Clin Oral Invest (2013) 17:379–388
DOI 10.1007/s00784-012-0711-7

ORIGINAL ARTICLE

Clinical and microbiological effects of ozone nano-bubble water irrigation as an adjunct to mechanical subgingival debridement in periodontitis patients in a randomized controlled trial

Sae Hayakumo · Shinichi Arakawa · Yoshihiro Mano · Yuichi Izumi

Received: 30 September 2011 / Accepted: 4 March 2012 / Published online: 17 March 2012

Abstract
Aim Ozone nano-bubble water (NBW3) seems to be suitable as an adjunct to periodontal treatment owing to its potent antimicrobial effects, high level of safety, and long storage stability. The aim of the present study was to evaluate the clinical and microbiological effects of NBW3 irrigation as an adjunct to subgingival debridement for periodontal treatment.
Methods Twenty-two subjects were randomly assigned to one of the two treatment groups: full-mouth mechanical debridement with tap water (WATER) or full-mouth mechanical debridement with NBW3 (NBW3). Clinical examination was performed at baseline and 4 and 8 weeks after treatment. Microbiological examination was carried out just before and after treatment and at 1 and 8 weeks posttreatment.
Results There were significant improvements in all clinical parameters after 4 weeks in both groups. The reduction in the probing pocket depth and the clinical attachment gain after 4 and 8 weeks in the NBW3 group were significantly greater than those in the WATER group. Moreover, only the NBW3 group showed statistically significant reductions in the mean total number of bacteria in subgingival plaque over the study period.
Conclusions The present study suggests that subgingival irrigation with NBW3 may be a valuable adjunct to periodontal treatment.
Clinical relevance This study verified the potential of new antimicrobial agent, MNW3, as an adjunct to periodontal treatment.

Keywords Adjunctive antimicrobial agents · Irrigation · Ozone nano-bubble water · Periodontitis · Subgingival debridement

S. Hayakumo · S. Arakawa (✉) · Y. Izumi
Section of Periodontology, Department of Hard Tissue Engineering, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University,
1-5-45 Yushima,
Bunkyo-ku, Tokyo 113-8549, Japan
e-mail: shinperi@tmd.ac.jp

Y. Mano
Hyperbaric Medical Center, Hospital of Medicine, Tokyo Medical and Dental University,
Tokyo, Japan

Y. Izumi
Global Center of Excellence (GCOE) Program, Tokyo Medical and Dental University,
Tokyo, Japan

Introduction

Periodontitis is a chronic inflammatory disease caused by microorganisms existing in a subgingival biofilm. *Porphyromonas gingivalis* and *Tannerella forsythia* are the specific pathogens most frequently associated with this disease, being present in high numbers within deep periodontal pockets in severe periodontal conditions [1–4]. Elimination of pathogen-containing biofilms remains the primary goal of periodontal treatment. Supra- and subgingival mechanical debridement is traditionally used as the initial phase of treatment to achieve this goal even though it is rarely capable of complete removal of putative periodontal pathogens. A review of studies evaluating the effectiveness of various subgingival debridement procedures showed that 5–80 % of treated roots harbor residual plaque or calculus, and as the pockets and furcation involvements get deeper, more deposits are left behind [5].

To augment mechanical debridement, adjunctive antimicrobial agents in the form of topical or systemic antibiotics or topical antiseptics have been employed [6, 7]. Considering the potential problems with selectivity of antimicrobial action and

possible development of resistant bacteria and adverse host reactions, topical and systemic antibiotic therapies seem to constitute an inferior choice to the topical use of low-cost, broad spectrum antiseptic agents with low potential for adverse reactions. Subgingival irrigation with various antiseptic agents such as povidone-iodine, chlorhexidine (CHX), or hydrogen peroxide has been performed in conjunction with scaling and root planing and produced significant clinical benefits relative to conventional mechanical root debridement alone [8]. However, these existing antiseptic agents have serious shortcomings, e.g., the prolonged use of CHX may cause mucosal desquamation, tooth staining, and altered taste sensation. In addition, an increasing number of immediate type allergies to this agent such as anaphylactic shock have been reported [9]. Povidone-iodine should not be administered to individuals who are allergic to iodine, suffering from thyroid dysfunction, or are pregnant or nursing [10–12]. Moreover, a huge amount of povidone-iodine may be dispersed into the air, linen, and other nearby surfaces when used as an adjunct to ultrasonic debridement and the surroundings can be stained brown by the iodine. Therefore, an alternative adjunctive antiseptic with high antimicrobial potential, a good safety profile, fewer contraindications, and a higher degree of usability would be beneficial for periodontal therapy.

Currently, ozone is attracting attention as a possible alternative antiseptic in the dental field as well as food industries. Ozone has strong antimicrobial effects against bacteria, fungi, protozoa, and viruses [13] and does not induce microbial resistance, characteristics that were originally noted in the fields of water purification and food preservation [14–16]. Recent clinical investigations have reported antimicrobial effects of both gaseous and aqueous forms of ozone on oral pathogens associated with caries and endodontic infections [17–22]. Furthermore, ozone therapy has been reported to considerably reduce the growth of *Aggregatibacter actinomycetemcomitans*, *P. gingivalis*, and *T. forsythia* [23, 24]. Regarding cytotoxicity, ozone gas has been found to decrease the viability of oral cells significantly in the concentrations currently used in dentistry [25]. In comparison, it has been reported that aqueous ozone possesses a high level of biocompatibility to fibroblasts, cementoblasts, and epithelial cells [25–28], suggesting the properness of its use against oral infectious diseases such as periodontal disease, apical periodontitis, and peri-implantitis. So far, positive clinical effects of ozonated water in reducing signs of gingivitis and periodontitis have been noted in two clinical studies [29, 30]. However, ozonated water has a half-life of about only 20 min and will degrade back into oxygen very quickly, so it should be used within the first 5–10 min to assure its potency.

To overcome this disadvantage of ozonated water, ozone nano-bubble water (NBW3) was developed using nano-bubble generating technology by Chiba et al. The procedure of generating nano-bubble water has been protected by patent [31]. The ozone concentration of NBW3 is 1.5 mg/l that is equivalent to the oxidation titer determined by electron spin resonance. Nano-bubble is a form of gas nucleus which is less than 100 nm in diameter and is produced by collapsing micro-bubble (less or equal to 50 μm in diameter) in the electrolyte under ultra-high temperature and pressure. During the collapse of micro-bubble by applying physical stimulus in the electrolyte, the ions in aqueous solution become concentrated around the gas nucleus and prevent the gas from dispersion (the salting-out phenomenon). Due to this nature of the ions, nano-bubble is stabilized over a long period in aqueous solution. NBW3 retains ozone gas in the form of gas nucleus and exerts antimicrobial effect for more than 6 months if it is protected against ultraviolet rays [32].

Although the entire mechanisms of NBW3 to inactivate bacteria are as yet not well known, it might be basically similar to those of the existing ozonated water. Ozone is a potent oxidizing agent [33] and ozone in ozonated water has been reported to react with various organic substances and decompose them by free radical-mediated oxidation reactions [34]. Ozone per se also changes to oxygen when it reacts with organic substances. In this process, hydroxyl radicals (·OH) are generated, which are among the most reactive oxidizing species. These free radicals might play a role in the destruction of bacteria by NBW3.

Previously, we confirmed that NBW3 had strong bactericidal effects against multiple-drug-resistant bacteria and representative periodontopathic bacteria in vitro (data not shown). Besides, we confirmed that NBW3 did not possess significant cytotoxicity against human oral tissues in vitro (data not shown). Also, it is authorized to be used as drinking water by the Ministry of Health, Labour and Welfare in Japan (No. 10-S1-2393), which indicates its high level of safety for use in humans. NBW3 seems to be suitable for adjunctive antiseptic in periodontal treatment because of the advantages mentioned above, that is, powerful antimicrobial effect, long storage stability, ease of handling, and high level of safety for human use. The aim of the present study was to evaluate the clinical and microbiological effects of NBW3 irrigation as an adjunct to single visit full-mouth ultrasonic subgingival debridement for the treatment of periodontitis. This is the first report on the clinical application of NBW3 as an adjunct to mechanical subgingival debridement for the treatment of periodontitis.

Methods

Selection of subjects

A total of 22 healthy, non-smoking subjects, aged 26–72 years (6 females and 16 males, mean age 45.9±14.8 years), were recruited from new referrals to the periodontics clinic of the Tokyo Medical and Dental University Dental Hospital and

participated in this single center, placebo-controlled, randomized trial with blind allocation to two parallel groups. All subjects exhibited mild to moderate chronic periodontitis, based on clinical findings. Each study subject had a minimum of 20 teeth present and exhibited at least one pocket site with a probing depth of 4 mm or more in each quadrant. All subjects were in good general health, and none had received periodontal treatment and/or antibiotic therapy within the preceding 6 months. Subjects who were pregnant or lactating were excluded from this randomized controlled study. The characteristics of the subjects are shown in Table 1. The study protocol was approved by the Ethics Committee of the Tokyo Medical and Dental University, Tokyo, Japan (#234), and all patients signed a written informed consent form.

Treatment protocol

After screening, all subjects received thorough oral hygiene instructions including the use of inter-proximal cleaning aids such as floss and inter-dental brushes, depending on individual needs. They were then randomly assigned to one of the two treatment groups based on the treatment protocol and the examiner was blinded to the allocation. The random sequence was computer-generated, with no stratification of balancing factors. The subjects chose a sequentially numbered opaque, sealed envelope, which enclosed the code for the treatment protocol they were to receive. The number of envelopes was the same as the number of subjects. The treatment groups were coded so that only the operator was aware of the protocol and the examiner remained blinded throughout the study. After random allocation, the patients in this study were subjected to one of the two treatment protocols: full-mouth mechanical debridement with tap water in a single visit (WATER) or full-mouth mechanical debridement with NBW3 in a single visit (NBW3). Mechanical debridement included supra- and subgingival ultrasonic instrumentation, which was performed by one experienced and trained periodontist with an ultrasonic scaler (Varios 750; Nakanishi Inc., Tochigi, Japan) equipped with a P10 tip. Local anesthesia was administered, if necessary, during debridement. The time spent on the full-mouth mechanical debridement was nearly 1 h in each patient. At each re-examination visit (1, 4, and 8 weeks after treatment), plaque control was reinforced in all subjects on an individual basis and they were subjected to professional tooth cleaning with a rubber cup and polishing paste. During the experimental period, no additional pocket instrumentation was allowed.

Clinical examination

Clinical examination was performed at baseline (before treatment) and 4 and 8 weeks after treatment by a single blinded examiner. Full-mouth clinical measurements of probing pocket depth (PPD), clinical attachment level (CAL), and the percentage of bleeding on probing (BOP (%)) were recorded using a manual constant load periodontal probe (TUCL probe, Williams type; Shioda Dental Co., Tochigi, Japan) at all six sites per tooth (disto-buccal, buccal, mesio-buccal, disto-lingual, lingual, mesio-lingual). PPD was measured to the nearest 1 mm from the base of the pocket to the gingival margin. CAL was assessed by measuring the distance between the cemento-enamel junction and the bottom of the pocket. BOP (%) was recorded as present or absent after completing the probing on the buccal or lingual sites of each quadrant. The research schedule is shown in Fig. 1. All recordings were made without knowledge of the previous measurements.

Microbiological analysis

Microbiological examination was performed just before and after treatment and at 1 and 8 weeks posttreatment. Subgingival plaque samples were collected from one selected pocket site with a probing depth of 4 mm or more around a maxillary single-rooted tooth in each patient. These data were obtained with no knowledge of the type of treatment rendered. Samples were collected as described below. First, the tooth was isolated with cotton rolls and gently air-dried. Supragingival plaque along the gingival margin of the pocket site was wiped gently with a sterile cotton pellet. After that, subgingival plaque was collected by inserting two sterile paper points (No. 30) into each selected pocket site until resistance was felt and kept in

Table 1 Patient characteristics at baseline

Treatment group	No. of subjects	Age[a] (years)	Full-mouth mean BOP (%)[a]	Full-mouth mean PPD (mm)[a]	Full-mouth mean CAL (mm)[a]
NBW3	10	45.90±13.8	32.95±15.7	2.59±0.3	2.73±0.3
WATER	11	46.00±16.4	30.20±14.8	2.58±0.3	2.79±0.5

No significant difference between groups ($p>0.05$)

NBW3 full-mouth debridement with ozone nano-bubble water, *WATER* full-mouth debridement with water, *BOP (%)* bleeding on probing (percent), *PPD* probing pocket depth, *CAL* clinical attachment level.

[a] Mean ± standard deviation

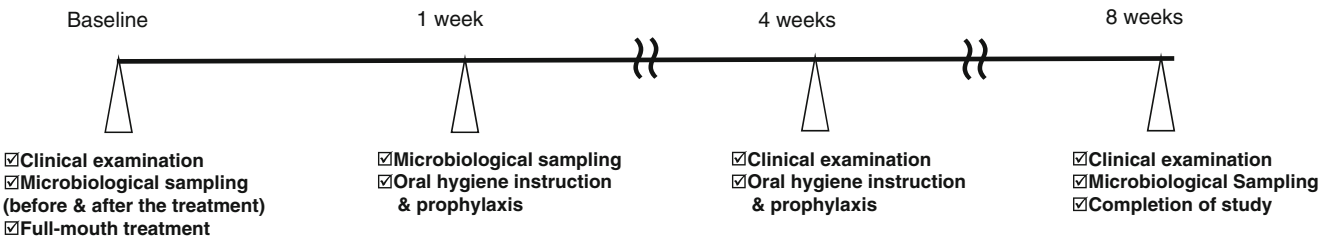

Fig. 1 Treatment schedule in this study

place for 10 s. Following removal, the paper points were transferred into a screw-capped sterile vial.

The bacterial species identified were *P. gingivalis* and *T. forsythia*. Using a real-time polymerase chain reaction (PCR) assay, the relative and absolute numbers of these bacteria in subgingival plaque were determined. The following oligonucleotide primers were used—*P. gingivalis* and *T. forsythia* with a sequence of 16S ribosomal RNA—5′-TACCCATCG TCGCCTTGGT-3′ and 5′-CGGACTAAAACCGCATACA CTTG-3′, and 5′-TACGCATACCCATCCGCAA-3′ and 5′-CGCTAGTAATCGTGGATCAGAATG-3′, respectively.

Statistical analyses

The data were analyzed with the subject as unit. Mean and standard deviations of each parameter were calculated for each patient and compared to determine any differences within and between groups. PPD and CAL were considered as the primary outcome variables.

For a comparison between groups (at each follow-up visit), Student's *t* test was applied. The level of significance was set at 0.05. Also, a comparison within each group (baseline versus follow-up visit) was made by means of a paired *t* test. As multiple comparisons were conducted in that analysis, Bonferroni correction was performed with the alpha-level for the clinical parameters and microbiological parameters of 0.025 and 0.05/3, respectively. Furthermore, improvements in each parameter from baseline to posttreatment were calculated and compared to determine any differences between groups at 4 and 8 weeks with Student's *t* test. With respect to the sample size, we first calculated that we would need a sample size of at least 11 patients to detect a 0.5-mm true difference between two groups at 5 % type I error and 80 % power for a two-tailed Student's *t* test. All statistical analyses were carried out with the aid of statistical software (IBM® SPSS® Statistics 19, SPSS Inc., Chicago, USA).

Results

Patient characteristics at baseline

The study population was randomly divided into two groups with 11 subjects in each group. During the follow-up period, one subject in the NBW3 group withdrew from the trial for reasons unrelated to the study. The data of this subject were not included in the assessment of the outcome of the treatment.

The demographic characteristics and clinical parameters of the two treatment groups at baseline (before treatment) are presented in Table 1. The mean age of the subjects was 45.9±13.8 in the test group (NBW3) and 46.0±16.4 in the control group (WATER). The mean PPD and CAL were 2.59±0.3 and 2.73±0.3 mm in the test group and 2.58± 0.3 and 2.79±0.5 mm in the control group. The BOP (%) of the test and control groups was 32.95 and 30.20 %, respectively. No statistically significant differences in the demographic and clinical variables were found between the two treatment groups at baseline ($p>0.05$).

BOP (%)

The changes over time in BOP (%), sorted per treatment group, are shown in Fig. 2a. BOP (%) decreased significantly from baseline to 4 weeks in both groups ($p=0.003$ in the test group; $p=0.009$ in the control group). However, only the test group (NBW3) showed a statistically significant improvement from baseline to 8 weeks ($p=0.001$). When the treatment groups were compared with each other, there were no statistically significant differences in BOP (%) at any re-examination period.

The amount of reduction in BOP (%) from baseline to 4 and 8 weeks after treatment is shown in Table 2. BOP (%) was reduced by 15.69 and 8.98 % at 4 weeks and 13.47 and 6.97 % at 8 weeks in the NBW3 and WATER groups, respectively. When the treatment groups were compared with each other, there were no significant differences in the amount of reduction in BOP (%) at both 4 and 8 weeks, although numerically greater reductions were observed in the test group.

Changes in probing depth

The changes in full-mouth mean PPD during the experimental period are shown in Fig. 2b. There were statistically significant improvements in the full-mouth mean PPD in both groups from baseline to 4 weeks ($p<0.001$ in the test group and $p=0.002$ in the control group) and 8 weeks ($p<$

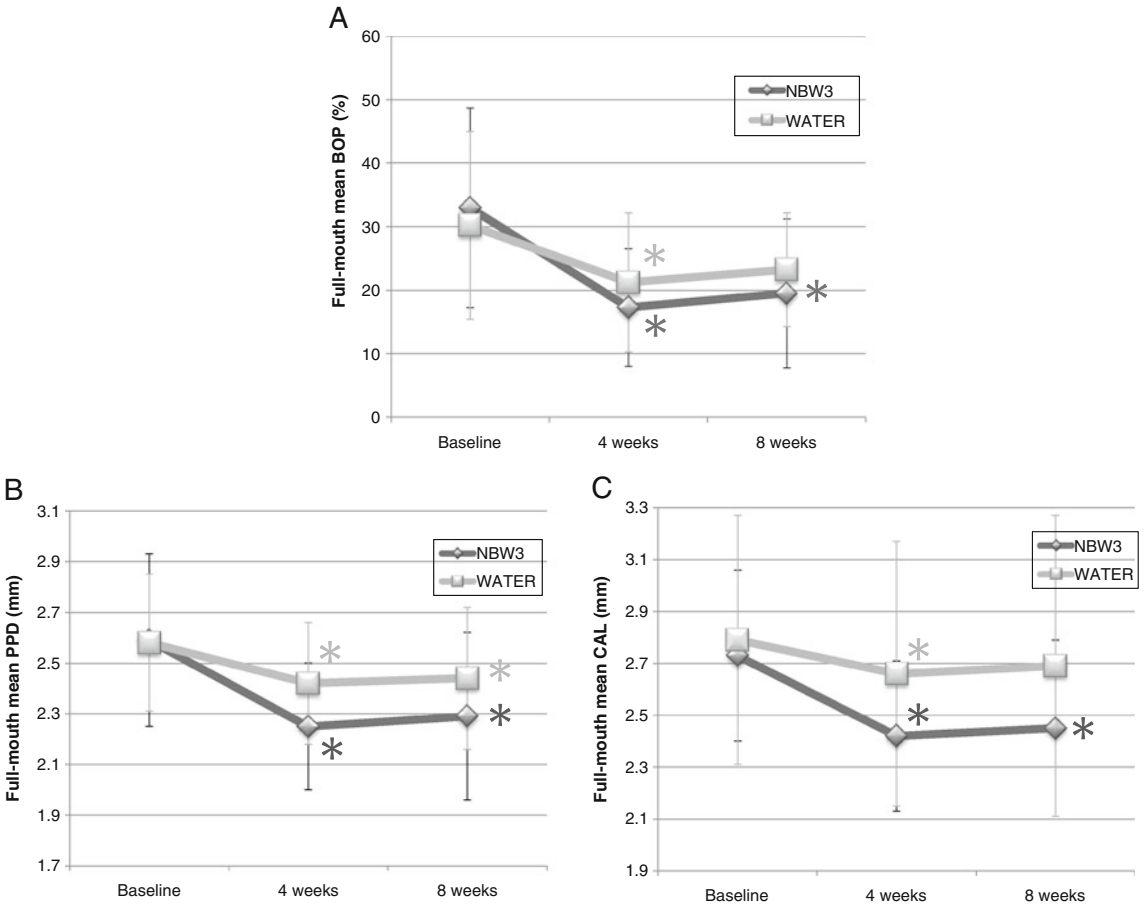

Fig. 2 Changes in the full-mouth mean (± standard deviation) BOP (percent) (**a**), PPD (millimeter) (**b**), and CAL (millimeter) (**c**) in each treatment group from baseline to 8 weeks. *NBW3* full-mouth debridement with ozone nano-bubble water, *WATER* full-mouth debridement with tap water, *BOP* bleeding on probing, *PPD* probing pocket depth, *CAL* clinical attachment level. No significant differences in all the clinical parameters assessed were seen between groups at any examination period ($p>0.05$). *$p<0.05$, within the groups, full-mouth mean BOP (percent), PPD (millimeter), and CAL (millimeter) decreased significantly from baseline to re-examination periods (paired *t* test)

0.001 in the test group and $p=0.018$ in the control group). When the two treatment groups were compared with each other, the differences in the full-mouth mean PPD were not statistically significant at each follow-up visit.

Table 2 Improvements in clinical parameters at 4 and 8 weeks of re-examination

		ΔBOP (%)[a]	ΔPPD (mm)[a]	Attachment gain (mm)[a]
4 weeks	NBW3 ($N=10$)	15.69±12.5	0.34±0.2*	0.31±0.1*
	WATER ($N=11$)	8.98±9.2	0.17±0.1	0.10±0.2
8 weeks	NBW3 ($N=10$)	13.47±9.2	0.29±0.2*	0.27±0.2*
	WATER ($N=11$)	6.97±10.8	0.14±0.2	0.09±0.2

NBW3 full-mouth debridement with ozone nano-bubble water, *WATER* full-mouth debridement with water, *BOP (%)* bleeding on probing (percent), *PPD* probing pocket depth

[a] Mean ± standard deviation

*$p<0.05$, significantly greater improvements were observed in the NBW3 group, compared to the WATER group

The reduction in the full-mouth mean PPD from baseline to 4 and 8 weeks is shown in Table 2. Mean PPD was reduced by 0.34 and 0.17 mm at 4 weeks and 0.29 and 0.14 mm at 8 weeks in the NBW3 and WATER groups, respectively. When the treatment groups were compared with each other, there were significant differences in the reduction of the full-mouth mean PPD at both 4 and 8 weeks ($p=0.013$ and 0.037, respectively).

Clinical attachment level

The changes in full-mouth mean CAL during the experimental period are shown in Fig. 2c. Full-mouth mean CAL decreased significantly from baseline to 4 weeks in both groups ($p<0.001$ in the test group; $p=0.009$ in the control group). However, only the test group (NBW3) showed a statistically significant improvement from baseline to 8 weeks ($p<0.001$). When the treatment groups were compared with each other, there were no statistically significant differences in the full-mouth mean CAL at any re-examination period.

The CAL gain recorded at 4 and 8 weeks is shown in Table 2. The CAL gain was 0.31 and 0.1 mm at 4 weeks and 0.27 and 0.09 mm at 8 weeks in the NBW3 and WATER groups, respectively. When the treatment groups were compared with each other, there were significant differences in the amount of CAL gain at both 4 and 8 weeks (p=0.002 and 0.021, respectively).

Changes in the mean total number of bacteria in subgingival plaque

Figure 3 shows the mean total number of bacteria in the NBW3 and WATER groups in subgingival plaque. The NBW3 group showed statistically significant reductions in the mean total number of bacteria over 8 weeks of re-examination period (p=0.005 just after the treatment; p=0.006 at 1 week; p=0.009 at 8 weeks). However, the control group did not show significant reductions in the mean total number of bacteria at any re-examination period. When the treatment groups were compared with each other, no statistical differences were found in the total number of bacteria at any time point during the study.

Percentage reductions in the total number of bacteria in subgingival plaque over the study period are tabulated in Table 3. When the treatment groups were compared with each other, differences in the mean percentage reductions in the total number of bacteria were not statistically significant at any re-examination period (data not shown). However, the NBW3 group showed a reduction of 95 % or more in the total number of bacteria in 40 and 20 % of subgingival plaque samples at 1 and 8 weeks, respectively, whereas the WATER group revealed similar reductions in only 9.10 and 0 %, respectively, of subgingival plaque samples.

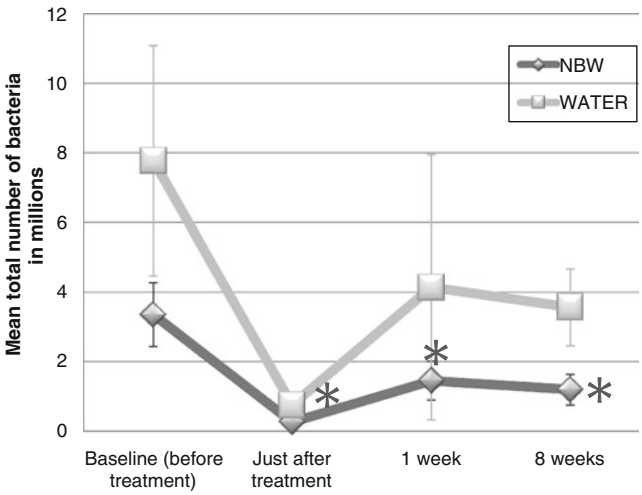

Fig. 3 Changes in the mean (± standard deviation) total number of bacteria in subgingival plaque in each treatment group from baseline (before treatment) to 8 weeks. *NBW3* full-mouth debridement with ozone nano-bubble water, *WATER* full-mouth debridement with water. No significant differences were seen between groups at any examination period (p>0.05). *p<0.05, within the groups, mean total number of bacteria in subgingival plaque decreased significantly from baseline (before treatment) to re-examination periods (paired t test)

Changes in the mean number and percentage of *P. gingivalis* and *T. forsythia* in subgingival plaque

The changes in the mean number and percentage of *P. gingivalis* and *T. forsythia* in subgingival plaque for the NBW3 and WATER groups over time are depicted in Figs. 4 and 5, respectively. The subjects initially negative for each pathogen were excluded from the statistical analyses. For *P. gingivalis* (N=4 and 6 in the test and control groups, respectively), insignificant reductions in the mean number and percentage of *P. gingivalis* were found in both groups over 8 weeks (Figs. 4a and 5a). When the treatment groups were compared with each other, there were no statistically significant differences in the mean number and percentage of *P. gingivalis* at any re-examination period. Comparable observations were made for *T. forsythia* (N=6 and 9 in test and control groups, respectively) (Figs. 4b and 5b).

Percentages of subgingival plaque samples showing >95 % reduction in the number of *P. gingivalis* and *T. forsythia* are shown in Table 4. When the treatment groups were compared with each other, differences in the mean percentage reductions in the number of *P. gingivalis* and *T. forsythia* were not statistically significant at any re-examination period (data not shown). However, *P. gingivalis* was reduced by at least 95 % in all the initially positive subgingival plaque samples over the study period in the test group (N=4), whereas the pathogen was reduced by more than 95 % only in 66.7 and 50 % of subgingival plaque samples at 1 and 8 weeks in the control group, respectively (N=6). On the other hand, the percentage of subgingival plaque samples showing >95 % reduction in the number of *T. forsythia* was similar between the test and control groups.

Discussion

Because of the microbial etiology of periodontitis [35], a variety of antimicrobial agents have been employed as adjuncts to conventional therapy to further suppress periodontal pathogens [36]. In this study, a new antiseptic agent, NBW3, was used as an adjunct to mechanical debridement in the treatment of periodontitis. Although there are no existing papers on NBW3, we previously performed various in vitro studies and confirmed its potent antimicrobial effects against representative periodontopathic bacteria (data not shown), good safety profile (data not shown), and high degree of usability.

The method of the preparation of nano-bubble water has been protected by patent [31]. NBW3 encapsulates ozone

Table 3 Percentage reductions in the total number of bacteria in subgingival plaque

		Percentage reductions in the total number of bacteria in subgingival plaque		
		% of subgingival plaque samples showing >95 % reduction	% of subgingival plaque samples showing 95–33 % reduction	% of subgingival plaque samples showing <33 % reduction
NBW3 (N=10)	Just after treatment	40	60	0
	1 week	40	40	20
	8 weeks	20	40	40
WATER (N=11)	Just after treatment	45.5	45.5	9.1
	1 week	9.1	45.5	45.5
	8 weeks	0	45.5	54.5

NBW3 full-mouth debridement with ozone nano-bubble water, *WATER* full-mouth debridement with water

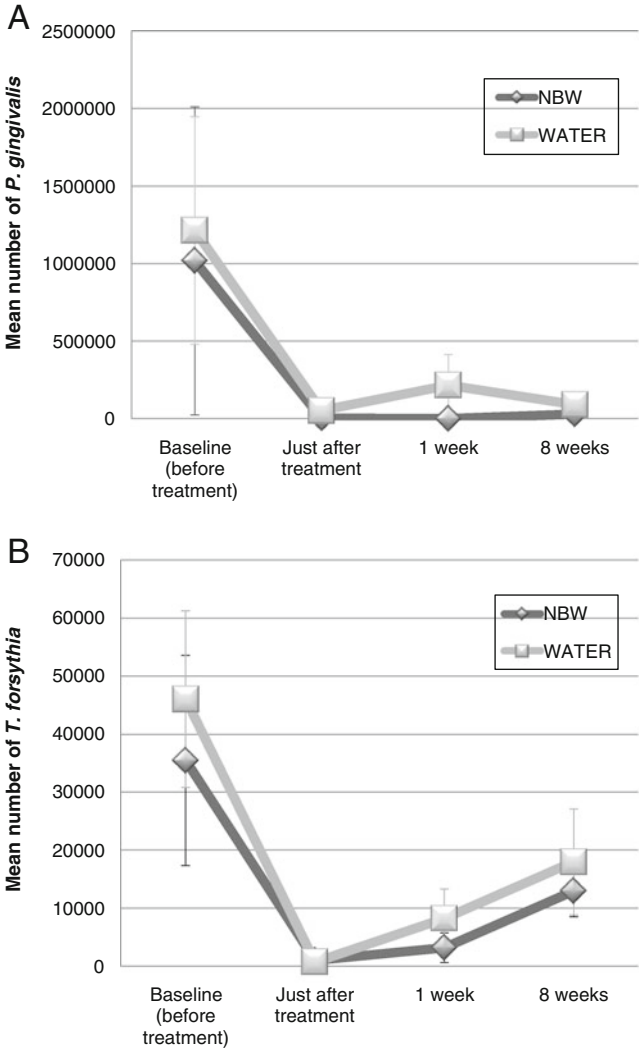

Fig. 4 Changes in the mean (± standard deviation) number of *P. gingivalis* (**a**) and *T. forsythia* (**b**) in subgingival plaque in each treatment group from baseline (before treatment) to 8 weeks. *NBW3* full-mouth debridement with ozone nano-bubble water, *WATER* full-mouth debridement with water. No significant differences were seen between groups at any examination period (p>0.05). No significant differences between baseline and any re-examination period were seen within both groups (p>0.05)

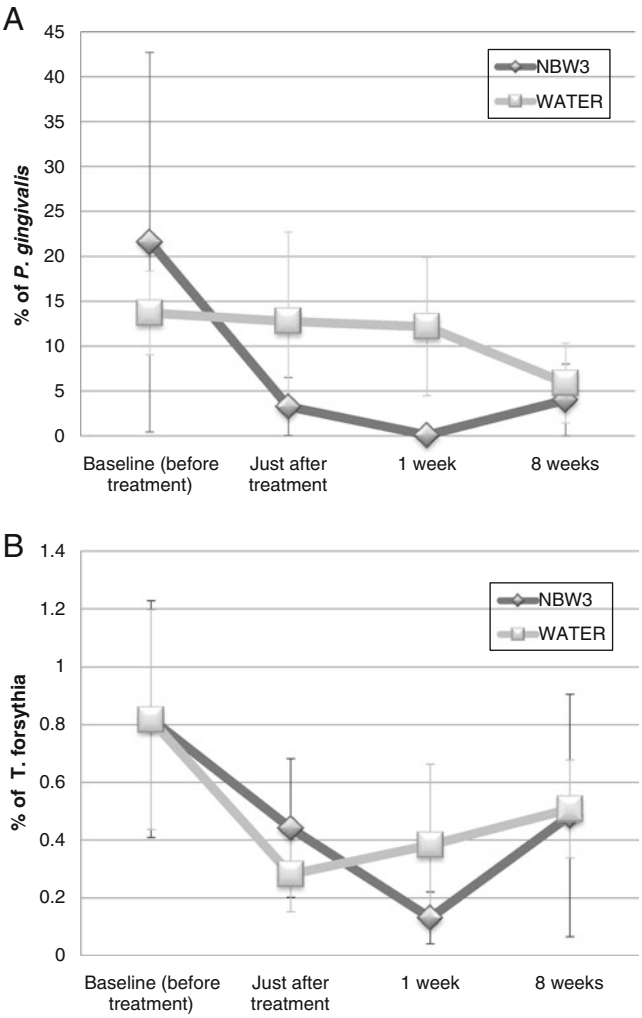

Fig. 5 Changes in the mean (± standard deviation) percentage of *P. gingivalis* (**a**) and *T. forsythia* (**b**) in subgingival plaque in each treatment group from baseline (before treatment) to 8 weeks. *NBW3* full-mouth debridement with ozone nano-bubble water, *WATER* full-mouth debridement with water. No significant differences were seen between groups at any examination period (p>0.05). No significant differences between baseline and any re-examination period were seen within both groups (p>0.05)

Table 4 Percentage of subgingival plaque samples showing >95 % reduction in the number of *P. gingivalis* and *T. forsythia*

		% of subgingival plaque samples showing >95 % reduction	
		P. gingivalis	*T. forsythia*
NBW3	Just after treatment	100	83.3
	1 week	100	50
	8 weeks	100	33.3
WATER	Just after treatment	83.3	88.9
	1 week	66.7	55.6
	8 weeks	50	33.3

NBW3 full-mouth debridement with ozone nano-bubble water, *WATER* full-mouth debridement with water

gas in the form of gas nucleus which is less than 100 nm in diameter and is produced by collapsing ozone micro-bubble (less or equal to 50 μm in diameter) in the electrolyte under ultra-high temperature and pressure. During the collapse of ozone micro-bubble in the electrolyte by applying physical stimulus, the ions in electrolyte become concentrated around the gas nucleus and prevent the ozone gas from dispersion (the salting-out phenomenon). Due to this nature of the ions, ozone nano-bubble is stabilized over a long period in aqueous solution. Therefore, NBW3 exerts antimicrobial effect for more than 6 months if it is protected against ultraviolet rays [32]. This stability is a great advantage of NBW3 over the existing ozonated water when used as a disinfecting solution. Originally, ozone is unstable and its stability in the existing ozonated water is very low. Ozone dissipates very quickly in ozone demand free water at room temperature over 5 min [37, 38]. Therefore, the existing ozonated water has to be freshly prepared before each use and it is impossible to market ozonated water as a medical product.

Although the entire mechanisms of NBW3 to inactivate bacteria are as yet not well known, it might be basically similar to those of the existing ozonated water. Ozone is a potent oxidizing agent [33], and ozone in ozonated water has been reported to react with various organic substances and decompose them by free radical-mediated oxidation reactions [34]. Ozone per se also changes to oxygen when it reacts with organic substances. In this process, hydroxyl radicals (·OH) are generated, which are among the most reactive oxidizing species. These free radicals might play a role in the destruction of bacteria by NBW3. Since there is no investigation regarding the detailed antimicrobial mechanisms of NBW3, close examinations must be progressed.

In the present single-blind randomized controlled clinical trial, mechanical therapy was completed in a single visit with an ultrasonic scaler running NBW3 or tap water as irrigant. This combined approach was to augment the effect of NBW3 against periodontopathic bacteria in subgingival biofilms. It is well known that serum proteins are essential compounds of subgingival bacterial biofilm matrix [39]. A recent study demonstrated that the efficacy of ozone might be decreased in the presence of human serum in vitro [40]. For this reason, the lower effectiveness of NBW3 against biofilm-associated bacteria could be assumed. In order to achieve optimal efficacy of NBW3, we performed mechanical (scaling and root planing) disruption of subgingival biofilm concurrently with NBW3 irrigation and allowed NBW3 to react directly with the planktonic microorganisms.

Previously, various antiseptic agents such as povidone-iodine, sodium hypochlorite, or chlorhexidine have been used as subgingival irrigants in conjunction with mechanical debridement to treat periodontitis patients. These previous studies demonstrated that periodontal sites with probing depths ≥7 mm benefited the most from the adjunctive local antimicrobial agent treatment [8]. However, in the present study, the results were obtained in adults with mild-to-moderate periodontitis. The percentage of pocket sites ≥7 mm was only 0.9 % in the NBW3 group (N=1,569 sites) and 0.4 % in the WATER group (N=1,775 sites). The present study might have underestimated the therapeutic potential of NBW3 because the study subjects exhibited only a mild form of periodontitis.

Moreover, no further application of the NBW3 was performed in the NBW3 group during the re-examination period in order to monitor the long-term effect of one-time irrigation with NBW3. However, the use of NBW3 for mouth rinsing during the re-examination period with the intention of delaying supragingival plaque formation and preventing recolonization of the pockets and oral niches might have supplemented patient-dependent mechanical plaque control and resulted in better clinical and microbiological outcomes in the NBW3 group.

Despite the above-mentioned limitations, the present study showed that the reductions in mean PPD from baseline to 4 and 8 weeks in the NBW3 group were significantly greater than those in the WATER group, with corresponding higher gain in attachment level in the NBW3 group. However, there were no statistically significant differences between the NBW3 and WATER groups in any of the clinical parameters assessed at any re-examination period.

Real-time PCR was employed for detection and quantification of the periodontopathic bacteria in this study. Real-time PCR assay was recently developed for the quantitative detection of bacteria and this has actually contributed to the diagnosis and monitoring of periodontal disease [41, 42].

In the present study, the NBW3 group showed statistically significant reductions in the mean total number of bacteria in subgingival plaque over the entire study period, whereas the WATER group did not show significant reductions at any re-examination period. Moreover, as many as 40 % (at 1 week) and 20 % (at 8 weeks) of subgingival

plaque samples in the NBW3 group revealed a reduction of least 95 % in the total number of bacteria, whereas only 9.1 % (at 1 week) and 0 % (at 8 weeks) of subgingival plaque samples in the WATER group exhibited similar bacterial suppression. Regarding each periodontal pathogen, both groups did not show significant reductions in the mean number and percentage of *P. gingivalis* and *T. forsythia* at any re-examination period (even immediately after treatment). This seems to be in contrast to previous reports, which had shown that non-surgical mechanical therapy could reduce the prevalence and levels of the main periodontal pathogens [43–45]. The lack of statistically significant reductions might be caused by the small sample size. The subjects initially negative for each pathogen were excluded from the statistical analyses; therefore, the sample size was 4 and 6 in the test and control groups for *P. gingivalis*, and 6 and 9 for *T. forsythia*, respectively. However, in the present study, *P. gingivalis* was reduced by at least 95 % in all the initially positive subgingival plaque samples over the study period in the NBW3 group, whereas the pathogen was reduced by more than 95 % only in 66.7 and 50 % of subgingival plaque samples at 1 and 8 weeks in the WATER group, respectively. Thus, mechanical subgingival debridement concurrently with NBW3 irrigation showed more potent and prolonged microbiological benefits than mechanical debridement with water. This additional reduction of the microbial load in subgingival pockets by NBW3 irrigation might lead to further improvement of clinical parameters (e.g., probing pocket depth, clinical attachment gain) in the NBW3 group compared to the WATER group in this study.

The disadvantage of the PCR technique is that it detects both viable and non-viable bacteria. If the non-viable microorganisms are detected following antimicrobial therapy, then the effectiveness of the antimicrobial agent is difficult to assess, i.e., prognostic value of PCR is compromised because it detects even non-viable organisms. In such case, the culture method is still the gold standard. Although only PCR was carried out in this study, the culture technique ought to have used further so that live bacterial estimation should be helpful [46].

Currently, ozone is attracting attention as a possible alternative antiseptic in the dental field because it is strongly antimicrobial and does not induce microbial resistance, characteristics that were originally noted in the fields of water purification and food preservation [14–16]. Recently, both gaseous and aqueous ozone have been reported to have antimicrobial activity against specific periodontal pathogens. It has been reported that ozone therapy reduced the growth of *A. actinomycetemcomitans*, *T. forsythia*, *Treponema denticola*, *P. gingivalis*, and *Prevotella intermedia*; however, no information was provided about application time or dose [23]. Moreover, high concentrations of aqueous ozone (20 μg/ml) has been reported to be equally or more effective in the reduction of periodontal pathogens than CHX in vitro [24]. Only two clinical studies reporting the use of aqueous ozone to treat gingivitis and periodontitis have been published [29, 30]. The periodontal pockets of 40 patients were irrigated with 10 ml of ozonated bi-distilled water, daily, for 4 weeks, resulting in an enhancement of the sulcus bleeding index, plaque index, and sulcus fluid rate, without any observed adverse effects [29]. The exact concentration of ozone in the water and the contact time were not reported. A recent clinical and microbiological study of 16 patients over an 18-day time period found a higher percentage reduction of several clinical indices (plaque, gingival, and bleeding indices) upon subgingival irrigation of periodontal pockets with ozonated water (0.082 mg/h, 5–10 min for a split mouth), when compared with 0.2 % CHX [30]. The antimicrobial results, however, were conflicting. Antimicrobial effects of ozonated water were observed against *A. actinomycetemcomitans* and *Candida albicans*, but no effect on *P. gingivalis* or *T. forsythia* was seen. Due to the difference of study design and application method of ozonated water, it would be difficult to compare the effects of the ozonated water used in that study with those of NBW3. It would be required to determine the antimicrobial activity of NBW3, in comparison with the existing ozonated water under the same condition. Furthermore, in proposing NBW3 as another potential antimicrobial for use in periodontal therapy, it is important to compare its potential with that of the established agents such as CHX or povidone-iodine.

In conclusion, the present study suggests that subgingival irrigation with NBW3 may be a valuable antimicrobial adjunct to mechanical instrumentation in the management of periodontal infections. The observed beneficial effects of NBW3 were, however, minor and of unknown clinical significance. Further studies with more severe cases of periodontitis, longer follow-up periods, and the comparison of NBW3 with the established agents would be needed to fully delineate the utility of NBW3 in the treatment of periodontitis.

Acknowledgments This work was supported by a grant-in-aid for scientific research (B) #18390561 to Y.I. and a grant-in-aid for scientific research (C) (2) #21592620 to S.A. from the Ministry of Education, Culture, Sports, Science, and Technology.

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

References

1. Dzink JL, Socransky SS, Haffajee AD (1988) The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. J Clin Periodontol 15:316–323

2. Ali RW, Velcescu C, Jivanescu MC, Lofthus B, Skaug N (1996) Prevalence of 6 putative periodontal pathogens in subgingival plaque samples from Romanian adult periodontitis patients. J ClinPeriodontol 23:133–139
3. Ashimoto A, Chen C, Bakker I, Slots J (1996) Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. Oral Microbiol Immunol 11:266–273
4. Haffajee AD, Socransky SS, Patel MR, Song X (2008) Microbial complexes in supragingival plaque. Oral Microbiol Immunol 23:196–205
5. Petersilka GJ, Ehmke B, Flemmig TF (2002) Antimicrobial effects of mechanical debridement. Periodontol 2000 28:56–71
6. Drisko CH (1996) Non-surgical pocket therapy: pharmacotherapeutics. Ann Periodontol 1:491–566
7. Greenstein G, Tonetti M (2000) The role of controlled drug delivery for periodontitis. The Research, Science and Therapy Committee of the American Academy of Periodontology. J Periodontol 71:125–140
8. Rams TE, Slots J (1996) Local delivery of antimicrobial agents in the periodontal pocket. Periodontol 2000 10:139–159
9. Krautheim AB, Jermann TH, Bircher AJ (2004) Chlorhexidine anaphylaxis: case report and review of the literature. Contact Dermatitis 50:113–116
10. Fleischer W, Reimer K (1997) Povidone-iodine in antisepsis—state of the art. Dermatology 195(Suppl 2):3–9
11. NobukuniK HN, Namba R et al (1997) The influence of long-term treatment with povidone-iodine on thyroid function. Dermatology 195(Suppl 2):69–72
12. Linder N, Davidovitch N, Reichman B et al (1997) Topical iodine-containing antiseptics and subclinical hypothyroidism in preterm infants. J Pediatr 131:434–439
13. Kim JG, Yousef AE, Dave S (1999) Application of ozone for enhancing the microbiological safety and quality of foods: a review. J Food Prot 62:1071–1087
14. Restaino L, Frampton EW, Hemphill JB, Palnikar P (1995) Efficacy of ozonated water against various food-related microorganisms. Appl Environ Microbiol 61:3471–3475
15. Unal R, Kim JG, Yousef AE (2001) Inactivation of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Lactobacillus leichmannii* by combinations of ozone and pulsed electric field. J Food Prot 64:777–782
16. Paraskeva P, Graham NJ (2002) Ozonation of municipal wastewater effluents. Water Environ Res 74:569–581
17. Baysan A, Whiley RA, Lynch E (2000) Antimicrobial effect of a novel ozone-generating device on micro-organisms associated with primary root carious lesions in vitro. Caries Res 34:498–501
18. Baysan A, Lynch E (2004) Effect of ozone on the oral microbiota and clinical severity of primary root caries. Am J Dent 17:56–60
19. Nagayoshi M, Kitamura C, Fukuizumi T, Nishihara T, Terashita M (2004) Antimicrobial effect of ozonated water on bacteria invading dentinal tubules. J Endod 30:778–81
20. Arita M, Nagayoshi M, Fukuizumi T, Okinaga T, Masumi S, Morikawa M et al (2005) Microbicidal efficacy of ozonated water against *Candida albicans* adhering to acrylic denture plates. Oral Microbiol Immunol 20:206–210
21. Hems RS, Gulabivala K, Ng YL, Ready D, Spratt DA (2005) An in vitro evaluation of the ability of ozone to kill a strain of *Enterococcus faecalis*. Int Endod J 38:22–29
22. Huth KC, Quirling M, Maier S, Kamereck K, Alkhayer M, Paschos E et al (2009) Effectiveness of ozone against endodontopathogenic microorganisms in a root canal biofilm model. Int Endod J 42:3–13
23. Bezrukova IV, Petrukhina NB, Voinov PA (2005) Experience in medical ozone use for root canal treatment. Stomatologiia (Mosk) 84:20–22
24. Huth KC, Quirling M, Lenzke S, Paschos E, Kamereck K, Brand K et al (2011) Effectiveness of ozone against periodontal pathogenic microorganisms. Eur J Oral Sci 119:204–210
25. Huth KC, Jakob FM, Saugel B, Cappello C, Paschos E, Hollweck R et al (2006) Effect of ozone on oral cells compared with established antimicrobials. Eur J Oral Sci 114:435–440
26. Filippi A (2001) The effects of ozonized water on epithelial wound healing (in German). Deutsche Zahnärztliche Zeitschrift 56:104–108
27. Ebensberger U, Pohl Y, Filippi A (2002) PCNA-expression of cementoblasts and fibroblasts on the root surface after extraoral rinsing for decontamination. Dent Traumatol 18:262–266
28. Nagayoshi M, Fukuizumi T, Kitamura C, Yano J, Terashita M, Nishihara T (2004) Efficacy of ozone on survival and permeability of oral microorganisms. Oral Microbiol Immunol 19:240–246
29. Brauner A (1991) Clinical studies of therapeutic results from ozonized water for gingivitis and periodontitis. Zahnarztl Prax 42:48–50
30. Kshitish D, Laxman VK (2010) The use of ozonated water and 0.2 % chlorhexidine in the treatment of periodontitis patients: a clinical and microbiologic study. Indian J Dent Res 21:341–348
31. Chiba K, Sugai T. Functional water production method. JP, 2010-167365, A. 2010-08-05
32. Grebenshchikov SY, Qu ZW, Zhu H, Schinke R (2007) New theoretical investigations of the photodissociation of ozone in the Hartley, Huggins, Chappuis, and Wulf bands. Phys Chem Chem Phys 9:2044–2064
33. Broadwater WT, Hoehn RC, King PH (1973) Sensitivity of three selected bacterial species to ozone. Appl Microbiol 26:391–393
34. Staehelin J, Hoigne J (1985) Decomposition of ozone in water in the presence of organic solutes acting as promoters and inhibitors of radical chain reactions. Environ Sci Technol 19:1206–1213
35. Haffajee AD, Socransky SS (1994) Microbial etiological agents of destructive periodontal diseases. Periodontol 2000 5:78–111
36. Slots J, Feik D, Rams TE (1990) Prevalence and antimicrobial susceptibility of *Enterobacteriaceae*, *Pseudomonadaceae* and *Acinetobacter* in human periodontitis. Oral Microbiol Immunol 5:149–154
37. Wilson M (1996) Susceptibility of oral bacterial biofilms to antimicrobial agents. J Med Microbiol 44:79–87
38. Shechter H (1973) Spectrophotometric method of determination of ozone in aqueous solutions. Water Res 7:729–739
39. Kuboniwa M, Lamont RJ (2010) Subgingival biofilm formation. Periodontol 2000 52:38–52
40. Eick S, Tigan M, Sculean A (2012) Effect of ozone on periodontopathogenic species—an in vitro study. Clin Oral Investig. doi:10.1007/s00784-011-0515-1
41. Maeda H, Fujimoto C, Haruki Y et al (2003) Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillusactinomycetemcomitans*, *Porphyromonasgingivalis*, *Prevotellaintermedia*, *tet*Q gene and total bacteria. FEMS Immunol Med Microbiol 39:81–86
42. Yoshida A, Suzuki N, Nakano Y, Kawada M, Oho T, Koga T (2003) Development of a 5′ nuclease-based real-time PCR assay for quantitative detection of cariogenic dental pathogens *Streptococcus mutans* and *Streptococcus sobrinus*. J Clin Microbiol 41:4438–4441
43. Haffajee AD, Cugini MA, Dibar S, Smith C, Kent RL, Socransky SS (1997) The effect of SRP on the clinical and microbiological parameters of periodontal diseases. J Clin Periodontol 24:324–334
44. Ali RW, Lie T, Skaug N (1992) Early effects of periodontal therapy on the detection frequency of four putative periodontal pathogens in adults. J Periodontol 63:540–547
45. Shiloah J, Patters MR (1994) DNA probe analyses of the survival of selected periodontal pathogens following scaling, root planing, and intra-pocket irrigation. J Periodontol 65:568–575
46. Boutaga K, van Winkelhoff AJ, Vandenbroucke-Grauls CM, Savelkoul PH (2003) Comparison of real-time PCR and culture for detection of *Porphyromonas gingivalis* in subgingival plaque samples. J Clin Microbiol 41:4950–4954