

Study of photodynamic effects on NIH 3T3 cell line and bacteria

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Background. Bacterial resistance to antibiotics is a constantly growing challenge. Photodynamic therapy (PDT) offers a new approach to the treatment of bacterial and viral diseases. The aim of this study was to compare the efficacy of photosensitizers used in PDT applied to cell lines and bacterial strains.

Methods. We tested the cytotoxicity and phototoxicity of 3 photosensitizers: TPPS₄, ZnTPPS₄ and TMPyP applied to the NIH3T3 cell line using two established methods for measuring ROS production and, MTT viability assay. Bacterial viability was determined spectrophotometrically over 24 h following PDT.

Results. The most efficient photosensitizer was TMPyP as it reduced the viability of the NIH3T3 cell line by more than 85%. In general, the photosensitizers were more phototoxic to the two Gram-positive bacterial strains, *Enterococcus faecalis* and *Staphylococcus aureus*. The viability of *E. faecalis* was reduced to 78 % by a dose radiation 0.5 J/cm² and concentration of TMPyP 1.562 μmol/L. The viability of bacterium *S. aureus* was reduced to 23 % when exposed to a radiation dose 0.5 J/cm² and 100 μmol/L concentration of ZnTPPS₄. The highest viability decrease (15 %) for *Pseudomonas aeruginosa* was caused by 0.5 J/cm² radiation dose and 50 μmol/L TMPyP concentration. *Escherichia coli* proved to be PDT resistant as the bacterial viability was higher than 90%.

Conclusions. The goal of the present study was to test the efficiency of photosensitizers on the NIH 3T3 cell line and bacterial cells. Subsequently we would like to study effectiveness of photosensitizers bound to carriers (for example cyclodextrins) on other cell line and bacterial strain.

Key words: phototoxicity, porphyrin, Gram-positive bacteria, Gram-negative bacteria, antibacterial therapy

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INTRODUCTION

Photodynamic therapy (PDT) is a relatively new modality for the treatment of illnesses. The first application of PDT research was successfully used in the treatment of cancer. Currently, it is being studied for its application to various other diseases such as cardiovascular disorders and antimicrobial therapy. PDT is now used as a treatment for localized microbial infections. From the time of the discovery of antibiotics in the last century, photodynamic therapy was abandoned. In recent decades however, the worldwide rise in antibiotic resistance has driven research to the development of new anti-microbial strategies. A number of human pathogens are increasingly resistant to antibiotics and the issue is still growing. PDT appears to be the new treatment for a number of microbial diseases. Studies confirm the differences in perception between Gram-positive and Gram-negative bacteria to PDT (ref.¹⁻³). The main reason for the differential sensitivity and response to PDT is the varied structure of bacterial cells. Anionic and neutral photosensitizers are effective in the case of Gram-positive bacteria, inducing growth inhibition or killing by visible light

whereas Gram-negative bacteria are not killed in this way. However, direct photokilling of Gram-negative bacteria is also possible. In recent years, a large number of chemical classes of positively charged photosensitizers, including porphyrins and phthalocyanines, have been successfully tested as photoinactivating agents against Gram-positive and Gram-negative bacteria⁴⁻⁷.

The phototoxicity of chemical compounds towards microorganisms was first published at the turn of the 20th century. Oskar Raab observed that the toxicity of acridine hydrochloride against *Paramecia candatum* was dependent on the amount of light⁸.

Another type of application of PDT is treatment of non-tumour diseases affecting the musculoskeletal system due to uncontrollable proliferation of synovial tissue cells, rheumatoid arthritis. This is an autoimmune disorder involving severe degenerative inflammation of the joints, and hyperproliferative growth of the synovial membrane into the synovial space. It limits the range of motion, as well as causing irreversible erosive damage of the joint by invasion of cartilagenous surfaces⁹. We used the fibroblastic NIH3T3 cell line as a tissue model to test the effectiveness of PDT.

The first generation of PDT photosensitizers to win the approval of the regulatory agencies in several countries was Protopfrin. This is a complex mixture of the more active porphyrin oligomers that comprises the hematoporphyrin derivate (HpD) (ref.¹⁰). However, Protopfrin has not been sufficient for all the criteria for an ideal photosensitizer, mainly due to its complexity and poor absorption of tissue-penetrating red light. HpD is also retained in the skin and thus characterization extended to produce cutaneous photosensitivity. The unsatisfactory features of the first generation photosensitizers stimulated development of a second generation photosensitizers. This includes: benzoporphyrins, porphyrins, purpurins, texaphyrins, phthalocyanines and endogenously generated photosensitive metabolites – protoporphyrin IX (PPIX). Currently, a third generation of photosensitizers represents an emerging class of compounds in the shape of second generation photosensitizer with the addition of carrier molecules to deliver photosensitizer to tumor tissue.

This alternative therapeutic approach employs non-toxic dye, photosensitizer, and visible light which, in the presence of oxygen, combine to produce cytotoxic oxygen species. Antimicrobial photodynamic therapy involves the killing of pathogenic cells by light in the presence of a photosensitizing agent. Excitation of the photosensitizer by absorption of light of appropriate wavelength converts the photosensitizer into its photoactive triple state. This in turn, reacts with either a local substrate (type I reaction) to form cytotoxic radicals or with molecular oxygen (type II reaction) to produce cytotoxic singlet oxygen (1O_2) and free radicals¹¹⁻¹³. The reactive oxygen species lead to cell death or initiate it.

We conducted an experiment with photosensitizers ZnTPPS₄, TPPS₄ and TMPyP, applied them to NIH 3T3 cells and compared the result with the bacterial strains. We focused on the efficacy of photodynamic therapy to diminish cell viability.

MATERIAL AND METHODS

Photosensitizers

We used 3 photosensitizers: TPPS₄, ZnTPPS₄ and TMPyP, which were prepared by Jiri Mosinger at the Department of Inorganic Chemistry, Faculty of Sciences, Charles University in Prague, Prague, Czech Republic.

Cell line

The NIH 3T3 cells (Mouse embryonic fibroblast cell line, ATCC, USA) were grown in 96-well microplates (10⁴ cells/well), using cultivation medium DMEM with additions of 0.5, 1, 5, 10, 50 and 100 μmol/L photosensitizers at 37 °C, 5% CO₂ for 24 hours in darkness. The viability of cell line was then investigated using methods for evaluating cell damage¹⁴.

Irradiation

To produce the photodynamic effect, we used light emitting diodes (LEDs 414 nm) in a total dose of from 0.5 to 25 J/cm².

Determination of ROS

ROS are reactive oxygen species originating in mitochondria. When the cell diaphragmal (membrane) potential rises, respiration is low and the production of ROS increases. Production of ROS is an indicator of cell damage leading to cell death. The efficacy of photosensitizers to produce photoinactivation on bacterial strains was determined spectrophotometrically. The production of ROS in cells was investigated by molecular probe CM-H₂DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester) (Invitrogen) which is sensitive enough to detect intracellular ROS. This agent is a derivative of DCF-DA. ROS measurement was performed using 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) producing fluorescence dye CM-DCF in the presence of ROS. DCF-DA has been used as a detector of ROS in many applications. This dye is not fluorescent in reduced chemical form but after cellular oxidation and withdrawal of acetate groups by cellular esterases it converts into fluorescent. After incubation with photosensitizers, cells were treated with 10 μmol/L CM-H₂DCFDA for 30 min at 37 °C in darkness. Fluorescence of CM-DCF (excitation/emission: 495/530 nm) was recorded by 96-well microplate reader Synergy HT (BioTek, Winooski, Vermont, USA) (ref.¹⁴). Fluorescence of CM-DCF was calibrated according to the corresponding fluorescence response of the probe to the addition of external H₂O₂ (ref.¹⁵). Two columns of wells were used as negative control (photodynamically treated cell line in the absence of photosensitizer). The experiment was repeated three times.

Cell viability test

Phototoxicity was evaluated by the MTT test. This colorimetric assay measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. MTT enters the cells and passes into the mitochondria where is reduced to an insoluble, purple formazan product. The crystals in the cells are then dissolved with an organic solvent. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells. After photodynamic treatment, the cells were incubated at 37 °C, 5% CO₂ for 24 h in fresh DMEM. Before the start of the viability measurement, DMEM was replaced by 0.5 mg ml⁻¹ MTT (Sigma-Aldrich) dissolved in PBS and incubated for 3.5 h at 37 °C and 5% CO₂ atmosphere. The MTT solution was then carefully removed and replaced with 100 μl of DMSO to dissolve formazan crystals. The absorbance of the prepared solution was measured in 96-well microplate reader Synergy HT at 570 nm and 690 nm¹⁴. The experiment was repeated three times.

Bacteria

For our PDT study we used 2 Gram-positive bacteria strains: *Staphylococcus aureus* CCM 4223 (ATCC 29213) and *Enterococcus faecalis* CCM 4224 (ATCC 29212) and 2 Gram-negative strains: *Escherichia coli* CCM 3954 (ATCC

25922) and *Pseudomonas aeruginosa* CCM 3955 (ATCC 27853). The bacteria were used at a density of 10^6 cfu/mL and grown in Brain Heart Infusion (Himedia) in shaker BMT (Brněnská medicínská technika) aerobically at 37 °C for 2 h in darkness.

Photodynamic inactivation of bacterial strains

Suspensions of bacteria (10^6 cfu/mL) were tested in 96-well microplates (GAMA GROUP a.s., Czech republic), where photosensitizers were diluted by geometric series (concentrations from 100 to 0.78128 $\mu\text{mol/L}$). After 2 h in darkness, the bacterial culture appeared in the exponential growth phase. The microplates were irradiated from above by a light source (LEDs 414 nm) 0.5 – 25 J/cm² and after 24 h of culture incubation in darkness and aerobically at 37 °C, the absorbance was measured on a spectrophotometer (BioTek, Winooski, Vermont, USA) in program Gen5 at a wavelength of 630 nm. The experiments were repeated three times. Control samples were bacteria untreated with photosensitizers or light and bacteria exposed to light in the absence of photosensitizers.

Statistical analysis

The data were presented as mean \pm SD of three independent experiments. The statistical significance was determined by an analysis of variance with ANOVA. $P < 0.05$ (*) was considered statistically significant. If the ANOVA demonstrated for some quantities showed statistically significant differences, multiple comparison test of LSD (Least Significant Difference) was performed. If the value $P < 0.05$ (*), differences were statistically significant.

RESULTS

Cytotoxicity and phototoxicity determination of cell line NIH 3T3

Firstly, cell cytotoxicity of all photosensitizers were tested on NIH3T3 cell line. The cells were incubated with the tested photosensitizers for 24 h without irradiation and ROS production determination and MTT assay were performed. The viability of the cells inoculated with all tested compounds was reduced by only 10% which was confirmed by MTT assay. The highest relative fluorescence units and concentration of H₂O₂ were detected for TMPyP at a concentration 100 $\mu\text{mol/L}$. Higher concentrations of photosensitizer can lead to cytotoxicities of their own¹⁶.

According to our results, the most efficient light dose to NIH 3T3 cell line was from 0.5 J/cm² to 10 J/cm² and the concentration of TMPyP was from 0.5 to 100 $\mu\text{mol/L}$ (Fig. 1, 2, 3). It was also demonstrated that high phototoxicity was achieved at a light dose of 1 J/cm² and concentration of TMPyP 10 $\mu\text{mol/L}$ (Table 1). In this regard, the viability of the cell line decreased to 13%. However, the observed phototoxicity of TMPyP was higher than the phototoxicity of the other two photosensitizers. In order to determinate hydrogen peroxide values it was necessary to carry out calibration of the dependence of fluorescence on concentration of H₂O₂ (Table 2). From the graphs

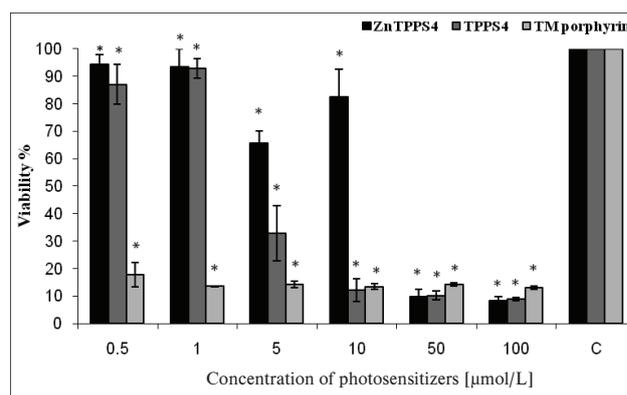


Fig. 1. Dependence of cell viability NIH 3T3 on the photosensitizers concentration at light dose of 1 J/cm²; C - control plate (without photosensitizers).

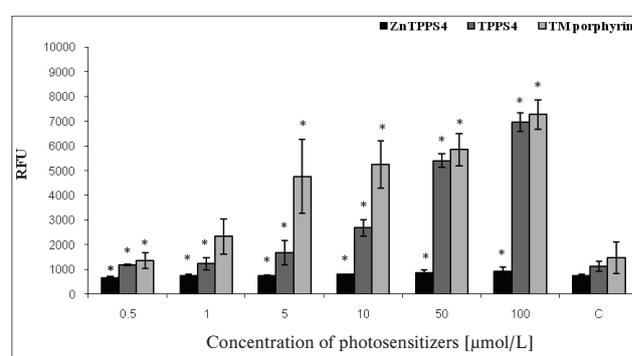


Fig. 2. Dependence of relative fluorescence unit of NIH 3T3 cell line on concentration of photosensitizers and light dose of 1 J/cm²; RFU - Relative Fluorescence Unit; C - control plate (without photosensitizers).

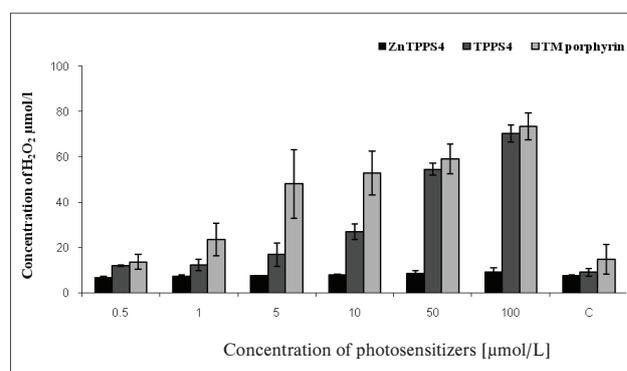


Fig. 3. Dependence of H₂O₂ production in NIH 3T3 cell line on concentration of photosensitizers at light dose of 1 J/cm²; C - control plate (without photosensitizers).

suggest that the photosensitizer TMPyP is very efficient on the NIH 3T3 cell line. We can conclude that bacteria show higher resistance against applied photodynamic therapy. The phototoxicity of these photosensitizers is the subject of ongoing investigation along with other photosensitizers to be tested in conjunction with chemical carriers towards increase efficiency of antimicrobial photodynamic effect.

(Fig. 2, 3) it is obvious that production of cytotoxic ROS is highest for photosensitizer TMPyP.

Bacterial strains

From these results (Fig. 4, 5, 6 and 7) it is also clear that PDT applied to strains of Gram-positive bacteria showed greater efficiency than to Gram-negative bacteria. For *Pseudomonas aeruginosa* we achieved the best results for concentrations of TMPyP 50, 25, 12.5 and 6.25 $\mu\text{mol/L}$ and light dose of 0.5 J/cm^2 (Fig. 4), (Table 4). However, the viability of microorganism remained high (85%). The viability of Gram-positive bacteria *Enterococcus faecalis* was lowest (78%) at a light dose of 0.5 J/cm^2 and concentration of TMPyP 1.562 $\mu\text{mol/L}$ (Fig. 5), (Table 5). We found that ZnTPPS₄ appears to be an appropriate photosensitizer for PACT. Bacteria *Staphylococcus aureus* exposed to a radiation dose 0.5 J/cm^2 and concentration 100 $\mu\text{mol/L}$ of ZnTPPS₄ is represented by a decline in viability to 23% under these conditions (Fig. 6), (Table 3). Photosensitizer TMPyP was not so efficient, as the viability of bacteria decreased to 91% at a light dose of 0.5 J/cm^2 and concentration at 0.78128 $\mu\text{mol/L}$. The photodynamic effect with the 3 tested photosensitizers was not evident for *Escherichia coli* (Fig. 7). Our development was performed to compare the photosensitivity and the antimicrobial effectivity of cell line NIH 3T3 and the pathogen microbial cell. The testing of the ability was focused on investigating the cytotoxic effect of photosensitizers and the decrease in viability of 4 bacterial strains. The concentration of TMPyP 1.562 $\mu\text{mol/L}$ and light dose 0.5 J/cm^2 were efficient in the case of Gram-positive bacteria *Enterococcus faecalis* (Fig. 5). The most efficient photosensitizer for destroying Gram-positive bacteria *S. aureus* was ZnTPPS₄ at a concentration of 100 $\mu\text{mol/L}$ and light dose of 0.5 J/cm^2 while TMPyP was less efficient for use in antimicrobial photodynamic therapy (Fig. 6). On the other hand TMPyP was efficient at a concentration of 50 $\mu\text{mol/L}$ and light dose of 0.5 J/cm^2 in the case of Gram-negative bacteria *Pseudomonas aeruginosa* (Fig. 4). The other Gram-negative bacteria strain *Escherichia coli* was not strongly sensitive to PDT with any of the 3 tested photosensitizers.

DISCUSSION

In this study, the cytotoxic effect of ZnTPPS₄, TPPS₄ and TMPyP was tested on NIH 3T3 cells by MTT test and determination of ROS production. Our results show that ROS production and cell viability in NIH 3T3 cell line and bacterial strains are dependent on photosensitizer concentration and dose of irradiation. Although all tested photosensitizers were able to induce photodynamic effects, TMPyP was found to be the most efficient photosensitizer to the NIH 3T3 cell line. Fig. 1, 2 and 3 demonstrate the accumulation of fluorescence in cells corresponding to production of ROS and consequently decrease in viability. Based on our results with 4 bacte-

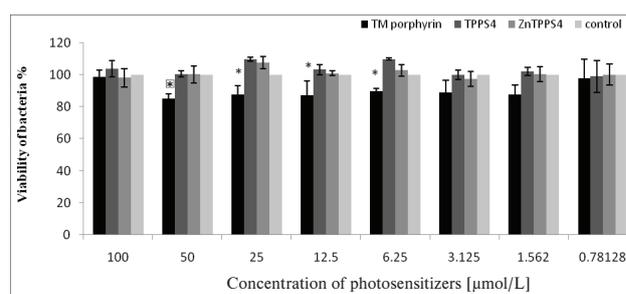


Fig. 4. Dependence of viability bacterial strain *Pseudomonas aeruginosa* on concentration of photosensitizers at light source of 0.5 J/cm^2 .

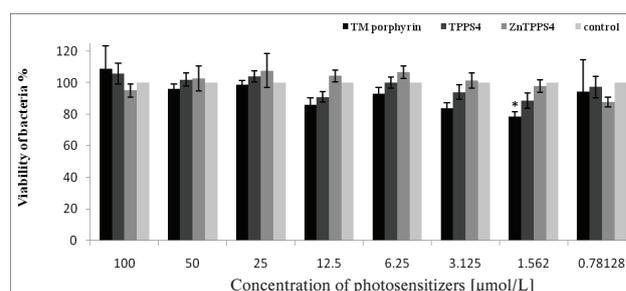


Fig. 5. Dependence of viability bacterial strain *Enterococcus faecalis* on concentration of photosensitizers at light dose of 0.5 J/cm^2 .

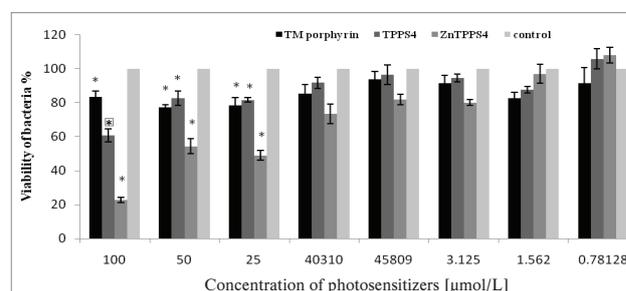


Fig. 6. Dependence of viability bacterial strain *Staphylococcus aureus* on concentration of photosensitizers at light dose of 0.5 J/cm^2 .

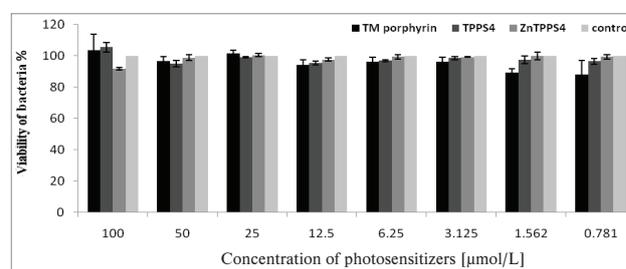


Fig. 7. Dependence of viability bacterial strain *Escherichia coli* on concentration of photosensitizers at light dose of 0.5 J/cm^2 .

rial strains, we conclude that photosensitizers are more antimicrobial toward Gram-positive bacteria than to less photosensitizing Gram-negative bacteria. The structure and organization of the cell wall are the presumed cause

Table 1. Statistics data for viability of cell line NIH3T3 after exposure light of dose 1 J/cm².

Photosensitizers	Concentration (μmol/L)	F (ANOVA)	Sig. (P)
Between TPPS ₄ , ZnTPPS ₄ , TMPyP	10	206.939	<0.0001
Photosensitizers	Concentration (μmol/L)	LSD Sig. (P)	-
TPPS ₄ x ZnTPPS ₄		<0.0001	
TPPS ₄ x TMPyP	10	<0.0001	
TMPyP x ZnTPPS ₄		<0.0001	

F - test statistics; Sig. (P) - significance.

Table 2. Statistics data for RFU of cell line NIH3T3 after exposure light of dose 1 J/cm².

Photosensitizers	Concentration (μmol/L)	F (ANOVA)	Sig. (P)
Between TPPS ₄ , ZnTPPS ₄ , TMPyP	0.5	12.820	0.005
Between TPPS ₄ , ZnTPPS ₄ , TMPyP	1	11.850	0.006
Between TPPS ₄ , ZnTPPS ₄ , TMPyP	5	18.012	0.005
Between TPPS ₄ , ZnTPPS ₄ , TMPyP	10	47.780	<0.0001
Between TPPS ₄ , ZnTPPS ₄ , TMPyP	50	132.634	<0.0001
Between TPPS ₄ , ZnTPPS ₄ , TMPyP	100	216.305	<0.0001
Photosensitizers	Concentration (μmol/L)	LSD Sig. (P)	-
TPPS ₄ x ZnTPPS ₄		0.006	
TPPS ₄ x TMPyP	0.5	0.005	
TMPyP x ZnTPPS ₄		0.005	
TPPS ₄ x ZnTPPS ₄		0.01	
TPPS ₄ x TMPyP	1	0.01	
TMPyP x ZnTPPS ₄		0.002	
TPPS ₄ x ZnTPPS ₄		0.01	
TPPS ₄ x TMPyP	5	0.001	
TMPyP x ZnTPPS ₄		0.002	
TPPS ₄ x ZnTPPS ₄		0.003	
TPPS ₄ x TMPyP	10	0.001	
TMPyP x ZnTPPS ₄		<0.0001	
TPPS ₄ x ZnTPPS ₄		<0.0001	
TPPS ₄ x TMPyP	50	<0.0001	
TMPyP x ZnTPPS ₄		<0.0001	
TPPS ₄ x ZnTPPS ₄		<0.0001	
TPPS ₄ x TMPyP	100	0.005	
TMPyP x ZnTPPS ₄		<0.0001	

RFU - Relative Fluorescence Unit; F - test statistics; Sig. (P) - significance.

of the impact of PDT on bacteria. During the last 10 years, advances in PDT have been made: short time of antimicrobial therapy, possibility to use relatively low intensity, significant reduction of pathogens, broad spectrum of photosensitizers for treatment, lack of induced resistance after repetitive treatment and lack of mutagenicity¹⁷. Furthermore the advantages are that bacteria are eradicated in a short time, resistance is improbable and microflora are not disrupted¹⁷. This study reports the success of photodynamic therapy with photosensitizers ZnTPPS₄, TPPS₄ and TMPyP. All these photosensitizers were efficient on the NIH 3T3 cell line. The best results were achieved by TMPyP. This photosensitizer decreased

the viability of the NIH 3T3 cell line more than 85% (Fig. 1).

CONCLUSION

The goal of the present study was to test the efficiency of photosensitizers on the NIH 3T3 cell line and bacterial cells. Due to the rapid emergence of antibiotic resistance between pathogenic bacteria, the search for possible photosensitizer to resolve this problem is ongoing. Study of the effect of photodynamic therapy on the cell line was performed for comparison to bacterial strains. Our results

Table 3. Statistics data for viability of *S. aureus* after exposure light of dose 0.5 J/cm².

Photosensitizers	Concentration (μmol/L)	F (ANOVA)	Sig. (P)
Between TPPS ₄ , ZnTPPS ₄ , TMPyP	25	45.513	<0.0001
Between TPPS ₄ , ZnTPPS ₄ , TMPyP	50	79.421	<0.0001
Between TPPS ₄ , ZnTPPS ₄ , TMPyP	100	110.477	<0.0001
Photosensitizers	Concentration (μmol/L)	LSD Sig. (P)	-
TPPS ₄ x ZnTPPS ₄	25	<0.0001	-
TPPS ₄ x TMPyP		<0.0001	-
TMPyP x ZnTPPS ₄		<0.0001	-
TPPS ₄ x ZnTPPS ₄	50	<0.0001	-
TPPS ₄ x TMPyP		<0.0001	-
TMPyP x ZnTPPS ₄		<0.0001	-
TPPS ₄ x ZnTPPS ₄	100	0.02	-
TPPS ₄ x TMPyP		<0.0001	-
TMPyP x ZnTPPS ₄		<0.0001	-

F - test statistics; Sig. (P) - significance.

Table 4. Statistics data for viability of *P. aeruginosa* after exposure light of dose 0.5 J/cm².

Photosensitizers	Concentration (μmol/L)	F (ANOVA)	Sig. (P)
TMPyP	50	72.447	<0.0001
	25	99.906	<0.0001
	12.5	22.844	0.00049
	6.25	12.820	0.005

F - test statistics; Sig. (P) - significance.

Table 5. Statistics data for viability of *E. faecalis* after exposure light of dose 0.5 J/cm².

Photosensitizers	Concentration (μmol/L)	F (ANOVA)	Sig. (P)
TMPyP	1.562	11.850	0.006

F - test statistics; Sig. (P) - significance.

ABBREVIATIONS

CM-H₂DCFDA, 5-(6-)chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; DMSO, Dimethyl sulfoxide; LEDs, Light emitting diodes; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium-2H-tetrazolium bromide; NIH 3T3, Mouse embryonic fibroblast cell line; PACT, Photodynamic antimicrobial chemotherapy; PDT, Photodynamic therapy; ROS, Reactive oxygen species; TMPyP, 5,10,15,20-tetrakis(N-methylpyridinium-4-yl)porphyrin; TPPS₄, Meso-tetra (4-sulfonatophenyl) porphyrin; ZnTPPS₄, Zinc-5,10,15,20-tetrakis(4-sulphonatophenyl) porphyrin.

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