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CLINICAL STUDY

Efficiency of photodynamic inactivation *Actinomyces israelii* and *Prevotella melaninogenica*

Iryna VORONKINA[1], Adriana LIPTAKOVA[1], Valentina DYACHENKO[2], Svetlana DERKACH[2], Nadejda SKLYAR[2]

Institute of Microbiology, Faculty of Medicine, Comenius University Bratislava and University Hospital Bratislava, Bratislava, Slovakia. **adriana.liptakova@fmed.uniba.sk**

ABSTRACT

BACKGROUND: The goal of our work was to develop a composition for antimicrobial photodynamic inactivation (aPDI) of anaerobic periodontopathogenic pathogens.

METHODS: The three test groups were as follows: light plus doxycycline (L+DOX+), light plus doxycycline and hypericin (L + DOX + HYP +), and control groups. aPDI was evaluated by the number of grown colonies on a dense nutrient medium after 12, 24, and 48 hours of bacterial suspension cultivation.

RESULTS: Based on the results of microbiological studies, the combined photosensitising effect of a subinhibitory dose of doxycycline and hypericin was determined. The delay of growth of *A. israelii*, *P. melaninogenica* in the second group (L+DOX+HYP) was significantly significant compared to the first group (L+DOX+), and the statistical difference in colony formation activity was found for both gram-positive and gram-negative cultures ($p<0.05$).

CONCLUSIONS: aPDT is a promising therapeutic alternative for the local treatment of purulent-inflammatory diseases of various localisation caused by antibiotic-resistant bacteria, including in the oral cavity *(Fig. 3, Ref. 57)*. Text in PDF www.elis.sk

KEY WORDS: peri-implant diseases, photodynamic therapy, photosensitiser, anaerobic bacteria.

Introduction

One of the most common diseases facing dentists recently is inflammatory and dystrophic periodontal disease. According to the WHO, severe periodontal disease is widespread and affects 19% of people over 15 years of age, which is more than 1 billion cases worldwide (1, 2).

The experience of studying the etiology of inflammatory periodontal diseases accumulated over the past decades shows the leading role of the facultatively anaerobic obligate and microaerophilic microflora in the development of inflammatory processes in the oral cavity. Currently, such microorganisms as *Aggregatibacter actinomycetemcomitans, Actinomyces naeslundii, Tannerella forsythia, Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum, Treponema denticola*, as well as *Parvimonas micra* have been proven etiological in the development of inflammatory and dystrophic periodontal diseases (3–6).

Systemic antibiotic therapy is one of the additional methods of periodontal treatment (7–9). One of the negative consequences of antibiotic use is the selection of polyresistant strains of microorganisms. According to scientists' predictions, the constant increase in the resistance of pathogenic agents to the action of antibiotics causes the threat of the "end of the antibiotic era". The search for ways to solve this problem encourages the creation/study of new alternative methods of fighting infections, which allow the destruction of pathogens with multiple antimicrobial resistance but do not themselves lead to the development of such resistance (10–14).

aPDI of microorganisms is considered one of these methods, which in its essence is the selective oxidative destruction of microorganisms due to the combined effect of a photosensitiser (PS) and radiation of a certain wavelength that corresponds to its absorption spectrum. When local irradiation is performed with light of a certain wavelength, PS goes into an excited state and transfers energy to the third component, oxygen. The interaction of these components provides the fundamental photobiological process on which aPDI is based. In microbial cells, a photochemical reaction begins with the formation of singlet oxygen and oxygenfree radicals, creating a toxic effect on pathogenic microorganisms (15, 16). Microorganisms don't develop resistance to photodynamic exposure; the bactericidal effect is local, limited to the area of laser irradiation of tissues, and does not create a harmful effect on the normal microflora of the human body. All this reveals broad prospects for the improvement and spread of the aPDI method

[1]Institute of Microbiology, Faculty of Medicine, Comenius University Bratislava and University Hospital Bratislava, Bratislava, Slovakia, and [2]Laboratory of anaerobic infection, State Institution I. Mechnikov Institute of Microbiology and Immunology National Academy of Medical Sciences of Ukraine, Kharkiv, Ukraine

Address for correspondence: Adriana LIPTAKOVA, MD, Assoc Prof, PhD, MPH, Institute of Microbiology, Faculty of Medicine, Comenius University, Bratislava, Sasinkova 4, SK-811 08 Bratislava, Slovakia. Phone: +02/9011 9315

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for the treatment of purulent inflammatory diseases of various localisations (17–19).

Various compounds are most often used as PS, which have intense absorption bands in the visible and ultraviolet regions of the spectrum and are capable of transitioning into long-lasting triplet states after exposure to light. An ideal photosensitiser should be non-toxic and exhibit local toxicity only after activation by light. Today, more than 1000 compounds are known, which are considered PS. Among them are specially developed dyes and traditional drugs capable of performing PS functions and are used for aPDT). Natural products are also used: hypericin, riboflavin, and curcumin (20–21).

An important role in the development of aPDI was played by the discovery of radiationsensitive, phototoxic properties of natural antibiotics of the tetracycline group. It has been confirmed that tetracyclines have PS properties and are activated under the influence of blue or ultraviolet radiation. Most drugs of this group have an absorption peak in the UV spectrum. The antibiotic doxycycline, like tetracycline, has a bacteriostatic effect; in experiments on *A. actinomycetemcomitans*, it was proven that doxycycline can block collagenase enzymes and inhibit the growth of these bacteria. Also, doxycycline can act as an exogenous photosensitizer at a wavelength of 375–780 nm. The study of the chemical processes of photosensitivity of tetracyclines showed that they can act as light-activated antibiotics due to the participation of oxygen-dependent and independent mechanisms. Covalent bonds appear between the PS molecule and the microbial structure, which can be called the creation of "photoadductors" (22–23).

Nowadays, biologically active substances of plant origin are part of many drugs. St. John's wort (*Hypericum perforatum L.*), which is widely used worldwide, can be a source of such compounds. The red pigment of St. John's wort (hypericin) exhibits photosensitising properties, and by its nature is a condensed derivative of anthraquinone. It was established that such derivatives have a chromophoric groups of atoms in their composition, because of which they can be widely used as PS in the treatment of various diseases, including oncological. The quantitative content of hypericin in different types of St. John's wort ranges from 0.03% to 0.34% (24–25). However, the use of hypericin, as a biological compound in a chemically pure form, is hindered by the possibility of unwanted side effects. Hypericin in its pure chemical form has been established to be phototoxic to human skin and eyes, can lead to macular degeneration, and has other toxic properties (26).

We aimed to develop a composition for aPDI of anaerobic periodontopathogens, which, because of the use of natural PS and a photosensitive antibiotic, will ensure the effectiveness of the reaction regardless of the presence of oxygen in the tissues.

Materials and methods

Growth and culture conditions of A. israelii and P. melaninogenica

We selected two types of bacteria for experiencing the genus *Actinomyces* and *Prevotella* (*A. israelii, P. melaninogenica*) which are periodontopathogens and have different tinctorial properties. The strains were obtained from the SI «I. Mechnikov Institute of Microbiology and Immunology National Academy of Medical Sciences of Ukraine». *A. israelii* and *P. melaninogenica* were stored in Microbank™ and placed at −80°C until use. The agar plating medium used was Schaedler agar. The determination of biochemical activity of test cultures was carried out in Rapid ID 32A.

Preparation of the culture suspension for aPDI

The *A. israelii* and *P. melaninogenica* strains were grown for 24 hours on Schaedler agar at 37°C in anaerobic conditions. Microbial suspension for aPDI was prepared on phosphate-buffered saline (PBS) with a density of 0.5 McF ($5{,}0*10^7$ CFU/ml *A. israelii*) and ($1{,}5*10^8$ CFU/ml *P. melaninogenica*).

Preparation of PS

To conduct aPDI experiments, a stock solution of doxycycline in distilled water at a concentration of 100,0 mg/l was used, from which a series of tenfold dilutions were prepared and the minimal inhibition concentration was determined. Subinhibitory concentrations were used to establish the reaction aPDI, which were equal to 3.0 mg/l for *A. israelii* and 6,0 mg/l for *P. melaninogenica*. As an additional PS, a standardised alcoholic extract of St. John's wort (*Hypericum perforatum L.*) containing hypericin in a concentration of at least 0.08% was used (27).

Source of radiation

The source of irradiation was a highly intense LED with a light wavelength of 460–480 nm and a radiation power of 1200 mW/cm^2. The LED was placed above the plate in such a way that the distance between the surface of the bacterial suspension and the light-emitting end was 4–5 mm, which allowed covering the entire well during irradiation. The duration of irradiation was 2 and 4 min (Fig. 1).

Photosensitisation procedure

The three test groups were as follows: light plus doxycycline (L+DOX+), light plus doxycycline and hypericin (L + DOX + HYP +), and control groups. In previous studies, we selected the optimal irradiation regimens for *A. israelii* and *P. melaninogenica.* We followed two groups: one light alone (L+PS−) and a second group without PS and light (L−PS−). The duration of irradiation was 2 min for *A. israelii* and 4 min for *P. melaninogenica* (28).

For the L+DOX+ group, a solution of doxycycline (3,0 mg/l) was added to the suspension of the daily culture of the studied strain of *A. israelii* in PSB. The bacteriological suspension was applied in triplicate wells on a 96 well flat bottomed plate in 200 µl portions and the culture was exposed to irradiation. Tablet with the bacterial suspension was cultivated under anaerobic conditions at 37°C. To carry out the aPDI reaction of *P. melaninogenica*, a doxycycline solution at a concentration of 6,0 mg/l was added to the daily culture suspension in PSB.

For the L + DOX + HIP + group, 100 µl of microbial suspension in PSB, 100 µl of doxycycline solution, and 100 µl of St. John's wort alcoholic extract were added to the wells of

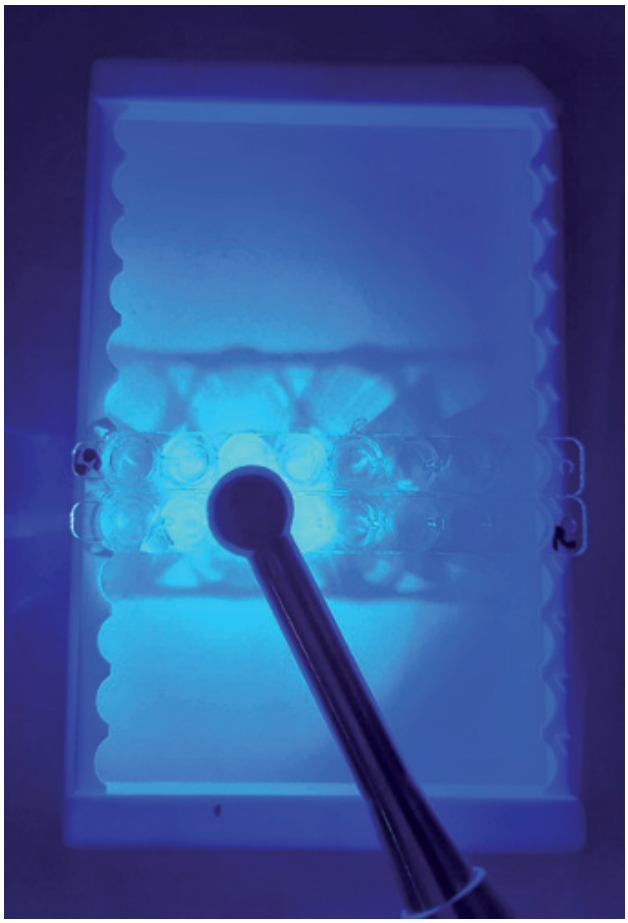

Fig. 1. The process of the irradiation of *A. israelii* with a blue laser (λ=460–480 nm, 1200 mW/cm²).

a 96 well flat bottomed plate. Next, irradiation and cultivation was carried out.

aPDI was evaluated by the number of grown colonies on a dense nutrient medium after 12, 24, and 48 hours of bacterial suspension cultivation.

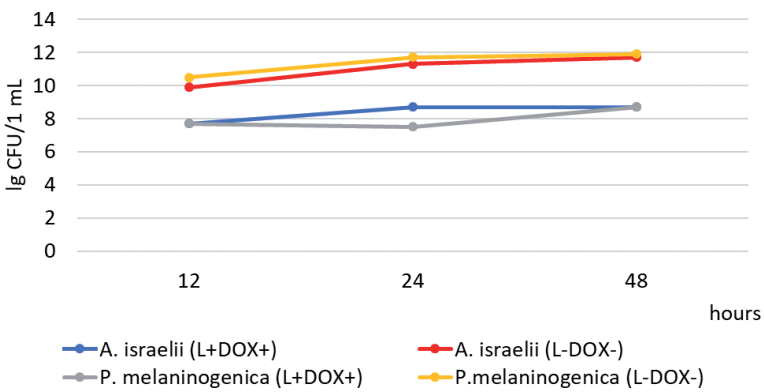

Fig. 2. Effectiveness of antimicrobial PDI of periodontopathogenic pathogens in the presence of doxycycline (CFU – colony forming unit; L+DOX+ – light plus doxycycline; L−DOX− £ control group).

For the control group (L−PS−), 200 µl of microbial suspension in PSB was introduced into the wells of a 96 well flat bottomed plate and incubated (37°C). The sowing of the control wells was carried out in parallel with the sowing of the studied wells.

Statistical analysis

Results were reported as mean values±standard deviations (SD) performed at least in triplicate (n=3). Significant differences were estimated by applying a t-test. In all cases, differences were considered significant at $p<0.05$. All statistical tests were performed with Microsoft Exel 2019.

Results

As the results of the study show, the effect of the combined action of irradiation and PS (doxycycline) was observed in the first observation group (L+DOX+). Thus, in experimental wells with *A. israelii* after 12 hours of incubation, the number of bacteria (lg CFU/1 mL) was 7.7±0.8, after 24 and 48 hours – 8.7±0.3, which was significantly less compared to the control wells that were not exposed to irradiation and PS action (Fig. 2). aPDI was also observed in wells with *P. melaninogenica*. After 12 hours of incubation, the number of colonies was 9.9±0.5 lg CFU/1 mL, after 24 and 48 hours – 7.5±0.3 and 8.7±0.5, which also significantly less compared to the control wells (in the control wells the number of bacteria was 10.5±0.7 after 12 hours of cultivation; 11.7±0.5 and 11.9±0.5 after 24 and 48 hours of cultivation, respectively) ($p<0.05$).

The results obtained showed that the delay in culture growth, compared to the control, was observed after 12 hours of cultivation of the microbial suspension cultivation and was maintained throughout the observation period, that is, up to 48 hours. It should be noted that a statistical difference in colonyforming activity was found for both gram-positive and gram-negative cultures ($p<0.05$).

Thus, based on the results obtained, it can be stated that aPDI of periodontopathogenic cultures is possible when the antibiotic doxycycline is used as a PS even in anaerobic cultivation conditions, which is especially important for studies with anaerobic periodontopathogenic pathogens.

To obtain the optimal photosensitizing composition, we compared the effectiveness of two compositions. L+DOX+ and L+DOX+HYP+ (Fig. 3).

As can be seen from the results shown in Figure 3, the difference in the number of colonies between the culture inactivated in the presence of hypericin and the culture in which hypericin was not added was revealed after 12 hours of bacterial cultivation. Thus, in the first group (L+DOX+), the number of microorganisms *A. israelii* after 12 hours of incubation was 7.3±0.2 lg CFU/1 mL, in the second group (L+DOX+HYP) – 6.5±0.3 lg CFU/1 mL, seeds after 24 hours of incubation showed the number of bacteria in the first group 8.6±0.3 lg CFU/1 mL, in the second – 4.5±0.2 lg CFU/1 mL, the same trend was maintained in both groups was maintained even after 48 hours of incubation.

About *P. melaninogenica*, the photosensitising composition L+DOX+HYP was also several times more effective than the composition without hypericin after 12 hours of incubation. The number of bacteria when seeded after 12 hours in the first group was 7.8±0.2 lg CFU/1 mL, in the second – 6.7±0.4 lg CFU/1 mL, after 24 hours – 6.7±0.4 and 4.3±0.3 lg CFU/1 mL, respectively, after 48 hours – 7.7±0.4 lg CFU/1 mL in the first group (L+DOX+) and 3.7±0.3 lg CFU/1 mL in the second (L+DOX+HYP).

Therefore, it can be stated that the effect of the combined photosensitising effect of the subinhibitory dose of doxycycline and hypericin was observed during exposure to the cultures studied. The delay in the growth of cultures in the second group (L+DOX+HYP) was significantly significant compared to the first group (L+DOX+), the statistical difference in colonyforming activity was found for both gram-positive and gram-negative cultures ($p<0.05$).

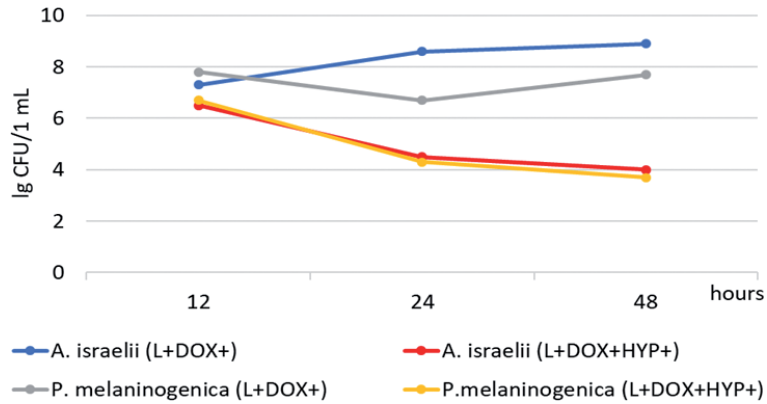

Fig. 3. Effectiveness of antimicrobial PDI of periodontopathogenic pathogens in the presence of doxycycline and hypericin (L+DOX+ – light plus doxycycline; L+DOX+HYP+ – light plus doxycycline and hypericin; CFU – colony forming unit).

Discussion

Modern periodontal treatment consists of removing tartar, rinsing with antiseptics, and using systemic antibiotics. Among antiseptics, povidone-iodine, chlorhexidine, and sodium hypochlorite, which also have a hemostatic effect, are used actively in dentistry (29–34). Despite the high effectiveness of antiseptics, it is already known about the development of resistance to these drugs in representatives of the oral microbiome. *S. sanguinis, S. mitis, E. faecalis, Capnocytophaga spp., P, gingivalis, A. actinomycetemcomitans, F. nucleatum,* and *C. albicans* isolates with reduced sensitivity to chlorhexidine were identified (35–38). The existence of microorganisms in the oral cavity in the form of a biofilm contributes to the development of resistance to antiseptics. Living in the deep layers of dental plaque, bacteria are less accessible to the action of antiseptics and can adapt to low concentrations of biocides, which contributes to resistance development of resistance (39, 40).

Antibacterial therapy is often used to treat inflammatory processes in periodontal tissues and can stop aggressive periodontitis. Systemic antibiotic therapy is prescribed simultaneously with mechanical treatment or immediately after it, using combinations of antibiotics amoxicillin-metronidazole, azithromycin-metronidazole, and clindamycin, which turned out to be more effective than regimens with doxycycline (41–43). To avoid the side effects of systemic antibiotic therapy, Szulc et al suggest a local application of antibiotics, directly in periodontal pockets (29). Considering the growth of antibiotic resistance of bacteria all over the world, there is a need to find therapeutic alternatives.

Interest in the possibility of using aPDT in dentistry has recently been growing, which is related to the safety of the method, ease of use, and lack of development of resistance in bacteria. aPDT studies include the study of the cytotoxicity of various PSs, their efficacy against biofilms, and their effects on macroorganism immunological responses. aPDT has already shown its effectiveness against microorganisms resistant to antimicrobial agents (44–46). Studies by Legéňová et al have proven the effectiveness of aPDI against the biofilm of *S. mutans*. aPDI using PS methylene blue was shown to be more effective than the antimicrobial activity of an antiseptic (0.1% chlorhexidine digluconate), the latter being more toxic and less effective against the biofilm of *S. mutans* (47). In oral candidiasis and subgingival yeast colonisation, aPDT reduced the number of infected an-atomical sites and reduced soft tissue inflammation (48, 49).

The positive effects of aPDT are also the immunomodulatory effect, which is provided by neutrophil stimulation, and the acceleration of wound healing, due to rapid regeneration and remodeling (14). The effects of aPDT on rat pulp tissue were evaluated by Takahashi et al, showing that aPDT used to sterilize carious dentin could induce pulpal tissue reversal by laser penetration and the generation of singlet oxygen with subsequent healing. and the formation of tertiary dentin (50).

In systematic review studies, Lopez et al showed that the reduction of bacterial load after aPDT in the treatment of periodontal and peri-implant diseases can reach 99%. But when studying antimicrobial action in vitro, parameters such as constant formation and accumulation, variable salivation, and factors of the immune system are not taken into account, due to the difficulty of reproducing such conditions in an experiment, so the development of adequate models to study aPDT is also promising (51, 52).

The effectiveness of aPDT on bacteria of the genus *Actinomyces* was shown in studies by Hafner in patients with osteonecrosis of the jaw. Photodynamic inactivation during 10 seconds decreased the bacterial load by more than 4 orders of magnitude and was superior to polyhexanide and chlorhexidine exposure for 60 seconds (53).

Although scaling and root planing are the mainstay of treatment for periodontal and peri-implant diseases, the combined use of scaling and root planing and aPDT produces better results in terms of reduced probing bleeding, reduced probing depth, and improved clinical attachment than scaling and root planing alone. Haas in their review concluded that repeated use of aPDT in addi-

tion to scaling and root planing is more effective for the treatment of deep pockets in aggressive periodontitis than scaling and root planing alone (54).

It is believed that aPDT is better used in chronic periodontitis or as an additional method of treatment of periodontal and peri-implant diseases. aPDT can be combined with systemic antibiotic therapy for the treatment of stage III-IV periodontitis (55–57).

Conclusions

Our studies have shown that the original composition developed for the photodynamic inactivation of anaerobic periodontopathogens pathogens *A. israelii* and *P. melaninogenica* provides aPDI regardless of the presence of oxygen.

Although dentists now actively use aPDT as an adjuvant for the treatment of periodontal and peri implant diseases and has its positive results. aPDT remains a relevant object of study to understand better the issues related to the mechanisms of antibacterial action of lasers, the search for new safe PS and their combinations, optimal models for studying aPDT, and studying the effect of aPDT on the immune system of the macroorganism. The development of bacterial resistance to aPDT is currently considered unlikely; therefore, aPDT is a promising therapeutic alternative for the local treatment of purulent-inflammatory diseases of various localisation caused by antibioticresistant bacteria, including in the oral cavity.

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