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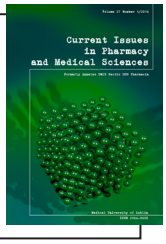

Application of low-level laser radiation with TiO_2, Ag/TiO_2 and S/TiO_2 on *Streptococcus salivarius* isolated from the oral cavity

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ABSTRACT
In our research, we determine the effect of low-level laser irradiation with nanoparticles on *Streptococcus salivarius*. Photodynamic killing of periodontopathogenic bacteria may be an alternative to the systemic application of antibacterial drugs used in the treatment of periodontal diseases. The application of photosensitizing nanoparticles and their excitation by visible light of blue spectra enables effective killing of periodontopathogens. This data combined with the results demonstrates that TiO_2, $AgTiO_2$ and S/TiO_2 can inhibit the proliferation of *Streptococcus salivarius* due to its high photocatalytic activity, which irreversibly damages the cell walls and membranes.

INTRODUCTION

Microorganisms found in the oral cavity play a fundamental role in the aetiology of dental caries, gingivitis, and periodontitis pathology. Mechanical instrumentation of infected caries lesions provides the gross removal of cavity pathogens and is the first step in the combat against cariogenic oral pathogen invasion. In order to achieve a higher level of bacterial eradication for the *Streptococci* group found in the diseased tissue, a variety of antimicrobial agents is used [15]. The growing resistance against antibacterial agents has generated a search for alternative antimicrobial treatments. In particular, the use of topical antibiotics is under discussion since it has been suggested that such an approach induces antibiotics resistance faster than the use of oral antibiotic [1-3]. In recent years, photodynamic inactivation (PDI) has been proposed as an alternative treatment for localized bacterial infections in response to the problem of antibiotic resistance. The bactericidal effect of photodynamic treatment (PDT) has been known for a long time [4-7]. The method consists in illumination of microorganisms treated with nontoxic photosensitizers (PSs) by low-power visible (red, blue, or white) light.

Photocatalysis is a suitable method for disinfection of pathogenic bacteria. There are some reports concerning the photocatalytic removal of organic, inorganic and microbial pollutants. The TiO_2 catalyst has been found to be a widely used component in various photocatalytic applications. The bactericidal activity upon addition of Ag to TiO_2 was tremendously enhanced [8-10].

The purpose of our study was to determine the effect of low-level laser irradiation with nanoparticles on *Streptococcus salivarius*.

MATERIAL AND METHODS.

During the research the oral microflora was examined in 80 patients aged 25-45 years who reported to the clinical diagnosis.

The material for microbiological examination was dental plaque and the content of caries cavities. To prevent material contamination by environmental microflora, a sterile excavator was used aseptically. The biological substrate was streaked during one hour after it had been taken on the following culture media Mitis-Salivarius Agar (Hi-media, India) and placed in 37°C incubator for 20 h.

Aerobic bacteria were determined on the basis of morphological characteristics, cultural properties and the establishment of biochemical properties. Streptococcus strains were determined according to the type of hemolysis (α - or β - hemolysis), esculin hydrolysis; also the disks with bacitracin and bile and Streptotest (Lachema, Czech Republic) were used.

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Fifty-nine strains of *S.salivarius* were allocated from patients with dental caries and periodontitis, which were identified on the basis of biochemical activity. Pure cultures isolated from patients and healthy individuals were used for further studies of the effects of the blue spectra and nanoparticles. Quantitative parameters of the microflora study were presented in the colony forming units per 1ml (CFU / ml).

Commercial TiO_2, Ag/TiO_2 and S/TiO_2 powders were ground in a porcelain mortar with a small amount of water (1.0 ml). After the powder had been dispersed by the high shear forces in the paste, it was diluted by slow addition of water (1.0; 5.0 and 10 ml)under the influence of continuous grinding.

S.salivarius cultures with 1.0 McFarland turbidity were diluted to 10^{-4} CFU/ml and added the concentration of nanoparticles 0.1, 0.5 and 1.0 mg/ml with the following irradiation in the logarithmic phase of growth in sterile 0.1 ml plates by a laser of blue spectra with exposition of 5 minutes.

Laser light source was a laser diode module of the blue spectrum with a wavelength of 445 nm diode power 1000 mW and an output power of 700 mW (AixiZ, USA).

After the irradiation, the entire volume of the culture (0.1 ml) was passaged with micropipette onto a solid nutrient medium, streaked with a spatula and incubated at 37°C. After 24 hours, the number of colonies was counted and the results were compared with the streptococci isolates of the control group that were subjected to the exposure.

Statistical calculation of the results was performed using the software package for statistical data analysis of biomedical research «Instat» (GraphPad Software Inc., 1993). During the statistical processing, the results were obtained in the form of the medium value of the test parameter (M), standard error (deviation) of the test parameter (m) and reliability index (p).

Results and discussion. The data are reported as the mean of a triplicate measurement. Survival fractions in each well were calculated by counting the colonies on the plates and dividing these by the number of colonies from the control group. Numerous studies have demonstrated the success of PDT against oral bacteria but none of these studies evaluated the susceptibility of *S. salivarius* to PDT using nanoparticles as a photosensitizer. The results of this study show that nanoparticles in combination with blue light had a phototoxic effect on *S. salivarius.*

According to the data it was established that upon irradiation with a laser beam with a wavelength of 445 nm, a decrease of quantitative indicators was achieved while maintaining properties of streptococci isolated from the oral cavity. When applying the low-level laser therapy (LLLT) blue light during the irradiation for 5 min., the intensity of microorganisms growth constituted 18.5 ± 1.1 CFU/ml ($p < 0.001$). The microbial number for the control group (irradiated) constituted 29.8 ± 1.2 CFU/ml ($p < 0.001$).

The effects of PDT on *S. salivarius* can be seen in Fig. 1, and show that the group that used Ag/TiO_2 with concentrations of 0.5 mg/ml followed by blue light illumination contained a significantly lower number of bacteria ($p > 0.05$) than did any other group. Differences among mean survival for the groups were quite low 2.7 ± 2.2 CFU/ml for 0.1 mg/ml, 0.4 ± 0.7 CFU/ml and 2.4 ± 2.3 CFU/ml were obtained for 0.5 and 1.0 mg/ml, respectively.

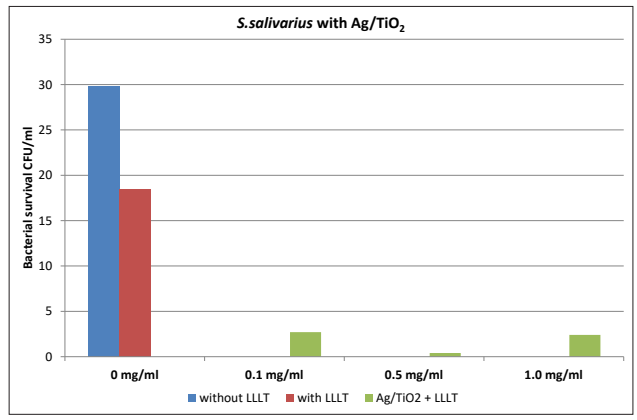

Figure 1. *Streptococcus salivarius* survival of the studied samples after photosensitization with 0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml of Ag/TiO_2 by irradiation with blue light at a wavelength of 445 nm ($p > 0.05$)

Figure 2 shows the effect of the exposure of the *S. salivarius* suspension of 0.1 mg/ml TiO_2 and subsequent illumination with light, resulting in a 4.7 ± 4.3 CFU/ml ($p > 0.05$) of viable cells. When the TiO_2 concentration was 0.5 mg/ml, a decrease to 2.1 ± 1.7 CFU/ml for the bacterial survival was obtained($p > 0.05$). Using TiO_2 with concentrations of 1.0 mg/ml by blue light, survival for the *S. salivarius* was 4.1 ± 5.0 CFU/ml ($p > 0.05$).

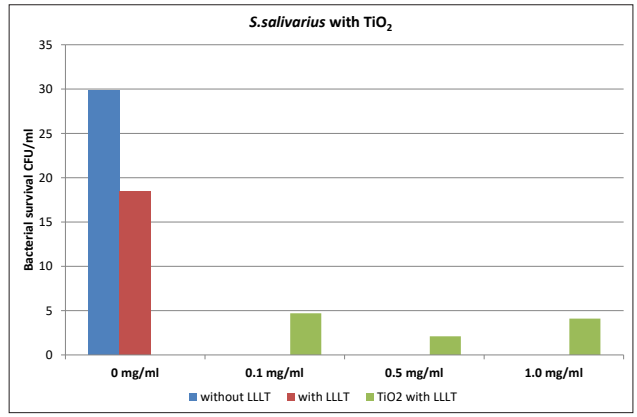

Figure 2. *Streptococcus salivarius* survival of studied samples after photosensitization with 0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml of TiO_2 by irradiation with blue light at a wavelength of 445 nm ($p > 0.05$)

A small reduction to 6.2 ± 4.5 CFU/ml and 5.0 ± 4.0 CFU/ml was observed when *S. salivarius* suspension was exposed to 0.1 mg/ml and 1.0 mg/ml of S/TiO_2 and subsequently illuminated with light ($p > 0.05$), respectively. However, at S/TiO_2 concentration of 0.5 mg/ml, a slightly higher reduction to 2.6 ± 2.5 CFU/ml ($p > 0.05$) was observed (Fig. 3).

The results of this study show that TiO_2, Ag/TiO_2 and S/TiO_2 in combination with blue light had a phototoxic effect on *S. salivarius*. The main observation made during the tests to photoinactivate bacteria with photosensitizers, was the relative sensitivity of gram-positive strains to photodynamic inactivation. Additionally, we observed that the photodynamic effect was dose-dependent for

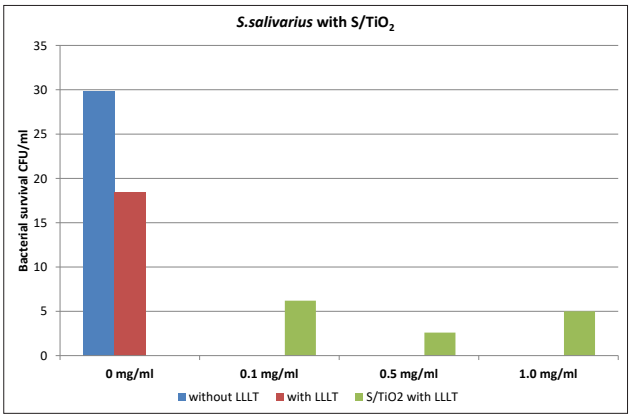

Figure 3. *Streptococcus salivarius* survival of studied samples after photosensitization with 0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml of S/TiO_2 by irradiation with blue light at a wavelength of 445 nm ($p > 0.05$)

different nanoparticles concentrations [10,11]. For species of *S. salivarius*, elimination with efficacy was obtained with Ag/TiO_2 at 0.5 mg/ml after irradiation with blue light. Despite the achieved reduction ($p > 0.05$) of TiO_2, under the same assay conditions as for *S. salivarius*, PDT had a lower effect on cell viability. *S. salivarius*, tested as a species and using the same concentrations of S/TiO_2, was less susceptible to PDT than TiO_2 was [12-13].

In addition, phototoxicity tests of the current study revealed that Ag/TiO_2, TiO_2 and S/TiO_2 particles, when irradiated with blue light, elicited lower cell viability as compared to non-irradiated particles. Again, particles with different concentrations also tended to trigger various phototoxicity as compared with particles, suggesting the possible role that particle concentrations may play in influencing phototoxic effects of nanoparticles. Unlike in the absence of blue light, the generation of hydroxyl radicals was chiefly responsible for the phototoxicity of Ag/TiO_2, TiO_2 and S/TiO_2 particles. During photodynamic inactivation, a number of reactive oxygen species (ROS) is generated. *S. salivarius* responds to extraneous stresses caused by oxidation and heat or by specific regulations of the protein levels [14,15].

CONCLUSIONS

The results of antibacterial study indicated that the Ag/TiO_2 under blue light had better results for bactericidal ability in comparison with two other tested nanoparticles due to photocatalytic property of TiO_2 and S/TiO_2, which did not have influence on killing of *S.salivarius* bacteria. The current study demonstrates that Ag/TiO_2 particles are able to elicit more phototoxic effects in the presence of low-level laser radiation than TiO_2 and S/TiO_2, respectively.

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