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Development and Study of Bacteriophage-containing Dosage Form for the Treatment of the Outer Ear Infections

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Abstract

Introduction. Infectious otitis externa and middle ear can cause hearing loss, which significantly reduces the quality of life of patients. The main causative agents of acute bacterial otitis media are *Pseudomonas aeruginosa* and *Staphylococcus aureus*. This article is devoted to the development and study of a novel dosage form for treatment of infectious diseases of the external ear containing bacteriophages that lyse bacterial strains of *Pseudomonas aeruginosa*. Ear drops were considered as a promising dosage form for instillation into the ear canal.

Aim. The aim of the work is to develop a dosage form of *Pseudomonas aeruginosa* bacteriophages for the local treatment of infectious otitis media.

Materials and Methods. The active substances of the developed drug are bacteriophages that lyse bacterial strains of Pseudomonas aeruginosa: PA5 and PA10, which were obtained by growing on a solid growth medium in mattress flasks with subsequent sterilizing filtration through a membrane filter (0,22 μm) and elimination of endotoxins on a chromatographic column. To obtain experimental compositions, excipients that do not cause a drop in the titer of bacteriophages were used – purified water as the solvent, viscosity modifiers: glycerol (CHIMMED, Russia) and a mix of macrogol 6 and glyceryl caprilocaprate brand Softigen 767 (Cremer, Germany), antioxidant Ethylenediaminetetraacetic acid (EDTA), preservatives nipagin and nipazole. The obtained samples were standardized according to pharmacopoeial indicators, recommended for the dosage form "drops" – density, pH, viscosity. For all experimental compositions, the stability of the titer of bacteriophages was studied by the Gratia method for 6 months. The local irritation and systemic effects were also studied on five chinchilla male rabbits.

Results and discussion. As a result of the conducted research, four experimental compositions of ear drops with a cocktail of bacteriophages PA5 and PA10 were obtained. The optimal technological characteristics were observed in the composition containing glycerol as a viscosity modifier at a concentration of 10,0 %. For optimal composition, the stability of the bacteriophages cocktail titer, local irritating and systemic effects were analyzed. The study revealed stability of the bacteriophages PA5 and PA10 titers in the composition of dosage form, and absence of local irritating and systemic effects of ear drops.

Conclusion. The dosage form can be recommended for preclinical studies.

Keywords: ear drops, bacteriophages, otitis externa, *Pseudomonas aeruginosa*, rabbits, local irritation, systemic effect

Conflict of interest. The authors declare that they have no obvious and potential conflicts of interest related to the publication of this article.

Contribution of the authors. Elena O.Bakhrushina, Maria N. Anurova and Alexey M. Vorobiev and Yulia O. Shcherbina came up with and developed an experiment. Svatlana S. Bochkareva and Yulia O. Shcherbina conducted studies of local irritant and systemic effects on animals. Alexey M. Vorobiev, Maria A. Pasivkina and Lika O. Krekhtunova carried out microbiological studies *in vitro*. Maria A. Pasivkina and Lika O. Krekhtunova participated in data processing. Elena O. Bakhrushina carried out theoretical calculations. Elena O. Bakhrushina, Maria N. Anurova and Alexey M. Vorobiev participated in writing the text of the article. Natalia B. Demina and Andrey V. Aleshkin participated in the review and approval of the article. All authors participated in the discussion of the results.

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Разработка и изучение ушных капель с бактериофагами для лечения инфекционных отитов, осложненных *P. aeruginosa*

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Резюме

Введение. Инфекционные отиты наружного и среднего уха способны вызывать потерю слуха, что существенно снижает качество жизни пациентов. Основными возбудителями острого бактериального отита являются *Pseudomonas aeruginosa* и *Staphylococcus aureus*. Статья посвящена разработке и изучению новой лекарственной формы для лечения инфекционных отитов, содержащей бактериофаги, лизирующие бактериальные штаммы *Pseudomonas aeruginosa*. Препараты бактериофагов могут быть альтернативой препаратам антибиотиков местного действия, использующихся в оториноларингологической практике по всему миру. В качестве перспективной лекарственной формы для инстилляции в ушной проход рассматривались ушные капли.

Цель. Целью работы является разработка лекарственной формы бактериофагов *Pseudomonas aeruginosa* для местного лечения инфекционных отитов.

Материалы и методы. Действующими веществами разрабатываемого препарата являются бактериофаги, лизирующие бактериальные штаммы *Pseudomonas aeruginosa*: PA5 и PA10, полученные методом выращивания на плотной питательной среде в матрасах с последующей стерилизующей фильтрацией через мембранный фильтр (0,22 мкм) и освобождением от эндотоксина на хроматографической колонке. Для получения экспериментальных составов использовались эксципиенты, не вызывающие падения титра бактериофагов – растворитель вода очищенная, модификаторы вязкости глицерин (ООО ТД «ХИММЕД», Россия) и смесь макрогола 6 и глицерил каприлокапрата марки Softigen® 767 (CREMER, Германия), антиоксидант этилендиаминтетрауксусная кислота (ЭДТА), консерванты нипагин и нипазол. Полученные образцы стандартизовали по фармакопейным показателям, рекомендуемым для лекарственной формы капли: плотность, pH, вязкость. Для всех экспериментальных составов изучали стабильность титра бактериофагов по методу Грациа в течение 12 месяцев, также исследовали местораздражающее и системное действие на пяти кроликах самцах породы шиншилла.

Результаты и обсуждение. В результате проведенных исследований получено четыре экспериментальных состава ушных капель с коктейлем бактериофагов PA5 и PA10. Оптимальным технологическими характеристиками обладал состав, содержащий глицерин в качестве модификатора вязкости в концентрации 10,0 %. Для этого образца установлены стабильность титра бактериофагов PA5 и PA10, отсутствие местораздражающего и системного действия.

Заключение. По итогам проведенных исследований лекарственная форма может быть рекомендована к проведению доклинических исследований, а после проведения дополнительных испытаний на других бактериофагах – для применения в персонализированной фаготерапии.

Ключевые слова: ушные капли, бактериофаги, инфекционный отит, *Pseudomonas aeruginosa*, персонализированная терапия

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Вклад авторов. Е. О. Бахрушина, М. Н. Анурова, А. М. Воробьев и Ю. О. Щербина придумали и разработали эксперимент. С. С. Бочкарева и Ю. О. Щербина провели исследования местораздражающего и системного действия на животных. А. М. Воробьев, М. А. Пасивкина и Л. О. Крехтунова проводили микробиологические исследования *in vitro*. М. А. Пасивкина и Л. О. Крехтунова участвовали в обработке данных. Е. О. Бахрушина проводила теоретические расчеты. Е. О. Бахрушина, М. Н. Анурова и А. М. Воробьев участвовали в написании текста статьи. Н. Б. Демина и А. В. Алешкин участвовали в рецензировании и одобрении статьи. Все авторы участвовали в обсуждении результатов.

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INTRODUCTION

Infectious otitis externa and media are a socially significant disease – affecting about 31 million people worldwide every year, it can cause hearing loss, which significantly reduces the quality of life of patients [1–3].

The main causative agents of acute bacterial otitis are *Pseudomonas aeruginosa* and *Staphylococcus aureus*. These bacterial agents form biofilms with other otopathogens and induce heightened congenital inflammatory responses that can contribute to the chronic development of otitis media and the progression of purulent inflammation [2]. In some cases, *Pseudomonas aeruginosa* also causes malignant otitis externa, a serious disease in which the mortality rate is up to 10 %. In high-income countries, clinical guidelines for the treatment of otitis externa and media include antibiotic therapy and the use of pneumococcal conjugate vaccine [2].

However, infections caused by *Pseudomonas aeruginosa* and *Staphylococcus aureus* often respond to antibiotic therapy poorly due to multiple resistance,

it is resistant to the action of many aminoglycosides, cephalosporins and fluoroquinolones, which complicates the effectiveness of therapeutic measures in patients [3,4].

The use of topical drugs makes it possible to achieve a higher concentration of antibacterial drug in the focus of infection, and due to the absence of long-term exposure to the bacterial agent of the drug in a subtherapeutic dose, the possibility of developing resistance is minimized [3]. Local treatment of infectious otitis is recommended in cases of otitis externa and media with sufficient perforation of the tympanic membrane [2, 3].

Ear drops with bacteriophages can be an alternative to topical antibiotics used in ENT practice all over the world. Ear drops are convenient delivery system that is suitable for patients of different ages and providing maximum concentration in the inflammation focus in a short time [5,6]. Phage therapy is successfully used in many countries of the world as a tool for overcoming antibiotic resistance and can be used as part of a personalized approach to patient treatment.

The purpose of this study is development of the bacteriophages *Pseudomonas aeruginosa* delivery system for the topical treatment of infectious diseases.

MATERIALS AND METHODS

The active substances of the developed drug are bacteriophages that lyse bacterial strains of *Pseudomonas aeruginosa*: PA5 and PA10, which were obtained by growing on a solid growth medium in mattress flasks with subsequent sterilizing filtration through a membrane filter (0,22 μm) and elimination of endotoxins on a chromatographic column [7].

Based on scientific literature research, the most common and well-studied excipients were selected for dosage form development [7–9]. Analysis of drugs in the dosage form of ear drops demonstrated that most often their composition includes the following excipients: aqueous-alcoholic solutions of various concentrations and purified water as solvents, glycerol in a concentration of 10 to 40 % and macrogols as viscosity modifiers, antioxidants – ethylenediaminetetraacetic acid and its salts, sodium thiosulfate, preservatives – chlorobutanol hydrate, benzalkonium chloride, thiomersal, ethanol, as well as pH regulators.

The possibility of using mixtures of purified water and glycerol (CHIMMED, Russia) in the ratios of 1/2.5 and 1/1.25, macrogol-400 (CHIMMED, Russia) in the ratio of 1/2.5 and the mixture of macrogol-6 and glyceryl caprylocaprate of the Softigen 767 brand (Cremer, Germany) in a ratio of 1/2.5 as solvents and formative substances was considered. Ethylenediaminetetraacetic acid (EDTA) was used as an antioxidant, parabens as the only group of stabilizers of antimicrobial resistance, allowed to use in ear dosage forms, providing activity in the pH range from 4 to 8 and not providing a lytic effect on bacteriophages, were used as preservatives [7, 8]. Also, depending on the production possibilities, the finished dosage form can be poured into bottles with a nozzle that provides filtration during the evacuation of each subsequent dose, which allows to make the drug sterile and excludes preservatives from the pharmaceutical composition.

The compatibility of chosen excipients with a cocktail of bacteriophages was studied during the first phase of this research. The sterile water solutions of excipients in working concentrations were prepared and a bacteriophages cocktail was introduced, the absence of bacteriophages titer declining was controlled by the Gratia method right after the manufacturing, one day and 14 days after manufacturing of dosage form.

Then experimental samples of ear drops were obtained, bacteriophages were introduced after thermal sterilization of dosage form base during 15 minutes at a temperature of 121 °C. The lytic activity of bacteriophages in one drop (0.05 ml) was 2×10^8 PFU for PA5 and 2.2×10^8 PFU for PA10.

The following criterions were studied for all experimental compositions: the stability of bacteriophages titer that was determined by the Gratia method during 12 months and the technological characteristics of the dosage form – the pH value by the potentiometric method with the Aquilon pH-meter pH-410 at a temperature of 20–25 °C, density and viscosity with an Ostwald capillary viscometer.

The assessment of local irritating and systemic effects were evaluated on five male chinchilla breed rabbits weighing 3,5–4 kg. The care and maintenance of the animals implemented in accordance to the recommendations and requirements of Directive 2010/63/EU of the European Parliament and of the EU Council of 22 September 2010 on the protection of animals used for scientific purposes. All animal experiments were approved by the ethics committee of FBIS MRIEM of G. N. Gabrichevsky.

For the local irritating effect evaluation, 3 drops of experimental composition were instilled once into one ear, while the second ear was left for control. The skin condition was recorded visually daily for five days. The functional morphological disorders of the skin (ery-

thema, edema, cracks, necrosis, desquamation, dryness, ulcerations) were noted.

The design of the experiment assumes only local action for developed delivery system in the form of ear drops. To prove the absence of systemic effect, blood was taken from the rabbit's ear vein every 15 minutes, beginning from the moment the drops were administered. The titer of bacteriophages in the blood was determined by the Gratia method. Titration was conducted till the 5th dilution. Inoculation was conducted at "0", 1st, 2d, 3d, 4th, and 5th dilutions [7].

For the results processing, the statistical analysis package for Microsoft Office Excel 2007 was used.

RESULTS AND DISCUSSION

The research of bacteriophages titer stability in the presence of the studied excipients, showed reliable absence of a titer declining, therefore experimental samples of ear drops were obtained based on all selected excipients, the compositions are shown in Table 1. All samples are transparent, colorless or light brown colored liquids that meet the requirements of state normative documentation.

For bacteriophages PA 5 and PA 10, the pH ensuring titer stability is 6–8 [5]; only composition 2 with a pH equal 6.63 ± 0.02 satisfy this requirement. Indicators of the viscosity and density for compositions 1,3 and 4 are similar, composition 2 has the maximum density and viscosity values. From a consumer point of view, ear drops should have enough viscosity which ensure no leakage from the auricle cavity; therefore, composition 2, which has the highest viscosity value, is considered the most preferable for the further study.

For this composition, the stability of the bacteriophages titer was studied. The research found that the selected composition ensures the stability of the titer of bacteriophages during the 12 months of storage (Table 2).

Table 2. Stability study of the titer of bacteriophages in ear drops (PFU / 0,05 ml) when stored for 12 months

Strains \ Exposition	24 hours	3 months	6 months	12 months
PA5	2.0×10^8	1.9×10^8	1.5×10^8	3.0×10^7
PA10	2.2×10^8	2.2×10^8	1.0×10^8	1.0×10^7

The next phase of the study was the determination of local irritation and systemic effects.

The erythema and edema rate in the inner surface tissues of rabbit's ear after a after a single administration of three drops of the test sample equals 0, which corresponds to the absence of local irritating effects. To assess the systemic effect, blood was taken from the ear vein of the test animals with an interval of 15 minutes for 1 hour. The titer of bacteriophages in the blood was determined by the Gratia method. For strains PA 5 and PA 10 the absence of negative colonies was shown.

Thus, the absence of locally irritating and systemic effects of the drug was confirmed.

Table 1. The compositions of the experimental samples of ear drops and pharmacopeial quality indicators

№	Composition of excipients, g	Density, g/ml	pH	Viscosity, mPa · s
1	Glycerin 10,0 Nipagin/nipazol (1 : 3) 0,015 EDTA 0,025 Purified water up to 50,0	1.0339 ± 0.05	5.38 ± 0.03	1.0339 ± 0.05
2	Glycerin 20,0 Nipagin/nipazol (1 : 3) 0,015 EDTA 0,025 Purified water up to 50,0	1.1622 ± 0.06	6.63 ± 0.02	3.9950 ± 0.2
3	Вода очищенная до 50,0 Macrogol-400 10,0 Nipagin/nipazol (1 : 3) 0,015 EDTA 0,025 Purified water up to 50,0	1.0338 ± 0.05	3.67 ± 0.02	1.2923 ± 0.06
4	Softigen® 767 10,0 Nipagin/nipazol (1 : 3) 0,015 EDTA 0,025 Purified water up to 50,0	1.0195 ± 0.05	5.74 ± 0.03	1.5675 ± 0.08

CONCLUSION

During the research, a dosage form with a cocktail of bacteriophages PA5 and PA10 was developed for use in the treatment of infectious diseases of the middle ear caused by *Pseudomonas aeruginosa*, which has a proven topical effect and the absence of a local irritating effect. Further development of the topic is study of stability issues in the developed delivery system for other bacteriophages to use it as a standard basis and in personalized phage therapy for patients with infectious otitis media.

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