Chapter 4

Photodynamic therapy has no adverse effects in vitro on human gingival fibroblasts and osteoblasts
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Abstract:

**Aim:** Invasion of periodontal pockets with persistent microorganisms and subsequent development of a biofilm are the main cause of periodontal infections. In severe cases, additional use of antibiotics to the standard therapy of scaling and root planing (SRP), is necessary, but the use of antibiotics may lead to resistance. As an alternative, the combination of SRP and adjunct photodynamic therapy (PDT) is applied in the treatment of periodontal infections to improve periodontal therapy. The aim of this study was to determine possible side effects of PDT on human gingival fibroblasts (GF) and human osteoblasts (OB).

**Material and Methods:** GF and OB were either untreated or treated with methylene blue (MB) only, with MB and subsequently irradiation with a soft laser (PDT) or irradiated with a soft laser only. All cells were analyzed for viability using the MTT test, migration capacity using Boyden chambers and the scratch wound assay.

**Results:** Viability and migration capacity of GF and OB were not affected by PDT whereas soft laser irradiation only improved cell viability and migration and MB treatment only reduced cell viability and migration.

**Conclusion:** In vitro, PDT did not affect viability and migration capacity of GF and OB whereas soft laser treatment only had an positive effect on GF and OB. Therefore, PDT seems to be a safe method in the treatment of periodontal infections without significant side effects.

**Keywords:** PDT, periodontitis, in vitro study

**Running head:** PDT and periodontal cells
Introduction

Periodontal diseases occur more frequently in time, for example in Germany its incidence has risen by approximately 27% over the past decades [1]. Thirty to 50% of adults in industrialized countries are affected by periodontitis [2]. Today, periodontitis is defined as a multifactorial disease and several risk factors have been described. Standard therapy such as scaling and root planing (SRP) is often not sufficient. Supporting therapy with antibiotics is considered in difficult cases. The main problem of antibiotics is that their use can lead to resistance. Furthermore, systemic treatment with antibiotics may cause unwanted side effects such as allergies or gastrointestinal complaints.

Photodynamic therapy (PDT) seems to be an attractive bactericidal alternative to support and improve periodontal therapy with minor side effects. PDT is a well-established method that is used in, for example, oncology, dermatology and dentistry. The history of PDT goes back to the ancient Egyptians who treated skin diseases with light-absorbing compounds. Oxygen-dependent photodynamic reactions were discovered by Oscar Raab in 1897-1898. The mechanism of PDT is based on interactions between a photosensitizer and a soft laser. Today, there are over 400 compounds that are known that have photosensitizing properties. Most of the sensitizers used for medical purposes are either tricyclic dyes, tetrapyrroles or furocoumarines [2].

After application of a sensitizer that attaches to the membrane of bacteria (e.g. methylene blue (MB)), the sensitizer is irradiated with a non-thermal diode laser (630-690 nm). The energy of the excited sensitizer in its triple state is then transferred to molecular oxygen to generate oxygen-derived free radicals that are reactive and destroy the membrane of bacteria. The use of PDT for desinfection of intra-oral regions is based on various studies by Wilson et al. [3-10]. PDT is also used for indications such as periodontology, endodontology or peri-implantitis therapy [2, 11-16].

The purpose of periodontal treatment is the elimination of bacteria that are present in the periodontal pockets. As a consequence, a relatively large area has to be irradiated and possible unwanted side effects of PDT may occur in cells of the periodontium. Qiao et al. [17] reported an in vitro study of the effects of PDT on human periodontal ligament cells and human gingival cells. PDT showed to have positive effects rather then cytotoxic effects to the cells in
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Materials and Methods

Cell cultures

Human gingival fibroblasts
GF (3H Biomedical, Uppsala, Sweden; AB # SC2620) were obtained and cultured in an incubator (Heraeus Instruments, Hanau, Germany) under standard conditions (5% CO₂ and 95% air at 37°C) in Dulbecco’s Modified Eagle Medium, (DMEM; Invitrogen™, Carlsbad, CA, USA) supplemented with 0.05% penicillin-streptomycin-neomycin mixture (PSN; Invitrogen, Paisley, UK) and 10% fetal bovine serum (FBS; PAA, Pasching, Austria).

Human osteoblasts
OB (PromoCell, Heidelberg, Germany, proliferating cells # C-12760) were cultured under standard conditions (5% CO₂ and 95% air at 37°C) in OB growth medium (Promo Cell) supplemented with 1% PSN, 10% FBS and 1% L-glutamine in an incubator (Heraeus Instruments).

Laser irradiation and application of photosensitizer
We have simulated PDT treatment of periodontal patients in our in vitro study as much as possible by using the same soft laser irradiation settings and photosensitizer applications.

The laser device that we used was the diode laser Periowave™ (Ondine Biopharma, Vancouver, Canada) with an emission spectrum of 650-675 nm and a maximum power of 160 mW. An optic fiber with a diameter of 350 µm delivered the light to the cell cultures. The fiber was positioned at a distance of exactly 25 mm from the cell monolayers without the use of the flexible periodontal tip with an irradiation power of 890 mW/cm² that covered the entire surface of each well [18]. Irradiation time per well was set at 60 sec. In this setting, the power density of the target surface was 53.4 J/cm².

The photosensitizer applied in this study was methylene blue (MB; Ondine Biopharma, Vancouver, Canada) with an absorbance maximum at 670 nm.

Four treatment groups of GF and OB were studied. As control group, we used untreated cells (Control group). The cells of the second group (MB group) were incubated in the presence of 1 µM MB for 60 sec, cells of the third group (PDT group) were treated with 1 µM MB for 60
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Cells in all treatment groups were analyzed for cell viability using a MTT viability test and migration capacity in a Boyden chamber as well as a scratch wound assay. All experiments were carried out in triplicate with all measurements performed in triplicate.

MTT assay

For the MTT assay, 5 x 10⁴ human GF or OB in 10 µl DMEM in the presence of 10% FCS were seeded in 24-well plates. To examine the viability of GF and OB, 100 µl cell suspension was added to 1 ml medium containing MTT (Sigma, Deisenhofen, Germany). Lysis buffer (2 ml) was used to lyse cells (49 ml isopropanol and 1 ml 1N hydrochloric acid). The absorbance of MTT was measured using a spectrophotometer (T & O LAB Systems, Henstedt - Ulzburg, Germany) at 550 nm as a measure of cell viability.

Migration test

To investigate the migration behavior of GF and OB, Boyden chambers (Greiner Bio-One GmbH, Frickenhausen, Germany) were used. A porous polyethyleneterephthalate membrane (pore size, 8 µm) separated the 2 compartments of the Boyden chamber. Cells (5 x 10⁴ human GF or OB) were added in the upper well and absorbance at 550 nm in the upper and lower wells was determined as a measure of migration of the cells.

Scratch wound assay

For the scratch wound assay, cells were seeded in 6-well plates (one plate per cell group). When 80% confluency was reached after 24 h incubation, a straight scratch using a 200 µl sterile pipette tip was made to simulate a wound and the size of the gap was measured and set at 100%. Cell cultures were observed and photographed using a stereomicroscope after 0, 24, 48 and 72 hours. The distance of the front of migrating cells to the center of the wound was measured.

Statistical analysis

Statistical analyses were performed using SPSS 22.0 (Chicago, IL, USA). Continuous parameters were described by mean and standard deviation and the Mann-Whitney U-Test was used for continuous data. Bonferroni correction with a local significance level of α = 0.0125 was used to account for multiple testing.
Results

Figures 1-3 show viability and migration activity of GF and OB in culture after treatment in 3 tests, the MTT test, the Boyden chamber migration test and the scratch wound assay.

Cell viability
There was no significant difference in the MTT test of GF between the Control group and the PDT group and the Laser group (Fig. 1A). The MB group showed a significantly lower absorbance in comparison to the other groups.

There was no significant difference in the MTT test of OB between the Control group and the PDT group (Fig. 1B). The MB group showed a significant reduction in absorbance as compared to the Control group, whereas the Laser group showed a significantly higher absorbance compared to all other groups.

Migration capacity in Boyden chambers
In the migration test of GF, significant differences between the Control group and the PDT group were not found (Fig. 2A). The only significant difference was found between the MB group and the Laser group with the MB group showing reduced migration activity.

The Laser group of OB showed a significantly higher migration capacity in comparison to the other groups (Fig. 2B). There was no significant difference between the PDT group and the Control group, whereas a reduction was observed in migration in the MB group as compared to the Control and Laser groups.

Scratch wound assay
In Fig. 3, migration of GF and OB is shown as measured as gap-closing activity after 24, 48 and 72 h of incubation. After 72 h of incubation of both GF and OB, the gaps were almost closed. The scratch wound assay showed no significant differences between groups (Fig. 3).
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Discussion

Numerous studies of periodontitis, endodontitis and peri-implantitis have demonstrated a positive additional effect of PDT on elimination of bacteria [11, 19-25]. Therefore, PDT may be effective in periodontal therapy and an alternative to systemic application of antibacterial drugs [26]. One of the major challenges in periodontal therapy is to reach and destroy all pathogenic microorganisms in the biofilm in the periodontal pockets. Several studies have reported antibacterial effectivity of PDT in biofilms [6, 27, 28]. One of the advantages of PDT is that treatment to eliminate bacterial activity is local, so that eventual side effects are limited. The other advantage of bactericidal activity of PDT is that the development of resistance by bacteria against reactive oxygen species is unlikely due to the non-specific activity of reactive oxygen species [27].

Some bacteria species such as A. actinomycetemcomitans or P. gingivalis show an active invasive behavior [29-31]. Thus, all colonies should be eradicated effectively including hidden colonies to limit recolonization [2]. However, such radical treatment increases potential phototoxic and/or photoallergic side effects. In order to develop an effective therapy, it is important to address this issue of toxic side effects in the patient [32]. That is why we have selected 2 cell types that occur abundantly in periodontium, GF and OB, to check for adverse effects of PDT. Qiao et al [17] have performed a similar study but mixed cell populations obtained from periodontal ligaments and gingiva were used. These cells were not characterized and may have well consisted of different cell types such as fibroblasts, endothelial cells, epithelial cells, immune cells and others. Moreover, Qiao et al. used a power density on the target cell monolayers of 19.7 J/cm2, whereas we applied almost 3-fold more power. We selected this setting because it is the same as we apply to patients.

All 3 tests of viability and migration activity of GF and OB show that the combination of MB followed by soft laser irradiation (PDT) does not have a significant effect on these parameters. Soft laser treatment only had a significantly positive effect on viability and migration of both GF and OB and in particular of OB, where as MB treatment only had a negative effect. The negative effect of MB on viability of GF and OB, particularly in comparison with soft laser only treatment is likely due to interference with electron chain reactions such as oxidative phosphorylation in mitochondria. MB is an electron carrier that
can by-pass these electron chain reactions [33] and thus inhibit processes such as mitochondrial ATP generation.

Positive effects of soft laser therapy, such as increased cell proliferation and activity of soft and hard tissues have been previously described [34-37]. Furthermore, analgesic effects of soft laser irradiation are also known [38-42] indicating that it may reduce postoperative pain. Furthermore, increased viability, mitosis [43], DNA synthesis [34] and mitochondrial membrane potential [35] are some of the effects ascribed to low-level laser irradiation of cells. Yu et al. (1994) found increased basic fibroblast growth factor (bFGF) levels in the supernatant of lasered fibroblast cultures [36]. Apparently, MB annihilates this stimulating soft laser effect. It is intriguing that we found a reduced viability and migration activity when using MB only whereas viability and migration was increased when applying soft laser irradiation only and the combination of MB and soft laser irradiation (PDT) leveled these effects out.

It remains to be proven whether positive effects of soft laser irradiation on cells also occurs in patients, but these effects on viability and migration activity of cells may stimulate tissue regeneration in patients because of reduced inflammation and postoperative pain and accelerated wound healing.

**Conclusion**

Our in vitro study indicates that PDT as is used in periodontal patients has no negative side effects on GF and OB in vitro, suggesting that PDT can be safely used for antimicrobial treatment in periodontal diseases.
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Figure legends

**Figure 1.** Cell viability test. The MTT test was used to measure the cell viability of GF (A) and OB (B) in the 4 treatment groups. P-values of the differences between groups are indicated.

![Figure 1](image1.png)

**Figure 2.** Migration test. The migration test in Boyden chambers was used as a measure of viability of GF (A) and OB (B) in the 4 treatment groups. P-values of the differences between groups are indicated.

![Figure 2](image2.png)
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**Figure 2.** Migration test. The migration test in Boyden chambers was used as a measure of viability of GF (A) and OB (B) in the 4 treatment groups. P-values of the differences between groups are indicated.
Figure 3. Scratch wound assay. The scratch wound assay was used as a measure of viability of GF (A) and OB (B) in the 4 treatment groups. P-values of the differences between groups are indicated.