Infection and Drug Resistance

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

Expression patterns of *oxyR* induced by oxidative stress from *Porphyromonas gingivalis* in response to photo-activated disinfection

This article was published in the following Dove Press journal:
Infection and Drug Resistance

Maryam Pourhajibagher[1–3]
Roghayeh Ghorbanzadeh[4]
Abbas Bahador[1,3,5]

[1]Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran; [2]Dental Implant Research Center, Dentistry Research Institute, Tehran University of Medical Sciences, Tehran, Iran; [3]Dental Research Center, Dentistry Research Institute, Tehran University of Medical Sciences, Tehran, Iran; [4]Private Practice, Tehran, Iran; [5]Laser Research Center, Dentistry Research Institute, Tehran University of Medical Sciences, Tehran, Iran

Introduction: *Porphyromonas gingivalis*, an important endodontic pathogen, may be exposed to sublethal doses of photo-activated disinfection (sPAD) during root canal therapy. Such an exposure can affect bacterial survival and virulence features. In this study, we evaluated the effect of sPAD-related oxidative stresses on the expression of oxidative stress response gene (*oxyR*) in *P. gingivalis* clinical isolates surviving in vitro photodynamic treatment.

Materials and methods: To determine the sPAD, 16 clinical *P. gingivalis* isolates photosensitized with toluidine blue O (TBO), methylene blue (MB), and indocyanine green (ICG) were irradiated with specific wavelength and energy density of diode laser corresponding to the photosensitizers following bacterial viability measurements. The effects of sPAD on the expression ratio of *oxyR* of 16 clinical *P. gingivalis* isolates were then assessed using quantitative real-time PCR (qRT-PCR) assay.

Results: Maximum values of sPAD against *P. gingivalis* were 6.25, 15.6, and 25 µg/mL at fluencies of 171.87, 15.6, and 93.75 J/cm^2, respectively, for TBO-, ICG-, and MB-sPAD (P>0.05). ICG-, MB-, and TBO-sPAD could increase the *oxyR* gene expression of the clinical *P. gingivalis* isolates 12.3-, 5.6-, and 8.5-fold, respectively. ICG-sPAD increased the expression of *oxyR* gene in clinical isolates of *P. gingivalis* ~1.5- and 2-fold higher than TBO- and MB-sPAD, respectively.

Conclusion: Our results showed that upregulation of *oxyR* during sPAD may lead to better survival and increased pathogenicity of *P. gingivalis* isolates. Therefore, selection of appropriate photo-activated disinfection dosage should be considered for the successful treatment of endodontic infection.

Keywords: antimicrobial photodynamic therapy, indocyanine green, methylene blue, oxidative stress, *Porphyromonas gingivalis*, toluidine blue O, low-level laser therapy

Introduction

Infected root canals are a unique niche for colonization of endodontic pathogens.[1] *Porphyromonas gingivalis* as a Gram-negative anaerobic bacterium is one of the most prominent endodontic pathogens.[2] *P. gingivalis* in the infected root canals can form biofilms consisting of a complex network with other microorganisms.[3] Additionally, *P. gingivalis* has a sophisticated regulation system that protects the bacteria against harsh environments. In these circumstances, oxidative stress response gene (*oxyR*) encodes a tetrameric DNA-binding protein that is known to be regulated under oxidative stresses including reactive oxygen species (ROS) and is an important virulence factor for the survival of *P. gingivalis* against oxidative stresses in the root canal system. On the other hand, the expression of *oxyR* is essential for the aerotolerance of *P. gingivalis* and the regulation of other major virulence factors such as biofilm formation in this bacterium.

Correspondence: Abbas Bahador
Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Keshavarz Blvd, 100 Poursina Avenue, Tehran 14167-53955, Iran
Tel +98 216 405 3210
Fax +98 218 895 5810
Email abahador@sina.tums.ac.ir

submit your manuscript | www.dovepress.com
Dovepress
http://dx.doi.org/10.2147/IDR.S152834

Photo-activated disinfection (PAD), also known as antimicrobial photodynamic therapy, is an effective adjuvant treatment strategy in eliminating endopathogenic microorganisms.[4] The principle of PAD is based on a nontoxic photosensitizer which is activated by a specific wavelength of light.[5] The photosensitizers such as toluidine blue O (TBO), methylene blue (MB), and indocyanine green (ICG) are used for inactivation and eradication of both Gram-positive and Gram-negative oral bacteria in the presence of specific corresponding wavelengths of light due to production of cytotoxic ROS, such as the superoxide radical (O2·), hydroxyl radical (HO·), hydrogen peroxide (H_2O_2), and oxides of nitrogen that pose a significant threat to cell integrity and gene expression.[6]

Endodontic pathogens such as *P. gingivalis* may be exposed by a sublethal dose of PAD (sPAD; i.e., a combination of sub-minimum inhibitory concentration of photosensitizers and/or a sublethal dose of diode laser irradiation time) at the site of infection during endodontic treatment, which not only does not eradicate or inactivate the *P. gingivalis* but also may affect a broad range of its features and virulence factors.[7,8] Some studies[9–11] have provided evidence for the effects of sPAD with different photosensitizers and the corresponding wavelengths of light on the count and biofilm formation ability of *P. gingivalis*, but the sPAD effects on the expression of *P. gingivalis* virulence factor genes, including those that are involved in response to oxidative stress, are still missing. The aim of this study was to assess the effects of sPAD treatment in vitro on *oxyR* gene expression of clinical *P. gingivalis* isolates surviving photodynamic treatment.

Materials and methods

Bacterial strains and growth conditions

In a previous study,[12] 16 clinical isolates of *P. gingivalis,* which did not disappear through PAD, were collected from patients who attended a private dental clinic in Tehran, Iran. After admission, written informed consent were obtained from the patients. The approval for the study protocol was obtained from the Ethics Committee of the Iranian Registry of Clinical Trials (IRCT ID: IRCT2015031321455N1). These *P. gingivalis* isolates were cultured in brain heart infusion (BHI) agar plate (Merck, Darmstadt, Germany) supplemented with 5% defibrinated sheep blood, 0.6% (wt/vol) yeast extract (Merck), 5 mg/L hemin, and 1 mg/L menadione (both purchased from Sigma-Aldrich, Steinheim, Germany) at 37°C in an anaerobic atmosphere comprising 10% CO_2, 10% H_2, and 80% N_2. These conditions were generated using the Mart® Anoxomat Mark II anaerobic system (Mart® Microbiology BV, Drachten, The Netherlands).

Fresh BHI broth (Merck) supplemented with 0.6% (wt/vol) yeast extract and 1 mg/L menadione was used to prepare fresh suspensions of 16 clinical *P. gingivalis* isolates. The suspensions were incubated for 4–5 h in anaerobic conditions at 37°C, and OD_{600} measurements were taken at specific intervals to assess growth of the cells, which requires a concentration of 1.0×10^6 colony-forming units (CFUs)/mL, as verified by colony counting after incubation for 48 h. The flowchart of experiment steps is shown in Figure 1.

Preparation of photosensitizers and selection of light sources

According to the previous studies,[9–11] stock solutions of TBO and MB (both items were purchased from Sigma-Aldrich) at final concentrations of 0.1 mg/mL and ICG (Santa Cruz Biotechnology Co., Ltd; Shanghai, China) stock solution at a final concentration of 1.0 mg/mL were prepared as photosensitizers in sterile 0.9% (wt/vol) NaCl. Prior to use, the photosensitizers were filtered with a sterile syringe filter of 0.22 μm pore size and were kept under dark conditions.

A diode laser (Klas DX82; Konftec, New Taipei City, Taiwan) at wavelengths of 635 nm for 1, 2, 3, 4, and 5 min with 23.43, 46.87, 70.31, 93.75, and 117.18 J/cm^2, respectively; 660 nm for 1, 2, 3, 4, and 5 min with 34.37, 68.75, 103.12, 137.5, and 171.87 J/cm^2, respectively; and 810 nm for 0.5, 1, and 2 min with 15.62, 31.25, and 62.5 J/cm^2, respectively, at maximum output power of 220, 150, and 250 mW/cm^2 was used for TBO, MB, and ICG activation, respectively. The output powers of all wavelengths examined were measured by a power meter (Laser Point Srl, Milano, Italy). The irradiated area had a 0.384 cm^2 surface with a diameter of 6.39 mm, and the diode laser probe was placed 1 mm above the top plate surface using a microphone stand during experiments. Experimental groups are presented in Table 1.

Determination of sPAD

sPAD was determined by measuring the minimum inhibitory concentration (MIC) of photosensitizers and sublethal dose of diode laser irradiation time against *P. gingivalis* isolates according to our previous study.[9] In short, the wells of 96-well, flat-bottomed, sterile polystyrene microplates (TPP, Trasadingen, Switzerland) were filled with 100 μL of 2×BHI broth and 100 μL of each photosensitizer at 2×MIC, and individually diluted in serial 2-fold to 1/8× MIC. One hundred microliters of each fresh suspension of the 16 clinical *P. gingivalis* isolates in final concentrations of 5.0×10^5 CFU/mL was then inoculated into each well. Eventually, the microplates were incubated in an anaerobic atmosphere

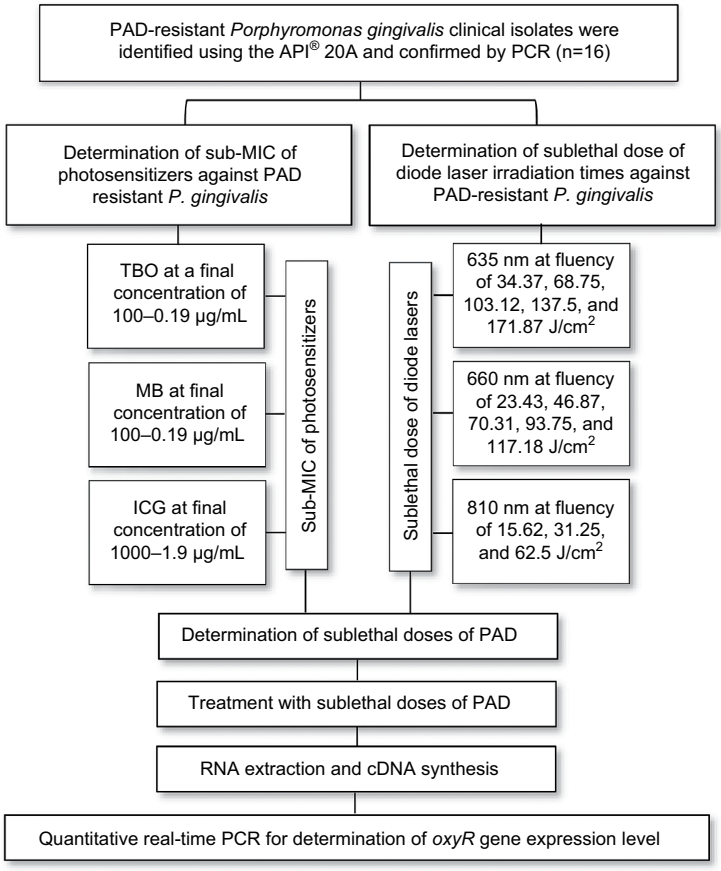

Figure 1 Flowchart of the testing steps in the current study.
Abbreviations: ICG, indocyanine green; MB, methylene blue; MIC, minimum inhibitory concentration; PAD, photo-activated disinfection; PCR, polymerase chain reaction; TBO, toluidine blue O.

Table 1 Experimental groups

Evaluated groups based on the assay	Description
Sub-MIC of photosensitizers	Treatment with TBO only in the range of 0.19–100 μg/mL
	Treatment with MB only in the range of 0.19–100 μg/mL
	Treatment with ICG only in the range of 1.9–1000 μg/mL
Sublethal dose of diode laser irradiation times	Diode laser at a wavelength of 635 nm for 1, 2, 3, 4, and 5 min with 23.43, 46.87, 70.31, 93.75, and 117.18 J/cm^2, respectively
	Diode laser at a wavelength of 660 nm for 1, 2, 3, 4, and 5 min with 34.37, 68.75, 103.12, 137.5, and 171.87 J/cm^2, respectively
	Diode laser at a wavelength of 810 nm for 0.5, 1, and 2 min with 15.62, 31.25, and 62.5 J/cm^2, respectively
Sublethal doses of PAD	1.5–6.25 μg/mL TBO at a fluency of 137.5 J/cm^2
	12.5–25 μg/mL MB at a fluency of 93.75 J/cm^2
	3.9–15.6 μg/mL ICG at a fluency of 15.6 J/cm^2
sPAD treatment on *oxyR* gene expression	6.25 μg/mL TBO at a fluency of 171.87 J/cm^2
	25 μg/mL MB at a fluency of 93.75 J/cm^2
	15.6 μg/mL ICG at a fluency of 15.6 J/cm^2

Abbreviations: ICG, indocyanine green; MB, methylene blue; MIC, minimum inhibitory concentration; PAD, photo-activated disinfection; sPAD, sublethal doses of photo-activated disinfection; TBO, toluidine blue O.

in the dark at room temperature for 5 min and were then immediately exposed to a sublethal dose of light sources, depending on the type of photosensitizer according to our previous study.[9] We placed a black paper sheet under the microplates to avoid beam reflection from the table top during irradiation. Instantly, 10 μL of each well containing the diluted series was cultured in enriched BHI plates and incubated in anaerobic conditions at 37°C for 48 h, and the

CFU/mL values were determined using the method of Miles et al.[13] The lowest concentrations of photosensitizers with the shortest irradiation time of diode laser in the last well showing growth were defined as the (maximal) sPAD. Each of the 16 clinical *P. gingivalis* isolates was tested in separate panels under the same conditions.

sPAD treatment on clinical *P. gingivalis* isolates

Based on a previous study,[11] 16 clinical *P. gingivalis* isolates were exposed to sPAD treatment. Briefly, 100 µL of 2× MIC of each photosensitizer was individually diluted in the wells of 96-well, flat-bottomed, sterile polystyrene microplates that were filled with 100 µL 2× BHI broth. The wells were then inoculated with 100 µL/well of a fresh bacterial suspension of each clinical *P. gingivalis* isolate adjusted to a concentration of 1.0×10^6 CFU/mL. The microplates were then inoculated for 5 min in anaerobic conditions in the dark at room temperature and quickly exposed to a sublethal dose of diode laser irradiation time, depending on the type of photosensitizer. The clinical *P. gingivalis* isolates were treated with 1 mM (final concentration) H_2O_2 as a positive control group, and the MIC of H_2O_2 was determined as described in previous study.[14] Also, untreated clinical *P. gingivalis* isolates that did not receive sPAD treatment were used as a negative control group.

RNA extraction and cDNA synthesis

Total RNA was directly extracted from all of the treated and untreated (as control groups) clinical *P. gingivalis* isolates using a GeneAll Hybrid-R™ RNA purification kit (Geneall Biotechnology Co. Ltd, Seoul, Korea), as described by the manufacturer's recommendations. RNA was visualized on 1% agarose gel to evaluate RNA integrity. Additionally, the quantity and the quality of the total extracted RNA were tested using NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Genomic DNA was eliminated from all of the total extracted RNA samples by RNase-free DNase I treatment (Thermo Fisher Scientific), and first-strand cDNA synthesis was performed using a Revert Aid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific) with random primers, according to the manufacturer's protocol.

Quantitative real-time PCR (qRT-PCR) condition and gene expression analysis

qRT-PCR analysis was performed after cDNA synthesis on a Line-GeneK Real-Time PCR Detection System and Software (Bioer Technology, Hangzhou, China). The target qRT-PCR primer sets that were designed using Primer3 software version 4.0 (http://bioinfo.ut.ee/primer3/) were *oxyR* (fwd: 5′-CCACAACTGACCGTAGAGCA-3′, rev: 5′-CCTGTCTGCAACTTGTGCAT-3′) and *16S rRNA* (fwd: 5′-TGACACTGAAGCACGAAAGC-3′, rev: 5′-TCCTTGAGTTTCACCGTTGC-3′). Thermocycling conditions consisted of an initial denaturation of 5 min at 95°C, followed by 35 cycles of 95°C for 15 s, 59°C for 30 s, and 72°C for 30 s. The melting curve profile was analyzed to verify the specificity of primers. The expression levels of target gene were analyzed using the method of Livak and Schmittgen.[15]

Data evaluation and statistical analysis

All assays were carried out in triplicate. The $\Delta\Delta C_t$ method was used to calculate fold induction of target gene by comparison with calibrator gene. The expression levels were defined as n-fold differences relative to the calibrator. A variation of >2-fold difference in the expression levels of the target gene was considered to be significant. One-way analysis of variance with Tukey's post hoc test was used to reveal the significant differences for sPAD examinations, and *P* values <0.05 were considered significantly different.

Results

Sub-MIC of photosensitizers, sublethal dose of diode laser irradiation times, and sPAD against *P. gingivalis* are shown in Table 2. According to the results of our study, 6.25 µg/mL TBO at fluency of 171.87 J/cm^2, 15.6 µg/mL ICG at fluency of 15.6 J/cm^2, and 25 µg/mL MB at fluency of 93.75 J/cm^2 were maximal sPAD that displayed an insignificant reduction in clinical *P. gingivalis* isolates cell counts when compared with the control groups (untreated isolates; $P>0.05$; Figure 2A–C).

Table 2 Sub-MIC of photosensitizers, sublethal dose of diode laser irradiation times, and sublethal doses of antimicrobial photodynamic therapy against *Porphyromonas gingivalis*

Groups	Treatment	Results
Sub-MIC of photosensitizers	TBO only	12.5 µg/mL
	MB only	50 µg/mL
	ICG only	31.2 µg/mL
Sublethal dose of diode laser irradiation times	635 nm irradiation only	4 min (137.5 J/cm^2)
	660 nm irradiation only	5 min (117.18 J/cm^2)
	810 nm irradiation only	0.5 min (15.6 J/cm^2)
Sublethal doses of PAD	TBO plus 635 nm irradiation	6.25 µg/mL TBO at a fluency of 137.5 J/cm^2
	MB plus 660 nm irradiation	25 µg/mL MB at a fluency of 93.75 J/cm^2
	ICG plus 810 nm irradiation	15.6 µg/mL ICG at a fluency of 15.6 J/cm^2

Abbreviations: ICG, indocyanine green; MB, methylene blue; MIC, minimum inhibitory concentration; PAD, photo-activated disinfection; TBO, toluidine blue O.

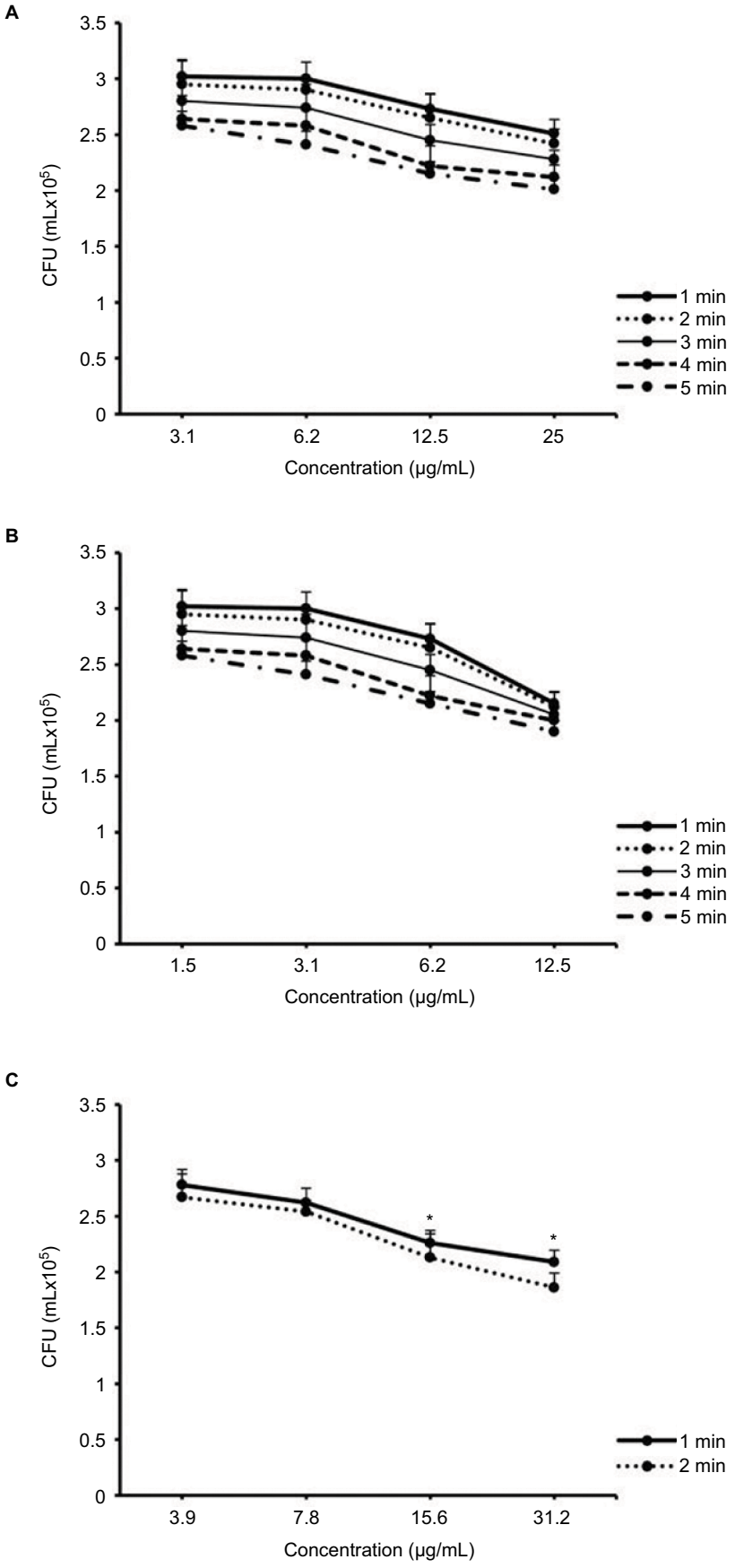

Figure 2 Effects of sublethal doses of photo-activated disinfection on *Porphyromonas gingivalis* CFUs/mL: (**A**) MB; (**B**) TBO; (**C**) ICG.
Note: *Significantly different from the control group (no treatment), $P<0.05$.
Abbreviations: CFU, colony-forming units; ICG, indocyanine green; MB, methylene blue; TBO, toluidine blue O.

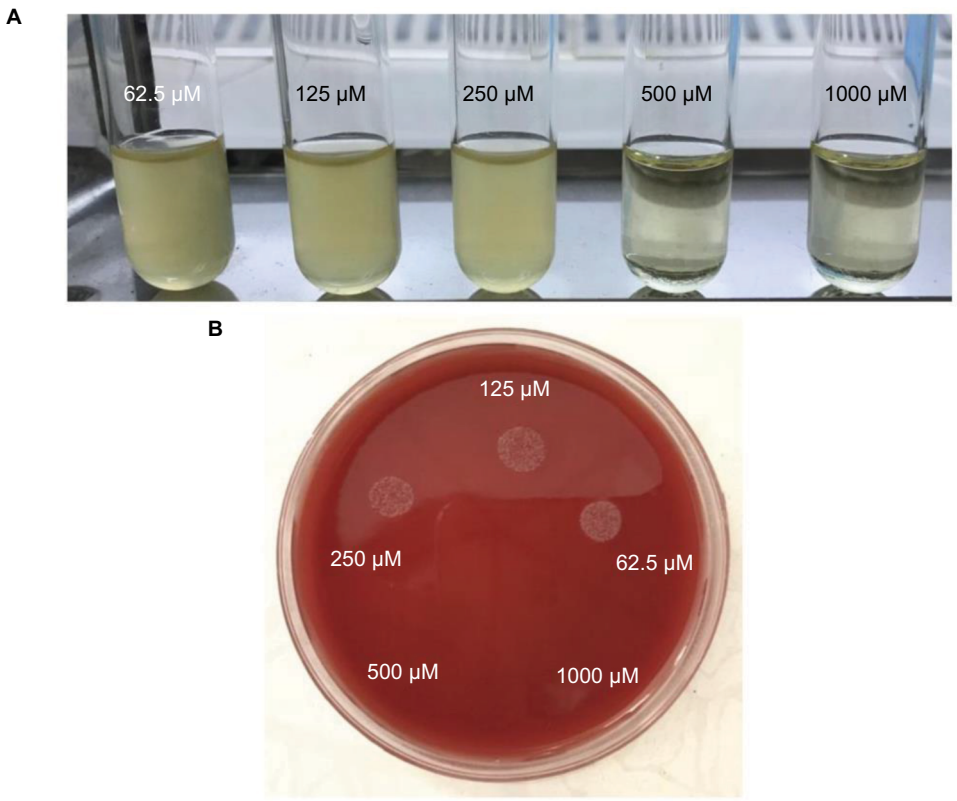

Figure 3 Two-fold broth microdilution method for determination of MIC of H_2O_2 against *P. gingivalis*.
Notes: (**A**) Growth responses of *Porphyromonas gingivalis* to different concentrations of H_2O_2. Recovery of growth after inhibition with H_2O_2. (**B**) Ten microliters from undiluted cultures was plated on each plate and grown overnight at 37°C in anaerobic atmosphere, followed by further checking on the next day.

On the other hand, as no visible growth of *P. gingivalis* was detected at 500 µM H_2O_2, this concentration was taken as the MIC (Figure 3).

Extracted RNA integrity and primers specificity are shown in Figure 4A and B, respectively. The concentration of total RNA was >350 ng/µL ± 30.8 among all samples. In Figure 4A, clear bands corresponded to *16S* and *23S rRNA*, indicating total RNA integrity and minimal degradation of total extracted RNA. Also, in Figure 4B, the presence of a single curve for each primer implied the formation of a single product (i.e., specificity of primers for target genes).

MB-, TBO-, and ICG-mediated sPAD increased the expression level of *oxyR* gene significantly when compared to the non-treated group. As shown in Figure 5, the expression levels of *oxyR* were increased to almost 5.6-, 8.5-, and 12.3-fold following MB-, TBO-, and ICG-sPAD, respectively.

As the results showed, the level of gene expression in ICG-sPAD compared with MB-sPAD was remarkably different ($P<0.05$), while there was no significant difference in the expression of *oxyR* following ICG-sPAD with TBO-sPAD ($P>0.05$). On the other hand, there was a nonsignificant difference between ICG-sPAD and TBO-sPAD treatments

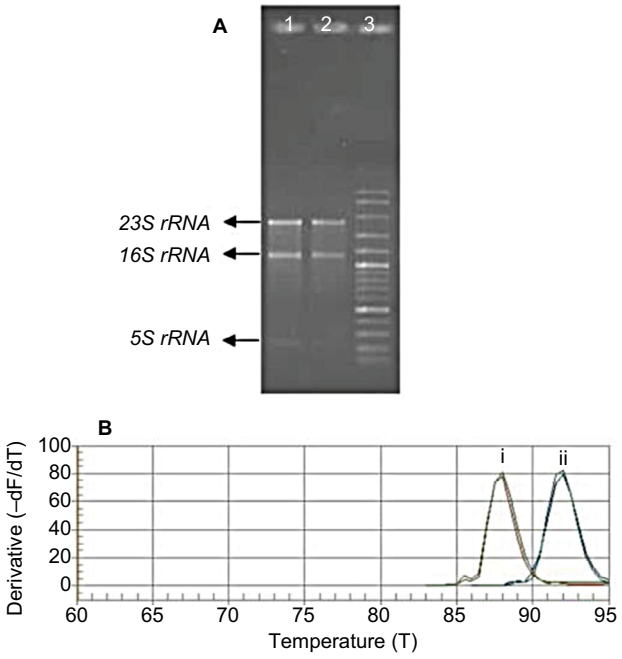

Figure 4 Determination of RNA integrity and specificity of primers.
Notes: (**A**) Agarose gel electrophoresis of extracted RNA: (1) and (2) extracted RNAs and (3) ladder 100 bp. (**B**) The melting curve profiles generated following real-time amplification to assess potential primer–dimer artifacts (or nonspecific PCR product): (i) *16S rRNA* and (ii) *oxyR*.
Abbreviation: PCR, polymerase chain reaction.

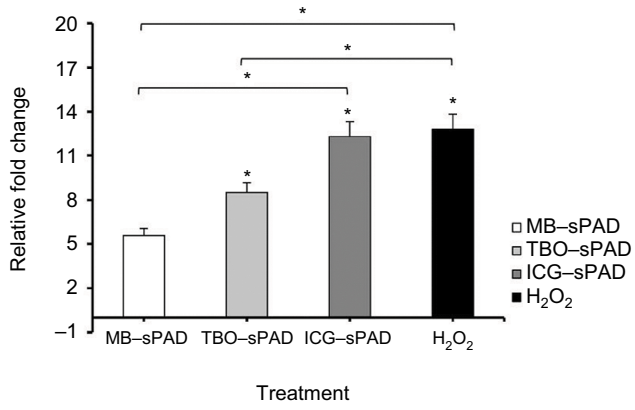

Figure 5 The relative fold change in mRNA expression levels of *Porphyromonas gingivalis oxyR* gene following sPAD with MB, TBO, and ICG.
Note: *Significantly different from the control group (no treatment), $P<0.05$.
Abbreviations: ICG, indocyanine green; MB, methylene blue; PAD, photo-activated disinfection; sPAD, sublethal doses of photo-activated disinfection; TBO, toluidine blue O.

($P>0.05$). As mentioned previously, the gene expression profiling was enhanced in *P. gingivalis* cells, with the greatest increase seen for ICG-sPAD, which was ~1.5- and 2-fold higher than TBO- and MB-sPAD, respectively. It is noteworthy that under treating condition with H_2O_2 as a positive control, the increased expression level of *oxyR* was ~12.8-fold. According to the results, the level of gene expression in H_2O_2 and ICG-sPAD compared with TBO- and MB-sPAD was significantly different ($P<0.05$), while there was no remarkable difference in the expression of *oxyR* following H_2O_2 with ICG-sPAD ($P>0.05$).

Discussion

PAD is a promising technology with a local antimicrobial effect in treatment of infected dental root canals. During PAD, photosensitizing molecules become excited when light of an appropriate wavelength is captured by photosensitizers and ROS are generated as oxidative stress. ROS induce degradation of nucleotides, proteins, lipids, and carbohydrate macromolecules of the microbial cell.[5,6]

The major factor that affects the survival of microorganisms during PAD treatment in the infected dental root canals is oxidative stress.[7] Antioxidant and DNA repair systems as well as chaperone/protease systems are the three major protective cellular systems against oxidative stress in several bacteria. In contrast, *P. gingivalis* uses another unique defense mechanism, OxyR response, in its protection against ROS damage.[16] The *oxyR* gene is widely distributed in most Gram-negative bacteria including *P. gingivalis*.[17] Results of a previous study indicated that OxyR represents the first redox-regulatory protein in the LysR family of DNA-binding transcription modulators, and it is required for activation of the oxidative stress-related genes of *P. gingivalis* and it functions in cellular resistance to H_2O_2.[16] The previous study reported that *P. gingivalis oxyR* is critical not only for resistance to ROS, but also for the aerotolerance of the microorganism.[18] On the other hand, *P. gingivalis oxyR* gene expression is positively associated with the expression regulation of other virulence factors in this bacterium. As the previous studies have reported, the expression of *fimA*, a gene encoding a major subunit protein of fimbriae that is related to biofilm formation in *P. gingivalis*, and *sod*, as an important virulence factor that is required for modest aerotolerance of *P. gingivalis*, are coordinately regulated through *oxyR* gene expression.[19,20] Therefore, the pathogenicity and biofilm formation ability of *P. gingivalis* can be enhanced, and the emergence of resistance to antimicrobial agents can develop due to the increasing *P. gingivalis oxyR* gene expression level.

Sometimes, the exposure of infected root canals to sublethal doses of sPAD during treatment can occur due to various reasons such as lower energy density of laser and/or lower photosensitizer concentrations in comparison to optimal treatment condition. In the infected root canals the photosensitizer may be diluted by the remaining fluid in the root canals after root canals rinsing and/ or presence of the apical tissue fluid.[19] In these circumstances, sPAD can not only eliminate the microorganisms but also considerably influence microbial pathogenicity through changes in gene expression of the virulence factor and increase the resistance to antimicrobial agents.[9,11,12] Nevertheless, in addition to sPAD not helping the treatment of root canal infections, it also may cause treatment failure and the emergence of secondary endodontic infections.

To the best of our knowledge, this is the first study that has evaluated sPAD effects with three types of photosensitizers, TBO, MB, and ICG, on the *P. gingivalis oxyR* gene expression level. As the results of this study have shown, the expression of *oxyR* gene in *P. gingivalis* was significantly increased after sPAD treatment with different photosensitizers. This observation indicated that sPAD is not only able to upregulate the virulence factor expression of *P. gingivalis* strains, but can also lead to the bacterial survival and withstand the presence of ROS.

Collectively, the data clearly reveal that the upregulation in *oxyR* gene expression as a gene corresponding to responses to oxidative stress in *P. gingivalis* can occur when exposed to sPAD treatments and under H_2O_2 stress conditions. Together, these data reveal that there was no notable difference between ICG-sPAD groups and H_2O_2 in *oxyR* gene expression.

The robustness of *P. gingivalis* against sPAD is not only due to the strong antioxidative stress mechanisms which preserve its DNA and proteins from oxidative damage, but also because it can repair DNA and remove the deleterious lesions.[15] So, it is essential to use the appropriate concentration of photosensitizer along with irradiation time of light to produce a sufficient amount of ROS to disrupt the bacterial cell structure and function. On the other hand, ultrasonic activation and chemical modification of photosensitizers can enhance the effect of PAD against the infected root canal isolated.[20] Nevertheless, further studies are needed to examine the effect of PAD on various virulence factors of multispecies microorganisms involved in endodontic infections, in order to establish an optimal protocol for using this new therapeutic alternative in therapy of infected root canals.

Despite the use of a high-throughput protocol to evaluate the PAD effects on gene expression in *P. gingivalis* strains, this study has certain limitations. As is well known, endodontic infections have a polymicrobial nature characterized by frequently anaerobic bacteria, and in future studies we should examine the multi-microbial model involved in endodontic infections.

Conclusion

In this study, we found the upregulation of *oxyR* gene expression in *P. gingivalis* isolates during exposure to sPAD treatment. Since the *oxyR* gene is involved in oxidative stress response and regulation of several virulence factors, its upregulated expression may lead to increased survival and pathogenicity of *P. gingivalis*. Thus, selection of appropriate concentration of photosensitizer and irradiation time of light during PAD treatment should be considered for successful endodontic infection therapy, in order to prevent the onset of secondary endodontic infections.

Acknowledgment

This research was supported by the Tehran University of Medical Sciences and Health Services (grant No. 93-04-30-27681).

Disclosure

The authors report no conflicts of interest in this work.

References

1. Ng R, Singh F, Papamanou DA, Song X, et al. Endodontic photodynamic therapy ex vivo. *J Endod*. 2011;37(2):217–222.
2. Chiniforush N, Pourhajibagher M, Shahabi S, Bahador A. Clinical approach of high technology techniques for control and elimination of endodontic microbiota. *J Lasers Med Sci*. 2015;6(4):139–150.
3. Gerits E, Verstraeten N, Michiels J. New approaches to combat *Porphyromonas gingivalis* biofilms. *J Oral Microbiol*. 2017;9(1):1300366.
4. Chiniforush N, Pourhajibagher M, Shahabi S, Kosarieh E, Bahador A. Can antimicrobial photodynamic therapy (aPDT) enhance the endodontic treatment? *J Lasers Med Sci*. 2016;7(2):76–85.
5. Pourhajibagher M, Boluki E, Chiniforush N, et al. Modulation of virulence in Acinetobacter baumannii cells surviving photodynamic treatment with toluidine blue. *Photodiagnosis Photodyn Ther*. 2016; 15:202–212.
6. Sperandio FF, Huang YY, Hamblin MR. Antimicrobial photodynamic therapy to kill Gram-negative bacteria. *Recent Pat Antiinfect Drug Discov*. 2013;8(2):108–120.
7. Pourhajibagher M, Monzavi A, Chiniforush N, et al. Real-time quantitative reverse transcription-PCR analysis of expression stability of Aggregatibacter actinomycetemcomitans fimbria-associated gene in response to photodynamic therapy. *Photodiagnosis Photodyn Ther*. 2017;18:78–82.
8. Pourhajibagher M, Chiniforush N, Shahabi S, et al. Monitoring gene expression of rcpA from Aggregatibacter actinomycetemcomitans versus antimicrobial photodynamic therapy by relative quantitative real-time PCR. *Photodiagnosis Photodyn Ther.* 2017;19:51–55.
9. Pourhajibagher M, Chiniforush N, Raoofian R, Ghorbanzadeh R, Shahabi S, Bahador A. Effects of sub-lethal doses of photo-activated disinfection against *Porphyromonas gingivalis* for pharmaceutical treatment of periodontal-endodontic lesions. *Photodiagnosis Photodyn Ther*. 2016;16:50–53.
10. Pourhajibagher M, Chiniforush N, Ghorbanzadeh R, Bahador A. Photo-activated disinfection based on indocyanine green against cell viability and biofilm formation of *Porphyromonas gingivalis*. *Photodiagnosis Photodyn Ther*. 2017;17:61–64.
11. Pourhajibagher M, Chiniforush N, Raoofian R, et al. Evaluation of photo-activated disinfection effectiveness with methylene blue against *Porphyromonas gingivalis* involved in endodontic infection: an in vitro study. *Photodiagnosis Photodyn Ther*. 2016;16:132–135.
12. Pourhajibagher M, Ghorbanzadeh R, Parker S, Chiniforush N, Bahador A. The evaluation of cultivable microbiota profile in patients with secondary endodontic infection before and after photo-activated disinfection. *Photodiagnosis Photodyn Ther*. 2017;18:198–203.
13. Miles AA, Misra SS, Irwin JO. The estimation of the bactericidal power of the blood. *J Hyg (Lond)*. 1938;38(6):732–749.
14. Zubko EI, Zubko MK. Co-operative inhibitory effects of hydrogen peroxide and iodine against bacterial and yeast species. *BMC Res Notes*. 2013;6:272–278.
15. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25(4):402–408.
16. Henry LG, McKenzie RM, Robles A, Fletcher HM. Oxidative stress resistance in *Porphyromonas gingivalis*. *Future Microbiol*. 2012;7(4): 497–512.
17. Leclerc J, Rosenfeld E, Trainini M, et al. The cytochrome *bd* oxidase of *Porphyromonas gingivalis* contributes to oxidative stress resistance and dioxygen tolerance. *PLoS One*. 2015 2;10(12):e0143808.
18. Xie H, Zheng C. OxyR activation in *Porphyromonas gingivalis* in response to a hemin-limited environment. *Infect Immun*. 2012;80(10): 3471–3480.
19. Wu J, Lin X, Xie H. OxyR is involved in coordinate regulation of expression of *fimA* and sod genes in *Porphyromonas gingivalis*. *FEMS Microbiol Lett*. 2008;282(2):188–195.
20. Tennert C, Drews AM, Walther V, et al. Ultrasonic activation and chemical modification of photosensitizers enhances the effects of photodynamic therapy against *Enterococcus faecalis* root-canal isolates. *Photodiagnosis Photodyn Ther*. 2015;12(2):244–251.