Journal of
Periodontology
&
Implant Dentistry

Research Article

Efficacy of Photodynamic Therapy as an Adjunct to Full-mouth Root Planing in the Treatment of Periodontitis Assessed by Real-time PCR: A Microbiological and Clinical Study

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Received: 19 December 2014; Accepted: 21 March 2015
J Periodontal Implant Dent 2015; 7(1):26–32 | doi: 10.15171/jpid.2015.005
This article is available from: http://dentistry.tbzmed.ac.ir/jpid

Abstract

Background and aims. The aim of this investigation was to compare clinical and microbiological effectiveness of adjunctive photodynamic therapy (PDT) in the treatment of periodontitis.

Materials and methods. Twenty-four subjects (14 women and 10 men) diagnosed with moderate to severe chronic periodontitis underwent scaling and root planing (SRP). One tooth in each quadrant (probing depth >4 mm) was selected for combined PDT and SRP (PDT group) with the contralateral tooth (SRP group), as a control site (SRP-treated site). Clinical assessment was carried out at baseline and 1 and 3 month later. Microbial assessment was carried out by real-time PCR. Periodontal probing depth (PPD) was considered as the primary outcome.

Results. Baseline PPDs were 4.86±0.77 and 4.04±0.65 in the SRP and PDT groups, respectively (P>0.05), which decreased to 3.65±0.58 in the SRP and 3.86±0.56 in the PDT groups after one month and to 3.20±0.68 in the SRP and 3.34±0.56 in the PDT groups three months later. Although values decreased significantly in both groups after one (P=0.001 in the SRP and P=0.001 in the PDT groups) and three months (P=0.001 in the SRP and P=0.001 in the PDT groups) the inter-group differences were not significant after one (P=0.25) and three months (P=0.51). Clinical measurements showed significant decreases after one and three months at both sites, without inter-group differences, except for bleeding on probing after one (P=0.004) and three months (P=0.0001).

Conclusion. Subgingival application of PDT combined with scaling and root planing could not improve clinical and microbiological results.

Key words: **Chronic periodontitis, photochemotherapy, periodontal disease/therapy, randomized controlled trial.**

Introduction

Periodontitis is a chronic inflammatory disease of tooth and its supporting structures with clinical signs of bone and connective tissue loss and is mediated by combination of periodontal pathogens and host defense system.[1]

Aggregatibacter actinomycetemcomitans (*A.a.*), *Porphyromonas gingivalis* (*P.g.*) and Tannerella forsythensis (*T.f.*) are known as the main pathogens of periodontal diseases and treatment of periodontal disease is associated with successful removal and reduction of these microorganisms.[2-4]

The main treatment for periodontal disease is nonsurgical treatment by scaling and root planing as a gold standard treatment of periodontitis.[5] However, some patients may not respond favorably to nonsurgical treatment. This can be due to recolonization and reinfection of microorganisms remaining in soft and hard tissues. Other reasons are associated with difficulty of access to deep periodontal pockets, furcation areas and root concavities.[6,7]

In these clinical situations numerous novel approaches have been utilized as a mono therapy or as an adjunct to scaling and root planing in order to improve effectiveness of nonsurgical therapy and achieving long-term periodontal health, especially in patients who do not adequately respond to conventional scaling and root planing. In this field, application of local and systemic antibiotics and different laser systems have been proposed and utilized in recent years.[8,9]

Photodynamic therapy (PDT) was introduced as a medical therapy for inactivation of microorganisms on the basis of photosensitizer attachment to target cells. PDT can be activated by a suitable wavelength of light,[10] but even broad-spectrum light can activate photosensitizers such as toluidine blue.[11] Established photosensitizers such as toluidine blue have been reported to be antibacterial, antiviral and antiprotozoal since World War II.[12] Toluidine blue has been shown to be highly effective when used with a soft laser irradiation.[13-15]

Photodynamic therapy uses two components including a visible light source of a specific wavelength and a dye or photosensitizer (PS) that binds to the target cell and is activated by a light source. The resulting reaction induces cell death due to the production of toxic oxygen species, like singlet oxygen and free radicals. Susceptibility of microorganism to photodynamic therapy have been shown in different studies. However, there is no conclusive evidence to confirm other effects of this technique in destroying periodontal pathogens in human studies and many of the results are controversial, requiring more studies in this field.[11,16]

This clinical and microbiological study used real-time PCR monitoring to answer the question whether topical application of photodynamic therapy as an adjunct to scaling and root planing can improve the effectiveness of scaling and root planing or not.

Materials and Methods

Patient Selection

A study by Panos[17] was used to calculate the sample size at an error of 5%, power of 80% and an SD of 0.07 mm of CAL (primary outcome); a difference of 0.2 mm between the groups was considered clinically significant. It was indicated that a sample of 12 patients per group would be needed. Twenty-four subjects (14 women and 10 men) diagnosed with localized or generalized moderate to severe chronic periodontitis were included in the study in the Department of Periodontics, Faculty of Dentistry, Tabriz University of Medical Sciences, Iran. All the subjects submitted their informed consent. The patients were enrolled in a longitudinal study, lasting from October 2011 to October 2012. The study design was approved by the Ethics Committee and supported by Tabriz Dental and Periodontal Research Center and also registered in the Iranian Registry of Clinical Trials (IRCT) under the code IRCT2012070210155N1. The nature of this investigation was explained to the participants in detail and the patients signed an informed consent form. The participants with the following inclusion criteria were included in the study: chronic periodontitis, no active periodontal treatment during the past 6 months, presence of at least one site per quadrant exhibiting pocket depth of ≥4 mm with bleeding on probing, no use of antibiotics for the past 12 months. The exclusion criteria consisted of smokers, pregnant or lactating women, subjects with any systemic condition that might influence the course of periodontal disease or treatment (HIV/AIDS, uncontrolled diabetes) and subjects with any active malignancy of any sort.

Treatment

Before any nonsurgical treatment, subgingival plaque sampling was carried out. One of the teeth in one quadrant with probing depth of >4 mm was selected for plaque sampling. The selected tooth was isolated by sterile cotton rolls and dried gently by air. Supragingival plaque was removed carefully by sterile periodontal curettes. After that a sterile paper point

#40 was inserted in the deepest part of the pocket and remained there for 60 seconds. Then the paper point was removed from the pocket and pooled in the transportation vial (Thin Prep-PAP TEST- Hologic, Inc UK) and sent immediately for DNA real-time PCR. Quantification of *Porphyromonas gingivalis* was carried out by Primer Dedign^{TM} genesig KIT. The *fimbbrillinfim A* (I) gene, which has been identified as a highly specific marker for *P. gingivalis* was used to detect and quantify *P. gingivalis* genome.[16] Real time PCR analysis included the following:

DNA Extraction

A total of 4 µL of internal extraction control DNA was added to each sample in DNA lysis/extraction buffer per sample and completed the extraction according to the manufacturer's protocol.

Real-time PCR Detection

The reaction mix was prepared that included sufficient reactions for the standard curve wells (6 samples in duplicate) and also for the negative control. A total of 15 µL of this mix was pipetted into each well according to manufacturer's real-time PCR experimental plate setup. Sample DNA templates for each of the samples (suggested concentration of 5 ng/µL) in RNAse/DNAse free water was prepared. A total of 5 µL of diluted DNA template was pipetted into each well, according to the experimental plate setup and standard curve dilution series were prepared. A total of 5 µL of standard template was pipetted into each well, according to the experimental plate setup. Amplification conditions used Primer Design 2X Precision TM Master Mix.

Measurements

Measurement of clinical parameters was carried out by a single examiner blinded to the treatment allocation of each patient. Clinical parameters of probing depth (PD), clinical attachment level (CAL), bleeding on probing (BOP) and gingival recession (REC) were measured at baseline and one month and three months after treatment. O'Leary plaque index was used as a control variable. Positive BOP was measured in terms of the presence of bleeding 30 seconds after gentle probing of four sites of each tooth sulcus or pocket and BOP score in these experimental teeth was measured. Quantitative measurement of *Porphyromonas gingivalis* was carried out at baseline and three months after treatment. All the clinical parameters were measured with manual Williams periodontal probe (PWD, Hu-Friedy Immunity, USA). Randomization envelope was opened and each tooth in one quadrant was randomized to one of the two treatment modalities: Photodynamic therapy combined with SRP (PDT + SRP) or SRP alone.

Alginate impressions were taken from the upper or lower teeth of each patient in order to customize splints. Splints were made precisely to ensure close adaptation with teeth and simplify reproducibility of measurements. In addition, a groove was made at the site of the pockets to dictate the placement of the periodontal probe in the same place during the examination period.

At the beginning, all the patients received mechanical nonsurgical therapy by a sonic scaler (Varios 350. NSK, Japan). After the experimental site was isolated by cotton rolls, tolonium chloride (Cumdente GmbH, Germany) was equipped with a blunt needle, applied by walking movement in the entire pocket space and remained there for 60 seconds. The laser unit (Handy Laser Sprint, USA, FDA approved) was equipped with a flexible applicator tip (PACT, Endo tip 0.04 mm) and applied in the deepest part of the pocket, followed by irradiated with the specific wavelength of 638 nm and an energy density of 8–10 j/cm^2 for 120 seconds (Figure 1).

No additional procedure was carried out on the con-

Figure 1. Application of tolonium chloride in the deepest part of the pocket (left), followed by laser irradiation (right).

tralateral tooth. Calibration exercise was performed to obtain acceptable intra-examiner reproducibility for probing depth. Prior to the study and after 3 months, five patients, each with ten teeth with probing depth of >5 mm on at least one aspect of each tooth, were used for calibration. The examiner evaluated the patients on two occasions 48 hours apart. Calibration was accepted if >90% of the recording could be reproduced within a 1.0-mm difference. The mean of intra-examiner Kappa score value was 0.73 for assessment of PD, when PD=5 mm served as the cut-off point.

Statistical Analysis

Because the normality of the data was not confirmed by Kolmogorov-Smirnov test, non-parametric test was used to analyze data. Data were analyzed using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). Significance of difference of clinical parameters within groups before and after treatment was evaluated by Friedman test. In addition, Wilcoxon test was used for significance of differences in pathogens before and after treatment. Mann-Whitney test was used to compare the clinical and microbiological parameters in each group. Statistical significance was set at $P<0.05$.

Results

Of 24 patients enrolled in the study, two patients did not attend the follow-up sessions and were excluded from the study. Twenty-two patients (12 females and 10 males) with a mean age of 46.1 years completed the study. No adverse reactions from both interventions were reported by patients.

Probing depth was the primary outcome. According to Table 1, at baseline PPD was 4.86±0.77 in the SRP group and 4.04±0.65 in the gel group ($P>0.05$). One month after treatment these values decreased to 3.65±0.58 in the SRP group and 3.86±0.56 in the PDT group and three month later, to 3.20±0.68 in the SRP group and 3.34±0.56 in the PDT group. Although the values decreased significantly in both groups after one ($P=0.001$ in the SRP group and $P=0.001$ in the PDT group) and three month ($P=0.001$ in the SRP group and $P=0.001$ in the PDT group) the inter-group differences were not statistically significant one month ($P=0.25$) and three months ($P=0.51$) after baseline.

Secondary outcomes were CAL, BOP, REC and microbial evaluation. Clinical measurements of CAL, REC and BOP in the treated sites are shown in Table 2. All of these clinical measurements showed significant decreases after one month and three months in both treated sites (Table 2). No statistically significant differences were observed between the two treated sites in terms of CAL and REC during both follow-up periods.

The only clinical parameter that showed statistically significant inter-group differences was BOP three months ($P=0.0001$) after baseline (Figure 2) in the PDT group compared to the SRP group.

Discussion

Nonsurgical periodontal therapy, consisting of scaling and root planing, has been confirmed as a gold standard in periodontal therapy and this treatment is particularly critical in the esthetic zone.[5]

Numerous novel approaches have been utilized as a mono therapy or as an adjunct to scaling and root planing in order to improve the efficacy of nonsurgical therapy and achieve long-term periodontal health, with different results being reported in this field.[8,9]

Photodynamic therapy has been utilized as an adjunctive to scaling and root planing, but it has not been shown to yield superior results compared to scaling and root planing alone and considering that human studies are limited, there are not sufficient data to confirm its superiority.

This clinical and microbiological study was conducted by monitoring real-time PCR to answer the question whether topical application of PDT as an adjunct to scaling and root planing can improve the effectiveness of scaling and root planing.

The results of the current study showed that the probing depth decreased significantly in both groups one month and three months after baseline but inter-group differences were not significant, consistent with the results of a study of Polansky et al, who did not report significant differences in probing pocket depth between the SRP group and the PDT–SRP group three months after baseline, although the PPD reduction in the test group was higher than that in the control group.[19] Similar results were reported by Yilmaz et al after 3 and 6 months.[20]

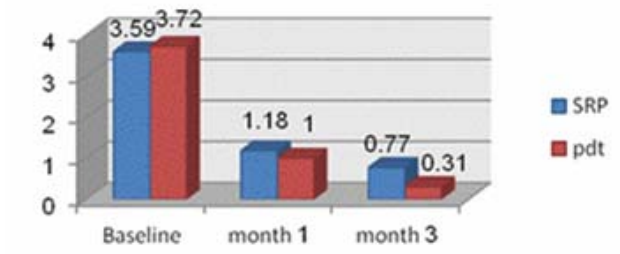

Figure 2. Mean levels of BOP at the treated sites at baseline and one month and three months after treatment.

Table 1. Clinical assessment of PPD in the treated sites

Parameter	baseline	at 1 month	at 3 month	Difference 0-1 month	p	Difference 0-3 month	p
PPD (mm)							
PDT + SRP	4.04 ± 0.65	3.86 ± 0.56	3.34 ± 0.56	1.18	0.001	1.7	0.001
SRP	4.86 ± 0.77	3.65 ± 0.58	3.20 ± 0.68	1.21	0.001	1.66	0.001
P- value	0.281	0.258	0.510				

Table 2 . Clinical assessment of, CAL, REC, BOP at treated sites.

Parameter	baseline	at 1 month	at 3 month	Difference 0-1 month	P	Difference 0-3 month	
CAL (mm)							
PDT + SRP	4.00 ± 0.53	3.65 ± 0.49	3.31 ± 0.54	0.35	0.001	0.69	0.001
SRP	3.93 ± 0.56	3.63 ± 0.49	3.4 ± 0.59	0.3	0.001	0.53	0.001
P value	0.454	0.951	0.759				
REC (mm)							
PDT + SRP	-0.86 ±0.58	-0.06 ± 0.54	0.18 ± 0.60	0.8	0.001	-0.92	0.001
SRP	-0.79 ± 0.57	-0.09 ± 1.18	0.38 ± 0.53	-0.7	0.001	-1.17	0.001
p-value	0.582	0.273	0.241				
BOP							
PDT + SRP	3.72 ± 0.45	1.00 ±0.69	0.31 ± 0.476	2.72	0.001	3.41	0.001
SRP	3.59 ± 0.5	1.18 ± 0.66	0.77 ± 0.42	2.41	0.001	2.82	0.001
p-value	.346	.257	.003				

On the other hand, Anderson et al reported significant decreases in PPD with the use of PDT as an adjunct to SRP.[21] Similar results have been reported by Christodoulides et al after 3 and 6 months.[22]

One of the reasons for insignificant inter-group PPD reduction is good plaque control by the patients. In this study, except for 2 patients with poor oral hygiene, the remaining patients had good plaque control, with a plaque index less than 10%. Therefore, administration of PDT in patients with good plaque control and healthy patients with no systemic disease can be questioned as it could not have additional benefits over SRP. As a result, monitoring the application of PDT can be focused on patients with compromised cooperation, like compromised healthy patients, elderly patients, and also in more severe forms of periodontitis, like aggressive periodontitis or periodontitis modified by systemic factors.

In the present study BOP reduction in the PDT group was significantly higher than that in the control group three months after the study, consistent with the results of a study by Christodialides et al, in which significant differences were observed in BOP reduction in the PDT–SRP group three and six months after baseline, compared to the SRP group.[22] Similar findings were reported by Braun et al.[23]

In spite of the fact that positive BOP is not indicative of periodontal disease, negative BOP is an excellent predictor of periodontal health.[19] Since positive BOP in multiple examinations with increasing probing depth is correlated with the progression of periodontitis,[19] controlling gingival inflammation by maintaining pockets at least in a healthy condition can be a successful treatment option, especially in patients in which periodontal surgery is contraindicated. The positive results of PDT on BOP reduction may provide some advantages to achieve this goal. No adverse reaction was observed from PDT. It is of crucial importance because it can facilitate its use in clinical practice. Moreover, it does not have some of the disadvantages of systemic antibiotics, such as the emergence of resistant bacteria or GI disturbances.[25,26]

Considering that periodontal disease is caused by disequilibrium between dental plaque and host defense system, monitoring the number of pathogens like *P. gingivalis* is necessary before and after treatment.[27-30] Detection of pathogens is influenced by detection methods. Real-time polymerase chain reaction by double fluorescent probes provides precise quantification of bacteria and is one of the most accurate technologies in this field.[30-32]

In the current study, *P. gingivalis* counts decreased significantly after three months, with no significant differences between the two groups. In a study by Chondros[22] *P. gingivalis* counts decreased three months after treatment but intra-group differences were not significant. In a study by Polanski et al, *P. gingivalis* counts decreased in both PDT and SRP groups, without significant inter-group differences. Conventional PCR in this study provided endpoint measurement or qualitative measurements and was not able to quantify the number of bacteria, which is one of the limitations of conventional PCR compared to real-time PCR used in this study.[33-35]

Although the susceptibility of periodontal pathogens to photodynamic therapy has been shown in

vivo, its bactericidal effects in human studies is not well established and there is limited data in this field.

Conclusion

Within the limitations of the current study, subgingival application of photodynamic therapy combined with scaling and root planing may reduce bleeding of periodontal pockets. Other clinical and microbiological parameters were not different between the two groups. It is suggested that clinical trials be designed to evaluate the efficacy of this gel in aggressive periodontitis and severe periodontitis modified by systemic factors.

Reference

1. Page RC, Offenbacher S, Schroeder HE, Seymour GJ, Kornman KS. Advances in the pathogenesis of periodontitis: summary of developments, clinical Implications and future directions. *Periodontol* 2000 1997;14:216-48. doi: 10.1111/j.1600-0757.1997.tb00199.x
2. Slots J, Rams TE. New views on periodontal microbiota in special patient categories. *J Clin Periodontol* 1991;18:411–20. doi: 10.1111/j.1600-051x.1991.tb02309.x
3. Socransky SS, Haffajee AD. The bacterial etiology of destructive periodontal disease: current concepts. *J Periodontol* 1992;63:322–31. doi: 10.1902/jop.1992.63.4s.322
4. Cugini MA, Haffajee AD, Smith C, Kent RL Jr, Socransky SS. The effect of scaling and root planing on the clinical and microbiological parameters of periodontal diseases: 12-month results. *J Clin Periodontol* 2000; 27:30-6.
5. Loesche WJ, Gossman NS. Periodontal disease as a specific, albeit chronic, infection: diagnosis and treatment. *Clinical Microbiology Reviews* 2002;14:727-52. doi: 10.1128/cmr.14.4.727-752.2001
6. Kaldahl WB, Kalkwarf KL, Patil KD, Molvar MP. Relationship of gingival bleeding, gingival suppuration, and supragingival plaque to attachment loss. *J Periodontol* 1990;61:347–51. doi: 10.1902/jop.1990.61.6.347
7. Adriaens PA, De Boever JA, Loesche WJ. Bacterial invasion in root cementum and radicular dentin of periodontally diseased teeth in humans. A reservoir of periodontopathic bacteria. *J Periodontol* 1988;59:222–30. doi: 10.1902/jop.1988.59.4.222
8. Cobb CM. Non-surgical pocket therapy: mechanical. *Ann Periodontol* 1996:443-90. doi: 10.1902/annals.1996.1.1.443
9. Socransky SS, Haffajee AD. Dental biofilms: difficult therapeutic targets. *Periodontol 2000* 1996;28:12-55. doi: 10.1034/j.1600-0757.2002.280102.x
10. Slots J. Selection of antimicrobial agents in periodontal therapy. *J Periodontal Res* 2002;37:389-98. doi: 10.1034/j.1600-0765.2002.00004.x
11. Azarpazhooh A, Shah PS, Tenenbaum HC, Goldberg MB. The effect of photodynamic therapy for periodontitis: a systematic review and meta-analysis. *J Periodontol* 2010;81:4–14. doi: 10.1902/jop.2009.090285
12. Peng Q, Moan J, Nesland JM. Correlation of subcellular and intratumoral photosensitizer localization with ultrastructural features after photodynamic therapy. *Ultrastruct Pathol* 1996;20:109-29. doi: 10.3109/01913129609016306
13. Dobson J, Wilson M. Sensitization of oral bacteria in biofilms to killing by light from a low-power laser. *Arch Oral Biol* 1992;37:883-7. doi: 10.1016/0003-9969(92)90058-g
14. Wilson M, Burns T, Pratten J, Pearson GJ. Bacteria in supragingival plaque samples can be killed by low-power laser light in the presence of a photosensitizer. *J Appl Bacteriol* 1995;78:569-74. doi: 10.1111/j.1365-2672.1995.tb03101.x
15. Shibli JA, Martins MC, Nociti FH, Jr., Garcia VG, Marcantonio E, Jr. Treatment of ligature-induced peri-implantitis by lethal photosensitization and guided bone regeneration: a preliminary histologic study in dogs. *J Periodontol* 2003;74:338-45. doi: 10.1902/jop.2003.74.3.338
16. Sharman WM, Allen CM, van Lier JE .Photodynamic therapeutics: basic principles and clinical applications. *Drug Discov Today* 1999;4:507–17. doi: 10.1016/s1359-6446(99)01412-9
17. Panos C, Dimitris N, Nicos C, Ralf, Norbert G, Anton S. Photodynamic therapy as adjunct to non-surgical periodontal treatment in patients on periodontal maintenance: a randomized controlled clinical trial. *Lasers in Medical Science* 2009;24:681-6. doi: 10.1007/s10103-008-0565-z
18. Quantification of *Porphyromonas gingivalis* fimbrilin Fimbrillinfim A (I) gene for general laboratory and research use only Standard kit handbook. available from: http://www.primerdesign.co.uk/Download%20material/P.gingivalis-std.pdf
19. Polansky R, Haas M, Bertha G, Wimmer G. Clinical effectiveness of photodynamic therapy in the treatment of periodontitis. *J Clin Periodontol* 2009;36:575-80. doi: 10.1111/j.1600-051x.2009.01412.x
20. Yilmaz S, Kuru B, Kuru L, Noyan U, Argun D, Kadir T. Effect of gallium arsenide diode laser on human periodontal disease: a microbiological and clinical study. *Lasers Surg Med* 2002;30,60-6. doi: 10.1002/lsm.10010
21. Andersen R, Loebel N, Hammond D, Wilson M. Treatment of periodontal disease by photodisinfection compared to scaling and root planing. *J Clin Dent* 2007;18,34-8.
22. Christodoulides N, Nikolidakis D, Chondros P, Becker J, Schwarz F, Rössler R, et al. Photodynamic therapy as an adjunct to nonsurgical periodontal treatment: a randomized, controlled clinical trial. *J Periodontol* 2008;79,1638-44.
23. Braun A, Dehn C, Krause F, Jepsen S. Short-term clinical effects of adjunctive antimicrobial photodynamic therapy in periodontal treatment: a randomized clinical trial. *J Clin Periodontol* 2008;35:877-84. doi: 10.1111/j.1600-051x.2008.01303.x
24. Armitage GC. Periodontal diseases: diagnosis. *Ann Periodontal* 1996;1:37-215.
25. Preshaw PM. Antibiotics in the treatment of periodontitis. *Dent Update* 2004;31:448-50, 453-4, 456.
26. Baltacioglu E, Aslan M, Saraç Ö, Saybak A, Yuva P. Analysis of clinical results of systemic antimicrobials combined with nonsurgical periodontal treatment for generalized aggressive periodontitis - a pilot study: *J Can Dent Assoc* 2011; 77:b97.
27. Eick S, PfisterW. Comparison of microbial cultivation and a commercial PCR based method for detection of periodontopathogenic species in subgingival plaque samples. *J Clin Periodontol* 2002;29:638–44. doi: 10.1034/j.1600-051x.2002.290708.x
28. Loesche WJ, Lopatin DE, Stoll J, van Poperin N, Hujoel PP. Comparison of various detection methods for periodontopathic bacteria: can culture be considered the primary reference standard? *J Clin Microbiol* 1992;30:418–26.

29. Socransky SS, Haffajee AD, Smith C, Dibart S. Relation of counts of microbial species to clinical status at the sampled site. *J Clin Periodontol* 1991;18:766–75. doi: 10.1111/j.1600-051x.1991.tb00070.x
30. Nonnenmacher C, Dalpke A, Mutters R, Heeg K. Quantitative detection of periodontopathogens by real time PCR. *J Microbiol Methods* 2004;59:117-25. doi: 10.1016/j.mimet.2004.06.006
31. Boutaga K, van Winkelhoff A J, Vandenbroucke-Grauls CM, Savelkoul PH. The additional value of real-time PCR in the quantitative detection of periodontal pathogens. *J Clin Periodontol* 2006;33:427-33. doi: 10.1111/j.1600-051x.2006.00925.x
32. Nonnenmacher C, Dalpke A, Rochon J, Flores-de-Jacoby L, Mutters R, Heeg K. Real-time polymerase chain reaction for detection and quantification of bacteria in periodontal patients. *J Periodontol* 2005;76:1542-9. doi: 10.1902/jop.2005.76.9.1542
33. Soskolne WA, Heasman PA, Stabholz A, Smart GJ, Palmer M, Flashner M, et al. Sustained local delivery of chlorhexidine in the treatment of periodontitis: a multi-center study. *J Periodontol* 1997;68:32-8. doi: 10.1902/jop.1997.68.1.32
34. Löe H, Schiott CR. The effect of mouth rinses and topical application of chlorhexidine on the development of dental plaque and gingivitis in man. *J Periodontal Res* 1970;5:79-83. doi: 10.1111/j.1600-0765.1970.tb00696.x
35. Quirynen M, Avontroodt P, Peeters W, Pauwels M, Coucke W, van Steenberghe D. Effect of different chlorhexidine formulations in mouth rinses on de novo-plaque formation. *J Clin Periodontol* 2001;28:1127-36. doi: 10.1111/j.1600-051x.2001.281207.x