also indicate that after one application, ozone effectively diffuses through dentin to pulp tissue.

Although randomized clinical trials have shown that under simply sealed cavities, the viability of sealed bacteria is significantly reduced [1], a number of questions remain. Survival of sealed bacteria depends on their number, especially of those whose inactivation is difficult and those which metabolize substrate from pulpal fluids, justifying the cavity disinfection. The use of ozone as a method of cavity disinfection is cost-dependent regarding the use of classical disinfectants and

Table 5 VEGF, nNOS, and SOD in ozone-treated dental pulp and control group—study II

Treatment	n	VEGF concentration (pg/ml) (mean ± SD)	nNOS concentration (ng/ml) (mean ± SD)	SOD activity (%) (mean ± SD)
Ozone	19	1023.39 ± 176.59^a	0.28 ± 0.18^a	88.69 ± 3.76^a
Control group	19	776.18 ± 207.13^b	0.12 ± 0.12^b	92.04 ± 2.39^b

Mean values represented with same superscript lowercase letters (column) are not significantly different ($p > 0.05$)

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method of simply sealing of residual bacteria, but its observed effects on VEGF, nNOS, and SOD reflect not only its biocompatibility but also its potential on pulp tissue reparation, especially in deep carious lesions, in contrast to mentioned methods.

Conclusion

In conclusion, our study suggests that local application of ozone in deep carious lesions after incomplete dentin caries removal provides significant antibacterial effect (comparable to chlorhexidine), measured by total number of bacteria and *Lactobacillus* spp. Results obtained from immediate effect of local application of ozone in deep cavities from healthy teeth provide, for the first time, evidence of its biocompatibility as cavity disinfectant measured by its effect on VEGF, nNOS, and SOD in healthy pulp tissue.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in study were in accordance with the ethical standards of the Institutional research committee and with the 1964 Helsinki declaration and its later amendments.

Informed consent Informed consent was obtained from all individual participants included in the study.

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