



Photodynamic effects on human periodontal-related cells *in vitro*



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KEYWORDS

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Summary

Background: Photodynamic therapy (PDT) may be especially effective in combination with conventional periodontal therapy by its antimicrobial activities, but PDT may also exhibit other mechanisms that promote the healing of periodontal tissue. Therefore, the purpose of the present study was to evaluate the photodynamic effect of PDT on human periodontal ligament cells (hPDLs) and human gingival fibroblasts (hFBs) *in vitro* and other possible mechanisms to promote periodontal healing.

Methods: The proliferation of hPDLs and hFBs was assessed by MTT assay. Cell attachment on cementum slices of hPDLs and hFBs was evaluated by MTT assay. Type I collagen synthesis of hPDLs and hFBs was analyzed using enzyme linked immunosorbent assay. The alkaline phosphatase (ALP) activity in hPDLs was measured by p-nitrophenyl phosphate substrate reactions.

Results: PDT treatment induced constant time-dependent growth of hPDLs and hFBs at 24 h, 72 h and 6 days ($P < 0.05$). PDT treatment also promoted time-dependent hPDLs and hFBs attachment on the cementum slices at 24 h, 72 h and 6 days compared to the controlled cells ($P < 0.05$). Type I collagen synthesis of hPDLs and hFBs was markedly stimulated by PDT in a time-dependent manner ($P < 0.05$). Likewise, a significant increase in the specific ALP activity in hPDLs was observed ($P < 0.05$).

Conclusions: The findings of this study indicate that PDT exhibited no cytotoxicity to hPDLs or hFBs. Instead, it stimulated proliferation, attachment and collagen synthesis of hPDLs and hFBs and ALP activity of hPDLs. These effects might signal similar PDT activity on periodontal-related cells, and expanding the scope of its potential therapeutic utilization is very appealing.

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Introduction

Periodontitis is a chronic inflammatory disease triggered by periodontopathogens that leads to the destruction of supporting structures of the teeth, including pocket formation in the gum tissue, attachment loss, bone destruction and, ultimately, possible tooth loss [1]. The main approach to treat periodontitis involves the removal of supragingival and subgingival plaque biofilm by mechanical therapies, such as scaling and root planing [2]. Mechanical debridement aims at improving clinical conditions by lowering the microbial load either by physical removal of plaque or by radical alteration of the subgingival habitat. However, the use of mechanical instrumentation has its limitations. Indeed, the efficacy of subgingival instrumentation is largely dependent on the experience of the operator. Complete removal of bacterial deposits therefore remains very difficult to accomplish [3,4]. To further facilitate bacterial reduction, complementary methods, such as the use of systemically and locally administered antibiotics, directly target subgingival species residing in the plaque biofilm or in the adjacent epithelial tissues lining the periodontal pocket [5,6]. However, the emergence of resistance to antibiotics is increasingly concerning. In addition, an organized biofilm protects periodontal pathogens and greatly limits the antibiotic action [7–10]. For these reasons, innovative and efficacious approaches for the efficient removal of periodontal bacteria are currently being sought.

Photodynamic therapy (PDT) was first introduced as a medical therapeutic strategy around 100 years ago [11]. PDT utilizes the activation of a photoactivatable agent, the photosensitizer, bound to its target, with a certain wavelength of light to produce singlet oxygen and other highly reactive agents that are extremely toxic to target cells and bacteria [12–14].

Its characteristic mode of action makes PDT particularly appealing as an alternative to conventional antibiotic therapy for conditions such as periodontitis. Although several *in vitro* studies have demonstrated the effectiveness of PDT in suppressing periodontal pathogens, the usefulness of the clinical application of PDT in the treatment of periodontal diseases is still questionable [15–17]. Furthermore, it is important to determine whether host tissues would be affected by light doses and sensitizer concentrations that are effective against bacteria. A few studies have reported the photodynamic effects on host periodontal tissues. Soukos et al. [18] investigated the photodynamic effects of toluidine blue on human oral keratinocytes and fibroblasts *in vitro*, and Luan et al. [19] evaluated the safety of toluidine blue-mediated photosensitization on periodontal tissues in mice.

However, more proof of the effects of PDT on human periodontal related cells and tissues is needed. Current research has proven that PDT treatment may be especially effective in combination with conventional periodontal therapy, helping to increase bacterial killing and inactivate bacterial virulence factors and host cytokines that may impair periodontal restoration, but PDT may also exhibit other mechanisms that promote the healing of periodontal tissue [20–22].

Therefore, the purpose of the present study was to evaluate the photodynamic effect of PDT on human periodontal ligament cells and human gingival fibroblasts *in vitro*. This was an attempt to identify and characterize the effects of PDT on adjacent normal tissue and any mechanisms to promote periodontal healing it may exhibit.

Materials and methods

Cell isolation and cell culture

Human periodontal ligament cells (hPDLs) were obtained from extracted teeth removed for orthodontic reasons from young healthy volunteers. The periodontal ligament tissues attached to the middle one-third of the roots were removed by a surgical scalpel and then minced, placed in 35-mm culture dishes in Dulbecco's modified minimum essential medium (DMEM; Gibco BRL, Grant Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/ml penicillin G and 100 µg/ml streptomycin and were overlaid with sterile cover slips. Cultures were maintained at 37 °C in an incubator with an atmosphere consisting of 95% air, 5% CO₂ and 100% relative humidity. Cells were passaged until confluence was reached. Then cells were sub-cultured in fresh DMEM containing 10% FBS under the standard incubation conditions. Cells of the third passage were used for the experiment.

Human gingival fibroblasts (hFBs) were obtained from gingiva removed during crown lengthening surgeries from periodontal healthy patients. Gingival tissue samples were minced into small pieces using microdissection scissors and washed with serum-free DMEM. The gingival tissue was then digested in serum-free medium containing antibiotics and 1 mg/mL type IV collagenase (Worthington Biochemical, Freehold, NJ, USA) at 37 °C in a 5% CO₂ humidified atmosphere for 3 h. The supernatant was then removed, and the remaining gingival tissue was placed in six-well culture dishes, allowing cells to migrate from the explants. After confluence was reached, cells were trypsinized and cultures expanded. Cells of the third passage were used for the experiment.

In all cases, patients were informed of the nature and extent of the study, and their informed consent was obtained according to the Helsinki Declaration. The study was approved by the Ethics Committee of the Peking University School and hospital of Stomatology, Beijing, China.

Preparation of cementum slices

Healthy cementum slices were obtained from teeth freshly extracted for orthodontic reasons. These teeth had no caries or fillings. The soft tissue on the root surfaces was gently removed with a scaler, and then the middle one-third of the tooth roots was taken and prepared with emery milestone. The final size of cementum slices was 4 mm × 4 mm. These slices were disinfected with penicillin and streptomycin, and then stored in DMEM medium for later use.

Table 1 The parameters of laser irradiation.

Parameter	Units
Fiber diameter	320 μm
Beam diameter	10.4 mm
Distance from the light tip to cells in culture	9 mm
Fiber steady or moved	Moved
Illuminated surface diameter	34.8 mm
Power emitted toward the surface	329 mW/cm^2
Power density at the target surface	19.7 J/cm^2

Laser and photosensitizer selection

A non-thermal diode laser (Periowave, Ondine Biopharma Corporation, Canada) (675 nm, $P_{\text{max}} = 280 \text{ mW}$) was used for all the tests. The parameters of laser irradiation are shown in Table 1. Methylene blue (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in growth medium to give a stock solution at a concentration of $1000 \mu\text{g}/\text{mL}$. The solution was kept in the dark and further diluted in growth medium prior to use.

Treatment of cells

Cells were trypsinized in the exponential growth phase and counted using a hemocytometer. The hPDLCs and hFBs were seeded in 6-well culture plates (Corning Life Sciences, Acton, MA, USA) at a density of 5×10^6 cells/well and were incubated for 24 h in 2 mL of DMEM containing 10% FBS to allow cells to attach and resume exponential growth.

After 24 h, the baseline of the experiment, the medium was removed and replaced with 2 mL of DMEM containing 10% FBS and 0.01% methylene blue (MB). Control cultures received only medium without MB. Ten seconds after the addition of MB, the cells of the test group received a continuous 60 s of illumination. The starting room temperature was 22°C , and the temperature increased about 2°C from light illumination for about 30 s, which would not affect cell mitochondrial activity [23]. An air conditioner was used to control the room temperature.

Cell proliferation

hPDLCs and hFBs were grown for 24 h, 72 h and 6 days following treatment. Cell proliferation analysis was performed using the MTT assay according to the cell proliferation kit protocol (Sigma–Aldrich, St. Louis, MO, USA) at different time points. In the MTT test, tetrazolium salts were transformed by active enzymes of the cells into intracellular formazan deposits; the amount of color produced was directly proportional to the number of viable cells. Absorbance was determined at 490 nm with a microplate reader (Bio-Rad Model 550, Hercules, CA, USA).

Cell growth on cementum

hPDLCs and hFBs were grown together with the cementum slice for 24 h, 72 h and 6 days following treatment. At different time points, cementum slices were taken out and washed with sterile PBS solution to remove the unattached

cells. Then $20 \mu\text{L}$ of trypsin was added to each slice to digest the cells on the slice surface. After adding $200 \mu\text{L}$ of MTT to each well, the slices were incubated for 4 h. Then the supernatant was discarded, and 1 mL of DMSO was added to each well. After shocking for 10 min, absorbance was determined at 490 nm with a microplate reader.

Collagen extraction and analysis

hPDLCs and hFBs were grown for 24 h, 72 h and 6 days following treatment. The collagen type I produced by cells was extracted and analyzed using enzyme linked immunosorbent assay (ELISA) (MD Biosciences, Gewerbestrasse, Switzerland). At different time points, the cell culture media was removed, and $500 \mu\text{L}$ of 0.05 M acetic acid (pH 2.8–3.0 with formic acid) was added to the cell layer. The cells were transferred to a microcentrifuge tube using a cell scraper. Then $50 \mu\text{L}$ of pepsin solution was added which digested the collagen at $2\text{--}8^\circ\text{C}$ overnight with gentle mixing. After the addition of $50 \mu\text{L}$ of 10X TBS and the adjustment of pH to 8.0 with 1 N NaOH, $50 \mu\text{L}$ of pancreatic elastase solution was added, and the mixture was incubated at $2\text{--}8^\circ\text{C}$ overnight with gentle mixing. Then the samples were centrifuged at 10,000 rpm for 5 min at room temperature. Twenty-five microliters of the supernatant was aspirated and diluted with $225 \mu\text{L}$ of assay diluent. The competitive enzyme immunometric assay (EIA), a colorimetric based immunoassay, utilized a biotinylated polyclonal antibody to collagen type I and purified human collagen type I as an antigen. Bound collagen type I coated on the surface of the microwells competed for the biotinylated antibody with free antigen in the standard and samples. This system created an inverse relationship between OD values and concentration: the higher the OD value, the lower the concentration of collagen type I in the sample. The plate was then read at 450 nm using a microplate reader (Bio-Rad Model 550). The assay kit instructions were followed for this experiment.

Alkaline phosphatase activity in hPDLCs

hPDLCs were grown for 24 h, 72 h and 6 days following treatment. At different time points, alkaline phosphatase (ALP) activity in the hPDLC cell layer was measured by p-nitrophenyl phosphate (p-NPP) substrate reactions (Sigma–Aldrich, St. Louis, MO, USA). After the medium was removed, cells were washed twice with PBS and solubilized by the addition of $200 \mu\text{L}/\text{well}$ of 0.2% Triton X-100 and agitation on a plate-shaker for 20 min. The lysates were then collected and centrifuged at $13,000 \times g$ for 5 min at 4°C , and the supernatants were used for the determination of ALP activity. The reaction mixture contained cell extract and the substrate (0.1 M diethanolamine buffer pH 10.5, 0.5 mM MgCl_2 , 12 mM p-nitrophenyl phosphate) in a final volume of 0.5 mL. The mixture was incubated at 37°C for 30 min, and the reaction was stopped by the addition of 0.5 mL of 0.5 M NaOH. The p-nitrophenol formed was spectrophotometrically measured at 405 nm using a microplate reader (Bio-Rad Model 550).

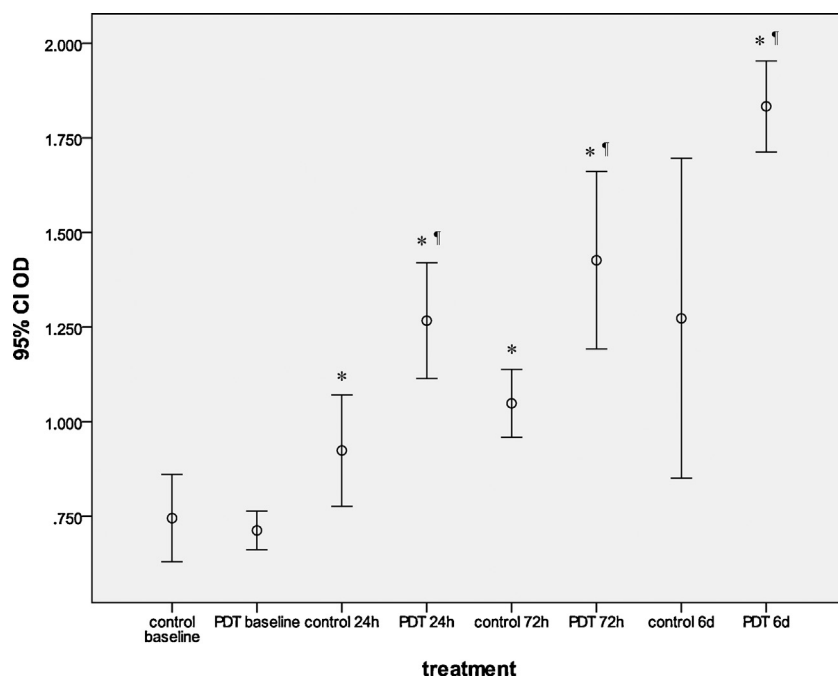


Figure 1 Time-dependent increase of hPDLC proliferation after PDT treatment. *: compared with the other time points, $P < 0.05$; †: compared with the controlled cells at the same time points, $P < 0.05$.

Statistical analysis

All reported values are the means of triplicate samples, and tests were repeated twice. Data were analyzed using SPSS version 10.0 (Chicago, IL, USA). Statistical analysis of the data was performed by Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Tukey *post hoc* test for comparisons. For all tests, statistical significances were accepted for P values lower than 0.05.

Results

Effect of PDT on hPDLC and hFB proliferation

The effect of PDT on hPDLC and hFB cell growth was determined by MTT assay. PDT treatment induced a strong time-dependent increase of hPDLC proliferation (Fig. 1). A significant difference between PDT-treated cells and control cells became apparent at 24 h and was further increased at 72 h and 6 days ($P < 0.05$). The increased growth rate of PDT-treated hPDLCs presented an evident multilayered disposition of the cells (Fig. 2). The growth of the controlled hPDLCs significantly increased at 24 and 72 h compared with the baseline ($P < 0.05$), while the difference between the proliferation of the controlled hPDLCs at 72 h and 6 days was not statistically significant ($P > 0.05$).

Similarly, PDT treatment also induced a time-dependent increase of hFB proliferation compared to the control group (Fig. 3). Treatment with PDT resulted in a constant significant increase in hFB proliferation at 24 h, 72 h and 6 days ($P < 0.05$). The growth of the controlled cells also increased at 24 h, 72 h and 6 days compared to the baseline ($P < 0.05$), though there was no significant difference between the

proliferation of controlled cells at 72 h and 6 days. The growth of hFBs is shown in Fig. 4.

Effect of PDT on hPDLC and hFB growth on cementum

PDT treatment promoted time-dependent hPDLC growth on the cementum slices at 24 h, 72 h and 6 days compared to the controlled cells ($P < 0.05$) (Fig. 5). However, the controlled cells only showed increased growth on the cementum slices at 24 h. The growth of controlled cells at 72 h and 6 days showed no significant increase compared to 24 h ($P > 0.05$).

A similar phenomenon was observed in the growth of hFBs on the cementum slices (Fig. 6). PDT-treated hFBs showed increased time-dependent growth compared to the controlled cells ($P < 0.05$). The controlled cells showed an increasing tendency at 24 h, 72 h and 6 days compared to the baseline, while the difference between different time points was not statistically significant ($P > 0.05$).

H&E staining of hPDLC growth on the cementum slices is shown in Fig. 7.

Effect of PDT on hPDLC and hFB collagen levels

Type I collagen synthesis of hPDLCs and hFBs was markedly stimulated by PDT.

As shown in Fig. 8, collagen levels were significantly higher in hPDLCs treated with PDT than in controlled cells at different time points ($P < 0.05$), and these changes were time-dependent ($P < 0.05$). For the controlled cells, only the collagen level at 72 h was significantly higher than that at 24 h ($P < 0.05$).

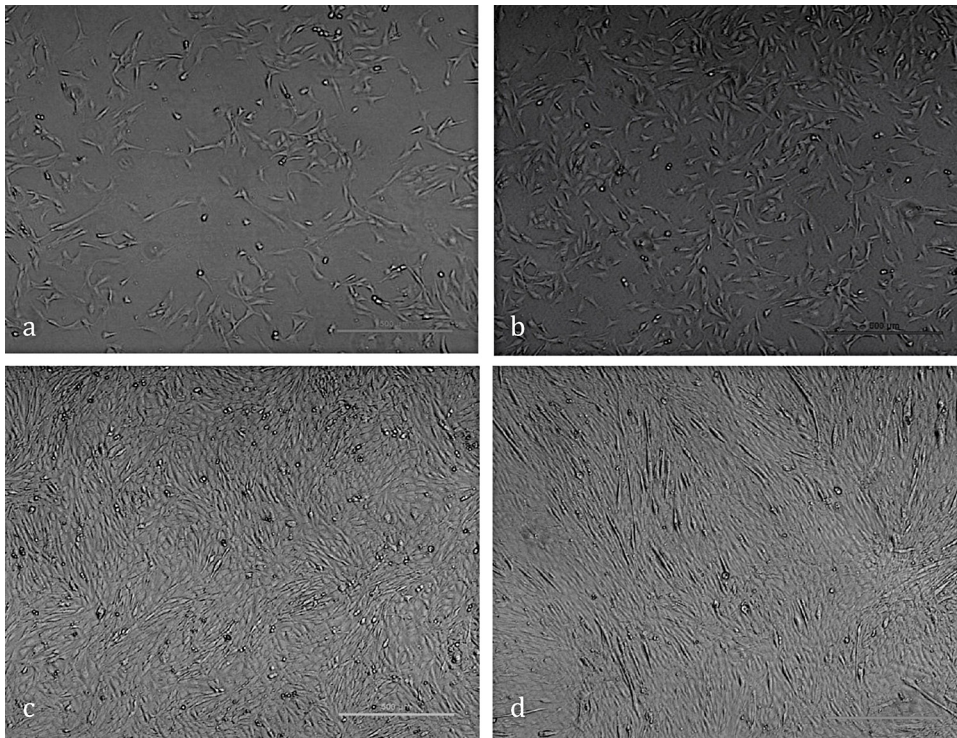


Figure 2 Increased growth of hPDLC cells after PDT treatment. (a) Baseline; (b) 24 h after PDT treatment; (c) 72 h after PDT treatment; (d) 6 d after PDT treatment.

PDT also increased type I collagen synthesis of hFBs in a time-dependent manner ($P < 0.05$) (Fig. 9). Furthermore, the collagen levels of PDT-treated cells at different time points were significantly higher than that of the controlled cells ($P < 0.05$).

Effect of PDT on hPDLC ALP activity

hPDLCs treated with PDT showed a significant increase of ALP activity compared to controlled cells at 24 h, 72 h and 6

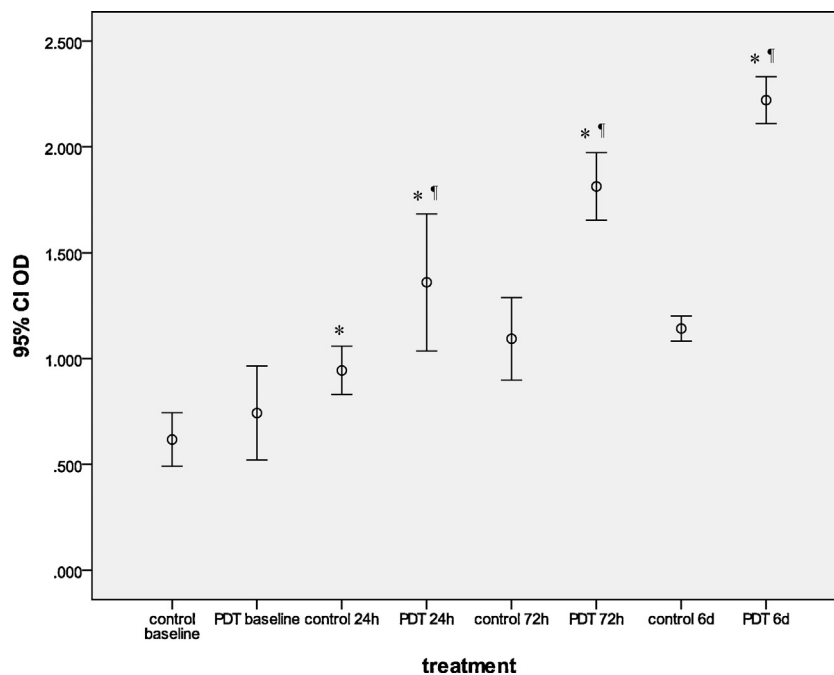


Figure 3 Time-dependent increase of hFB proliferation after PDT treatment. *: compared with the other time points, $P < 0.05$. †: compared with the controlled cells at the same time points, $P < 0.05$.

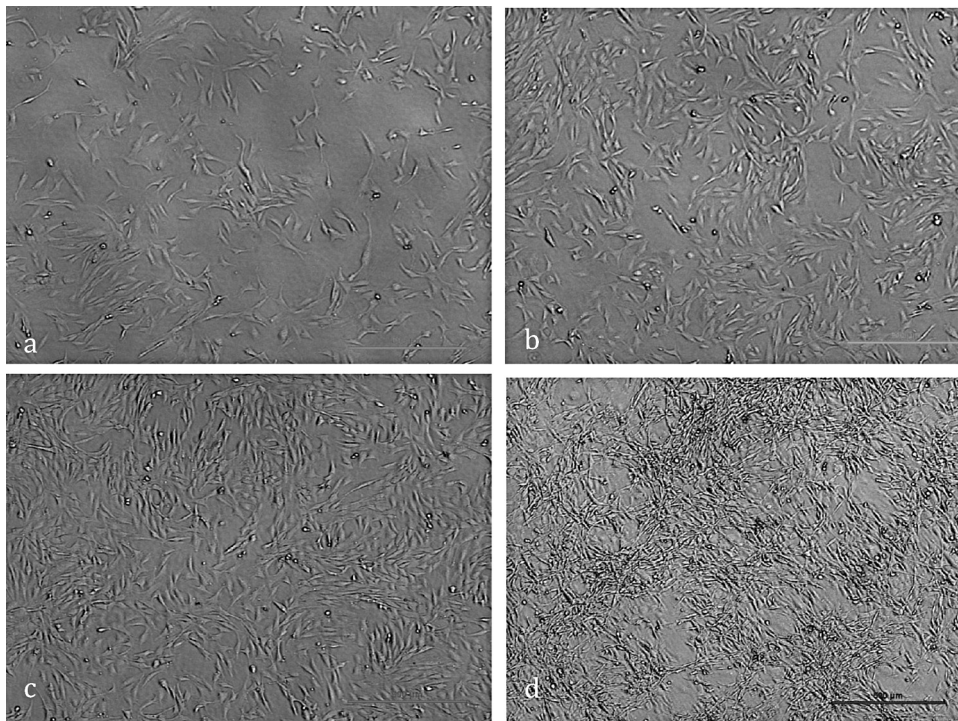


Figure 4 Increased growth of hFB cells after PDT treatment. a. baseline; b. 24 h after PDT treatment; c. 72 h after PDT treatment; d. 6d after PDT treatment.

days ($P < 0.05$) (Fig. 10), while the controlled cells showed no significant increase in ALP activity ($P > 0.05$). ALP activity reached its maximum after 6 days of PDT treatment ($P < 0.05$).

Discussion

This study was designed to test the safety and effect of PDT on the function of hPDLCs and hFBs, the two cell types

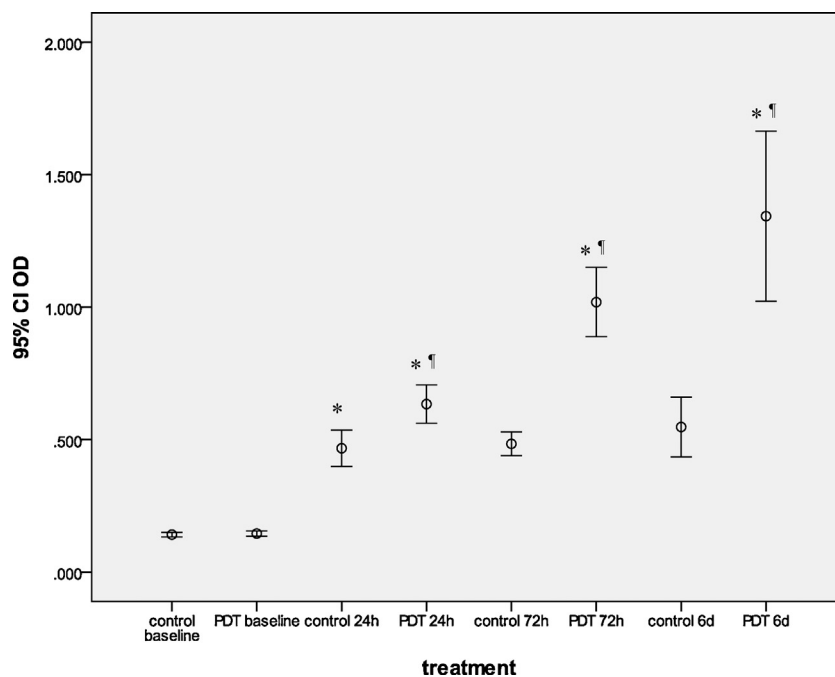


Figure 5 Increased time-dependent growth of hPDLC cells on cementum after PDT treatment. *: compared with the other time points, $P < 0.05$. †: compared with the controlled cells at the same time points, $P < 0.01$.

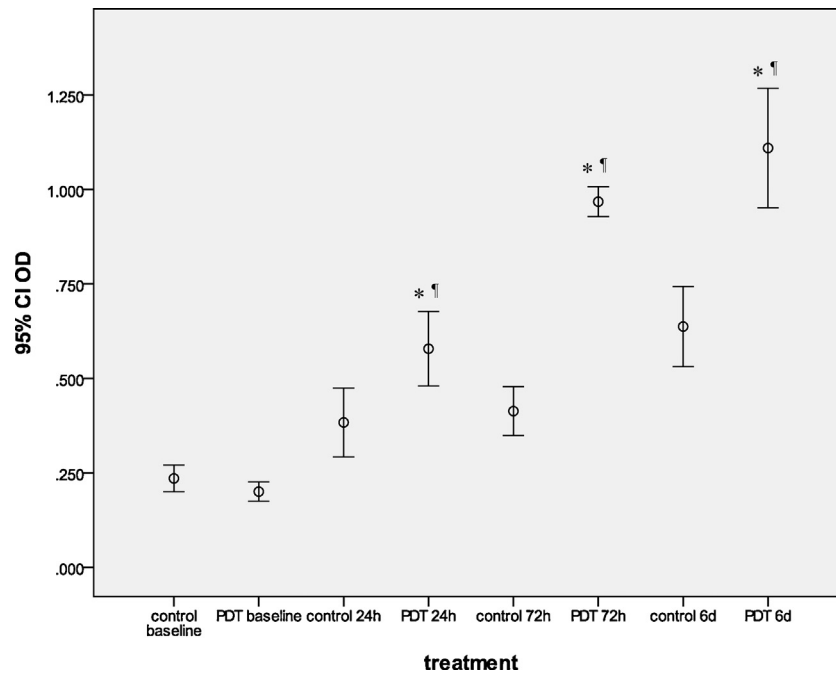


Figure 6 Increased time-dependent growth of hFB on cementum after PDT treatment. *: compared with the other time points, $P < 0.05$. †: compared with the controlled cells at the same time points, $P < 0.01$.

critical for wound healing of periodontal tissues, under *in vitro* conditions. The findings of this study indicate that PDT exhibited no cytotoxicity to hPDLCs or hFBs. Instead, it stimulated proliferation, attachment and collagen synthesis of hPDLCs and hFBs and ALP activity of hPDLCs.

PDT showed a time-dependent effect on the function of hPDLCs and hFBs, albeit to different extents. PDT treatment induced a strong time-dependent increase of hPDLC and hFB proliferation, especially at 24 h, and time-dependent attachment compared to controlled cells after 24 h. PDT also induced collagen synthesis in hPDLCs after 24 h and in hFBs after 72 h. hPDLCs treated with PDT showed a significant increase of ALP activity after 72 h.

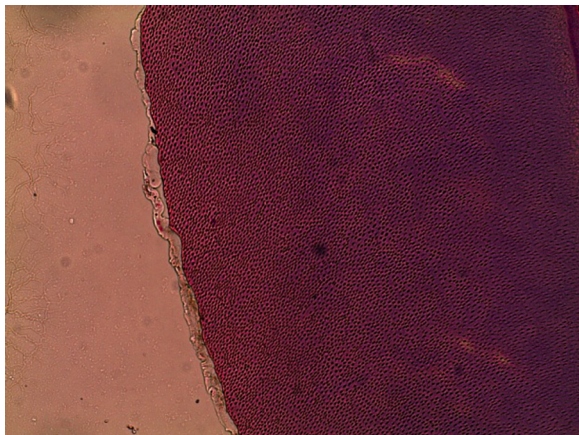


Figure 7 H&E staining of hPDLC growth on the cementum slices.

PDT acts through the light-induced excitation of the photosensitizer and its subsequent conversion from the ground state to the triplet state. The lifetime of the triplet state is in the microsecond to the millisecond range. Movement from the triplet state to the ground state results in the emission of light (phosphorescence). Alternatively, molecules in the triplet state can react further to produce free radicals and/or singlet oxygen species via Type I and Type II photoprocesses, respectively. Electron transfer between the photosensitizer and a substrate produces radical ions which, upon reacting with oxygen, create numerous cytotoxic species in the target region. The Type II process involves energy transfer from the photosensitizer triplet state to ground state molecular oxygen (triplet) to produce an excited state singlet oxygen. This singlet oxygen can then oxidize proteins, nucleic acids and lipids, leading to cytotoxicity. Singlet oxygen, probably the major damaging species in photodynamic therapy, is short-lived in biological systems ($< 0.04 \mu\text{s}$) and has a short radius of action ($< 0.02 \mu\text{m}$) [24–26]. Due to the tendency of the singlet oxygen to remain near the site of its formation, most cell damage related to PDT treatment remains localized to regions of the sensitizer, thus limiting the effects of PDT on distant systems.

Biofilms of oral bacteria have been shown to be one of the primary agents responsible for chronic periodontitis [27,28]. It has been demonstrated that gram-negative bacterial species are more resistant to PDT than gram-positive bacteria [29,30]. However, modification of the cell wall or the selection of special positively charged photosensitizers that bind more readily to the cell wall may act to circumvent this resistance [31,32]. In clinical periodontal therapy, each site (periodontal pocket) is thoroughly irrigated with PDT photosensitizer solution, followed by a continuous 60 s of laser illumination in both apico-coronal and mesiodistal

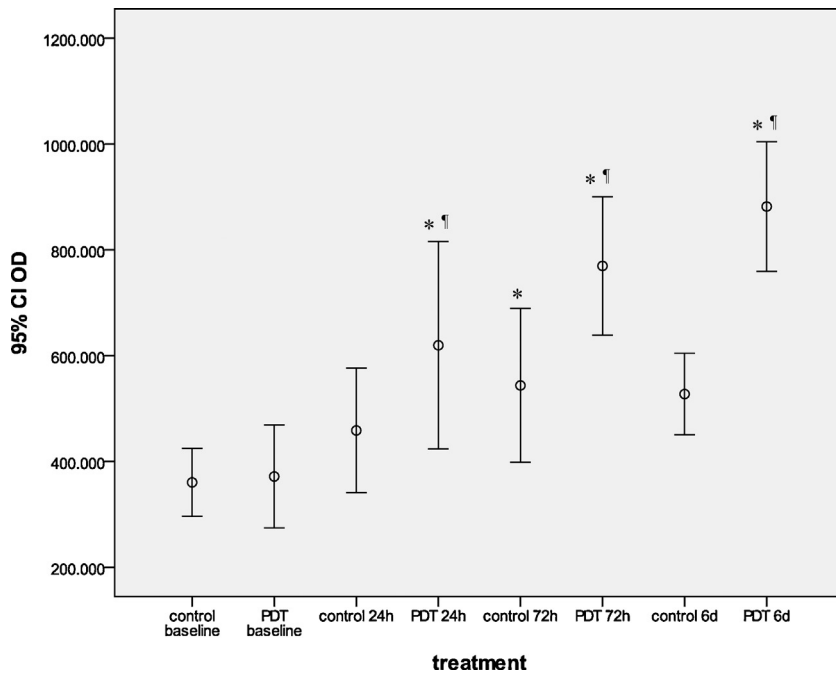


Figure 8 Increased hPDL collagen levels after PDT treatment. *: compared with the other time points, $P < 0.05$. †: compared with the controlled cells at the same time points, $P < 0.05$.

directions, exciting photosensitizer molecules bound to subgingival bacterial films.

Previous studies investigated the effect of PDT on periodontitis and host periodontal tissues. Garcia et al. found that PDT, using low concentrations of methylene blue or toluidine blue, was the most effective adjuvant therapy to scaling and root planing, acting indirectly as a

down-regulator of the molecular mechanisms that control bone resorption in periodontitis [33]. Franco et al. compared conventional mechanical debridement with conventional mechanical treatment followed by PDT in patients with severe periodontitis. The results suggested that PDT acted in part by controlling bone resorption and increasing the expression of genes important for tissue repair [34].

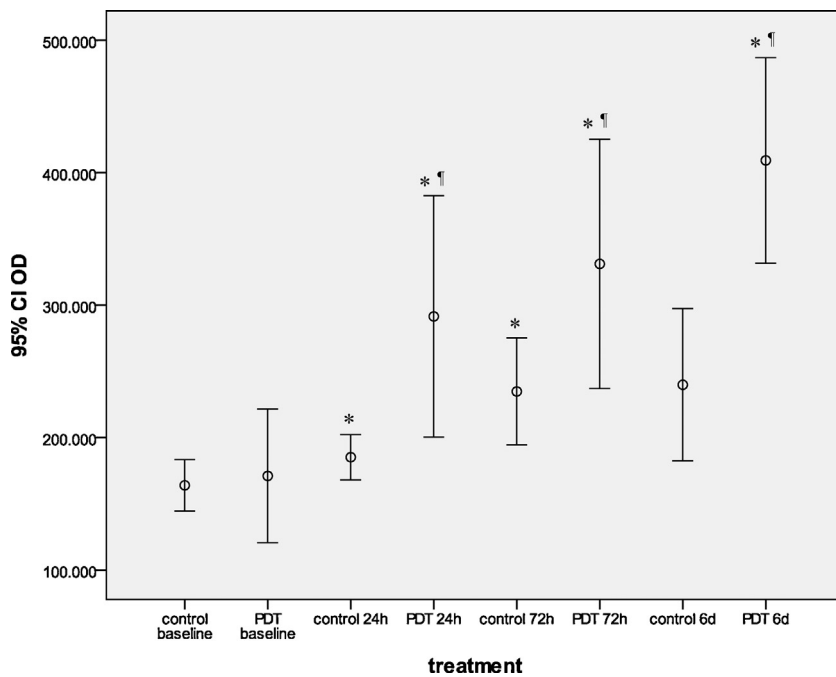


Figure 9 Increased hFB collagen levels after PDT treatment. *: compared with the other time points, $P < 0.05$. †: compared with the controlled cells at the same time points, $P < 0.05$.

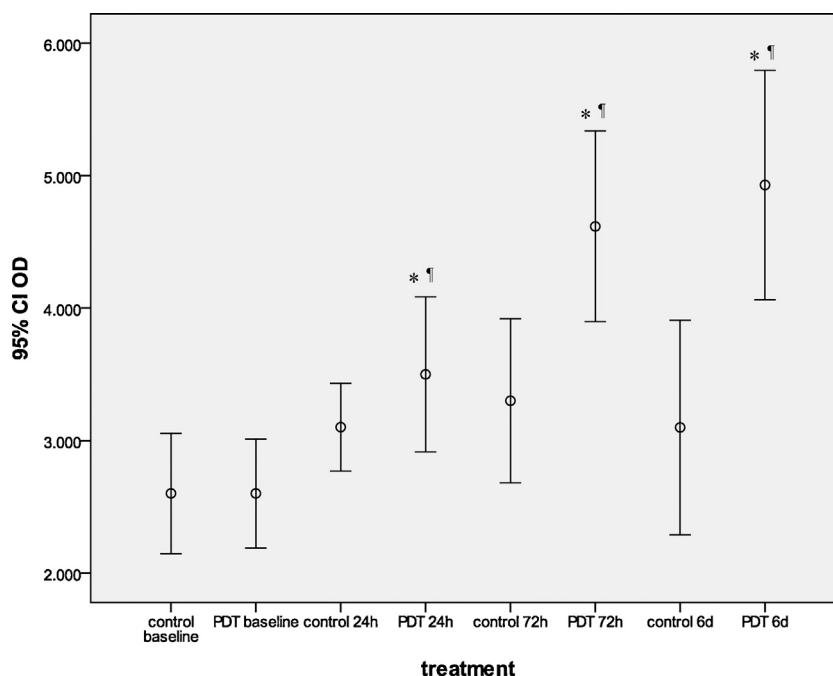


Figure 10 Increased ALP activity in hPDLC cells after PDT treatment. *: compared with the other time points, $P < 0.05$. †: compared with the controlled cells at the same time points, $P < 0.05$

The present study showed that PDT was safe for the adjacent periodontal cells – PDLCs and FBs. More importantly, PDT could improve the function of these two cell types *in vitro*. Proliferation, attachment and extracellular matrix formation are crucial for the periodontal healing process, especially in the early phase (days 1–6). PDT-mediated stimulation of ALP, an early marker of the osteoblastic phenotype expressed at high levels by PDLCs, might enhance periodontal regeneration, offering to hPDLCs the instruments required for the mineralization of hard tissues composing the deep periodontium.

PDT might also improve periodontal healing and tissue regeneration, which, in addition to its antimicrobial effects, makes PDT a valuable periodontal therapeutic option. Indeed, the use of PDT in periodontal therapy may be expanded beyond antimicrobial therapy to other fields, such as periodontal regeneration. However, further studies are still needed to investigate the other mechanisms by which PDT may act on periodontal cells and tissues.

Notably, some studies do not support the benefits of PDT. Pourabbas et al. compared the clinical parameters and cytokine profiles in gingival crevicular fluids of patients with moderate to severe chronic periodontitis who had been treated using SRP alone or SRP + PDT. The results showed that PDT did not provide any additional benefit to SRP in terms of clinical parameters or inflammatory markers three months following the intervention [35]. More research is needed to identify the time-dependent effects of PDT on periodontal tissues and periodontitis.

In conclusion, within the limits of the present study, PDT treatment enhanced the proliferation, attachment and collagenous matrix synthesis of hPDLCs and hHBs *in vitro*, and enhanced the ALP activity of hPDLCs *in vitro*. Time-dependent improvement was also noted. These effects

might signal similar PDT activity on periodontal-related cells, and expanding the scope of its potential therapeutic utilization is very appealing.

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