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Impact of methylene blue and LED radiation on microbial biofilms

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Antibiotics play a key role in treating and controlling infectious diseases caused by pathogenic bacteria. Overconsumption and misuse of antibiotics have led to an increase in bacterial drug resistance. Resistant microorganisms are responsible for infections that are more difficult and expensive to treat; besides multidrug-resistant microorganisms cause hundreds of thousands of deaths annually. To combat antibiotic resistance, physicochemical methods, including antimicrobial photodynamic therapy, are becoming increasingly widespread. The purpose of the study was to determine the complex effect of methylene blue as a photosensitizer, and LED radiation on the biofilms of some opportunistic microorganisms. To examine biofilm formation and the impact of photodynamic therapy on formed biofilms, the microtiter plate method was used. The obtained results showed that 20-minute exposure of LED light of red-infrared spectrum did not cause statistically significant effect on formed biofilms. Photosensitizer methylene blue alone caused a certain antibiofilm effect which was expressed in a decrease in the growth intensity of biofilm scrapings after inoculation of appropriate dilutions into the Petri dishes on average by 33.2–60.0%, compared to the control group. The combined action of the photosensitizer with subsequent irradiation of biofilms by LED radiation demonstrated pronounced antibiofilm activity, which was much higher than the separate effects of specified factors. In this case, the growth intensity of the studied microorganisms at similar dilutions of biofilm scrapings decreased on average by 65.8–92.5%. Investigated strains of *Staphylococcus aureus* and *Candida albicans* were more susceptible than *Escherichia coli*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*. Due to the lack of pronounced side effects and the presence of several advantages, including wide-spectrum antimicrobial and antibiofilm activity, antimicrobial photodynamic therapy can be used in the treatment of inflammatory-purulent processes particularly in dentistry and dermatology.

Keywords: opportunistic microorganisms; antibiofilm effect; antimicrobial photodynamic therapy; antibiotic resistance.

Introduction

The discovery of antibiotics is one of the greatest achievements of science and their therapeutic application is a miracle in medical history (Uddin et al., 2021, Watkins & Bonomo, 2016). Since being introduced into medical practice, they have saved numerous of human lives (Banin et al., 2017). At the same time, antimicrobial resistance (AMR) is an inevitable phenomenon due to the ability of microorganisms to develop genetic mutations. The problem gets complicated due to the overuse and misuse of antibiotics in clinical practice and wide use of antimicrobial means for purposes other than medical treatment (Serwecińska, 2020). AMR is a major cause of death and economic burden around the world and is thus considered to be one of the greatest global public health threats (Lin et al., 2015, Nwobodo et al., 2022). Therapy of infectious diseases caused by resistant microorganisms has become challenging, difficult and expensive (Murugaiyan et al., 2022). Multi-drug resistant bacteria cause hundreds of thousands of deaths every year (Urban-Chmiel et al., 2022) and the spread of global AMR shows no signs of slowing down despite the fact that in recent decades different approaches have been taken to solve this issue. However, (Dadgostar, 2019, Cella et al., 2023).

The causes of AMR are related to natural reasons such as genetic mutations, transfer of genetic material, as well as inadequate use of antibiotics – improper antibiotic prescribing, false diagnoses, widespread agricultural use (Watkins & Bonomo, 2016), etc. Biofilm formation is one of the modes by which bacteria exert AMR. Biofilm can be defined as an aggregate of microbial cells surrounded by a self-produced polymer matrix (Costerton et al., 1999, Høiby, 2017). The hallmark of the biofilm mode of existence is increased resistance to a wide range of factors including the defense mechanisms of immune system, disinfectants and antibiotics (Banin et al., 2017). Biofilm formation is also a key virulence factor for a wide range of microorganisms that cause chronic infections (Koo et al., 2017).

Due to these factors, the search and study of some alternatives to antibiotics is of great relevance and importance. Among these can be highlighted the therapeutic use of bacteriophages (Kwiatek et al., 2020), vaccines, monoclonal antibodies, quorum-quenching (Murugaiyan et al., 2022), antimicrobial peptides (Browne et al., 2020), novel chemical compounds with antimicrobial properties (Kut et al., 2024, Pantyo et al., 2024), photochemical methods (Pantyo et al., 2020, Gonçalves et al., 2024).

One of the photochemical methods that are developing most dynamically nowadays is antimicrobial photodynamic therapy (APDT). This is a promising approach to fight the growing problem of antimicrobial resistance that threatens health care, food security, and agriculture. APDT is based on the use of photosensitizers with the subsequent effect of low-intensity radiation of a certain wavelength, which triggers a photodynamic response leading to the generation of reactive oxygen species (Piksa et al., 2023). The main advantages of APDT are action on the wide range of both Gram-positive and Gram-negative bacteria, fungi and viruses. Besides, APDT does not induce resistance in microorganisms even in sub-inhibitory doses (Pantyo et al., 2020, Polat & Kang, 2021, Murugaiyan et al., 2022). Due to these advantages, APDT can be considered as an alternative or complement to traditional antibiotic therapy.

Although APDT is already widely used in many branches of medicine, some aspects still require more detailed further study. Particularly questions about the direct impact of such light parameters as polarization and coherence on biological objects remain open. That is why examining the complex impact of photosensitizers and different sources of low-power light – LED, laser, polarized radiation, etc. – is relevant.

The aim of the work was to determine the complex impact of photosensitizer 0.1% aqueous solution of methylene blue and LED radiation of the red and infrared spectrum on the biofilms of some opportunistic microorganisms.

Material and methods

The impact of photosensitizer – 0.1% aqueous solution of methylene blue and LED radiation of red-infrared spectrum on clinical isolates of *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Candida albicans* was studied. Specified clinical strains were isolated from patients with chronic generalized periodontitis who underwent treatment in Uzhhorod University dental clinic. One strain of each species was studied. For the identification of microorganisms, generally accepted methods were used (Franco-Duarte et al., 2019) with the use of microscopical, bacteriological, and biochemical techniques. For final identification, the test systems STAPHYtest 16, NEFERMtest 24, ENTEROtest 24, and CANDIDAtest 21 ("PLIVA-Lachema a.s.", Czech Republic) were used. In addition to clinical isolates, collection test strains *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231 (Selectrol®, UK) were also studied.

To examine biofilm formation in the investigated strains, the microtiter plate method was used (Djordjevic et al., 2002, Azeredo et al., 2017). For that, 16–24-hours agar cultures of microorganisms were prepared with subsequent standardization in liquid media to the optical density 0.5 by McFarland using the device Densi-La-Meter (DEN-1, Biosan, Latvia, 2016). After that, microbial inoculum in a volume of 10 μL was added into the wells of a 96-well ELISA plate (C bottom, middle binding, detachable, sterile, DNase and RNase free) with 190 μL of sterile meat-peptone broth. Plates were cultivated 5 days in the thermostat at 37^{0}.

After the cultivation, we removed planktonic form from the wells and washed them three times with distilled water in a volume of 200 μL. To determine the density of biofilms, the wells were stained with crystal violet for 10 minutes with subsequent double washing by distilled water (200 μL into each well) and the addition of 96% ethyl alcohol for 45 minutes. Optical density of biofilms was measured on an ELx800 (BioTek, USA) reader at wavelengths of 630 and 492 nm. As a control of biofilm formation, sterile meat peptone broth was used, which was added into wells of microtiter plates in a volume of 200 μL.

To determine the complex impact of photosensitizer and LED radiation on microbial biofilms, they were divided into 4 groups. The first group – control – consisted of 5-day biofilms grown on microtiter plates, which were not affected by any factors. The second group included biofilms that, after removing their planktonic form and washing with distilled water, were irradiated by LED radiation of the red infrared spectrum with application duration of 20 minutes and frequency 8000 Hz. Into the wells with microbial biofilms of the third group, photosensitizer 0.1% aqueous solution of methylene blue in a volume of 200 μL was added, after which the wells were kept in the dark for 20 minutes. Then the methylene blue was removed and the wells were washed with distilled water. The fourth group biofilms underwent action of the photosensitizer (similarly to the third group) with subsequent irradiation (after the dark phase) by LED radiation (similarly to the second group). It should be noted that arrangement of experimental groups of microorganisms was analogous to our previous research (Pantyo et al., 2020) in which we studied the impact of the same factors on planktonic forms, as well as a similar experimental study of other authors (Souza et al., 2010).

Since adding methylene blue to the biofilms of the third and the fourth groups significantly influenced their optical density, its evaluation using a reader would not show the true impact on biofilm viability. To determine biofilm density, after the impact of the factors mentioned above, we added 200 μL of distilled water to the wells of all examined groups, after carefully scraping microbial biofilms from the bottom of the wells with the tip of the pipette dispenser. The resulting suspension was then titrated to obtain 10-fold dilutions from 1/10 to $1/10^{4}$ and plated in a volume of 10 μL onto Petri dishes with solid nutrient media. After that the Petri dishes were cultivated in the thermostat at 37^{0} for 24 hours (48 hours for *C. albicans* strains). Thus size of biofilms in different groups was compared according to the growth intensity of microorganisms (number of CFU) on the nutrient media in Petri dishes.

The source of LED radiation of the red and infrared spectrum (λ = 640 ± 30 and 880 ± 30 nm) was the Medolight Red device by Bioptron light therapy system (Zepter, Switzerland, 2017). The power density of irradiation was equal to 5.35 mW/cm^2, so with a 20-minute exposure dose density was equal to 6.42 J/cm^2. Irradiation of biofilms was conducted directly above the wells of microtiter plates (Fig. 1).

Fig. 1. Irradiation of microbial biofilm by LED light generated by Medolight Red device (Zepter, Switzerland, 2017)

The obtained data were statistically processed to determine the arithmetic mean (x) and standard deviation (SD) of the samples. To determine the significance of the difference between the control and experimental groups the ANOVA program (analysis of variance) was used. Differences were considered significant at $P < 0.05$.

Results

It was established that all investigated clinical and collection strains were able to produce biofilms on microtiter plates. As the mean optical density of the sterile liquid medium (meat peptone broth) was 0.054 ± 0.010, the optical density of 0.100 and higher was considered biofilm formation. Table 1 compares the optical densities of the first (control) and second groups of investigated microorganisms.

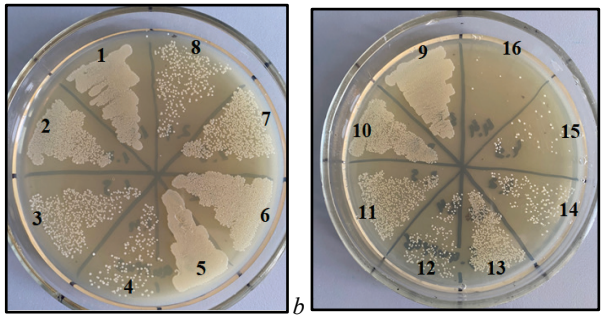
Table 1
Impact of LED radiation on the optical density of microbial biofilms (x ± SD; n = 8)

Species of microorganisms	Group 1 (control)	Group 2 (LED light irradiation)
Staphylococcus aureus (clinical isolate)	0.266 ± 0.049	0.276 ± 0.051
S. aureus ATCC 25923	0.286 ± 0.042	0.294 ± 0.058
Escherichia coli (clinical isolate)	0.168 ± 0.030	0.184 ± 0.030
E. coli ATCC 25922	0.201 ± 0.026	0.210 ± 0.031
Klebsiella pneumonia (clinical isolate)	0.253 ± 0.044	0.258 ± 0.054
K. pneumonia ATCC 13883	0.267 ± 0.032	0.268 ± 0.038
Pseudomonas aeruginosa (clinical isolate)	0.247 ± 0.051	0.241 ± 0.056
P. aeruginosa ATCC 27853	0.226 ± 0.048	0.214 ± 0.044
Candida albicans (clinical isolate)	0.357 ± 0.053	0.340 ± 0.057
C. albicans ATCC 10231	0.332 ± 0.058	0.316 ± 0.038

Note: ANOVA showed that the difference between groups is not considered to be statistically significant ($P > 0.05$).

The obtained data indicate that the most dense biofilms were produced by *S. aureus* and *C. albicans*. 20-minute application of the LED light did not cause a significant effect on biofilm density. In the case of *C. albicans* and *P. aeruginosa* (for both clinical and collection strains) there was a slight decrease of biofilm density in irradiated groups – on average 2.5–5.3%. For *S. aureus* and *E. coli* strains the opposite pattern was noted – irradiated biofilms' density was 2.7–9.5% higher, compared to the control group. In the case of investigated clinical and collection strains of *K. pneumonia*, the near absence of irradiation effect on biofilm density was stated.

The abovementioned was confirmed by the growth intensity of biofilms, scraped from the wells of microtiter plates after their inoculation on the Petri dishes (Fig. 2).



a *b*

Fig. 2. Growth of *Staphylococcus aureus* on Petri dishes after subculture of different dilutions: *a* – first (1–4) and second (5–8) groups; *b* – third (9–12) and fourth (13–16) groups

Figure 2 shows the growth intensity of different dilutions (1/10 to 1/10000) of a clinical isolate of *S. aureus* biofilms. There are clearly similar growth patterns of control (sectors 1–4), second (sectors 5–8), and third (sectors 9–12) groups. This indicates that neither low-power LED radiation nor the photosensitizer methylene blue alone induced significant antibiofilm activity against the strains studied. The growth intensity of the fourth group (sectors 13–16) was significantly lower compared to the other groups. Quantitative data of the growth (amount of colonies) of the investigated strains after the inoculation of the highest dilution (1/10000) of biofilm scrapings on the Petri dishes with solid media are represented in Table 2.

Analyzing data of the growth intensity of clinical isolate *S. aureus* we can state that there was no significant difference in the number of colonies between the 2nd and 1st groups ($P = 0.5244$). Exposure of methylene blue to biofilms of the 3rd group caused a decrease in the number of colonies by 40.2% compared to the control ($P = 0.0011$). The complex impact of photosensitizer and LED light decreased the number of colonies in 10^4-fold biofilm scraping dilution by 92.5% compared to the 1st group ($P < 0.0001$). For the collection test strain *S. aureus* ATCC 25923 we also noted no impact of LED radiation on the growth intensity, compared to the control ($P = 0.327$). The number of colonies in the 3rd and 4th groups decreased on average by 35.0% ($P = 0.0051$) and 91.9% ($P < 0.0001$) respectively. The same regularities were noted concerning other investigated strains – the impact of LED radiation did not cause a statistically significant effect on the biofilms; growth intensity of the 3rd group decreased by 33.2% in *P. aeruginosa* ATCC 27853 ($P = 0.0048$) to 60.1% in *C. albicans* ($P < 0.0001$); finally the number of the 4th group colonies was smaller by 65.8% in *K. pneumonia* ATCC 13883 ($P < 0.0001$) and 85.9% in *C. albicans* ($P < 0.0001$) than in the control group.

Table 2

Number of colonies of investigated microorganisms on solid nutrient media (x ± SD; n = 8)

Species of microorganisms	Group 1 (control)	Group 2 (LED light irradiation)	Group 3 (addition of methylene blue)	Group 4 (methylene blue+LED)
Staphylococcus aureus (clinical isolate)	85.6 ± 19.4	91.9 ± 19.2	51.1 ± 13.7**	6.4 ± 4.4**
Staphylococcus aureus ATCC 25923	97.8 ± 22.3	109.1 ± 22.5	63.5 ± 18.9**	7.9 ± 5.6**
Escherichia coli (clinical isolate)	72.1 ± 14.1	74.4 ± 12.6	39.9 ± 8.3**	20.9 ± 7.8**
Escherichia coli ATCC 25922	77.6 ± 11.0	80.9 ± 8.1	44.9 ± 9.2**	21.9 ± 8.2**
Klebsiella pneumonia (clinical isolate)	39.8 ± 8.7	40.1 ± 9.5	25.9 ± 7.1**	12.5 ± 5.0**
Klebsiella pneumonia ATCC 13883	44.6 ± 7.0	46.9 ± 5.5	29.1 ± 6.8**	15.3 ± 6.5**
Pseudomonas aeruginosa (clinical isolate)	59.3 ± 10.4	57.0 ± 10.2	36.6 ± 9.5**	19.4 ± 8.8**
Pseudomonas aeruginosa ATCC 27853	47.4 ± 10.2	46.5 ± 7.4	31.6 ± 8.5**	14.9 ± 4.6**
Candida albicans (clinical isolate)	66.7±19.8	61.2 ± 15.6	26.2 ± 5.3**	9.4 ± 6.5**
Candida albicans ATCC 10231	56.9 ± 9.9	52.9 ± 14.0	29.6 ± 9.1**	10.9 ± 8.0**

Note: for clinical isolate *C. albicans* and collection test strain *C. albicans* ATCC 10231 showed the number of colonies after the inoculation of dilution 1/100; the difference between control and experimental groups is significant (ANOVA): ** – $P < 0.01$.

Comparing the antibiofilm effect of 0.1% aqueous methylene blue solution alone and in complex with LED radiation, we noted a much more pronounced effect of the latter. The least difference in colony number between the 3rd and 4th groups was in clinical strains *E. coli*, *P. aeruginosa*, and collection strain *K. pneumonia* ATCC 13883 and was 47.6% ($P = 0.0003$), 47.0% ($P = 0.0020$), and 47.4% ($P = 0.0009$) respectivelly. The highest difference between the growth intensity of the third and fourth groups was noted for clinical isolate *C. albicans* – 64.2% ($P < 0.0001$) and both clinical and collection strains of *S. aureus* – 87.5% ($P < 0.0001$).

Discussion

The obtained results correspond with the results of similar experimental works by other authors (Souza et al., 2010, Fu et al., 2013, Soria-Lozano et al., 2015, Mahmoudi et al., 2018) as well as with our previous research (Pantyo et al., 2020) in which we studied the effect of methylene blue and LED radiation on planktonic form of microorganisms. In the case of the antimicrobial effect on planktonic form examination, we used much lower irradiation exposure – 5 minutes. 20 minutes of light exposure on biofilms is explained by their much higher resistance to physical and chemical factors. The biofilms' resistance is mostly associated with the presence of extracellular matrix that consists of proteins, polysaccharides, lipids, extracellular DNA, etc., that provides protection by making penetration of antimicrobials difficult, as well as with the host's defensive mechanisms factors (Stewart, 2015, Singh et al., 2017).

Another important aspect is the study of the influence of low-power radiation on biofilm formation. In our previous research (Pantyo et al., 2024) we studied the impact of 10-minute exposure to LED radiation generated by Medolight Red device on microbial inoculum with subsequent examination of its ability to produce biofilms on microtiter plates. Low doses of low-power light did not inhibit biofilm formation by the examined microbes and even stimulated this process. At the same time, according to some research (Pousty et al., 2024) high power shortwave radiation efficiently reduced biofilms. The abovementioned again proves the dependence of biological activity of light on its parameters, in particular on the wavelength and power density.

At present among different light sources, laser radiation is the most widely used in various fields of medicine and, as a result, is the most studied. The stimulating effect of low-level laser therapy on biological objects is explained by producing higher rates of ATP, RNA, and DNA synthesis in stem cells and other cell lines (AlGhamdi et al., 2012). It is precisely the stimulating effect on microorganisms that can also explain the increased sensitivity of microorganisms

to some antimicrobial agents when the microbial inoculum is irradiated with low-intensity radiation (Pantyo et al., 2024). Thus, investigating the impact of different types of low-power light alone and in combination with photosensitizers can clarify the understanding of the interaction of irradiation with biological objects, some of which are still not completely comprehensible.

The high effectiveness of antimicrobial photodynamic therapy on both planktonic and biofilm forms of opportunistic and pathogenic bacteria, as well as a number of other advantages, allow this method to be used in the treatment of various pathological conditions as a supplement or even an alternative to traditional antimicrobial therapy. APDT is already widely used in the dentistry, particularly for disinfection of infected root canals (Cheng et al., 2012, Bago et al, 2013). At the same time, also the increase in the role of non-coherent light sources such as LED for low-energy phototherapy deserves attention (Lee et al., 2022). It is important to perform further research on the antimicrobial and antibiofilm effects of APDT with the use of coherent and non-coherent, polarized and non-polarized low-power light sources, as well as a variety of photosensitizers on a wide range of microorganisms for the wider introduction of this method in clinical practice.

Conclusions

Application of photosensitizer methylene blue (0.1% water solution) and subsequent 20-minute irradiation with LED light of the red-infrared spectrum expresses significant antibiofilm activity against the investigated clinical and collection strains *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia*, and *Candida albicans*. The complex impact of specified factors is much more pronounced compared to the effects of photosensitizer and LED radiation when used alone and caused a decrease in growth intensity of biofilm scrapings on average by 65.8–92.5% compared to control. Methylene blue alone induced a decrease in the growth rate of the investigated strains by 33.2–60.1%. Irradiation of formed biofilms by LED radiation did not cause a statistically significant effect either on biofilms' density or on their growth intensity. Due to its expressed antimicrobial and antibiofilm activity against a wide range of opportunistic and pathogenic microorganisms, antimicrobial photodynamic therapy can be used to treat a variety of inflammatory-purulent processes caused by bacteria or microscopic fungi.

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