Научно-практический журнал
Scientific and Practical Journal

ISSN 2307-9266
e-ISSN 2413-2241

ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

PHARMACY & PHARMACOLOGY

2022 Том / Volume X

№ 5

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АРТЛЕГИА

Олокизумаб

Одобренное показание — патогенетическая терапия синдрома высвобождения цитокинов при новой коронавирусной инфекции (COVID-19) среднетяжелого и тяжелого течения [1]

Артлегиа включена в рекомендованные схемы терапии госпитализированных пациентов с COVID-19 [2]:

легкого течения
(пациенты с высоким индексом коморбидности)

среднетяжелого течения

тяжелого и крайне тяжелого течения

[1] Инструкция по медицинскому применению лекарственного препарата Артлегиа, регистрационное удостоверение ЛП-006218 от 21.05.2020.
[2] Временные методические рекомендации МЗ РФ «Профилактика, диагностика и лечение новой коронавирусной инфекции (COVID-19)». Версия 16 от 18.08.2022

Краткая инструкция по медицинскому применению лекарственного препарата АРТЛЕГИА

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Гиперчувствительность к олокизумабу, любому компоненту препарата в анамнезе. Активные инфекционные заболевания (в том числе и туберкулез). Детский возраст до 18 лет. Наследственная непереносимость фруктозы (препарат содержит сорбитол). Период грудного вскармливания.
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Scientific and Practical Journal

PHARMACY & PHARMACOLOGY

Scientific and practical journal

Volume X, Issue 5, 2022

The mass media registration certificate:
ПИ №ФС77–67428 от 13.10.2016

ISSN 2307-9266 e-ISSN 2413-2241

Manuscripts presented in sections **Reviews, Lectures / Pharmaceutical Education / Brief Reports / Discussions, Referee Reports, Anniversaries, School of Thought, History of Pharmacy and Pharmacology** can be considered by any members of the editorial board.

Founder: Volgograd State Medical University. 1, Pavshikh Bortsov Sq., Volgograd, Russia, 400131
Editors office address: 11, Kalinin ave., Pyatigorsk, Russia, 357532
Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University
Phone number: +7(8793) 32-44-74. E-mail: pharmjournal@mail.ru
www.pharmpharm.ru
Union catalogue. Russian Press / Newspapers an journals. Code 94183
A4 size, 1000 issues circulation. Price free

Journal "Pharmacy & Pharmacology" is recommended International Comittee Of Medical Journal Editors and included in Higher Attestation Commission, Scopus, Web of Science (ESCI), Russian citation database, eLibrary, ARISTI (All-Russian Institute of Scientific and Technical Information), RSL (Russian State Library), CyberLeninka, Socionet, EMBASE, Chemical Abstracts (CAS), Directory of Open Access Journals (DOAJ), EBSCO Discovery Service, RNMJ, University of CAMBRIDGE, Ulrich'sWeb, Google Scholar, Biefeld Academic Search Engine (BASE), Directory of Open Access Scholarly Resources (ROAD), Research Bible, Open Archives Initiative, Academic Keys, JournalTOCs, WorldCat, OpenAIRE, University of Oxford, The British Library, Universitait Gent, Université de Montréal, University of Saskatchewan.

Printed in the LLC "Amirit" in accord with provided materials, 410004, Saratov, 88, Chernishevsky Str.

Научно-практический журнал

ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

Периодичность 6 номеров в год

Том 10, Выпуск 5, 2022

Свидетельство регистрации СМИ:
ПИ №ФС77–67428 от 13.10.2016 г.

ISSN 2307-9266 e-ISSN 2413-2241

Учредитель: Федеральное государственное бюджетное образовательное учреждение высшего образования «Волгоградский государственный медицинский университет» Минздрава России.
400131, Россия, г. Волгоград, площадь Павших Борцов, д. 1
Адрес издательства: 357532, г. Пятигорск, пр-кт Калинина, 11.
Пятигорский медико-фармацевтический институт – филиал ФГБОУ ВО ВолгГМУ Минздрава России
Телефон: +7 (8793) 32-44-74. E-mail: pharmjournal@mail.ru
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Объединенный каталог. Пресса России . Газеты и журналы. Индекс 94183
Формат А4, тираж 1000 экз. Цена свободная.

Журнал «Фармация и фармакология» включен в перечень рецензируемых научных изданий, входящих в международные реферативные базы данных и системы цитирования, и в соответствии с пунктом 5 правил формирования перечня рецензируемых научных изданий, в которых должны быть опубликованы основные научные результаты диссертаций на соискание ученой степени кандидата наук, на соискание ученой степени доктора наук (Перечень ВАК), Scopus, Web of Science (ESCI), РИНЦ, eLibrary, ВИНИТИ, РГБ, Киберленинка, Соционет, EMBASE, Chemical Abstracts (CAS), Directory of Open Access Journals (DOAJ), EBSCO Discovery Service, RNMJ, University of CAMBRIDGE, Ulrich'sWeb, Google Scholar, Biefeld Academic Search Engine (BASE), Directory of Open Access Scholarly Resources (ROAD), Research Bible, Open Archives Initiative, Academic Keys, JournalTOCs, WorldCat, OpenAIRE, University of Oxford, The British Library, Universitait Gent, Université de Montréal, University of Saskatchewan.

Отпечатано в соответствии с предоставленными материалами в ООО «Амирит», 410004, г. Саратов, ул. Чернышевского, 88.

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

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REVIEWS

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

MOLECULAR MECHANISMS DEFINING APPLICATION OF GLYCINE AND ZINC COMBINATION IN CORRECTION OF STRESS AND ANXIETY MAIN MANIFESTATIONS

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Received 10 Sep 2022 After peer review 25 Oct 2022 Accepted 30 Oct 2022

The aim of the work was to carry out a systematic analysis of the molecular mechanisms that determine the possibility of a combined use of amino acid glycine and zinc compounds for the treatment of patients with manifestations of stress and anxiety.

Materials and methods. Information retrieval (Scopus, PubMed) and library (eLibrary) databases were used as research tools. In some cases, the ResearchGate application was applied for a semantic search. The analysis and generalization of references was carried out on the research topic, covering the period from 2000 to the present time.

Results. It has been shown that amino acid glycine, along with gamma-aminobutyric acid (GABA), is a key neurotransmitter that regulates physiological inhibition processes in the central nervous system (CNS) by increasing transmembrane conductance in specific pentameric ligand-gated ion channels. The introduction of zinc ions can potentiate the opening of these receptors by increasing their affinity for glycine, resulting in an inhibitory processes increase in CNS neurons. The replenishment of the glycine and zinc combined deficiency is an important element in the correction of a post-stress dysfunction of the central nervous system. A balanced intake of zinc and glycine is essential for most people who experience daily effects of multiple stresses and anxiety. This combination is especially useful for the people experiencing a state of chronic psycho-emotional stress and maladaptation, including those who have a difficulty in falling asleep.

Conclusion. A balanced maintenance of the zinc and glycine concentration in the body of a healthy person leads to the development of a stable anti-anxiety effect, which is accompanied by the normalization of the sleep-wake rhythm, which makes it possible to have a good rest without any loss of working efficiency after waking up.

Keywords: glycine; zinc; anxiolytic agents; brake action; anxiety states

Abbreviations: GABA – gamma-aminobutyric acid; pLGICs – pentameric ligand-gated ion channels; CNS – central nervous system; GlyR – glycine receptor; MT – metallothioneins; ROS – reactive oxygen species; RNS – reactive nitrogen species; SHMT – serine hydroxymethyltransferase; GCS – glycine cleavage system; VIAAT – vesicular inhibitory amino acid transporter; BBB – blood-brain barrier.

For citation: V.N. Shishkova, Y.R. Nartsissov, V.Y. Titova, E.V. Sheshegova. Molecular mechanisms defining application of glycine and zinc combination in correction of stress and anxiety main manifestations. *Pharmacy & Pharmacology.* 2022;10(5):404-415. **DOI:** 10.19163/2307-9266-2022-10-5-404-415

Для цитирования: В.Н. Шишкова, Я.Р. Нарциссов, В.Ю. Титова, Е.В. Шешегова. Молекулярные механизмы, определяющие применение комбинации глицина и цинка в коррекции основных проявлений стресса и тревоги. *Фармация и фармакология.* 2022;10(5):404-415. **DOI:** 10.19163/2307-9266-2022-10-5-404-415

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

ОБЗОРЫ

DOI: 10.19163/2307-9266-2022-10-5-404-415

МОЛЕКУЛЯРНЫЕ МЕХАНИЗМЫ, ОПРЕДЕЛЯЮЩИЕ ПРИМЕНЕНИЕ КОМБИНАЦИИ ГЛИЦИНА И ЦИНКА В КОРРЕКЦИИ ОСНОВНЫХ ПРОЯВЛЕНИЙ СТРЕССА И ТРЕВОГИ

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Получена 10.09.2022 После рецензирования 25.10.2022 Принята к печати 30.10.2022

Цель. Работа посвящена системному анализу молекулярных механизмов, определяющих возможность комбинированного использования аминокислоты глицин и соединений цинка для лечения пациентов с проявлениями стресса и тревоги.

Материалы и методы. В качестве инструментов проведения исследования использовались информационно-поисковые (Scopus, PubMed) и библиотечные (eLibrary) базы данных. В ряде случаев для семантического поиска использовалось приложение ResearchGate. В работе осуществлялся анализ и обобщение научной литературы по теме исследования, охватывающей период с 2000 по настоящее время.

Результаты. Показано, что аминокислота глицин, наравне с гамма-аминомасляной кислотой (ГАМК) является ключевым нейромедиатором, регулирующим процессы физиологического торможения в центральной нервной системы (ЦНС) путем увеличения трансмембранной проводимости в специфических гетеропентамерных лигандзависимых хлорных каналах. Введение ионов цинка способно потенцировать открытие данных рецепторов путем увеличения их сродства к глицину, в результате чего происходит усиление процессов торможения в нейронах ЦНС. Восполнение сочетанного дефицита глицина и цинка является важным элементом коррекции постстрессорной дисфункции ЦНС. Сбалансированное потребление цинка и глицина имеет важное значение для большинства людей, ежедневно испытывающих последствия многочисленных стрессов и находящихся в тревожном состоянии. Особенно полезна данная комбинация для лиц, испытывающих состояние хронического психоэмоционального напряжения и дезадаптации, в том числе имеющих сложности с засыпанием.

Заключение. Сбалансированное поддержание концентрации цинка и глицина в организме здорового человека приводит к развитию стойкого противотревожного эффекта, который сопровождается нормализацией ритма сон-бодрствование, что дает возможность полноценного отдыха без потерь работоспособности после пробуждения.

Ключевые слова: глицин; цинк; анксиолитические средства; торможение; тревожные состояния

Список сокращений: ГАМК – гамма-аминомасляная кислота; pLGICs – гетеропентомерные лиганд-зависимые хлорные каналы; ЦНС – центральная нервная система; GlyR – глициновый рецептор; МТ – металлотионеины; ROS – реактивные формы кислорода; RNS – реактивные формы азота; SHMT – серин гидроксиметилтрансфераза; GCS – митохондриальная система расщепления глицина; VIAAT (vesicular inhibitory amino acid transporter) – везикулярный переносчик тормозных аминокислот; ГЭБ – гематоэнцефалический барьер.

INTRODUCTION

A negative impact of stress and anxiety is experienced by an increasing number of people in the modern world, regardless of age and gender [1]. It is known that stress is also called a state of acute or chronic psycho-emotional tension. It should be also notified that anxiety disorders are significant psychosocial risk factors for the development of many chronic noncommunicable diseases [2]. Taking into account the growing need for timely therapy and prevention of disorders associated with the development of stress and anxiety, the search and development of safe and effective means for their correction are becoming increasingly important.

Treatment regimens for anxiety states of various

ISSN 2307-9266 e-ISSN 2413-2241

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origins are based on the use of a number of anxiolytic psychotropic drugs [3]. The molecular mechanism of their anti-anxiety action is based on a long-term increase in the activity of subclass A gamma-aminobutyric acid (GABA) receptors [4]. This class of membrane receptors responsible for the inhibition in neurons belongs to the family of pentameric ligand-gated ion channels (pLGICs) [5, 6]. The interaction of the agonist with the receptor, leading to the opening of a selective anion channel on the surface of the excitable membrane, leads to an increase in the Cl^- flux, which causes hyperpolarization of the neuron [7]. This activation of the transmembrane anionic current through the GABA receptors makes it possible to consider GABA as the main inhibitory neurotransmitter in the central nervous system (CNS) in the classical approach to neurophysiological processes [8]. Along with this, the second most physiologically important mediator that causes inhibition in the neurons of the spinal cord and brainstem is amino acid glycine [9]. This neurotransmitter, along with GABA, is present both in specific glycinergic and mixed synapses and is widely distributed in different parts of the brain. It also activates the transmembrane conductivity of chloride ions in the glycine receptor (GlyR), which belongs to the already mentioned pLGICs family [10, 11] (Fig. 1).

It is noteworthy that the structures of transmembrane proteins are isolated together with zinc ions, which are present in the analyzed recombinant proteins [12, 13]. Zinc belongs to the group of the most significant trace elements in the body along with iron, magnesium, and iodine. A decrease in the content of this divalent cation leads to significant problems for patients in both developing and developed countries [14, 15].

Zinc is the second most common micronutrient in the body after iron. On average, the body of an adult contains 2–3 grams of zinc [16]. In the body, it is distributed according to the skeletal type – 63% in the skeletal muscles, 22% in the skeletal system. The maximum concentration of zinc is also observed in the muscles and bones, as well as in the prostate gland in men. The concentration of zinc in the brain is estimated at 150 µmol/l, which, in turn, is 10 times higher than the content of zinc in blood serum [17]. Zinc is involved in all types of metabolism: it is assumed that it binds to about 3000 enzymes *in vivo*, which corresponds to about 10% of the human proteome [18]; regulates the cell stability and permeability and participates in membrane transport [19]; it has a pronounced immunomodulatory effect on hematopoiesis, osteogenesis, respiration processes and programmed cell death (apoptosis) [16, 20]. The role of Zn^{2+} as a neurotransmitter and modulator of the neurons state has been experimentally proven, since this ion is able to accumulate in presynaptic vesicles with a subsequent release into the synaptic cleft [21]. In addition, the level of zinc affects the susceptibility to learning and memory [22]. These results show that zinc ions, along with well-known neurotransmitters, can directly affect the state of neurons and participate in the regulation processes of CNS excitation and inhibition.

THE AIM of the work was to carry out a possible combined application of glycine and zinc compounds to change the metabolism and correct the conditions of patients with anxiety disorders and manifestations of stress.

MATERIALS AND METHODS

Information retrieval (Scopus, PubMed) and library (eLibrary) databases were used as research tools. In some cases, the ResearchGate application was used for a semantic search.

The analysis and synthesis of the scientific literature on the research topic, was carried out covering the period from 2000 to September 2022.

The following keywords and word combinations were used in the search: anxiety, anxiolytic properties, neuron metabolism, a synaptic cleft, inhibitory mediators, glycine metabolism, glycine receptor, GABA, GABA receptors, glycine transporters, chloride ion properties, chloride connectivity, zinc metabolism, tissue zinc levels, zinc levels, zinc transport, zinc effects, allosteric regulation, reactive oxygen species, antioxidant effects, metabolic levels of glycine, metabolic level of zinc, blood-brain barrier, vasodilatation, cerebral blood flow, anti-anxiety effects of glycine, glycine effects on stress, clinical trials of glycine.

Visualization of membrane receptors was carried out using the data from the Protein Data Bank (PDB) (https://www.rcsb.org/). To make up chemical formulas and illustrations, the libraries of the ACD/ChemSketch 2020.2.0 software package were used.

RESULTS AND DISCUSSION

A feature of the molecular mechanisms underlying the therapeutic effect of glycine and zinc ions on patients suffering from anxiety disorders is the combined effect of these metabolites on various biochemical and signaling systems. In fact, it is necessary to discuss a complex effect touching upon several systems at once.

In the context of accumulation and transformation, in neurons and other types of human cells, there are fundamental differences between glycine and zinc due to their chemical nature. Glycine is a non-essential amino acid that is actively involved in many metabolic processes, while Zn^{2+} is a part of the trace elements, the level of which is always regulated by an influx from an external source.

Processes of zinc transport and storage in human cells and tissues

In enterocytes of the small intestine, zinc buffer proteins determine the process of this ion transfer into the bloodstream. Further, Zn^{2+} is redistributed between albumin (the main zinc carrier, binds up to 80% in blood), α-microglobulin and transferrin [22, 24]. The protein content of food, as well as the condition of the mucosal layer of the small intestine, determine the absorption of zinc. Only 10% of zinc is excreted from the body with sweat and urine, the rest – with fecal masses [25].

DOI: 10.19163/2307-9266-2022-10-5-404-415

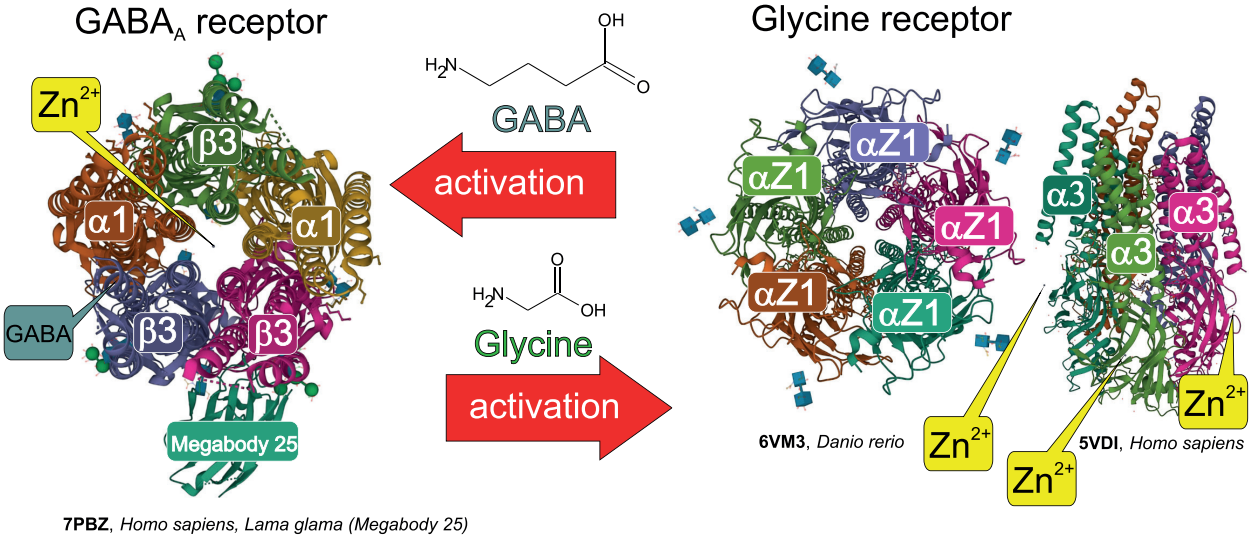

Figure 1 – Pentamers activation of pLGICs family by CNS inhibitory neurotransmitters

Note: Structural images of membrane proteins are presented based on the Protein Data Bank (PDB) (https://www.rcsb.org) in parallel planes (GABA$_A$ receptor, 7PBZ, [12]; glycine receptor, 6VM3, [23]) and perpendicular (glycine receptor, 5VDI, [13]) plane of the membrane.

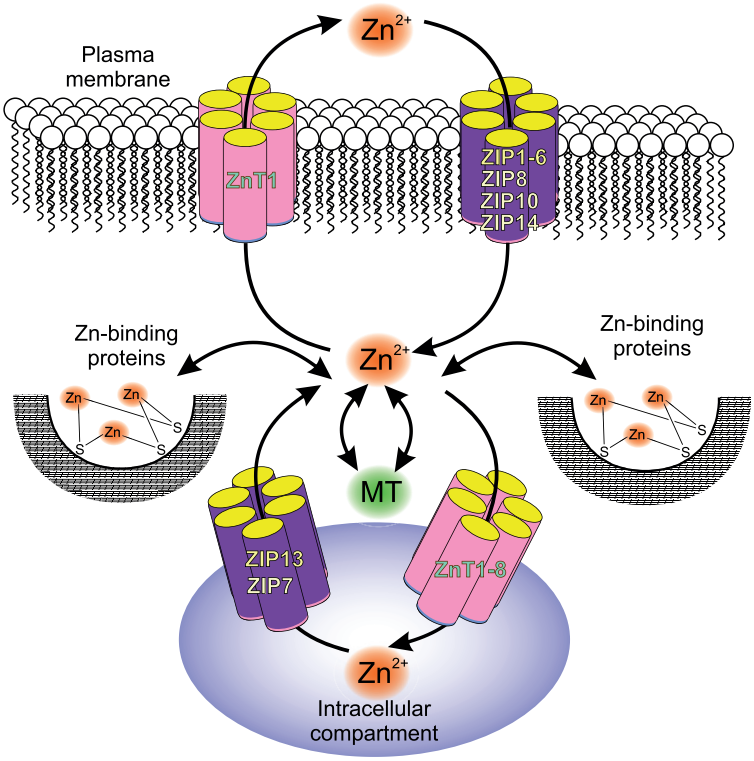

Figure 2 – Main ways of zinc ions transport and deposition in human cells

Note: Intracellular compartments are: endoplasmic reticulum (ZnT1, ZIP7), Golgi apparatus (ZnT5-7, ZIP13), endosomes (ZnT4), lysosomes (ZnT2), insulin granules (ZnT5, ZnT8) and synaptic vesicles (ZnT3).

ISSN 2307-9266 e-ISSN 2413-2241

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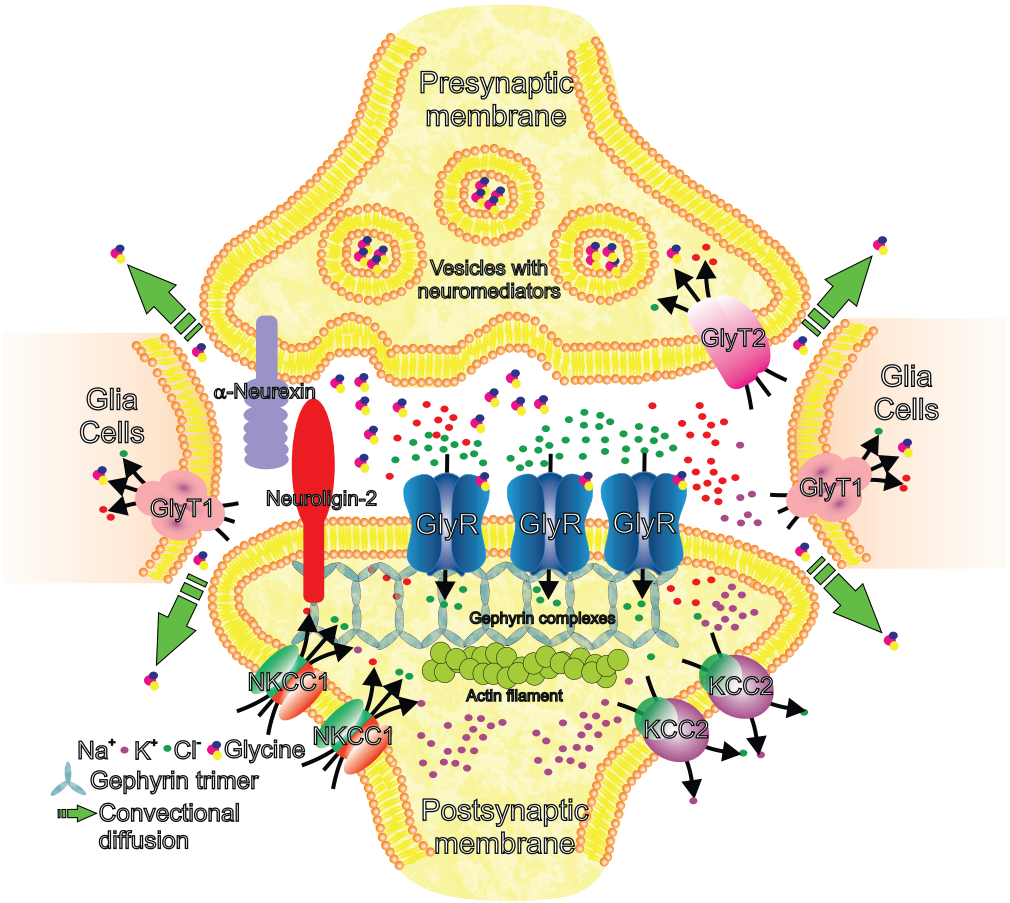

Figure 3 – Schematic representation of glycinergic synapse

Note: A neurotransmitter release from synaptic vesicles is accompanied by a subsequent diffusion into the synaptic cleft and the activation of structured GlyR and gephyrin clusters on the postsynaptic membrane. The increasing concentration of chloride ions in the postsynaptic terminal is regulated by transport through the KCC2 transporter. Structural plasticity of the synapse is mediated by the interaction of α-neurexin (presynaptic membrane) and the neuroligin-2 complex with the structural network of gephyrin trimers in the postsynaptic terminal [43].

At the cellular level, 30–40% of zinc is localized in the nucleus, 50% in the cytoplasm and organelles, and the rest – in the cell membrane. Cellular zinc homeostasis is mediated by three main mechanisms [26]. First, this is transport across the plasma membrane by importer proteins from the ZIP and ZnT families (Fig. 2). Second, this is due to the sequestration mediated by the transporter into intracellular organelles, including endoplasmic reticulum, a Golgi complex and lysosomes. To maintain the cell viability, a strict control of zinc homeostasis is necessary, since dysregulation leads to the cell death. The third mechanism for maintaining homeostasis is the metallothionein/thionein system [18]. Metallothioneins (MTs) form complexes with about 20% of intracellular zinc. MTs are ubiquitous proteins characterized by a low molecular weight, a high cysteine content and the ability to form complexes with metal ions.

One MT molecule can bind up to seven zinc ions. Due to the different affinity of metal ion binding sites, Zn can act as a powerful cellular zinc buffer. Free and weakly bound zinc ions interact with the apoprotein thionein (T_{red}) to form MTs [27]. An increase in the level of free zinc ions triggers the transcription factor-1 (MTF), thus inducing the expression of thionein [18]. In addition, oxidation of thiols by reactive oxygen species (ROS) or nitrogen (RNS) triggers the formation of oxidized protein thionine (Tox) with a concomitant zinc release [28].

Since there is no zinc storage system in the body, its level in cells must be constantly maintained. Both vegetable (mushrooms, nuts, cereals, legumes) and animal (meat, liver, seafood, cheese) products can be used as sources to maintain the normal level of this ion [25, 29].

In accordance with the norms of physiological needs for energy and nutrients for various population groups in the Russian Federation (Methodological recommendations MP 2.3.1.0253-21), the recommended daily allowance is from 3 to 12 mg of zinc for children and 12 mg for adults. In the US, the daily allowance of zinc for men is 11 mg, for women – 8 mg. In Germany, it is 10 mg for men and 7 mg for women [26].

Glycine metabolism in human cells

As mentioned above, unlike zinc, amino acid glycine, being both a substrate and a product of enzymatic reactions, is actively involved in the metabolic processes

DOI: 10.19163/2307-9266-2022-10-5-404-415

of human cells. In most cases, glycine is synthesized by serine hydroxymethyltransferase (SHMT), which uses serine supplied with food or obtained as a product of anabolic reactions from glucose and glutamate, as a substrate [30]. SHMT is a pyridoxal phosphate and a tetrahydrofolate dependent protein that is present in both the cytoplasm (SHMT1) and mitochondria (SHMT2), with the mitochondrial enzyme being more active [31]. Alternative metabolic pathways are the synthesis of glycine from threonine (with the participation of threonine aldolase and threonine dehydrogenase), choline (initiated by choline oxidase), and glyoxylate (catalyzed by alanine glyoxylate aminotransferase) [32, 33]. In general, the balance and dominance of these anabolic pathways is highly dependent on conditions, diet, and a body state. As catabolic reactions, one can consider the reversibility of the SHMT reaction, as well as the mitochondrial glycine cleavage system (GCS), which is a combination of four proteins (glycine decarboxylase (P-protein), aminomethyltransferase (T-protein), dihydrolipoamide dehydrogenase (L-protein) and a protein containing lipoic acid (H-protein) [34]. It should be notified that despite the complexity of the process, GCS is considered as a reversible system and its activity is unevenly distributed in human tissues: the glycine cleavage system is more represented in the liver and kidneys and, to a lesser extent, in the brain, testicles, and the small intestine [30].

Role of glycine as neurotransmitter in neurons

Synthesized glycine is pumped into vesicles *via* the vesicular inhibitory amino acid transporter (VIAAT), which is associated with the transport of chloride ions into synaptic particles [35]. Such an activity is typical for both glycinergic and GABAergic neurons, as well as for terminal endings of a mixed type [36]. Exocytosis of synaptic vesicles leads to the diffusion of glycine into the postsynaptic membrane with a subsequent activation of GlyR, which, in turn, leads to the depletion of the chloride ion gradient [37, 38]. For most mature CNS neurons, the intracellular concentration of chloride ions is maintained at a low level (about 5 mM) [39], which is achieved due to the activity of K^+/Cl^- carriers known as KCC2 [40, 41], which function along with $Na^+/K^+/Cl^-$ transporter (NKCC1), as well as glycine transporters – GlyT1 and GlyT2 [42] (Fig. 3).

It should be emphasized that a distinctive structure feature of the postsynaptic region containing glycine and GABAA receptors is their cluster organization on the membrane surface. A similar effect is achieved due to the interaction of GlyR with a specific protein, gephyrin [44], which consists of three subunits [43] and forms trimers associated with cytoskeleton (Fig. 3). This protein is a part of a multistage system that ensures the formation and development of neuroplasticity of the neurons postsynaptic membrane containing receptors activated by inhibitory neurotransmitters (glycine and GABA) [45]. This process is dynamic and can be regulated in various ways, in particular, by the level of a specific brain-derived neurotrophic factor [46]. Glycine released into the synaptic cleft is subsequently captured back into neurons and glial cells through the already mentioned GlyT carriers, and some of the neurotransmitter molecules are carried away by convection diffusion into the interstitial fluid. This process is important for the formation of spatial heterogeneity in the distribution of glycine and the explanation of the molecular mechanisms of its effects in neurons [33].

Other concomitant and metabolic effects of glycine

The considered molecular activation mechanism of chlorine ions transmembrane currents indicates the direct participation of glycine in the formation of inhibitory processes in CNS neurons and is the basis for the formation of various treatment regimens aimed at reducing anxiety and reducing the manifestation of stress. Thus, it has been experimentally shown that high doses of glycine when taken orally (3 g once before falling asleep) improve the subjective and objective assessment of the quality of sleep in the group of patients under consideration [47]. The oral intake of glycine reduces metabolic disorders in patients with cardiovascular diseases, inflammation of various origins in a number of cancers, as well as in obesity and diabetes [48]. Glycine protects against oxidative stress caused by a wide range of toxic compounds (including drugs) at the level of cells or an entire organ in the liver, kidneys, intestines, and a vascular system [49]. It is noteworthy that glycine has a direct effect on the arteriole dilatation [50, 51], which is the most important aspect of this amino acid overall effect on the CNS state [52]. The impact on the blood flow system in microvessels and capillaries leads to a theoretically substantiated [53–55] and experimentally confirmed increase in the glucose content in tissues [56].

Allosteric regulation of GlyR by zinc ions

A number of experimental works have shown the allosteric regulation of GlyR by zinc ions [57, 58]. The effect of zinc on the activity of glycine receptors depends on the level of the ion content and has a biphasic form. At low concentrations of Zn^{2+} (<10 μM), the receptor is activated, while at high concentrations (>10 μM), it is inhibited. These multidirectional processes involve different sites on the receptor and have different molecular mechanisms. Potentiation is achieved by increasing the affinity of the receptor for glycine, while inhibition is achieved by decreasing its efficiency [57]. These effects should be considered as a consequence of zinc physicochemical properties; zinc is the only ion among transition metals that does not have a biological redox activity. It is the lack of the zinc redox activity, along with its relatively strong affinity for proteins that has made zinc a suitable ion to play the role of a

ISSN 2307-9266 e-ISSN 2413-2241
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structural cofactor that modulates the activity of the glycine receptor.

Antioxidant effects of glycine and zinc

In addition to the immediate direct combined effect on the state of the neuron membrane polarization, glycine and Zn^{2+} have many effects on metabolic processes that directly affect the condition of patients with anxiety disorders. In particular, it has been experimentally shown that an increase in the concentration of glycine has a protective effect on the oxidative phosphorylation system in the mitochondria of neurons under anoxia and hypoxia conditions [59–61], which is a part of the global regulatory mechanism of the metabolism switching state depending on the level of the tissue amino acids [62]. In addition, a direct increase in the content of glycine reduces the generation level of reactive oxygen species initiated by glutamate excitotoxicity [63]. The antioxidant effect is supported by the mediated participation of glycine in the glutathione tripeptide in the system of protection against the oxidative stress, which is the basis of this amino acid protective effect in various ischemic conditions and acute cerebrovascular accidents [64]. Under normal physiological conditions, Zn^{2+} is redox-inactive; therefore, it takes part in the processes of receiving and transmitting electrons indirectly. The antioxidant properties of zinc are the result of several indirect mechanisms, i.e. the inhibition of ROS formation by transition metals and sulfhydryl stabilization [65, 66].

The above-mentioned molecular mechanisms of the glycine and zinc effect on the cellular and subcellular systems of the brain tissue indicate the need for the combined use of these metabolites to achieve a more pronounced effect in patients suffering from anxiety disorders. At the same time, both the ability to maintain the concentration of the amino acid and microelement in question, as well as their effectiveness and bioavailability, are important.

Bioavailability and maintenance of glycine and zinc levels in human body

Despite the fact that glycine is a non-essential amino acid, Melendes-Hevia E. et al. point at the need for its supply from outside as a source to meet the biological needs of the cells [67]. It should be notified that to date, the ability of glycine to penetrate the BBB with the help of nonspecific amino acid carriers when administered orally has been experimentally proven [47]. Nevertheless, the doses used in this method of administration are quite high [68] and, therefore, it is necessary to take into account the specificity of local changes in the concentration of glycine, which is achieved by an effective choice of this metabolite route of delivery.

To maintain full zinc homeostasis, a sufficient daily intake is necessary, because the systems of the intracellular zinc ions localization discussed above, are rather dynamically filled compartments and traps, which ultimately can lead to the absence of a deposition system for this microelement in the body.

Unfortunately, zinc deficiency does not show any specific symptoms. With its deficiency, such nonspecific conditions as sleep disturbances, deterioration of the skin, hair and nails, decreased appetite, increased hair loss, impaired night vision, decreased mood, increased duration of wound healing, and others can be observed [69].

Zinc deficiency is more common among people on the diet high in phytates [15]. Most often, they are residents of developing countries. Phytates are found in grains, seeds, nuts, legumes, cocoa beans and cocoa powder, and coffee beans. Phytates bind to zinc, thereby reducing its bioavailability [25, 26]. It is worth noting that zinc derived from animal products has a higher bioavailability compared to plant foods. Therefore, vegetarians are usually recommended to increase the zinc norm by 1.5 times [26].

To increase its bioavailability in vegetarian diets, legumes should be used in the sprouted form, or grains and legumes should be soaked in water for several hours before cooking.

According to the unified sanitary-epidemiological and hygienic requirements for goods subject to sanitary-epidemiological supervision (control), the recommended adequate level of the daily zinc intake for an adult is 12 mg; the upper allowable intake level is 25 mg [70]. The physiological need for children is from 3 to 12 mg/day (depending on age). Breastfeeding until at least 6 months of age provides an adequate level of zinc intake in the child's body [71].

Interestingly, some authors point at the need for sublingual zinc in the treatment of colds [14]. A slow drug dissolution in the mouth will allow zinc ions to be released, absorbed and transported to the nose – the source of infection. The chemical composition of the preparation is also important so that zinc can be ionized in the oral cavity at pH 7.4: citric acid, glycine and tartrate prevent zinc ionization [14].

In biologically active food supplements, zinc can be present in the form of compounds: acetate, sulfate, chloride, citrate, gluconate, lactate, oxide, carbonate, L-ascorbate, L-aspartate, bisglycinate, L-lysinate, malate, mono-L-methionine sulfate, picolinate, L-pyroglutamate, as well as amino acid complexes (in accordance with the Unified Sanitary-Epidemiological and Hygienic Requirements). Biological zinc supplements have a varying bioavailability. Zinc bound to amino acids such as aspartate, cysteine and histidine, has the highest absorption concentration, followed by zinc chloride, sulfate and acetate, while zinc oxide has the lowest bioavailability [14, 26, 72]. A comparison of various saccharides and their combinations effect on the zinc uptake by vesicles with brush border membranes showed that the addition of maltose and a mixture of galactose

DOI: 10.19163/2307-9266-2022-10-5-404-415

with glucose did not significantly reduce the level of the zinc uptake compared with the control. The addition of a glucose polymer or lactose significantly increased the bioavailability of zinc [73]. The addition of glucose to lactose or mannitol to the glucose polymer had the same effect as lactose or polymer alone, respectively. The galactose-only buffer had no effect on zinc binding. In another study, a low molecular weight lactose-zinc complex was found out to have a higher bioavailability *in vitro* [74].

The use of Zn^{2+} together with glycine will allow the formation of chelated forms of zinc, the undeniable advantages of which include a maximum bioavailability even under the conditions where the assimilation of the components is impaired (the lack of interaction with food, other minerals and gastric hydrochloric acid, the absence of adverse reactions) [75].

It has been established that zinc, as one of the most important trace elements, plays an important role in various pathological conditions. Various diseases of the gastrointestinal tract, such as malabsorption, cirrhosis of the liver, a celiac disease, Crohn's disease and chronic diarrhea, can also lead to zinc deficiency, due to the impaired absorption [19, 26].

Low zinc levels have been shown to be associated with metabolic syndrome and diabetes [76, 77], as well as decreased immunity [26, 78, 79]. Large amounts of iron from supplements can interfere with the zinc absorption. Disruption of zinc homeostasis, leading to either depletion or excess zinc, causes severe damage to neurons [80]. Zinc-induced cell death and changes in brain zinc status are associated with a wide range of diseases, including many neurodegenerative disorders, such as Alzheimer's disease, and mood disorders, including depression, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and prion diseases, as well as autism spectrum disorders. [66, 81–83].

The considered molecular mechanisms of the metabolites action are reflected in the clinical practice of the anxiety states treatment. In particular, it was shown that such anxiety symptoms as anxious mood, tension, and sleep disturbances were subjected to the greatest reverse dynamics during glycine therapy [85]. In addition, a randomized placebo-controlled study demonstrated the effectiveness of glycine in the treatment of mild anxiety in patients with an adjustment disorder with a predominance of disturbance of other emotions [86].

All major metabolic pathways are regulated by zinc metalloenzymes. The functions of these enzymes include catalytic, structural and regulatory roles. The status of zinc, whether deficient or abundant, is able of influencing each of this element's diverse roles in human biology.

CONCLUSION

Thus, deficiencies of certain essential trace elements and amino acids, such as glycine and zinc, especially their combined deficiencies, are one of the frequent causes of various adverse effects, including post-stress CNS dysfunctions. Given the accumulated experience of these micronutrients positive impact on the processes of recovery and maintenance of the central nervous system normal functioning, an adequate intake of zinc and glycine may be important for most people who experience the consequences of numerous stresses and anxiety on a daily basis. This combination can be especially useful for the people experiencing a state of chronic psycho-emotional stress and maladaptation, including those who have difficulty in falling asleep. Replenishment of zinc and glycine deficiency in the body of a healthy person is manifested by the development of a persistent anti-anxiety effect, which is accompanied by the normalization of the sleep-wake rhythm, which makes it possible to have a good rest without any loss of working efficiency after waking up.

FUNDING

The work was carried out with the financial support of LLC "MNPK "BIOTIKI".
The sponsor had no influence on the choice of material for publication, analysis and interpretation of the data.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

VNS – writing and editing the text, analyzing literary sources and interpreting the results, analyzing glycine and zinc clinical effects, approval of the text; YRN – writing and editing the text, analyzing literary sources and interpreting the results, conducting a database search in the Protein Data Bank (PDB) (https://www.rcsb.org/), selecting material on the glycine action, developing design and making illustrations using graphic tools and the library of the ACD/ChemSketch 2020.2.0 software package, approval of the article final version for publication; VYT – writing and editing the text, analyzing literary sources and interpreting the results, selecting material on the metabolic zinc action; EVS – writing and editing the text, analyzing literary sources and interpreting the results, analyzing pharmaceutically acceptable zinc compounds and bioavailability of combinations, approval of the text.

ISSN 2307-9266 e-ISSN 2413-2241

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REVIEWS

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

CONVENTIONAL APPROACHES TO THE THERAPY OF HEREDITARY MYOPATHIES

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Received 05 Sep 2022 After peer review 10 Oct 2022 Accepted 25 Oct 2022

The aim of the work was to analyze the available therapeutic options for the conventional therapy of hereditary myopathies.
Materials and methods. When searching for the material for writing a review article, such abstract databases as PubMed and Google Scholar were used. The search was carried out on the publications during the period from 1980 to September 2022. The following words and their combinations were selected as parameters for the literature selection: “myopathy”, “Duchenne”, “myodystrophy”, “metabolic”, “mitochondrial”, “congenital”, “symptoms”, “replacement”, “recombinant”, “corticosteroids”, “vitamins”, “tirasemtiv”, “therapy”, “treatment”, “evidence”, “clinical trials”, “patients”, “dichloracetate”.
Results. Congenital myopathies are a heterogeneous group of pathologies that are caused by atrophy and degeneration of muscle fibers due to mutations in genes. Based on a number of clinical and pathogenetic features, hereditary myopathies are divided into: 1) congenital myopathies; 2) muscular dystrophy; 3) mitochondrial and 4) metabolic myopathies. At the same time, treatment approaches vary significantly depending on the type of myopathy and can be based on 1) substitution of the mutant protein; 2) an increase in its expression; 3) stimulation of the internal compensatory pathways expression; 4) restoration of the compounds balance associated with the mutant protein function (for enzymes); 5) impact on the mitochondrial function (with metabolic and mitochondrial myopathies); 6) reduction of inflammation and fibrosis (with muscular dystrophies); as well as 7) an increase in muscle mass and strength. The current review presents current data on each of the listed approaches, as well as specific pharmacological agents with a description of their action mechanisms.
Conclusion. Currently, the following pharmacological groups are used or undergoing clinical trials for the treatment of various myopathies types: inotropic, anti-inflammatory and antifibrotic drugs, antimyostatin therapy and the drugs that promote translation through stop codons (applicable for nonsense mutations). In addition, metabolic drugs, metabolic enzyme cofactors, mitochondrial biogenesis stimulators, and antioxidants can be used to treat myopathies. Finally, the recombinant drugs alglucosidase and avalglucosidase have been clinically approved for the replacement therapy of metabolic myopathies (Pompe’s disease).

Keywords: hereditary myopathies; Duchenne’s muscle dystrophy; metabolic therapy; pharmacological correction

Abbreviations: ETC – electronic transport chain; mRNA – matrix ribonucleic acid; tRNA – transport ribonucleic acid; siRNAs – small interfering ribonucleic acids; NAD, nicotineamide-adenine dinucleotide; FAD – flavin adenine dinucleotide; NADP – nicotinamide adenine dinucleotide phosphate; ATP – adenosine triphosphate; ADP – adenosine diphosphate; CTGF – connective tissue growth factor; TGFβ – transforming growth factor-beta; NSAIDs – non-steroidal anti-inflammatory drugs; XLMTM – X-linked myotubular myopathy; TCA – tricarboxylic acid cycle; TNF-α – tumor necrosis factor-alpha; CTGF/CCN2 – connective tissue growth factor.

For citation: M.V. Pokrovsky, M.V. Korokin, A.M. Krayushkina, N.S. Zhunusov, K.N. Lapin, M.O. Soldatova, E.A. Kuzmin, O.S. Gudyrev, I.S. Kochkarova, A.V. Deikin. Conventional approaches to the therapy of hereditary myopathies. *Pharmacy & Pharmacology.* 2022;10(5):416-431. **DOI:** 10.19163/2307-9266-2022-10-5-416-431

Для цитирования: М.В. Покровский, М.В. Корокин, А.М. Краюшкина, Н.С. Жунусов, К.Н. Лапин, М.О. Солдатова, Е.А. Кузьмин, О.С. Гудырев, И.С. Кочкарова, А.В. Дейкин. Конвенциональные подходы к терапии наследственных миопатий. *Фармация и фармакология.* 2022;10(5):416-431. **DOI:** 10.19163/2307-9266-2022-10-5-416-431

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

ОБЗОРЫ

DOI: 10.19163/2307-9266-2022-10-5-416-431

КОНВЕНЦИОНАЛЬНЫЕ ПОДХОДЫ К ТЕРАПИИ НАСЛЕДСТВЕННЫХ МИОПАТИЙ

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Получена 05.09.2022 После рецензирования 10.10.2022 Принята к печати 25.10.2022

Цель. Проанализировать доступные терапевтические опции для конвенциональной терапии наследственных миопатий.

Материалы и методы. При поиске материала для написания обзорной статьи использовали такие реферативные базы данных, как PubMed и Google Scholar. Поиск осуществлялся по публикациям за период с 1980 г. по сентябрь 2022 г. Параметрами для отбора литературы были выбраны следующие слова и их сочетания: “myopathy”, “Duchenne”, “myodystrophy”, “metabolic”, “mitochondrial”, “congenital”, “symptoms”, “replacement”, “recombinant”, “corticosteroids”, “vitamins”, “tirasemtiv”, “therapy”, “treatment”, “evidence”, “clinical trials”, “patients”, “dichloracetate”.

Результаты. Врожденные миопатии представляют собой гетерогенную группу патологий, которые вызваны атрофией и дегенерацией мышечных волокон вследствие мутаций в генах. На основании ряда клинических и патогенетических особенностей наследственные миопатии разделяют на: 1) врожденные миопатии; 2) мышечные дистрофии; 3) митохондриальные и 4) метаболические миопатии. При этом, подходы к лечению значительно варьируют в зависимости от типа миопатии и могут быть основаны на 1) замещении мутантного белка; 2) увеличении его экспрессии 3) стимуляции экспрессии внутренних компенсаторных путей; 4) восстановлении баланса соединений, связанных с функцией мутантного белка (для ферментов); 5) воздействии на функцию митохондрий (при метаболических и митохондриальных миопатиях); 6) снижении воспаления и фиброза (при мышечных дистрофиях); а также на 7) увеличении мышечной массы и силы. В текущем обзоре представлены современные данные о каждом из перечисленных подходов, а также конкретные фармакологические агенты с описанием их механизмов действия.

Заключение. В настоящее время для лечения разных типов миопатий используются или проходят клинические исследования следующие фармакологические группы: инотропные, противовоспалительные и антифибротические препараты, антимиостатиновая терапия и препараты, способствующие трансляции через стоп-кодоны (применима при нонсенс-мутациях). Кроме того, для лечения миопатий могут быть применены метаболические препараты, кофакторы метаболических ферментов, стимуляторы митохондриального биогенеза и антиоксиданты. Наконец, клинически одобрены рекомбинантные препараты алглюкозидаза и авалглюкозидаза для заместительной терапии метаболических миопатий (болезнь Помпе).

Ключевые слова: наследственные миопатии; миодистрофия Дюшенна; метаболическая терапия; фармакологическая коррекция

Список сокращений: ЭТЦ – электронно-транспортная цепь; мРНК – матричная рибонуклеиновая кислота; тРНК – транспортная рибонуклеиновая кислота; миРНК – малая интерферирующая рибонуклеиновая кислота; НАД – никотинамидадениндинуклеотид; ФАД – флавинадениндинуклеотид; НАДФ – никотинамидадениндинуклеотидфосфат; АТФ – аденозинтрифосфат; АДФ – аденозиндифосфат; CTGF – фактор роста соединительной ткани; TGFβ – трансформирующий фактор роста-бета; НПВП – нестероидные противовоспалительные препараты; XLMTM – X-сцепленная миотубулярная миопатия; ЦТК – цикл трикарбоновых кислот; ФНО-α – фактор некроза опухоли-альфа; CTGF/CCN2 – фактор роста соединительной ткани.

ISSN 2307-9266 e-ISSN 2413-2241

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INTRODUCTION

Hereditary myopathies are a clinically, histologically and genetically heterogeneous group of muscle pathologies that are caused by atrophy and degeneration of striated muscles due to mutations in genes whose role is closely related to the functioning of myocytes. Most often, the proteins encoded by these genes are involved in the formation or maintenance of the structural integrity of the cytoskeleton and plasma membrane. At the same time, myopathies associated with the pathology of cytoskeletal proteins are characterized by a progressive course (muscular dystrophies), and myopathies caused by a function loss of membrane proteins are fully manifested from birth (congenital myopathies). In addition, hereditary myopathies can be caused by mutations in the genes associated with the work of mitochondria (mitochondrial myopathies), or the genes encoding enzymes of intracellular metabolism (metabolic myopathies) [1].

Initially, classifications of hereditary myopathies were based on the clinical presentation or typical histological features found in muscle biopsy specimens. However, according to the current recommendations, the diagnosis of myopathy should be accompanied by the data from molecular genetic studies. In addition to precision diagnostics, this approach leads to an expansion list of the nosological group genetic correlates [2].

THE AIM of the work was to analyze the available therapeutic options for the conventional therapy of hereditary myopathies.

MATERIALS AND METHODS

When searching for the material for writing a review article, such abstract databases as PubMed and Google Scholar were used. The search was carried out on the publications during the period from 1980 to September 2022. The following words and their combinations were selected as parameters for the literature selection: “myopathy”, “Duchenne”, “myodystrophy”, “metabolic”, “mitochondrial”, “congenital”, “symptoms”, “replacement”, “recombinant”, “corticosteroids”, “vitamins”, “tirasemtiv”, “therapy”, “treatment”, “evidence”, “clinical trials”, “patients”, “dichloracetate”.

RESULTS AND DISCUSSION

1. General characteristics of hereditary myopathies

The most typical symptoms of myopathies are muscle weakness, myalgia, myopenia, and exercise intolerance. The clinical picture of myopathies can vary from asymptomatic forms with an increase in serum creatine kinase values and an increased tendency to hyperthermia to severe forms leading to skeletal deformities, as well as respiratory and heart failures. The groups of affected muscles can differ significantly – from an isolated lesion of the oculomotor muscles [3] to a systemic muscle atrophy involving myocardium and diaphragm. The variability of clinical signs is associated both with the diversity of causative genes and with the degree of their function loss. For example, a severe muscle phenotype in Duchenne’s muscle dystrophy is associated with neuropsychiatric disorders [4], and muscle symptoms in the phosphofructokinase deficiency (Tarui disease) are accompanied by hemolytic anemia and hyperuricemia [5]. Mitochondrial myopathies are characterized by especially high clinical heterogeneity [6]. Since multisystem disorders, nervous, digestive, urinary, cardiovascular, endocrine and reproductive systems, as well as the organs of vision and hearing often accompany a mitochondrial dysfunction, they can be involved in the pathological process (Table 1).

1.1. Muscular dystrophies

More than 30 muscular dystrophies have been identified, the most common of which are as follows: Duchenne’s muscle dystrophy, facioscapulohumeral muscular dystrophy, Becker muscular dystrophy, limb-girdle and myotonic kinds of muscular dystrophy. Etiologically, these diseases are very heterogeneous. For example, Duchenne’s muscle dystrophy and Becker dystrophy are caused by dystrophin mutations, while lumbar-limb muscular dystrophies can be caused by an impaired function of calpain, dysferlin, sarcoglycan, lamin, anoctamine, etc. [7]. In all cases, early signs of degeneration and then regeneration of some muscle fibers are usually found. The fibers that regenerate become larger than usual, and eventually the muscle is almost completely replaced by a fibrous scar tissue and fat.

The most classic type of such muscle disorders is Duchenne’s dystrophy. It is caused by frameshift mutations in the MDD gene encoding the dystrophin protein, which is a plasma membrane-associated protein that plays a critical role in sarcolemma stabilizing in mechanical shifts during the muscle contraction or stretching [8, 9]. The dystrophin absence leads to a decrease in the resistance of sarcolemma and the subsequent necrosis of the muscle fibers [10]. The muscle fibers destruction is exacerbated by a mechanical stress and improves while the muscle immobilization [11, 12]. Thus, the accumulation of the damaged muscle fibers is the cause of the progressive course of Duchenne’s myodystrophy. At the same time, the exact molecular mechanisms by which dystrophin plays the role of a mechanical stabilizer, are still unclear [13].

1.2. Congenital myopathies

Unlike muscular dystrophies, congenital myopathies are already manifested in the neonatal period [14]. This is due to the fact that the function of defective proteins is not associated with maintaining the integrity of already differentiated myocytes, but with

DOI: 10.19163/2307-9266-2022-10-5-416-431

the structural organization of the muscle tissue even at the stage of histogenesis. Basically, these are the proteins involved in the formation of the cytoskeleton or intercellular substance. At the same time, these can be such multifunctional proteins as myotubularin, which is involved in the transfer of endosomes, coupling of excitation and contraction, the organization of intermediate filaments, and apoptosis.

Although the exact epidemiology of congenital myopathies is unknown, researchers estimate their incidence to be around 1:25 000 [15]. The classification of congenital myopathy is constantly being revised as more genes that are associated with its various phenotypic and histological manifestations, are identified. At the moment, it continues to be based mainly on the features observed in muscle biopsy [16]. Accordingly, congenital myopathy can be divided into the following five forms: rod myopathy; cardiac myopathy; centronuclear myopathy; congenital myopathy of a fiber type imbalance; myosin storage myopathy.

Clinically, congenital myopathies are manifested by muscle hypotonia and weakness, present at birth or appearing in infancy and not progressive during life. Depending on the causative gene and the nature of the mutation, the clinical spectrum varies from severe neonatal forms with congenital arthrogryposis to mild forms with isolated hyposthenia [14, 16]. In the neonatal period, symptoms tend to be more pronounced and may include reduced fetal movement and a subsequent development of arthrogryposis and clubfoot. Severe muscular hypotonia is often present at birth and in the first months of life (a sign of a lethargic baby) along with a frog-like posture, difficulty sucking, and a respiratory failure [17].

1.3. Metabolic myopathies

Metabolic myopathies are associated with mutations in the genes encoding energy metabolism enzymes. Biochemical disorders include disorders of fatty acids, glucose, or glycogen oxidation. As a result, the functional reserves of the muscle tissue are reduced, which is manifested by hypotension, increased fatigue, myalgia, convulsions, episodes of rhabdomyolysis, etc. [18]. At the same time, fatty acids utilization defects are characterized by a low tolerance to long-term endurance exercises, while disorders of glucose and glycogen metabolism are manifested by an intolerance to fast high-intensity exercises [19]. A separate feature of myopathies also associated with mutations of glycogenolysis enzymes, is the accumulation of intracellular glycogen inclusions [20].

1.4. Mitochondrial myopathies

The pathogenetic basis of mitochondrial myopathies is a violation of energy metabolism processes due to defects in the oxidative phosphorylation. In this regard, some authors consider mitochondrial myopathies as a subtype of metabolic ones. Nevertheless, a number of features of inheritance and pathogenesis, as well as some clinical characteristics, make it possible to distinguish them into a separate group. Thus, mitochondrial myopathies are always associated with impaired functioning of the electronic transport chain (ETC), most often with defects in complex 1 [21–23]. In addition, mitochondrial myopathies can be caused by mutations in both nuclear and mitochondrial genes. In case of mitochondrial DNA mutations, inheritance occurs almost exclusively maternally [24]. The severity of symptoms is determined not only by the pathogenicity of the mutation, but also by the number of the mutant mitochondrial DNA copies that the body has inherited from the mother [25]. The fact is that the mitochondrial genome is heterogeneous (the phenomenon of heteroplasmy) and, along with mutant ones, healthy mitochondria are always present in the cell. Thus, the proportion of defective mitochondria is determined randomly with a random distribution of mitochondria between the daughter cells, which is called the “bottleneck” phenomenon [26].

In general terms, mitochondrial myopathies are mitochondrial diseases, in the spectrum of clinical manifestations of which there are pronounced symptoms from the muscle tissue. Mitochondrial myopathies are characterized by a progressive course and a wide range of associated symptoms, including epilepsy, neuropathy, sensory impairments, etc. [27].

2. Treatment of hereditary myopathy

Treatment of hereditary myopathies varies widely depending on the type and specific disease. A significant proportion of therapeutic interventions in the treatment of myopathies patients are the approaches based on diet, exercise therapy and massage. For example, in metabolic myopathies associated with impaired glucose utilization, the most important therapeutic approach is a low-carbohydrate ketogenic diet [53]. When correcting congenital myopathies, patients are recommended a controlled physical activity, as well as the use of special corsets to prevent the development of bone deformities.

Pharmacological approaches occupy an important place in symptomatic, supportive, and pathogenetic-oriented kinds of therapy. In addition, high rates development of antisense and gene therapy have recently made it possible to focus on etiotropic approaches in the treatment of myopathies patients.

As with most monogenic diseases associated with the gene function loss, conventional specific therapy can be aimed at: 1) replacement of the mutant protein; 2) increase in its expression; 3) stimulation of the expression of internal compensatory pathways; 4) restoration of the balance of compounds associated with the function of the mutant protein (for enzymes).

REVIEWS
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Table 1 – General clinical characteristics of hereditary myopathies

Group of congenital myopathies	Examples of diseases	Clinical manifestations	Proteins with impaired function	Pathogenesis
Muscular dystrophies	Miyoshi myopathy	Distal skeletal muscle weakness Elevated levels of creatine kinase in the blood First symptoms occur during adolescence [28, 29]	Dystrophin [30]	Dysferlin is a membrane-associated linker protein; its function is to mediate calcium-dependent regeneration of mechanical damage to sarcolemma. With mutations that disrupt the dysferlin function; the accumulation of damage to sarcolemma occurs, which leads to progressive dystrophy of skeletal muscles [31].
	Limb-girdle muscular dystrophy type 2B (LGMD2B)	Proximal skeletal muscle weakness Elevated levels of creatine blood kinase Manifestation at the age from 10 to 30 years [32, 33]		
	Duchenne's myodystrophy	Muscular hypotension Heart failure Respiratory distress Debut in early postnatal or postnatal age Death before age of 20 [34, 35]	Dystrophin [36]	Dystrophin is involved in the mechanical stabilization of sarcolemma. In case of loss of the protein product dysferlin due to large deletions or a shift in the reading frame, sarcolemma becomes vulnerable to mechanical deformations that occur during muscle contraction or stretching [37]. Since dystrophin plays an important role in the processes of mitotic division, Duchenne's disease disrupts cell polarity and myogenic differentiation of stem cells. Stem cells lacking functional dystrophin undergo aberrant asymmetric division with centrosome amplification, spindle orientation errors, and an extended cell cycle [38, 39].
Congenital myopathies	Myosin storage myopathy	Muscular hypotension Hypertrophic or dilated cardiomyopathy Manifestation in the neonatal or postnatal period	MYH7 (heavy chain of slow/β-cardiac myosin)	MYH7 is the main myosin isoform in slow oxidative type 1 muscle fibers of skeletal muscles and myocardium. Numerous missense mutations in the MYH7 globular head lead to disruption of the structural protein function and the formation of large inclusions consisting of myosin chains.
	Bethlem muscular dystrophy	Weakness of proximal muscles Joints contracture Hypotension progresses slowly, and more than two-thirds of patients older than 50, continue to move independently. Possible damage to the respiratory muscles [40].	Type VI collagen	Collagen VI is an extracellular matrix protein that forms a microfibrillar network. The protein consists of three different α-chains encoded by separate genes named COL6A1, COL6A2 and COL6A3 in humans. Potential effects on muscles include progressive dystrophic changes, fibrosis, and signs of increased apoptosis [41].
Metabolic myopathies	Pompe's disease	Muscular hypotension Hepatomegaly Heart failure Neurological disorders Debut at any age (an early debut correlates with a more severe course) [42]	Acid maltase [43]	After entering the lysosomes, acid maltase mediates the catalytic breakdown of glycogen by interacting with the mannose-6-phosphate receptor [44]. More than 500 mutations including insertions, deletions, splicing site mutations, nonsense and missense mutations, have been found. They disrupt the functional activity of acid maltase, leading to glycogenosis and energy deficiency of the muscle tissue [45, 46].
	Tarui disease	Muscle weakness Muscle cramps Encephalopathy Hemolytic anemia Rhabdomyolysis risk Debut at any age [47]	Phosphofructokinase [48]	Phosphofructokinase catalyzes the transfer of a phosphate group from ATP to fructose-6-phosphate, which is one of the key elements of glycolysis. In humans, three isozymes named M (muscle), L (liver), and P (platelets), have been identified, Mutations in phosphofructokinase-M lead to muscle weakness due to an energy deficiency in working muscles [49].
Mitochondrial myopathies	Myoclonus epilepsy with myopathy and sensory ataxia (MEMSA)	Proximal and/or distal myopathy Muscular hypotension Myoclonic epilepsy Encephalopathy Sensory ataxia Debut at any age [50]	Polymerase gamma (POLG) [51]	Gamma polymerase is a key enzyme of a mitochondrial DNA replication Mutations in the POLG gene lead to the energy deficiency due to the accumulation of defective mitochondria and a decrease in the number of mtDNA copies (mtDNA depletion), especially in muscle, brain, or liver cells [52].

DOI: 10.19163/2307-9266-2022-10-5-416-431

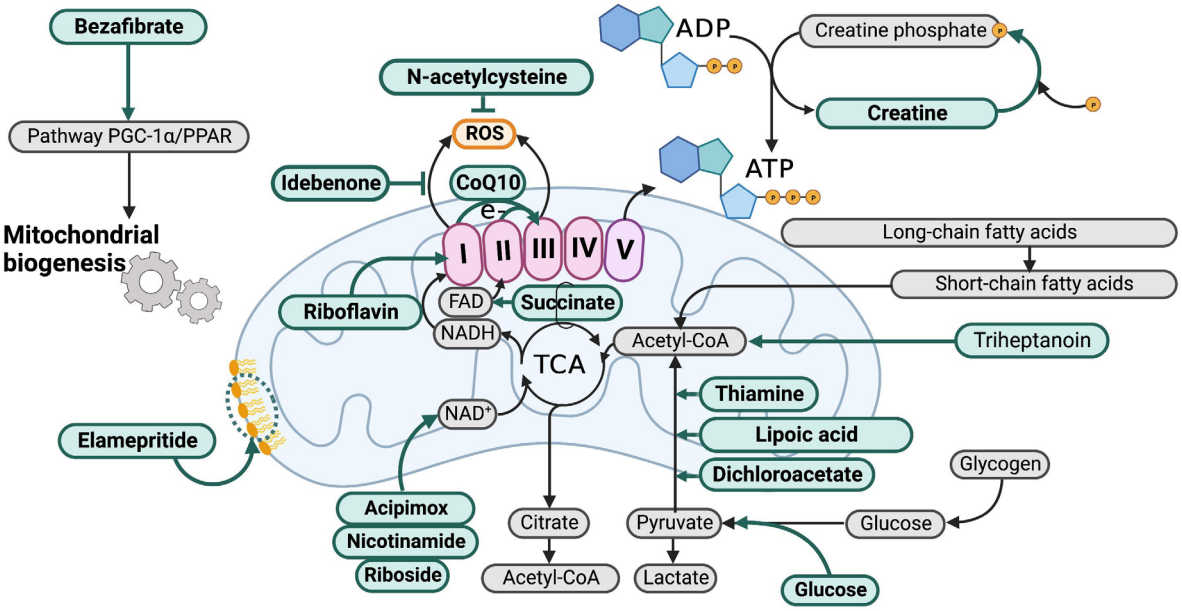

Figure 1 – Classical pharmacological methods to compensate for inadequate functioning of mitochondria

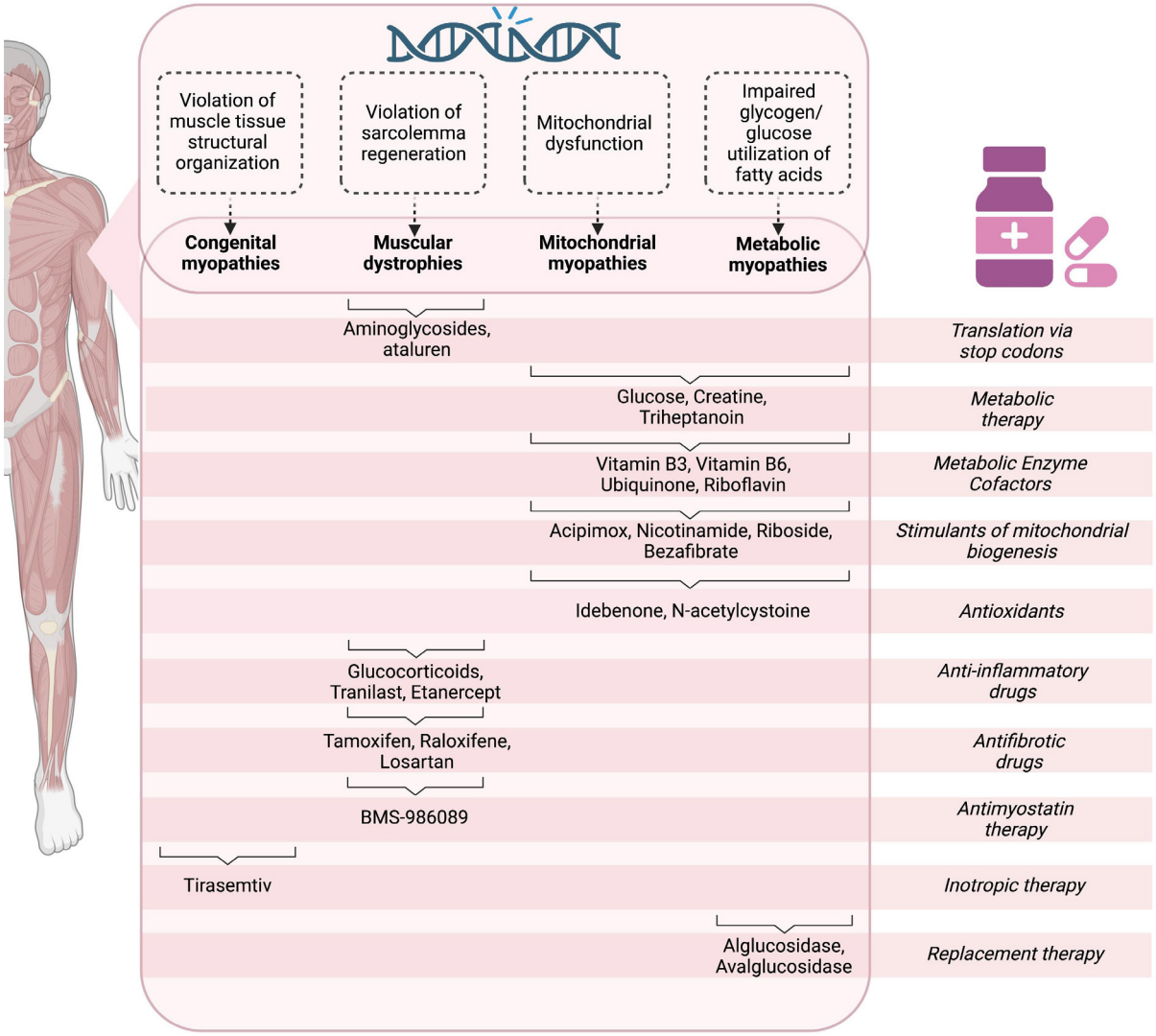

Figure 2 – Varies of existing pharmacological methods for hereditary myopathies correction depending on disease type

ISSN 2307-9266 e-ISSN 2413-2241

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2.1. Mutant protein substitution

Currently, a number of recombinant enzymes have been approved for the specific myopathies therapy. One of the approved approaches is the enzyme replacement therapy for type II glycogenosis (Pompe's disease) with recombinant human alglucosidase alfa (rhGAA; Myozyme© (ex-US) and Lumizyme© (USA), which has been available since 2006, or with avalglucosidase alfa (NEXVIAZYME™; avalglucosidase alfa-ngpt) available since 2021 [54, 55].

Obviously, replacement therapy with recombinant forms of proteins is not the main strategy, since most exogenous proteins cannot penetrate intracellularly to carry out their functions. However, the approaches to directly modify proteins and peptides to enhance cytosolic translocation, continue to be a promising method for improving the delivery efficiency and extending the viability of intracellular protein therapeutics. Among the proposed approaches to improve the cytosolic delivery of exogenous proteins, there have been such as chemical recharging or the inclusion of intracellular internalization motifs [56]. For example, the enzyme replacement therapy with a modified recombinant protein has been proposed for the treatment of X-linked myotubular myopathy. In the preclinical study on Mtm1 δ4 mice with a myotubularin gene knockout, the replacement therapy with recombinant 3E10Fv-MTM1 protein (0.1 mg/kg) into the tibialis anterior muscle twice a week significantly improved the muscle function [57].

2.2. Increase in expression

Some nucleotide substitutions called nonsense mutations, lead to the formation of a stop codon in the coding gene region, resulting in the premature termination of the desired protein synthesis. In addition, mRNA resulting from nonsense mutations is destabilized by a nonsense-mediated decay [58]. Similar mutations are often the cause of hereditary myopathies. Such mutations are found in approximately 10% of Duchenne's patients [59] and in 20% of individuals with X-linked myotubular myopathy (XLMTM) [60].

To restore the expression of the full amino acid sequence, the drugs that force the reading of termination codons were proposed [61]. For example, these were aminoglycosides containing a 2-deoxystreptamine ring bind to the small ribosomal RNA subunit, reducing the accuracy of translation [62]. This property made it possible to propose the use of aminoglycosides for the treatment of Duchenne's myodystrophy [63] and a number of other monogenic diseases caused by premature stop codon mutations [64, 65].

However, serious side effects of aminoglycosides, such as nephrotoxicity and ototoxicity, limit their long-term use. In this regard, alternative agents have been proposed, including suppressor tRNAs and small interfering RNAs (siRNAS) [66] and ataluren [67]. However, only ataluren is currently approved for a clinical use [68].

Theoretically, the termination forcing approach could help treat all hereditary myopathies associated with premature stop codons. Restoration of translation does not always lead to the formation of a functional protein, which, apparently, is associated with impaired intracellular traffic and post-translational modifications of the product [69]. To date, the termination-forcing strategy has been approved for only use with nonsense mutations that cause Duchenne's muscular dystrophy. In addition, despite a high proportion of nonsense mutations in myopathies, their heterogeneity and a low prevalence of each specific disease in the general population make it difficult to conduct full-fledged clinical studies [57].

2.3. Stimulation of internal compensatory pathways expression

In some cases, a decrease or absence of protein expression can be partially compensated for hyperactivation of internal pathways that can functionally mitigate the defect. For example, the severity of muscle pathology in dystrophin defects can be reduced due to the myogenic stimulation, which leads to an increase in the expression of myocyte structural proteins. Non-clinical studies demonstrate that inhibitors of histone deacetylases have a pronounced therapeutic effect in some myopathies. Apparently, due to the regulatory activity in relation to epigenetic modifications, such compounds increase the activity of myogenic differentiation of myocyte precursors. *In vitro* studies have found out that inhibitors of histone deacetylases enhance myogenesis and the formation of enlarged skeletal myotubes [70, 71]. When administered to the dystrophy mice, the drugs had similar beneficial effects. In the mdx mice, the inhibitors increased the cross-sectional area of myofibrils, reducing the histological signs of inflammation and remodeling [72]. Interestingly, among the compounds with an inhibitory activity against histone deacetylases, such well-known drugs as trichostatin A and valproic acid can be distinguished. Herewith, computational biology methods have shown that trichostatin A has the ability to weaken the posttranscriptional repression of utrophin, which has a significant similarity in sequence and functional motifs with dystrophin, including the ability to bind the same dystrophin-associated glycoprotein complex [73, 74]. Utrophin is expressed in fetal tissues at high levels and is inhibited during its development in adults. It was found out that in mice, a decrease in the level of utrophin programmed in embryogenesis corresponds to the onset of muscle necrosis [75]. At the same time, the gene therapy approaches aimed at the

DOI: 10.19163/2307-9266-2022-10-5-416-431

delivery of utrophin, significantly improve the condition of Duchenne's myodystrophy mice [76]. Trichostatin A is currently undergoing clinical trials for the treatment of Duchenne's muscular dystrophy. At the same time, the specific utrophin modulator ezutromid/SMT C1100 demonstrated unsatisfactory results in phase II clinical trials and was withdrawn [77]. At present, the search for an optimal candidate for increasing utrophin expression continues [78].

2.4. Restoring Balance of Compounds Associated with Mutant Protein Function

In some cases, in addition to repairing the deficiency of the protein itself, a strategy for the delivery of compounds related to its catalytic function can be used (Fig. 1). Obviously, such an approach can be implemented in only metabolic and mitochondrial myopathies, where the cause of the disease is the metabolic enzyme deprivation function, and not the one of structural protein or kinase. The main principle of this approach is based on the fact that the use of an exogenous metabolite compensates for its endogenous deficiency, restoring the efficiency of the entire biochemical chain. For example, it has been known since the 1960s that intravenous glucose improves the exercise tolerance in patients with McArdle's disease, which is associated with a defect in the conversion of glycogen to glucose [79]. Glucose therapy is also effective in some other diseases associated with mutations in the proximal enzymes of glycogen catabolism [80–82]. Another example is the use of triheptanoin, a synthetic medium-length triglyceride that restores the energy efficiency of long-chain fatty acid oxidation in the presence of mutations in proximal catabolism enzymes [83]. Triheptanoin has demonstrated a significant improvement in cardiac and muscle symptoms in VLCAD syndrome patients and in patients with carnitine palmitoyltransferase 2 deficiency [84, 85]. In some cases, an effective strategy to increase the concentration of compounds that serve as substrates for the bypass or an alternative biochemical cascade is also used. Thus, for example, in case of a defect in the formation of ATP along the pathway of fatty acid oxidation, an increase in glucose concentration can compensate for the total energy deficiency due to glycolysis [86]. A similar effect can be achieved with the use of creatine. Creatine is a skeletal muscle amino acid that serves as a substrate for the formation of creatine phosphate, a phosphate group donor for the conversion of ADP to ATP by the enzyme creatine kinase. In a number of studies, the administration of exogenous creatine has shown a therapeutic effect on the muscle symptoms in metabolic myopathies [62, 63].

2.5. Mitotropic drugs

Various cofactors, including riboflavin, coenzyme Q10, vitamins B6 and B3, can be used to partially compensate for the disorders caused by a dysfunction of one of the metabolic pathways (Fig. 1). These drugs can partially increase the energy efficiency of cells due to a positive effect on oxidative phosphorylation in mitochondria [88]. It is known that vitamin B3 (nicotinic acid) serves as a substrate for the formation of NAD and NADP, thereby facilitating the transfer of hydrogen from the tricarboxylic acid cycle to complex 1. Coenzyme Q10 (ubiquinone), in turn, is directly involved in the transfer of electrons from the NADH dehydrogenase complex (complex I) and succinate dehydrogenase complex (II) to complex III.

The use of cofactors is one of the main therapeutic options for mitochondrial myopathies. However, due to the lack of full-fledged clinical studies, it is impossible to judge the effectiveness of this approach in terms of evidence-based medicine. Moreover, the vast majority of these compounds are registered as food additives [89]. Obviously, the approaches based on the use of cofactors do not have a dramatic clinical effect due to e thweak mitochondrial transport, nonselectivity of the action, and a weak overlap with the pathogenetic mechanisms of the disease [90, 91]. The use of vitamin and cofactor cocktails is more justified when the number of factors considered is reduced due to their deficiency or transport defect now when this approach can be considered as replacement therapy [42, 68, 69].

In general, there are still not so many effective methods for restoring a mitochondrial function in mitochondrial mutations from the point of view of evidence-based medicine. In addition to cofactors, mitotropic compounds are represented by antioxidants, mitoprotectors, incl. dichloroacetate, arginine, coenzyme Q10, idebenone, etc. [70, 71]. Pharmacological approaches aimed at improving the function of mitochondria are based on the use of a very wide range of drugs [89, 90, 94]. Some of the most requested connections are shown in Fig. 1.

Classical pharmacological methods of compensating for inadequate functioning of mitochondria are based on increasing the activity of mitochondrial metabolic cascades and reducing the content of toxic agents such as lactate and reactive oxygen species (ROS). E.g., bezafibrate has been shown to stimulate mitochondrial biogenesis by activating the PGC-1a/PPAR pathway. In addition, acipimox, nicotinamide and riboside restore the content of NAD^+, increasing the efficiency of electron transfer to the ETC.

Thiamine, lipoic acid and dichloroacetate activate pyruvate dehydrogenase, which leads to a decrease in the lactate accumulation due to the conversion of pyruvate to another metabolite, acetyl-CoA. Succinate, riboflavin, and CoQ10 promote the ETC electron transfer or restore the function of complexes I and II. Some

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compounds, such as idebenone, N-acetylcysteine, and lipoic acid, have the ability to reduce or inactivate the ROS production. Elamepritide stabilizes mitochondrial membrane lipids, preventing a mitochondrial destruction.

In case of a deficiency of certain lipid or carbohydrate metabolism enzymes, the deficiency replenishing strategy of compounds in the biochemical chain after the reaction catalyzed by the mutant enzyme, has a therapeutic efficacy. E.g., with a defect in the utilization of long-chain fatty acids, the use of heptanoin, a more proximal component included in the tricarboxylic acid (TCA) cycle, is justified. Similarly, in case of glycogen cleavage defects, the therapeutic potential is the use of exogenous glucose. Finally, the defects in the oxidative phosphorylation and mitochondrial function can be partially compensated for by the use of creatine, which acts in muscles as an alternative carrier of the high-energy phosphate bond in the formation of creatine phosphate.

2.6. Anti-inflammatory therapy

Anti-inflammatory therapy is one of the key approaches to the treatment of muscular dystrophies [96]. Inflammatory changes may accompany other types of myopathies, but this is extremely rare [97].

Currently, the only approved approach aimed at suppressing the inflammatory process in myodystrophy is corticosteroid therapy. However, it is important to emphasize that, despite the progressive death of muscle fibers, anti-inflammatory therapy is not necessary for all muscular dystrophies. E.g., treatment with deflazacort in dysferlinopathies patients neither improved nor showed any trend towards a decrease in muscle strength [98].

Corticosteroids have been shown in clinical trials to improve muscle strength and function without clinically serious side effects [99, 100]. Moreover, glucocorticoids have been shown to increase the utrophin expression [101].

In view of the serious side effects developed during a long-term use of corticosteroids, the search for other anti-inflammatory therapy strategies continues. For example, among the strategies tested in myodystrophy, inhibitors of cyclooxygenase (COX), tumor necrosis factor-alpha (TNF-α) and its receptor, as well as TRPV2 channels, can be distinguished.

Non-steroidal anti-inflammatory drugs (NSAIDs) have shown a relatively modest efficacy in a mouse model of Duchenne's dystrophy. Despite the fact that the use of aspirin and ibuprofen improved the morphological picture of muscles and reduced the inflammatory infiltration and necrosis, the percentage of regenerating myofibrils and isometric tension did not change significantly [102].

The spectrum of the pharmacological activity of the antiallergic drug tranilast includes blockade of TRPV2 [103], therefor its use led to a decrease in fibrosis in skeletal muscles and an increase in the exercise tolerance [104, 105].

Inhibitors of TNF-α have shown some potential in the treatment of myodystrophy. The use of etanercept or an anti-TNF-α antibody slowed down the course of the disease and also reduced the inflammation and destruction of dystrophic muscles in mdx mice without the development of pronounced side effects [106, 107].

2.7. Antifibrotic therapy

An extracellular matrix is an important component of skeletal muscles. It provides a scaffold structure that holds the myofibrils and vessels. In addition, it plays a major role in the processes of biomechanical contraction, as well as in maintaining the integrity and repair of muscle fibers. An excessive accumulation of extracellular matrix components, especially collagen, is defined as fibrosis. An excess formation of the connective tissue as a result of death and defect in muscle cells proliferation is the most important distinguishing feature of muscular dystrophies. Since the dynamics of fibrotic replacement in myodystrophy strongly correlates with the development of muscle symptoms, antifibrotic therapy is one of the main approaches to treating such patients [108].

Tamoxifen is a prodrug, and some of its metabolites interact with the nuclear estrogen receptor, mediating antifibrotic and myoprotective effects. A multicenter, prospective study in 13 outpatient boys aged 6–14 years with genetically confirmed Duchenne's muscular dystrophy demonstrated that patients treated with tamoxifen 20 mg/daily maintained a motor and respiratory functions, compared with a significant deterioration in the patients of the same history of age who had been administrated with only corticosteroids [109].

A similar approach has also shown off its efficacy in a mouse model of dystroglycanopathy. In the studies by Wu B. et al. it has been demonstrated that tamoxifen and raloxifene significantly alleviate a disease progression in the animals with the c.1343C>T mutation of the FKRP gene, demonstrating a pronounced phenotype of a limb-girdle muscular dystrophy [110].

A primary profibrotic signal in skeletal muscles, as in other tissues, is a transforming growth factor-beta (TGFβ) [111]. A high expression of TGFβ is a characteristic feature of dystrophic muscles [112] and is considered one of the main therapeutic targets for reducing fibrosis. It has been shown that Wnt-TGFβ2 is one of the key factors mediating the differentiation of dystrophin-deficient muscle cell precursors in the fibrogenic direction. Antibodies stabilizing LTBP4, which is a TGFβ binding factor, demonstrated a high efficiency.

DOI: 10.19163/2307-9266-2022-10-5-416-431

Anti-LTBP4 treatment also reduced muscle fibrosis and increased muscle strength, including the ones in the diaphragm muscles [113].

The renin-angiotensin system plays an important role in the transmission of profibrotic signals. In particular, the activation of the angiotensin 1 receptor stimulates fibrosis. At the same time, it has been shown that the antihypertensive drug with an inhibitory activity against TGFβ2 losartan led to an increase in the level of myogenic factors with a reduced expression of fibrogenic genes in mdx mice (Duchenne's myodystrophy model) [112].

Interestingly, another drug that blocks the renin-angiotensin-aldosterone axis, enalapril, also exhibits inhibitory effects on the connective tissue growth factor (CTGF/CCN2) [114] and is another regulator of profibrotic signaling [115, 116]. Pharmacological blockade of CTGF has been shown to slow the progression of fibrosis and improve a muscle function in mdx mice [114]. Moreover, anti-muscle CTGF therapy is currently undergoing clinical trials for the treatment of Duchenne's muscular dystrophy [117].

2.8. Means with a positive effect on muscle strength

A decreased muscle strength is the main symptom of myopathies. In this regard, in addition to other approaches, strategies have been developed to increase the effectiveness of the muscle contraction or the prevention of myopenia.

For example, tirasemtiv, fast skeletal troponin activator acting on thin filaments, has been shown to be effective as an agent that increases muscle strength, and can be used to compensate for hypotension in the muscle dysfunction. In the studies on genetically modified mice and cells from a patient with rod myopathy carrying an actin mutation (ACTA1H40Y), treatment with tirasemtiv increased inotropic parameters to those comparable to healthy controls [118].

One of the most popular targets for regulating a muscle mass is myostatin. A decrease in signaling of this myokine leads to a sharp increase in the muscle mass due to the intensification of the muscle fiber growth [119]. The first similar drug, domagruzumab (PF-06252616, Pfizer), which is a recombinant humanized antibody to myostatin, was withdrawn during the second phase of clinical trials, despite the fact that in the first phase, a 6.1% increase in the muscle mass after treatment was shown compared with a placebo group [120]. Another antimyostatin drug, BMS-986089, has demonstrated its high efficacy in preclinical test systems in mice and cynomolgus monkeys, and is currently undergoing clinical trials. However, in general, despite the theoretical promise of the approach and positive initial results, the recent clinical data demonstrate that antimyostatin therapy is less effective than expected. In addition, the long-term effects of antimyostatin therapy require a particularly close study, due to the possible negative impact on the pool of myosatellite cells [121].

CONCLUSION

Hereditary myopathies are a group of incurable diseases with a wide range of symptoms and a high variability in the clinical course. Currently, a large number of therapeutic approaches have been developed and approved for the use in various types of myopathies (Fig. 2). The most developed are the methods for the correction of muscular dystrophies, which, due to the progressive nature of the course, have the largest number of pathogenetic pathways that can be targets for therapy. At the same time, the smallest number of therapeutic options is available for the treatment of congenital myopathies, where the hereditary defect is permanently manifested throughout life, and there are no secondary alteration factors, such as inflammation and fibrosis. In addition, with the exception of some nosologies, there are no effective approaches to correct metabolic and mitochondrial myopathies.

In the treatment of all myopathies, an important role is played by symptomatic and supportive therapy aimed at treating pain and symptoms from other organs and systems. Osteoporosis [122] and pneumonia are regular consequences arising from hypodynamia myopathies, which are treated according to standard schemes.

It should be notified that in recent years, gene therapy approaches that correct or compensate for a defect at the gene level have become increasingly important. These approaches were not been covered in the work, the aim of which was to analyze the existing conventional strategies. However, to date, it is gene and cell therapy that constitute the most growing and promising layer of pharmacological agents for the treatment of hereditary myopathies.

In congenital myopathies, tirasemtiv, rapid skeletal troponin activator acting on thin filaments, has been shown to be effective. Theoretically, this approach can be effective in other types of myopathies. For the treatment of muscular dystrophies, anti-inflammatory and antifibrotic drugs, as well as antimyostatin therapy and a strategy aimed at translation through stop codons (applicable for nonsense mutations), can be used. In addition, metabolic drugs, metabolic enzyme cofactors, mitochondrial biogenesis stimulants, and antioxidants can be used to treat mitochondrial and metabolic myopathies. Finally, the recombinant drugs alglucosidase and avalglucosidase have been clinically approved for the replacement therapy of metabolic myopathies (Pompe's disease).

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

FUNDING

The study was financially supported by the Ministry of Education and Science of the Russian Federation, Agreement No. 075-15-2021-1346.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

MVP – idea creating, article concept planning, advising on writing of individual manuscript sections; MVK – idea development, article writing; AMK –literature analysis, article writing; NSZ – literature analysis, article writing; KNL – article writing, graphic material preparing; MOS – literature analysis, article writing; EAK– literature analysis, article writing; OSG – literature analysis, article writing; ISK – literature analysis, article writing; AVD – article concept planning, article writing.

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ISSN 2307-9266 e-ISSN 2413-2241

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DOI: 10.19163/2307-9266-2022-10-5-416-431

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ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

CURRENT ASPECTS OF ETIOTROPIC COVID-19 THERAPY

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Received 01 Sep 2022 After peer review 03 Oct 2022 Accepted 08 Oct 2022

Since the beginning of the pandemic, repeated attempts have been made to develop etiotropic therapy for a novel coronavirus infection. Hydroxychloroquine, lopinavir/ritonavir, etc. derivatives were used as antiviral agents, however, they demonstrated a low efficiency and an insufficient safety. In this connection, other groups of drugs with a more effective and safe pharmacological profile are currently being actively used.

The aim of the study was to analyze the literature references on the efficacy and safety of antiviral drugs for the COVID-19 treatment.

Materials and methods. When searching for the materials for the review article writing, such abstract databases as PubMed, Google Scholar, e-Library were used. The search was carried out on publications for the period from January 2020 to September 2022. The key queries were: COVID-19, etiotropic therapy; immunological drugs; antiviral drugs; interferons.

Results. Currently, there are various degrees of effective etiotropic drugs for the treatment of COVID-19 patients. The review has considered a few groups of drugs that are of interest from the point of view of etiotropic therapy: immunological drugs (anticovid plasma, the drugs based on antiviral antibodies, the drugs of recombinant interferons-α2 and -β1, as well as interferon inducers, i.e., the drugs based on double-stranded RNA sodium salt, and others); drugs that block the penetration of the virus into the cell (umifenovir); the drugs that disrupt the process of the viral replication (favipiravir, remdesivir, molnupiravir, nirmatrelvir/ritonavir).

Conclusion. Synthetic antivirals, in particular favipiravir, molnupiravir, remdesivir, and nirmatrelvir/ritonavir, have the largest evidence base for their efficacy and safety. The search for new effective and safe etiotropic drugs for the treatment of COVID-19, as well as the collection and analysis of post-registration data on the drugs already used in clinical practice, continues.

Keywords: COVID-19; interferons; molnupiravir; favipiravir; nirmatrelvir/ritonavir; etiotropic therapy

Abbreviations: IFN – interferon; II – interferon inducers; IVIG – intravenous immunoglobulin; dsRNA – double-stranded ribonucleic acid; siRNA – small interfering RNA; ARDS – acute respiratory distress syndrome; OR – odds ratio; CI – confidence interval; RR – risk ratio; AE - adverse events; ALV – artificial lung ventilation.

АКТУАЛЬНЫЕ АСПЕКТЫ ЭТИОТРОПНОЙ ТЕРАПИИ COVID-19

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Получена 01.09.2022 После рецензирования 03.10.2022 Принята к печати 08.10.2022

For citation: D.N. Zemskov, L.A. Balykova, O.A. Radaeva, K.Ya. Zaslavskaya, P.A. Bely, E.V. Semenova, M.V. Shirmankin, K.N. Koryanova. Current aspects of etiotropic COVID-19 therapy. *Pharmacy & Pharmacology.* 2022;10(5):432-445. **DOI:** 10.19163/2307-9266-2022-10-5-432-445

Для цитирования: Д.Н. Земсков, Л.А. Балыкова, О.А. Радаева, К.Я. Заславская, П.А. Белый, Е.В. Семенова, М.В. Ширманкина, К.Н. Корянова. Актуальные аспекты этиотропной терапии COVID-19. *Фармация и фармакология.* 2022;10(5):432-445. **DOI:** 10.19163/2307-9266-2022-10-5-432-445

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

ОБЗОРЫ

DOI: 10.19163/2307-9266-2022-10-5-432-445

С начала пандемии предпринимались неоднократные попытки разработки этиотропной терапии новой коронавирусной инфекции. В качестве противовирусных средств использовались производные гидроксихлорохина, лопинавир/ритонавир и др., однако они продемонстрировали невысокую эффективность и недостаточную безопасность. В связи с чем, в настоящее время активно применяются другие группы препаратов, обладающих более эффективным и безопасным фармакологическим профилем.

Цель. Анализ литературных данных по эффективности и безопасности противовирусных препаратов для лечения COVID-19.

Материалы и методы. При поиске материала для написания обзорной статьи использовали такие реферативные базы данных, как PubMed, Google Scholar, e-Library. Поиск осуществлялся по публикациям за период с января 2020 по сентябрь 2022 гг. Ключевые запросы: COVID-19, этиотропная терапия/etiotropic therapy; иммунологические препараты/immunologic drugs; противовирусные препараты/antiviral drugs; интерфероны/interferons.

Результаты. В настоящее время имеются в разной степени эффективные этиотропные препараты для лечения пациентов с COVID-19. В обзоре рассмотрены несколько групп лекарственных препаратов, представляющих интерес с точки зрения этиотропной терапии: иммунологические препараты (антиковидная плазма, препараты на основе противовирусных антител, препараты рекомбинантных интерферонов-α2 и -β1, а также индукторы интерферона, например, препараты на основе РНК двуспиральной натриевой соли и др.); препараты, блокирующие проникновение вируса в клетку (умифеновир); препараты, нарушающие процесс репликации вируса (фавипиравир, ремдесивир, молнупиравир, нирматрелвир/ритонавир).

Заключение. Наиболее объемную доказательную базу данных по эффективности и безопасности имеют синтетические противовирусные средства, в частности фавипиравир, молнупиравир, ремдесивир и нирматрелвир/ритонавир. Поиск новых эффективных и безопасных этиотропных препаратов для лечения COVID-19 продолжается, также как сбор и анализ пострегистрационных данных об уже применяющихся в клинической практике средствах.

Ключевые слова: COVID-19; интерфероны; молнупиравир; фавипиравир; нирматрелвир/ритонавир; этиотропная терапия

Список сокращений: ИНФ – интерферон; ИИ – индукторы интерферонов; ВВИГ – внутривенный иммуноглобулин; дсРНК – двуспиральная рибонуклеиновая кислота; миРНК – малая интерферирующая рибонуклеиновая кислота; ОРДС – острый респираторный дистресс-синдром; ОШ – отношение шансов; ДИ – доверительный интервал; ОР – отношение рисков; НЯ – нежелательные явления; ИВЛ – искусственная вентиляция легких.

INTRODUCTION

The novel coronavirus infection has challenged all of humanity, showing the global vulnerability of the society to infectious diseases. The main target of SARS-CoV-2 is the respiratory system, however, in addition to the fatal pulmonary complications of COVID-19, the patients have a variety of dangerous extrapulmonary manifestations, including thrombotic complications, an acute kidney injury, and “acute” cardiovascular disorders [1, 2]. The prognosis for COVID-19 is determined by a combination of individual risk factors (age, comorbidities, healthcare organizations).

In the retrospective study by Magleby R. et al., comprising 678 hospitalized COVID-19 patients, it was demonstrated that an independent risk factor for mortality (odds ratio (OR)=6.05; p <0.001) and the crossover to the artificial lung ventilation (ALV) (OR=2.73; p <0.001) is a high viral load [3]. An early initiation of the antiviral therapy contributes to an effective reduction in the viral load, reduces the risk of the disease progression and improves the prognosis [4]. In this regard, in the early phase of the disease, when the maximum replication rate of SARS-CoV-2 is notified, the antiviral therapy is of primary importance, while in later periods, the hyperinflammatory syndrome and coagulopathy take the leading places in the pathogenesis of the disease, respectively, the role of anti-inflammatory drugs (glucocorticosteroids), immunomodulating agents, anticoagulants and their combinations, increases [5]. However, it should be notified that the antiviral therapy remains a significant even at the late stages of the disease, due to the long-term (from 17 to 27 days) viral shedding in patients, especially those with a severe infection [6].

Since the beginning of the pandemic, repeated attempts have been made to develop etiotropic therapy for a novel coronavirus infection. Hydroxychloroquine, lopinavir/ritonavir, etc. derivatives were used as antiviral agents, however, they demonstrated a low efficiency and an insufficient safety [7–10]. In this connection, other groups of drugs with a more effective and safe pharmacological profile are currently being actively used.

THE AIM of the study was to analyze the literature references on the efficacy and safety of antiviral drugs for the COVID-19 treatment.

MATERIALS AND METHODS

When searching for the materials for the review article writing, such abstract databases as PubMed, Google Scholar, e-Library were used. The search was carried out on publications for the period from Jan 2020 to Sep 2022. The key queries were: COVID-19, etiotropic therapy; immunological drugs; antiviral drugs; interferons. The data from both clinical and *in vitro* trials, were considered as references.

ISSN 2307-9266 e-ISSN 2413-2241
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RESULTS AND DISCUSSION

At the moment, the following groups of etiotropic drugs for the treatment of COVID-19 can be distinguished (Table 1):

1) immunological drugs (anticovid plasma, preparations based on antiviral antibodies, preparations of recombinant interferons-α2 and -β1, as well as interferon inducers, i.e., the drugs based on double-stranded RNA sodium salt, and others);

2) drugs that block the penetration of the virus into the cell (umifenovir);

3) drugs that disrupt the process of the viral replication (favipiravir, remdesivir, molnupiravir, nirmatrelvir/ritonavir).

1. Immunological drugs

1.1. Anticovid plasma

Plasma from the patients who have been cured of the COVID-19 infection, is a source of antiviral antibodies and is considered as a treatment option backed by a significant historical experience, but still promising in the context of SARS-CoV-2. In addition to the antiviral (virus-neutralizing) effect, plasma reduces an antibody-dependent cellular cytotoxicity, a complement activation, and phagocytosis [11]. Theoretically, the administration of convalescent plasma at an early stage of the disease is more effective [12], since the peak of viremia is observed in the first week of the infection, and the native primary immune response usually develops on the 10–14th days [13]. In addition to direct antiviral effects, plasma components can also restore the activity of the hemostasis system [14].

Against the background of the conventional therapy and in the controlled study, in patients with severe COVID-19, in the description of individual series of plasma clinical cases, positive results were obtained in 76–90% [15–17]. A donor selection according to the titers or the activity of neutralizing antibodies can further increase the efficacy of anticovid plasma [18]. Clinical and biochemical predictors of the plasma efficacy are lymphopenia, elevated levels of procalcitonin, ferritin, D-dimer and C-reactive protein. It is believed that the preference should be given to the patients who are in a non-critical condition, at the early stage of the disease [19]. A potential danger lies in the intensification of the disease in the presence of certain antibodies – an antibody-dependent increase in the penetration of coronavirus [20]. An analysis of more than 5 000 patients with a severe or life-threatening COVID-19 infection treated with anticovid plasma, showed that serious adverse events (AEs) occurred in <1% of patients in the first 4 h after the infusion [21].

1.2. Intravenous immunoglobulin

Intravenous immunoglobulin (IVIG) can inhibit the complement cascade activation of pro-inflammatory cytokines, differentiation and activation of dendritic cells, as well as the activation of neutrophils and the formation of neutrophil extracellular traps [22]. Considering that these mechanisms can play an important role in the pathogenesis of a novel coronavirus infection, IVIG is one of the options for treating COVID-19 [23]. In a multicenter, double-blind, placebo-controlled (Phase 3) study of 146 patients (69 of whom 69 had received IVIG, 77 – placebo) with an acute respiratory distress syndrome (ARDS) due to COVID-19, the use of IVIG did not improve clinical outcomes (on day 28) and was associated with a slight increase in the incidence of thromboembolic complications [24].

1.3. Interferons

Based on the pathogenetic mechanisms of infection caused by SARS-CoV-2, a possible drug target is the interferon (IFN) system. The SARS-CoV-2 virus can inhibit the induction of type I and type III IFNs [25]. In the study by Contoli M. et al., the hospitalized COVID-19 patients with a respiratory failure had 3.8 times lower levels of IFN-α compared to the controls. Herewith, the improvement in the patients' condition was accompanied by an increase in the blood level of the same IFN-α [26]. In addition, the patients with congenital defects in the type I IFN system (with the presence of autoantibodies) have a predisposition to a severe COVID-19 [27]. In the treatment of COVID-19, the antiviral effect of IFN-α2b is determined by the time of the therapy initiation [28].

1.4. Double-stranded RNA sodium salt

The data accumulated by now, show that interferon inducers (IIs) of double-stranded ribonucleic acid (dsRNA), sodium ribonucleonate, being a multiclonal stimulator, induces the synthesis of IFN by several cell populations (cells of the mononuclear phagocytic system, granulocytes, neutrophils, endothelial cells and fibroblasts), characterized by a high (specific) activity and safety.

By activating a number of Toll-like receptors, dsRNA stimulates the synthesis of endogenous IFNs (α, β, γ), which block the ability of the cells to support a viral reproduction by both activating the synthesis of proteins that inhibit the production of viral copies in affected cells and, possibly, damaging the genetic virus material when interacting with the host cell (similar to siRNA effects). Subsequently, both NK cells and mechanisms of adaptive immunity are activated.

In the Russian Federation, a medicinal product based on the dsRNA sodium salt (Radamin® Viro

DOI: 10.19163/2307-9266-2022-10-5-432-445

LS-000381[1] dated 03 Aug 2010, date of renewal 27 Dec 2021), is registered. When administrated into the body, dsRNA stimulates the formation of endogenous IFN I (IFN-α, IFN-β) and IFN II (IFN-γ) types, which are the most important cytokines of the immune response, induce differentiation of myeloid cells, stimulate phagocytosis of neutrophils and macrophages, activate NK cells, enhance the Th1-type T-helper response, thus triggering the innate and adaptive immune response. The antiviral effect of the drug is associated with the activation of the proteins synthesis inhibiting the production of viral copies in the affected cells [29].

DsRNA belongs to the "early type" of interferon inducers, while the production of IFN occurs within 2-6 hours after the administration of the drug with a return to the background values within 2 days. The drug inhibits the reproduction of viruses and various microorganisms (including chlamydia) at the cellular level, prevents the development of the infectious process by activating the body's nonspecific resistance, optimizing inflammatory reactions. Due to its mechanism of action, the drug provides a high protection of the body at already early stages of viral or bacterial infections, has a pronounced anti-inflammatory effect, and also indirectly stimulates reparative and regenerative processes in the body, has antiviral, antibacterial and immunostimulating effects, and also increases the body's resistance to infections [29].

As an II, dsRNA itself has been known for more than 10 years. However, a new technology for the production of dsRNA sodium salt has made it possible to obtain a highly purified biological product, which significantly increases the safety of the drug and opens up broad prospects for its use in clinical practice [29].

2. Drugs that block virus penetration into cell

2.1. Umifenovir

Since the start of the pandemic, umifenovir has been one of the first widely used synthetic antiviral drugs in our country. It is a broad-spectrum antiviral drug that blocks the entry of viruses into host cells by inhibiting the fusion of the lipid envelope of the virus with the cell membrane. Initially, umifenovir was developed for the prevention of the influenza treatment [30]. It has demonstrated an activity against SARS-CoV-2 *in vitro* [31]. In the meta-analysis assessing the efficacy and safety of umifenovir in COVID-19, it was found out that the use of the drug was associated with a higher incidence of negative PCR results on the 14th day of illness (OR=1.27; 95% CI=1.04–1.55) compared with the control group, however, was not associated with a reduction in the risk of the COVID-19 progression, a clinical improvement, and a duration reduction in hospital stay [32].

3. Drugs that disrupt viral replication process

Recently, the drugs able of inhibiting RNA-dependent RNA polymerase of direct action, which is an important enzyme of RNA-containing viruses, have been of primary importance in the development of the antiviral therapy strategy for COVID-19 and ensuring their replication [33].

3.1. Favipiravir

Favipiravir, synthesized and patented by Japanese scientists Y. Furuta and H. Egawa in the late 1990s, is a broad-spectrum antiviral drug proposed for the treatment of severe viral infections, including influenza A, B and C, as well as Ebola. [34]. In 2014, this drug was approved in Japan for the treatment of the infection caused by a pandemic variant of the influenza virus or when other drugs had failed. The subsequent studies have shown that favipiravir is highly active against a large group of RNA-containing viruses, such as influenza viruses, bunya-, arena-, flavi-, picoranaviruses, etc. [35]. In an experimental study by Yamada K. et al., favipiravir has been shown to be effective for a post-exposure prophylaxis of rabies and may be a suitable alternative to immunoglobulin [36]. Favipiravir has shown a good inhibitory activity *in vitro* against SARS-CoV-2, but relatively high doses of the drug are required to obtain effective inhibitory concentrations and provide an antiviral activity [35].

Favipiravir is a prodrug, its active form is ribofuranosyl triphosphate. As a nucleoside analogue, it inhibits the SARS-CoV-2 RNA-dependent RNA polymerase complex by binding to its catalytic domain and preventing the incorporation of nucleotides for a viral RNA replication, which leads to an increase in the mutation frequency and a possible lethal mutagenesis. Also important note that RNA-dependent RNA polymerase is absent in human cells, so the drug is active only contrary virus [37, 38].

The Ministry of Health of the Russian Federation has issued an accelerated permission to use favipiravir preparations for the treatment of COVID-19 [37]. Similar approvals have been obtained in China, India and other countries. In phase II/III of the clinical study in sixty patients, favipiravir therapy was well tolerated and safe, resulting in viral clearance in 62.5% of COVID-19 patients after four days. On the fifth day, twice as many patients treated with favipiravir, received a negative PCR result for SARS-CoV-2 compared with the patients in the control group ($p < 0.05$) [39].

A lot of clinical trials and observatory studies which reported on the effectiveness and safety of

[1] Russian State Register of Medicines. Instructions for Radamin® Viro. Available from: https://grls.rosminzdrav.ru/Grls_View_v2.aspx?routingGuid=27d5a81d-b2e9-49d2-a9eb-1f1c9eacbaa4

ISSN 2307-9266 e-ISSN 2413-2241
Scientific and Practical Journal
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Favipiravir in the treatment of COVID-19 patients, have been conducted [38–44]. Alamer A. et al. assessed the effectiveness of Favipiravir in the treatment of COVID-19 (n=457). It has been established that the average time from the onset of the disease to discharge was 10 days (95% CI = 9–10) in the group of patients receiving favipiravir (n=234), versus 15 days (95% CI=14–16) in the comparison group, receiving supporting therapy (n=223) [38]. In the prospective open multicenter clinical study, including 240 COVID-19 patients (120 patients received Favipiravir, 120 – Umifenovir), in the group of favipiravir patients, there was a faster decrease in the temperature and a decrease in the cough severity [40].

According to the results of the open randomized multicenter comparative study (N = 206), the use of favipiravir for the COVID-19 treatment contributed to a more rapid improvement of the condition (6-8 days) compared with the use of the standard therapy (7–12 days), also demonstrating a favorable security profile. According to the PCR, the Elimation of SARS-COV-2 to the 10th day of therapy was recorded in 98% of the favipiravir patients, and in 80% in the control group (p=0.00007). AEs were observed in 24.04% of the patients of the main group and in 27.45% – the control group [41].

In a number of meta-analyses that summarize the data of clinical studies, the benefits of adding favipiravir to the standard therapy, were confirmed [43, 44]. In the hospitalized patients, Favipiravir, compared with the control group that were receiving only the standard therapy, contributed to a faster elimination of the virus – an average of 5 days (OR=1.60; p=0.02), an earlier temperature decrease – an average of 3 by an average of the 3–4th day (OR=1.99; p <0.01), an improvement in the radiological picture in the lungs (OR=1.33; p <0.01) and an earlier discharge from the hospital (OR=1.19; p <0.01). As for the AEs, the Favipiravir group recorded a higher frequency of hyperuricemia (OR=9.42; p <0.01), increased levels of alanineine-veransferase (OR=1.35; p <0.01), but a lower frequency of nausea (OR=0.42; p <0.01) and vomiting (OR=0.19; p=0.02). The authors arrived at the conclusion that the addition of Favipiravir to the standard therapy is beneficial to the hospitalized COVID-19 patients. At the same time, it has been notified that pregnant women and patients with hyperuricemia in an anamnesis should avoid the use of phavipiral [43].

Favipiravir for *per os* administration has proved to be quite effective and safe for the treatment of a novel coronavirus infection in both mild and moderate courses and has occupied its niche in the outpatient practice. However, in complicated cases, the parenteral therapy has advantages over the oral route of the drug delivery. This therapy can be used in the situations where the patient is in a serious condition or unconscious, has swallowing difficulties or conditions that prevent swallowing. It may also be important in patients with gastrointestinal COVID-19 symptoms, in patients with antibiotic-associated diarrhea (uncontrolled use of antibiotics combinations on an outpatient basis), the exacerbation of chronic gastrointestinal diseases and pseudomembranous colitis, and other situations where the p. o. administration is difficult). The intravenous route of the drug administration is used for a quick and pronounced result, since it immediately enters the bloodstream, its quickly provides maximum bioavailability and the pharmacokinetics are generally more predictable – there is no interaction with food and digestive enzymes [42]. In view of this, in 2021 in the RF was developed and registered a new dosage form of favipiravir for the parenteral administration – Areplivir® (RU LP-007598 dated 18 May 2022), was registered. In the clinical centers of Moscow, Smolensk, Yaroslavl, St. Petersburg, Saransk and Ryazan, an open randomized multicenter comparative study of favipiravir for the parenteral administration (n=209) was conducted in the hospitalized patients aged 18–80 years with moderate form of the coronavirus infection. Based on the results of the study, the data were obtained confirming a high efficacy and safety of the parenteral form of favipiravir for the treatment of COVID-19. In the main group, by the 10th day of therapy, an improvement in the clinical status by 2 or more points on the World Health Organization (WHO) scale was observed in 56.86% of patients, which corresponds to mild symptoms or the complete absence of signs of the disease, and in the control group (the patients receiving the standard therapy) – in 28.04% (p <0.0001). In the group of favipiravir patients, the clinical status improved faster (median=5 days) than in the control group (7 days). On days 5 and 14 of the treatment (visits 2 and 4), a more pronounced improvement in the clinical status was recorded in the main group, in contrast to the patients in the comparison group [42].

A faster and more enhanced favipiravir action for the parenteral use is aimed at increasing the effectiveness of therapy and preventing the development of an extremely severe COVID-19 course, getting to the resuscitation and intensive care unit and death [42, 45].

3.2. Remdesivir

One of the first drugs in the group of RNA-dependent RNA polymerase inhibitors was remdesivir, originally developed for the treatment of the infection caused by the Ebola virus. It is a prodrug that inhibits the reproduction of a wide range of viruses, including filo-, paramyxo-, pneumo- and ortho-coronaviruses (SARS-

DOI: 10.19163/2307-9266-2022-10-5-432-445

CoV and a Middle East respiratory syndrome coronavirus [MERSCoV]) [46–48]. This drug is administered parenterally, which makes it difficult to be used on an outpatient basis[2]. Remdesivir in high doses inhibits the enzyme RNA-dependent RNA polymerase of the virus, causing a delayed termination of the RNA chain without affecting the activity of human polymerases[3] [49, 50].

The National Institute of Allergy and Infectious Diseases (NIAID) in the United States has initiated a placebo-controlled, double-blind, randomized Phase III trial to evaluate the efficacy and safety of remdesivir versus placebo (NCT04280705). This study included 1062 hospitalized patients with COVID-19 and signs of a lower respiratory tract infection (541 patients in the remdesivir group, 521 patients in the placebo group). In the patients treated with remdesivir, the median recovery time was 10 days (95% CI=9-11) compared with 15 days (95% CI=13-18) in the placebo group (OR=1.29; 95% CI = 1.12–1.49; p <0.001). Serious AEs were reported in 131 of 532 patients treated with remdesivir (24.6%) and in 163 of 516 patients treated with placebo (31.6%). The authors concluded that remdesivir is superior to placebo in terms of its effect on the duration of the disease and the severity of clinical symptoms [46].

In patients with moderate COVID-19 who received a 10-day course of remdesivir, there was no statistically significant difference in the clinical status compared to the standard therapy by day 11 of treatment. The patients treated with a 5-day remdesivir course, had a statistically significant difference in clinical status compared to the standard therapy (OR=1.65; 95% CI =1.09–2.48; p=0.02), but this difference, according to the researchers, had no clinical significance [50]. In some other randomized trials, it was not possible to obtain any convincing evidence of the remdesivir effectiveness, either.

Despite the mixed trial results, the FDA has approved remdesivir for the use in hospitalized adult patients with severe COVID-19. Subsequently, the range of indications was expanded and remdesivir was also recommended for the treatment of COVID-19 children aged ≥28 days and weighing ≥3 kg[4]. The data from a number of other clinical studies have also been published in support of the final approval [51–56]. In the double-blind, placebo-controlled study (n=562) of unvaccinated outpatients aged ≥12 years (with one or more risk factors for severe COVID-19), the risk of hospitalization was 87% lower in the remdesivir group (n=279) compared with placebo (n=283) (95% CI=0.03-0.59) [51]. In the study by Goldman D.L. et al., in 77 children with severe COVID-19, the remdesivir therapy was characterized by a favorable safety profile with a high clinical recovery rate [52].

To date, many additional randomized controlled trials and meta-analyses have been obtained, though their conclusions are still conflicting. Among all these works, the most authoritative is the independent WHO Solidarity study, which, according to the results of the interim analysis, did not reveal a significant effect of remdesivir (as well as other antiviral drugs) on mortality rates in hospitalized COVID-19 patients [54]. For this reason, the WHO did not initially recommend the use of remdesivir in these patients. However, the continuation of the study found out that remdesivir had no effect on the survival of ventilated COVID-19 patients, while it slightly reduced the risk of death (up to 14.6% compared to 16.3% in the control group) or the crossover to the artificial lung ventilation (ALV) (14.1% versus 15.7% in the control group) of the hospitalized patients [54]. Based on these data, the WHO has revised its conclusions regarding the use of remdesivir, and now remdesivir is recommended for the treatment of mild to moderate COVID-19, where there is a high risk of hospitalization[5]. Singh S. et al. summarized the data from 4 studies involving 7,324 patients. No reduction in mortality was observed with remdesivir compared with controls (OR=0.92; 95% CI=0.79–1.07; p=0.30). The authors concluded that, given the lack of a significant effect on mortality and a high cost of the drug, its use in COVID-19 is not appropriate, especially in low-income countries [54].

3.3. Molnupiravir

Molnupiravir has become another innovative drug that has not been previously used in clinical practice and received an accelerated approval during the COVID-19 pandemic. It is a prodrug, an analog of N-hydroxycytidine, which is phosphorylated to form N-hydroxycytidine triphosphate and is integrated into viral RNA with the help of RNA polymerase, leading to the accumulation of mutations in the virus genome and, as a result, inhibiting a replication [57]. Molnupiravir is active against RNA-containing viruses, including SARS-CoV-2, which has been shown in experiments *in vitro* and *in vivo* [58]. The results of phase I/II/III clinical trials confirmed the efficacy and safety of molnupiravir in COVID-19 [59, 60].

During conducting a phase I clinical study on healthy volunteers (n=130), the data on a good tolerability of the

[2] Cohen P, Gebo K. COVID-19: Outpatient evaluation and management of acute illness in adults. UpToDate. Literature review current through: Jun 2022.

[3] Coronavirus (COVID-19) update: FDA approves first COVID-19 treatment for young children. Available from: https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-approves-first-covid-19-treatment-young-children.

[4] Ibid.

[5] Remdesivir for COVID-19. Available from: https://apps.who.int/iris/bitstream/handle/10665 /359753/WHO-2019-nCoV-Therapeutics-Remdesivir-Poster-A-2022.1-eng.pdf

drug were obtained. 35.4% and 43.8% (control group) of patients experienced mild side effects with a single dose, 42.9% and 50.0% (control group) – with multiple increasing doses, respectively [59]. By PCR, in a phase IIa clinical trial (n=202), SARS-CoV-2 virus clearance was shorter in the study group compared with placebo (median = 14 days for molnupiravir and 27 days for placebo; p=0,01)[6].

The Phase III, double-blind, randomized, placebo-controlled MOVe-OUT trial included 1 433 non-hospitalized adult patients with mild to moderate COVID-19 (the most common SARS-CoV-2 variants were delta (58.1%), mu (20.5%), gamma (10.7%) and the presence of at least one risk factor for a severe novel coronavirus infection (716 participants received molnupiravir, 717 – placebo). Patients from 15 Russian centers also participated in the MOVe-OUT study. The risk of hospitalization or death was lower in the molnupiravir group (6.8%) compared with placebo (9.7%) (95% CI=5.9−0.1%). The frequency of the AEs registration (including viral pneumonia) in the group of patients receiving molnupiravir was comparable to that in the placebo group (30.4% and 33.0%, respectively). The most common side effects were: diarrhea (1, 7% and 2.1%), nausea (1.4% and 0.7%) and dizziness (1.0% and 0.7%) [60].

Due to the increase in the incidence of COVID-19 and the need to introduce effective drugs for its treatment into clinical practice, the Russian Federation has also developed and registered the drug molnupiravir (Esperavir®) in the oral dosage form capsules (LP-007856 dated 18 May 2022)[7]. According to the results of the clinical study involving 240 outpatients with mild to moderate COVID-19 from 12 Russian centers, the use of molnupiravir for 5 days at the dose of 800 mg 2 times a day led to a 4-fold reduction in the risk of worsening the disease course to the 2nd study week compared with the standard therapy (p=0.0149). It should be notified that about 70% of the patients who participated in the study had concomitant diseases (mainly obesity of degree 2 and above, as well as arterial hypertension).

An important indicator for predicting a COVID-19 course is the virus elimination rate. In 71.67% of patients treated with molnupiravir, SARS-CoV-2 RNA in a swab from the nasopharynx and / or oropharynx was not detected already 6–7 days after the therapy start. In 19% of patients in the molnupiravir group, a complete clinical recovery had been achieved by days 6-7. In the standard therapy group, only 6% of patients (p=0.0039) had been cured by this point.

The treatment of COVID-19 with molnupiravir also led to a significant decrease compared to the standard therapy in the frequency and severity of the disease symptoms, such as cough, changes in osphresis and taste sensitivity over the latest 24 hours after 6–7 days from the therapy start. The data obtained indicate significant advantages of molnupiravir compared to the standard therapy in terms of the dynamics of the COVID-19 symptoms disappearance, the viral load reduction, the improvement in the condition of patients and their clinical status. Therapy with molnupiravir was well tolerated, most of the AEs were of a mild severity, there were no cases of therapy discontinuation or changes in the dose of the study drug due to the development of AEs [61].

Molnupiravir is contraindicated during pregnancy and lactation, and is also prohibited in patients under 18 years [57].

3.4. Nirmatrelvir/ritonavir

The data on the antiviral efficacy of the nirmatrelvir and ritonavir combination in the treatment of COVID-19 are being accumulated. The combination with a commercial product name Paxlovid, was developed by Pfizer and approved by the FDA for an emergency use in mild to moderate COVID-19 in adults and children over 12 years of age at high risk of developing a severe disease. This drug is included in the WHO recommendations for the treatment of COVID-19 [62, 63]. Nirmatrelvir is an inhibitor of the 3-chymotrypsin-like enzyme of SARS-CoV-2 cysteine protease (M^{pro}), which is involved in the viral replication. It has a high antiviral activity against different types of SARS-CoV-2, including alpha (B.1.1.7), beta (B.1.351), gamma (P.1), delta (B.1.617.2) and omicron (B.1.1.529) variants [64]. Ritonavir, an inhibitor of cytochrome P450 3A4, acts as a pharmacokinetic booster, slowing down the nirmatrelvir metabolism of [62, 63]. In December 2021, the combination medicine nirmatrelvir/ritonavir was first approved in the UK for the treatment of COVID-19 in adults who did not require supplemental oxygen and are at the increased risk of progression to severe COVID-19. In January 2022, this drug was approved for the same indications in the European Union, then in the United States, as well as in several other countries.

To date, two randomized trials have shown that the use of nirmatrelvir/ritonavir in outpatients with mild to moderate COVID-19 for 5 days leads to a reduction in hospitalization and mortality [62, 64]. The double-blind, randomized, placebo-controlled EPIC-HR Phase

[6] US Food and Drug Administration. Fact sheet for healthcare providers: emergency authorization for Paxlovid. 2022. Available from: https://www.fda.gov/media/155050/download. Accessed 30 April 2022.
[7] Russian State Register of Medicines. Instructions for molnupiravir (Esperavir®). Available from: https://grls.rosminzdrav.ru/Grls_View_v2.aspx?routingGuid=62a879e9-2c06-4028-8a58-5bac4e01d9ef

DOI: 10.19163/2307-9266-2022-10-5-432-445

2/3 trial evaluated the efficacy of nirmatrelvir/ritonavir in 1,120 outpatient unvaccinated patients at a high risk of a severe novel coronavirus infection compared with 1,126 placebo-treated patients. The use of nirmatrelvir/ritonavir resulted in an 88.9% (95% CI=75%, 8 of 1039 [0.8%]) reduction in the risk of severe COVID-19 (hospitalizations and all-cause mortality) vs. 66 of 1046 [6.3%] in the placebo group). There were no deaths in the nirmatrelvir/ritonavir group (0/1039), while 12 deaths (12/1046) were described in the placebo group (12/1046) by day 28 of the observation. Herewith, the incidence of AEs was comparable in both groups (22.6% and 23.9% in the study and control groups, respectively) [64].

The second study (n=180 351 patients) was conducted in January-February 2022 in Israel, when the omicron strain predominated; 2.6% of participants received nirmatrelvir/ritonavir, resulting in a reduced risk of a severe COVID-19 mortality (OR 0.54 (95% CI=0.39–0.75). This was comparable to an adequate vaccine status (OR=0.20; 95% CI=0.17–0.22). The combined antiviral drug appeared to be more effective in elderly and immunocompromised patients, as well as patients with concomitant neurological and cardiovascular diseases (p <0.05 for all), regardless of vaccination status [62].

Currently, there are insufficient clinical data on the use of the nirmatrelvir/ritonavir combination in children under 12 years of age (<40 kg). Gangfeng Y. et al. conducted a cohort study on a small sample of patients (n=5 – the main group, n=30 – the comparison group) aged 6-14 years with comorbidities and found out that this combination may be one of the options for treating COVID-19 in children with comorbidities. Despite the drug is recommended for use in children by the EU from 12 years and older, the efficacy and safety of the nirmatrelvir/ritonavir combination requires a further study in pediatric practice [63].

In a recent review by Saravolatz L.D. et al., the authors analyzed the available data from FDA clinical trials of oral antivirals, concluded that the nirmatrelvir/ritonavir combination showed a greater reduction in the risk of hospitalization and death than molnupiravir compared with placebo [65]. They also notified that this combination had a better safety profile (it does not have a proven teratogenic effect). The WHO considers this drug “today’s best therapeutic agent for the treatment of COVID-19”[8].

In the Russian Federation, a unique technology was developed; that made it possible to combine both active ingredients (nirmatrelvir and ritonavir) into one fixed dosage form (Skyvira® LP-008056 from 20 Apr 2022)[9], which lead to the reduction of the number of tablets used, by 6 times compared to the American analogue. This provides a reduction in polypharmacy and increases the adherence and safety of therapy in general.

According to the results of the Russian open two-stage multicenter study, the considered fixed combination has a high efficacy and a favorable safety profile when used in COVID-19 patients (including the patients with comorbid pathology). The proportion of patients receiving Skyvira® who had achieved a complete recovery by the 6th day of observation was twice higher than in the comparison group. In the main group, there were no cases of COVID-19 transition to a severer course, in contrast to the patients who had received the standard therapy (8 patients were hospitalized) (p=0.0035, i.e. p <0.0275) [66].

CONCLUSION

Thus, to varying degrees, etiotropic drugs are currently available for the treatment of COVID-19 patients. Synthetic antivirals, in particular favipiravir, molnupiravir, remdesivir, and nirmatrelvir/ritonavir, have the largest evidence base for efficacy and safety. In the latest version, in addition to the above, the 16th one of the Russian interim recommendations for the prevention, diagnosis and treatment of a novel coronavirus infection (dated 18 Aug 2022), the following immunotropic drugs are marked: anticovid plasma, monoclonal antibodies and intranasal interferon alfa, umifenovir and the original domestic development – a MIR 19 preparation (synthetic small interfering ribonucleic acid, siRNA)[10]. It should be notified that both the search for new effective and safe etiotropic drugs for the COVID-19 treatment as well as the collection and analysis of post-registration data on the drugs already used in clinical practice, are being continued.

[8] WHO recommends highly successful COVID-19 therapy and calls for wide geographical distribution and transparency from originator, 22 April 2022 Statement, Geneva. Available from: https://www.who.int/news/item/22-04-2022-who-recommends-highly-successful-covid-19-therapy-and-calls-for-wide-geographical-distribution-and-transparency-from-originator.

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ISSN 2307-9266 e-ISSN 2413-2241
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Table 1 – Preparations for etiotropic COVID-19 therapy

Drug groups	INN	Mechanism of action	Brief information on efficacy and safety	References
Immunological preparations	Anticovid Plasma	Antiviral (virus-neutralizing action), antibody-dependent cellular cytotoxicity, complement activation and phagocytosis	Efficacy in patients with severe COVID-19 is 76-90%. Safety: serious adverse events were observed in <1% of patients in the first 4 hours after infusion.	11–21
	Intravenous immunoglobulin	Inhibitor of pro-inflammatory complement cytokine cascade activation, dendritic cell differentiation and activation, and neutrophil activation	Efficacy in patients with ARDS due to COVID-19: no improvement in clinical outcomes (on day 28); association with a slight increase in the incidence of thromboembolic complications was found out.	22–24
	Interferons	Block virus replication via stimulation of antiviral immunity	Hospitalized patients with COVID-19 and respiratory failure had 3.8 times lower levels of interferon-α compared with control group, while improvement of patients' condition was accompanied by an increase in the level of blood interferon-α	25, 26
	Double stranded RNA sodium salt	Inducer of IFN-α (lymphocytic) and IFN-β (fibroblast)	Stimulates formation of endogenous IFN I (IFN-α, IFN-β) and IFN II (IFN-γ) types, which are the most important cytokines of immune response, induce differentiation of myeloid cells, stimulate phagocytosis of neutrophils and macrophages, activate natural killers, enhance T-helper a Th1-type response thus trigger an innate and adaptive immune response.	29
Drugs that block the entry of the virus into the cell	Umifenovir	Blocks virus penetration into "host cells" by inhibiting fusion of the virus cell membrane lipid envelope	Efficacy in patients with COVID-19: higher incidence of negative PCR results on day 14 of illness (OR=1.27; 95% CI=1.04–1.55) compared with control group; no association with reduced risk of COVID-19 progression, clinical improvement, and reduced length of hospital stay	30–32
Drugs that interfere with viral replication	Favipiravir	Inhibitor of SARS-CoV-2 RNA-dependent RNA polymerase complex by binding to its catalytic domain and preventing incorporation of nucleotides for viral RNA replication, leading to increased mutation rates and possible "lethal mutagenesis"	Efficacy of favipiravir in hospitalized COVID-19 patients according to meta-analysis: faster elimination of virus in favipiravir group – on average, on day 5 (OR=1.60; p=0.02), earlier decrease in temperature – on average on days 3–4 (OR=1.99; p <0.01), improvement of X-ray picture in the lungs (OR=1.33; p <0.01) and earlier discharge from hospital (OR=1.19; p <0.01). Efficacy of Areplivir® for parenteral administration in hospitalized patients with moderate and severe COVID-19: improvement in clinical status by 2 or more points on WHO scale by visit 3, was observed in 56.86% of patients, and in control group (patients receiving standard therapy) – in 28.04% (p <0.0001).	34–45
	Remdesivir	SARS-CoV-2 RNA-dependent RNA polymerase enzyme inhibitor	Meta-analysis of 4 studies involving 7 324 patients hospitalized with COVID-19: use of remdesivir compared with control group did not lead to a decrease in mortality (OR=0.92; 95% CI=0.79–1.07; p=0.30)	46–56
	Molnupiravir	Prodrug, N-hydroxycytidine analogue, which is phosphorylated to form N-hydroxycytidine triphosphate and integrated into viral RNA with the help of RNA polymerase, leading to accumulation of mutations in virus genome and "lethal mutagenesis"	MOVe-OUT study (n=1,433): lower risk of hospitalization or death in molnupiravir group (6.8%) compared with placebo (9.7%) (95% CI=5.9–0.1%). Efficacy of molnupiravir in outpatients with COVID-19 (n=240): 4-fold reduction in the risk of worsenig the disease course by week 2 of the study compared with standard therapy (p=0, 0149); in 71.67% of patients treated with molnupiravir, SARS-CoV-2 RNA in a swab from the nasopharynx and/or oropharynx was not determined on already days 6-7 from the start of therapy; there were no cases of therapy discontinuation or changes in the study drug dose due to AEs development.	57–61
	Nirmatrelvir/ ritonavir	SARS-CoV-2 3-chymotrypsin-like cysteine protease inhibitor	EPIC-HR study in COVID-19 outpatients (n=1120): 88.9% (95% CI=75%, 8 out of 1039) reduction in the risk of developing severe COVID-19 (hospitalizations and all-cause mortality) (95% CI=75%, 8 out of 1039 [0.8%]) vs. 66 of 1046 [6.3%] in placebo group). Efficacy of nirmatrelvir/ritonavir in COVID-19 outpatients: proportion of patients who achieved complete recovery by day 6 of observation was twice as many than in the comparison group; in the main group, there were no cases of transition of COVID-19 to a severer course, in contrast to patients receiving standard therapy (8 patients were hospitalized) (p=0.0035, i.e. p <0.0275).	62–66

DOI: 10.19163/2307-9266-2022-10-5-432-445

FUNDING

This review was made with the support of OOO PROMOMED RUS.
The sponsor had no influence on the choice of materials for publication, analysis and data interpretation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

DNZ – literature references collecting, data processing, article writing; LAB – review idea and concept, text writing and editing; OAR – literature references collecting, data processing, article writing; KYaZ – literature references collecting, data processing, article writing; PAB – literature references collecting, data processing, article writing; EVS – literature references collecting, data processing, article writing; MVSh – literature references collecting, data processing, article writing; KNK – literature references, collecting, data processing, article writing.

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DOI: 10.19163/2307-9266-2022-10-5-432-445

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RESEARCH ARTICLE

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

CONSUMPTION DETAILS OF SYSTEMICALLY ACTING ANTIVIRAL AND ANTIMICROBIAL PREPARATIONS IN PERIOD OF NOVEL CORONAVIRUS INFECTION SPREAD IN RETAIL SECTOR OF SAMARA REGION PHARMACEUTICAL MARKET

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Received 25 April 2022 After peer review 05 July 2022 Accepted 08 Sep 2022

An analysis of the medicinal preparation consumption structure in the period of the COVID-19 pandemic in the pharmacy network reflects the existing outpatient practice and makes it possible to draw generalized conclusions about its compliance with the pharmacotherapy standards.

The aim. Comparative analysis of population consumption of antimicrobial and antiviral medicines sold in the retail pharmacies of the Samara region in 2015–2021.

Materials and methods. The study was conducted in the retail sector of the Samara region pharmaceutical market. The material of the study was the information on the list of items and dispensing volumes of antibacterial and individual antiviral drugs during the novel coronavirus infection spread (in 2020) in the network of the Samara region pharmacies. The data are compared with the indicators of the drug sales in 2015–2019. Methods of retrospective, comparative, graphical, methodological, content analyzes and statistical methods of analyses were used.

Results. The authors have established a significant distortion in the consumption of systemic antimicrobial preparations in the Samara region pharmacy segment in the period of 2015–2019 with the predominance of the ATC (Anatomical Therapeutic Chemical Classification System) J01D group, primarily cephalosporins (38%), mainly by the parenteral administration route. The share of macrolides (J01F) consumption in volume terms was 14.9%, of fluoroquinolones (J01M) – 11.3%, beta-lactam antibiotics with beta-lactamase inhibitors – 10.7%, beta-lactam antibiotics penicillins (J01C) – 8.1%. Compared to 2019, in 2020, under the conditions of the COVID-19 pandemic, the total consumption of AMPs increased by 2.1 times. In the "Other beta-lactam antibiotics" group with a predominant proportion of cephalosporins, there was an increase by 3.2 times, in the "Macrolides and lincosamides" group – by 3.5 times, in "Quinolone derivatives" – by 2.6 times. The noted facts should be assessed as the phenomenon that can have a direct impact on the growth of an antibiotic resistance on a population scale. Among antivirals, the largest consumption increase was noted for oseltamivir and rimantadine. In absolute terms, the volume of antiviral preparations consumption in 2020 increased by 2.4 times, which was accompanied by an increase in the cost of one package by 55.8%.

Conclusion. In the period of spreading a novel coronavirus infection, a significant increase in the consumption of antimicrobial and antiviral preparations (up to 20 times for certain pharmacotherapeutic groups and names) was notified, which may negatively affect the growth of the antibiotic resistance in the population.

Keywords: systemic antimicrobial preparations; antiviral drugs; pharmacy segment; consumption; COVID-19 pandemic

Abbreviations: AMPs – antimicrobial preparations; MP – medicinal preparations; DDD – Defined Daily Dose; INN – international non-proprietary name; ATC – Anatomical Therapeutic Chemical Classification System; ARVI – acute respiratory viral infection; RNA – ribonucleic acid.

For citation: I.K. Petrukhina, P.A. Lebedev, I.I. Sirotko, T.K. Ryazanova, E.P. Gladunova, A.A. Garanin. Consumption details of systemically acting antiviral and antimicrobial preparations in period of novel coronavirus infection spread in retail sector of Samara region pharmaceutical market. *Pharmacy & Pharmacology.* 2022;10(5):446-459. **DOI:** 10.19163/2307-9266-2022-10-5-446-459

Для цитирования: И.К. Петрухина, П.А. Лебедев, И.И. Сиротко, Т.К. Рязанова, Е.П. Гладунова, А.А. Гаранин. Особенности потребления противовирусных и антимикробных препаратов системного действия в период распространения новой коронавирусной инфекции в розничном секторе фармацевтического рынка Самарской области. *Фармация и фармакология.* 2022;10(5):446-459. **DOI:** 10.19163/2307-9266-2022-10-5-446-459

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

ОРИГИНАЛЬНАЯ СТАТЬЯ

DOI: 10.19163/2307-9266-2022-10-5-446-459

ОСОБЕННОСТИ ПОТРЕБЛЕНИЯ ПРОТИВОВИРУСНЫХ И АНТИМИКРОБНЫХ ПРЕПАРАТОВ СИСТЕМНОГО ДЕЙСТВИЯ В ПЕРИОД РАСПРОСТРАНЕНИЯ НОВОЙ КОРОНАВИРУСНОЙ ИНФЕКЦИИ В РОЗНИЧНОМ СЕКТОРЕ ФАРМАЦЕВТИЧЕСКОГО РЫНКА САМАРСКОЙ ОБЛАСТИ

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Получена 25.04.2022 После рецензирования 05.07.2022 Принята к печати 08.09.2022

Анализ структуры потребления лекарственных препаратов в период пандемии COVID-19 в аптечной сети отражает существующую амбулаторную практику и позволяет сделать обобщенные выводы о соответствии ее стандартам фармакотерапии.

Цель. Сравнительный анализ популяционного потребления антимикробных и противовирусных лекарственных препаратов, реализованных в розничном секторе фармацевтического рынка Самарской области в 2015–2021 гг.

Материалы и методы. Исследование проведено в розничном секторе фармацевтического рынка Самарской области. В качестве материала исследования использовали сведения о номенклатуре и объемах отпуска антибактериальных и отдельных противовирусных лекарственных препаратов в период распространения новой коронавирусной инфекции (в 2020 г.) в сети аптек Самарской области. Данные сопоставлены с показателями реализации лекарственных препаратов в 2015–2019 гг. Использованы методы ретроспективного, сравнительного, графического, методологического, контент-анализ и статистические методы анализа.

Результаты. Авторами установлена значительная деформация потребления антимикробных препаратов системного действия в аптечном сегменте Самарской области в период 2015–2019 гг. с преобладанием группы АТХ J01D с доминированием цефалоспоринов (38%) преимущественно парентерального пути введения. Доля потребления в натуральном выражении макролидов (J01F) составила 14,9%, фторхинолонов (J01M) – 11,3%, бета-лактамных антибиотиков с ингибиторами бета-лактамаз – 10,7%, бета-лактамных антибиотиков-пенициллинов (J01C) – 8,1%. В сравнении с 2019 г., в 2020 г. в условиях пандемии COVID-19 общее потребление АМП увеличилось в 2,1 раз. В группе «Другие бета-лактамные антибиотики» с преимущественной долей цефалоспоринов произошло увеличение в 3,2 раза, «Макролиды и линкозамиды» – в 3,5 раз, «Производные хинолона» – в 2,6 раза. Отмеченные факты следует оценивать как фактор, который может оказать непосредственное влияние на рост антибиотикорезистентности в популяционном масштабе. Среди противовирусных препаратов наибольший рост потребления отмечен для осельтамивира и римантадина. В абсолютном выражении объем потребления противовирусных лекарственных препаратов в 2020 г. увеличился в 2,4 раза, что сопровождалось увеличением стоимости одной упаковки на 55,8%.

Заключение. В период распространения новой коронавирусной инфекции отмечен значительный рост потребления антимикробных и противовирусных лекарственных препаратов (по отдельным фармакотерапевтическим группам и наименованиям – до 20 раз), что может негативным образом отразиться на росте антибиотикорезистентности у населения.

Ключевые слова: антимикробные препараты системного действия; противовирусные препараты; аптечный сегмент; потребление; пандемия COVID-19

Список сокращений: АМП – антимикробные препараты; ЛП – лекарственные препараты; DDD – суточная поддерживающая доза; МНН – международное непатентованное наименование; АТХ – анатомо-терапевтическо-химическая классификация; ОРВИ – острая респираторная вирусная инфекция; РНК – рибонуклеиновая кислота.

INTRODUCTION

A new coronavirus infection has become an unprecedented challenge for the health care system of the Russian Federation (RF), the pharmaceutical industry, and the regional drug supply system [1–3]. The greatest difficulty is the search for the effective methods of etiotropic treatment. In the absence of new medicinal preparations (MPs) that effectively suppress the SARS-Cov-2 replication, screening of the known antiviral agents seems relevant. Obtaining reliable data on the clinical benefit of drugs has proved to be very problematic in the current situation and has led to the fact that the preference of physicians and patients has become a criterion for benefit [4–8].

The consumption structure of antimicrobial and antiviral preparations by the population through the pharmacy network, reflecting the outpatient practice in the period preceding the pandemic, as well as the

dynamics of the MPs consumption during the pandemic, seems important in terms of compliance with the directions of modern recommendations[1]. Herewith, the scope of use of antimicrobial and antiviral preparations in the outpatient practice of the Russian Federation regions is not reliably known; such data are rare in the press. At the same time, the problem of the antibiotic resistance may become particularly acute in the near future in case of an irrational increase in the AMPs use [9–15]. The AMPs prescribtion and use should always be justified, since the irrational use of this pharmacotherapeutic group MPs can lead to a noticeable increase in the antibiotic resistance on a population-based study [1, 12, 16]. The dynamics of the antimicrobial and antiviral preparations consumption makes it possible to indirectly assess the optimality of pharmacotherapy for the novel coronavirus infection, as well as to establish compliance with current guidelines. The results of such an analysis can be used to improve the medicinal preparations efficiency and safety at the population level [17–26].

In this regard, marketing research is becoming highly relevant, making it possible to identify trends in the population consumption of antimicrobial and antiviral preparations in the retail sector of the pharmaceutical market.

THE AIM. Comparative analysis of population consumption of antimicrobial and antiviral medicines sold in the retail pharmacies of the Samara region in 2015–2021.

MATERIALS AND METHODS

The study was conducted on the retail sector example of the Samara region pharmaceutical market. According to a number of demographics, medical, social, economic and infrastructural indicators, this region is among the most developed ones of the Volga Federal District and the Russian Federation. The regional pharmaceutical market is highly concentrated with a high degree of competition.

The material of the study was information on the list of items and volumes of antimicrobial and antiviral preparations sale during the spread of the coronavirus infection (in 2020–2021) in the pharmacy network of the Samara region. The analyzed pharmacy network includes 30 pharmacies located in different municipalities of the Samara region. These network pharmacies have a wide range of medicines and other pharmacy products (about 30 thousand items).

The following analytical methods were used: methods of the retrospective analysis (changes in the indicators of the retail sale of medicines to the population during 2015–2021), a comparative analysis (the one of individual groups and intragroup indicators), a graphical analysis (presentation pharmaceutical sales time series), a methodological analysis (the identification of common characteristics for the objects, the analysis relationships between the phenomena), content analysis (the analysis of the text arrays content about the medicinal preparations implementation in the analyzed period) and statistical methods of the analysis. A statistical processing was performed using IBM SPSS Advanced Statistics 24.0 No. 5725-A54 (IBM, USA).

The representativeness assessment of the sample in the conducted studies was carried out by assessing the number of the medicinal preparations purchases of the groups under consideration in the analyzed retail network of the Samara region pharmaceutical market. For this purpose, the following formula was used:

$$m = 2\sqrt{n},$$

where: m is the resulting sample size; n is the size of the general totality.

In the conducted studies, the general totality is understood as the Samara region population (n in 2021 was 3 154 200 people). Therefore, to ensure the representativeness of the sample size, it should be 3 552 purchases of antibacterial medicinal preparations in 2020. In the studied pharmacy chain, in 2015-2021, about 50 thousand purchases were made annually, which confirms the representativeness of the data obtained, i.e. the correspondence of the characteristics of the sample to the characteristics of the general population.

As for the medicinal preparations, the cost of one defined daily dose (DDD) was calculated by dividing the total cost of the medicinal preparations packages with one INN by the total number of DDDs.

RESULTS

Consumption of systemic antimicrobials

For the period of 2015–2021, in the studied retail segment sector of the Samara region pharmaceutical market, about 18 million packages of MPs and other pharmacy products were sold, 2.57% of which were accounted for AMPs. In the total volume of the dispensed packages, the average share of AMPs for the period of 2015–2021 was 3.38%. For comparison, in the Russian pharmaceutical market, the share of AMPs sales by volume was about 11.69%. Herewith, 43.7% of purchases were made at the expense of the population's personal funds. In general, in 2015–2021, the range of AMPs averaged (± standard deviation, SD) 54±3 international non-proprietary names (INN), which corresponds to 138±3 trade names.

[1] Interim guidelines "Prevention, diagnosis and treatment of a new coronavirus infection (COVID-19). Version 3 (03.03.2020). Available from: http://edu.rosminzdrav.ru/fileadmin/user_upload/specialists/COVID-19/Vremennye_MR_COVID-19_03.03.2020__versija_3__6-6_ver1.pdf. Russian

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DOI: 10.19163/2307-9266-2022-10-5-446-459

A noticeable increase in the consumption of antibacterial medicinal preparations in the retail sector of the Samara region pharmaceutical market was notified in 2020 (compared to 2019, the sales in packages increased by 2.12 times), which exceeds the average annual fluctuations in realized demand for this group of MPs in 2015-2019. The dynamics study of the realized demand for individual systemic AMPs subgroups (in accordance with subgroups in the anatomical-chemical-therapeutic [ATC-]classification) revealed a significant increase in the number of dispensed packages in 2020 for the following subgroups: J01D "Other beta-lactam antibacterials", J01F "Macrolides, lincosamides and streptogramins", J01M "Quinolone antibacterials" (Table 1, Fig. 1). In the authors' opinion, this circumstance is due to the influence of the novel coronavirus infection spread and, in some cases, the rush demand for drugs from certain pharmacotherapeutic groups.

Herewith, at the end of 2021, the level of the consumer demand returned to the values of 2015–2019. Possible reasons for this trend may be as follows: the formation of AMPs stocks in home medicine cabinets by the end of 2020; the implementation of programs providing the COVID-19 patients with the MPs prescribed for them at the expense of the federal budget; a change in the algorithm for treating outpatients (in 2021, at the outpatient stage of medical care, AMPs were excluded from pharmacotherapy regimens).

In 2020, against the background of a significant increase in demand for drugs of ATC subgroups J01D "Other beta-lactam antibacterials", J01F "Macrolides, lincosamides and streptogramins", J01M "Quinolone antibacterials", a decrease in the share of the AMPs packages total sales for the drugs of J01A subgroups "Tetracyclines" was notified. Besides, there were J01C "Beta-lactam antibacterials, penicillins", J01G "Aminoglycoside antibacterials", J01X "Other AMPs", and "Beta-lactam antibacterials with beta-lactamase inhibitors". As Fig. 2 shows, in 2021, there was a return to the existing picture of demand for AMPs in 2015–2019, with the exception of aminoglycosides, the number of sold packages decreased in 2019–2021 by an average of 35% annually (Fig. 2).

In 2015–2021, the maximum share of the total volume of the realized demand was accounted for the ATCs subgroup J01D "Other beta-lactam antibiotics" (Fig. 3). The average value of the share (± SD) of the volume of the realized demand in real terms for this ATCs subgroup was 38.5±5.6%.

In 2020, against the background of the beginning of a novel coronavirus infection pandemic, an increase in the proportion of the medicines of this ATCs subgroup to 47.6% was notified. In 2021, the value of the share of J01D MPs in the total volume of the realized demand returned to the previous average annual values (39.0%) (Table 1, Fig. 1). Over the past two years, an extraordinary demand for these drugs was notified in October, although in 2021 it was less pronounced compared to 2020 (Fig. 4).

In 2020, as in the previous period (2015–2019), in the "Other beta-lactam antibacterials" group, ceftriaxone preparations had the largest volumes of consumption in volume terms (median 72.2%), ranging from 63.5 % in 2020 to 76.8% in 2017 of the total realized demand for drugs from this ATC subgroup. At the same time, in 2020, in the overall structure of the dispensed drugs packs of the ATC J01D subgroup, there was a sharp increase in the share and number of the dispensed packs for the drugs of cefazolin, cefditoren and meropenem, which returned to their previous values in 2021. The greatest demand in the outpatient practice is for parenteral preparations from the group of cephalosporins "Other β-lactam antibacterials", including ceftriaxone, as well as cefazolin (8.3–12.9%) and cefotaxime (7.7–5.4%). In accordance with the current recommendations, amoxicillin and its combination with clavulanic acid (β-lactam antibiotics and β-lactam penicillins with β-lactamase inhibitors) should be the basic treatment for the vast majority of bacterial infections in the outpatient practice. However, the frequency of amoxicillin sales from 2015 to 2021 decreased from 14.1% to 6.1% (Table 1). In combination with β-lactamase inhibitors, where the main share of MPs is accounted for amoxicillin (96.9–81.6%), the frequency of this antibacterial sales does not exceed 10% in total in 2020 (Table 1).

According to the literature data, in outpatient practice, the first-line MPs of choice is amoxicillin, administered orally for pneumonia, exacerbation of chronic obstructive pulmonary disease, acute rhinosinusitis, bronchitis, acute tonsillitis, and uncomplicated skin and soft tissue infection [8, 15, 17]. At the same time, a high consumer demand for β-lactam antibiotics was notified in the retail sector of the Samara Region pharmaceutical market.

In 2015–2021, a significant share in the structure of consumption volume in physical terms was also occupied by medicines of J01F ATC subgroups "Macrolides, lincosamides and streptogramins" (15.0%) and J01M "Quinolone antibacterials" (11.4%) (Table 1, Fig. 1). In 2020, for the group "Macrolides, lincosamides and streptogramins", an increase in the share was notified (in the total structure of the dispensed packages – up to 19.9%). In 2021, this share decreased compared to 2020, but continued to be high compared to the average annual data for the period of 2015–2019 (14.4%). When analyzing the demand by months, it was found out that in 2020 and 2021, the demand for these AMPs, as in the case of the ATC subgroup J01D "Other beta-lactam antibacterials", peaked in October (Fig. 5).

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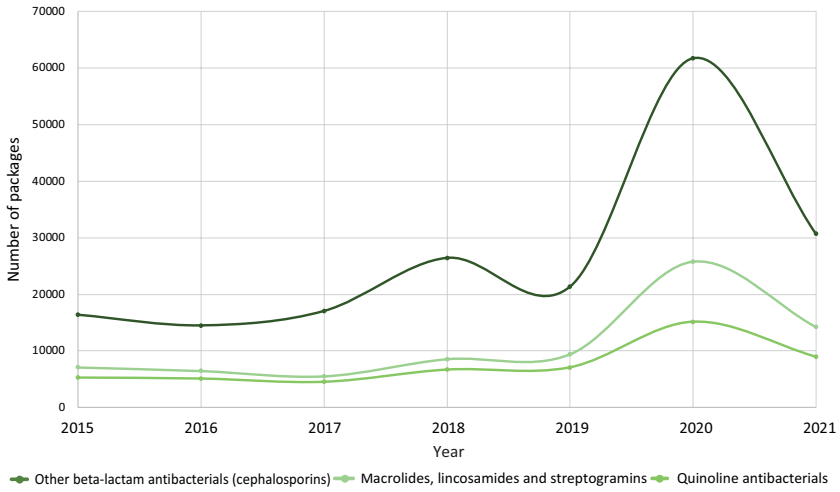

Figure 1 – Dynamics of realized demand for some groups of antibacterial medicinal preparations, pandemic sales of which increased

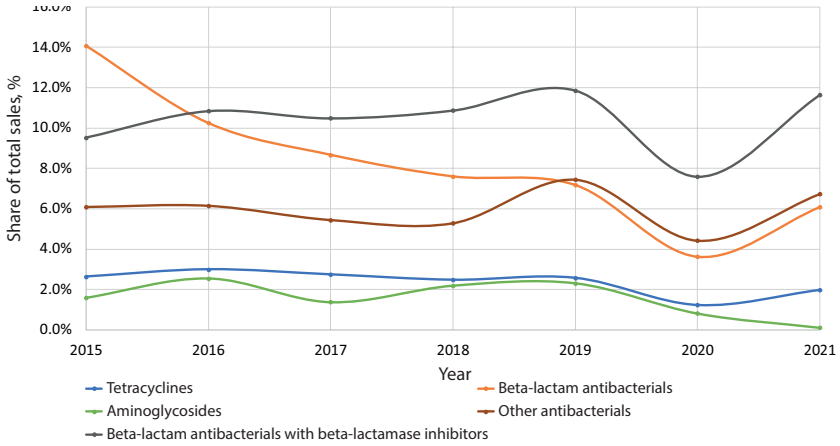

Figure 2 – Dynamics of realized demand for some groups of antibacterials, sales of which decreased or did not change during pandemic

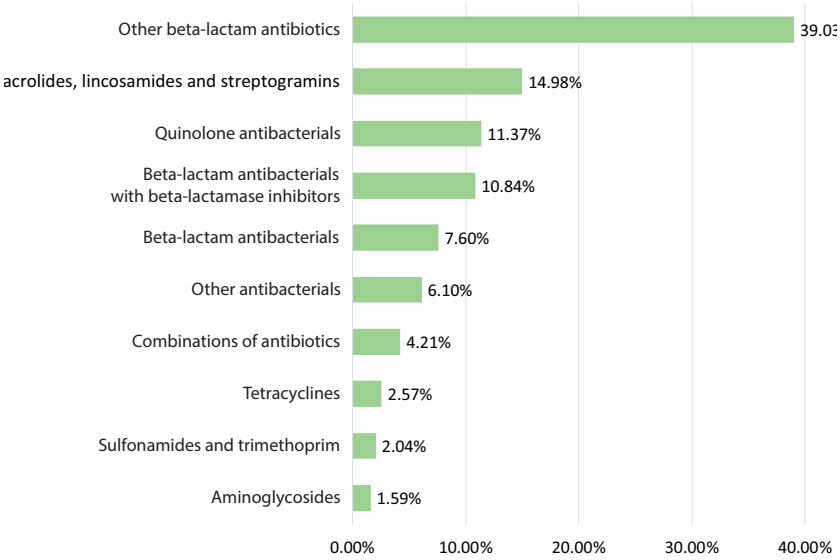

Figure 3 – Median shares of realized demand volume for antibacterial MPs in 2015–2021

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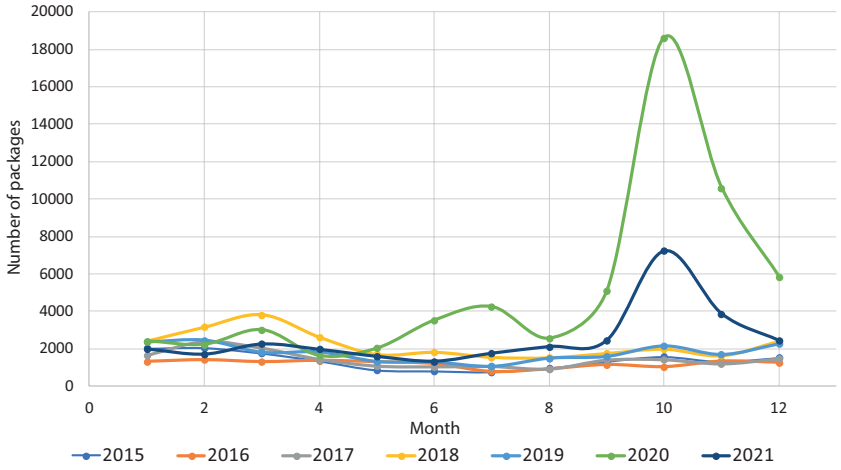

Figure 4 – Dynamics of demand for ATC subgroup medicinal preparations – J01D "Other beta-lactam antibacterials" in 2015–2021 (by months)

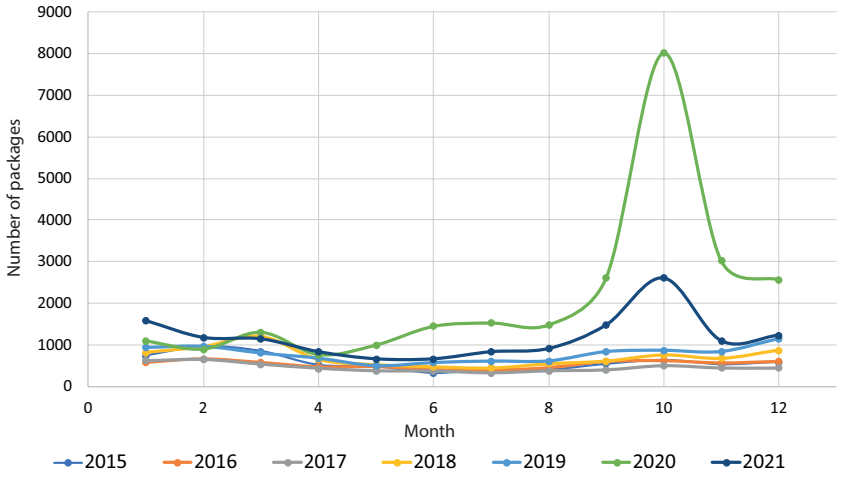

Figure 5 – Dynamics of demand for ATC subgroup medicinal preparations – J01F "Macrolides, lincosamides and streptogramins" in 2015–2021 (by months)

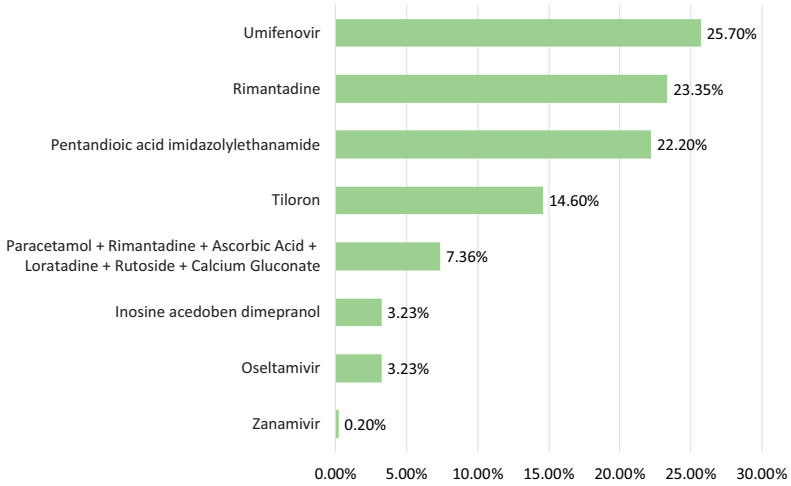

Figure 6 – Median volume shares of realized demand for antiviral medicinal preparations in 2015–2020

ISSN 2307-9266 e-ISSN 2413-2241

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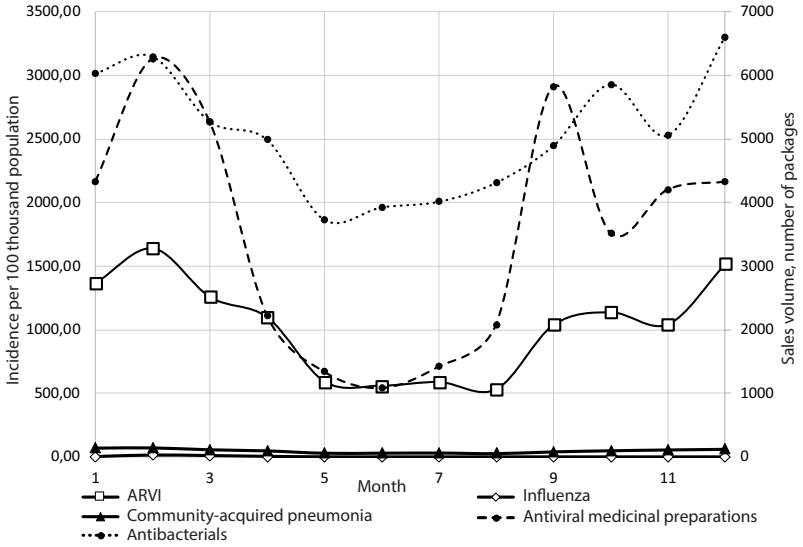

Figure 7 – Incidence of acute respiratory viral infections, influenza, COVID-19, community-acquired pneumonia in 2019 (according to Samara region Rospotrebnadzor), volumes of realized antiviral MPs and AMPs packages

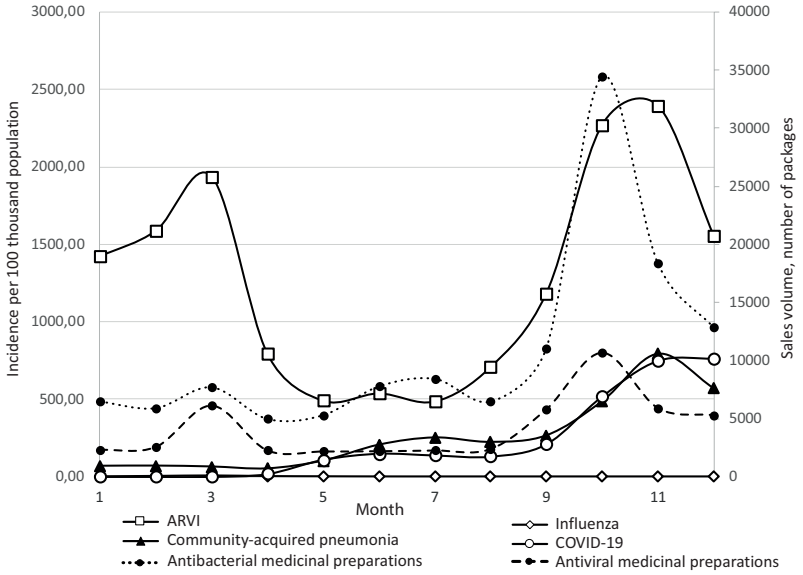

Figure 8 – The incidence of acute respiratory viral infections, influenza, COVID-19, community-acquired pneumonia in 2020 (according to Samara region Rospotrebnadzor), volumes of realized of antiviral MPs and AMPs packages

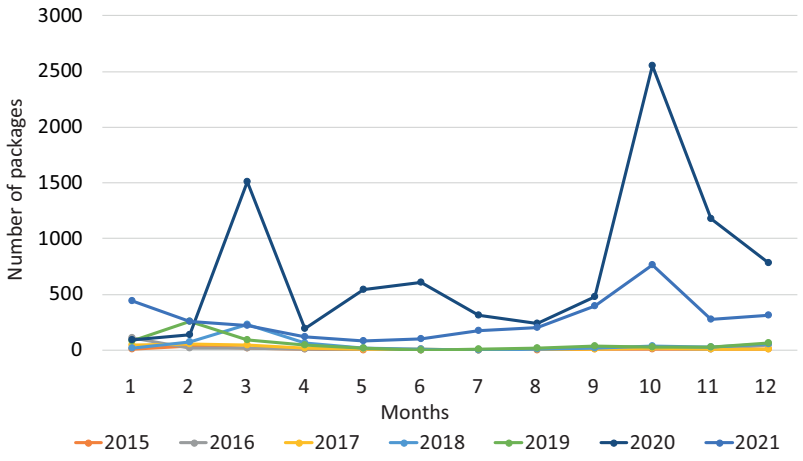

Figure 9 – Dynamics of oseltamivir demand in 2015–2021

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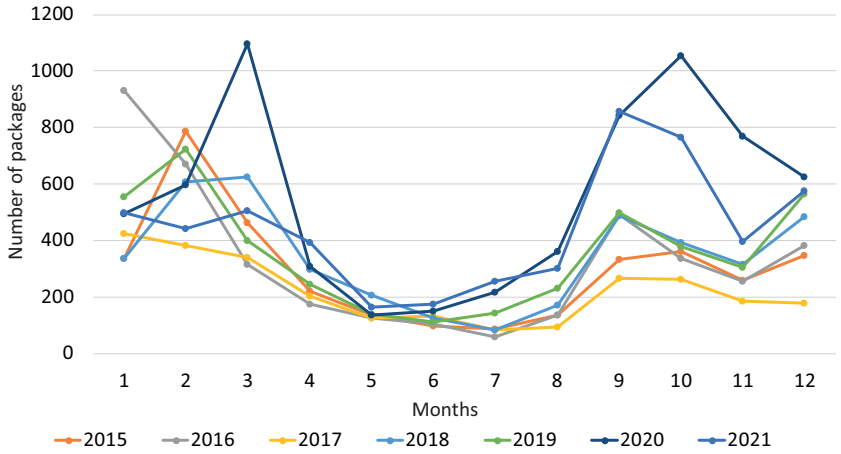

Figure 10 – Dynamics of rimantadine demand in 2015–2020

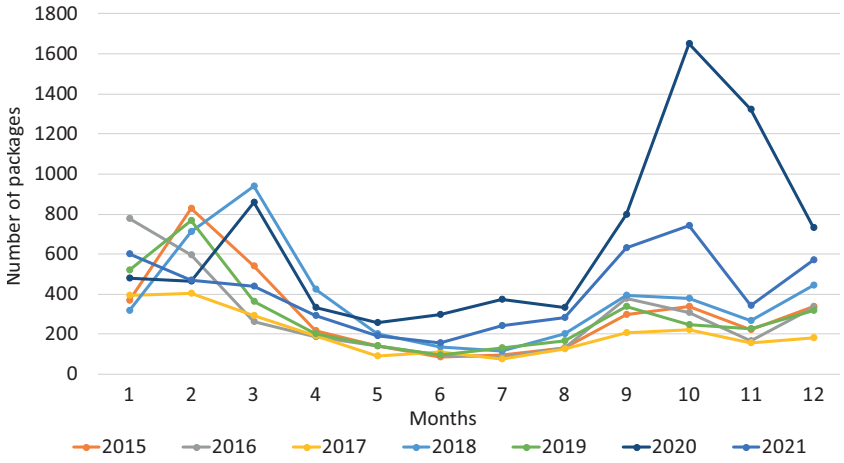

Figure 11 – Dynamics of imidazolylethanamide pentadioic acid demand in 2015–2020

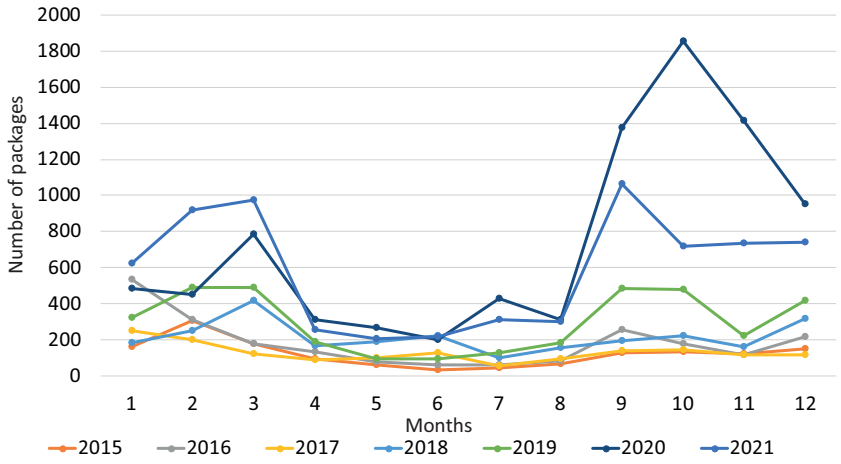

Figure 12 – Dynamics of thyrolon demand in 2015–2020

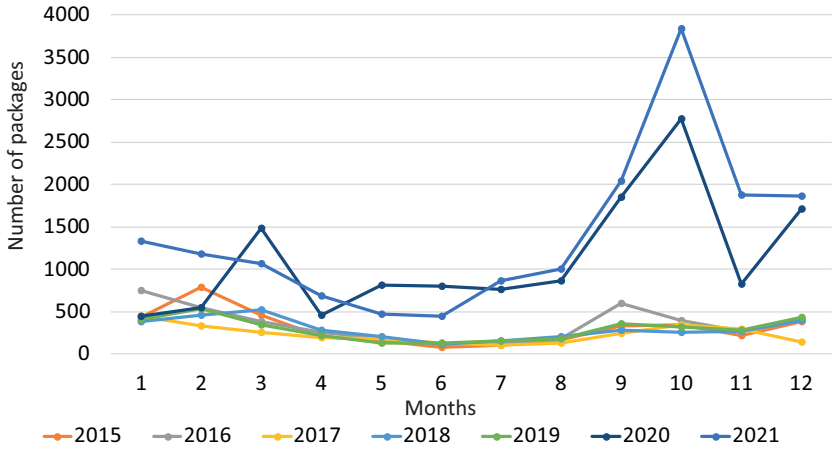

Figure 13 – Dynamics of umifenovir demand in 2015–2020

Table 1 – Consumption structure of antibacterials in retail sector of Samara region pharmaceutical market

ATC-subgroups and medicines (INN)	Share of realized demand volume (in packages), %*							
	Year							Median (minimum-maximum) in 2015–2021
	2015	2016	2017	2018	2019	2020	2021	
J01A Tetracyclines	**2.6**	**3.0**	**2.7**	**2.5**	**2.6**	**1.2**	**2.0**	**2.6 (1.2–3.0)**
Doxycycline	85.5	88.7	85.6	84.3	86.5	80.2	87.7	87.6 (80.2–88.7)
Tetracycline	14.5	11.1	12.8	15.3	13.3	19.1	9.9	13.3 (9.9–19.10)
J01C Beta-lactam penicillins	**14.1**	**10.3**	**8.7**	**7.6**	**7.2**	**3.6**	**6.1**	**7.6 (3.6–14.1)**
Amoxicillin	58.6	81.3	85.4	93.3	91.3	95.5	100.0	91.3 (58.6–100.0)
J01D Other beta-lactam antibacterials	**34.0**	**33.5**	**41.0**	**41.4**	**35.0**	**47.6**	**39.0**	**39.0 (33.5–47.6)**
Ceftriaxone	71.8	65.8	76.8	74.0	72.5	63.5	75.7	72.6 (63.5–76.8)
Cefazolin	8.5	8.3	5.1	4.0	3.5	12.8	4.5	5.1 (3.5–12.8)
Cefotaxime	5.5	7.8	3.1	5.4	5.1	6.3	2.5	5.4 (2.5–7.8)
Cefixime	4.6	5.4	5.9	6.8	8.1	3.9	8.3	5.9 (3.9–8.3)
Cefditoren	0.0	0.4	0.3	1.0	1.8	8.1	2.1	1.0 (0.00–8.1)
Beta-lactam antibacterials with beta-lactamase inhibitors	**9.5**	**10.8**	**10.5**	**10.9**	**11.9**	**7.6**	**11.7**	**10.8 (7.6–11.9)**
Amoxicillin + clavulanic acid	88.2	94.6	95.4	96.9	91.9	81.6	93.3	93.3 (81.6–96.9)
J01E Sulfonamides and trimethoprim	**2.1**	**2.0**	**1.9**	**2.2**	**2.3**	**1.2**	**1.5**	**2.0 (1.2–2.3)**
Co-trimoxazole	85.8	85.7	81.8	85.9	89.6	91.2	98.8	98.9 (81.8–98.8)
Sulfadimethoxine	12.8	12.9	10.6	12.8	9.8	8.4	1.1	10.6 (1.1–12.9)
J01F Macrolides, lincosamides and streptogramins	**14.8**	**15.0**	**13.3**	**13.4**	**15.5**	**19.9**	**18.1**	**15.0 (13.3–19.9)**
Azithromycin	48.2	46.1	44.6	50.4	49.4	79.7	68.7	49.4 (44.6–79.7)
Clarithromycin	23.9	25.3	28.6	27.3	30.2	12.4	20.0	25.3 (12.4–30.2)
Josamycin	8.4	10.4	10.1	7.8	8.2	3.4	5.5	8.2 (3.4–10.4)
Lincomycin	9.0	8.1	8.0	7.4	7.5	2.7	4.2	7.5 (2.7–9.0)
J01G Aminoglycosides	**1.6**	**2.5**	**1.4**	**2.2**	**2.3**	**0.8**	**0.1**	**1.6 (0.1–2.5)**
Gentamicin	19.0	14.1	18.1	13.0	11.1	29.2	72.4	18.1 (11.1–72.4)
Amikacin	51.5	37.9	65.8	86.8	88.5	70.8	27.6	65.8 (27.6–88.5)
J01M Quinolone antibacterials	**10.9**	**11.8**	**10.9**	**10.5**	**11.6**	**11.7**	**11.4**	**11.4 (10.5–11.8)**
Ciprofloxacin	37.1	37.4	37.3	36.1	35.6	21.8	30.4	36.1 (21.8–37.4)
Levofloxacin	25.9	31.1	33.7	37.1	38.1	59.8	49.6	37.1 (25.9–59.8)
Norfloxacin	18.3	17.6	15.4	15.2	15.6	7.8	13.0	15.4 (7.8–18.3)
J01X Other antibacterials	**6.1**	**6.2**	**5.4**	**5.3**	**7.4**	**4.4**	**6.7**	**6.1 (4.4–7.4)**
Metronidazole	96.0	92.8	93.6	91.8	94.8	91.7	92.9	92.9 (91.7–96.0)
Antibiotic combinations	**4.3**	**4.9**	**4.2**	**4.1**	**4.3**	**2.0**	**3.4**	**4.2 (2.0–4.9)**
Benzathine benzylpenicillin + Benzylpenicillin procaine	37.6	35.3	23.9	33.3	21.1	16.3	18.5	23.9 (16.3–37.6)
Benzathine benzylpenicillin + Benzylpenicillin procaine + Benzylpenicillin sodium	18.7	11.7	16.7	13.7	13.3	18.0	13.6	13.7 (11.7–18.7)
Ciprofloxacin + tinidazole	39.8	44.2	51.6	48.2	57.9	63.5	66.5	51.6 (39.8–66.5)

Note: * – indicators of realized demand in packages were used for the analysis. For pharmacotherapeutic groups, the shares of the total volume of realized demand are indicated, for INN it is the share of the volume of realized demand for a particular pharmacotherapeutic group; the table shows INNs with the largest volumes of realized demand within each pharmacotherapeutic group.

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Table 2 – Consumption structure of antiviral medicinal preparations prescribed for treatment of respiratory viral infections in retail sector of Samara region pharmaceutical market

Antiviral medicinal preparations (INN)	Share in realized demand structure, %						
	2015	2016	2017	2018	2019	2020	2021
Zanamivir	0.10	0.40	0.20	0.34	0.23	0.19	0.06
Pentandioic acid imidazolylethanamide	24.97	22.20	22.64	25.63	16.80	15.85	11.96
Inosine acedoben dimepranol	3.49	2.58	2.95	4.63	4.82	2.09	3.23
Oseltamivir	0.71	1.60	2.11	3.23	3.25	17.26	8.04
Rimantadine	24.74	25.55	24.69	23.35	20.35	13.34	12.81
Tiloron	10.26	14.10	14.34	14.60	20.56	17.71	17.00
Umifenovir	25.61	26.97	25.70	19.97	17.13	26.79	40.13
Favipiravir*	–	–	–	–	–	0.44	1.18
Paracetamol + Rimantadine + Ascorbic Acid + Loratadine + Rutoside + Calcium Gluconate	10.11	6.61	7.36	8.23	0.17	6.22	5.52

Note: for the analysis, indicators of realized demand in packages were used; * – Favipiravir preparations were registered in Russia in 2020.

Table 3 – Average cost of treatment for one maintenance daily dose of antiviral MPs in 2019–2020

INN	Median (minimum-maximum) cost of 1 DDD, rub. (2019)	Median (minimum-maximum) cost of 1 DDD, rub. (2020)	Median (minimum-maximum) cost of 1 DDD, rub. (2021)	Median (minimum-maximum) cost of 1 DDD in treatment with original drugs, rub. (as exemplified by 2021)
Zanamivir	234.09 (234.09–234.09)	276.33 (256.33–300.10)	280.32 (280.32–280.32)	280.32 (280.32–280.32)
Pentandioic acid imidazolylethanamide	82.21 (77.34–87.08)	103.38 (85.86–120.70)	102.09 (73.33–122.79)	102.09 (73.33–122.79)
Inosine Acedoben Dimepranol	192.43 (93.19–417.22)	209.75 (115.25–374.58)	216.98 (145.00–312.09)	247.40 (187.88–245.72)
Oseltamivir	174.88 (133.92–291.74)	202.99 (167.22–288.68)	210.53 (126.46–248.71)	245.38 (242.05–248.71)
rimantadine	11.64 (6.87–255.80)	20.65 (11.32–261.82)	16.92 (7.72–157.55)	–
Tiloron	102.09 (41.66–143.53)	102.26 (51.99–151.42)	92.27 (46.29–134.74)	133.43 (132.12–134.74)
Umifenovir	169.06 (97.77–291.56)	188.25 (99.85–335.25)	238.62 (119.60–384.22)	253.10 (246.88–384.22)
Favipiravir	–	2 073.68 (1 795.27–2 073.68)	1 255.79 (1 001.78–2 382.38)	–

In 2020–2021, in the group "Macrolides, lincosamides and streptogramins", there was an increase in the share of azithromycin-containing medicines in the total number of the dispensed packages of this ATC subgroup, which amounted to 79.7% and 68.7%, respectively (compared to the average value of 47.8±2.4% in 2015–2019). The demand for other medicines within this ATC subgroup remained at the same level or slightly decreased. According to the document of the World Health Organization (WHO) and domestic recommendations, macrolides should be considered as second-line drugs in the treatment of respiratory infections [16].

The group of fluoroquinolones is considered as reserve AMPs and is not recommended for the treatment of acute uncomplicated infections in the outpatient practice. In this study, their share of the total number of the dispensed packages remained approximately at the same level and averaged 11.4±0.5%. The drugs of the ATC subgroup "Quinolone antibacterials" were more often applied for. They were the medicines containing the active ingredients ciprofloxacin, levofloxacin or norfloxacin (medians for the period of 2015–2021 – 36.1%, 37.1% and 15.4% of the volume of this group realized demand, respectively). At the same time, in 2020–2021, there was an increase in demand for "respiratory" drugs of this group: levofloxacin and moxifloxacin.

In 2020, for the ATC subgroups "Other beta-lactam antibacterials", the number of the dispensed packages increased by 3.2 times, for "Macrolides, lincosamides and streptogramins" – by 3.5 times, for "Quinolone antibacterials" – by 2.6 times in 2020 (compared to the average values of the dispensed MPs packages of these subgroups in 2015–2019). A similar increase was notified for individual INNs: ceftriaxone – by 2.9 times, cefazolin – by 7.5 times, by cefotaxime – by 3.3 times, cefditoren –

by 33 times, meropenem – by 90 times, azithromycin – by 5.8 times, levofloxacin – by 4.7 times, moxifloxacin – by 7.0 times. Interest in cefditoren is obviously due to the fact that it was included in the domestic clinical guidelines for the treatment of community-acquired pneumonia in 2018. The number of dispensed packages of the remaining drugs was approximately at the level of the previous period, respectively, their share in the total consumption structure in 2020 slightly decreased.

For most of the groups and individual items of the MPs under consideration, there was an increase in the cost of 1 DDD in 2020 compared to 2019. The cost of one package increased by 15%, the median cost of 1 DDD increased by 20%. In 2021, the corresponding values compared to 2020, were 3.5% and 5.0%, respectively.

Antiviral medicinal preparations

The range of antiviral medicinal preparations approved for use in the treatment of acute respiratory viral infections was represented by 8 out of 9 INNs registered in the Russian Federation (except baloxavir carboxyl, registered in September 2020), which corresponded to 35 trade names. At the end of 2021, the share of antiviral medicinal preparations in the total structure of the dispensed packages amounted to 0.92% (for comparison, in 2018 and 2020 – 0.93% and 1.02%, respectively). In absolute terms (in terms of the package quantity), in 2020, the volume of consumption of medicinal preparations increased by 2.38 times, which exceeds the average annual fluctuations in the consumer demand in the period preceding the start of the novel coronavirus infection spread (an average increase by 1.15 times).

Among antiviral medicines, the largest consumption volume in physical terms was accounted for the MPs of imidazolylethanamide pentadioic acid (median 22.2% of the realized demand volume, the range of 15.9% – 25.6%), umifenovir (25.7%, 16.7% – 40.1%) and rimantadine (23.4%, 12.8% – 25.6%) (Table 2, Fig. 6). In 2020–2021, the consumption of rimantadine naturally decreased to 13.3 and 12.8%, respectively, since the indications for its use do not refer to ARVI, SARS-Cov-2 infections, while the incidence of influenza in 2020, compared to 2019, decreased by 25%.

In the period of the new coronavirus infection spread (2020), a significant increase in the share of oseltamivir was notified in the overall consumption structure (up to 17.26% compared to 0.71% in 2015, 1.60% in 2016, 2, 11% – in 2017, 3.23% – in 2018 and 3.25% – in 2019), and the number of the dispensed packages (by 23.5 times compared to the average value in 2015–2019). Oseltamivir, a neuraminidase inhibitor approved for the treatment of influenza, has no documented *in vitro* activity against SARS-CoV-2. It seems that understanding of oseltamivir ineffectiveness in the SARS-CoV-2 infection and diagnostics advance led to a decrease in the share of the total oseltamivir demand to 8.0% in 2021.

In addition, in 2020, a number of the dispensed imidazolylethanamide pentanedioic acid, tilorone and umifenovir packages increased by 2.2, 3.9 and 3.8 times, respectively, compared with the average values in 2015–2019). Inosine acedoben dimepranol has been registered in more than 70 countries as an antiviral and immunomodulatory MP that has received a good evidence base since 1971. It has been shown to inhibit the replication of herpes simplex virus, cytomegalovirus and Epstein-Barr virus, human papillomavirus, human immunodeficiency virus, influenza viruses and SARS [22, 24]. Nevertheless, the positive qualities of the medicines did not affect its consumption frequency, which remains one of the lowest in the considered segment of the pharmacy market (Table 2).

Umifenovir has a high sales rating in this study (Table 2, Fig. 6), since it is officially recommended by the Ministry of Health of the Russian Federation to be used in patients with mild COVID-19, as well as in patients with signs of SARS and unconfirmed SARS-Cov-2 [25, 27]. In 2021, umifenovir MPs were accounted for 40.1% of total antiviral MPs sales.

Among the antiviral medicinal preparations used to treat acute respiratory viral infections, the highest cost per 1 DDD was for medicines containing favipiravir, a substance active against the novel coronavirus infection (according to the results of 2021, the median was 1,255.79 rubles, in the range from 1,001.78 to 2,382.38 rub.).

Taking into account the fact that in 2019 the MPs containing favipiravir was not introduced in the retail sector, the increase in the cost of 1 DDD of antiviral MPs in 2020, compared to 2019, occurred by 43.1% (at the same time, the average cost of one package increased by 55.8%). Excluding these medicines, the average cost of 1 DDD increased by 9.4% (the average price of one package increased by 13.7%). In 2021, there were no significant changes in the price level compared to 2020, with the exception of umifenovir MPs, for which the cost of 1 DDD increased by 26.8% (Table 3). In all the cases, the cost of treatment with original MPs exceeded the cost of treatment with generic MPs (if available on the pharmaceutical market).

Fig. 7 and 8 show the incidence of acute respiratory viral infections, influenza, community-acquired pneumonia in 2019 and 2020, as well as COVID-19[2] in 2020. In addition to the well-known dynamics of

[2] [Information materials of the Office of Rospotrebnadzor of the Samara Region on the epidemiological situation of the incidence of SARS and influenza in the Samara Region for the period 2019 and 2020]. Available from: https://www.63.rospotrebnadzor.ru

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DOI: 10.19163/2307-9266-2022-10-5-446-459

a decrease in the incidence in the summer period, an increase in the incidence of the acute respiratory viral infections by 24% in 2020 and a decrease in the incidence of influenza by 25% compared to 2019 should be notified. The sales volumes of antiviral MPs presented in these graphs in volume terms show clearly defined seasonal fluctuations with maximum values in the autumn-winter-spring period, which corresponds to the period of the highest incidence of SARS. As expected, the smallest volumes of antiviral MPs sales during 2015–2020 were recorded in July and August. It should be notified that the volume of AMPs sales is significantly higher than that of antiviral MPs, and this trend is most pronounced in 2020.

Fig. 9–13 show a monthly dynamics of antiviral MPs realization in 2015–2021. Despite different ranges of the MPs, their consumption dynamics is exactly the same. The dynamics of demand for typical anti-influenza MPs, oseltamivir and rimantadine, is absolutely consistent with other antiviral drugs and does not correlate with the incidence of influenza among the Samara region population. In 2020–2021, in all cases, an extraordinary demand for antiviral drugs was observed in the autumn period, however, in 2021 it was lower compared to 2020.

Thus, a significant increase in demand for antibacterial and antiviral MPs was recorded in the autumn period of 2020 against the backdrop of an increase in the incidence of the novel coronavirus infection.

CONCLUSION

There was a 2.12-fold increase in the AMPs consumption in 2020 compared to the average values of the dispensed MD packages of these subgroups in 2015–2019. For the ATC subgroups “Other β-lactam antibacterials”, “Macrolides, lincosamides and streptogramins”, “Quinolone antibacterials” in 2020, this indicator increased by 3.2, 3.5 and 2.6 times, respectively, which has adverse consequences for the bacterial resistance.

In 2020, the dynamics of the SARS, COVID-19 and community-acquired pneumonia incidence have largely a similar pattern, which is probably more due to the difficulty of recognizing these respiratory infection forms, mainly based on the results of the PCR method for detecting SARS-Cov-2 RNA. The AMPs and antiviral MPs consumption is closely related to the incidence of acute respiratory viral infections and has clear maxima in the spring and autumn-winter periods. At the same time, the AMPs consumption is higher than that of antiviral MPs. In 2020, the cumulative influenza incidence was 25% lower than in 2019, which can be explained by the curing effect of sanitary measures during the COVID-19 pandemic.

In absolute terms, in 2020, the volume of antiviral MPs consumption increased by 2.38 times, which was accompanied by an increase in the average cost of one package by 55.8%. In the initial period of the novel coronavirus infection spread (2020), a significant increase in the share of oseltamivir was notified in the overall consumption structure (up to 17.26% compared to 0.71%, 1.60%, 2.11%, 3.23% and 3.25% in 2015–2019, respectively), and the number of the dispensed packages (by 23.5 times compared to the average value in 2015–2019). In 2021, the share of umifenovir MPs in the total volume of the realized demand increased (up to 40.1%).

In the authors’ opinion, the results of the study confirm the need to strengthen control over the implementation of AMPs. Other measures may include timely informing outpatient medical specialists about the new editions appearance of methodological recommendations of the Russian Ministry of Health for the treatment of a novel coronavirus infection. By no means unimportant is the educational work with the population about the inadmissibility of following false algorithms for COVID-19 therapy. They periodically appear in the public domain on the Internet, social networks and instant messengers, and contain information about the need to take two, and sometimes three AMPs at the same time, even with mild course of the novel coronavirus infection.

FUNDING

This study did not receive financial support from outside organizations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS’ CONTRIBUTION

IKP, PAL – concept and design of the study, editing, approval of the article final version;
IIS, EPG – collection and processing of material, statistical data processing;
TKR – collection and processing of material, writing a text, compiling a references;
AAG – writing a text, compiling a references.

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RESEARCH ARTICLE

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

CHARACTERISTICS OF OLOKIZUMAB PHARMACOKINETICS IN PATIENTS WITH NOVEL CORONAVIRUS INFECTION COVID-19

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Received 10 Sep 2022 After peer review 15 Sep 2022 Accepted 06 Okt 2022

The aim of the article is to study pharmacokinetic characteristics of intravenous olokizumab in patients with moderate COVID-19 to relieve a hyperinflammation syndrome.

Materials and methods. The pharmacokinetic study was conducted as a part of a phase III clinical study (RESET, NCT05187793) on the efficacy and safety of a new olokizumab regimen (intravenous, at the doses of 128 mg or 256 mg) in COVID-19 patients. Plasma concentrations of olokizumab were determined by the enzyme immunoassay. The population analysis was performed using a previously developed pharmacokinetic model based on a linear two compartment.

Results. The pharmacokinetic analysis included the data from 8 moderate COVID-19 patients who had been administrated with olokizumab intravenously at the dose of 128 mg. According to the analysis results in this population, there was an increase in the drug clearance, compared with the data obtained in healthy volunteers and the patients with rheumatoid arthritis: 0.435, 0.178 and 0.147 l/day, respectively. The parameters analysis within the framework of a population pharmacokinetic model showed that the main factors for the increased olokizumab clearance are a high body mass index. In addition, the presence of COVID-19 itself is an independent factor in increasing the drug clearance.

Conclusion. After the intravenous olokizumab administration, an increase in the drug clearance is observed in moderate COVID-19 patients against the background of the disease course. The main contribution to the increased clearance is made by the characteristics of the population of COVID-19 patients associated with the risk of a severe disease and inflammation. When administered intravenously at the dose of 128 mg, a therapeutically significant olokizumab level was maintained throughout the acute disease phase for 28 days.

Keywords: COVID-19, olokizumab; clearance; pharmacokinetic model

Abbreviations: IL(s) – interleukins; PAIT – proactive anti-inflammatory therapy; Ig(s) – immunoglobulins; PK – pharmacokinetics; RA – rheumatoid arthritis; RESET – hyperinflammation; CRP – C-reactive protein; CT – computer tomography; RR – respiratory rate; ALT – alanine aminotransferase; AST – aspartate aminotransferase; ULN – upper limit of normal; BMI – body mass index; ELISA – enzyme-linked immunosorbent assay; $T_{1/2}$ – half-life; AUC_{0-t} – area under the concentration-time pharmacokinetic curve from zero to the last blood draw; Kel – elimination constant; $AUC_{0-\infty}$ – area under the concentration-time curve from time zero to infinity; CL – clearance; T_{max} – time to reach the maximum concentration of olokizumab in blood plasma; C_{max} – maximum concentration of olokizumab in blood plasma; MRT – Mean Resident Time.

For citation: E.V. Tavlueva, E.V. Zernova, M.P. Kutepova, N.E. Kostina, V.S. Lesina, D.R. Mould, K. Ito, A.V. Zinchenko, A.N. Dolgorukova, M.V. Nikolskaya, M.C. Lemak, O.V. Filon, M.Yu. Samsonov. Characteristics of olokizumab pharmacokinetics in patients with novel coronavirus infection COVID-19. *Pharmacy & Pharmacology.* 2022;10(5):460-471. **DOI:** 10.19163/2307-9266-2022-10-5-460-471

Для цитирования: Е.В. Тавлуева, Е.В. Зернова, М.П. Кутепова, Н.Э. Костина, В.С. Лесина, Д.Р. Моулд, К. Ито, А.В. Зинченко, А.Н. Долгорукова, М.В. Никольская, М.С. Лемак, О.В. Филон, М.Ю. Самсонов. Особенности фармакокинетики олокизумаба у пациентов с новой коронавирусной инфекцией COVID-19. *Фармация и фармакология.* 2022;10(5):460-471. **DOI:** 10.19163/2307-9266-2022-10-5-460-471

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

ОРИГИНАЛЬНАЯ СТАТЬЯ

DOI: 10.19163/2307-9266-2022-10-5-460-471

ОСОБЕННОСТИ ФАРМАКОКИНЕТИКИ ОЛОКИЗУМАБА У ПАЦИЕНТОВ С НОВОЙ КОРОНАВИРУСНОЙ ИНФЕКЦИЕЙ COVID-19

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Получена 10.09.2022 После рецензирования 15.09.2022 Принята к печати 06.10.2022

Цель. Изучение особенностей фармакокинетики олокизумаба при внутривенном введении у пациентов с COVID-19 среднетяжелого течения для купирования синдрома гипервоспаления.

Материалы и методы. Изучение фармакокинетики проводилось в рамках клинического исследования III фазы (исследование RESET, NCT05187793) эффективности и безопасности нового режима применения олокизумаба (внутривенно, в дозах 128 мг или 256 мг) у пациентов с COVID-19. Определение концентрации олокизумаба в плазме крови проводили методом иммуноферментного анализа. Популяционный анализ выполнен с помощью ранее разработанной фармакокинетической модели на основе линейной двухкамерной модели.

Результаты. В анализ фармакокинетики были включены данные 8 пациентов с COVID-19 среднетяжелого течения, получавшие олокизумаб в дозе 128 мг внутривенно. Согласно результатам анализа в данной популяции наблюдалось увеличение клиренса препарата, по сравнению с данными, полученными у здоровых добровольцев и пациентов с ревматоидным артритом: 0,435, 0,178 и 0,147 л/сут, соответственно. Анализ параметров в рамках популяционной фармакокинетической модели показал, что основными факторами повышенного клиренса олокизумаба являются высокий индекс массы тела. Кроме того, независимым фактором повышения клиренса препарата является само наличие COVID-19.

Заключение. У пациентов со среднетяжелым течением COVID-19 после внутривенного введения олокизумаба наблюдается увеличение клиренса препарата на фоне течения заболевания. Основной вклад в повышенный клиренс вносят особенности популяции пациентов с COVID-19, связанные с риском тяжелого течения заболевания и выраженным воспалением. При внутривенном введении в дозе 128 мг терапевтически значимый уровень олокизумаба сохранялся в течение всей острой фазы заболевания на протяжении 28 дней.

Ключевые слова: COVID-19; олокизумаб; клиренс; фармакокинетическая модель

Список сокращений: ИЛ – интерлейкины; УПТ – упреждающая противовоспалительная терапия; Ig – иммуноглобулины; ФК – фармакокинетика; РА – ревматоидный артрит; RESET – гипервоспаление; СРБ – С-реактивный белок; КТ – компьютерная томография; ЧДД – частота дыхательных движений; АЛТ – аланинаминотрасфераза; АСТ – аспартатаминотрансфераза; ВГН – верхняя граница нормы; ИМТ – индекс массы тела; ИФА – иммуноферментный анализ; $T_{1/2}$ – период полувыведения; AUC_{0-t} – площадь под фармакокинетической кривой «концентрация-время» от нуля до последнего отбора крови; K_{el} – константа элиминации; $AUC_{0-\infty}$ – площадь под фармакокинетической кривой «концентрация-время», начиная с нулевого значения времени, экстраполированная до бесконечности; CL – клиренс; T_{max} – время достижения максимальной концентрации олокизумаба в плазме крови; C_{max} – максимальная концентрация олокизумаба в плазме крови; MRT – среднее резидентное время.

INTRODUCTION

In December 2019, a large outbreak of a disease caused by a novel coronavirus (SARS-CoV-2) affecting the lower respiratory tract, occurred in Wuhan, China [1].

Most patients suffer from a mild form of the disease (like an acute respiratory viral infection), but the infection can turn into an acute respiratory distress syndrome. In this case, there is a rapid replication of the virus, a rapid release of pro-inflammatory cytokines against the background of the inflammatory infiltrates formation in the lung parenchyma and pulmonary vascular endothelium, damage to the alveoli, vascular microthrombosis, etc. There is a pattern of systemic hyperinflammation with increased levels of interleukins (ILs) cytokines such as IL-1β, IL-1Ra, IL-6 and the IL-2

receptor. The progressive development of systemic pathological inflammation results in a pronounced increase in the severity of the disease and the development of multiorgan damage [2–4].

According to the Interim Guidelines (IGs) of the Russian Ministry of Health "Prevention, diagnosis and treatment of novel coronavirus infection (COVID-19)"[1], the use of proactive anti-inflammatory therapy (PAIT) in combination with active anticoagulant therapy is currently the standard of care. Monoclonal antibodies – blockers of the IL-6, IL-6, IL-1 receptors, can be used among others as PAIT.

Olokizumab (Artlegia®) is a humanized monoclonal antibody of the immunoglobulin (Ig) G4/kappa isotype that can specifically bind to the IL-6 molecule. The drug has a unique action mechanism, since it directly binds IL-6 and thus blocks the pathological cascade of inflammatory reactions. In this, it differs from tocilizumab, sarilumab, and levilimab, which are antagonists of the IL-6 receptor [5–7]. Due to the high affinity for IL-6 and the mode of action (inhibition of the interaction between IL-6 and the glycoprotein gp130), the pharmacodynamic effects of olokizumab are realized at lower doses [8, 9].

Olokizumab was originally developed as a drug for the treatment of rheumatoid arthritis (RA) and has successfully passed a full-fledged clinical development program that included phase II studies [10] in 380 patients, and phase III studies in 2443 patients (CREDO 1[2] , CREDO 2[3], CREDO 3[4] and CREDO 4[5]), as well as post-marketing studies [11]. According to the studies, the recommended olokizumab dose in RA is 64 mg once every 2 or 4 weeks when administered subcutaneously. In case of pathogenetic therapy of a cytokine release syndrome in a new coronavirus infection (COVID-19) – 64 mg subcutaneously once.

Subsequently, the effect of olokizumab was studied in COVID-19 patients. The use of olokizumab as a part of the complex therapy for COVID-19 revealed a number of pharmacokinetic characteristics of the drug in this population, compared with healthy volunteers and RA patients. In general, the patient population in which olokizumab was prescribed as proactive anti-inflammatory therapy (PAIT) is characterized by a number of trends in both demographic data and laboratory parameters. In particular, a body mass index, or rather overweight, which, in turn, is a risk factor for severe COVID-19, has a known effect on the pharmacokinetics of drugs. In COVID-19, a typical pattern of deviations in the biochemical analysis of blood is observed: an increase in the levels of inflammatory markers, a change in the levels of protein fractions, reflecting the course and severity of the inflammatory process, which also affects the drugs pharmacokinetics.

THE AIM of the article is to study pharmacokinetic characteristics of intravenous olokizumab in patients with moderate COVID-19 to relieve hyperinflammation syndrome.

MATERIALS AND METHODS

Study design

Currently, according to the Interim guidelines (version 16 dated 18 Aug 2022), the intravenous administration of olokizumab is included in the recommended standards of COVID-19 therapy. The pharmacokinetics of the drug when administered intravenously in COVID-19 patients, was evaluated as a part of a multicenter, open, randomized phase III research, the aim of which was to study the efficacy and safety of a new olokizumab regimen (at the doses of 128 and 256 mg, respectively, administered intravenously) in COVID-19 patients with signs of hyperinflammation (RESET). The randomization of patients in the study was central and it was performed using an electronic system. The patients were randomized into 2 groups at the ratio of 1:1 – the olokizumab group (group 1) and the comparison group (group 2). In order to evenly distribute patients into the treatment groups, the stratification was carried out according to the following criteria:

- according to the need for the oxygen support at screening (yes / no),
- the presence of a concomitant disease that is a risk factor for severe COVID-19 (no risk factors or there is one or more risk factors).

Thus, as a result of the stratification, the patients in groups will be equivalent in terms of the presence of respiratory failure and risk factors for severe COVID-19.

Selection criteria for the study

The RESET study was conducted with the approval

[1] Interim Guidelines of the Russian Ministry of Health "Prevention, diagnosis and treatment of novel coronavirus infection (COVID-19), version 16, 18.08.2022. Available from: https://static-0.minzdrav.gov.ru

[2] Evaluation of the Effectiveness and Safety of Two Dosing Regimens of Olokizumab (OKZ), Compared to Placebo, in Subjects With Rheumatoid Arthritis (RA) Who Are Taking Methotrexate But Have Active Disease (CREDO 1). Available from: https://grlsbase.ru/clinicaltrails/clintrail/2763

[3] Evaluation of the Efficacy and Safety of Two Dosing Regimens of Olokizumab (OKZ), Compared to Placebo and Adalimumab, in Subjects With Rheumatoid Arthritis (RA) Who Are Taking Methotrexate But Have Active Disease (CREDO 2). Available from: https://clinicaltrials.gov/ct2/show/NCT02760407

[4] Evaluation of the Efficacy and Safety of Two Dosing Regimens of Olokizumab (OKZ), Compared to Placebo, in Subjects With Rheumatoid Arthritis (RA) Who Were Taking an Existing Medication Called a Tumour Necrosis Factor Alpha Inhibitor But Had Active Disease (CREDO 3). Available from: https://clinicaltrials.gov/ct2/show/NCT02760433

[5] Efficacy and Safety of Olokizumab in Subjects With Moderately to Severely Active Rheumatoid Arthritis (CREDO 4). Available from: https://clinicaltrials.gov/ct2/show/NCT03120949

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of the Ethics Council of the Department for Regulation of the Circulation of Medicines (Ministry of Health of the Russian Federation, Protocol No. 273 dated 20 Apr 2021); the local ethics committees of Voronezh Regional Clinical Hospital No. 1 (Protocol No. 117 dated 22 Jul 2021) and Inozemtsev City Clinical Hospital (Protocol No. 11 dated 28 May 2021). The results of the pharmacokinetic study in the subgroup of COVID-19 patients, compared with the data from the previous studies in healthy volunteers and RA patients, are presented in this paper. The study included hospitalized patients with a confirmed moderate-to-severe coronavirus infection and signs of hyperinflammation, aged over 18 years.

The main inclusion criteria were: moderate COVID-19, pneumonia on computer tomography (CT) and the body temperature > 38°C, in combination with 1 or more features, including the saturation level (SpO_2) < 95%, respiratory rate (RR) > 22, dyspnea on exertion, C-reactive protein (CRP) >10 mg/l; the presence of one of the risk factors (diabetes mellitus, severe cardiovascular pathology, chronic renal failure, oncological pathology, obesity, or age ≥65 years); the presence of the hyperinflammation signs (a body temperature ≥38°C for 2 days or more, in combination with 1 or more signs: a CRP level >3×upper limit of normal (ULN), the leukocyte count – 2.0–3.5 × 10^9/l, the absolute number of lymphocytes – 1.0–1.5×10^9/l).

The main exclusion criteria were: a severe or extremely severe COVID-19 course, the presence of severe laboratory abnormalities (hemoglobin <80 g/l, an absolute neutrophil count <0.5×109/l, a leukocyte count <2.0×109/l, a number of platelets <50 x 109/L, alanine aminotransferase (ALT) ≥ 3.0 × ULN and/or aspartate aminotransferase (AST) ≥ 3.0 × ULN), the creatinine clearance < 30 ml/min, confirmed sepsis by pathogens other than COVID- 19, a high probability of a disease progression to death within the next 24 hours.

Selection criteria for the pharmacokinetics subgroup

The pharmacokinetics (PK) study subgroup included patients with a body mass index (BMI) in the range of 18.5–35.0 kg/m^2 who had signed an additional voluntary informed consent form for the inclusion in the PK study. A total of 9 patients were included in the PK subgroup. These patients were administrated with Artlegia® (INN: olokizumab), a solution for the subcutaneous administration, 160 mg/mg, as an intravenous 60-minute infusion, at the dose of 128 mg (8 patients were administrated with the drug once at the dose of 128 mg), 1 patient was not included into statistical analysis (total dose 256 mg). 1 patient was administrated with the drug twice with a total dose of 256 mg).

In addition to olokizumab, the patients received baricitinib (4 mg/day, for 7 days) and low-doses of glucocorticosteroids (dexamethasone at the doses of 4–20 mg/day IV or IM, or methylprednisolone at the dose of 1 mg/kg intravenously every 12 hours), as well as etiotropic therapy for COVID-19 (favipiravir or remdesivir), symptomatic and anticoagulant therapy drugs.

In the patients included in the PK assessment subgroup, blood biosamples were taken to study olokizumab concentrations as follows: before the start of the infusion, then after 2, 4, 8, 24, 48 and 72 hours; then every day, starting from 4 to 10 days; at the end on days 14 and 28 after the first administration of the drug (i.e. from the moment the infusion began). After the selection, plasma biosamples were frozen and stored at the temperature not exceeding –65°C.

For the analysis of biosamples, a bioanalytical method based on enzyme immunoassay (ELISA) was developed. The method is based on the interaction of olokizumab with IL-6 associated with goat antibodies to human IL-6 immobilized on the surface of the plate. The method was validated in the concentration range of 2.5–100 μg/ml.

Statistical analysis

To assess a possible influence of various factors on the olokizumab clearance, the results were combined with a previously created phases 1 and 2 database of olokizumab clinical trials, including the data from the pharmacokinetic samples analysis of 30 healthy volunteers and 30 RA patients, and the ones who had received a single intravenous olokizumab injection at various concentrations [12].

The description of the olokizumab pharmacokinetics was performed using a linear 2 compartment model with the absorption kinetics and the first order elimination. The model parameterization included pharmacokinetic parameters such as clearance (CL), an distribution volume (V), an elimination rate constant (K_{el}) and rate constants of exchange between compartments (Q/Vc, Q/Vp) (Fig. 1). Interindividual variability (IIV) parameters were included in the final model for the volume of distribution parameters of the central (Vc) and peripheral compartments (Vp). The influence of the following covariates was assessed: age, sex, body weight, serum albumin, liver enzymes, bilirubin, creatinine clearance. Since study CL04041094 did not collect data on participants' albumin levels, for modeling purposes, missing individual albumin levels were reconstructed using the following formula (ALB = –0.4714×CRP+50.714) based on the literature data [13].

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

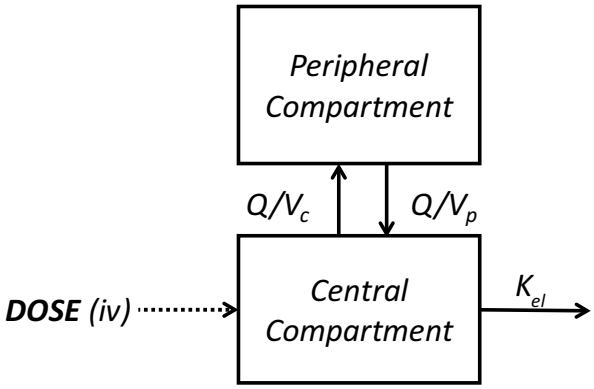

Figure 1 – Diagram of linear two compartment model to describe olokizumab pharmacokinetics
Note: Q/Vc, Q/Vp – speed constants of exchange between cameras; K_{el} – elimination rate constant.

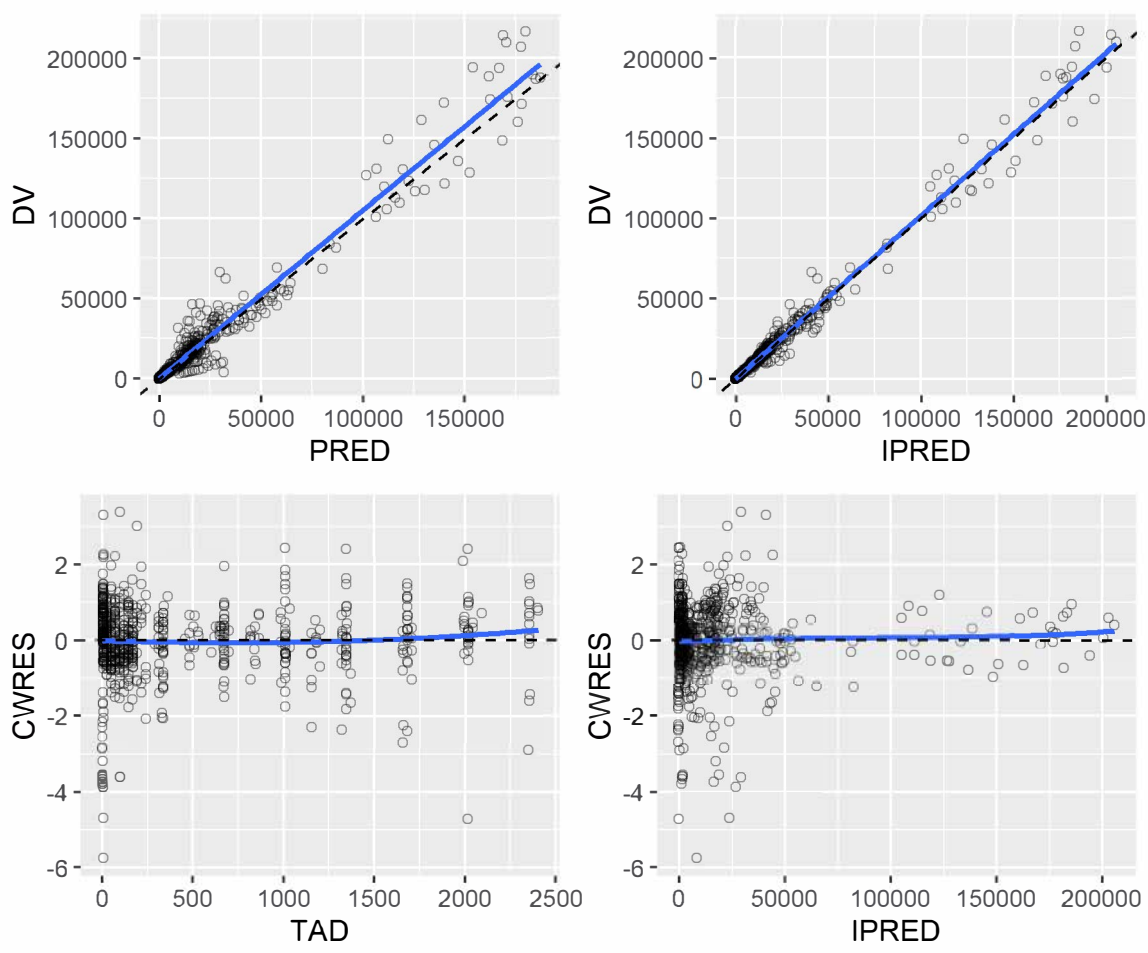

Figure 2 – Goodness of fit plots for the final model
Note: DV – dependent variable; PRED – predicted values; IPRED – individual predicted values; TAD – time after the last dose; CWRES – conditional weighted residuals calculated using the FOCI algorithm.

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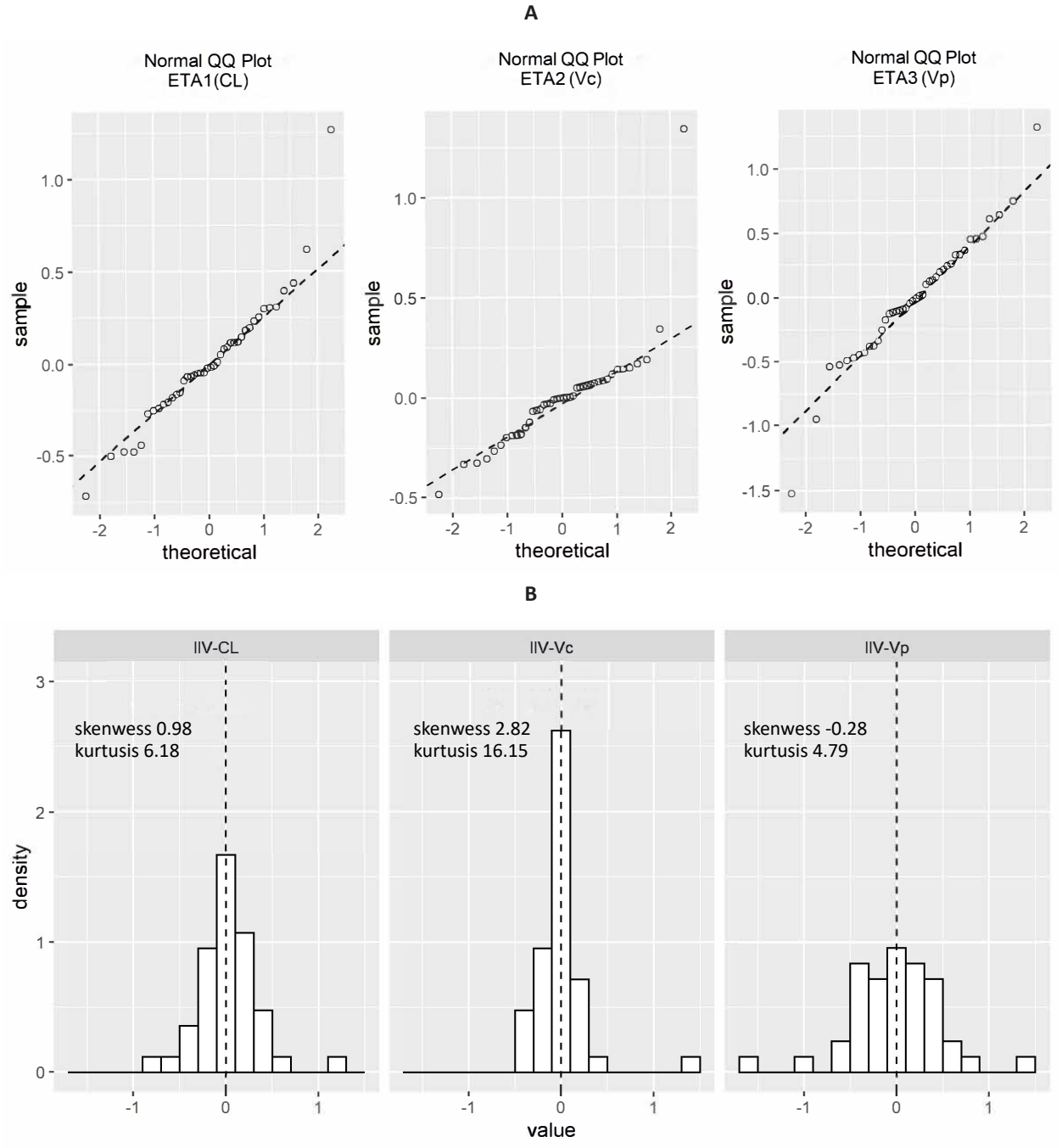

Figure 3 – Assessments of the normality assumption of interindividual variability

Note: ETA = η; IIV – interindividual variability; CL - clearance; Vc – volume of the central chamber; Vp – volume of the peripheral chamber; Normal QQ Plot – quantile-quantile plot for assessing normality of the distribution.

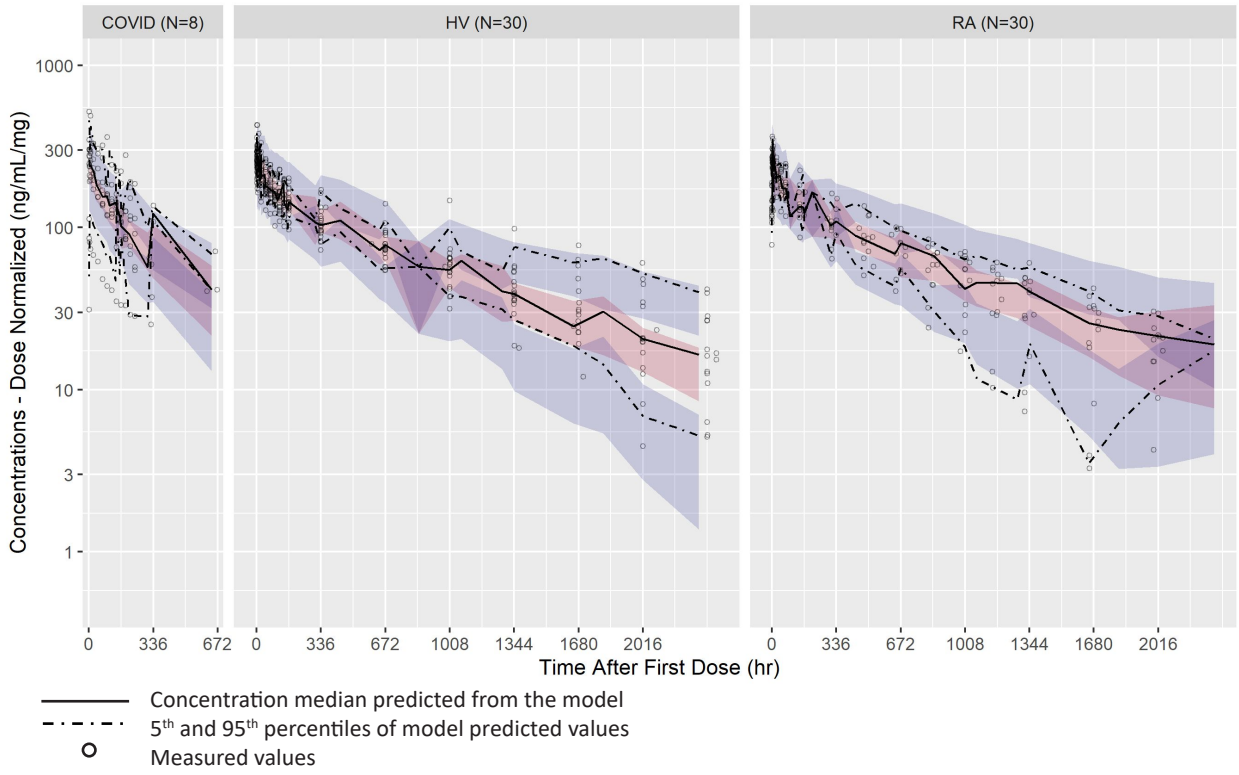

Figure 4 – Graphical representation of observed and predicted pharmacokinetic olokizumab profiles for COVID-19 patients, healthy volunteers, and RA patients

Note: The area marked in pink is the boundaries of the 95% confidence interval for estimating the median; the areas marked in gray are the boundaries of the 95% confidence interval for estimating the 5th and 95th percentiles.

Table 1 – Individual pharmacokinetic olokizumab parameters and mean values

PK parameter	Healthy volunteers [15] 1 mg/kg (76 mg) IV, n = 3[1]	RA patients [16] 1 mg/kg (75 mg) IV, n = 7	COVID-19 patients (RESET study) 128 mg, IV, n=8
C_{max} (µg/ml)			
Mean (±SD)	21.4 (±0.842)	22.28 (±3.9)	40.20 (±18.06)
Geometric mean (CV%)	–	21.98 (17.53)	35.69 (44.93)
T_{max} (h)			
Median	4.00	2.00	6.00
L.Qu., U.Qu.	2.03–4.00	2.0 – 14.0	2,0–18,0
Geometric mean (CV%)	–	4.49 (123.26)	7.44 (159,72)
AUC_{0-t} (h×mcg/ml)			
Mean (±SD)	9 427 (±524)	7 001.58 (±1,259.89)	7 802,19 (±4005,29)
Geometric mean (CV%)	–	6 911.9 (17.99)	6 855.77 (51.34)
$AUC_{0-\infty}$ (h×mcg/ml)			
Mean (±SD)	10 435 (±1,266)	13 979.67 (±3,267.86)	13 117.51 (±9,777.32)
Geometric mean (CV%)	–	13 633.52 (23.38%)	10 600.21 (74.54)
$T_{1/2}$ (days)			
Mean (±SD)	27.9 (±12)	30.66 (±14.2)	13.8 (±10.86)
Geometric mean (CV%)	–	28.13 (46.31)	10.35 (78.65)
CL, l/day			
Mean (±SD)	0.177 (±0.020)	0.145 (±0.03)	0.349 (±0.214)
Geometric mean (CV%)	–	0.143 (20.372)	0.289 (61.275)
Vd, l			
Mean (±SD)	7.08 (±3.04)	6.29 (±2.97)	5.26 (±4.56)
Geometric mean (CV%)	–	5.79 (47.23)	4.33 (86.81)

Notes: SD – standard deviation; CV% – coefficient of variation; L.Qu. – lower quartile (25%); U.Qu. – upper quartile (75%); IV – intravenously; max. – maximum; min. – minimum; n is the number of patients; CL – clearance; Vd – volume of distribution; 1 – the comparison table includes only the data of volunteers administrated with olokizumab at the dose of 1 mg/kg intravenously. A total of 87 volunteers participated in the phase 1 studies, 67 – in the European population (RA0001), 20 – in the Asian (Japanese) population (RA0074).

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Table 2 – Mean pharmacokinetic olokizumab parameters in general population of RA and COVID-19 patients

Parameter		Final model		RSD (%)
		Value	η-shrinkage	
CL (l/day)	θ1	0.154	–	6.8
Vc (l)	θ2	4.1	–	5.6
Q (l/day)	θ3	0.348	–	16.3
Vp (l)	θ4	1.67	–	12.2
Residual error – frequent sampling	θ7	0.167	5.9	–
IIV CL (CV%)	η1	34.2	2.2	–
IIV Vc (CV%)	η2	27.3	2.6	–
IIV Vp (CV%)	η3	56.7	16.0	–
Correlation of random effects				
IIV CL – IIV Vc	$CORR_{1,2}$	0.651	–	–
IIV CL – IIV Vp	$CORR_{1,3}$	0.110	–	–
IIV Vc – IIV Vp	$CORR_{2,3}$	0.512	–	–

Note: RSD – relative standard deviation; CL – total clearance; Vc, Vp – volume of distribution of the central, peripheral, respectively; Q/Vc, Q/Vp – speed constants of exchange between cameras; IIV(CV%) – interindividual variability (coefficient of variation %); θ is a parameter with a fixed value; η is the variability parameter given by a value with a normal distribution; CORR – correlation between random effects.

Table 3 – Effects of individual patients' characteristics

Covariates		Final model	
		Value	RSD (%)
Impact of body weight on CL and Q	θ8	0.654	57.3
Impact of body weight on Vc andVp	θ14	0.498	60.0
Impact of COVID-19 disease on CL	θ16	0.965	23.4

Note: θ – parameter with a fixed value; RSD – relative standard deviation; CL – total clearance; Q – intercompartmental clearance; Vc, Vp – volume of distribution of the central, peripheral, respectively.

Diagnostic plots were used to assess model assumptions and goodness-of-fit; satisfactory η-shrinkage values were obtained, and Visual Predictive Check was performed. The stability of the model, the asymmetry and kurtosis of the η distribution were also evaluated [13, 14].

Population pharmacokinetic olokizumab parameters were evaluated using the First Order Condition Estimation (FOCE) algorithm in NONMEM 7.4 software. The construction of diagnostic plots, the exploratory analysis, and post-processing of the NONMEM output data were performed using R version 3.5.3 software. The analysis was performed in accordance with FDA[6] and EMEA[7] guidelines for population pharmacokinetics.

RESULTS

The mean age of the patients was 56.4 (±10.0) years [45 to 74 years], the majority were males (87.5%), the average body weight of the patients was 87.0 (±15.1) kg, and BMI – 26.8 (±3.4). All patients were Caucasian. In five patients, biosamples were taken at all planned points, in 2 patients the sampling was completed at the point of 366 hours and in 1 – at the point of 240 hours (a withdrawal due to death).

Standard non-compartmental PK parameters from individual studies are presented in Table 1, compared with the results of the previous studies in healthy volunteers and RA patients. After the administration, the drug was distributed fairly fast. In the studied population, C_{max} was reached quite fast and was about 36 μg/ml, the median T_{max} was 6 hours. Further on, the concentration decreased during the entire subsequent observation period. Despite a faster decrease in the concentration compared with the intravenous (IV) olokizumab administration at the dose of 1 mg/kg (mean 75 mg) in RA patients [10], 7 days after the administration, in 7 out of 8 patients, the plasma concentration of olokizumab exceeded 10 mcg/ml; after 14 days, in 5 out of 7 patients, the concentration was above 5 mcg/ml. In this case, the mean $T_{1/2}$ was about 13.8 days, which was significantly lower than when administered intravenously to healthy volunteers (27.9 days) and RA patients (30.66 days).

[6] Guidance for Industry: Population Pharmacokinetics. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), February 2022.
[7] EMEA report. Guideline on Reporting the Results of Population Pharmacokinetic Analyses, 2007.

Analysis of individual characteristics influence on olokizumab clearance

A graphical analysis of the goodness-of-fit criteria and a visual assessment of the compliance with the model predictions demonstrated the satisfactory ability of the selected population pharmacokinetic model to describe plasma concentrations of olokizumab (Fig. 2–4).

The average pharmacokinetic olokizumab parameters in the general population of RA and COVID-19 patients, determined on the basis of the developed population pharmacokinetic model, generally corresponded to the previously obtained individual pharmacokinetic parameters in the RA population (clearance 0.153 l/day vs. 0.147 l/day)[8].

The covariates analysis showed that a body weight has the greatest effect on the drug elimination rate (Table 3). After adjusting for albumin and body weight, a COVID-19 disease was found to be an independent significant factor in increasing olokizumab clearance by 96.5% (θ16).

DISCUSSION

The peak intravenous concentration in COVID-19 patients was about 36 μg/mL; it was consistent with the previous data in healthy volunteers and RA patients however, in only two patients T_{max} exceeded 8 hours. Thus, in general, the time to peak concentration was comparable in all studied populations, the observed differences may be associated with differences in the speed and techniques of intravenous infusion of the drug in different studies. Although the rate of elimination and the volume of olokizumab distribution did not significantly differ between healthy volunteers and RA patients, the patients with moderate COVID-19 showed a significantly faster clearance of the drug. The median of $T_{1/2}$ in COVID-19 patients was about 6 days compared to about 30 days in healthy volunteers and RA patients. For a more detailed analysis, a previously developed population pharmacokinetic model based on the results intravenous administration of olokizumab in healthy volunteers and patients with RA program, was adapted to assess the impact of individual patients' characteristics on the drug clearance in COVID-19 patients. It has been shown that a decrease in albumin levels and an increase in body weight are associated with an increase in the rate of olokizumab clearance. Decreased albumin levels are a hallmark of a COVID-19 disease: hypoalbuminemia is observed in 30–50% of hospitalized patients and can serve as an independent predictor of a severe disease and death [23, 24], while average levels of albumin in healthy volunteers and RA patients do not differ. An increased body weight is a risk factor for severe COVID-19, and therefore such patients are more likely to be hospitalized and are disproportionately represented in the study populations. The median body weight of patients in the COVID-19 patient cohort was higher compared to RA patients and healthy volunteers (92, 78 and 76 kg, respectively). Thus, a faster olokizumab clearance in COVID-19 patients may be partly explained by a higher incidence of hypoalbuminemia and the greater body weight of patients. A COVID-19 disease was also independently associated with increased olokizumab clearance, which may be due to the acceleration of protein metabolism in infectious and inflammatory diseases [25], one of the markers of which can serve as a reduced level of albumin. Hypoalbuminemia is a characteristic feature of a COVID-19 disease: it is observed in 30–50% of hospitalized patients and plays an independent predictor of a severe disease and death [17–22], while mean albumin levels do not differ between healthy volunteers and RA patients.

A similar effect, comparable in magnitude, was previously demonstrated for another inhibitor of the IL-6 signaling pathway, tocilizumab, in patients with severe COVID-19 [21]. In the review by Leung E. et al. (2022), 2 routes for the elimination of monoclonal antibodies are described. The first pathway, providing a linear clearance, is associated with proteolytic catabolism of drugs after the administration. The second pathway involves a specific a ligand-receptor (e.g., IL-6 receptor and tocilizumab) binding to both soluble and membrane-bound targets, followed by an internalization and an intracellular degradation. This process provides a non-linear clearance and depends on the relative expression of the target. Therefore, this mechanism may be influenced by patient-specific factors such as the type and severity of the disease. In this case, the linear part of the tocilizumab clearance, apparently, to some extent depends on a body weight.

In the study by Moes D.J.A.R. et al (2021), in patients with severe COVID-19, the clearance (CL) estimate was 0.725 l/day and it was higher than the estimate in adult RA patients (0.2–0.3 l/day), children with systemic juvenile idiopathic arthritis (0.17 l/day), children and adults with a CAR T-induced cytokine release syndrome (0.5 l/day) [26]. Similar trends are shown by the ratio of the olokizumab clearance in patients with moderate

[8] Instructions for drug use Artlegia®. Available from: https://artlegia.com/#close

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DOI: 10.19163/2307-9266-2022-10-5-460-471

COVID-19 (0.349 l/day) and RA patients (0.145 l/day). However, it should be taken into account that the study did not show the feasibility of calculating the dose of tocilizumab based on the body weight of patients, the use of fixed doses is preferable. Given these data, a caution should be exercised when interpreting the study results of this population olokizumab pharmacokinetics. Thus, until further pharmacokinetic data are obtained in COVID-19 patients, a revision of the dosing olokizumab regimen seems unreasonable in this population.

CONCLUSION

After the intravenous administration of olokizumab, during the disease course, in patients with moderate COVID-19, an increase in clearance was demonstrated compared with previously studied populations of healthy volunteers and RA patients. The main contribution to the increased olokizumab clearance is made by the characteristics of the COVID-19 patients population associated with the risk of the severe disease (overweight) and the effect of accelerated protein metabolism due to the severe inflammation, characterized by hypoalbuminemia. At the same time, the contribution of the unidentified factors of the increased clearance associated with a COVID-19 disease, and probably due to the interaction of the mechanism of olokizumab action and COVID-19 pathogenesis, was also observed. However, when administered intravenously at the dose of 128 mg, a therapeutically significant olokizumab level was maintained throughout the acute phase (28 days) of the disease.

FUNDING

The work was carried out with the financing of the JSC “R-Pharm” group of companies.

CONFLICT OF INTERESTS

The clinical trial was organized by the sponsor, the JSC “R-Pharm”, the manufacturer and owner of the registration certificate for the drug Artlegia® (olokizumab) dated May 21, 2020. The authors of the article Zinchenko A.V., Dolgorukova A.N., Nikolskaya M.V., Lemak M.S., Filon O.V., Samsonov M.Yu. are employees of the JSC “R-Pharm”. The authors of the article Tavlueva E.V., Zernova E.V., Kutepova M.P., Kostina N.E., Lesina V.S. are physicians-researchers of the scientific centers of Voronezh Regional Clinical Hospital No. 1 (Center No. 03) and Inozemtsev City Clinical Hospital, Department of Health of the City of Moscow (Center No. 04) according to the protocol “Multicenter open randomized study of efficacy and safety of a new regimen for the use of the drug Artlegia® (INN: olokizumab) in patients with a coronavirus infection (COVID-19) with signs of hyperinflammation, sponsored by the JSC “R-Pharm”.

AUTHORS’ CONTRIBUTION

Tavlueva E.V., Zernova E.V., Kutepova M.P., Kostina N.E., Lesina V.S. – implementation of the experimental part of the study; Mould D.R., Ito K. – development of population pharmacokinetic model; Zinchenko A.V. – analytical processing of the obtained results; Dolgorukova A.N. – statistical processing of the study results; Nikolskaya M.V. – text writing and editing; Lemak M.C. – planning and description of the pharmacokinetic model; Filon O.V. – development of research design, text writing and editing; Samsonov M.Yu. – aim setting, research design development.

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DOI: 10.19163/2307-9266-2022-10-5-460-471

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RESEARCH ARTICLE

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

HEMOSTIMULATING PROPERTIES OF THE CONJUGATES OF GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR WITH ALENDRONIC ACID

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Received 04 Aug 2022 After peer review 20 Oct 2022 Accepted 30 Oct 2022

The aim of the work is to evaluate the hemostimulating activity of recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) conjugates with alendronic acid (ALN) in the model of cytostatic myelosuppression and the dynamics of rhGM-CSF accumulation as a part of the conjugate in the bone tissue and bone marrow of mice.

Materials and methods. The conjugates obtained by a solid-phase synthesis using 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide or periodate oxidation, were used. A hemostimulating activity was evaluated in a model of a cytostatic myelosuppression induced by the administration of cyclophosphamide to CBA/Calac mice. RhGM-CSF preparations were injected subcutaneously for 4-5 days at the dose of 90 μg/kg. After the injections cycle had been completed, the total leukocyte and segmented neutrophil counts were carried out in the blood samples, and the total karyocyte count was carried out in the bone marrow samples.

The tissue distribution of rhGM-CSF preparations was assessed in outbred CD-1 mice after a single intravenous administration at the effective dose. The content of rhGM-CSF in blood, femoral tissue and bone marrow was determined by enzyme immunoassay.

Results. RhGM-CSF conjugates with ALN have been shown to retain the ability of the original protein to increase the number of leukocytes, segmented blood neutrophils, and bone marrow karyocytes under the action of conjugates. The stimulation of the neutrophil production used to be observed at earlier times than in the case of rhGM-CSF. The increase in the total number of bone marrow cells after the introduction of all three conjugates was more pronounced compared to the original protein (by 34%). The increased hemostimulatory effect of the AEG conjugate was accompanied by a more intense accumulation of rhGM-CSF in the bone tissue and bone marrow of mice. The rhGM-CSF introduced into the conjugate was detected in the bone tissue for 24 h and it circulated in the bloodstream for a longer time compared to the original protein.

Conclusion. The data obtained make it possible to conclude that further work on the development of effective hemostimulating drugs based on rhGM-CSF conjugates with ALN, is promising.

Keywords: recombinant human granulocyte-macrophage colony-stimulating factor; alendronic acid; conjugate; hemostimulating activity; accumulation in bone tissue and bone marrow.

Abbreviations: ALN – alendronic acid; GM-CSF – granulocyte-macrophage colony-stimulating factor; rhGM-CSF – recombinant human granulocyte-macrophage colony-stimulating factor; HAP – hydroxylapatite; EDC – 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide; CP – cyclophosphan; ELISA – electronic intelligence search and analysis; TKC – total karyocyte count.

For citation: G.G. Shimina, A.V. Bateneva, E.S. Tsyplenkova, S.G. Gamaley, T.I. Esina, E.A. Volosnikova, E.D. Danilenko. Hemostimulating properties of the conjugates of granulocyte-macrophage colony stimulating factor with alendronic acid. *Pharmacy & Pharmacology.* 2022;10(5): 472-482. **DOI:** 10.19163/2307-9266-2022-10-5-472-482

Для цитирования: Г.Г. Шимина, А.В. Батенева, Е.С. Цыпленкова, С.Г. Гамалей, Т.И. Есина, Е.А. Волосникова, Е.Д. Даниленко. Гемостимулирующие свойства конъюгатов гранулоцитарно-макрофагального колониестимулирующего фактора с алендроновой кислотой. *Фармация и фармакология.* 2022;10(5):472-482. **DOI:** 10.19163/2307-9266-2022-10-5-472-482

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

ОРИГИНАЛЬНАЯ СТАТЬЯ

DOI: 10.19163/2307-9266-2022-10-5-472-482

ГЕМОСТИМУЛИРУЮЩИЕ СВОЙСТВА КОНЪЮГАТОВ ГРАНУЛОЦИТАРНО-МАКРОФАГАЛЬНОГО КОЛОНИЕСТИМУЛИРУЮЩЕГО ФАКТОРА С АЛЕНДРОНОВОЙ КИСЛОТОЙ

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Получена 04.08.2022 После рецензирования 20.10.2022 Принята к печати 30.10.2022

Цель. Оценка гемостимулирующей активности конъюгатов рекомбинантного гранулоцитарно-макрофагального колониестимулирующего фактора человека (рчГМ-КСФ) с алендроновой кислотой (АЛН) на модели цитостатической миелосупрессии и динамики накопления рчГМ-КСФ в составе конъюгата в костной ткани и костном мозге мышей.

Материалы и методы. В работе использовали конъюгаты, полученные методом твердофазного синтеза с помощью 1-этил-3-[3-диметиламинопропил]карбодиимида или реакции периодатного окисления. Гемостимулирующую активность оценивали на модели цитостатической миелосупрессии, вызванной введением мышам CBA/Calac циклофосфана. Препараты рчГМ-КСФ вводили подкожно в течение 4–5 дней в дозе 90 мкг/кг. По окончании курса инъекций в образцах крови подсчитывали общее количество лейкоцитов, сегментоядерных нейтрофилов, в образцах костного мозга – общее число кариоцитов. Оценку распределения препаратов рчГМ-КСФ по тканям проводили на аутбредных мышах CD-1 после однократного внутривенного введения в эффективной дозе. Содержание рчГМ-КСФ в крови, ткани бедренной кости и костном мозге определяли иммуноферментным методом.

Результаты. Показано, что конъюгаты рчГМ-КСФ с АЛН сохраняли присущую исходному белку способность повышать число лейкоцитов, сегментоядерных нейтрофилов крови и кариоцитов костного мозга. Стимуляция продукции нейтрофилов под действием конъюгатов наблюдалась в более ранние сроки, чем в случае рчГМ-КСФ. Увеличение общего числа клеток костного мозга после введения всех трех конъюгатов было более выраженным по сравнению с исходным белком (на 34%). Повышенный гемостимулирующий эффект конъюгата AEG сопровождался более интенсивным накоплением рчГМ-КСФ в костной ткани и костном мозге мышей. Введенный в состав конъюгата рчГМ-КСФ обнаруживался в костной ткани в течение 24 ч и более длительно циркулировал в кровеносном русле по сравнению с исходным белком.

Заключение. Полученные данные позволяют сделать вывод о перспективности дальнейших работ по созданию эффективных гемостимулирующих препаратов на основе конъюгатов рчГМ-КСФ с АЛН.

Ключевые слова: рекомбинантный гранулоцитарно-макрофагальный колониестимулирующий фактор человека; алендроновая кислота; конъюгат; гемостимулирующая активность; накопление в костной ткани и костном мозге

Список сокращений: АЛН – алендроновая кислота; ГМ-КСФ – гранулоцитарно-макрофагальный колониестимулирующий фактор; рчГМ-КСФ – рекомбинантный гранулоцитарно-макрофагальный колониестимулирующий фактор человека; ГАП – гидроксилапатит; EDC – 1-этил-3-[3-диметиламинопропил] карбодиимид; ЦФ – циклофосфан; ИФА – иммуноферментный анализ; ОКК – общее количество кариоцитов.

INTRODUCTION

The relevance of the search for new drugs and methods for the treatment of neutropenia of various etiologies, arising, in particular, as a result of chemotherapy and radiotherapy in cancer patients, is extremely high. Chemotherapy-induced disorders of hematopoiesis and, above all, suppression of leukocyte production, in some cases become the main indicators for interrupting the treatment, despite a distinct oncolytic therapy effect [1].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a natural cytokine protein that regulates the proliferation and differentiation of stem cells with the formation of colonies of neutrophilic, eosinophilic leukocytes, and macrophages [2–4]. The ability of GM-CSF to enhance hematopoiesis served as a basis for the development and introduction of drugs based on it, Leukine and Leucomax (USA), into clinical practice, to reduce the side effects of the antitumor therapy, increase resistance to infections, during bone marrow transplantation. However, despite a high level of GM-CSF preparations safety, their use leads to the development of adverse reactions such as fever, chills, lethargy, myalgia, bone pain, fever, body weight fluctuations,

generalized pruritus, redness and an aerythematous reaction around the site of the subcutaneous injection [5–8]. In this regard, the improvement of GM-CSF preparations in terms of increasing their affinity for the target tissues, reducing the therapeutic dose and, as a result, side treatment effects, is of undoubted interest.

Literature references describe examples of various vector molecules used for the targeted delivery of immunoregulatory ligands, in particular, cytokines [9–11]. As a means of delivering biologically active substances to the bone tissue and bone marrow, bisphosphonates which are characterized by a high affinity for calcium ions and the ability to rapidly accumulate in the bone, are used [12–17]. One of the methods for obtaining the targeted drugs based on bisphosphonates, is their conjugation with medicinal preparations [12, 13].

Taking into account all of the above, conjugates of a human tumor necrosis factor alpha with alendronic acid aminobisphosphonate (ALN), which demonstrated the ability to accumulate in the foci of bone metastasis of the tumor, thereby exhibiting an antitumor activity, was obtained [18, 19]. These data became a basis for the use of ALN as a vector molecule for a delivery of a recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) to bone marrow cells.

A technology for the production of rhGM-CSF in a prokaryotic expression system (recombinant strain E. coli SG20050/p280_2GM) [20], as well as methods for conjugating rhGM-CSF with ALN using different types of cross-linking agents, has been developed at the Institute of Medical Biotechnology of the State Research Center for Virology and Biotechnology “Vector”, Federal Service for Surveillance on Consumer Rights Protection and Human Well-being (IMBT FBRI SRC VB "Vector", Rospotrebnadzor) [21]. It has been shown that the resulting conjugates, compared to rhGM-CSF, have an increased affinity for hydroxylapatite, an analog of the mineral bone tissue matrix. The evaluation of the specific conjugates activity *in vitro* confirmed the preservation of the biological activity of rhGM-CSF in the composition [21].

THE AIM of the work is to evaluate the hemostimulatory activity of recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) conjugates with alendronic acid (ALN) in the model of cytostatic myelosuppression and the dynamics of rhGM-CSF accumulation as a part of the conjugate in the bone tissue and bone marrow of mice.

MATERIALS AND METHODS

Study drugs

Experimental preparations were conjugates of rhGM-CSF with ALN obtained by the described methods [21]. The key point in the synthesis process was the choice of conditions that would minimize conformational changes in protein molecules in order to preserve their biological activity. For this, equimolar amounts of the rhGM-CSF protein and ALN were used. 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) was chosen as the crosslinking agent; dextran with a molecular weight of 40,000 Da, activated with periodate, was used as a linker. The conjugation with EDC was carried out in two ways: a direct (rhGM-CSF →EDC → ALN) and reverse (ALN →EDC → rhGM-CSF) sequence of components application to the solid phase (hydroxyapatite sorbent, HAP).

For the direct sequence conjugation, the HAP chromatographic column was equilibrated with 2 mM potassium phosphate buffer, pH 7.0. A rhGM-CSF protein solution was applied to the balanced column, after the sorption of which the EDC solution was also fed to the column. The outlet of the solution from the column was blocked for 2 h to bind the components, then the column was washed with 2 mM potassium phosphate buffer (pH 7.0), and the ALN solution was applied. The column was repeatedly washed with phosphate buffer to remove the unbound components. The resulting conjugate was eluted from the column with 0.2 M potassium phosphate buffer, pH 7.0. The synthesized conjugate was transferred into a *physiological saline solution* by dialysis.

When dextran was used as a linker during the conjugation, it was added to the solution containing sodium periodate to form reactive aldehyde groups in the ratio of 1:40 (mol/mol), mixed, and incubated at 20°C for an hour. The activated dextran was separated by a gel filtration on the Sephadex G-25 column. The solutions of protein and ALN were added to the dextran solution in equimolar ratios: 1 mol of protein and 1 mol of ALN per 1 mol of dextran. The resulting mixture was incubated for 3 h at 20°C. To remove the unreacted components, the gel filtration was performed on the Sephadex G-25 column. The synthesized conjugate was transferred into the physiological saline by dialysis.

Three types of conjugates were obtained:

1 – GEA – rhGM-CSF conjugate with ALN, obtained by a solid-phase synthesis through the carboxyl group of the protein using EDC by the method of a direct (protein→EDC→ALN) sequence of applying components to the sorbent, with a protein concentration of 1.09 mg/ml (Fig. 1, track 1);

2 – AEG – conjugate of rhGM-CSF with ALN, obtained by a solid-phase synthesis through the carboxyl group of the protein using EDC by the reverse (ALN→EDC→protein) sequence of applying components to the sorbent, with a protein concentration of 1.45 mg/ml (Fig. 1, track 2);

3 – DGA – conjugate of rhGM-CSF with ALN, obtained by a synthesis through the protein amino group *via* a dextran molecule as a linker using the Malaprade reaction [22], with a protein concentration of 0.86 mg/ml (Fig. 2, track 1).

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Reference drug

The rhGM-CSF protein, obtained at the IMBT FBRI SRC VB "Vector", Rospotrebnadzor according to the described method [23], was used as a reference drug and to obtain conjugates with ALN.

The protein substance was characterized by quality indicators in accordance with the requirements of regulatory documentation[1]. The protein concentration in the used substance was 1.5 mg/ml, the homogeneity of the preparation was 99.2%.

Experimental animals

The study was carried out on 66 healthy male CBA/Calac mice weighing 19–23 g, obtained from the nursery of Goldberg Research Center of Pharmacology and Regenerative Medicine (Tomsk), and 45 female white outbred CD-1 (ICR) mice weighing 22–25 g from the nursery of the Federal Budgetary Institution of the State Research Center "Vector" of Rospotrebnadzor (Koltsovo, the Novosibirsk region). The age of the animals was 2.0–2.5 months. Before the start of the study, the animals had gone through a period of the adaptive quarantine. The mice were kept under standard vivarium conditions at the constant temperature and humidity; food and drink were available at any time of the day. The animals' keeping and experimental studies were carried out in accordance with the requirements of the International Convention for the Protection of Vertebrate Animals used for Experimental and Scientific Purposes (Strasbourg, 1986), as well as in compliance with Directive 2010/63/EU of the European Parliament and Council of the European Union dated September 22, 2010, for the protection of the animals used for scientific purposes. The studies were approved by the Bioethical Commission of "Vector" (protocol No. 5 dated 01.10.2020).

Method for assessing hemostimulating activity of conjugates

The hemostimulating activity of the drugs was studied in a model of cytostatic myelosuppression induced by the administration of cyclophosphamide (CP, Sigma-Aldrich, USA) to CBA/Calac mice [24].

The animals were divided into 5 experimental groups (4 experimental and 1 control); there were 12 male mice in each one. All the experimental animals received a single intraperitoneal injection of a CP solution at the maximum tolerated dose (250 mg/kg) in the volume of 0.25 ml per 20 g of body weight.

24 hours after the CP injection, the mice of the experimental groups were injected subcutaneously for 4–5 days with one of the following drugs: 1) rhGM-CSF; 2) GEA conjugate; 3) AEG conjugate; 4) DGA conjugate. The administration dose, previously determined as an effective hemostimulating agent using the rhGM-CSF preparation [25], was 90 μg/kg; the volume of the administration was 0.2 ml per 20 g of the animal body weight. The mice of the control group received subcutaneous saline in the equivalent volume according to the similar scheme. Six intact animals were used as an additional control group (without the administration of rhGM-CSF and CF preparations). All manipulations with the animals were carried out at the same time (in the morning).

One day after the drug administration completion (on the 5th day after the CP administration), the blood samples from the tip of the tail were taken for the analysis from each group in half of the animals, and after the cervical dislocation of the cervical vertebrae, the bone marrow samples were taken. On that day, the second half of the animals received another injection; the biomaterial was taken for the analysis 24 hours after injection.

In the blood samples, the total leukocyte count was determined by a light microscopy (the blood was diluted 20 times with a 3% solution of acetic acid, the calculation was carried out in Gorjaev's chamber), the relative and absolute contents of neutrophils and other morphological forms of leukocytes were also detremined. In the bone marrow samples, the total karyocyte count (TKC) was carried out and the number of cells per femur was calculated. To obtain the bone marrow, the mouse femur was isolated, it was cleaned from the soft tissues of the femur, and the bone marrow canal was thoroughly washed with a 3% acetic acid solution in the volume of 1 ml. The TKC was carried out using Gorjaev's chamber.

Method for studying rhGM-CSF accumulation dynamics

To study the rhGM-CSF accumulation dynamics in bone tissue and bone marrow, CD-1 female mice were divided into 3 groups: a control group (5 individuals) and two experimental groups (20 individuals in each). On the eve of the experiment, at the end of the working day, the mice were transferred to clean cages without food. The animals were given food 2 hours after the drugs administration, there was water without restrictions.

The animals of the first experimental group received an intravenous rhGM-CSF injection; the mice of the second experimental group were injected with the AEG conjugate. The preparations were administered at the dose of 90 μg/kg of body weight, 0.2 ml per 20 g of animal weight. The intact mice served as control animals (the third group).

[1] GPM.1.7.1.0007.15 "Medicines obtained by recombinant DNA methods". Russian State Pharmacopeia ed 14. 2018;4: 2575-95. Available from: https://docs.rucml.ru/feml/pharma/v14/vol2/763/

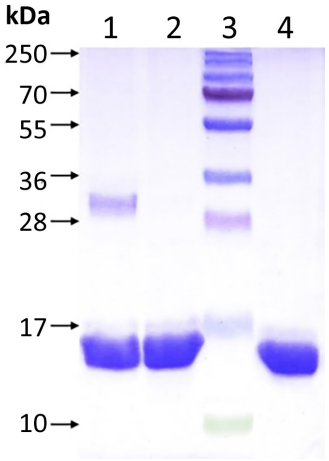

Figure 1 – Electrophoregram of conjugates obtained by direct sequence of applying components, GEA (1) and reverse sequence, AEG (2)

Note: Electrophoresis in 15% polyacrylamide gel under reducing conditions, R-250 Coomassie staining. Tracks: 3 – protein marker 10–250 kDa; 4 – rhGM-CSF protein, 20 µg.

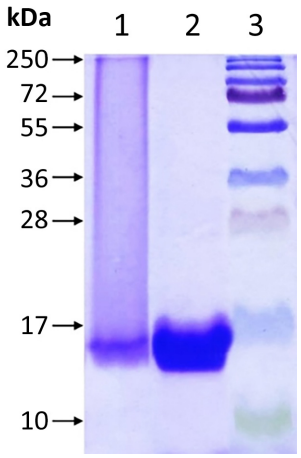

Figure 2 – Electropherogram of conjugate obtained using dextran (1)

Note: Electrophoresis in 15% polyacrylamide gel under reducing conditions, R-250 Coomassie staining. Tracks: 2 – rhGM-CSF protein, 20 µg; 3 – protein marker 10–250 kDa.

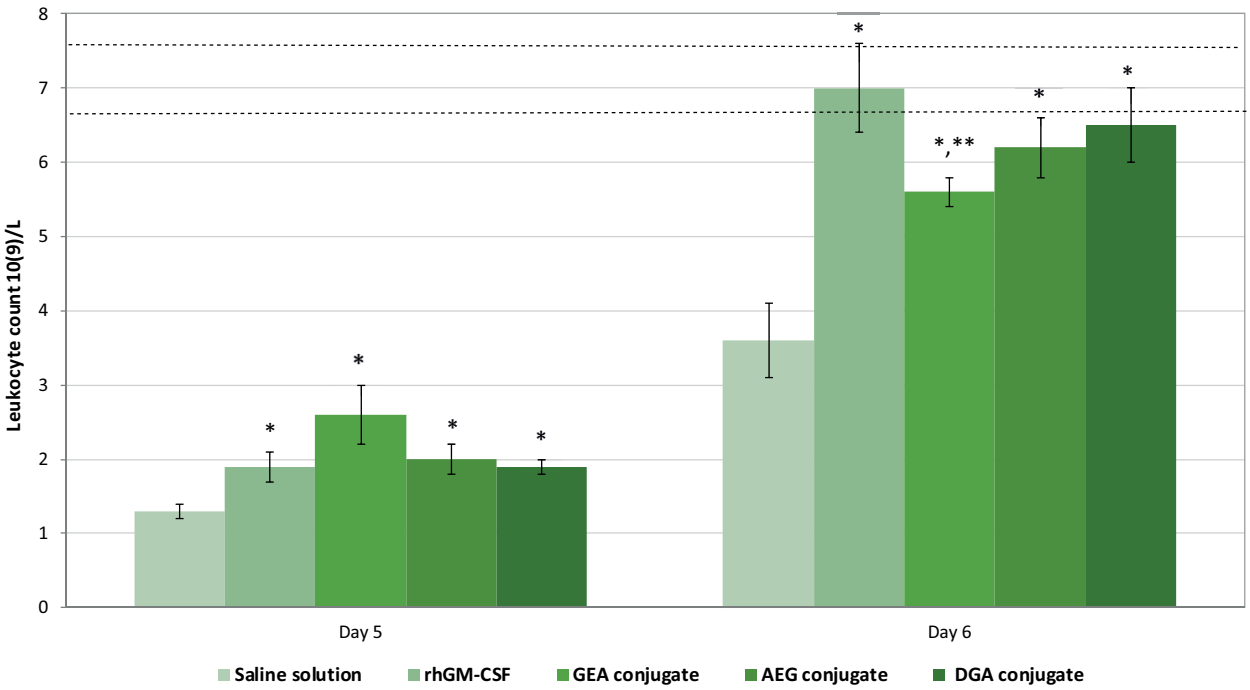

Figure 3 – Leukocyte count in peripheral CBA mice blood against the background of CP administration, rhGM-CSF drug and its conjugates with ALN

Note: abscissa shows study time (days); * – statistically significant difference in relation to the control (saline); ** – statistically significant difference in relation to rhGM-CSF at p ≤0.05. Area between dotted lines is confidence interval of indicator in intact mice.

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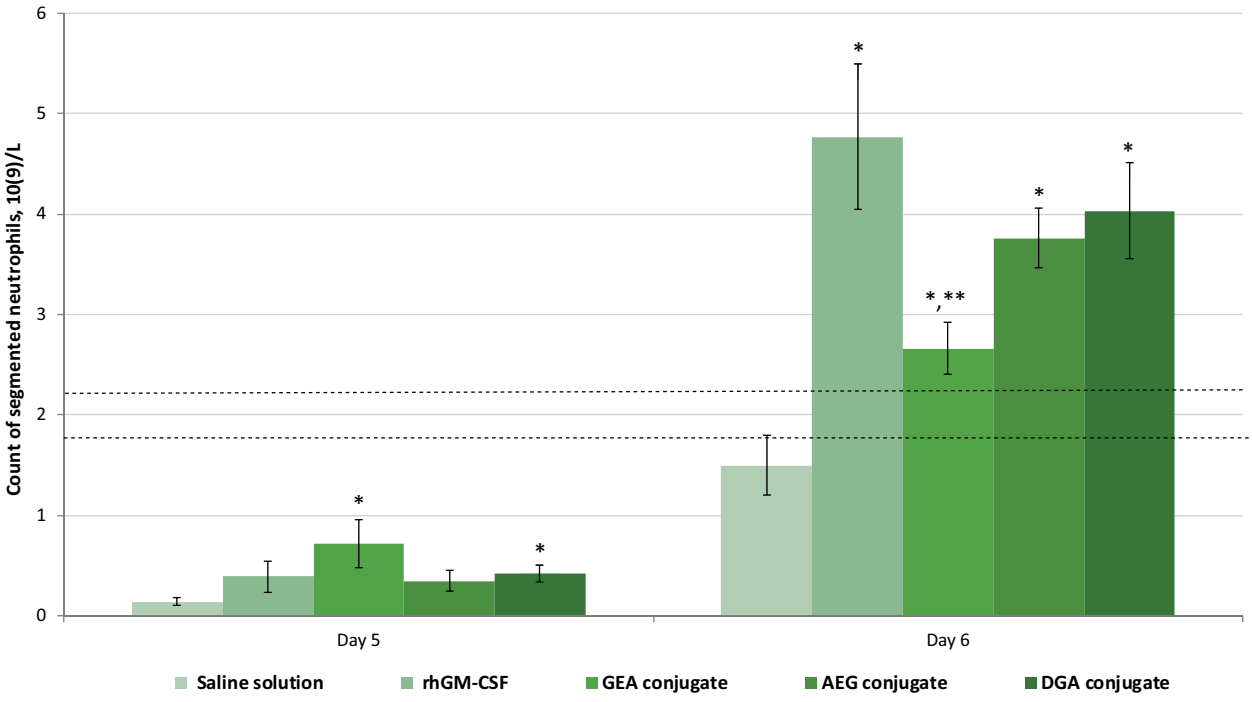

Figure 4 – Count of segmented neutrophils in peripheral CBA mice blood against the background of CP administration, rhGM-CSF drug and its conjugates with ALN

Note: abscissa shows study time (days); * – statistically significant difference in relation to the control (saline); ** – statistically significant difference in relation to rhGM-CSF at p ≤0.05. Area between dotted lines is confidence interval of indicator in intact mice.

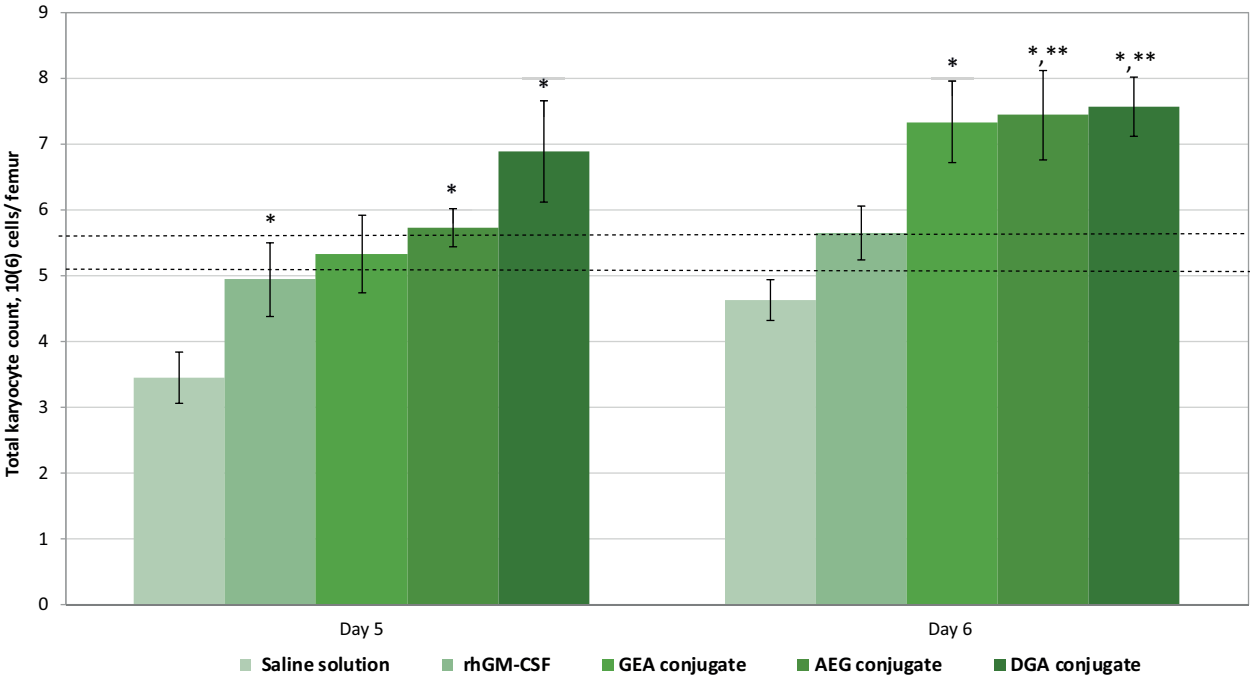

Figure 5 – Karyocyte counts in CBA mice bone marrow against the background of CP, rhGM-CSF its conjugates with ALN administration

Note: abscissa shows study time (days); * – statistically significant difference in relation to the control (saline); ** – statistically significant difference in relation to rhGM-CSF at p ≤0.05. Area between dotted lines is confidence interval of indicator in intact mice.

Table 1 – Dynamics of changes in the level of rhGM-CSF in the blood, bone tissue and bone marrow of mice after a single intravenous administration of rhGM-CSF preparations

Drug	Concentration of rhGM-CSF in samples			
	Blood serum, pg/ml			
	3 minutes	1 hour	4 hours	24 hours
rhGM-CSF	151 715±32 571	3 138±214	24.5±2.3	0.174±0.148
AEG conjugate	406 468±54 586**	8 500±2 539**	48.8±9.0**	0.676±0.676
	Femur, pg/g			
Control	184±139			
rhGM-CSF	3 869±458*	322±151	436±52	137±105
AEG conjugate	11 154±1 613*,**	1 108±387	1 706±374*,**	1 652±449
	Bone marrow of femur, pg/femur			
Control	3.52±2.03			
rhGM-CSF	237±36*	9.84±7.47	0±0	1.20±1.20
AEG conjugate	567±127*	20.3±12.8	5.64±5.64	15.7±9.7

Note: experimental data are presented as arithmetic mean and standard error (M±m); * – differences are statistically significant compared to control; ** – differences are statistically significant compared to mice that were injected with rhGM-CSF ($p<0.05$ for blood; $p<0.017$ for tissues).

3 minutes later, 1, 4 and 24 hours after the drugs administration, the blood and one femur was taken from 5 animals from each experimental group after euthanasia. On the first day of the experiment, a similar material was taken from the mice in the control group. The serum was obtained from the blood; the bone marrow was extracted from the femur by washing the bone marrow canal with a 0.9% sodium chloride solution in the volume of 1 ml. The bone marrow cells were resuspended with a dispenser until a homogeneous suspension. After the bone marrow removal, the femur was weighed and a 10% homogenate was prepared in 0.1 M potassium phosphate buffer (pH 7.2) using a GlasCol homogenizer (USA). The resulting biomaterial was stored at the temperature not exceeding –20°C. On the day of the analysis, the bone marrow and femur homogenates were thawed, centrifuged (5810R centrifuge, Eppendorf, Germany) at 5000 g and the temperature of 2–8°C for 5 min, and the supernatants were collected.

In the blood serum and supernatants of femur homogenatesthe content of rhGM-CSF was determined by electronic intelligence search and analysis (ELISA) using reagent kits for the determination of human GM-CSF in serum, blood plasma, supernatants of cell cultures and organ homogenates "Human Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) ELISA Kit", "CUSABIO", China. The range of the standard sample determined concentrations included in the kit, was 15.6–1000 pg/ml, the sensitivity was less than 3.9 pg/ml.

Statistical processing of obtained results

The results obtained were processed using the "Statgraphics, Version 5.0" software package ("Statistical Graphics Corp.", USA). Due to the small sample sizes, non-parametric tests were used to assess the significance of intergroup differences – the two-sample Mann-Whitney U-test and the Kruskal-Wallis H-test of multiple comparisons with a critical level of statistical significance (p) equal to 0.05. When statistically significant differences were found in the H-test, post-hoc comparisons were performed using the U-test, while the adjusted critical significance level (p) for three pairwise comparisons was taken equal to 0.0170 [26, 27]. The experimental data are presented as the arithmetic mean and standard error (M±m). The figures are constructed using Microsoft Excel.

RESULTS AND DISCUSSION

The study showed that a single CP administration to the mice at the dose of 250 mg/kg led to the regular development of myelosuppression. The total karyocyte count of the bone marrow in the control group mice on the 5th day after the CP administration decreased by 1.5 times, the blood total leukocyte count- by 5.5 times, the count of segmented neutrophils – by 13.9 times.

The change in the counts of leukocytes, segmented neutrophils, and bone marrow cells in the mice after the drug administration was evaluated in comparison with the indicators of the control group (physiological saline), the values of which were taken as 100%. The administration of both rhGM-CSF and its conjugates with ALN to the animals resulted in a faster statistically significant ($p \leq 0.05$) increase in the blood leukocyte count compared to the control values (Fig. 3). On the 5th day after the CP administration, the blood leukocyte count in the mice treated with rhGM-CSF preparations exceeded the control value by 46–100%, on the 6th day – by 56–94%. Significant differences in the level of the leukopoiesis stimulation in the groups that had been injected with different types of conjugates or rhGM-CSF, were not observed.

On the 5th day after the CP administration, in the mice groups that had been injected with GEA and

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DGA conjugates, a statistically significant increase in the number of segmented neutrophils compared with the control ones, was notified by 414 and 200%, respectively, p≤0.05). On the 6th day, with the conjugates administration, the increased values of the indicator were registered in all the two groups (by 77 and 169%). A significant stimulating effect of the initial rhGM-CSF was observed only on the 6th day, the count of the segmented neutrophils in the blood during this period increased by 218% compared with the index of the animals treated with saline (Fig. 4).

The data presented in Fig. 5, shows that 5 days later, the rhGM-CSF administration led to a statistically significant increase in the total karyocytes count, by 43% after the CP administration compared with the control values. Similar, but more pronounced, changes were notified in the groups of mice that had been injected with the conjugates. At the same time, the DGA effect (an increase of 100% compared to the control) was maximal. Six days after the cytostatic administration, the karyocytes count in the bone marrow of the mice that had been injected with conjugates, was 58–63% higher than the control level, and 30–34% higher than the level recorded after the rhGM-CSF administration.

Thus, the conjugation of rhGM-CSF with ALN did not lead to a decrease in the hemostimulatory protein activity. No significant differences were found in the level of the leukostimulating activity of the studied preparations. The GEA and DGA conjugates accelerated the recovery of the murine blood neutrophil count. The AEG and DGA conjugates had a more pronounced stimulating effect on the production of bone marrow cells compared to GM-CSF, which was manifested in a more intense and earlier increase in the total bone marrow cellularity after the exposure to the cytostatic.

The enhancement of the stimulating rhGM-CSF effect in the conjugate on the production of karyocytes can obviously be associated with an increased affinity of the protein to the bone tissue cells.

To confirm the targeted rhGM-CSF delivery in the composition of the ALN conjugate to the bone tissue, a comparative study of the drug accumulation in the bone tissue and bone marrow after the intravenous rhGM-CSF and its AEG conjugate administration, was carried out.

The dynamics in protein concentration changes in the blood, the bone tissue and bone marrow of mice after a single intravenous rhGM-CSF and its conjugate administration at the effective hemostimulating dose (90 μg/kg), is presented in Table 1.

The obtained data indicate that the highest rhGM-CSF blood values in the mice were registered 3 min after the drug administration and amounted to 19.2% of the administered dose in the mice treated with AEG conjugate, and to 7.2% in the mice after the rhGM-CSF administration. (Table 1). The protein was retained in the blood serum of mice of the both experimental groups for 4 hours of the observation. 1 hour after the drugs administration, the values of the indicator decreased by 48 times compared to the “3 min” point, and in the subsequent periods, their further decrease to the background level was notified. The rhGM-CSF concentration in the blood of mice that had been administrated with the conjugate, exceeded the corresponding indicator after the rhGM-CSF administration by 2.7 times after 3 minutes and 1 hour, and twice after 4 hours after the administration (the differences are statistically significant, p ≤0, 05). 24 hours after the injection, rhGM-CSF was detected in small amounts in the blood of two out of five animals after the rhGM-CSF administration, and in one out of five mice that had been administrated with the AEG conjugate. In the blood serum of the control animals, the values of the indicator did not differ from the zero in any of the observation periods.

The highest rhGM-CSF level in the mice femoral bone homogenates was detected 3 min after the drug administration. At the same time, the protein content in the bone tissue of the mice treated with the conjugate at this point was 60 times higher than that of the animals administrated with rhGM-CSF. In the subsequent periods, the protein concentration in all the mice femurs that had been administrated with rhGM-CSF, decreased to the control level (1 hour after the administration, Table 1). In the bone tissue samples of the mice that had been administrated with AEG conjugate, the concentration of rhGM-CSF 1 hour after the injection was 6 times higher than the control level (the differences are not statistically significant, p ≤0.017), in the subsequent periods (4 and 24 hours) – by 9 times (the statistial differences were observed at the 4-hour point). 24 hours after injection, only two out of five mice that had been administrated with rhGM-CSF, had an increased rhGM-CSF content in the bone tissue (541 and 146 pg/g of the tissue), while a higher level of rhGM-CSF than in the control CSF (from 1408 to 2460 pg/g) after the administration of the AEG conjugate was observed in four out of five animals.

The maximum rhGM-CSF concentration in the bone marrow of the mice from the both experimental groups, as well as in the blood and femoral bone homogenates, was recorded at the first point, 3 minutes after the administration (Table 1). The values of the indicator in the mice that had been administrated with the AEG conjugate, were 161 times higher than the control values, and 2.4 times higher than the indicator of the mice that had been administrated with rhGM-CSF. In the subsequent periods, the protein content in both experimental groups decreased. However, it should be notified that 4 and 24 hours after the rhGM-CSF administration of all five mice, the protein in the bone marrow was not detected, while in the group that had been administrated with the AEG conjugate, the trace amounts of rhGM-CSF were registered in 2 mice (from 28.2 to 42.2 pg/femur).

Thus, the introduction of alendronic acid into the

composition of rhGM-CSF contributed to an increase in the accumulation and distribution of rhGM-CSF in the bone tissue and bone marrow of the mice, which is consistent with the available literature data on the accumulation of alendronic acid preparations in the bone tissue, their strong binding and retention by the bone matrix [28-34]. The concentration of the conjugated protein in the blood in the first hours after the administration was higher than that of the free rhGM-CSF, which may be due to an increase in the protein resistance to proteolytic enzymes as a result of modification and, as a result, the appearance of its ability to circulate in the bloodstream for a longer time.

CONCLUSION

It has been established that rhGM-CSF conjugates with ALN have a hemostimulating activity comparable to the activity of the original protein. In the composition of conjugates, the effect of rhGM-CSF on the bone marrow cells was more pronounced and prolonged, which is apparently due to the longer presence of the protein in the conjugate in the blood and its increased accumulation in the bone tissue and bone marrow. The data obtained make it possible for us to conclude that further work on the development of hemostimulating drugs based on rhGM-CSF conjugates with alendronic acid, is promising.

FUNDING

The study was carried out as a part of the work on the state task implementation of the of State Research Center of Virology and Biotechnology "VECTOR", Rospotrebnadzor, subject GZ-39/21.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

GGSh – conducting experimental studies, statistical data processing, diagramming, writing the article text;
AVB – literature search, experimental research, statistical data processing, writing the article text;
EST – conducting experimental studies; SGG – conducting a literature search, forming the purpose and objectives of the study, developing the design of the study, finalizing the article text;
TIE – obtaining drugs for research; EAV – obtaining drugs for research; EDD – approval of the pilot study plan, editing and revision of the article text content, approval of the final article text version.

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Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

ОРИГИНАЛЬНАЯ СТАТЬЯ

DOI: 10.19163/2307-9266-2022-10-5-483-496

B6.A-DYSF^{PRMD}/GENEJ MICE AS A GENETIC MODEL OF DYSFERLINOPATHY

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Received 05 July 2022 | After peer review 10 Sep 2022 | Accepted 18 Oct 2022

The aim of the work was behavioral and pathomorphological phenotyping of the mice knockout for the DYSF gene, which plays an important role in the development and progression of dysferlinopathy.
Materials and methods. A B6.A-Dysf^{prmd}/GeneJ (Bla/J) mice subline was used in the work. During the study, a muscle activity was determined basing on the following tests: "Inverted grid", "Grip strength", "Wire Hanging", "Weight-loaded swimming", Vertical Pole". Histological and immunofluorescent examinations of skeletal muscles *(m. gastrocnemius, m. tibialis)* were performed. The presence and distribution of the dysferlin protein was assessed, and general histological changes in the skeletal muscle characteristics of mice at the age of 12 and 24 weeks, were described. A morphometric analysis with the determination of the following parameters was performed: the proportion of necrotic muscle fibers; the proportion of fibers with centrally located nuclei; the mean muscle fiber diameter.
Results. The "Grip strength" test and the "Weight-loaded swimming" test revealed a decrease in the strength of the forelimbs and endurance in the studied mice of the Bla/J subline compared to the control line. The safety of physical performance was checked using the "Wire Hanging" test and the "Vertical Pole" test, which showed a statistically significant difference between the studied mice and control. The coordination of movements and muscle strength of the limbs examined in the "Inverted Grid" test did not change in these age marks. Decreased grip strength of the forelimbs, decreased physical endurance with age, reflects the progression of the underlying muscular disease. Histological methods in the skeletal muscles revealed signs of a myopathic damage pattern: necrotic muscle fibers, moderate lympho-macrophage infiltration, an increase in the proportion of fibers with centrally located nuclei, and an increase in the average fiber diameter compared to the control. The dysferlin protein was not found out in the muscle tissues.
Conclusion. Taking into account the results of the tests performed, it was shown that the absence of Dysf$^{-/-}$ gene expression in Bla/J subline mice led to muscular dystrophy with the onset of the development of phenotypic disease manifestations at the age of 12 weeks and their peak at 24 weeks. Histopathological phenotypic manifestations of the disease are generally

Для цитирования: М.В. Корокин, Е.В. Кузубова, А.И. Радченко, Р.В. Деев, И.А. Яковлев, А.В. Дейкин, Н.С. Жунусов, А.М. Краюшкина, В.М. Покровский, В.О. Солдатов, О.А. Пученкова, К.Д. Чапров, Н.В. Екимова, С.Н. Бардаков, О.Н. Чернова, А.М. Емелин, И.С. Лимаев. Использование линии трансгенных мышей B6.A-DYSF^{PRMD}/GENEJ для моделирования дисферлинопатии. *Фармация и фармакология.* 2022;10(5):483-496. **DOI:** 10.19163/2307-9266-2022-10-5-483-496

For citation: M.V. Korokin, E.V. Kuzubova, A.I. Radchenko, R.V. Deev, I.A. Yakovlev, Deikin A.V., N.S. Zhunusov, A.M. Krayushkina, V.M. Pokrovsky, V.O. Soldatov, O.A. Puchenkova, K.D. Chaprov, N.V. Ekimova, S.N. Bardakov, O.N. Chernova, A.M. Emelin, I.S. Limaev. Use of transgenic B6.A-DYSF^{PRMD}/GENEJ mice for dysferlinopathy modeling. *Pharmacy & Pharmacology.* 2022;10(5):483-496. DOI: 10.19163/2307-9266-2022-10-5-483-496

RESEARCH ARTICLE

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

nonspecific and corresponded to the data of intravital pathoanatomical examination in diferlinopathy patients. The mice of the studied subline Bla/J are a representative model of dysferlinopathy and can be used to evaluate new therapeutic agents for the treatment of this disease.

Keywords: dysferlinopathy; DYSF gene; Myoshi's myopathy; muscular dystrophy; phenotyping; knockout; genotyping; animals; mouse model; B6.A-Dysf^{prmd}/GeneJ

Abbreviations: Bla/J – B6.A-Dysf^{prmd}/GeneJ; CK – creatine kinase; DNA – deoxyribonucleic acid; PCR – polymerase chain reaction; bp – base pairs; WT – wild-type mice; EDTA – ethylenediaminetetraacetic acid; Gas M – *M. gastrocnemius medialis*; Gas L – *M. gastrocnemius lateralis*; tib – *M. tibialis.*

МЫШИ B6.A-DYSF^{PRMD}/GENEJ КАК ГЕНЕТИЧЕСКАЯ МОДЕЛЬ ДИСФЕРЛИНОПАТИИ

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Получена 05.07.2022 После рецензирования 10.09.2022 Принята к печати 18.10.2022

Цель. Поведенческое и патоморфологическое фенотипирование мышей, нокаутных по гену Dysf, играющему важнейшую роль в развитии и прогрессировании дисферлинопатии.

Материалы и методы. В работе использована сублиния мышей B6.A-Dysf^{prmd}/GeneJ (Bla/J). В ходе исследования определяли мышечную активность при помощи следующих тестов: «Перевернутая сетка», «Сила хватки», «Удержание на проволоке», «Вынужденное плавание с грузом», «Удержание животного на скользком вертикальном стержне». Выполнено гистологическое и иммунофлюоресцентное исследование скелетной мускулатуры (*m. gastrocnemius*, *m. tibialis*). Оценено наличие и распределение белка дисферлина, описаны общие гистологические изменения скелетной мышцы, характерные для мышей в возрасте 12 и 24 недель. Также выполнен морфометрический анализ с определением следующих параметров: доля некротизированных мышечных волокон; доля волокон с центрально расположенными ядрами; средний диаметр мышечного волокна.

Результаты. Тест «Сила хватки» и «Принудительное плавание с грузом» выявили снижение силы передних конечностей и выносливости у исследуемых мышей сублинии Bla/J по сравнению с контрольной линией. Сохранность физической работоспособности проверена при помощи тестов «Проволочный тест» и «Удержание животного на скользком вертикальном стержне», которые показали статистически значимое различие между исследуемыми мышами и контролем. Координация движений и мышечная сила конечностей, исследованных в тесте «Перевёрнутая сетка», в данных возрастных метках не изменена. Уменьшение силы хватки передних конечностей, снижение физической выносливости с возрастом отражает прогрессирование основного мышечного заболевания. Гистологическими методами в скелетной мускулатуре выявлены признаки миопатического паттерна повреждения: некротизированные

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DOI: 10.19163/2307-9266-2022-10-5-483-496

мышечные волокна, умеренная лимфо-макрофагальная инфильтрация, увеличение по сравнению с контролем доли волокон с центрально расположенными ядрами, среднего диаметра волокна. Белок дисферлин в тканях мышц не обнаружен.

Заключение. С учетом результатов проведенных тестов показано, что отсутствие экспрессии гена Dysf$^{-/-}$ у мышей сублинии Bla/J приводило к мышечной дистрофии с началом развития фенотипических проявлений болезни в 12 недель жизни и их пиком к 24 неделе. Патогистологические фенотипические проявления болезни в целом неспецифичны и соответствовали данным прижизненного патологоанатомического исследования у пациентов с диферлинопатией. Мыши исследуемой сублинии Bla/J являются репрезентативной моделью дисферлинопатии и могут быть использованы для оценки новых терапевтических средств для лечения данного заболевания.

Ключевые слова: дисферлинопатия; ген Dysf; миопатия Миоши; мышечная дистрофия; фенотипирование; нокаут; генотипирование; трансгенные животные; мышиная модель; B6.A-Dysf^{prmd}/GeneJ

Список сокращений: Bla/J – B6.A-Dysf^{prmd}/GeneJ; КК – креатинкиназа; ДНК – дезоксирибонуклеиновая кислота; ПЦР – полимеразная цепная реакция; п.о. – пар оснований, WT – мыши дикого типа; ЭДТА – этилендиаминтетрауксусная кислота; Gas M – *M. gastrocnemius medialis*; Gas L – *M. gastrocnemius lateralis*; tib – *M. tibialis*.

INTRODUCTION

Dysferlinopathy is phenotypically heterogeneous progressive muscular dystrophy caused by mutations in the DYSF (2p13) gene, which encodes the transmembrane protein dysferlin (230 kDa) involved in the sarcolemma repair. [1]. Dysferlinopathy includes a presymptomatic stage of an asymptomatic increase in the level of creatine kinase (CK) in the blood, and a manifest stage, characterized by a progressive lesion of the proximal and/or distal muscles of the extremities [2]. There are five main phenotypes of dysferlinopathy: Miyoshi's distal myopathy (OMIM # 254130), limb-girdle muscular dystrophy R2 (LGMD R2, OMIM # 253601); distal myopathy of the anterior bed of the leg (distal, with the origin in the anterior tibial muscle (DMAT, OMIM # 606768); a proximal-distal form (transitional form) and a congenital phenotype [3]. Dysferlin consists of seven C2 domains and is a transmembrane protein of skeletal muscles, also expressed by cardiomyocytes and monocytes [4]. The main role of dysferlin is Ca^{2+} dependent repair of the sites of the sarcolemma damage [5]. Dysferlinopathy is manifested in the range from late adolescence to early adulthood, followed by a steady increase in disease manifestations [6].

The disease manifestation occurs in late adolescence – early adulthood, followed by a steady progression of muscle weakness and an outpatient status loss at 35–45 years [7].

A proper conduct of a clinical trial requires understanding of disease progression patterns and response to various outcome measures over time [8]. The complexity of choosing indicators for clinical studies of dysferlinopathy is determined by the variability of the manifestation age, a phenotype, a severity of the muscular dystrophic process, a variable progression rate, and modifying factors that have not been fully identified [9]. It has been previously shown that the assessment of a motor function in dysferlinopathy is a reliable method of objectification, however, the variability in the rate of progression of the disease makes it difficult to demonstrate a response in small cohorts [10, 11]. It has been established that, despite the absence of dysferlin protein expression in skeletal muscles, there is a variation in the age of manifestation, clinical manifestations, and a disease severity. Similarly, dysferlinopathy can progress at different rates, even in the presence of the same mutation [12, 13]. A number of factors that modify a phenotype and a dysferlinopathy progression rate have been described: a physical activity degree and duration and a type of mutation (homozygosity for nonsense mutations), in the absence of stable clinical and genetic correlations [14, 15].

THE AIM of the work was behavioral and pathomorphological phenotyping of the mice knockout for the DYSF gene, which plays an important role in the development and progression of dysferlinopathy.

MATERIALS AND METHODS

Laboratory animals

The subline of mice B6.A-Dysf^{prmd}/GeneJ (Bla/J), obtained from the testing center "Vivarno-experimental complex LLC "Mitoengineering Research Institute of Moscow State University", was used in the work. The animal cohorts were obtained by crossing A/J mice (#:000646), in which a spontaneous insertion in intron 4 was accidentally detected, with C57BL/6J wild-type mice. Maintenance and reproduction of the colony was carried out by crossing mutant animals with each other from the same litter.

Experimental and control (Bla/J, n=20; C57BL/6J, n=10 and n=13) animals were kept in a pathogen-free vivarium of Belgorod State National Research University under conditions of artificially regulated

daylight hours (12 hours of darkness and 12 hours of daylight) at the temperatures from +22 to +26°C, and had a free access to food and water. The work was guided by ethical principles for the treatment of laboratory animals in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS No 170). All painful manipulations with the animals were carried out in accordance with regulatory standards: Directive 2010/63/EU of the European Parliament and of the Council of the European Union dated September 22, 2010 on the protection of the animals used for scientific purposes. Experimental studies were approved by the Bioethical Commission of Belgorod State National Research University (protocol No. 15/10 dated 2021 Oct 29). Vivisection was carried out in accordance with the ethical principles for the treatment of laboratory animals as set out in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (CETS No. 123).

Behavioral tests were carried out mainly in the morning hours with fixed "home" lighting (to minimize the stress factor). Representative groups of the experimental animals were formed (homozygous knockouts – Bla/J, n=20). The groups were tested at two age points – 12 and 24 weeks – and the control animals without a genome modification (WT, n=10 and 13, respectively).

Inverted Grid Test

The inverted grid is a 45×45 cm wire grid with a mesh size of 12×12 mm and a wire diameter of 1 mm, surrounded by a 4 cm partition preventing a mouse from attempting to climb over to the other side. The test is used to assess the coordination of movements and muscle strength of both pairs of limbs. The mice were placed in the center of a wire grid, which was inverted and placed 50 cm above a soft surface. The fall time of the animal was recorded or the animal was removed from the grid if the time reached 180 sec. The test was evaluated in points: the more time the animal managed to hold on the higher its score was [16–18].

Grip strength test

The setup was a stainless-steel grid connected to a sensor to measure the grip strength (in grams) of a mouse's forelimbs. The animal was allowed to grasp a horizontal grid with its front paws, and then the mouse was pulled back by the tail until its grip was weakened, while the hind legs of the mouse should not touch the grid. The force measurement sensor stored the peak value of the thrust force. The test was used to study the function of the neuromuscular system. For the analysis, the mean values from 5 successful measurements of the forelimbs strength were used [19, 20].

Wire Hanging test

The test was based on the instinct of mice to avoid falling. The mouse was placed on a horizontally stretched wire with the capture of all four limbs (the diameter of 3 mm, the height above the surface of 60 cm). The ability to stay on the wire was measured by evaluating the time the mouse had been held until the moment of falling, using a stopwatch timer. The best result of two attempts was taken as the final value, the pause between which was 20 min [21, 22].

Weight-loaded swimming test

A physical performance of the animals in this test was assessed by the swimming duration with a load, which was 5% of the body weight (the weight of the load had been found out experimentally), attached to the root of the animal's tail with a rubber bandage. The weight of the animals was determined with an accuracy of 0.1 g; the load was selected with an accuracy of 0.01 g. The duration of testing (swimming) was recorded using a stopwatch timer with an accuracy of 1 sec. The end of the experiment was considered the moment of the animal's fatigue, the sign of which was the animal's inability to rise to the water surface within 5 seconds or a refusal to swim (submersion to the bottom for more than 5 sec). A sign of the animal's fatigue was a violation of the motor-coordination function (rotation around its axis and falling on its side in the water column).

Swimming was carried out in organic glass vessels with an inner diameter of 30 cm and a height of 60 cm. The height of the water column was 30 cm, and the water temperature was 23±1°C [23, 24].

Vertical Pole test

In the studies on laboratory animals for screening and assessing the safety of physical performance, this technique was mainly used. The setup consists of a tripod with a diameter of 7 mm and a height of 60 cm, with a plastic fence installed on the top. For the experiment, the animal was placed at the same distance from the top of the tripod strictly upside down, at least 1 m above the floor. The time of the animal's fall from the tripod using a

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DOI: 10.19163/2307-9266-2022-10-5-483-496

stopwatch, was recorded. The preservation of a physical performance was judged by comparison with the control group [25].

Genotyping of transgenic animals

For the analysis, genomic DNA was obtained by ear biopsy (about 30 mg of tissue). The biopsy material was placed in a lysing solution containing 100 mM of sodium chloride, 50 mM Tris-HCl (pH 8.0), 2 mM ethylenediaminetetraacetic acid (EDTA), and 2 mg/ml of proteinase K. The material was incubated at 55°C for 12–16 h, after that the reaction mixture was heated at 85°C for 40 min. The lysate was centrifuged using a Thermo SL16R laboratory centrifuge (Thermo Scientific, USA) for 1 min at 10,000 g, and 1 µl of the supernatant was used as a template in the PCR reaction.

Primer sequences from the JAX protocol (Protocol 26095) were used. They simultaneously amplified the retrotranspason area (if present) and the animal genomic DNA area. A mixture of 3 primers was used in the reaction:

1) reverse primer DYSF-R (5': CTT CAC TGG GAA GTA TGT CG), homologous to the sense strand sequence of intron 4 of the DYSF gene, is common;

2) forward primer DYSF-F (5': TTC CTC TCT TGT CGG TCT AG), homologous to the sequence of the antisense strand of intron 4 of the DYSF gene, is common;

3) specific forward primer ETn-oR (5': GCC TTG ATC AGA GTA ACT GTC), homologous to the sequence of long terminal repeats (3'LTR) in the retrotransposon inserted into intron 4.

The reaction mixture contained 1x Taq Turbo buffer (Evrogen, Russia), 0.2 mM of each dNTP (Evrogen, Russia), 0.5 µM of each forward primer and 1 µM of common reverse primer, 2 units of HS Taq DNA polymerase (Evrogen, Russia). The amplification program consisted of the following steps:

1. Activation of HS Taq DNA polymerase at 95°C for 3 min;
2. Denaturation – at 95°C for 20 sec;
3. Annealing – at 60°C for 20 sec;
4. Elongation – at 72°C for 20 sec;
5. Cyclic repetition of steps 2–4 – 30 times;
6. Final elongation – at 72°C for 2 min.

Amplification products were separated by electrophoresis in 3% agarose gel in 1x TAE buffer (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA, 0.5 µg/ml ethidium bromide) at 90 V for 60 min.

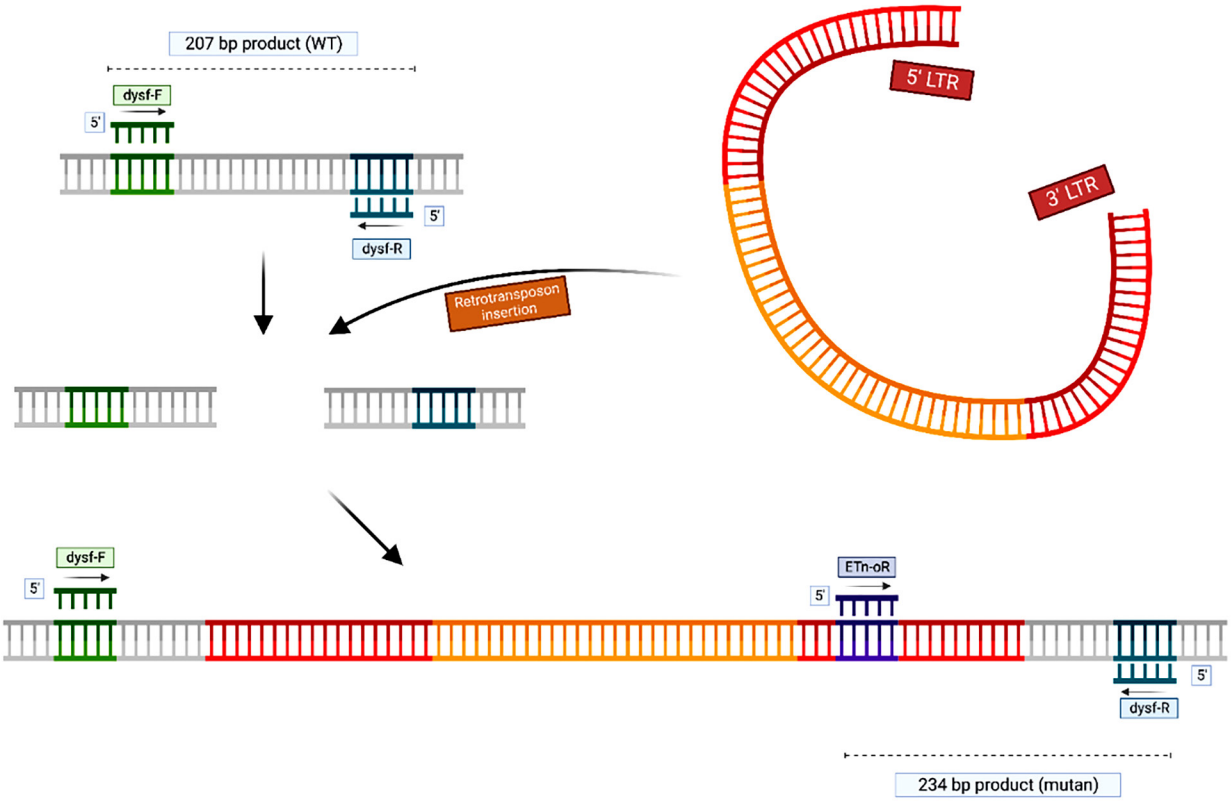

Figure 1 – Scheme of primers position for amplification of genomic area (wild type) and 3'LTR area of retrotransposon (mutant)

Note: WT – wild type; DYSF-F – common forward primer; DYSF-R – common reverse primer; ETn-OR – forward primer homologous to 3'LTR area of retrotransposon; 5'LTR, 5' – long terminal repeats; 3'LTR – 3' – long terminal repeats.

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
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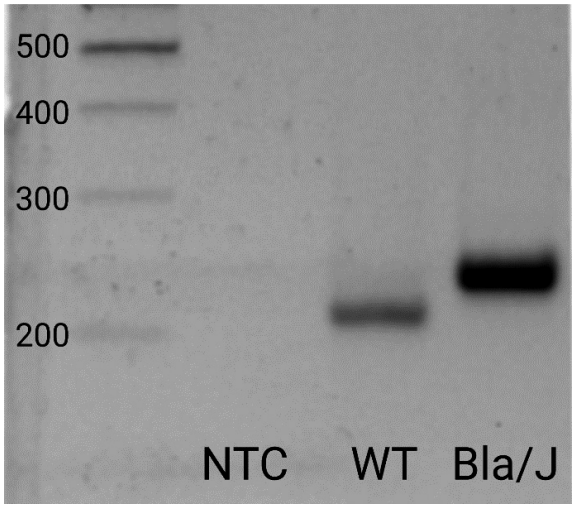

Figure 2 – Detection of retrotransposon presence in Bla/J mice by PCR

Note: product size WT (wild type) is 204 bps; product size Bla/J 237 – bps; NTC – negative control without template.

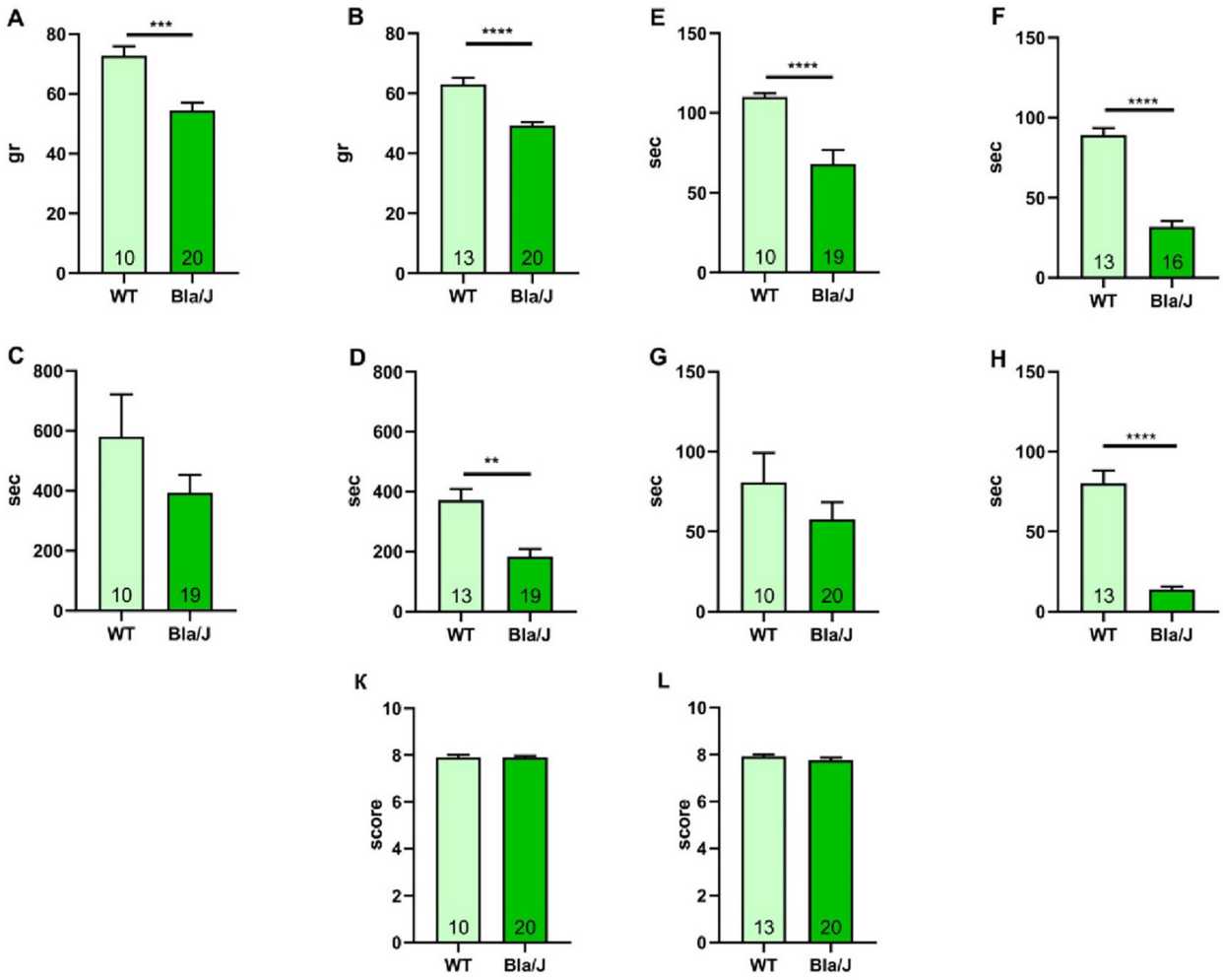

Figure 3 – Physiological parameters of Bla/J mice

Note: the analysis of forelimbs grip strength in the "Grip strength test" (A and B), endurance in the "Weight-loaded swimming test" (C and D), physical performance in the "Wire Hanging test" (E and F) and the "Vertical Pole test" (G and H), coordination of movements and limbs muscle strength in the "Inverted Grid test" (K and L). Experimental Bla/J and control mice without a genome modification (WT) were tested at the age of 12 (A, C, E, G, K) and 24 weeks (B, D, F, H, L). Medians and a standard error of the mean are presented, the number of animals is indicated at the bottom of the corresponding column. Samples were tested for normality, and statistical significance was assessed using the Mann-Whitney U-test (**p <0.01; ***p <0.0004, ****p <0.0001).

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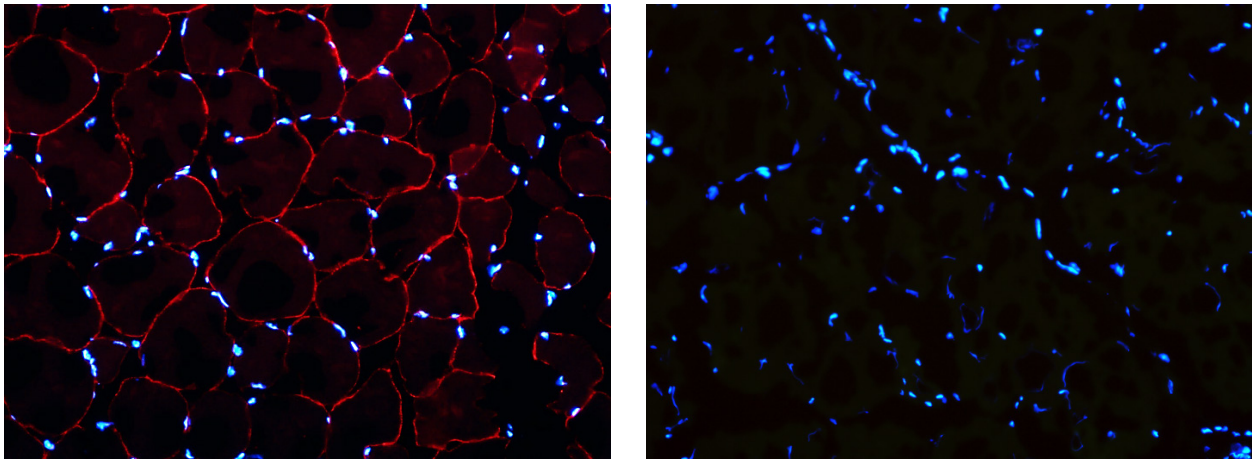

Figure 4 – Immunofluorescent reaction with antibodies to dysferlin
Note: A – C57Bl mouse; B –line Bla/J mouse. Red color – detectable dysferlin of sarcoplasmic localization, blue color – nuclei. Finishing dyeing: DAPI. Magnification ×200

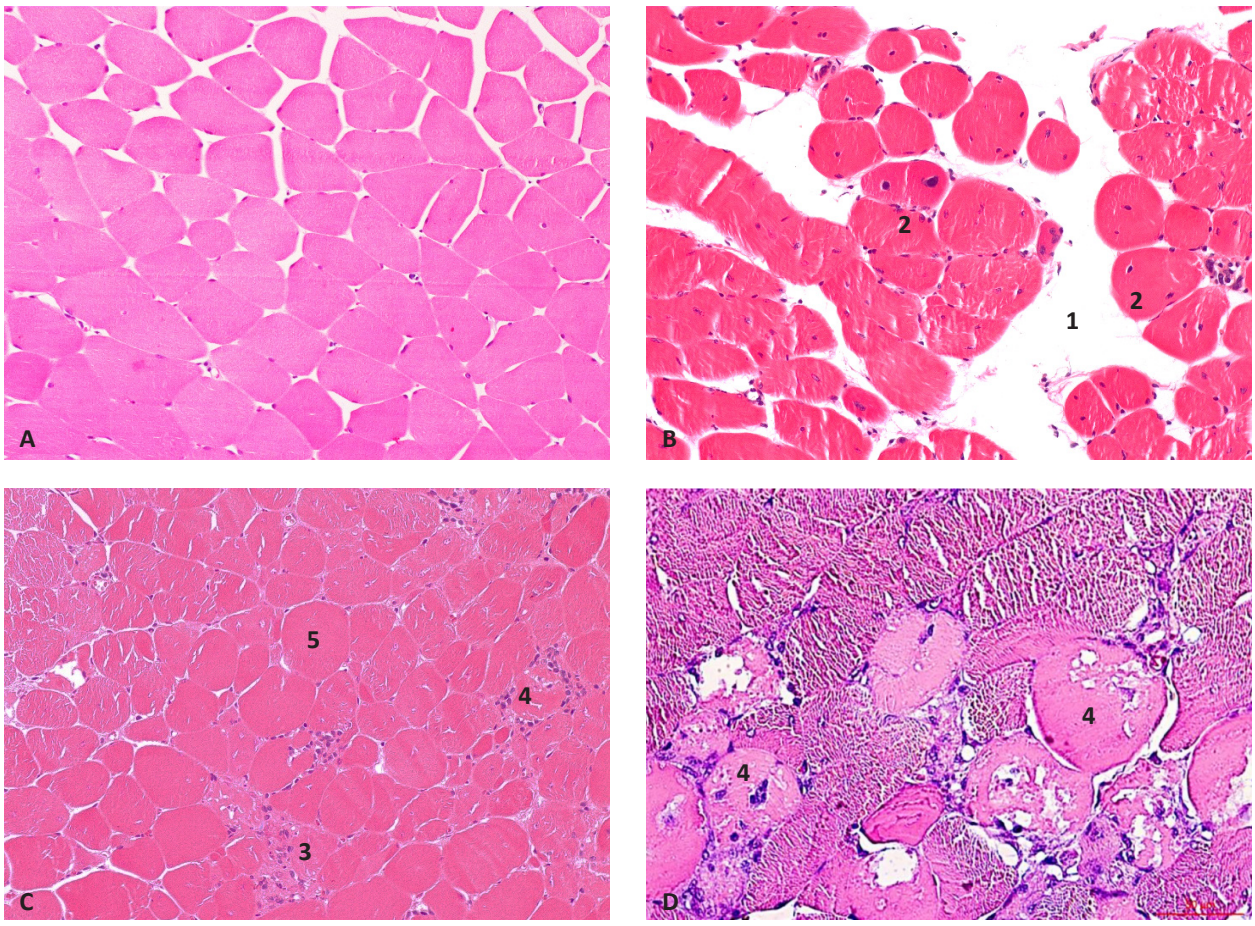

Figure 5 – Striated skeletal muscle tissue of studied animals
Note: A – control, C57Bl; B–D – Bla / J. 1 – endomysial and perimysial edema; 2 – fibers with centrally located nuclei; 3 – lympho-macrophage infiltration around necrotic muscle fibers; 4 – necrotic muscle fibers, incl. macrophage invasion; 5 – rounded muscle fibers. Colour: eosin. Magnification: A–C ×200, D ×400

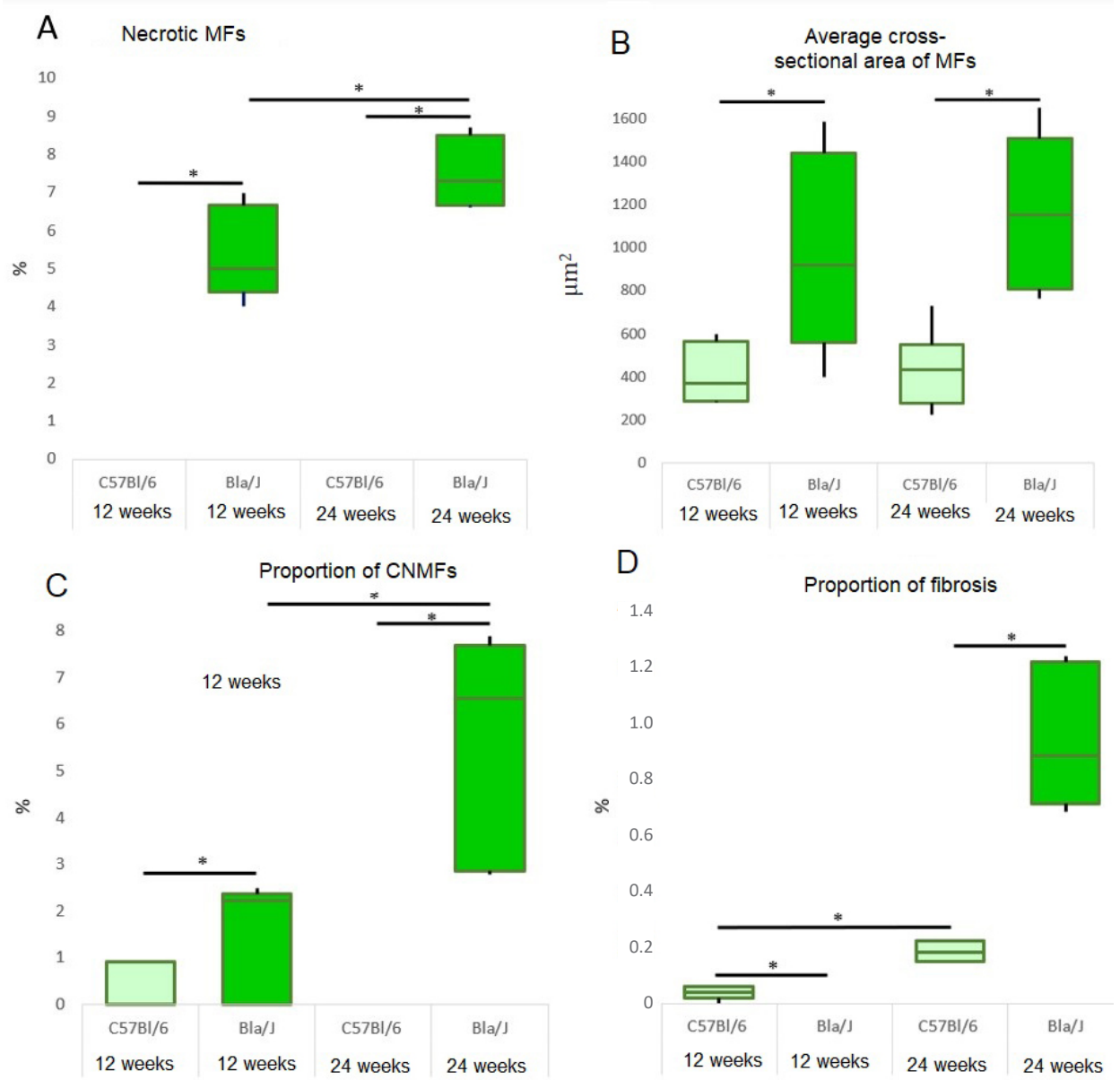

Figure 6 – Morphometric parameters of striated skeletal muscle tissue of Bla/J mice at 12 and 24 weeks of age
Note: A – proportion of necrotic muscle fibers; B – Average cross-sectional area of muscle fibers; C – proportion of central nuclear muscle fibers (CNMFs); D – proportion of connective tissue in skeletal muscle (*p < 0.05)

Pathological examination

The material included tissue fragments of *M. gastrocnemius medialis* (Gas M), *M. gastrocnemius lateralis* (Gas L), and *M. tibialis* (tib) skeletal muscles obtained from Bla/J mice and wild-type mice (WT) with a functional DYSF gene. The cryopreserved material was transferred to the chamber of a Thermo Fisher Scientific HM525 NX cryostat (USA), where it was oriented on a freezing platform in a mounting gel NEG-50 (Richard-Allan Scientific, TS, USA). The sections obtained were 3–7 microns thick. The slides with cryosections were fixed in 10% neutral buffered formalin (Biovitrum, Russia) for 10 min. Next, they were washed 3 times for 5 min in a Tris-HCL buffer solution pH 7.4 (Nevareaktiv, Russia); Background Block solution (Cell Marque, USA) was applied, the cryosections were transferred to a humid chamber, where they were incubated for 1 h. The protein block was removed by swiping, then primary recombinant rabbit monoclonal antibodies were applied to dysferlin (clone: JAI-1-49-3; ab124684; Abcam, UK) diluted 1:200 with Diamond: Antibody Diluent (Cell Marque, 938B-05, USA), in which cryosections were kept for 2 h at room temperature in a humid chamber. Over time, the sections were washed three times in a Tris-HCL buffer solution, after which a 1-hour incubation was performed at room temperature in a humid chamber with secondary goat anti-rabbit IgG H&L antibodies (Alexa Fluor 555, abcam, ab150078, UK) diluted in the

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ratio of 1:500 with Diamond:Antibody Diluent. After an hour, the sections were washed 4 times in a Tris-HCL buffer solution and incubated in a DAPI working solution (Thermo FS, D1306, USA) for 10 min. The conclusion was carried out in the mounting medium of glycerogel according to Kaiser.

For the paraffin sections manufacturing, after fixation, the tissue material was subjected to standard histological wiring, paraffin embedding, and microtoming. After deparaffinization, the sections were stained with hematoxylin and eosin according to the standard method. Photodocumentation was carried out using a computer video system (IBM PC + Leica DM 1000 microscope, Germany) and the ImageScope-M software package (Russia).

Based on the previous studies [26, 27], the estimated parameters for morphometry were chosen: the cross-sectional area of the muscle fiber, as well as the number of fibers with an internalized nucleus and the number of necrosis in relation to the total number of fibers in the field of view.

Statistical processing

Statistical processing was performed using GraphPad Prism Software 8.0 (GraphPad Software Inc, USA). Depending on the feature distribution type and equality of variances, the significance of the results obtained was assessed using a parametric (ANOVA) or nonparametric (Kruskal-Wallis test) one-way analysis of variance, and unpaired Student's t-test was used as a post-hoc analysis to identify differences in intergroup comparisons, the Mann-Whitney test, respectively, with a Benjamini-Hochberg correction for multiple hypothesis testing. The results were considered significant at $p \leq 0.05$.

RESULTS

To form representative groups of experimental and control animals synchronized by age, first the number of related sires corresponding to the objectives of the experiment were received. Crossing of homozygous mice with a mutation was performed with mice of the same genotype against the same genetic background obtained in the previous crosses. The offspring were genotyped by conventional PCR using three primers, one of which is specific for the mutation.

A schematic representation of the primers position for the amplification of the inserted retrotranspase is shown in Fig. 1.

In mutant mice, a retrotransposon larger than 6k bps was inserted into the fourth intron of the DYSF gene, which made it possible to use a system of three primers to identify a localized mutation in the allele. DYSF-F is homologous to the genomic sequence area of the intron located before the insertion sequence of the retrotransposon. Due to this, in case of the primer annealing on the mutant allele, the synthesized chain will not have time to amplify to the area homologous to the reverse primer – an amplicon with a size of more than 6 thousand bps will not form and accumulate in large numbers during PCR. ETn-oR is homologous to the retrotransposon area in the long terminal repeats (on the 3' LTR side). In combination with the reverse primer DYSF-F, which is homologous to the area of the intron genomic sequence that makes it possible for 193 nucleotide pairs to be flanked. This arrangement of primers led to the successful separation of mutant and wild type animals because of conventional PCR (Fig. 2) [28, 29].

Ho M. et al showed the insertion of a retrotransposon into a certain area of intron 4 (495 base pairs from exon 4 and about 4400 base pairs (bps) to exon 5) with a substitution of 6 bps [30]. The results of the genotyping confirmed the presence of the mutation in the homozygous form of the studied animals.

As a result of the amplification of wild-type animals images, a fragment of 204 bps was formed. In Bla/J animals homozygous for the mutant allele, a fragment 237 bps long was formed after the amplification of the DYSF gene sequence (marker 100+bp DNA Ladder).

To study a motor function, representative groups of experimental animals (homozygous knockouts – Bla/J) (n=20) were formed, the groups were tested at two age points – of 12 and 24 weeks and control animals without a genome modification (WT) (n=10 and n=13, respectively).

In the Grip Strength test, forelimb strength was significantly reduced in control mice compared to Bla/J mice at both age points (Fig. 3 A and B). In the Weight-loaded swimming test (Fig. 3 C and D), a difference was found out between the control and experimental mice at the age of 24 weeks and 12 weeks, respectively. This indicated a decrease in the endurance of mice at the age of 24 weeks with developing pathology.

The following physical performance tests were carried out: "Wire Hanging test" (Fig. 3 E and F) and "Vertical Pole test" (Fig. 3 G and H). As a result of the tests, it was found out that at the age of 24 weeks, there was a decrease in the physical performance of the Bla/J line mice compared to the C57BL/6J line.

In the "Inverted grid test" in the animals of both groups (Fig. 3 K and L), the coordination of movements and muscle strength of the limbs did not differ statistically significantly. Thus, in these age marks, the studied indicators did not change.

When setting up immunofluorescent reactions with antibodies to the dysferlin protein in the samples of

mutant animals, this protein was not detected; in the control tissues, sarcolemmal localization of dysferlin was detected, which corresponds to the canonical descriptions for skeletal muscle tissue (Fig. 4).

Histopathologically studied muscle tissue in the research of transverse sections was characterized by an age-related increase in the myopathic pattern and a violation of the tissue architecture. In contrast to the transverse sections of the WT mice muscle tissue, the fibers of the evaluated animals were characterized by polymorphism in cross sections and different sizes. In addition, compared to the WT mice, the cross section of Bla/J had a larger diameter: the so-called round muscle fibers were present in a significant amount. The muscle tissue of the studied animals was characterized by necrosis of single muscle fibers, the number of which increased by the age of 24 weeks. Around focal necrosis, accumulation of leukocytes can be notified, as well as invasion of macrophages under the basement membrane of the fiber and phagocytosis of the anesthetized part of the sarcoplasm. An important diagnostic criterion was a pronounced number of fibers with centrally located nuclei (central muscle fibers), which is interpreted in the scientific literature as a cytoskeleton destruction of the muscle fibers and/or an active regeneration process with the appearance of muscle tubules.

Attention is drawn to the edema of the endomysium and perimysium, as well as mild lymphomacrophage infiltration (Fig. 5). With an increase in the life expectancy of mutant animals, hyperplasia of the endomysium and perimysium was notified, which was expressed in an increase in the proportion of connective tissue in the composition of the muscular organ (Fig. 6).

A morphometric evaluation and statistical processing of the main tissue parameters revealed significant differences in the number of necrotic muscle fibers in WT mice, both when comparing the index at 12 and 24 weeks of age, and between the animal lines. Significant intra- and intergroup differences were established in terms of indicators: the proportion of central nuclear muscle fibers, the average cross-sectional area of the muscle fibers and the proportion of connective tissue (Fig. 6). As shown by the previous studies, an increase in the cross section of the muscle fibers should be considered as compensatory working hypertrophy due to the death of some of the structural units of the muscle. With a longer period of the animals observation, a breakdown of this process and a gradual atrophy of the muscle were established, which was later accompanied by a decrease in this indicator.

DISCUSSION

The identification of a representative *in vivo* model of dysferlinopathy is important for the search for new therapeutic targets and the study of the pharmacological activity of new drugs aimed at treating this pathology. Major mouse strains of dysferlinopathy with a complete lack of a dysferlin expression include the A/J^{dysf-}/$^{-}$ (A/J), SJL/J^{dysf-}/$^{-}$ (SJL/J), and BLA/J^{dysf-}/$^{-}$ (BLAJ; B6.A) animal strains. (Dysf^{prmd}/GeneJ), which have similar, with minor changes, phenotypic manifestations of the disease [31] and pronounced histopathology [32].

The studies examining a muscle function in the mice lacking a dysferlin (Dysf^{-}/$^{-}$) expression, have shown conflicting results. The lack of a sufficient repeatability and reproducibility of preclinical studies characterizing diferlinopathic mouse strains, may be partly due to the differences in a disease severity and phenotypic manifestations in specific strains of Dysf^{-}/$^{-}$ mice of different origins and/or the lack of suitable control colonies of wild-type mice for their comparison [33].

In the course of this study, representative groups of experimental (homozygous Dysf^{-}/$^{-}$ – Bla/J knockouts) and control animals (C57Bl/6), age-synchronized, which were further genotyped by conventional PCR, were formed. The authors used primer sequences from the JAX protocol (Protocol 26095), which simultaneously amplify the retrotranspason area (if present) and the animals' genomic DNA area. The absence of a DYSF gene expression in Bla/J mice led to muscular dystrophy, as a result of which the disease began to develop from the 12th week of life of the animal, and the peak of phenotepic manifestations could be observed at the 24th week.

When conducting behavioral testing, the authors showed that a physical activity and endurance of the studied animals decreases with age in comparison with the control group. This is confirmed by the "Grip Strength test", in which the indicators decreased in comparison with the control by 22.2% at 12 weeks, by 25.1% at 24 weeks and by 9.5% between the experimental groups at two age points (54.47±2.659 and 49.27±1.157, respectively). When conducting the "Weight-loaded swimming test", a difference was revealed in comparison with the control by 32.3% at 12 weeks, by 50.5% at 24 weeks and by 53.3% between the experimental groups at two age points (393.6±59.26 and 183.8±24.94). The preservation of physical performance was checked using the following tests: The "Wire Hanging test" (68.00± 8.809; 31.81±3.637) and the "Vertical Pole test" (13.67 ±1.875), which showed a decrease in results of the studied animals at 24 weeks compared with the control by 64.4% and 82.9%, respectively. The data on the criteria for the coordination of movements and muscle strength of the limbs, studied in the "Inverted Grid test", did not change at these ages.

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The results obtained in the course of behavioral testing, i.e., a decrease in the grip strength of the forelimbs and a decrease in physical endurance with age, reflect the progression of the underlying muscle disease in the studied animals. Based on this, it can be concluded that the studied experimental model describes the clinical picture of dysferlinopathy in patients with this disease.

The histological study also showed that with an increase in the life expectancy of mutant animals, hyperplasia of endomysium and perimysium was notified. That was expressed in an increase in the proportion of connective tissue in the composition of the muscular organ. Significant intra- and intergroup differences were established in terms of indicators: the proportion of central nuclear muscle fibers; the average cross-sectional area of muscle fibers; the proportion of connective tissue. As shown by the previous studies, an increase in the cross section of muscle fibers should be considered as compensatory working hypertrophy due to the death of some of the structural units of the muscle. With a longer period of observation, a breakdown of this process and gradual muscle atrophy were established, which was subsequently accompanied by a decrease in this indicator.

CONCLUSION

Using such tests, like: "Inverted Grid", "Grip strength", "Wire Hanging test", "Weight-loaded swimming", "Vertical Pole test", it was found out that the lack of Dysf$^{-/-}$ gene expression in the mice subline Bla/J B6.A-Dysf^{prmd}/GeneJ (Bla/J), can lead to muscular dystrophy. This pathology had been manifesting itself from the beginning of the disease development of phenotypic manifestations since the 12th week of life. The peak of phenotypic manifestations occurred at the 24th week of the animals' life. Histopathological phenotypic manifestations of the disease are generally non-specific and consistent with the data of intravital pathological examination in patients with diferlinopathy.

B6.A-Dysf^{prmd}/GeneJ (Bla/J) subline mice are a representative model of dysferlinopathy and can be used to evaluate new therapeutic agents for this disease treatment.

FUNDING

The study was financially supported by the Ministry of Education and Science of the Russian Federation, Agreement No. 075-15-2021-1346.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Mikhail V. Korokin – article writing, research design development; Elena V. Kuzubova – assessment and conduct of behavioral tests, results interpretation; Alexandra I. Radchenko – evaluation and conduct of behavioral tests, article writing; Kirill D. Chaprov – article writing, results interpreting; Alexey V. Deikin – literature analysis; Nikita S. Zhunusov – preparation of animals cohorts, gene therapy; Anastasia M. Krayushkina – preparation of animals cohorts, gene therapy; Natalya V. Ekimova – formalization of references list, observation and care of animals, animals handling; Olesya A. Puchenkova – histological examination sampling;
Roman V. Deev – consultation on planning, methodology and experiment implementation; Sergey N. Bardakov – literature analysis; Olga N. Chernova – sample preparation for histological examination, morphological description of muscle tissue sections; Vladimir M. Pokrovsky – research planning, stages of experimental work planning;
Ivan A. Yakovlev – design development and research program writing; Alexey M. Emelin – sample preparation for histological examination, morphological description of muscle tissue sections; Igor S. Limaev – sample preparation of histological sections, working with graphic materials.

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Современные препараты направленной этиотропной противовирусной терапии

эффективны против РНК-вирусов, вызывающих простудные заболевания*

*Е.П. Амон, Е.В. Эсауленко, А.В. Таганов, М.А. Ширяева, Е.Ю. Малинникова. Терапия острых респираторных вирусных инфекций в амбулаторной практике в условиях пандемии COVID-19. Терапия. 2022; 3: 14–26. Doi: https://dx.doi.org/10.18565/therapy. 2022. 3. 14–26. Петров В.А., Потапов А.Л., Ледовская Т.И., Таганов А.В. Опыт применения новых противовирусных препаратов у коморбидных пациентов в условиях пандемии. FOCUS Эндокринология. 2022. DOI: 10.47407/ef 2022. 3.1. p012. Новые возможности этиотропной терапии коронавирусной инфекции. В.П. Чуланов, Д.Ю. Пушкарь, А.В. Горелов, Л.А. Балыкова, Д.А. Лиознов, А.С. Дмитриев, К.Б. Колонтарев, А.Г. Малявин, А.В. Будневский, Е.В. Эсауленко, Н.Ю. Пшеничная, Х.Г. Омарова, А.В. Таганов, В.А. Петров. Инфекционные болезни, 2022, т. 20, №2. стр. 120-122.

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