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Effect of Photodynamic Therapy using Different Protocols on Human Gingival Fibroblasts Cultured on Treated Root Fragments: An *In Vitro* Study

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Abstract

Introduction: In periodontal therapy, the adhesion of fibroblasts and their attachment to cementum present a significant challenge. This study aims to evaluate the effects of photodynamic therapy (PDT) using various protocols on the adhesion and cell count of human gingival fibroblasts (HGFs) to root fragments using a scanning electron microscope (SEM).

Methods: Root fragments were divided into four groups and subjected to different treatments: a control group receiving scaling and root planing (SRP), SRP combined with PDT using a 630 nm light-emitting diode (LED) and toluidine blue O (TBO), SRP combined with PDT using a 660 nm diode laser and methylene blue (MB), and SRP combined with PDT using an 810 nm laser and indocyanine green (ICG) as the photosensitizer (PS). Then, HGFs were cultured on the tooth fragments and evaluated using SEM after 72 hours of incubation. The adhesion of fibroblasts was assessed by counting the number of spindle-shaped fibroblasts, while their cell count was determined by counting the total number of cells. Adhesion was analyzed using the Kruskal-Wallis test, and the cell count was analyzed using one-way ANOVA followed by Dunnett's test.

Results: PDT utilizing all three protocols significantly enhanced the adhesion of fibroblasts compared to the control group ($P<0.05$). However, there were no significant differences in adhesion between the PDT groups ($P>0.05$). Additionally, PDT with a 630 nm LED significantly promoted the cell count of fibroblasts compared to the control group ($P=0.05$).

Conclusion: PDT utilizing three distinct protocols has been demonstrated to enhance the attachment of fibroblasts. Specifically, PDT employing a 630 nm LED has been found to significantly promote the cell count of fibroblasts under SEM.

Keywords: Cell adhesion fibroblasts; Periodontal diseases; Photodynamic therapy.

Introduction

Successful periodontal therapy relies on the adhesion and proliferation of gingival fibroblasts on the root surface. However, after conventional scaling and root planing (SRP), the presence of a smear layer and residual surface irregularities can hinder fibroblast attachment and prevent optimal periodontal healing.[1,2] Various adjunctive treatments have been proposed to modify the root surface and enhance its biocompatibility, which promotes fibroblast adhesion and proliferation. Among these, antimicrobial photodynamic therapy (PDT) has gained attention as a minimally invasive approach that may improve the biological compatibility of root surfaces by altering their chemical and structural properties.[3]

PDT is a technique used for the inactivation of microorganisms and biological molecules.[4] It requires three essential components: light, a photosensitizer (PS), and oxygen.[5] PS is a non-toxic agent that absorbs light and is activated by a specific wavelength. In the presence of oxygen, the PS transfers energy from optical excitation to oxygen molecules, resulting in the generation of reactive oxygen species (ROS). These ROS can damage DNA and cell membranes, leading to the leakage of cellular components, the inactivation of intracellular transport systems, and ultimately, the death of microorganisms.[6] PDT is employed as an adjunct to SRP using various light wavelengths and PSs, and it has demonstrated promising results.[2,3,7-10] This non-invasive modality can effectively eliminate inaccessible pathogens in periodontal pockets within a short period, benefiting both patients and clinicians.[11,12] However, there are inconclusive data regarding the effects of PDT on the behavior of human gingival fibroblasts (HGFs), including proliferation, migration, and adhesion.[13-16] Some studies showed that PDT using 500 and 1000 µg/mL of indocyanine green (ICG) as a PS had no positive effect on the proliferation

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of HGFs and even induced their apoptosis.[14-16] The commonly used concentration of PSs in many studies is 100 µg/mL.[2,8,10,17] Therefore, this in vitro study aimed to evaluate the effects of PDT using three different protocols: the combination of toluidine blue O (TBO) with a 630 nm light-emitting diode (LED), methylene blue (MB) with a 660 nm diode laser, and ICG with an 810 nm laser, all at a commonly used concentration of 100 µg/mL on the cell count and adhesion of HGFs to root surfaces using scanning electron microscopy (SEM).

Materials and Methods

The study protocol was approved by the ethics committee of the School of Dentistry, Islamic Azad University, Tehran (No 005/1399).

Preparation of Root Fragments

This in vitro experimental study was conducted on twelve extracted sound teeth with a hopeless periodontal prognosis. The teeth exhibited no caries or restorations. After extraction, the teeth were cleaned with sterile saline and a soft brush to remove blood, saliva, and soft tissue debris. Subsequently, two sections were made at the cervical third of the root, oriented mesiodistally, resulting in root fragments each measuring 4 mm in length, 2 mm in width, and 2 mm in thickness, using a diamond disc with a water coolant. To ensure complete removal of contaminants and standardize the experimental conditions, all tooth fragments underwent scaling with a Gracey curette (20 strokes for each fragment), were rinsed with saline, and then were autoclave-sterilized.[2,8,17]

Photodynamic Protocols

A total of 24 root fragments were randomly divided into four groups (n = 6):

- The control group consisted of six root fragments that underwent SRP alone.
- Experimental group 1 (630 nm LED): In this group, TBO (Merck, Germany) was applied to the root fragments at a concentration of 100 µg/mL for 60 seconds. The fragments were then irradiated with an LED at a wavelength of 630 nm (Fotosan, CMS dental, Copenhagen, Denmark) in continuous mode of irradiation, with a power density of 2000 mW/cm² for a duration of 30 seconds, resulting in an energy density of 60 J/cm.[10,18]
- Experimental group 2 (660 nm laser): In this group, MB from Loffler's Company (USA) was applied to the fragments at a concentration of 100 µg/mL for 60 seconds. The fragments were then irradiated with a diode laser (Thor Photomedicine Ltd., London) in continuous mode of irradiation at a wavelength of 660 nm, with a power output of 200 mW and a power density of 769 mW/cm² for 30 seconds per fragment, resulting in an energy density of 23 J/cm².[19]
- Experimental group 3 (810 nm laser): In this group, ICG (Emundo®, Germany) was applied to tooth fragments at a concentration of 100 µg/mL. The samples were then irradiated with a diode laser (Fox, A.R.C. Laser GmbH, Nuremberg, Germany) operating at a wavelength of 810 nm in continuous mode of irradiation, with a power output of 200 mW and an energy density of 400 mW/cm². The irradiation lasted for 30 seconds, resulting in an energy density of 12 J/cm².[20]

All lasers were irradiated in continuous-wave mode.

Following PDT treatment, all root fragments were rinsed with sterile saline to remove any residual PS. To maintain a contamination-free environment, they were immediately transferred to cell culture plates under sterile conditions.

Culture of HGFs

HGFs (HGF1-PI 1; NCBI NO. C165) were obtained from the Pasteur Institute of Iran. The cells were placed in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Grand Island, NY, USA) and 1% antibiotic-antimycotic (10.000 UI penicillin, 0.05g/L streptomycin, and amphotericin B) (Sigma-Aldrich, St. Louis, USA) and incubated at 37 °C and 5% CO2. The 6th passage HGFs were used in 10^4 cells/root fragment density.[2,8] HGFs were placed over the root fragments in 24-well plates (six replicates). The cells and tooth fragments were incubated for 3 days, and they were then fixed with Karnovsky solution. Post-fixation was performed in 1% osmium tetroxide in 0.05 M cacodylate buffer solution.

Scanning Electron Microscopy

SEM has been demonstrated as a valuable tool for the evaluation of cell morphology and surface adhesion. It does not provide a direct measure of metabolic activity and cell proliferation, but it has been used as a method of cell count in previous studies.[3,8,17,21,22] Here, the specimens were first dehydrated and then coated with a thin gold film via sputter deposition and subsequently imaged using SEM. For each specimen, five distinct regions (center, upper right and left field, lower right and left field were obtained, aiming to cover the whole sample), each with an area of 0.25 mm², were analyzed. To ensure consistency, all images were acquired under the same magnification and lighting conditions. As an example, Figure 1 presents low-magnification images obtained from one region of each of the four specimens. The quality of cell adhesion to various surfaces can be assessed using different techniques.[23] Evaluation of cell morphology and adhesion to the surface can be conducted through SEM.[21, 22] Fibroblasts exhibiting a spindle-shaped morphology (see Figure 2a) are generally associated with stronger

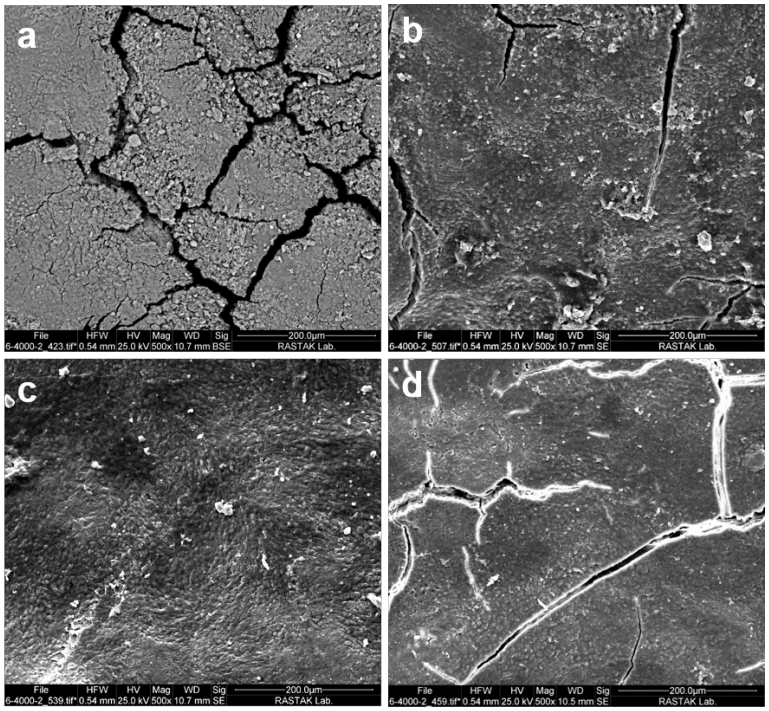

Figure 1. SEM Micrographs of the Groups at × 500 Magnification: (a) Control Group, (b) 630 nm Laser, (c) 660 nm Laser, and (d) 810 nm Laser

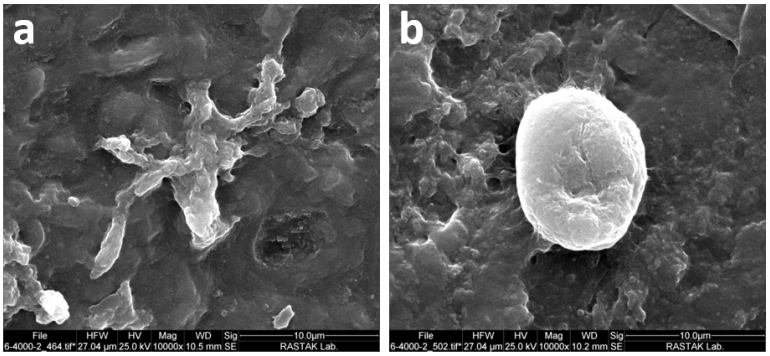

Figure 2. SEM Micrographs at × 10000 Magnification: (a) Spindle-Shaped Fibroblast, (b) Spherical Fibroblast

adhesion due to enhanced cytoskeletal organization and cell spreading, whereas fibroblasts with a spherical morphology (see Figure 2b) suggest a reduced adhesion potential.[24] The adhesion of fibroblasts was quantified by counting the number of spindle-shaped fibroblasts, while their relative cell coverage on the surface was determined by counting the total number of fibroblasts under SEM, conducted by a blinded examiner.

Statistical Analysis

The adhesion of fibroblasts was analyzed using the Kruskal-Wallis test. Additionally, the cell count of fibroblasts was compared among the three groups using a one-way ANOVA, followed by pairwise comparisons with Dunnett's test.[25]

Results

The box plots presented in Figure 3 illustrate the central dispersion of both total and spindle-shaped fibroblasts across each group. An assessment of fibroblast adhesion, using the Kruskal-Wallis test, revealed that the number of spindle-shaped fibroblasts was significantly higher in all three experimental groups compared to the control group ($P < 0.05$). However, there were no significant differences in the number of spindle-shaped fibroblasts between the experimental groups ($P > 0.05$, Figure 3b).

The results indicated that only PDT with a 630 nm LED resulted in a significant increase in the total number of fibroblasts compared to the control group ($P = 0.05$). The other groups did not show significant differences in this regard ($P > 0.05$, Figure 3a).

Discussion

This in vitro study evaluated the effects of PDT using a 630 nm LED with TBO, a 660 nm laser with MB, and an 810 nm laser with ICG as PSs on the adhesion and cell count

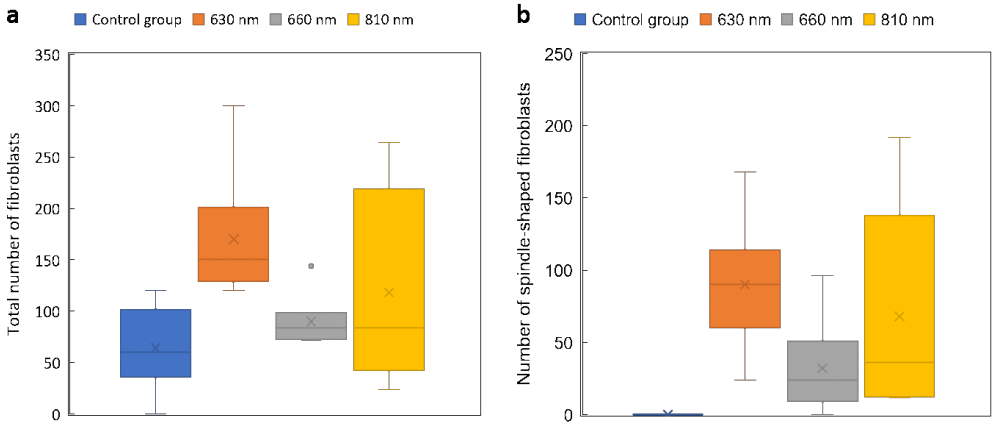

Figure 3. Box Plots Showing the Central Dispersion of the Number of Fibroblasts, Under a SEM, in Each Study Group. (a) The total number of fibroblasts; (b) The number of spindle-shaped fibroblasts

of HGFs on root fragments. The adhesion of fibroblasts was assessed by counting the number of spindle-shaped fibroblasts, while their cell count was evaluated by counting the total number of fibroblasts using SEM. Among various methods for the evaluation of fibroblast cell count and adhesion, we selected SEM analysis as it provides high-resolution micrographs enabling detailed quantification of cell number, morphology and adhesion to surfaces.[8,17,21,22] The results indicated that PDT with all three protocols significantly enhanced the adhesion of fibroblasts compared to the control group. However, only PDT with the 630 nm LED significantly increased the cell count of fibroblasts in comparison to the control group ($P=0.05$).

According to several studies, red light (600-760 nm) and near-infrared light (780-1000 nm) wavelengths penetrate the mucosa more effectively due to reduced absorption, resulting in favorable outcomes when utilized in low-level laser therapy.[26,27] Mitochondrial chromophores absorb low-level laser photons, including those in the near-infrared spectrum, which are not visible to the human eye. Cytochrome c oxidase, a key chromophore in the mitochondrial respiratory chain, is the first and most well-recognized light-absorbing chromophore. It activates various molecules, including nitric oxide, adenosine triphosphate, cyclic AMP, calcium ions, ROS, and numerous other signaling molecules. Additionally, it influences the cellular redox state,[28] initiates signaling cascades, and ultimately alters gene expression, leading to protein synthesis.[28,29] Diode lasers inhibit the production of pro-inflammatory cytokines, promote the synthesis of growth factors, and enhance cell proliferation.[30]

In the present study, PDT using a 630 nm LED at an energy density of 60 J/cm² significantly enhanced the cell count of fibroblasts after 72 hours compared to the control group. Huang et al[31] demonstrated that laser therapy with a 635 nm wavelength activated the copper-glycyl-L-histidyl-L-lysine complex, which promotes the synthesis of basic fibroblast growth factor and procollagen 1 carboxy-terminal propeptide. The latter possesses anti-inflammatory properties and directly facilitates wound healing. Sterczała et al[32] showed that laser irradiation of a 635 nm laser at an energy density of 64 J/cm² improved mitochondrial activity and significantly increased fibroblast proliferation compared to the control group after 48 hours. However, energy densities of 3 and 25 J/cm² did not significantly enhance fibroblast proliferation after 48 hours. The mitochondrial activity in all three groups decreased after 72 hours, which may be attributed to cellular saturation and a gradual reduction in the effects of the laser.[33] Nevertheless, in the current study, a significant increase in the fibroblast cell count was observed after 72 hours using a comparable energy density of 60 J/cm² compared to control groups. Our findings are consistent with a previous study that demonstrated increased proliferation of TBO-mediated PDT with an LED source of 630 nm on HGF on dentin blocks at 48- and 72-hour time points.[18] TBO–PDT has also been done in combination with a 660 nm laser in two studies.[2,8] SEM analysis revealed a significant increase in HGF proliferation following TBO-PDT at an energy density of 45 J/cm² on the root fragment, similar to our finding. However, only one of them[8] also investigated the effect of PDT on the adhesion of HGF, which showed enhanced attachment similar to our data.

In the current study, MB-mediated PDT using a 660 nm laser at an energy density of 23 J/cm² did not significantly alter the cell count of HGF; however, it did significantly enhance their adhesion. In a similar study, Ghasemi et al[34] demonstrated an increase in both proliferation and adhesion of HGF following MB-PDT. They applied a lower dose of irradiation (11.8 J/cm²) and utilized a different method for assessing proliferation, which may account for the observed differences. Our findings regarding ICG-mediated PDT were similar to those observed with MB in terms of cell proliferation and adhesion. According to a study by Mokhtari Asl et al,[14] PDT using an 810 nm laser with an energy density of 31.25 J/cm² and ICG as a PS at

concentrations ranging from 1000 to 2000 µg/mL resulted in a dose-dependent increase in the expression of the basic fibroblast growth factor (bFGF) gene. Conversely, PDT with 500 µg/mL ICG did not produce a significant change in bFGF gene expression. In the present study, PDT with an 810 nm laser at a lower energy density of 12 J/cm² and ICG at a concentration below 1000 µg/mL (specifically, 100 µg/mL) did not significantly enhance the fibroblast cell count; however, it did promote their adhesion to tooth fragments. It is important to note that, as SEM was used solely for visible cell counting, it does not provide a precise assessment of cell proliferation. Therefore, the findings related to the cell count should be interpreted with caution and validated in future studies using more specific methods for evaluating cell proliferation.

Rigi Ladiz et al [33] demonstrated the dose-dependent effect of an 810 nm laser on HGF. While an energy density of 0.5 J/cm² significantly increased cell viability, laser irradiation at energy densities of 1.5 and 2.5 J/cm² showed no significant difference in viability and proliferation when compared to the control group. Joshi et al[35] reported that PDT using an 810 nm wavelength, 200 mW power, and a 30-second irradiation time significantly reduced clinical attachment loss and periodontal probing depth when compared to SRP alone. In the present study, the laser with the same parameters improved the adhesion of HGFs to root fragments. In a study by Pourhajibagher et al,[36] the effect of PDT using ICG at various concentrations (500, 750, 1000, 1250, 1500, 1750, and 2000 µg/mL), and at three exposure regimens—30 seconds, 60 seconds, and two 30-second exposures with a 1-minute interval—was evaluated for its effect on the proliferation of monolayer HGF using the neutral red assay. The findings indicated that ICG significantly reduced cell viability at concentrations below 1000 µg/mL. In addition, cytotoxicity was higher when the cells were treated with two 30-second exposures. Different PDT protocols that utilize various PSs and light sources stimulate distinct chromophores and operate through different mechanisms of action. Consequently, they may exhibit varying effects.[37,38]

In this study, only SEM was used to evaluate cell adhesion and count. While SEM is a valuable tool for observing cell morphology and estimating the approximate number of cells on different surfaces, it does not directly measure metabolic activity or viability. Quantitative methods such as the MTT assay or DAPI staining could provide more precise data on cell proliferation and viability.[39] Therefore, future studies should incorporate additional techniques alongside SEM to obtain a more comprehensive evaluation of the effects of PDT on HGFs.

Conclusion

Within the limitations of this study, all PDT protocols, including TBO and 630 nm LED, MB and 660 nm Laser, and ICG and 810 nm laser, effectively increased the adhesion of fibroblasts. Among study groups, PDT employing a 630 nm LED in combination with TBO notably promoted the fibroblast cell count under SEM.

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Authors' Contribution

Conceptualization: Negin Barzegar Reyhani, Shabnam Aghayan.
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Project administration: Shabnam Aghayan.
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Software: Negin Barzegar Reyhani.
Supervision: Shabnam Aghayan.
Validation: Negin Barzegar Reyhani.
Visualization: Negin Barzegar Reyhani
Writing–original draft: Negin Barzegar Reyhani, Shabnam Aghayan, Neda Hakimiha.
Writing–review & editing: Negin Barzegar Reyhani, Shabnam Aghayan, Neda Hakimiha.

Competing Interests

All authors declare that they have no conflict of interest.

Ethical Approval

The study protocol was approved by the ethics committee of the School of Dentistry, Islamic Azad University, Tehran (No 005/1399).

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None.

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