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Photodynamic Inactivation of *Porphyromonas gingivalis* utilizing Radachlorin and Toluidine Blue O as Photosensitizers: An In Vitro Study

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Abstract

Introduction: *Porphyromonas gingivalis* is one of the major pathogens in the development and progression of periodontal disease. Antimicrobial photodynamic therapy (aPDT) is a new approach which is sorted in non-invasive phototherapy for bacterial elimination. This in vitro study was conducted to compare photodynamic inactivation using Radachlorin and Toluidine blue O (TBO) as photosensitizers on *P. gingivalis*.

Methods: Bacterial suspensions (200 µL) of *P. gingivalis* were exposed to either TBO with concentration of 0.1 mg/mL associated with portable light-emitting diode (LED) device (peak wavelength: 630 nm, output intensity: 2.000 mW/cm2, tip diameter: 6.2 mm) or 0.1% Radachlorin® and laser irradiation (InGaAlP, Peak wavelength: 662±0.1% nm, output power: 2.5 W, energy density: 6 J/cm2, fiber diameter: 2 mm). Those in control groups were subjected to laser irradiation or LED alone, Radachlorin® or TBO alone, and one group received neither photosensitizer nor light irradiation. Then counting of colony forming units (CFU) was performed to determine the bactericidal effects in each subgroup.

Results: LED-based aPDT reduced the colony count of P. gingivalis more than that of TBO ($P<0.001$) or LED group ($P=0.957$). Also, laser-based aPDT had a great reduction in colony count of P. gingivalis in comparison with Radachlorin® ($P<0.001$) or laser irradiation alone ($P=0.28$). In addition, the colony count reduction of laser-based aPDT was significantly more than LED-based aPDT ($P<0.05$).

Conclusion: Considering the results of this study, the viability of *P. gingivalis* was more affected by the combination of laser and Radachlorin® 0.1% in comparison with LED and TBO 0.1%

Keywords: Photochemotherapy; Photosensitizing agents; *Porphyromonas gingivalis*; Lasers; Periodontics.

Introduction

Periodontal disease is an inflammatory process in the tissues surrounding the teeth. Bacterial plaque is the main cause of periodontitis.[1] *Porphyromonas gingivalis* a black pigmented microorganism, is a major pathogen associated with initiation and progression of periodontitis.[2] *P. gingivalis* associated with Socransky's red complex (bacteria strongly related to periodontal disease) has many virulence factors including fimbriae, lipopolysaccharides, cysteine proteases.[3]

The first step in the treatment of periodontitis is scaling and root planning that can be combined with systemic or local antibiotics.[4] Antibiotic is associated with undesirable side effects and development of resistant bacteria to antibiotics as well as the disruption of the oral microflora.[5] Antimicrobial photodynamic therapy (aPDT) has been introduced as an alternative approach for antibacterial therapy. It is a combination of 2 nontoxic ingredients, a photosensitizer and light, which destruct the cell by photodamage, and finally leads to cell death. The benefits of this approach include local antimicrobial effect, selectivity, the killing of target microorganisms

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in short time, no drug resistance, less burdensome for patients.[6,7]

A photosensitizer (a photoactivable material) attaches to the target cells and gets stimulated by a proper wavelength of light. Then, singlet-oxygen and other reactive mediators are produced by the activated photosensitizer. These agents are toxic to certain cells and bacteria.[8,9]

It was demonstrated that *P. gingivalis* has endogenous photosensitizer molecules, such as porphyrins that in the presence of light generate reactive oxygen species leading to bacterial killing.[10,11] On the other hand, some studies using an exogenous photosensitizer implicated the capacity of aPDT for killing *P. gingivalis*.[12,13]

Toluidine blue O (TBO) is a thiazine type photosensitizer with little toxicity. Its highest absorption wavelength is 632 ± 8 nm. TBO is less expensive compared with the other existing photosensitizers. Its acidophilic condition with an affinity for nucleic acids helps it to bind to nuclear substance in tissues that contain DNA and RNA in high amounts and the tissues with acidicstate are dyed with it.[14,15]

Radachlorin®, a derivative of water-soluble chlorophyll α, acts as a photosensitizer. Sodium chlorin e6, sodium chlorin p6, and purpurine-5 are three chlorophyll substances combined to make Radachlorin®. It has noticeable benefits such as little toxicity in the dark and strong absorption band at relatively wide spectrum of wavelengths, especially at a wavelength of 662 ± 5 nm. Also, it accumulates in tumors with high contrast and causes high phototoxicity.[16-18]

Most commonly, laser device is used in aPDT. Recently, non-coherent light sources have been introduced. Light-emitting diode (LED) technology is a newer light source that is safer and less expensive than lasers.[16,19]

As we know, no study has been conducted to compare the laser-based aPDT (Radachlorin® as photosensitizer) and LED-based aPDT (TBO as photosensitizer) on *P. gingivalis*. This study was carried out to evaluate the influence of aPDT with Laser on the viability of *P. gingivalis* in comparison with LED.

Methods

Test Microorganism and Growth Conditions

The bacterial strain utilized in this study, Lyophilized *P. gingivalis* (*ATCC cultures* 33270 taken from Rayen Biotechnology Co. Ltd., Tehran, Iran) was rehydrated in SBHI broth, which was brain heart infusion broth (Merck, Darmstadt, Germany) complemented with vitamin K (1 μg/mL) and hemin (5 μg/mL). The incubation phase was done in an anaerobic atmosphere at 37°C for 48 hours. Both substances were provided by Sigma-Aldrich, Steinheim, Germany. For experiments in which cultures on plates were required, cultures developed in SBHI broth were passed on to sheep blood-agar (BHK) plates containing Brucella agar (Merck, Darmstadt, Germany) as the base medium, supplemented with hemin plus vitamin K.

A flat bottom microtitration plate with 96-wells (TPP, Switzerland) was utilized as a container. Each well was 500 μL in capacity with a diameter of 6 mm.

Bacterial Suspension

Fresh colonies of test bacteria from BHK plates were floating in SBHI broth, and bacterial density was attuned to a turbidity of 0.5 McFarland standard reagents. For each bacterial suspension, the accurate density (CFU/mL) was confirmed by serial tenfold dilutions and anaerobic culturing on BHI agar plates.

Light Sources and Photosensitizers

To determine the effect of light sources on bacterial growth inhibition, bacterial colonies were exposed to portable LED device (FotoSan®; CMS Dental, Copenhagen, Denmark, peak wavelength: 630 nm, output intensity: 2.000 mW/cm^2) and laser device with indium-gallium-aluminum-phosphate (InGaAlP; MILON LAHTA, Russia, output power: 2.5 W, Peak wavelength: 662 ± 0.1% nm, mode of irradiation: continuous).

The LED device had a tip that was 6.2 mm in diameter and it was kept at 1 mm distance from the surface of the plate. As the company suggested, irradiation time was 30 seconds. The photosensitizer used for LED device was TBO in syringe (0.1 mg/mL concentration, FotoSan® agent low, manufactured in Denmark).

The distance between the laser device tip (2 mm in diameter) and the exposure sample was 10 mm that covered the spot-size of 6 mm diameter. To obtain the optimum energy density (6 J/cm^2), with an optimal power density of 49.9 mW/cm^2, the irradiation time was adjusted to 34 seconds. Radachlorin® gel (0.1 mg/mL) (Rada-Pharma Ltd, Russia) was applied as the photosensitizer, specifically for this laser device.

Study Design and Photodynamic Therapy

The samples were divided into 7 groups:

1) Negative control (n = 15): 200 μL bacterial suspension + 200 μL Broth (no irradiation; no photosensitizer);
2) Laser irradiation alone (n = 15): 200 μL bacterial suspension + 200 μL Broth, with laser irradiation for 34 seconds;
3) Laser-based aPDT group (n = 15):200 μL bacterial suspension + 200 μL Radachlorin®, after shaking the samples were placed in the dark for 10 minutes and then laser irradiation was done for 34 seconds;
4) Radachlorin® alone (n = 15): 200 μL bacterial suspension + 200 μL Radachlorin®;
5) LED irradiation alone (n = 15): 200 μL bacterial suspension + 200 μL broth with LED irradiation for 30 seconds;
6) TBO alone (n = 15): 200 μL bacterial suspension + 200 μL TBO;
7) LED-based aPDT (n=15): 200 μL bacterial suspension

+ 200 µL TBO, after shaking the samples were placed in the dark for 10 minutes and then laser irradiation was done for 30 seconds.

In groups 1, 2 and 5, 200 µL of broth was used to have equal levels of wells. For even distribution of samples, all the plates were shaken for 1 minute. To have the photosensitizer absorbed by bacterial cells, all the plates were held in the dark for 10 minutes before irradiation.

Out of 96 wells, only 5 were utilized for samples; 4 of them in the corners and one in the centerplate, so keeping away from cross-irradiation and cross-contamination. Also, a light absorbing dye (Toluidine blue O, Kerr, Germany) was used in all wells adjacent to our sample containing wells. A sampler was used to transmit bacterial suspension and photosensitizer to containing wells. To determine the colony forming units per milliliter (CFU/mL), the cells dilution was performed serially with phosphate buffered solution (PBS) in microtiter plates and specified by means of the drop-plate method.[20] In this method, from each dilution, 20 µL drops were located onto BHK media. The plates were incubated for 72 hours anaerobically before CFU counting to verify the inhibitory effect of *P. gingivalis*. All tests were performed in triplicase.

Data Analysis

Subsequent to transforming the number of CFU/mL to logarithmic type, the data in each group (Laser/Radachlorin group and LED/TBO group) were evaluated and analyzed via two-way analysis of variance (ANOVA).

Table 1. Mean ± Standard Deviation of Logarithm of Concentration (CFU/mL) of *Porphyromonas gingivalis* in Each Group

Groups	Mean ± Standard Deviation
TBO	9.07±0.08
LED	9.28±0.07
LED+TBO	8.75±0.20
Radachlorin	7.93±0.58
Laser	8.45±0.57
Radachlorin + laser	6.20±0.87
Control	9.33±0.39

TBO: toluidine blue O; LED: light-emitting diode.

There was a significant interaction between the effects of photosensitizer and light on bacterial colonies. One-way ANOVA was used to verify significant differences between the test samples and the control group. A *t* test was carried out to analyze the data in each group. Groups 2, 3 and 4 were stated as "Laser subgroup", and groups 5, 6 and 7 were stated as "LED subgroup". The level of significance was put at 0.05.

Results

Table 1 demonstrates mean ± SD values of logarithm of CFU/mL in each group.

Table 2 shows the statistical comparison of bacterial concentration between different groups. There was a significant interaction between the effects of different groups and LED/Laser subgroups on colony of *P. gingivalis* ($P<0.05$). The results are displayed in Figure 1.

LED Subgroup

One-way ANOVA was used to compare the colony count of *P. gingivalis* among groups 5, 6, and 7. Application of LED light in conjunction with the TBO at low concentration showed the most colony count reduction of *P. gingivalis* (LED-based aPDT) ($P<0.05$). LED irradiation alone and the control group did not show any significant difference($P>0.05$). In the photosensitizer group (TBO), the colony count of *P. gingivalis* reduced significantly ($P<0.05$).

Laser Subgroup

The combination of Radachlorin® and laser (laser-based aPDT) resulted in a substantial reduction in colony count of *P. gingivalis* bacteria ($P<0.05$). The Laser alone group showed a significant reduction of microorganisms (p= 0.028). But, the decrease in Radachlorin® group was more significant ($P<0.05$).

Comparison of LED-Based and Laser-Based aPDT Group

According to *t* test analysis, laser-based aPDT was more effective in diminution of *P. gingivalis* bacteria compared with LED-based aPDT ($P<0.05$).

Discussion

Laser Subgroups

The outcomes of the present study demonstrated that

Table 2. Statistical Comparison of Bacterial Concentration Between Different Groups

Groups	TBO	LED	LED + TBO	Radachlorin	Laser	Laser + Radachlorin
TBO		*				
LED	*					
LED+ TBO	*	*				
Radachlorin					*	*
Laser				*		*
Laser+Radachlorin			*	*	*	
Control	*		*	*	*	*

TBO: toluidine blue O; LED: light-emitting diode.

*P value <0.05

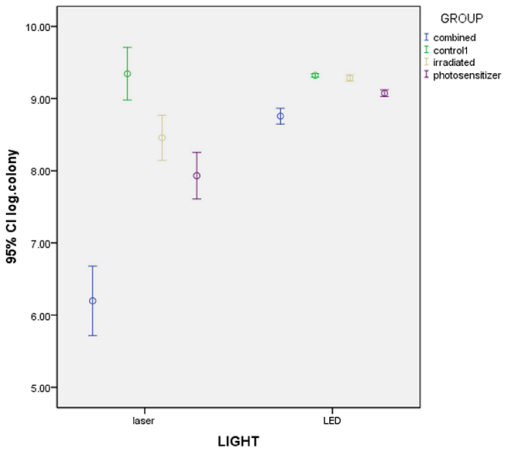

Figure 1. Error Bar of Mean and 95% CI for Mean Log Colony Count of P. gingivalis Bacteria.

aPDT using laser and Radachlorin reduces the viability of *P. gingivalis* more effectively than LED and TBO.

In our experiment, laser-based aPDT resulted in a substantial reduction of colony count of *P. gingivalis*. No study has been found to assess the effect of Radachlorin and laser on *P. gingivalis*. Vahabi et al observed that Radachlorin®-mediated aPDT using laser (12 J/cm^2) results in reducing the colony count of *Streptococcus mutans*.[17] Another study by Fekrazad et al showed that Radachlorin® 0.35% and diode laser (662 nm, 24 J/cm^2) reduced the colony count of *S. mutans*.[21]

The results of our study demonstrated that Radachlorin® reduced bacterial count significantly by itself. Vahabi et al reported that Radachlorin® (0.1%) alone had the ability to reduce *S. mutans* in dark conditions.[17] The chemical structure of the photosensitizer and the time of incubation might have an important role in the photosensitizer uptake before irradiation. During the incubation time, the photosensitizer is absorbed slowly by the microorganism.[9] In our experiment, Radachlorin® samples were incubated for 10 minutes in dark conditions before irradiation. The significant reduction of *P. gingivalis* in the presence of merely Radachlorin® might be due to the toxic effect of this photosensitizer.

We found that laser Irradiation (662±0.1% nm, 6 J/cm^2) alone resulted in a reduction of colony count of *P. gingivalis*. Soukos et al reported that black-pigmented bacteria that colonize the oral cavity contain large amount of endogenous porphyrins. In normal cultures and samples of dental plaque,these kinds of bacteria were affected by visible light irradiation.[22] So, the bactericidal effect of the laser light is species-dependent.[11] Umeda et al using red LED (650 nm, 1100 mW/cm^2) with dye solution methylene blue (MB) or TBO (10 mg/mL, 10 seconds) demonstrated that *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* have different susceptibilities to aPDT.[16]

On the other hand, the energy density and wavelength of laser significantly contributed to bacterial killing.[11,23]

LED Subgroups

Utilizing LED-based aPDT was more efficient compared with TBO alone or LED alone. These results confirmed the findings of Paardekooper et al. They reported that during illumination, the absorbance of TBO into the cell will be increased and this phenomenon leads to damage of the cellular membrane and intracellular structures.[12] Fekrazad et al stated that application of LED (with 630 nm wavelength) in the presence of TBO (0.1 mg/mL in concentration) resulted in significant reduction in *S. mutans* colonies.[21] Gois et al demonstrated that the combination of Photogem® and LED was more effective than LED alone (with 628 nm wavelength, power density: 14.6 mW/cm^2,40 J/cm^2, 60 J/cm^2 for energy density).[24] In addition, Umeda et al demonstrated that irradiation of red LED (650 nm, 1100 mW/cm^2) in the presence of dye solution (methylene blue or TBO 10 mg/mL 10 seconds) had a bactericidal effect on *P. gingivalis*.[16]

Analysis of our data showed a significant reduction of *P. gingivalis* colony count by using TBO alone. Previous studies demonstrated that TBO can directly start the photoinactivation of gram-positive bacteria as well as the gram-negative because of its positive charge in physiological pH.[25] Noteworthy, the effect of TBO toxicity on gram-negative bacteria is more characteristic.[26] Bhatti et al reported that penetration of TBO into the outer membrane and plasma membrane in *P. gingivalis* had the potential for cellular destruction.[27]

Based on our results, LED irradiation alone did not show any influence on the growth of *P. gingivalis*. This finding is in concurrence with that of Kim et al. They evaluated the relationship between the different wavelengths of LED (625, 525, and 425 nm) and its bactericidal effects on pathogenic bacteria. They concluded that LED (with 625 nm wavelength) did not have any bactericidal outcome on *P. gingivalis*.[26] Meanwhile, Chui et al made a similar conclusion in their study comparing the effects of blue and red LED irradiation on the growth of *P. gingivalis*. It was reported that the blue LED irradiation for 5 minutes did meaningfully reduced the expression of genes, DNA replication, and cell division whereas the irradiation of the red LED did not.[28] Gois et al observed a small reduction of colony count of *Staphylococcus aureus* using LED (628 nm, power density: 14.6 mW/cm^2, energy density: 60 J/cm^2) in the absence of photosensitizer.[24]

LED-Based aPDT Versus Laser-Based aPDT

Despite the high bactericidal effect of LED irradiation combined with TBO as a photosensitizer, the result of this study showed that laser-based aPDT was more effective than LED-based aPDT. We used an LED emitter with a peak wavelength of 630 nm, which almost coincided with the maximum absorbance of TBO (633 nm).[29]

Conclusion

In conclusion, based on the outcome of our research, laser-based aPDT (Radachlorin® as a photosensitizer) was more efficient than LED-based aPDT (TBO as a photosensitizer) in reducing the colony count of *P. gingivalis*. Also, a meaningful reduction of colony count of *P. gingivalis* was observed when laser, Radachlorin® or TBO were used alone.

Clinical investigations using different concentrations of the photosensitizers and different light source parameters are necessary prior to conclude any clinical benefit of aPDT.

Conflict of Interests

The authors state no conflict of interests.

Ethical Considerations

This study have been approved by ethical committee of Tehran University of Medical Sciences approved the study protocol (Approval number: 9101971719).

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