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Photodynamic Inactivation of Uropathogenic Biofilm-Forming Microorganisms: A Pilot Study

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ABSTRACT

BACKGROUND: Methods for microbial inactivation, including physical approaches aimed at the destruction of biofilms formed by uropathogenic microorganisms for the prevention of infectious and inflammatory diseases in urology, remain insufficiently studied. The development of new strategies in this field remains relevant.

AIM: To evaluate the feasibility of photodynamic inactivation of biofilms formed by typical representatives of uropathogenic microorganisms using an antiseptic agent with a bacteriostatic effect—methylene blue—possessing photochemical properties.

METHODS: Cultures of *Staphylococcus aureus* and *Escherichia coli* isolated from renal calculi of patients from a urology department were used. *In vitro* experiments on photodynamic inactivation of microorganisms were conducted on mature preformed biofilms. Irradiation was performed using a diode laser emitting at a wavelength of 662 nm through a sterile 0.1% methylene blue solution in continuous mode across five setups (three control, two experimental). After irradiation, biofilms on the cover glasses were fixed on microscope slides using colorless varnish. The prepared specimens were stained with acridine orange solution, dried in the dark, examined under a fluorescence microscope at ×100 magnification using an immersion system, and photographed with a digital camera. Images were digitally processed using 3D modeling technologies with ImageJ software version 1.52a.

RESULTS: The impact of the photoactive agent and laser irradiation was assessed at two power settings—450 mW and 1100 mW. In the first case, partial destruction of the biofilms was noted (41.9% of the original biofilm structure for *S. aureus* and 82.4% for *E. coli*), whereas in the second case, exposure at 1100 mW resulted in complete degradation of the mature multilayer biofilm into single cells without extracellular matrix, corresponding to 97.7% destruction of the original biofilm structure for *S. aureus* and 96.5% for *E. coli*.

CONCLUSION: This study is the first to demonstrate the feasibility of photodynamic inactivation of uropathogenic biofilm-forming microorganisms using a photochemically active agent—methylene blue. The promising results suggest that combined laser irradiation and methylene blue application may serve as an alternative or adjunct to systemic antibiotic therapy in urological practice.

Keywords: photodynamic inactivation of microorganisms; diode laser; methylene blue; biofilm; uropathogenic bacteria.

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Фотодинамическая инактивация уропатогенной микрофлоры в биопленках: пилотное исследование

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АННОТАЦИЯ

Обоснование. Методы инактивации микроорганизмов, включая физические методы воздействия с целью разрушения биопленок уропатогенных микроорганизмов в качестве профилактики инфекционно-воспалительных заболеваний в урологии, изучены недостаточно, и разработка новых подходов остается актуальной.

Цель — определить возможность фотодинамической инактивации биопленок, образованных типичными представителями уропатогенной микрофлоры, с использованием антисептического средства с бактериостатическим эффектом метиленового синего, обладающего фотохимическим свойством.

Материалы и методы. Использованы культуры уропатогенных бактерий *Staphylococcus aureus* и *Escherichia coli*, выделенные из почечных конкрементов пациентов урологического отделения. Эксперименты по фотодинамической инактивации микрофлоры проводили *in vitro* на выращенных зрелых сформировавшихся биопленках. Облучение выполняли диодным лазером, генерирующим излучение с длиной волны 662 нм через стерильный 0,1% раствор метиленового синего в непрерывном режиме в пяти (три контрольных, два опытных) вариантах. По окончании облучения биопленки на покровных стеклах фиксировали на предметном стекле с помощью бесцветного лака. Готовые препараты окрашивали раствором акридинового оранжевого, высушивали в темноте, просматривали с использованием микроскопа с люминесцентным модулем при увеличении ×100 в иммерсионной системе и фотографировали цифровой камерой. Проводили цифровую обработку полученных изображений с применением технологий 3D-моделирования при помощи программного комплекса ImageJ ver. 1.52a.

Результаты. Оценку воздействия фотоактивного препарата и лазерного излучения проводили в двух режимах — 450 и 1100 мВт. В первом случае отмечено частичное разрушение биопленок (41,9% изначальной структуры биопленки для *S. aureus* и 82,4% — для *E. coli*), во втором случае — воздействие в режиме 1100 мВт привело к полной деградации многослойной зрелой биопленки до единичных клеток, лишенных внеклеточного матрикса, то есть было разрушено 97,7% изначальной структуры биопленки для *S. aureus* и 96,5% — для *E. coli*.

Заключение. Впервые показана возможность фотодинамической инактивации уропатогенной биопленкообразующей микрофлоры с использованием препарата с фотохимическим эффектом — метиленового синего. Обнадеживающие результаты свидетельствуют, что комбинированное воздействие лазерного излучения и метиленового синего может быть использовано как альтернатива или дополнение к системной антибиотикотерапии в урологической практике.

Ключевые слова: фотодинамическая инактивация микроорганизмов; диодный лазер; метиленовый синий; биопленка; уропатогенные бактерии.

Как цитировать

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BACKGROUND

Modern urology is characterized by the rapid advancement of endoscopic surgery. However, the complexity of surgical equipment and instruments and the human factor at all stages of workflow contribute to the risk of postoperative complications. Among the risk factors are the need for urinary tract drainage and the growing ineffectiveness of antibiotic prophylaxis for urinary tract infection due to antibiotic resistance of uropathogens. Infections caused by resistant strains tend to have a more severe course, often require hospitalization, and increase the length of hospital stay. All this leads to increased treatment costs and worsens the prognosis for patients' health and life.

A category of so-called "problematic" microorganisms has emerged worldwide, among which strains resistant to one or several classes of antibiotics are particularly common [1]. These include *Staphylococcus* spp., *Enterococcus* spp., *Pseudomonas aeruginosa*, and several strains of the *Enterobacteriaceae* family [2]. Current understanding of the biology of microbial persistence allows the processes underlying the course of infection to be considered differently compared with the concepts of the past century. It has been established that the biofilm form of existence is an advantageous mode of organization for conditionally pathogenic prokaryotes during host colonization [3]. At the same time, biofilms play an important role in the persistence of clinically relevant pathogens in hospital settings. The problem of microbial biofilms in the hospital environment is associated with increased healthcare costs, prolonged hospitalization, and subsequent secondary infections with various complications. At present, the role of biofilms has been reliably established in at least 60% of all chronic or recurrent infections [4]. The formation of biofilms on various biotic and abiotic surfaces can lead to infection in many patients, whereas biofilms that develop on catheters, drains, endoprostheses, and other medical instruments and materials serve as foci of chronic infection in the patient's body. Evidence indicates that approximately 80% of all pathogenic strains infecting humans are associated with medical equipment, including urinary catheters [5]. It has also been noted that the difficulty of treating biofilm-related infections is caused by the fact that bacteria in biofilms are more resistant to antibiotics than their planktonic forms [6, 7].

In this study, we applied a method that has been known in science for more than 100 years—the method of photodynamic exposure, which is based on the use of photosensitive agents (photosensitizers) and optical irradiation. The first report in phototoxicology (the one on the action of photosensitizers on microorganisms) was delivered by the German researcher Oscar Raab in 1900, in relation to skin tumors, syphilis, and tuberculosis. However, with the discovery of antibiotics in the early 20th century, photodynamic research did not gain widespread application in clinical practice, except in oncology. Currently, the photodynamic effect is also used against a broad spectrum of bacteria, parasitic protozoa, fungi, and viruses [8]. A study by Tanaka et al. [9] demonstrated that the method can be employed for the inactivation of pathogenic microorganisms through the induction of oxidative stress and, via photodynamic therapy, indirect enhancement of the immune response against these bacteria. The photodynamic effect has been successfully introduced into the treatment of infectious diseases in otorhinolaryngology, dentistry, gastroenterology, and other fields of medicine [10–12].

In 2018, high efficacy of photodynamic inactivation of *Escherichia coli*, the most common causative agent of infections in urology, was demonstrated [13]. Encouraging experimental results on the application of photodynamic exposure to pathogenic microflora tropic to the urinary tract have been obtained in relation to planktonic microbial forms [14–16]. At the same time, the issue of developing new approaches and methods of microbial inactivation, including physical methods aimed at the destruction of uropathogenic biofilms for the prevention of infectious and inflammatory process in urology, has been insufficiently studied and remains relevant.

The study aimed to evaluate the feasibility of photodynamic inactivation of biofilms formed by typical representatives of uropathogenic microorganisms using an antiseptic agent with a bacteriostatic effect—methylene blue—possessing photochemical properties.

METHODS

The study was conducted using the cultures of uropathogenic bacteria such as *Staphylococcus aureus* and *Escherichia coli*, isolated from renal calculi of patients undergoing nephrectomy and nephrolitholapaxy, respectively. Antibiotic resistance of the isolates was determined using the disk diffusion method on Mueller–Hinton agar (according to EUCAST-2024). The biofilm-forming ability of the strains was assessed by adhesion to polystyrene plates following the O'Toole method [17]. Experiments on photodynamic inactivation of microorganisms were carried out *in vitro* on mature preformed biofilms prepared under the protocol described by Kropotov et al. [18]. For biofilm production, *S. aureus* and *E. colistrains* were cultivated on cover glasses placed in Petri dishes with nutrient agar overlaid with broth for three days, with daily replacement of the broth. The cover glasses were then removed, rinsed with 0.9% sodium chloride solution, immersed in 10 mL of sterile 0.1% methylene blue solution in a Petri dish, and irradiated after 10 minutes using the Latus-K diode laser (Aktus LLC, Russia), generating emission at a wavelength of 662 nm. The 0.1% methylene blue photoactive solution had an absorption

spectrum within the optical range of 618–668 nm, with an irradiation area of 78.5 cm^2 (beam diameter, 10 cm). Two continuous irradiation modes were applied: output power 0.45 W, dose 1.5 J/cm^2, power density 5.5 mW/cm^2, exposure time 4.5 min; output power 1.1 W, dose 4.75 J/cm^2, power density 14.4 mW/cm^2, exposure time 5.5 min. Irradiation was delivered using a light guide with a cylindrical diffusing tip 5 mm in length (Polironik LLC, Russia). Biofilms were irradiated in continuous mode across five setups (three controls, two experimental):

Control 1 (negative control): sterile 0.9% sodium chloride solution was used instead of the photosensitizer; laser exposure was simulated by contacting the biofilm with an inactive emitter;

Control 2: exposure to 0.1% methylene blue solution; laser exposure was simulated by contacting the biofilm with an inactive emitter;

Control 3: laser exposure with sterile 0.9% sodium chloride solution instead of the photosensitizer; laser parameters: output power, 450 mW; exposure time, 4.5 min; power density, 5.5 mW/cm^2; and irradiation dose, 1500 mJ/cm^2;

Experiment 1: laser irradiation through 0.1% methylene blue solution; laser parameters: output power, 450 mW; exposure time, 4.5 min; power density, 5.5 mW/cm^2; and dose, 1500 mJ/cm^2;

Experiment 2: laser irradiation through 0.1% methylene blue solution; laser parameters: output power, 1100 mW; exposure time, 5.5 min; power density, 14.4 mW/cm^2; and dose, 4750 mJ/cm^2.

Irradiation was performed in duplicate with a 1-hour interval.

After irradiation, biofilms on the cover glasses were fixed in 96% ethanol for 3 minutes, rinsed three times with water, and mounted on microscope slides using colorless varnish. The prepared specimens were stained with acridine orange solution, dried in the dark, examined under a Mikmed-6 microscope, version 11, with a fluorescence module (LOMO, Russia) at ×100 magnification in an immersion system, and photographed with a digital camera, at least 10 images were taken in multiple fields of view for each sample. Digital image processing was subsequently performed using 3D modeling techniques with ImageJ software version 1.52a.

RESULTS

The bacterial cultures used in this study exhibited multidrug resistance: the methicillin-resistant *S. aureus* strain was resistant to aminoglycosides and fluoroquinolones, whereas the *E. coli* strain produced extended-spectrum β-lactamases and was resistant to fluoroquinolones, nitrofurantoin, fosfomycin, and trimethoprim/sulfamethoxazole. Both cultures demonstrated biofilm-forming capacity.

In the photodynamic inactivation experiments, controls included exposure of the mature biofilm to either the photosensitizer alone or the laser alone. 3D-models of the studied biofilms showed that, compared with the negative control (control 1) (Fig. 1), exposure to either the photoactive agent alone or laser irradiation alone (controls 2 and 3) did not result in any significant alterations or disruption of biofilm structure (Fig. 2, Fig. 3).

Combined exposure to the photosensitizer and laser irradiation at 450 mW led to partial destruction of the biofilm, with the appearance of cavities and irregularly shaped open lacunae of approximately uniform size within its structure, indicating uniform exposure (Fig. 4).

Combined exposure to the photoactive agent methylene blue and laser irradiation at 1100 mW resulted in complete degradation of the mature multilayer biofilm into single cells devoid of extracellular matrix (Fig. 5).

Thus, a clear dose–effect relationship was observed in the action of laser irradiation. Quantitative image analysis using ImageJ software version 1.52a yielded the following results:

1) Exposure to the photoactive agent methylene blue and laser irradiation at 450 mW resulted in destruction of 41.9% of the original biofilm structure for *S. aureus* and 82.4% for *E. coli*;

2) Exposure to the photoactive agent methylene blue and laser irradiation at 1100 mW led to destruction of 97.7% of the original biofilm structure for *S. aureus* and 96.5% for *E. coli*.

DISCUSSION

To date, the use of antibacterial agents remains the primary method for the treatment and prevention of infectious and inflammatory complications aimed at eliminating pathogens in practical urology. However, there has been a rapid global increase in antibiotic resistance [19]. Recent studies have reported bacteriuria characterized by multidrug resistance (resistant to three or more classes of antibiotics recommended by both the Russian and American Urological Associations), which poses a substantial risk of infectious and inflammatory complications [20]. Importantly, the biofilm phenomenon must be considered as both an adaptive trait of microorganisms and a factor in their phenotypic response to antimicrobial agents. Thus, the search for new mechanisms of bacterial inactivation, targeting organisms released into the urinary tract during translocation from the human intestine, sexual activity, or endoscopic urological procedures, remains both timely and necessary. The most promising approach under current conditions is the development of new, and the optimization of existing, physical methods for treatment and for reducing urinary tract infections. Photodynamic therapy, with its pronounced

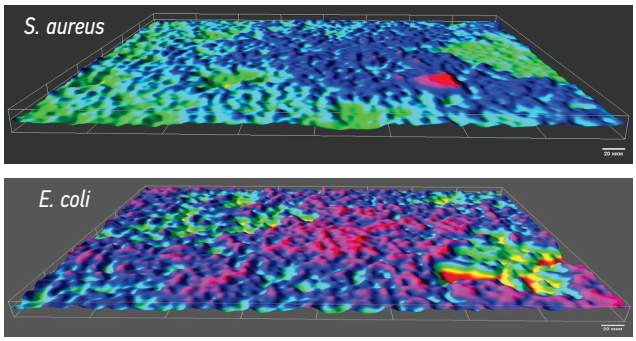

Fig. 1. 3D topographic model of a uropathogenic bacterial biofilm, control 1 (no photosensitizer, no irradiation).

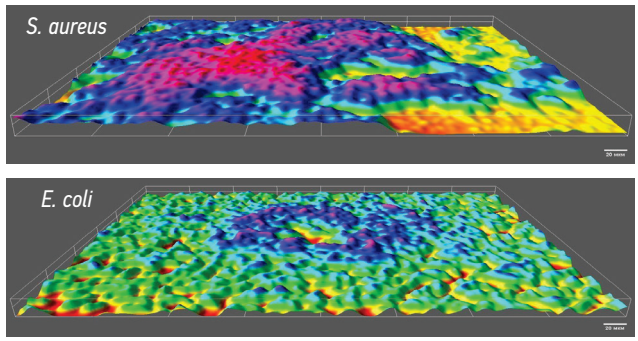

Fig. 2. 3D topographic model of a uropathogenic bacterial biofilm, control 2 (with photosensitizer).

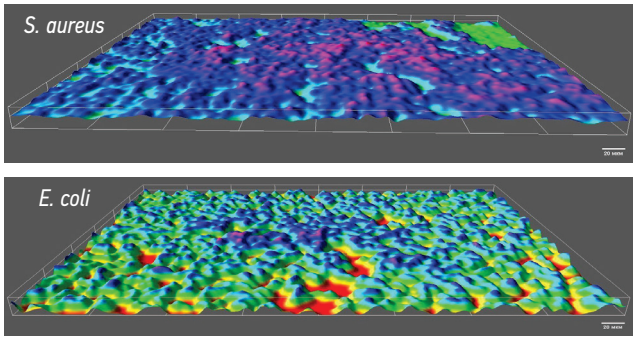

Fig. 3. 3D topographic model of a uropathogenic bacterial biofilm, control 3 (with laser irradiation).

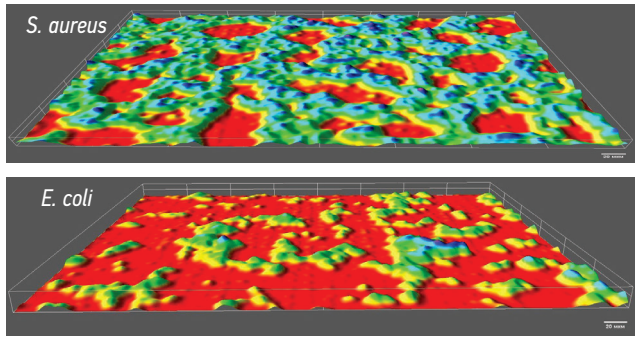

Fig. 4. 3D topographic model of a uropathogenic bacterial biofilm, experiment 1 (with photosensitizer and laser irradiation, 1500 mJ/cm^2).

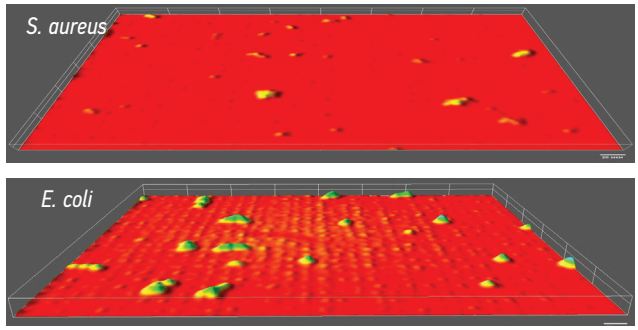

Fig. 5. 3D topographic model of a uropathogenic bacterial biofilm, experiment 2 (with photosensitizer and laser irradiation, 4750 mJ/cm^2).

bactericidal effect, may serve as an alternative or an adjunct to systemic antibiotic therapy in urological practice [21, 22].

In several studies, the photodynamic effect of methylene blue has been described [23, 24], notable for its strong cationic dye properties [25]. It is well established that the use of a positively charged ion as a photosensitizing agent facilitates uptake by Gram-negative microbial cells through intracellular sorption on functional structures, ultimately leading to lethal photosensitization [25]. Both Russian and international studies have demonstrated that cationic photosensitizers—specifically derivatives of meso-substituted porphyrins and phthalocyanines—exhibit photodynamic activity against Gram-positive and Gram-negative microorganisms as well as fungi. This work is the first to demonstrate high efficacy of photodynamic inactivation against biofilm forms of uropathogenic Gram-positive and Gram-negative microorganisms. It was also established that laser exposure and the photoactive agent applied separately exerted no significant destructive effect on microbial biofilms, whereas only their combined use resulted in nearly complete (~96.6%) degradation of mature multilayer biofilms of uropathogenic strains. Importantly, this combined effect was dose-dependent. Such large-scale biofilm destruction renders even the remaining viable bacterial cells a convenient “target” for antibiotic therapy [26].

Taking into account the global spread of resistant and multidrug-resistant pathogenic strains, the search for strategies to overcome microbial resistance continues. The phenomenon of biofilm formation in pathogenic microorganisms poses a significant threat to patients, as it hampers the effectiveness of chemotherapy and frequently contributes to increased infectious morbidity and chronicity. Thus, our findings of broad-spectrum bactericidal activity against biofilm forms of uropathogenic microorganisms may prove valuable in the empirical treatment of infections of the lower urinary tract.

CONCLUSION

This study is the first to demonstrate the feasibility of photodynamic inactivation of uropathogenic biofilm-forming microorganisms using a photochemically active agent—methylene blue. The encouraging results indicate that the combined use of laser irradiation and methylene blue may serve as an alternative or adjunct to systemic antibiotic therapy in urological practice.

ADDITIONAL INFO

Author contributions: D.V. Kryazhev: conceptualization, formal analysis, writing—original draft; O.S. Streltsova: supervision, methodology, writing— original draft; A.E. Antonyan: investigation; G.B. Ermolina: investigation; E.V. Belyaeva: investigation; V.N. Krupin: conceptualization, writing—review & editing. All the authors approved the version of the draft to be published and agreed to be accountable for all aspects of the work, ensuring that issues related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Data availability statement: All data generated during this study are available in this article.

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Provenance and peer-review: This paper was submitted unsolicited and reviewed following the standard procedure. The peer review process involved a single reviewer (an editorial board member, editorial council member, or an external reviewer); double-blind review was conducted.

ДОПОЛНИТЕЛЬНАЯ ИНФОРМАЦИЯ

Вклад авторов. Д.В. Кряжев — разработка концепции исследования, анализ полученных результатов, подготовка статьи; О.С. Стрельцова — общее руководство исследованием, разработка дизайна исследования, подготовка статьи; А.Э. Антонян — забор биологического материала; Г.Б. Ермолина — проведение модельных экспериментов; Е.В. Беляева — выделение чистых культур, определение фенотипических характеристик изолятов; В.Н. Крупин — идея исследования, редактирование текста статьи. Авторы одобрили версию для публикации, а также согласились нести ответственность за все аспекты работы, гарантируя надлежащее рассмотрение и решение вопросов, связанных с точностью и добросовестностью любой ее части.

Этическая экспертиза. Проведение исследования одобрено локальным этическим комитетом ФГБОУ ВО «Приволжский исследовательский медицинский университет» Минздрава России (протокол № 13 от 07.07.2021).

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Раскрытие интересов. Авторы заявляют об отсутствии отношений, деятельности и интересов за последние три года, связанных с третьими лицами (коммерческими и некоммерческими), интересы которых могут быть затронуты содержанием статьи.

Оригинальность. При создании настоящей работы авторы не использовали ранее опубликованные сведения (текст, иллюстрации, данные).

Доступ к данным. Все данные, полученные в настоящем исследовании, доступны в статье.

Генеративный искусственный интеллект. При создании настоящей статьи технологии генеративного искусственного интеллекта не использовали.

Рассмотрение и рецензирование. Настоящая работа подана в журнал в инициативном порядке и рассмотрена по обычной процедуре. В рецензировании участвовали один рецензент (член редакционной коллегии, член редакционного совета или внешний рецензент), рецензирование двойное слепое.

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