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## **In vitro evaluation of the oxidation efficacy of transgingival photodynamic therapy**

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**Short title:** Transgingival photodynamic therapy

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## **ABSTRACT**

**Objective:** To evaluate the capability of soft laser light to penetrate blood, serum, gingival connective tissue and pure collagen type I.

**Materials and Methods:** A 1:1 mixture of methylene blue (MB) and diphenylisobenzofuran (DPBF) was irradiated for 60 seconds with a diode laser (670 nm, 0.3 Watt) through blood, serum, gingival connective tissue and collagen type I (2 mm transillumination thickness). The oxidation of DPBF by MB was determined spectrophotometrically by measuring the optical density (oD) at 410 nm. The absorption spectra of DPBF/MB irradiated through MB (1 %) and strawberry red solution (3 %) served as control.

**Results:** The mean oD of non-irradiated DPBF/MB was  $1.98 \pm 0.04$ . Irradiation through MB showed no oxidation of DPBF ( $1.98 \pm 0.02$ ;  $p > 0.05$ ), while interposition of strawberry red and serum resulted in almost complete oxidation of DPBF ( $0.13 \pm 0.09$ ,  $0.06 \pm 0.03$ ;  $p \leq 0.0001$ ). Irradiation through gingiva and collagen reduced the oxidation of DPBF significantly ( $1.0 \pm 0.04$ ,  $0.7 \pm 0.04$ ;  $p \leq 0.0001$ ), accounting to 50 % to 35 % of the non-irradiated DPBF/MB solution.

**Conclusion:** Red light from a diode laser can penetrate blood and gingival tissues. However, light absorption for collagen and connective tissue can hamper the oxidation process.

**Keywords:** collagen, diode laser, gingiva, methylene blue, periodontitis

## **INTRODUCTION**

Photodynamic therapy (PDT) is a valuable approach to treat infectious diseases in medicine and dentistry. Its broad spectrum of action, which includes bacteria, fungi, yeast and parasitic protozoa and the relative simplicity of the procedure attracts increasing attention and clinical application [1]. PDT has further been proposed as a promising alternative treatment modality against selected benign diseases and some malignant tumors, and may improve wound-healing processes [2-4].

The basic principle in PDT requires the light activation of a photosensitizer (photoactive dye) at a specific wavelength in the presence of molecular oxygen. The energy transfer from the activated photosensitizer to the available oxygen results in its transition from a low energy ground state to a higher energy triplet state. Further, it leads to the formation of toxic oxygen species such as singlet oxygen and free radicals. The latter can damage proteins, lipids, nucleic acids, and other cellular components of stained target cells [5].

Neither the sensitizing dye nor the light alone has a cytotoxic effect [6]. However, in appropriate doses both factors together develop the desired antibacterial properties. Selective illumination results in localized photodamage and subsequent cell death, whereas damage to the surrounding normal tissues is kept low; the action is locally limited due to the limited diffusion path and the short half-life of singlet oxygen [7]. Further, laser light intensity decreases with penetration depth through the various tissue layers due to the combined effects of scattering and absorption. PDT is a specific therapy for target cells, which does not support resistant bacteria species selection, exerts limited collateral effects and initiates its activity only when light exposed [8]. However, antibacterial action can only be achieved in areas with sufficient dye concentration.

Periodontitis is an inflammatory disease caused by biofilms with a mixed microbial etiology and involves the progressive destruction of the teeth-supporting tissues [9,10]. It is a chronic infection that leads to periodontal pocket formation, bone destruction, gradual

attachment- and ultimately tooth loss. While current treatment protocols for chronic periodontitis involve the mechanical removal of the biofilm by non-surgical and surgical means, various adjunctive anti-infectious therapeutic possibilities have been proposed, including local disinfectants and antibiotics [11]. The application of systemic or local antibiotics, however, is not completely free from side effects and patient compliance is critical for its success [10]. Further it is known that extended use of antimicrobials can lead to the emergence of resistant microorganisms and an unwanted shift in the microflora [12,13]. Therefore transgingival PDT, where these disadvantages appear unlikely, has been proposed as a viable alternative treatment protocol for the topical antimicrobial treatment of periodontitis [14-16]. It has been shown that PDT is capable of killing oral bacteria in planktonic culture [17], plaque scrapings [18] and artificially formed biofilms in vitro [19,20]. This lethal effect could also be shown on natural oral plaque biofilms formed in vivo; PDT-treated biofilms were thinner than the control, with an altered structure and less dense biomass [21]. An in-vitro study has specifically proved that the anaerobic periodontopathogens *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Capnocytophaga gingivalis* can be completely photoinactivated by PDT [22]. Also, the biological activities of two key periodontopathogen bacterial virulence factors, namely LPS and proteases can be reduced significantly in a dose-dependent manner with respect to both light energy dose as well as the concentration of the photosensitizer [23]. No damage to the adjacent periodontal tissues could be found in an animal model [15].

The purpose of this in-vitro study was to evaluate the capability of soft laser light to penetrate blood, serum, gingival connective tissue and pure collagen type I. The liquid media methylene blue and strawberry red served as control. Light penetration was measured spectrophotometrically by the oxidation of diphenylisobenzofuran (DPBF) to *o*-dibenzoylbenzene in the presence of methylene blue. The research hypothesis was that body

fluids and gingival soft tissues do impair light transmission and therefore have an influence on the therapeutic oxidative process.

## 2. MATERIALS AND METHODS

In this in-vitro experiment, the phenothiazine dye methylene blue was used. The oxidation of diphenylisobenzofuran (DPBF) to *o*-dibenzoylbenzene was initiated by illumination with red laser light in the presence of methylene blue. The degree of oxidation was measured spectrophotometrically (U-2010 Spectrophotometer; Portmann Instruments AG, CH-4105 Biel-Benken, Switzerland) with the method described by Bell and MacGillivray [24].

The experiment was performed in the dark to minimize spontaneous degradation of the light-sensitive substances. A 1:1 mixture of 1ml 1% methylene blue (MB,  $1.6 \times 10^{-6}$  M; molecular weight=373.9) and 1ml diphenylisobenzofuran (DPBF,  $2 \times 10^{-4}$  M; molecular weight=270.3) was irradiated for 60 seconds with a diode laser with a wavelength of 670 nm and 0.3 Watt (Orcos medical Soft Power Laser MED-701; Orcos Medical AG, CH-8700 Küssnacht, Switzerland). The head of the laser light guide was centered directly on the top of the test plate. In a fixed set-up, which is shown in Figure 1, different media were interposed: human blood and serum, deepithelialized porcine gingival connective tissue and pure collagen type I. The amount of the interposed solutions human blood and serum was 0.375 ml, equaling 2 mm of height that had to be pervaded by laser light. They were kept on a vibrating unit (Porex Vibrator Standard; Renfert GmbH, D-78247 Hilzingen, Germany) before testing to prevent any sedimentation. Fresh deepithelialized porcine connective tissue was interposed in pieces of 2 mm thickness, which had the circumference of the test plates (Figure 2). In addition, pure collagen type I (Geistlich Mucograft®; Geistlich Pharma AG, CH-6110 Wolhusen, Switzerland) was prepared in 2 mm sections and was interposed under wet (24 hours of soaking in 3% strawberry red solution) conditions. Irradiation through 0.375 ml of methylene blue (1%) and strawberry red (3%), equaling 2 mm of height, served as control.

The oxidation of DPBF by MB was determined spectrophotometrically by measuring the optical density (oD) at the peak absorbance at 410 nm; the typical absorption spectrum of non-irradiated DPBF/MB can be seen in Figure 3. Methylene blue absorbs only very weakly at this wavelength, and did not interfere with the measurement. The laser-procedure was repeated 8 times for each material; all interposed substances were used just once and replaced for each of the 8 experiments.

The statistical analysis was done with a commercially available statistics computer software (StatView<sup>®</sup> 4.02, Abacus Concepts, Berkeley, CA, USA). Normality of data distribution was tested using Kolmogorov-Smirnov and Shapiro-Wilk tests. The results were presented in mean values and standard deviations. One-way ANOVA followed by Scheffé post-hoc test was used to determine the significant differences between groups. P values smaller than 5% were considered to be statistically significant.

### 3. RESULTS

The results can be seen in Table 1. The mean optical density  $\pm$  standard deviation (oD  $\pm$  SD) of non-irradiated DPBF/MB was  $1.98 \pm 0.04$ . Irradiation through the control solution MB showed no oxidation due to complete light attenuation ( $1.98 \pm 0.02$ ;  $p > 0.05$ ), while interposition of the control strawberry red resulted in almost complete oxidation of DPBF ( $0.13 \pm 0.09$ ;  $p \leq 0.0001$ ), indicating very high light transmission. The irradiation through serum led to almost complete conversion of DPBF to *o*-dibenzoylbenzene ( $0.06 \pm 0.03$ ;  $p \leq 0.0001$ ), as serum almost did not impair light transition either. Similar results were seen with the interposition of blood ( $0.19 \pm 0.1$ ;  $p \leq 0.0001$ ). However, irradiation through gingiva and collagen reduced oxidation significantly ( $1.0 \pm 0.04$ ,  $0.7 \pm 0.04$ ;  $p \leq 0.0001$ ), equivalent to approximately 50 % and 35 % of the oD of non-irradiated DPBF/MB solution. Therefore the null hypothesis that body fluids and gingival soft tissues do not impair light transmission was

only partly accepted. Body fluids do not seem to hamper the desired oxidation process whereas gingival soft tissues lead to significant reduction thereof.

#### **4. DISCUSSION**

There are limited clinical studies evaluating the effects of adjunctive use of PDT to scaling and root planing, and results are inconsistent [25]. Some reports claim a significant improvement of clinical parameters after adjunctive use of PDT in comparison to mechanical therapy alone [26], some trials found a limited effect [25], while others could not detect any additional microbiological and clinical advantages [27,28]. Study designs show great variety, and results therefore are not directly comparable. Nevertheless, these contradicting clinical findings point out the need of further investigations of basic PDT principles, followed by the development of effective treatment protocols.

In this study irradiation through serum and blood showed almost no reduction of oxidative potential, whereas irradiation through wet collagen and gingiva lead to reduced oxidation, equivalent to 35% and 50% of non-irradiated DPBF/MB control solution. Even though some oxidation took place after transition of 2 mm of gingival connective tissue, the question arises, whether this reduced laser light energy can still warrant a successful clinical treatment - meaning eradication or at least reduction of periodontopathogenic bacteria.

Prior to widespread use of any new technology or treatment modality it is important to investigate its security and efficacy first. Results from in-vitro studies cannot directly be translated into clinical practice, and every laboratory set-up has its limitations in what can be tested. However, in-vitro investigations show tendencies and serve as important basis for further research. In this experimental design, it was not possible to include perfused gingival tissues; potentially the results with vital gingiva would have been slightly different. Also, in clinic local anatomy plays a role; in some parts of the mouth gingiva will be thicker or thinner than the 2 mm of thickness tested in this experiment. Further, some bacterially contaminated

areas are very difficult to reach by laser light, such as molar furcations or infrabony defects. Even though treatment need is especially high in those places, light intensity and therefore PDT efficacy will be reduced because of their inaccessibility.

In this experiment light transmission of pure collagen type I was tested. Even though pure collagen never is encountered clinically, it was shown that attenuation with liquids enables higher light transmission (measurement after 24 hours of soaking in strawberry red, whose light transmissibility lay between that of blood and serum). This finding might be of special interest in the case of inflamed tissues, where perfusion rate and interstitial fluid-volumes are higher than normal. Therefore laser light efficacy might be superior in inflamed gingiva, a possibility that requires further investigation.

For different reasons no bacteria were included in this set-up. One reason was that bacterial biofilms have different properties than single planktonic microorganisms due to their protection within the polymer plaque matrix, and their adhesion to teeth or epithelia. Various biofilm models are available. However, they usually contain only few bacterial species, which do not represent the full diversity of the oral microflora and therefore permit only limited conclusions. Also, previous investigations showed that PDT was ineffective in an undisturbed biofilm model [29], possibly because the uptake of photosensitizers into dental plaque is impeded the same way as that of antibiotics [7]. Ultrasonic devices or photomechanical waves improve drug intake and consecutively treatment efficacy [30,31]. PDT therefore is normally applied as adjunct therapeutic intervention after mechanical debridement. The latter destroys or disturbs the biofilm and makes microorganisms more susceptible for the adjunct treatment. It is known that some periodontopathogens like *A. actinomycetemcomitans* and *P. gingivalis* are capable of invading host epithelial cells and gaining access to deeper periodontal tissue levels [32,33]. To avoid possible recolonization, these hidden colonies should also be a target, which may not be possible with PDT and therefore might put expectations of what is possible with this therapeutic modality to a limit. Further, it is not clear whether killing the entire oral

flora is beneficial, as this might lead to an overgrowth of a single resistant species [34] or leave the patient vulnerable to opportunistic infections [21].

Possible side effects of PDT are rare, but include phototoxic or photoallergic reactions [35]. In connection with patient acceptance, practical issues such as unwanted staining of crown margins, teeth and skin need to be raised. Nevertheless, PDT shows sufficient potential as cost-effective, non-invasive and painless antibacterial treatment and therefore deserves further attention in research and clinic.

Red light from a diode lasers can penetrate blood and gingival tissue. However, considerable light absorption was observed for collagen and connective tissue, which results in reduced oxidation and potentially reduced antibacterial efficacy of PDT. The described model may be suitable to test and screen PDT methodologies in different tissue environments *in vitro*.

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## TABLES

Table 1. Presentation of the different treatment groups (Light transmission barriers) in the experimental set-up. Different interposed light transmission barriers resulted in change in optical density of the reactive test solution (mean values and standard deviations) after induction of the oxidation process by red laser light. Identical superscript capitals represent values, which are *not* statistically significantly different. In addition, the percentage of reduction in oD was calculated as compared to the value obtained in the non-irradiated reactive solution (base value): A higher percentage relates to increased oxidation due to increased light transmissibility of the interposed light transmission barrier.

Laser light Exposure	-	+	+	+	+	+	+
Light transmission barrier	-	<b>Solution</b>				<b>Tissue</b>	
		Strawberry red (control)	Methylene blue (control)	Blood serum	Full blood	Gingiva	Type I collagen
Reactive solution	<b>Diphenylisobenzofuran (DPBF) + methylene blue</b>						
Optical density (oD)	1.98 <sup>B</sup> ± 0.04	0.13 <sup>AC</sup> ± 0.09	1.98 <sup>B</sup> ± 0.02	0.06 <sup>A</sup> ± 0.03	0.19 <sup>C</sup> ± 0.10	1.00 <sup>D</sup> ± 0.04	0.70 <sup>E</sup> ± 0.04
Reduction in oD (%)*		93.4%	0 %	99.7%	90.4%	49.5%	64.6%

## **FIGURE LEGENDS**

Figure 1: Schema of the experimental set-up

Figure 2: Test plates and gingival tissue samples used for measurements

Figure 3: Typical absorption spectrum of non-irradiated DPBF/MB control solution

Softlaser  $\lambda = 670\text{nm}$

$\varnothing$   
4.4mm

2 mm light transmission  
barrier (test solution/tissue)

1 ml DBPF + 1 ml MB





