IN VITRO STUDY OF THE PHOTODYNAMIC INACTIVATION OF CARIOGENIC BACTERIUM *STREPTOCOCCUS MUTANS* - REFERENCE STRAIN

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ABSTRACT

PURPOSE: The aim of this study is to investigate and compare the in vitro antimicrobial effect of 5 photosensitizers: methylene blue(MB), FotoSan® and 3 types of methalphtalocyanines on the reference strain *Streptococcus mutans*.

MATERIALS AND METHODS: A bacterial suspension at a density of 10^6 cells / ml. is used. An incubation mixture of 1 ml. bacterial suspension (10^6 Cl./ml.) and the corresponding PS is prepared, achieving a final concentration of PS 1-6 µl. Controls: bacterial suspension in the dark; with irradiation, bacterial suspension + PS in the dark. Sample: bacterial suspension + PS + irradiation. Diode laser with a wavelength of 600 nm is used for irradiation. There are also cultures from controls and samples after preliminary 10-fold dilutions (10^6; 10^5; 10^4; 10^3; 10^2;) 6 samples and controls for each tested PS after 24 h incubation at 37° C are reported.

RESULTS: The control samples in dark and toxicity only after laser radiation laser show a weak antimicrobial effect. Exception for the Ga(III)-phtalocyanines-serious dark toxicity. Samples of bacterial suspension + PS + diode laser irradiation showed strong antibacterial effect on *St. Mutans* with small differences in all tested PSs.

CONCLUSION: The conducted testing demonstrated that the six tested PS show strong antimicrobial effect in the strain reference of *S. Mutans*, with some small differences between the tested PS.

Key words: *S. Mutans, Photosensitizers, antimicrobial, dark toxicity*

INTRODUCTION

Dental caries is a multifactorial disease with pandemic spread. The main etiological factors consist of cariogenic diet, microbial dental plaque and non-resistant tooth structure (26). Of the all oral bacteria, *Mutans Streptococci*, such as, *Streptococcus mutans*, colonize the dental surface and are considered causative agents of dental caries in humans in the presence of fermentable carbohydrates e.g., sucrose and fructose (9). Presence of carious lesions is a sign of bacterial infection. Clinical practice shows that during caries treatment etiology is often neglected and attention focused entirely on replacement therapy (13). Symptomatic treatment is important

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but, underestimating the pathological condition, e.g. the infection, in any case stimulates the progression of the process. The importance of bacteria organized in biofilms in the genesis of the carious process is an undeniable fact, proven with the following scientific facts (26)**:**

1. Non contaminated teeth, either in sterile animals or pre-erupted human teeth do not develop caries.
2. Antibiotic drugs are effective in reducing caries activity in animals and in humans.
3. Oral bacteria are able to induce demineralization of enamel in vitro and lead to the occurrence of caries like lesions.

Specific microorganisms are isolated in various caries lesions. The group consists of 8 *S. mutans* serotypes, named from *a* to *h*. Some of them are called by specific names, such as S. rattus /b/, S. cricS. ferus /c/, S. Sobrinus /d,g,h/ (13).

All the serotypes demonstrate cariogenic potential. They are genetically and biochemically different and for accuracy's sake should be called *Mutans Streptococci*, instead of referring to them as *S. Mutans* (12). However probably based on the evidence that they all have similar behavior, e.g. produce a large amount of acid (acidogenic MO), live in an acidic environment (aciduric), their vitality is strongly stimulated by the presence of sucrose, are the major organisms associated with the development of caries in humans, the term S.mutans is the widely accepted way to refer to them. Infection in people with MS is pandemic-like spread, as these bacteria are found in merely each individual regardless of race, ethnicity, gender and geography (26). Dental caries control could be based upon management of disproportionate increase (often site-specific) in the dental plaque content of these strongly acidogenic and aciduric, Gram positive facultative aerobes (4,8,20).

We think that alternative ways of controlling the number St. mutans in dental biofilms is needed in order to promote better dental health, less carious lesions and less endodontic complications, due to advanced dental caries. This approach is an alternative to the well spread fluorid prophylaxis of dental caries. Among these alternative antimicrobial methods the following must be listed (2):

1. Prevention based on the impact of plant derived substances (22).
2. Probiotics. Probiotics are vital micro-organisms (MO) which, in certain quantities contribute to the health of the host (macro organism. Lactobacillus rhamnosus 19, Lactobacillus casei 20, Lactobacillus reuteri and Bifidobacterium DN 21 173 010 are probiotic cultures and have shown a potential to prevent the colonization of cariogenic MO and prevent the development of caries in all studies.
3. Biotechnological approaches. Examples of such are bacteriocins inhibiting the development of colonies of the same species.
4. Phage therapy. The use of phages must be mentioned as well. Their effect, however, is mainly against *Enterococcus faecalis*.
5. Prebiotics
6. Inhibition of the individual functions of bacteria.
7. Antibacterial photodynamic therapy (APDT) or photodynamic disinfection (FDD)

Since the discovery of penicillin by Fleming in 1928, many different families of antibiotics have been approved, most targeting either the cell wall or the protein synthesis processes (3) (Fig 1).

Antibiotics have become "the panacea" of medical practice and are being used to treat many common and trivial types of infections, many of which non-bacterial in nature. However, during the last few decades, the number of clinical drug-resistant isolates has significantly increased and grown to a pandemic and are an unavoidable problem in hospitals (7,10). Examples include Methicillin-resistant *Staphilococcus aureus*, Cephalosporin-resistant *Eshcerichia coli*, etc. The problem of increased resistance is not only restricted to bacteria, but involves fungal strains such as *Candida Albicans* and *Kodamaea* (3).

Therefore efforts aim at finding alternative antimicrobial therapies to which resistance will not develop easily. Among all the alternative treatments to antimicrobial agents, antimicrobial photodynamic therapy (APDT) seems to be a promising one (1); the first recorded observations using photodynamic processes to inactivate microbial cells were made by Raab *et al.* more than 100 years ago (21) APDT has

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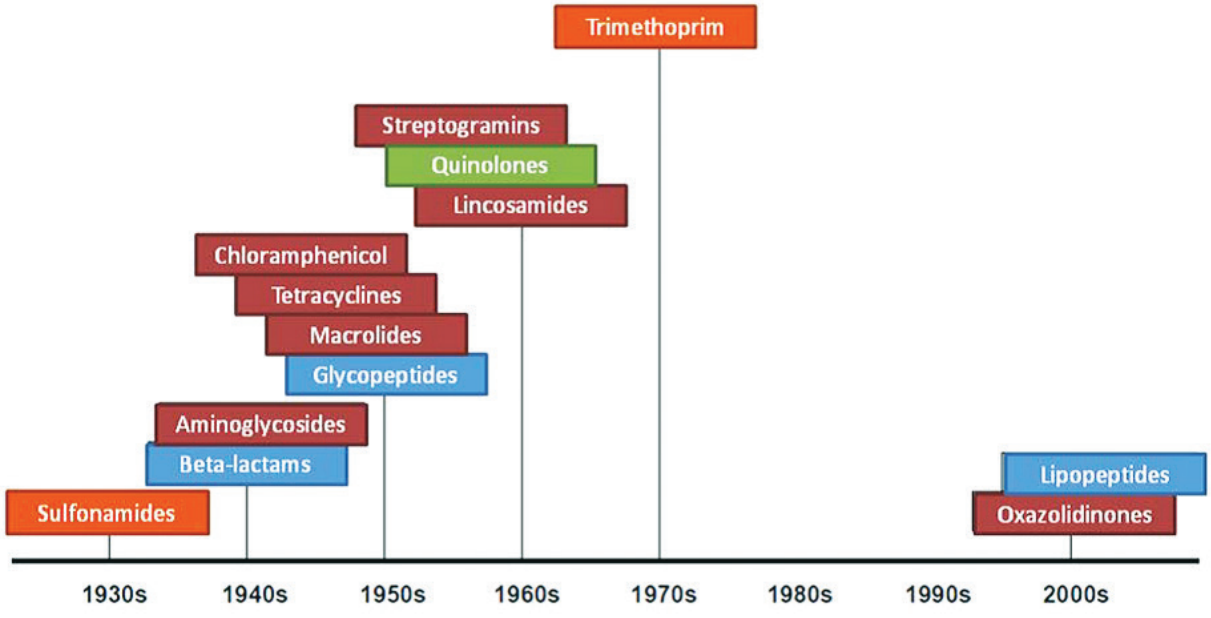


Fig. 1. Discovery of new classes of antibiotics. Color refers to the principal target. Orange: Inhibition of folic acid synthesis. Blue: Inhibition of cell wall synthesis. Maroon: inhibition of protein synthesis. Green:inhibition of DNA or RNA synthesis (3).

two main advantages: on the one hand, drug resistant microorganisms are as susceptible to APDT as their counterparts (14) or even more susceptible (23) On the other hand, it has not been possible to artificially induce resistance to APDT yet (5,11,24).

Photodynamic therapy (PDT) involves the administration of photoactive dye that is able to produce *reactive oxygen species* (ROS) upon irradiation with light (27). The ADPT has another advantage versus other antimicrobial agents and this is its boarder therapeutic window. It can be applied to many different infectious diseases in medicine as well as in dealing with dental pathogens (3) (Fig. 2.).

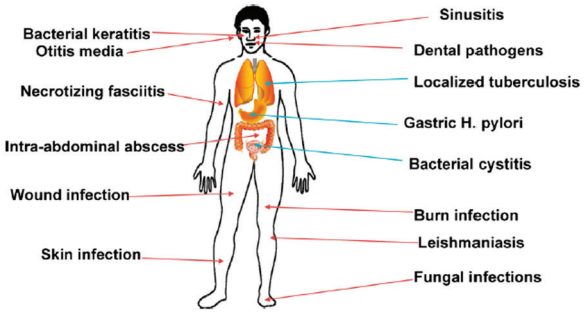


Fig. 2. Candidate infectious diseases for PDT. A wide variety of localized infections could be clinically treated by antimicrobial PDT (3).

There are many different possible applications of APDT against a wide range of pathogenic microorganisms, some of them in clinical trials.

Dental infections are the largest growth area of clinical APDT. Researches focus on treatment in cases of periodontitis (18), failed endodontic treatment, as well as caries prevention and treatment. Three main groups of photosensitizers are being observed and give positive results – metalphtalocyanines, porphirines and phenothiazines.

The study aims to prove the sensibility in vitro antimicrobial effect of 5 photosensitizers: 2 of them licensed for clinical use- methylene blue, FotoSan® and created by us three types of methalphtalocyanines on the reference strain *St. mutans* (15,16,17).

MATERIALS AND METHODS

- Reference strain Streptococcus mutans-20523 collection DSMZ- Germany
- Trypticase® Soy agar with 0.5% yeast extract
- Methylene blue stock solution
- FotoSan®

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- ZnPcMe
- GaPcMe
- SiPcMe
- Diode laser-600nm wavelength
- Wide-field dissecting microscope

The bacteria were incubated in microaerophilic conditions (5%CO_2) at 37°C for 48 hours on solid medium Trypticase® Soy agar, supplemented with 0,3% yeast extract. For the needs of antimicrobial photodynamic therapy is used a bacterial suspension at a density of 10^6 cells/ml. Samples of microbial suspensions were incubated for 20 min in the dark with 10 µL MPcs stock solution to final concentrations between 5-6 µM MPcs.

Controls are as follows: bacterial suspension in the dark; bacterial suspension with irradiation only, without photosensitizer added, bacterial suspension + PS in the dark.

Sample: bacterial suspension + PS + irradiation. For irradiation of the mixture (bacterial suspension + PS) after incubation in the dark 15 min into 200 µl, Placed in a standard 96-well polystyrene microtiter plate, where the irradiation was performed. Illuminated with a diode laser with a wavelength 600 nm was applied with irradiance of 100 mW cm^{-2} controlled during the experiment by photometer (Spectra Physics, USA), for 15minutes. Four samples groups with microbial cells were collected:

(1) light control (LC) - without photosensitizer, but illuminated;

(2) dark control (DC) - with PS, but no light (dark toxicity);

(3) only cells (C) - only bacterial suspension (no photosensitizer, no light) and

(4) PDT treated group.

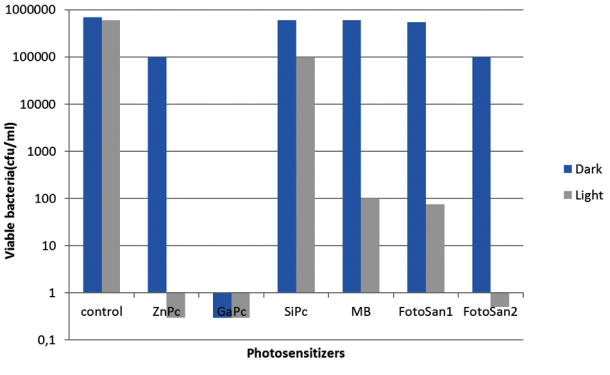
After the irradiation, 0.1 ml samples were taken off and serially diluted (10-fold) with Phosphate-Buffered Saline (PBS), pH 7.4, (10^6; 10^5; 10^4; 10^3; 10^2;). Aliquots (0.025 ml) were spread over Trypticase® Soy agar with 0.5% yeast extract. The number of colonies (CFU) formed on each plate was counted following 48 h incubation at 37°C in 5% CO_2.

Each experiment was carried out in triplicate and the data are presented as a mean ± standard deviation (SD). The difference between two means was compared by a two-tailed unpaired Student' test. The values of $P<0.05$ were considered as significant.

RESULTS

Examination of the number of the colonies that have grown in Petri dishes serves to analyze the effect of the PS subject of our study. The photodynamic inactivation of *S.mutans* 10^6 $CFUml^{-1}$ bacterial suspensions was compared for 5 different PS (Zn(II)-, Ga(III)-, and Si(IV), methylene blue(MB), FotoSan®.

The control samples made in dark show negligible dark toxicity at all dye concentrations used (< 0.5 log decrease of viable cells). Only Ga(III) showed high dark toxicity at concentrations used(>0.5 log decrease. The LC samples have similar results. The samples of bacterial suspension + PS + diode laser irradiation showed, on the contrary, strong antibacterial effect on *Streptococus mutans* with small differences in all tested PSs. Photodynamic response of bacteria showed full photo-inactivation with ZnPcMe (treated with 5,6 µM at a fluence rate of 100mWcm^{-2} after 15 min. irraditation). The bacteria treated with SiPcMe were deactivated approximately 1 log for the same experimental conditions. After irradiation of methylene blue treated bacteria the number of viable bacteria decreased 4 log. The photo inactivation of FotoSan® treated bacteria was concentration depending-full photo incativation at 136 µM, and 3,5 log at 34 µM (Diagr. 1).



Diagr. 1. Photodynamic inactivation of Streptococcus mutans with ZnPc Me(5,6µM), GaPc(5,6µM), SiPc(5,6 µM), Methilene blue(145 µM), FotoSan1(34 µM) and FotoSan2(136µM)

DISCUSSION

There is a growing bacterial resistance to antimicrobial drugs. A different approach to biofilm

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producing cariogenic *Streptococcus mutans* is needed and one plausible way to overcome the resistance problem is introducing the photodynamic therapy in caries control and treatment. PDT has the following advantages over conventional antimicrobial methods:noninvasive nature, repeatability, high selectivity, rapid inactivation of target microorganisms –minutes, depending on the energy densities, topical application and limited effects to the side of the lesion, further restriction of affected area achieved by the use of an optical fiber (25).

In the human oral cavity the species are organized in complex ecosystem. On the other hand they live forming biofilms and the response of biofilm microorganisms to photodynamic therapy may differ greatly from that of their in vitro isolates in many aspects, such as growth rate, metabolic activity and gene expression.

The photodynamic inactivation of microorganisms depends on the chemical structure of PS Photosensitizers are crucial elements in PDT; several studies have demonstrated the efficacy of a range of photosensitizers in the elimination or reduction of oral bacteria. FotoSan® is an attractive option because of its affordable cost and intense absorption wavelength in the red light spectrum (>600 nm). In our study this is demonstrated by the different effect of the three photosensitizers of the group of phtalocyanines - Zn ,Si, Ga and the different extent of bacterial inactivation.The last one shows unwanted dark toxicity at the used concentration. Further experiments are needed in order to determine the needed concentration that will have photodynamic disinfecting effect without dark toxicity. The damage to the bacterial cell consists of bacterial cell wall destruction, increased permeability of cytoplasmic membrane and probably nucleic acid strand breakage.

Based on these facts, several studies were carried out using PDT approving that oral bacteria are susceptible to PDT.

The advantages of photodynamic therapy over conventional antimicrobial agents are first, rapid killing of target organism depending mainly on the light energy dose delivered and therefore the power output of the light source used. Hence, resistance development would be unlikely as killing is mediated by singlet oxygen and free radicals and high concentrations of photosensitizer do not need to be maintained in the disease site for more than a few minutes, in contrast with hours or even days necessary in the case of conventional antimicrobial agents. Finally, antimicrobial effects can be confined to the site of the lesion by careful topical application of photosensitizer and the area of irradiation and can be restricted further by using an optical fibre (25).

CONCLUSION

The Gram-positive microaerophilic cariogenic bacterium *Streptococcus mutans*, was studied for susceptibility to antimicrobial PDT(APDT) with three phtalocyanines, methylene blue and FotoSan®. The new for APDT of cariogenic *Streptococcus mutans* (Zn(II)-, Ga(III)-, and Si(IV) phtalocyanines were studied in comparison to licensed for clinical use photosensitizers (methylene blue and FotoSan®) The highest effect was achieved with the complex of Zn(II)toward bacterial suspension. The so conducted microbiological testing demonstrated that the 5 tested PS put under irradiation with a diode laser show strong antimicrobial effect in the strain of reference of S. Mutans, with some small differences between the tested PS. Toxicity in dark use on this microorganism is to be neglected in all, but in Ga(III).

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